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Original article

Syntheses of new tetrasubstituted thiophenes as novel anti-inflammatory agents

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Abstract

A series of new tetrasubstituted thiophenes (**4a–4i**, **5a–5i** and **6a–6f**) have been synthesized as novel anti-inflammatory agents and were evaluated for their anti-inflammatory activity in carrageenin-induced rat hind paw oedema model at the doses of 10, 20 and 40 mg/kg body weight. Among ester series, the best compound **4c** showed 71% protection at 10 mg/kg, 72% at 20 mg/kg, and 76% at 40 mg/kg to inflamed paw; while in acid series **5a** showed 79% protection at 10 mg/kg, 80% at 20 mg/kg, and 70% at 40 mg/kg, and **5c** showed 72% protection at 10 mg/kg, 75% at 20 mg/kg, and 69% at 40 mg/kg, to inflamed paw. In case of oxime series **6a–6f**, the anti-inflammatory activities of the candidates were found to be poor as compared to acid and ester series. It was found on the basis of SAR studies of target compounds, that the presence of OCH_3 at R_2 position and H, OCH_3 at R_1 are one of the requirements for eliciting comparable anti-inflammatory activity in both tetrasubstituted thiophenes' ester and acid series. Compounds **4a–4i**, **5a–5i** were investigated for their analgesic activity in acetic acid induced writhing response model at 10 mg/kg dose. Among the ester series compound **4e** showed maximum protection of 60%, while **4a**, **4b**, and **4i** exhibited 55%, 45%, and 43% protection, respectively. The result showed that presence of H, Cl at R_1 and OCH_3, CH_3 at R_2 in tetrasubstituted thiophene ester series enhances their analgesic activity. The candidates of acid series **5a–5i** showed poor analgesic activity as compared to the standard drug ibuprofen. Compounds **4a–4i**, **5a–5i** were evaluated for their in vitro antioxidant nitric oxide radical scavenging assay. Among the ester series **4a** showed maximum in vitro nitric oxide radical scavenging activity having IC_{50} value 30.08 μg/ml while in acid series **5a** has IC_{50} value 25.20 μg/ml. The results showed that the presence of $R_1 = H$, $R_2 = OCH_3$ and $R_1 = R_2 = OCH_3$ enhances nitric oxide radical scavenging property in tetrasubstituted thiophenes' acid series.

Keywords: Tetrasubstituted thiophenes; COX-inhibitors; Anti-inflammatory activity; Analgesic activity; Antioxidant activity

1. Introduction

Inflammation is a local reaction of the vascular and supporting elements of a tissue to injury resulting in the formation of a protein-rich exudates; it is a protective response of the nonspecific immune system that serves to localize, neutralize, or to destroy an injurious agent in preparation for the process of healing. The cardinal signs of inflammation are rubor (redness), calor (heat), dolor (pain), tumor (swelling), and functio laesa (loss of function). Cause of inflammation includes physical agents, chemical agents, immunological reactions, and infection by pathogenic organism [1]. Inflammation is divided into acute and chronic patterns. The characteristics of acute inflammation are the exudation of fluid and plasma proteins (oedema) and the emigration of leukocytes, predominantly neutrophils. Chronic inflammation is

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considered to be inflammation of prolonged duration (weeks or months) in which active inflammation, tissue destruction, and attempts at repair are proceeding simultaneously. Chronic inflammation includes some of the most common and disabling human diseases, such as rheumatoid arthritis, atherosclerosis, tuberculosis, and chronic lung diseases [2].

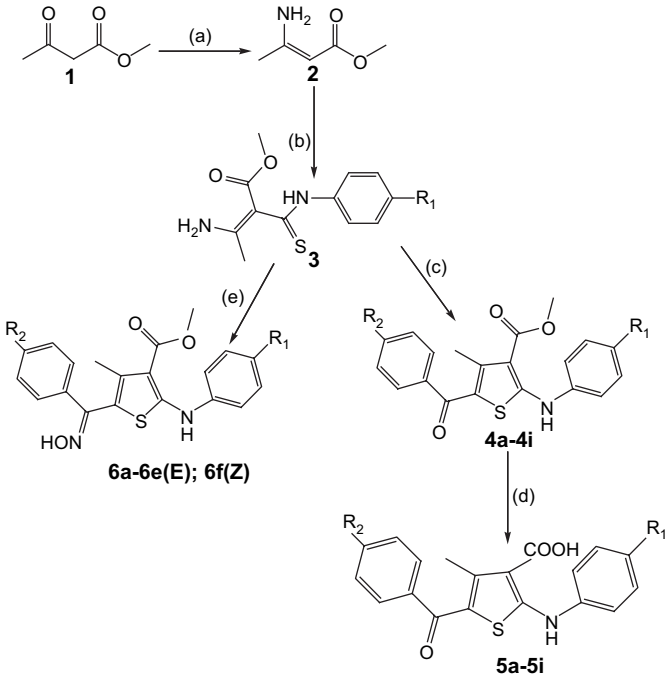
Nonsteroidal anti-inflammatory drugs (NSAIDs) are widely used for the choice treatment in various inflammatory diseases such as arthritis, rheumatisms as well as to relieve the aches and pain of everyday life [3]. Classical NSAIDs exhibit their action by restricting the biosynthesis of prostaglandin, some of which are pro-inflammatory. This is essentially brought about by inhibiting the rate limiting cyclooxygenase (COX) enzyme involved in the inflammatory cascade [4]. The acid group present in the classical NSAIDs binds to arginine, the 120th amino acid residue in the COX, and causes the inactivation of the enzyme [5]. Therefore classical NSAIDs act as preferential COX-1 inhibitors. NSAIDs cause side effects, namely, dyspepsia, gastrointestinal ulceration, bleeding, nephrotoxicity [6] and even death [7]. The discovery of the isoform of constitutive COX-1, namely, COX-2 (inducible) and its inhibition has proved to be beneficial in clinical situations had led to the introduction of many potent anti-inflammatory agents commonly referred to as COX-2 inhibitors [8]. COX-2 inhibitors, which were once thought to be a panacea for the treatment of inflammatory disease, have recently met with a measure of disrepute due to the emergence of serious side effects in long-term clinical use [9]. COX-3, a COX-1 variant and a new isozyme and its relation to analgesic/antipyretic drug has also been reported [10].

MAP kinases are signaling molecules that are activated by a number of extracellular stress stimuli. p38 MAP kinase is a member of a family of serine–threonine kinases that are activated by dual phosphorylation of a TGY motif [11]. The events that are regulated by p38 MAP kinase lead to the production of pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) [12]. Tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β), accumulation initiates a cascade of events leading to inflammation and tissue destruction in diseases such as rheumatoid arthritis [13], Crohn's disease [14], inflammatory bowel syndrome, and psoriasis. Agents having beneficial effects for the treatment of the inflammatory diseases and which inhibit the production of TNF-α antibody and IL-1β are also known [15]. Chimeric TNF-α antibody, infliximab [16], has been approved for the treatment of rheumatoid arthritis and Crohn's disease, while the TNF-α receptor fusion protein, etanercept [17] and IL-1 receptor antagonist, anakinra [18], have been approved for the treatment of rheumatoid arthritis. As such, p38 MAP kinase mediated regulation of TNF-α and IL-1β serves as an attractive target for the treatment of inflammatory diseases [19]. The p38 MAP kinase inhibitors such as SB203580 [20], BIRB-796, and SCIO-469 [21] are under clinical trial for the treatment of the rheumatoid arthritis. Aminobenzophenone derivatives have also been reported as a novel class of p38 MAP kinase inhibitors with high anti-inflammatory activity [22]. Recent years have witnessed the emergence of many candidates incorporating the oximino feature in their structure having anti-inflammatory activity. An attempt to generate a three point pharmacophore for designing better anti-inflammatory agents has been reported [23]. In continuation of our search for new tetrasubstituted thiophene as potent anti-inflammatory agents, we have reported optimization of electron withdrawing groups at the *para* position in anilino (second position) and benzoyl moiety (fifth position) in the bioisosteric tetrasubstituted thiophenes, falling in $+\sigma, \pm\pi$ quadrant of the Craig plot and its effect on their anti-inflammatory activity profile [24]. In present study we report synthesis, anti-inflammatory, analgesic and in vitro antioxidant nitric oxide radical scavenging activity of designed tetrasubstituted thiophene acid/ester molecules having the features of (a) COX-1 inhibitor and 5-LOX inhibitor (acid/ester) of the anthranilic acid type (fenamates), (b) p38 MAP kinase inhibitor, and (c) the selection of substituents at R_1 (both electron releasing and electron withdrawing) in anilino moiety and R_2 (electron releasing only) in benzoyl moiety. The selection of substituents at R_1 and R_2 was mainly guided by lipophilicity and electronic considerations as defined by $\sigma-\pi$ Craig plot chosen in such a way that they fall within the $-\sigma, \pm\pi$ quadrant of the Craig plot [25].

For optimization of the presence of electron releasing groups on anti-inflammatory activity of tetrasubstituted thiophenes we introduced, electron releasing groups such as *p*-OCH_3 ($\sigma = -0.27$; $\pi = -0.04$) and *p*-CH_3 ($\sigma = -0.17$; $\pi = +0.56$) in benzoyl moiety (R_2) and keeping them constant, electron releasing groups *p*-OCH_3 and *p*-CH_3 in anilino moiety (R_1) were introduced. Further keeping electron releasing groups *p*-OCH_3 and *p*-CH_3 at R_2 in benzoyl moiety as constant, electron withdrawing substituents *p*-F ($\sigma = +0.06$; $\pi = +0.14$) and *p*-Cl ($\sigma = +0.24$; $\pi = +0.71$) were introduced in anilino moiety (R_1), to study their effect on anti-inflammatory and analgesic activity profile of the designed compounds. Oximes of tetrasubstituted thiophene esters were synthesized to explore the introduction of oximino feature on the anti-inflammatory activity profile of the designed compounds. The role of the free radical (NO) in inflammatory processes is well known [26]. Free radicals liberated from phagocyte cells are important in inflammatory processes because they are implicated in the activation of nuclear factor kB (NF-kB), which induces the transcription of inflammatory cytokines and COX-2. Furthermore, antioxidants have been shown to be able to effectively block the activation of NF-kB through the stabilization of NF-kB/IkB-α complex [27]. In order to arrive at possible mechanism for anti-inflammatory activity of synthesized tetrasubstituted thiophene esters **4a–4i** and acids **5a–5i**, their in vitro antioxidant nitric oxide radical scavenging assay was performed.

2. Chemistry

The syntheses of tetrasubstituted thiophenes were carried out as shown in Scheme 1. The intermediate compound (**3**) was synthesized by the nucleophilic addition of arylisothiocyanate and enamine (**2**) in ether at 0 °C. Enamine (**2**) was synthesized by reacting ammonia with methyl acetoacetate (**1**) as per reported procedure [28–30]. Tetrasubstituted thiophene esters (**4a–4i**) were prepared by reaction of intermediate (**3**) with



Scheme 1. Synthesis of tetrasubstituted thiophenes. Reagents and conditions: (a) ammonia (25%), diethyl ether, 0–15 °C, 1 h, 62%; (b) ArNCS, diethyl ether, 0 °C–rt, 5 h, 50–55%; (c) 4-substituted phenacyl bromides, acetonitrile, rt, until no more starting material could be detected on TLC; (d) KOH, MeOH, rt, 1 h, then removed in vacuo, extracted with diethyl ether, aqueous layer acidified with dilute HCl; (e) oxime of 4-substituted phenacyl bromides, acetonitrile, rt, until no more starting material could be detected on TLC.

4-substituted phenacyl bromide in acetonitrile without adding base at room temperature. The corresponding acids (**5a–5i**) of tetrasubstituted thiophene esters (**4a–4i**) were prepared by hydrolyzing them in methanol with 1 eq of potassium hydroxide solution at room temperature. The intermediate (**3**) was separately treated with oxime of 4-substituted phenacyl bromide in acetonitrile at room temperature to obtained oximino compounds (**6a–6f**). All the synthesized compounds were characterized by spectroscopic data such as mass, IR, ^{1}H NMR and elemental analysis.

^{1}H NMR spectroscopy was used to assign *E*/*Z* configuration of oxime compounds **6a–6f**. The oxime compounds **6a–6e** were found to have *E* configuration while **6f** has *Z* configuration. *E* configuration of oxime compounds (**6a–6e**) was assigned on the basis of the absence of unshielding effect of oxime oxygen on the C–CH_3 singlet of the fourth position of thiophene ring. For compounds **6a**, **6b**, **6c**, **6d**, and **6e** shielded singlet values are $\Delta\delta = -0.01$, -0.05, -0.21, -0.86 and -0.06, respectively. In case of **6f** due to unshielding effect of oxime oxygen on the C–CH_3 singlet of the fourth position thiophene ring, shielded singlet value is $\Delta\delta = +0.23$ which indicates its *Z* configuration. These data agrees with previously published data for *E*/*Z* configuration of oximes [31].

3. Pharmacology

Albino rats (150–250 g) and mice (20–25 g) of either sex were supplied by Cadilla Pharmaceuticals, Ahmedabad, Gujarat, India, and kept under standard laboratory condition at 25 ± 2 °C. The animals were provided with pellet diet (Lipton, India) and water ad libitum. The institutional ethics committee constituted by the Ministry of Social Justice and Empowerment, Government of India approved the experimental protocol. All experiments were carried out at K.B. Institute of Pharmaceutical Education and Research, Gandhinagar, Gujarat, India.

3.1. Anti-inflammatory activity

3.1.1. Carrageenin-induced rat hind paw oedema model

The method adopted resembles essentially that described by Winter et al. [32]. The animals were studied for toxicity of DMSO up to 10% v/v in saline, and 5% DMSO was selected as a vehicle to suspend the standard drugs and the test compounds. Albino rats weighing between 150 and 250 g of either sex were starved for 18 h prior to the experiment. The animals were weighed, marked for identification and divided into groups of six. The standard drugs, ibuprofen (20 mg/kg body weight), mefenamic acid (100 mg/kg body weight) and three graded doses (10, 20 and 40 mg/kg body weight) of the test compounds were given orally as a suspension using 5% DMSO as a vehicle. One hour later foot paw oedema was induced by injecting 0.1 ml of 1% carrageenin subcutaneously into the planter portion of the right hind paw of each rat. Initial foot paw volume was measured immediately by mercury plethysmometer. Oedema was measured 3 h after carrageenin administration. The swelling in test group animals was used to calculate the % inhibition ± SEM of oedema achieved by the compound at the test dose compared with the vehicle control group. The % protection of oedema was calculated according to the formula, % anti-inflammatory activity = 100 × (1 − Vt/Vc) where Vt and Vc are the volume of oedema in test compounds and control groups, respectively.

3.2. Analgesic activity

3.2.1. Acetic acid induced writhing response model

The compounds of the ester series **4a–4i** and their counter parts in acid series **5a–5i** were selected for investigating their analgesic activity in acetic acid induced writhing response in albino mice. Following the method of Siegmund et al. [33], 10 mg/kg of the selected compounds was administered intra-peritoneally to six groups of mice (six in each group) starved for 16 h. The first four groups received the test compounds while the fifth and sixth groups, which served as positive and negative controls, respectively, received 10 mg/kg ibuprofen and 0.5 ml/100 g body weight of 1% DMSO solution. One hour after treatment, the animals in each group received 0.1 ml of 3% acetic acid to induce the characteristic writhing response. The number of writhing occurring within 30 min was recorded and the mean was compared with that of the control and converted into % inhibition.

3.3. Antioxidant activity

3.3.1. Nitric oxide radical scavenging assay

Nitric oxide generated from sodium nitroprusside in aqueous solution at physiological pH interacts with oxygen to produce

nitrite ions, which can be measured by Griess reagent [34]. The reaction mixture (3 ml) containing sodium nitroprusside (10 mmol) in phosphate buffered saline (PBS) and test compounds (**4a–4i** and **5a–5i**) and reference compound at different concentrations (5, 10, 15, 20, 25, 30, and 35 μg/ml) were incubated at 25 °C for 150 min. Each 30 min, 0.5 ml of the incubated sample was removed. Griess reagent of 0.5 ml (1% sulphanilamide, 0.1% naphthylethylene diamine dihydrochloride in 2% H_3PO_4) was added to the 0.5 ml aliquot of the sample removed. The absorbance of the chromophore formed was measured at 546 nm. The experiment was performed (in triplicate) and % scavenging activity was calculated using the formula 100 − [100/blank absorbance × sample absorbance]. The activity was compared with ascorbic acid at concentrations 0.1, 0.2, 0.4, 0.6, 0.8, 1.0 and 1.2 μg/ml, which was used as a standard antioxidant.

4. Results and discussion

All the synthesized compounds were tested in the carrageenin-induced 3 h rat paw oedema model, to determine their anti-inflammatory potential; the results are shown in Table 1. The selection of substituents on R_1 and R_2 in tetrasubstituted thiophene was mainly guided by lipophilicity and electronic considerations as defined by $\sigma-\pi$ Craig plot. All the selected substituents are spread over the three quadrants $-\sigma$, $\pm\pi$ of the Craig plot [25]. Initially keeping both R_1 and $R_2 = OCH_3$ ($\sigma = -0.02$; $\pi = -0.27$) and CH_3 ($\sigma = -0.17$; $\pi = +0.56$) as hydrophilic and electron releasing groups **4c**, **4g**, **5c**, and **5g**, were synthesized. Now keeping $R_2 = OCH_3$ and CH_3 as hydrophilic and electron releasing groups in benzoyl moiety we introduced at R_1 of the anilino moiety the representative lipophilic and electron withdrawing substituents *p*-Cl ($\sigma = +0.24$; $\pi = +0.71$), *p*-F ($\sigma = +0.06$; $\pi = +0.14$) **4d**, **4e**, **4h**, and **4i** and corresponding acids **5d**, **5e**, **5h**, and **5i** were synthesized to gain more insight in to the structural requirements for the anti-inflammatory activity. In order to study the anti-inflammatory effect of aryl oxime directly attached to the fifth position of thiophene, oximino compounds **6a–6f** were synthesized.

In the ester series, **4c** showed maximum anti-inflammatory activity at all the three graded doses employed, 71% at 10 mg/kg, 72% at 20 mg/kg and 76% at 40 mg/kg. For **4a** the % protection was found to be 69% at 10 mg/kg, 70% at 20 mg/kg and 73% at 40 mg/kg. Similarly, for **4b** the % protection was found to be 50% at 10 mg/kg, 63% at 20 mg/kg, and 70% at 40 mg/kg. Compounds **4d–4g** showed moderate to less activity as compared to **4a–4c**. In the case of **4h** and **4i** the increase in % protection was up to a dose of 20 mg/kg but % protection decreases at 40 mg/kg dose. To summarize these findings, the presence of an electron releasing substituents OCH_3 at R_2 and OCH_3, CH_3 at R_1 was the necessary condition to enhance the anti-inflammatory potential of the candidate. However, the presence of $R_1 = R_2 = CH_3$

Table 1

Compound	R_1	R_2	Anti-inflammatory activity[a] carrageenin-induced rat hind paw oedema % protection			Analgesic activity[b] acetic acid induced writhing test % protection
			10 mg/kg	20 mg/kg	40 mg/kg	10 mg/kg
4a	H	OCH_3	69	70	73	55
4b	CH_3	OCH_3	50	63	70	45
4c	OCH_3	OCH_3	71	72	76	40
4d	F	OCH_3	36	53	58	40
4e	Cl	OCH_3	42	40	39	60
4f	H	CH_3	43	49	53	27
4g	CH_3	CH_3	32	36	37	22
4h	F	CH_3	38	46	23	37
4i	Cl	CH_3	34	66	26	43
5a	H	OCH_3	79	80	70	30
5b	CH_3	OCH_3	56	63	62	26
5c	OCH_3	OCH_3	72	75	69	22
5d	F	OCH_3	57	57	54	28
5e	Cl	OCH_3	49	49	43	30
5f	H	CH_3	45	47	42	17
5g	CH_3	CH_3	35	38	38	18
5h	F	CH_3	38	44	42	22
5i	Cl	CH_3	63	68	59	24
6a	H	OCH_3	05	38	34	–
6b	CH_3	OCH_3	22	27	30	–
6c	F	OCH_3	20	25	30	–
6d	Cl	OCH_3	03	12	30	–
6e	H	CH_3	13	21	30	–
6f	Cl	CH_3	13	29	42	–

[a] Oral administration for all test compounds, $P < 0.05$, Student's *t*-test versus controls, the standard drugs (dose and % protection) were ibuprofen (20 mg/kg, 33%) and mefenamic acid (100 mg/kg, 39%).

[b] Intra-peritoneal administration for all test compounds, $P < 0.05$, Student's *t*-test versus controls, the standard drug (dose and % protection) was ibuprofen (10 mg/kg, 60%).

remarkably decreased the anti-inflammatory potential of the candidate **4g**. Keeping the electron donating groups OCH_3 and CH_3 at R_2 in benzoyl moiety as constant, introduction of the representative lipophilic and electron withdrawing substituents F and Cl at R_1 in anilino moiety leads to decrease in anti-inflammatory activity of the candidates **4d**, **4e**, **4h**, and **4i** at all the three graded doses as compared to **4a**–**4c**. However, these compounds showed comparable better activity than **4g**. To explore the effect of the acid function at C-3 of thiophene nucleus as compared to ester we have synthesized the acid analogues **5a**–**5i**. In acid series, there is a general trend of displaying increasing anti-inflammatory activity of compounds up to 20 mg/kg but decreasing % protection at 40 mg/kg dose. Compound **5a** was found to be most potent compound having % protection 79% at 10 mg/kg, 80% at 20 mg/kg and 70% at 40 mg/kg dose. Compound **5c** showed 72% protection at 10 mg/kg, 75% at 20 mg/kg and 69% at 40 mg/kg dose. In acid series also the presence of $-CH_3$ at both R_1 and R_2 (**5g**) remarkably decrease anti-inflammatory activity. Also when $R_1 = CH_3$ and $R_2 = OCH_3$ (**5b**) the activity was less as compared to **5a** and **5c**. Similar to ester series when $R_1 = F$, Cl and $R_2 = OCH_3$, CH_3 (**5d**, **5e**, **5h**, and **5i**) the anti-inflammatory activity was found to be moderate as compared to **5a** and **5c**. In case of oxime series **6a**–**6f**, the anti-inflammatory activities of the candidates were found to be poor as compared to acid and ester series. The best candidate among whole series was **4c**, which showed anti-inflammatory activity at a very low dose as compared to both of the standard drug mefenamic acid and ibuprofen.

The analgesic activity of ester and acid compound series was investigated using acetic acid induced writhing response test in albino mice at 10 mg/kg dose and results are shown in Table 1. Among the ester series compound **4e** showed maximum protection of 60%, while **4a**, **4b**, and **4i** exhibited 55%, 45%, and 43% protection, respectively, at 10 mg/kg dose. The result showed that presence of H, Cl at R_1 and OCH_3, CH_3 at R_2 in tetrasubstituted thiophene ester series enhances their analgesic activity. The candidates of acid series **5a**–**5i** showed poor analgesic activity as compared to the standard drug ibuprofen.

Taking into account, in vivo comparable anti-inflammatory activity results of esters **4a**–**4i** and acid compounds **5a**–**5i**, in vitro antioxidant nitric oxide radical scavenging assay of these compounds was performed at concentrations 5, 10, 15, 20, 25, 30 and 35 μg/ml and results are shown in Table 2. In the ester series, **4a** showed maximum in vitro nitric oxide radical scavenging activity having IC_{50} value 30.08 μg/ml. Compounds **4c** and **4d** showed nearly comparable activity having IC_{50} values 72.21 and 74.17 μg/ml, respectively. Compound **4f** showed very poor antioxidant activity. Compounds **4b**, **4e**, **4g**, **4h** and **4i** have showed no scavenging activity. In acid series, **5a** showed maximum in vitro nitric oxide radical scavenging activity having IC_{50} value 25.20 μg/ml. Compound **5c** was found to have comparable activity having IC_{50} value 36.57 μg/ml. Compounds **5d** and **5f** showed very poor antioxidant activity.

Compounds **5b**, **5e**, **5g**, **5h** and **5i** have showed no scavenging activity. The best candidate among whole series was **5a**; however, it was found to have poor in vitro antioxidant nitric oxide radical scavenging activity as compared to standard drug ascorbic acid which showed IC_{50} value 00.52 μg/ml at concentrations 0.1, 0.2, 0.4, 0.6, 0.8, 1.0 and 1.2 μg/ml. From the IC_{50} values of **4a**–**4i** and **5a**–**5i**, it can be concluded that the

Table 2

Compound	% Scavenging (mean ± SEM) of triplicates								
	5 μg/ml	10 μg/ml	15 μg/ml	20 μg/ml	25 μg/ml	30 μg/ml	35 μg/ml	[a]IC_{50} μg/ml	r[c]
4a	13.33 ± 0.002*	18.01 ± 0.001*	28.62 ± 0.003*	32.44 ± 0.001*	36.86 ± 0.002*	48.08 ± 0.003*	63.04 ± 0.002*	30.08	0.95
4b	–	–	–	–	–	–	–	–	–
4c	4.29 ± 0.001*	5.90 ± 0.002*	7.80 ± 0.002*	11.98 ± 0.003*	15.45 ± 0.001*	20.54 ± 0.002*	25.05 ± 0.001*	72.21	0.97
4d	5.28 ± 0.002*	14.03 ± 0.002*	15.24 ± 0.001*	18.15 ± 0.003*	20.18 ± 0.001*	23.25 ± 0.001*	25.58 ± 0.001*	74.17	0.93
4e	–	–	–	–	–	–	–	–	–
4f	2.08 ± 0.001*	3.16 ± 0.002*	4.59 ± 0.001*	6.06 ± 0.002*	8.16 ± 0.002*	9.35 ± 0.002*	10.25 ± 0.002*	171.43	0.99
4g	–	–	–	–	–	–	–	–	–
4h	–	–	–	–	–	–	–	–	–
4i	–	–	–	–	–	–	–	–	–
5a	19.60 ± 0.002*	24.71 ± 0.001*	36.47 ± 0.001*	41.17 ± 0.003*	52.58 ± 0.001*	59.45 ± 0.003*	61.22 ± 0.002*	25.20	0.97
5b	–	–	–	–	–	–	–	–	–
5c	20.15 ± 0.003*	23.47 ± 0.001*	30.63 ± 0.002*	32.47 ± 0.001*	38.47 ± 0.001*	41.23 ± 0.002*	50.98 ± 0.003*	36.57	0.97
5d	00.10 ± 0.003*	00.98 ± 0.002*	01.98 ± 0.003*	03.58 ± 0.003*	08.36 ± 0.002*	10.52 ± 0.001*	12.75 ± 0.001*	118.32	0.94
5e	–	–	–	–	–	–	–	–	–
5f	00.26 ± 0.003*	00.85 ± 0.001*	01.25 ± 0.002*	2.68 ± 0.001*	04.35 ± 0.002*	05.13 ± 0.003*	05.55 ± 0.003*	259.47	0.96
5g	–	–	–	–	–	–	–	–	–
5h	–	–	–	–	–	–	–	–	–
5i	–	–	–	–	–	–	–	–	–
Ascorbic acid[b]	02.98 ± 0.001*	11.54 ± 0.002*	29.51 ± 0.001*	39.33 ± 0.003*	41.62 ± 0.003*	58.34 ± 0.001*	70.24 ± 0.002*	0.52	0.98

*$P < 0.001$ compared to reagent blank.

– Showed no scavenging activity.

[a] IC_{50} = 50% Inhibitory concentration.

[b] Ascorbic acid tested at 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, and 1.2 μg/ml.

[c] Regration analysis.

presence of $R_1 = H$ and $R_2 = OCH_3$ enhances nitric oxide radical scavenging property. The presence of $R_1 = R_2 = OCH_3$ also enhances nitric oxide radical scavenging ability in acid derivative **5c** (IC_{50} value 36.57 μg/ml); while it enhances moderately the nitric oxide radical scavenging ability in ester derivative **4c** (IC_{50} value 72.21 μg/ml). The presence of CH_3 either at R_1 and R_2 leads to near or total loss of antioxidant activity of tested compounds.

5. Conclusion

A new series of tetrasubstituted thiophene analogues were designed, synthesized and characterized. The synthesized compounds **4a–4i**, **5a–5i**, and **6a–6f** were evaluated for their anti-inflammatory activity in carrageenin-induced rat hind paw oedema model; while **4a–4i** and **5a–5i** were investigated for their analgesic activity in acetic acid induced writhing response model at 10 mg/kg dose. As the synthesized compounds have the structural features of COX-1, 5-LOX (acid/ester, as in mefenamic acid) and the p38 MAP kinase inhibitor; the anti-inflammatory activity exhibited by the experimental candidates may be due to blocking of more than one rate limiting steps in the inflammatory cascade. In search of the optimum structural requirements for the anti-inflammatory activity around the thiophene scaffold it was possible to find, on the basis of SAR studies of target compounds, that the presence of OCH_3 at R_2 position and H, OCH_3 at R_1 in target compounds are the requirements for eliciting comparable anti-inflammatory activity in both the ester and acid series. The best candidate among whole series was **4c**, which showed anti-inflammatory activity at a very low dose as compared to both of the standard drug mefenamic acid and ibuprofen. It is, therefore, suggested that the compound to be studied further to explore its full potential particularly in the treatment of chronic inflammatory diseases. The best compound of the series was an ester derivative **4e** that showed maximum analgesic activity of 60% protection in acetic acid induced writhing response test in albino mice at a dose of 10 mg/kg dose. The result showed that presence of H, Cl at R_1 and OCH_3, CH_3 at R_2 in tetrasubstituted thiophene ester series enhances their analgesic activity. The best candidate among whole series was **5a** which was found to have maximum in vitro nitric oxide radical scavenging activity having IC_{50} value 25.20 μg/ml. The results showed the presence of $R_1 = H$, $R_2 = OCH_3$ and also $R_1 = R_2 = OCH_3$ enhances nitric oxide radical scavenging property in tetrasubstituted thiophene acid series. In vitro antioxidant nitric oxide radical scavenging assay of **4a–4i** and **5a–5i** compounds showed that the mechanism of anti-inflammatory activity of potent candidates in ester and acid series could be mediated through inhibition of nitric oxide burst in inflammatory situation.

6. Experimental

Melting points were recorded on capillary melting point apparatus and are uncorrected. The infrared spectra in KBr were recorded, on Buck Scientific M-500 Infrared spectrophotometer. ^{1}H NMR spectra were recorded on 300/200 MHz Bruker FT-NMR (Advance DPX200) spectrometer using tetramethylsilane as internal standard and the chemical shifts (δ) are reported in parts per million, coupling constants (J) are given in hertz. Masses of all the compounds were recorded on Perkin–Elmer Sciex atmospheric pressure ionization liquid chromatography mass instrument (LCMS). In this technique some of the compounds gave a molecular ion and an additional peak at (M + 23), which was due to (M + Na). Elemental analyses were carried out on Carlo-Erba 1108 instrument or Elementar's Vario EL III micro-analyzer. UV spectra were recorded in Shimadzu 1601 UV–vis spectrophotometer. All chromatographic purification was performed with silica gel 60 (100–200 or 200–400 mesh), whereas all TLC (silica gel) development was performed on silica gel coated (Merck Kiesel 60 GF-254, 0.2 mm thickness) sheets. Ibuprofen, mefenamic acid and ascorbic acid drugs were obtained from Baroda Chemist Association (Baroda, India). Griess reagent was procured from Aldrich Sigma Chemical Co. Ltd., USA. The chemicals used, namely, methyl acetoacetate, *p*-chloroaniline, aniline, carbon disulfide, triethylamine, acetonitrile, methanol, DMSO, DMF, dichloromethane, toluene, chloroform, sodium nitroprusside (Ranbaxy Fine Chemicals, Punjab, India), phenyl isothiocyanate (Lancester U.K.), substituted acetophenones, carrageenin, ethyl chloroformate and 4-fluoroaniline, (Spectrochem Mumbai, India) were used without further purification unless otherwise stated.

*6.1. General procedure for the synthesis of compounds **4a–4i***

As shown in Scheme 1, 1-(α-Carbomethoxy-β-aminothiocrotonoyl)-4-substituted aniline **3** was synthesized by nucleophilic addition of arylisothiocyanate and enamine **2**, which in turn was synthesized by reacting ammonia with methyl acetoacetate. Compounds **4a–4i** were synthesized by adding 0.001 mol of the respective substituted phenacyl bromide to a solution of **3** (0.001 mol) in 2 ml of acetonitrile without adding base at room temperature. The solution was stirred until the solid was separated from the reaction mixture or until no more starting materials could be detected on TLC. The solid that separated was filtered off, washed with chilled acetonitrile, dried, and yielded yellow coloured product corresponding to **4a–4i** characterized as per the analytical data.

*6.1.1. Methyl-2-anilino-5-(4-methoxybenzoyl)-4-methylthiophene-3-carboxylate (**4a**)*

Yield: 84%; m.p. 120–122 °C; MS: *m/z* 382 (M + 1); IR (KBr): 3250, 3050, 1666 (C=O stretching), 1600, 1445, 1364, 1199–1238, 1170, 996, 697–755 (strongest of thiophene bands, C–S stretching) cm^{-1}. ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.46 (s, 3H, CH_3 at fourth position), 3.87 (s, 3H, OCH_3 at fifth position), 3.91 (s, 3H, CH_3 of ester at third position), 6.92–6.95 (d, 2H, $J = 5.44$, aromatic protons *ortho* to OCH_3 at fifth position), 7.16–7.41 (m, 5H, aromatic protons of second position), 7.72–7.75 (d, 2H, aromatic protons *meta* to OCH_3 at fifth position), 10.59 (s, 1H, NH at second position). Anal. calcd. for $C_{21}H_{19}NO_4S$: C 66.15, H 4.98, N 3.67. Found: C 66.16, H 5.14, N 3.67%.

*6.1.2. Methyl-2-(4-methylanilino)-5-(4-methoxybenzoyl)-4-methylthiophene-3-carboxylate (**4b**)*

Yield: 65%; m.p. 247–249 °C; MS: *m/z* 395 (M^+); IR (KBr): 3452, 1615 (C=O stretching), 1577, 1428, 1339, 1191, 680–774 (strongest of thiophene bands, C–S stretching). ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.39 (s, 6H, CH_3 at fourth position and CH_3 at second position), 3.86 (s, 6H, CH_3 of ester at third position and OCH_3 at fifth position), 7.00–7.02 (d, 2H, $J = 5.44$, aromatic protons *ortho* to OCH_3 at fifth position), 7.26–7.28 (m, 4H, aromatic protons of second position), 7.72–7.75 (d, 2H, aromatic protons *meta* to OCH_3 at fifth position), 10.51 (s, 1H, NH at second position). Anal. calcd. for $C_{22}H_{21}NO_4S$: C 66.85, H 5.31, N 3.54. Found: C 66.54, H 5.65, N 3.42%.

*6.1.3. Methyl-2-(4-methoxyanilino)-5-(4-methoxybenzoyl)-4-methylthiophene-3-carboxylate (**4c**)*

Yield: 30%; m.p. 139–141 °C; MS: *m/z* 412 (M + 1); IR (KBr): 3250, 2890, 1667 (C=O stretching), 1552, 1517, 1364, 1263, 1138, 996, 697–755 (strongest of thiophene bands, C–S stretching) cm^{-1}. ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.48 (s, 3H, CH_3 at fourth position), 3.88 (s, 3H, CH_3 of ester at third position), 3.92 (s, 6H, OCH_3 at second position and fifth position), 6.91–6.94 (d, 4H, aromatic protons *ortho* to OCH_3 at second position and fifth position), 7.72–7.75 (d, 4H, aromatic protons *meta* to OCH_3 at second position and fifth position), 10.51 (s, 1H, NH at second position). Anal. calcd. for $C_{22}H_{21}NO_5S$: C 64.23, H 5.10, N 3.40. Found: C 63.59, H 4.92, N 3.71%.

*6.1.4. Methyl-2-(4-fluoroanilino)-5-(4-methoxybenzoyl)-4-methylthiophene-3-carboxylate (**4d**)*

Yield: 75%; m.p. 146–148 °C; MS: *m/z* 400 (M + 1); IR (KBr): 3100, 2363, 1660 (C=O stretching), 1607, 1431, 1363, 1238, 1174, 721 (strongest of thiophene bands, C–S stretching) cm^{-1}. ^{1}H NMR (200 MHz, $CDCl_3$): δ 2.57 (s, 3H, CH_3 at fourth position), 3.01 (s, 3H, CH_3 of ester at third position), 3.93 (s, 3H, OCH_3 at fifth position), 7.05–7.09 (d, 2H, $J = 5.28$, aromatic protons *ortho* to OCH_3 at fifth position), 7.26 (s, 4H, aromatic protons at second position), 7.49–7.53 (d, 2H, aromatic protons *meta* to OCH_3 at fifth position), 10.34 (s, 1H, NH at second position). Anal. calcd. for $C_{21}H_{18}FNO_4S$: C 63.17, H 4.50, N 3.50. Found: C 63.67, H 4.92, N 3.71%.

*6.1.5. Methyl-2-(4-chloroanilino)-5-(4-methoxybenzoyl)-4-methylthiophene-3-carboxylate (**4e**)*

Yield: 90%; m.p. 132–134 °C; MS: *m/z* 416 (M + 1); IR (KBr): 3120, 2365, 1670 (C=O stretching), 1593, 1441, 1368, 1245, 1169, 926, 692–780 (strongest of thiophene bands, C–S stretching) cm^{-1}. ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.46 (s, 3H, CH_3 at fourth position), 3.88 (s, 3H, OCH_3 at fifth position), 3.91 (s, 3H, CH_3 of ester at third position), 6.92–6.95 (d, 2H, $J = 5.65$, aromatic protons *ortho* to OCH_3 at fifth position), 7.26–7.38 (m, 4H, aromatic protons at second position), 7.72–7.75 (d, 2H, aromatic protons *meta* to OCH_3 at fifth position), 10.59 (s, 1H, NH at second position). Anal. calcd. for $C_{21}H_{18}ClNO_4S$: C 60.67, H 4.33, N 3.36. Found: C 60.91, H 4.16, N 3.47%.

*6.1.6. Methyl-2-anilino-5-(4-methylbenzoyl)-4-methylthiophene-3-carboxylate (**4f**)*

Yield: 35%; m.p. 138–140 °C; MS: *m/z* 388 (M + 23); IR (KBr): 3550, 2450, 1678 (C=O stretching), 1615, 1368, 1180, 987, 685–745 (strongest of thiophene bands, C–S stretching) cm^{-1}. ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.42 (s, 3H, CH_3 at fourth position), 2.46 (s, 3H, CH_3 at fifth position), 3.91 (s, 3H, CH_3 of ester at third position), 7.23–7.26 (d, 2H, $J = 5.61$, aromatic protons *ortho* to CH_3 at fifth position), 7.33–7.41 (m, 5H, aromatic protons of second position), 7.61–7.64 (d, 2H, aromatic protons *meta* to CH_3 at fifth position), 10.61 (s, 1H, NH at second position). Anal. calcd. for $C_{21}H_{19}NO_3S$: C 69.02, H 5.20, N 3.83. Found: C 68.70, H 5.35, N 4.01%.

*6.1.7. Methyl-2-(4-methylanilino)-5-(4-methylbenzoyl)-4-methylthiophene-3-carboxylate (**4g**)*

Yield: 43%; m.p. 204–206 °C; MS: *m/z* 379 (M^+); IR (KBr): 3450, 2370, 1627 (C=O stretching), 1602, 1363, 1142, 940, 685–706 (strongest of thiophene bands, C–S stretching) cm^{-1}. ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.28 (s, 3H, CH_3 at fourth position), 2.30 (s, 6H, CH_3 at second position and fifth position), 3.56 (s, 3H, CH_3 of ester at third position), 6.88–6.91 (d, 4H, $J = 6.03$, aromatic protons *ortho* to CH_3 at second position and fifth position), 7.09–7.12 (d, 4H, aromatic protons *meta* to CH_3 at second position and fifth position), 10.27 (s, 1H, NH at second position). Anal. calcd. for $C_{22}H_{21}NO_3S$: C 69.64, H 5.53, N 3.69. Found: C 69.78, H 5.15, N 3.81%.

*6.1.8. Methyl-2-(4-fluoroanilino)-5-(4-methylbenzoyl)-4-methylthiophene-3-carboxylate (**4h**)*

Yield: 35%; m.p. 129–131 °C; MS: *m/z* 383 (M^+); IR (KBr): 3359, 2365, 1670 (C=O stretching), 1612, 1491, 1363, 1180, 905, 680–744 (strongest of thiophene bands, C–S stretching) cm^{-1}. ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.35 (s, 3H, CH_3 at fourth position), 2.48 (s, 3H, CH_3 of ester at third position), 3.92 (s, 3H, CH_3 at fifth position), 7.18–7.21 (d, 2H, $J = 6.23$, aromatic protons *ortho* to CH_3 at fifth position), 7.25 (s, 4H, aromatic protons of second position), 7.62–7.65 (d, 2H, aromatic protons *meta* to CH_3 at fifth position), 10.48 (s, 1H, NH at second position). Anal. calcd. for $C_{21}H_{18}FNO_3S$: C 65.80, H 4.59, N 3.65. Found: C 66.01, H 4.92, N 4.01%.

*6.1.9. Methyl-2-(4-chloroanilino)-5-(4-methylbenzoyl)-4-methylthiophene-3-carboxylate (**4i**)*

Yield: 25%; m.p. 128–130 °C; MS: *m/z* 400 (M + 1); IR (KBr): 3540, 2455, 1670 (C=O stretching), 1592, 1441, 1368, 1242, 926, 683–780 (strongest of thiophene bands, C–S stretching) cm^{-1}. ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.13 (s, 3H, CH_3 at fourth position), 2.30 (s, 3H, CH_3 at fifth position), 3.88 (s, 3H, CH_3 of ester at third position), 7.15–7.17 (d, 2H, aromatic protons *ortho* to CH_3 at fifth position),

7.22–7.30 (m, 4H, aromatic protons of second position), 7.41–7.44 (d, 2H, $J = 5.26$, aromatic protons *meta* to CH_3 at fifth position), 10.41 (s, 1H, NH at second position). Anal. calcd. for $C_{21}H_{18}ClNO_3S$: C 63.07, H 4.50, N 3.50. Found: C 63.09, H 4.35, N 3.73%.

6.2. General procedure for the synthesis of compounds **5a–5i**

The corresponding acids (**5a–5i**) of the tetrasubstituted thiophene esters were synthesized by treating **4a–4i** in methanol with 1 eq of potassium hydroxide solution and stirring at room temperature until TLC showed the complete disappearance of the respective ester. The solvent was then removed in vacuo, treated with a small amount of distilled water and extracted with ether. The ether layer was discarded while the aqueous layer was cooled and treated with 1 eq of dilute hydrochloric acid to liberate the acids (**5a–5i**).

6.2.1. 2-Anilino-5-(4-methoxybenzoyl)-4-methylthiophene-3-carboxylic acid (**5a**)

Yield: 72%; m.p. 206–208 °C; MS: *m/z* 367 (M^+); IR (KBr): 3212, 2911, 1635 (C=O stretching), 1597, 1450, 1356, 1211, 1172, 1111, 868, 698–756 (strongest of thiophene bands, C–S stretching) cm^{-1}. ^1H NMR (200 MHz, DMSO-d_6): δ 2.40 (s, 3H, CH_3 at fourth position), 3.82 (s, 3H, OCH_3 at fifth position), 6.99–7.03 (d, 2H, $J = 4.89$, aromatic protons *ortho* to OCH_3 at fifth position), 7.19–7.42 (m, 5H, aromatic protons of second position), 7.62–7.66 (d, 2H, aromatic protons *meta* to OCH_3 at fifth position), 10.60 (s, 1H, NH at second position). Anal. calcd. for $C_{20}H_{17}NO_4S$: C 65.40, H 4.62, N 3.81. Found: C 65.01, H 4.92, N 4.01%.

6.2.2. 2-(4-Methylanilino)-5-(4-methoxybenzoyl)-4-methylthiophene-3-carboxylic acid (**5b**)

Yield: 49%; m.p. > 250 °C; MS: *m/z* 382 ($M + 1$); IR (KBr): 3037, 2360, 1692 (C=O stretching), 1555, 1454, 1240, 1169, 975, 674–714 (strongest of thiophene bands, C–S stretching) cm^{-1}. ^1H NMR (200 MHz, DMSO-d_6): δ 2.47 (s, 3H, CH_3 at fourth position), 2.48 (s, 3H, CH_3 at second position), 3.32 (s, 3H, OCH_3 at fifth position), 6.99–7.03 (d, 2H, $J = 4.97$, aromatic protons *ortho* to OCH_3 at fifth position), 7.33–7.48 (m, 4H, aromatic protons of second position), 7.62–7.66 (d, 2H, aromatic protons *meta* to OCH_3 at fifth position), 10.47 (s, 1H, NH at second position). Anal. calcd. for $C_{21}H_{19}NO_4S$: C 66.15, H 4.98, N 3.67. Found: C 66.16, H 4.80, N 3.97%.

6.2.3. 2-(4-Methoxyanilino)-5-(4-methoxybenzoyl)-4-methylthiophene-3-carboxylicacid (**5c**)

Yield: 40%; m.p. 210–212 °C; MS: *m/z* 397 (M^+); IR (KBr): 3180, 1661 (C=O stretching), 1589, 1495, 1351, 1237, 1122, 1002, 680–785 (strongest of thiophene bands, C–S stretching) cm^{-1}. ^1H NMR (200 MHz, DMSO-d_6): δ 2.48 (s, 3H, CH_3 at fourth position), 3.92 (s, 6H, OCH_3 at second position and fifth position), 7.17–7.21 (d, 4H, aromatic protons *meta* to OCH_3 at second position and fifth position), 7.85–7.89 (d, 4H, $J = 5.72$, aromatic protons *ortho* to OCH_3 at second position and fifth position), 10.89 (s, 1H, NH at second position). Anal. calcd. for $C_{21}H_{19}NO_5S$: C 63.48, H 4.78, N 3.52. Found: C 63.89, H 4.65, N 3.32%.

6.2.4. 2-(4-Fluoroanilino)-5-(4-methoxybenzoyl)-4-methylthiophene-3-carboxylic acid (**5d**)

Yield: 56%; m.p. 202–204 °C; MS: *m/z* 385 (M^+); IR (KBr): 2949, 2405, 1699 (C=O stretching), 1594, 1441, 1391, 1242, 1142, 672–723 (strongest of thiophene bands, C–S stretching) cm^{-1}. ^1H NMR (200 MHz, DMSO-d_6): δ 2.57 (s, 3H, CH_3 at fourth position), 3.93 (s, 3H, OCH_3 at fifth position), 7.05–7.09 (d, 2H, aromatic protons *ortho* to OCH_3 at fifth position), 7.26 (s, 4H, aromatic protons at second position), 7.49–7.53 (d, 2H, $J = 5.29$, aromatic protons *meta* to OCH_3 at fifth position), 10.28 (s, 1H, NH at second position). Anal. calcd. for $C_{20}H_{16}FNO_4S$: C 62.33, H 4.15, N 3.63. Found: C 61.78, H 4.54, N 3.88%.

6.2.5. 2-(4-Chloroanilino)-5-(4-methoxybenzoyl)-4-methylthiophene-3-carboxylic acid (**5e**)

Yield: 78%; m.p. 165–167 °C; MS: *m/z* 403 ($M + 2$); IR (KBr): 3747, 2364, 1670 (C=O stretching), 1590, 1431, 1256, 1174, 931, 691–775 (strongest of thiophene bands, C–S stretching) cm^{-1}. ^1H NMR (200 MHz, DMSO-d_6): δ 2.40 (s, 3H, CH_3 at fourth position), 3.82 (s, 3H, OCH_3 at fifth position), 6.99–7.03 (d, 2H, $J = 5.10$, aromatic protons *ortho* to OCH_3 at fifth position), 7.28–7.48 (m, 4H, aromatic protons of second position), 7.62–7.66 (d, 2H, aromatic protons *meta* to OCH_3 at fifth position), 10.75 (s, 1H, NH at second position). Anal. calcd. for $C_{20}H_{16}ClNO_4S$: C 59.80, H 3.98, N 3.48. Found: C 59.85, H 4.01, N 3.63%.

6.2.6. 2-Anilino-5-(4-methylbenzoyl)-4-methylthiophene-3-carboxylic acid (**5f**)

Yield: 55%; m.p. 194–196 °C; MS: *m/z* 351 (M^+); IR (KBr): 3124, 2501, 1663 (C=O stretching), 1601, 1358, 1188, 974, 673–760 (strongest of thiophene bands, C–S stretching) cm^{-1}. ^1H NMR (200 MHz, DMSO-d_6): δ 2.39 (s, 3H, CH_3 at fourth position), 3.82 (s, 3H, CH_3 at fifth position), 6.70–6.74 (d, 2H, aromatic protons *ortho* to CH_3 at fifth position), 7.37–7.43 (m, 5H, aromatic protons of second position), 7.70–7.74 (d, 2H, $J = 4.74$, aromatic protons *meta* to CH_3 at fifth position), 10.78 (s, 1H, NH at second position). Anal. calcd. for $C_{20}H_{17}NO_3S$: C 68.38, H 4.83, N 3.98. Found: C 68.54, H 4.78, N 3.61%.

6.2.7. 2-(4-Methylanilino)-5-(4-methylbenzoyl)-4-methylthiophene-3-carboxylic acid (**5g**)

Yield: 38%; m.p. 296–298 °C; MS: *m/z* 365 (M^+); IR (KBr): 3350, 2338, 1661 (C=O stretching), 1607, 1542, 1378, 1143, 958, 680–767 (strongest of thiophene bands, C–S stretching) cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$): δ 2.28 (s, 3H, CH_3 at fourth position), 2.30 (s, 6H, CH_3 at second position and fifth position), 6.88–6.91 (d, 4H, $J = 6.03$, aromatic protons *ortho* to CH_3 at second position and fifth position), 7.09–7.12 (d, 4H, aromatic protons *meta* to CH_3 at second

position and fifth position), 10.40 (s, 1H, NH at second position). Anal. calcd. for $C_{21}H_{19}NO_3S$: C 69.05, H 5.20, N 3.83. Found: C 69.25, H 4.80, N 3.84%.

*6.2.8. 2-(4-Fluoroanilino)-5-(4-methylbenzoyl)-4-methylthiophene-3-carboxylic acid (**5h**)*

Yield: 30%; m.p. 185–187 °C; MS: *m/z* 369 (M^+); IR (KBr): 3360, 2400, 1672 (C=O stretching), 1603, 1490, 1360, 1180, 910, 675–740 (strongest of thiophene bands, C–S stretching) cm^{-1}. ^1H NMR (200 MHz, $CDCl_3$): δ 2.28 (s, 3H, CH_3 at fourth position), 3.45 (s, 3H, CH_3 at fifth position), 7.05–7.26 (m, 8H, aromatic protons of second position and fifth position), 10.09 (s, 1H, NH at second position). Anal. calcd. for $C_{20}H_{16}FNO_3S$: C 65.04, H 4.33, N 3.79. Found: C 65.67, H 3.97, N 3.73%.

*6.2.9. 2-(4-Chloroanilino)-5-(4-methylbenzoyl)-4-methylthiophene-3-carboxylic acid (**5i**)*

Yield: 35%; m.p. 179–181 °C; MS: *m/z* 386 (M + 1); IR (KBr): 3280, 2363, 1700 (C=O stretching), 1588, 1438, 1352, 1246, 945, 680–740 (strongest of thiophene bands, C–S stretching) cm^{-1}. ^1H NMR (200 MHz, DMSO-d_6): δ 2.42 (s, 3H, CH_3 at fourth position), 2.66 (s, 3H, CH_3 at fifth position), 7.03–7.08 (d, 2H, aromatic protons *ortho* to CH_3 at fifth position), 7.28–7.48 (m, 4H, aromatic protons of second position), 7.63–7.68 (d, 2H, $J = 5.73$, aromatic protons *meta* to CH_3 at fifth position), 10.65 (s, 1H, NH at second position). Anal. calcd. for $C_{20}H_{16}ClNO_3S$: C 62.25, H 4.14, N 3.62. Found: C 62.65, H 4.01, N 3.63%.

*6.3. General procedure for the synthesis of compounds **6a–6f***

Compounds **6a–6f** were synthesized by adding 0.001 mol of the respective substituted oxime of 4-substituted phenacyl bromides to a solution 0.001 mol of **3** in 2 ml of acetonitrile without adding base at room temperature. The solution was stirred until the solid was separated from the reaction mixture or until no more starting materials could be detected on TLC. The solid that separated was filtered off, washed with chilled acetonitrile, and dried, yielded product corresponding to **6a–6f** characterized as per the analytical data.

*6.3.1. E-4-Methoxyphenyl-(2-anilino-3-methoxycarbonyl-4-methyl-5-thienyl)ketoxime (**6a**)*

Yield: 45%; m.p. 140–142 °C; MS: *m/z* 396 (M^+); IR (KBr): 3241, 3192, 1666 (C=O stretching), 1666, 1552, 1446, 1365, 1238, 1199, 1171, 1024, 697–756 (strongest of thiophene bands, C–S stretching) cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$): δ 2.47 (s, 3H, CH_3 at fourth position), 2.68 (s, 3H, OCH_3 at fifth position), 4.00 (s, 3H, CH_3 of ester at third position), 7.19–7.22 (d, 2H, aromatic protons *ortho* to OCH_3 at fifth position), 7.24–7.38 (m, 5H, aromatic protons of second position), 7.97–8.00 (d, 2H, $J = 4.56$, aromatic protons *meta* to OCH_3 at fifth position), 10.40 (s, 1H, NH at second position). Anal. calcd. for $C_{21}H_{20}N_2O_4S$: C 63.63, H 5.04, N 7.06. Found: C 63.16, H 5.14, N 7.01%.

*6.3.2. E-4-Methoxyphenyl-[2-(4-methylanilino)-3-methoxycarbonyl-4-methyl-5-thienyl]ketoxime (**6b**)*

Yield: 40%; m.p. 168–170 °C; MS: *m/z* 410 (M^+); IR (KBr): 3022, 2959, 1660 (C=O stretching), 1596, 1504, 1433, 1360, 1168, 688–780 (strongest of thiophene bands, C–S stretching) cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$): δ 2.34 (s, 3H, CH_3 at fourth position), 2.45 (s, 3H, CH_3 at second position), 3.87(s, 3H, OCH_3 at fifth position), 3.90 (s, 3H, CH_3 of ester at third position), 6.91–6.94 (d, 2H, $J = 6.08$, aromatic protons *ortho* to OCH_3 at fifth position), 7.17–7.26 (m, 4H, aromatic protons of second position), 7.71–7.74 (d, 2H, aromatic protons *meta* to OCH_3 at fifth position), 10.44 (s, 1H, NH at second position). Anal. calcd. for $C_{22}H_{22}N_2O_4S$: C 64.39, H 5.36, N 6.82. Found: C 64.65, H 5.78, N 6.73%.

*6.3.3. E-4-Methoxyphenyl-[2-(4-fluoroanilino)-3-methoxycarbonyl-4-methyl-5-thienyl]ketoxime (**6c**)*

Yield: 45%; m.p. 130–132 °C; MS: *m/z* 414 (M^+); IR (KBr): 3159, 1660 (C=O stretching), 1608, 1548, 1430, 1280, 1184, 825, 668–721 (strongest of thiophene bands, C–S stretching) cm^{-1}. ^1H NMR (200 MHz, $CDCl_3$): δ 2.36 (s, 3H, CH_3 at fourth position), 2.42 (s, 3H, OCH_3 at fifth position), 3.89 (s, 3H, CH_3 of ester at third position), 6.90–6.93 (d, 2H, $J = 5.86$, aromatic protons *ortho* to OCH_3 at fifth position), 7.17–7.26 (m, 4H, aromatic protons of second position), 7.72–7.75 (d, 2H, aromatic protons *meta* to OCH_3 at fifth position), 10.44 (s, 1H, NH at second position). Anal. calcd. for $C_{21}H_{19}FN_2O_4S$: C 60.88, H 4.58, N 6.75. Found: C 60.89, H 4.54, N 6.85%.

*6.3.4. E-4-Methoxyphenyl-[2-(4-chloroanilino)-3-methoxycarbonyl-4-methyl-5-thienyl]ketoxime (**6d**)*

Yield: 49%; m.p. 136–138 °C; MS: *m/z* 431 (M + 1); IR (KBr): 3242, 1669 (C=O stretching), 1602, 1593, 1552, 1398, 1368, 1288, 1169, 926, 690–786 (strongest of thiophene bands, C–S stretching) cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$): δ 1.60 (s, 3H, CH_3 at fourth position), 2.40 (s, 3H, OCH_3 at fifth position), 3.85 (s, 3H, CH_3 of ester at third position), 6.92–6.95 (d, 2H, aromatic protons *ortho* to OCH_3 at fifth position), 7.26–7.40 (m, 4H, aromatic protons of second position), 7.76–7.79 (d, 2H, aromatic protons *meta* to OCH_3 at fifth position), 10.46 (s, 1H, NH at second position). Anal. calcd. for $C_{21}H_{19}ClN_2O_4S$: C 58.53, H 4.40, N 6.49. Found: C 58.53, H 4.07, N 6.17%.

*6.3.5. E-4-Methylphenyl-(2-anilino-3-methoxycarbonyl-4-methyl-5-thienyl)ketoxime (**6e**)*

Yield: 80%; m.p. 125–127 °C; MS: *m/z* 380 (M^+); IR (KBr): 3021, 2959, 2450, 1661 (C=O stretching), 1615, 1595, 1370, 1188, 985, 684–740 (strongest of thiophene bands, C–S stretching) cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$): δ 2.36 (s, 3H, CH_3 at fourth position), 2.42 (s, 3H, CH_3 at fifth position), 3.88 (s, 3H, CH_3 of ester at third position), 7.11–7.14 (d, 2H, $J = 5.66$, aromatic protons *ortho* to CH_3 at fifth position), 7.23–7.39 (m, 5H, aromatic protons of second position), 7.66–7.69 (d, 2H, aromatic protons *meta* to CH_3 at fifth position), 10.41 (s, 1H, NH at second position). Anal. calcd.

for $C_{21}H_{20}N_3O_3S$: C 66.30, H 5.25, N 7.36. Found: C 66.70, H 5.35, N 7.42%.

*6.3.6. Z-4-Methylphenyl-[2-(4-chloroanilino)-3-methoxycarbonyl-4-methyl-5-thienyl]ketoxime (**6f**)*

Yield: 75%; m.p. 143–145 °C; MS: *m/z* 415 (M + 1); IR (KBr): 3183, 2364, 1662 (C=O stretching), 1591, 1443, 1374, 1246, 936, 673–780 (strongest of thiophene bands, C–S stretching) cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$): δ 2.36 (s, 3H, CH_3 at fourth position), 2.40 (s, 3H, CH_3 at fifth position), 3.83 (s, 3H, CH_3 of ester at third position), 7.22–7.25 (d, 2H, aromatic protons *ortho* to CH_3 at fifth position), 7.26–7.30 (m, 4H, aromatic protons of second position), 7.41–7.44 (d, 2H, aromatic protons *meta* to CH_3 at fifth position), 10.41 (s, 1H, NH at second position). Anal. calcd. for $C_{21}H_{19}ClN_3O_3S$: C 60.80, H 4.58, N 6.75. Found: C 60.98, H 4.78, N 3.48%.

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