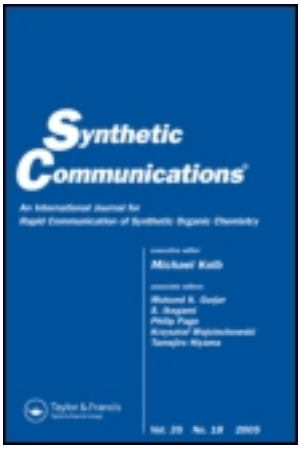

Synthetic Communications: An International Journal for Rapid Communication of Synthetic Organic Chemistry

Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/lsyc20

Synthesis and Evaluation of Substituted Pyrazoles: Potential Antimalarials Targeting the Enoyl-ACP Reductase of Plasmodium Falciparum

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Version of record first published: 15 Aug 2006.

To cite this article: Sanjay Kumar, Gyanendra Kumar, Mili Kapoor, Avadhesha Surolia & Namita Surolia (2006): Synthesis and Evaluation of Substituted Pyrazoles: Potential Antimalarials Targeting the Enoyl-ACP Reductase of Plasmodium Falciparum , Synthetic Communications: An International Journal for Rapid Communication of Synthetic Organic Chemistry, 36:2, 215-226

To link to this article: http://dx.doi.org/10.1080/00397910500334561

Synthetic Communications®, 36: 215–226, 2006

ISSN 0039-7911 print/1532-2432 online
DOI: 10.1080/00397910500334561

Synthesis and Evaluation of Substituted Pyrazoles: Potential Antimalarials Targeting the Enoyl-ACP Reductase of *Plasmodium Falciparum*

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Abstract: A series of 1,5- and 1,3-diarylsubstituted pyrazoles were designed, synthesized, and evaluated for their ability to inhibit enoyl-ACP reductase of *Plasmodium falciparum*. The inhibitory activity of these synthesized compounds was evaluated in a continuous spectrophotometric assay. Of all the compounds analyzed, NAS-81 and NAS-39 inhibited the enzyme with IC_{50} values of 30 μM and 50 μM, respectively. The mode of ligand binding was investigated by docking the synthetic inhibitors at the active site of the crystal structure of the enzyme.

Keywords: Antimalarials, fatty acid synthesis, inhibitor, substituted pyrazole

INTRODUCTION

Malaria is still a leading cause of death worldwide.[1] Fatty acid biosynthesis (FAS) has emerged as an important targeted pathway because of its different

Received in India July 11, 2005
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characteristics in the parasite, *Plasmodium falciparum*, and its human host. Enoyl-ACP (acyl-carrier protein) reductase (ENR; also known as FabI) catalyzes the final reduction step in the elongation phase of fatty acid synthesis to form acyl-ACP, which in turn serves as a substrate for another round of elongation. Because of the importance of ENR in type II dissociative FAS, it is an attractive target for developing antimalarials.[2]

The wide range of biological activities of pyrazoleshas made them popular synthetic inhibitors for different enzymes and pathways.[3–6] Numerous methods for the synthesis of these heterocyclic molecules have been described, which involve the direct C–C coupling by Grignard and Suzuki coupling[7] or the condensation of hydrazine with 1,3-diketone. Although the condensation of 1,3-diketone with aryl hydrazine yields both the 1,3 and 1,5-diarylsubstituted pyrazole, the 1,5-diaryl pyrazole can be generated exclusively by carrying out condensation in the presence of hydrochloride salt of the phenyl/substituted phenyl hydrazine by refluxing in ethanol.[8] Previously, the success of this method was demonstrated in the synthesis of a limited number of analogues only. Herein, we report the synthesis of new 1,3- and 1,5-disubstituted aryl pyrazoles that inhibit enoyl-ACP reductase of *P. falciparum*.

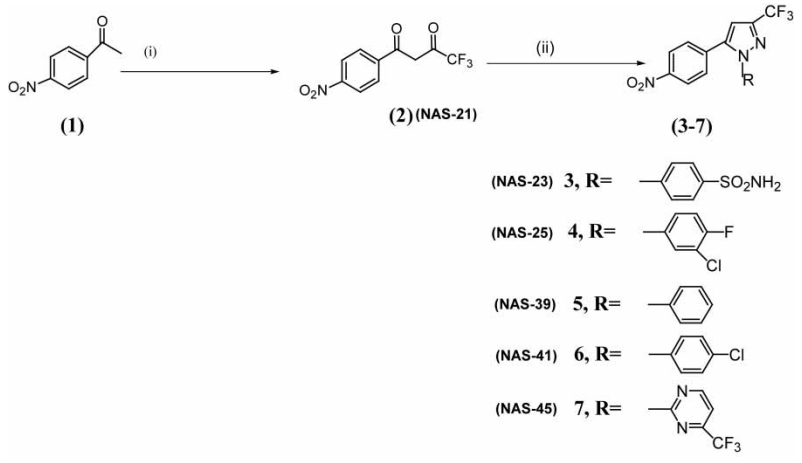
RESULTS AND DISCUSSION

Chemistry

The 1,3- and 1,5-diarylsubstituted pyrazole derivatives (**3**–**7** and **12**, **13**) were synthesized as shown in Schemes 1 and 2. The requisite intermediate 1,1,1-trifluoro-4-(4-nitrophenyl)-2,4-butanedione (**2**) and hydrazone (**10**, **11**) were synthesized according to our published procedure.[9] The 1,5-diarylsubstituted pyrazoles were synthesized by treating diketone (**2**) with different substituted hydrazine hydrochlorides in refluxing ethanol for 20 h to get good yields of **3**–**7**. The synthesis of 1,3-diarylsubstituted pyrazoles began with commercially available ketones (9: R = $-OCH_3/CH_3$) and hydrazine (**8**) as a starting material. The condensation between ketone and hydrazine provided hydrazones (**10**, **11**) in good yields. The Vilsmeier–Haack reaction using 2.5 equivalents of $POCl_3$-DMF performed a double addition at the methyl group of acetophenone pyrimidine hydrozone (**10**, **11**), which after hydrolysis gave the desired aldehydes (**12**, **13**) in 80–90% yield.[10] Although several 1,3-diarylsubstituted pyrazoles are known, these studies report for the first time the synthesis of trifluoromethyl pyrimidine–substituted pyrazoles.

Enzyme Inhibition

The pyrazole derivatives so synthesized were evaluated for their inhibitory activity on enoyl-ACP reductase in an in vitro spectrophotometric assay. As

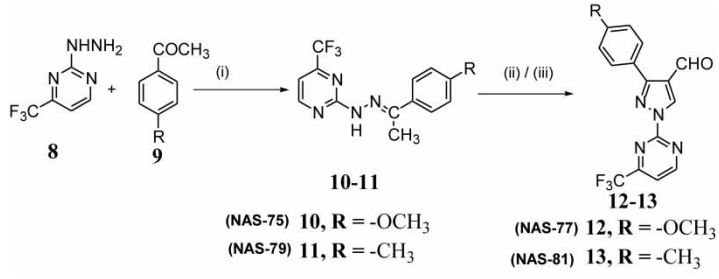


Scheme 1. Preparation of 1,5-disubstituted pyrazole derivatives: (i) MTBE, 25% NaOMe in MeOH, ethyl trifluro acetate, rt 18 h; (ii) ethanol, (4-sulphamoylpheny) hydrazine hydrachloride/3-cholro-4-fluoro pheny hydrazine hydrochloride/phenyl-hydrazine hydrochloride/phenylhydrazine hydodrochloride/4-chlorophenyl hydrazine hydrochloride/trifluoromethyl hydrazino pyrimidine.

shown in Table 1, some of the compounds inhibited the activity of the enzyme. NAS-81 and NAS-39 showed the maximum inhibition with IC_{50} values of 30 and 50 μM, respectively (Fig. 1).

Docking Studies

We docked the two best inhibitors with *P. falciparum* FabI (PfFabI) in silico using Autodock and Molecular Operating Environment (MOE) to investigate their mode of binding, which would provide a rationale for their



Scheme 2. Preparation of 1,3-disubstituted pyrazole derivatives: (i) acetic acid and water, rt 16 h; (ii) $POCl_3$ and dimethlyformamide at 0°C for 15 h; (iii) excess of water at rt for an additional 15 h.

Table 1. Inhibition of *P. falciparum* enoyl-ACP reductase by synthesized disubstituted pyrazoles

Entry	Compound	IC_{50} (μM)
1	NAS-21	NI[a]
2	NAS-23	200
3	NAS-25	100
4	NAS-39	50
5	NAS-41	NI[a]
6	NAS-45	100
7	NAS-77	200
8	NAS-81	30

[a]NI: No inhibition.

inhibitory activity (Fig. 2). Both the inhibitors bind right into the active site. Among the five 1,5-disubstituted pyrazoles synthesized, NAS-39 shows the best inhibition. NAS-39 has interactions with Tyr181 and Trp35 of the protein in the docked complex. The NO_2 group of NAS-39 makes hydrogen

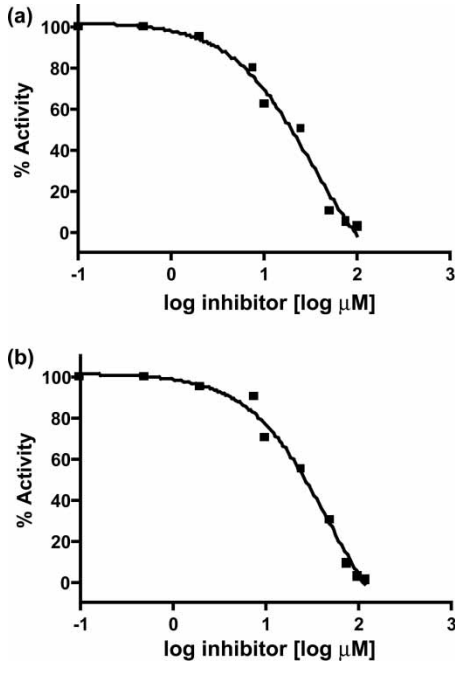


Figure 1. Dose response curves of the two most potent inhibitors: (a) NAS-81 and (b) NAS-39. The IC_{50} was calculated from a plot of percent activity versus log concentration of the inhibitor by fitting it to nonlinear regression analysis using GraphPad Prism® software.

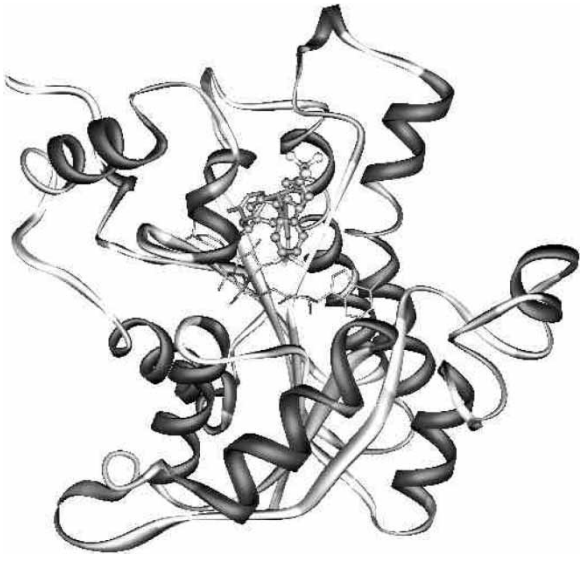


Figure 2. Ribbon diagram of *Plasmodium falciparum* enoyl-ACP reductase docked with NAS-81 (sticks) and NAS-39 (balls and sticks). NAD is also shown (thin sticks).

bonds with OH of Tyr181 and ribose on the nicotinamide side of NADH. The phenyl ring of Tyr181 also has an aromatic interaction with the phenyl ring of NAS-39. Similarly Trp35 has an aromatic interaction with the other phenyl ring (R-group) of NAS-39, contributing to the affinity of the molecule to the enzyme (Fig. 3a). Of the two 1,3-disubstituted pyrazoles synthesized, NAS-81 demonstrates better inhibitory activity and is also the best inhibitor among all the pyrazoles synthesized. On analysis of the docked complex of this inhibitor with the enzyme, we find that NH of the pyrazole ring forms a hydrogen bond with main chain C═O of Ala223, the oxygen of the CHO group is hydrogen bonded with NH of the Asn122 and sulphur of Met185, and one of the fluorine of CF_3 group is hydrogen bonded with OH attached to one of the phosphate groups of NADH. The phenyl ring with CH_3 as R-group of NAS-81 has an aromatic interaction with the phenyl ring of Tyr181, and the other phenyl ring makes an aromatic interaction with Tyr35 (Fig. 3b). The poor inhibitory activity of NAS-77 as compared to NAS-81 is probably due to bigger a R-group; that is, OCH_3 can destabilize the interactions occurring in the case of NAS-81.

CONCLUSION

In summary, we have described the synthesis of novel 1,3- and 1,5-disubstituted pyrazoles that inhibit *P. falciparum* enoyl-ACP reductase. One of the 1,3-disubstituted pyrazoles (NAS-81) and one of the 1,5-disubstituted

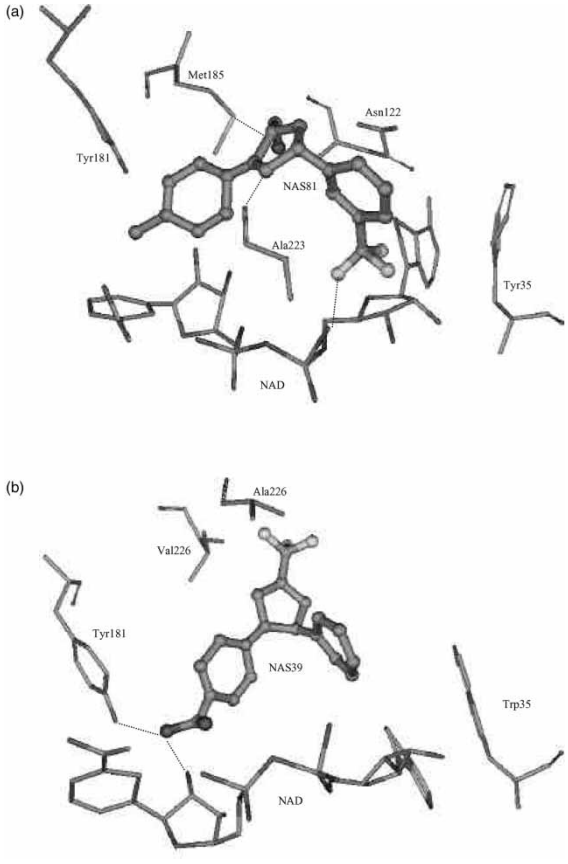


Figure 3. Interactions of (a) NAS-81 and (b) NAS-39 with *Plasmodium falciparum* enoyl-ACP reductase. Inhibitors are shown in balls and sticks. Hydrogens are not shown for sake of clarity.

pyrazoles (NAS-39) show significant in vitro inhibition of the enzyme. Docking simulations provide insight into the binding modes of these two molecules. These studies will pave the way for further development of more potent inhibitors in this series, leading to new antimalarial agents.

EXPERIMENTAL

Materials

Synthetic Chemistry

4-Nitrocetophenone, (4-sulphamoylphenyl) hydrazine-HCl, 3-chloro-4-fluoro phenyl hydrazine-HCl, phenyl hydrazine-HCl, 4-chloro phenyl hydrazine-HCl,

trifluoromethyl hydrazinopyrimidine, methyl-*ter*-butylether, and dimethylformamide were purchased from Sigma-Aldrich (USA). All other reagents were purchased locally and purified prior to use. The synthesized molecules were purified using 200-mesh silica-gel column chromatography and were characterized by ^{1}H NMR, mass spectra, ^{13}C NMR, and/or CHN analysis.

Biological Activity

Media components for *E. coli* cultures were from Hi-media (Delhi, India). β-NADH, crotonoyl-CoA, imidazole, and SDS-PAGE reagents were obtained from Sigma Chemical Co., St. Louis, MO, USA. His-bind resin was obtained from Novagen (Madison, USA).

General Procedure for Synthesis

The pyrazole derivatives (**3**–**7**) and (**10**–**13**) were synthesized as shown in Schemes 1 and 2. The requisite intermediate **2** was synthesized by following the published procedure.[9] During the course of synthesis we synthesized two different types of diarylsubstituted pyrazoles. The 1,5-diarylsubstituted pyrazoles (**3**–**7**) and 1,3-diarylsubstituted pyrazoles (**12, 13**) were different from each other in having a 3-trifluoromethyl and a 4-formyl group on the pyrazole ring, respectively. 1,5-diarylsubstituted pyrazoles (**3**–**7**) were synthesized by a one-step condensation of intermediate **2** and the substituted aryl hydrazine hydrochlorides. The 1,3-diaryl substituted-4-formyl pyrazoles (**12** and **13**) were synthesized by using intermediates (**10** and **11**), respectively. The intermediates **10** and **11** were synthesized by the reaction of 2-hydrazino-4-trifluoromethylpirimidine and p-methoxy/methyl acetophenone using the published procedure[11] and were characterized by ^{1}H NMR. All molecules (**3**–**7** and **12**, **13**) were purified by silica-gel column chromatography using a hexane/ethylacetate gradient and were characterized by ^{1}H NMR, mass spectra, and elemental analysis.

Synthesis of 4-[5-(4-Nitrophenyl)-3-(trifluoromethyl)-1*H*-pyrazole-1yl] benzenesulphonamide (NAS-23)

(4-Sulphamoylphenyl) hydrazine hydrochloride (112 mg; 0.5 mM) was added to a stirring solution of 4,4,4-trifluoro-1-(4-nitrophenyl)butane-1,3-dione (104 mg; 0.4 mM) in ethanol. The reaction mixture was refluxed for 20 h and, after cooling to room temperature, the mixture was concentrated in vacuo. The residue was taken in ethyl acetate, washed with water and brine, dried over Na_2SO_4, filtered, and concentrated in vacuo. The product was purified by column chromatography using a hexane : ethyl acetate gradient (yield 60%, 130 mg). The pure product was characterized by

^{1}H NMR. $CDCl_3$ δppm 8.26 (d, $J = 8.4$ Hz, 2H), 7.96 ($J = 8.00$ Hz, 2H), 7.61–7.43 (m, 4H), 5.00 (s, 2H). ESMS, m/z 412 (435 sodiated peak); calculated for $C_{16}H_{11}N_4O_4F_3S$, 412. C, H, N analysis found C, 46.34; N, 2.71; and N, 13.54; calculated for $C_{16}H_{11}N_4O_4F_3S$: C, 46.60%; H, 2.67%; N, 13.59%.

Synthesis of 5-(4-Nitrophenyl)-3-(trifluoromethyl)-1-(3-chlor-4-fluorophenyl)pyrazole (NAS-25)

(3-Chloro-4-fluorophenyl)hydrazine hydrochloride (97 mg; 0.5 mM) was added to a stirring solution of 4,4,4-trifluoro-1-(4-nitrophenyl)butane-1,3-dione (131 mg; 0.5 mM) in ethanol. The reaction mixture was refluxed for 20 h. After cooling to room temperature, the reaction mixture was concentrated in vacuo. The residue was taken in ethyl acetate washed with water and brine, dried over Na_2SO_4, filtered, and concentrated in vacuo. The product was purified using column chromatography in a hexane:toluene gradient (starting from hexane and then 10%, 20%, 30%, 40% toluene). The light yellow pure product (yield 55%; 110 mg) was characterized by ^{1}H NMR. $CDCl_3$ δppm 8.26 (d, $J = 7.80$ Hz, 2H), 7.43 (d, $J = 7.80$ Hz, 2H), 7.17–7.11 (m, 3H), 6.89 (s, 1H). ESMS m/z 385.5 (408 sodiated peak); calculated for $C_{16}H_8N_3O_2F_4Cl$; 385.5. C, H, N analysis found C, 49.66; H, 2.11; and N, 10.55; calculated for $C_{16}H_8N_3O_2F_4Cl$: C, 49.80%; H, 2.07%; N, 10.89%.

Synthesis of 5-(4-Nitrophenyl)-3-(trifluoromethyl)-1-phenyl Pyrazole (NAS-39)

Phenylhydrazine hydrochloride (116 mg; 0.8 mM) was added to a stirring solution of 4,4,4-trifluoro-1-(4-nitrophenyl)butane-1,3-dione (131 mg; 0.5 mM) in ethanol. The reaction mixture was refluxed for 20 h. After cooling to room temperature, the reaction mixture was concentrated in vacuo. The residue was taken in ethyl acetate washed with water and brine, dried over Na_2SO_4, filtered, and concentrated in vacuo. The product was purified by column chromatography using 1% ethyl acetate in hexane. The light yellow pure product (yield 50%; 130 mg) was characterized by ^{1}H NMR. $CDCl_3$ δppm 8.24 (d, $J = 8.86$ Hz, 2H), 7.66–7.22 (m, 7H), 6.89 (s, 1H). ESMS m/z 333; calculated for $C_{16}H_{10}N_3O_2F_3$, 333. C, H, N analysis found C, 57.48; H, 3.1; and N, 12.56%; calculated for $C_{16}H_{10}N_3O_2F_3$: C, 57.66; H, 3.02; N, 12.61%.

Synthesis of 5-(4-Nitrophenyl)-3-(trifluoromethyl)-1-(4-chlorophenyl) Pyrazole (NAS-41)

4-Chlorophenyl hydrazine hydrochloride (179 mg; 1 mM) was added to a stirring solution of 4,4,4-trifluoro-1-(4-nitrophenyl)butane-1,3-dione

(261 mg; 0.8 mM) in ethanol. The reaction mixture was refluxed for 20 h. After cooling to room temperature, the reaction mixture was concentrated in vacuo. The residue was taken in ethyl acetate washed with water and brine, dried over Na_2SO_4, filtered, and concentrated in vacuo. The product was purified by column chromatography using 1% ethyl acetate in hexane. The orange pure product (yield 45%; 160 mg) was characterized by ^{1}H NMR. $CDCl_3$ δ ppm 8.23 (d, $J = 8.40$ Hz, 2H), 7.51–7.18 (m, 7H), 6.89 (s, 1H). ESMS m/z 367; calculated for $C_{16}H_9N_3O_2F_3Cl$, 367.5. C, H, N analysis found C, 52.31; H, 2.51, and N, 11.39%; calculated for $C_{16}H_9N_3O_2F_3Cl$: C, 52.26; H, 2.47; N, 11.43%.

Synthesis of 5-(4-Nitrophenyl)-3-(trifluoromethyl)-1-{4-trifluoromethyl(2-pyrimidyl)} pyrazole (NAS-45)

2-Hydrazino-4-trifluoromethylpyrimidine (72 mg; 0.4 mM) was added to a stirring solution of 4,4,4-trifluoro-1-(4-nitrophenyl)butane-1,3-dione (105 mg; 0.4 mM) in ethanol and HCl (0.5 mL of 37% w/v). The reaction mixture was refluxed for 20 h. After cooling to room temperature, the reaction mixture was concentrated in vacuo. The residue was taken in ethyl acetate washed with water and sodium bicarbonate solution, dried over Na_2SO_4, filtered, and concentrated in vacuo. The product was crystallized in methanol. The orange pure product (yield 50%; 130 mg) was characterized by ^{1}H NMR. $CDCl_3$ δ ppm 9.09 (d, $J = 7.5$ Hz, 1H), 8.87 (d, $J = 7.8$ Hz, 1H), 8.29 (d, $J = 12.5$ Hz, 2H), 7.98 (d, $J = 11.7$ Hz, 2H), 6.90 (s, 1H). ESMS m/z 403; calculated for $C_{15}H_7N_5O_2F_6$, 404.24. C, H, N analysis found C, 44.61; H, 1.81; and N, 17.22%; calculated for $C_{15}H_7N_5O_2F_6$: C, 44.68; H, 1.75; N, 17.37%.

Synthesis of (NAS-77)

Phosphorous oxychloride (1.5 mM; 137 μL) was added to 10 mL of dimethylformamide at 0°C, and the reaction mixture was stirred for 30 min by maintaining 0°C temperature. The hydrazone (0.5 mM; 155 mg) was added as a solid slowly to the reaction mixture and stirred for 15 h at rt. The crude reaction mixture was quenched with excess water and stirred for an additional 15 h. The resulting solid was filtered and dried in vacuo and thereafter taken in dichloromethane. The resulting product was 95% pure. For testing biological activity it was purified further by 100–200–mesh silica-gel column chromatography using a gradient 10% ethyl acetate in petroleum ether (yield 60%; 100 mg). The pure product was characterized by ^{1}H NMR. $CDCl_3$ δ ppm 10.11 (s, 1H); 9.27 (s, 1H); 9.13 (d, $J = 4.8$ Hz, 1H); 7.87 (d, $J = 9.0$ Hz, 2H); 7.64 (d, $J = 2.1$ Hz, 1H); 7.02 (d, $J = 8.85$ Hz, 2H); 3.88 (s, 3H). ^{13}C NMR $CDCl_3$ δ ppm = 184.79, 162.23, 161.05, 157.69, 156.36, 155.69, 135.54, 130.66, 124.25, 123.37,

119.17, 118.58, 115.13, 114.15, 55.40. ESMS m/z found 348, calculated for $C_{16}H_{11}N_4O_2F_3$, 348.28.

Synthesis of (NAS-81)

Phosphorous oxychloride (1.5 mM, 137 μL) was added to 10 mL of dimethylformamide at 0°C, and the reaction mixture was stirred for 30 min and maintained at 0°C. The hydrazone (0.5 mM; 147 mg) was added as a solid slowly to the reaction mixture and stirred for 15 h at rt. The crude reaction mixture was quenched with excess water and stirred for an additional 15 h. The resulting solid was filtered and dried in vacuo and then taken in dichloromethane. The resulting product was 95% pure except for biological activity; it was purified by 100–200–mesh silica-gel column chromatography using a gradient of hexane : ethyl acetate :: 90 : 10 (yield 65%; 110 mg). The pure product was characterized by ^{1}H NMR. $CDCl_3$ δ ppm 10.12 (s, 1H); 9.29 (s, 1H); 9.14 (d, $J = 4.8$ Hz, 1H); 7.78 (d, $J = 6.3$ Hz, 2H); 7.66 (d, $J = 1.5$ Hz, 1H); 7.32 (d, $J = 7.2$ Hz, 2H); 2.44 (s, 3H). ^{13}C NMR $CDCl_3$ δ ppm = 184.96, 162.23, 162.00, 161.41, 140.03, 136.68, 135.06, 129.43, 128.48, 127.71, 115.18, 114.30, 108.56, 21.42. ESMS m/z found 332; calculated for $C_{16}H_{11}N_4OF_3$, 332.09.

Spectrophotometric Assay

PfFabI was expressed and purified as described previously.[12] All experiments were carried out on a Jasco V-530 UV-Vis spectrophotometer. Enoyl-ACP reductase was assayed at 25°C by monitoring the decrease in A_{340} as a result of the oxidation of NADH using crotonoyl-CoA as a substrate.[12] The standard reaction mixture in a total volume of 100 μL contained 20 mM of Tris-Cl buffer, pH 7.4; 500 mM of NaCl; 100 μM of crotonoyl-CoA; and 100 μL of NADH. For determining the IC_{50} of each compound, assays were performed as described, with the addition of various concentrations of the inhibitor to the reaction mixture. The concentration of inhibitor that reduced the enzyme activity by half was calculated as the IC_{50} of the inhibitor.

Docking Studies

All the docking studies were performed using the docking program AutoDock 3.05.[13,14]

Preparation of the Receptor and Ligand Molecules

The crystal structure of PfFabI submitted to PDB (www.rcsb.org) by Perozzo et al.[15] was used for docking studies. Triclosan was removed from the active

site of the pdb file 1NHG · pdb and the binary complex (PfFabI with cofactor) was converted into mol2 format with MMFF94 charges loaded using the MOE (Molecular Operating Environment) suite of programs.[16] The script molto2pdbqs (provided with AutoDock) was used to prepare the receptor file. Ligands were built using MOE and energy minimized with MMFF94 charges. The script AutoTors (provided with AutoDock) was used to define torsion angles in the ligand prior to docking. The receptor molecule was kept rigid while the ligands were flexible for docking.

Docking Simulations

Grid maps for docking simulations were generated with 80 grid points (with 0.375-Å spacing) in x, y, and z direction centered in the active site using the AutoGrid program. Lennard–Jones parameters 12–10 and 12–6 (supplied with the program package) were used for modeling H-bonds and van der Waals interactions, respectively. The distance-dependent dielectric permittivity of Mehler and Solmajer was used in the calculations of the electrostatic grid maps.[17] The genetic algorithm (GA) and Lamarckian genetic algorithm with the pseudo-Solis and Wets modification (LGA/pSW) methods were used with default parameters. For all simulations the starting population in the genetic algorithm was 50. Each simulation comprised 2.5×10^6 energy evaluations. Each docking experiment consisted of a series of 100 simulations. Finally, the docked inhibitors were energy minimized, keeping the cofactor and all the amino acids touching the radius of 4.5 Å around the inhibitor flexible on MOE. This accounts for the minor structural changes that might occur in amino acid side chains in the active site upon binding of the inhibitor.

ACKNOWLEDGMENTS

This work was supported by a grant from the Department of Biotechnology, Government of India, to N. S. and partially supported by grants from Shantha Biotechniques Pvt. Ltd., Hyderabad, to N. S. and A. S. S. K. is thankful to the Department of Biotechnology PDF program for a postdoctoral fellowship.

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