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Cyanoacetylation Of Substituted 2-Aminothiophenes And Evaluation for Antioxidant And Antibacterial Activities.

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ABSTRACT

Cyanoacetylation of multi-substituted 2-aminothiophenes was performed by using an effective cyanoacetylating agent, 1-cyanoacetyl-3,5-dimethylpyrazole. This method is more convenient and economical, as cyanoacetylation with 1-cyanoacetyl-3,5-dimethylpyrazole occurs at much faster rate and gives high yield of the product. The compounds prepared by this synthetic method were characterized with their physical and spectral data. All the synthesized compounds were evaluated for *in vitro* antioxidant activity by scavenging 1,1-diphenyl-2-picrylhydrazyl (DPPH) and nitric oxide free radicals at 100 µM concentration. They were also screened for antibacterial activity against gram negative bacteria *E. coli* and gram positive bacteria *S. aureus* using Agar well diffusion method. Among the evaluated compounds, 2-(2-cyanoacetamido)-4,5-dimethylthiophene-3-carboxamide was found to possess highest anti-oxidant activity in both models of free radical scavenging. The antibacterial activity data revealed that the evaluated compounds exhibited moderate to good antibacterial activity.

Keywords: Multi-substituted 2-Aminothiophenes, Cyanoacetylating agent, 1-Cyanoacetyl-3,5-dimethylpyrazole, Antioxidant activity, Antibacterial activity.

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INTRODUCTION

Cyanoacetylation of amines can be carried out by several reagents such as cyanoacetic ester, cyanoacetyl chloride, cyanoacetyl azide, mixed anhydride of cyanoacetic and acetic acids, 1-cyanoacetyl-3,5-dimethylpyrazole and N-(cyanoacetyl)imidazole [1]. Among these reagents 1-cyanoacetyl-3,5-dimethylpyrazole is economically cheap and nontoxic reagent that proved to be superior to other cyanoacetylating agents. The use of 1-cyanoacetyl-3,5-dimethylpyrazole is advantageous as N-cyanoacetylation products (N-substituted cyanoacetamides) can be isolated in higher yields from reaction mixture in crystalline form, while the 3,5-dimethylpyrazole by-product remains in mother liquor [2]. In the literature, various N-substituted cyanoacetamide derivatives and related heterocyclic moieties were reported to possess interesting biological and pharmaceutical activities [3]. Different drugs containing N-cyanoacetyl group such as Tofacitinib and Cephacetrile, a derivative of 7-aminocephalosporanic acid, are in use for a variety of medical treatments [4-6].

Multi-substituted 2-aminothiophenes are privileged structures, which attracted considerable attention in designing of biologically active molecules. Various substituted 2-aminothiophenes are reported as protein-tyrosine phosphatase 1B (PTP1B) inhibitors [7], antagonists of human glucagon receptor [8] and as antitubulin agents [9]. Some of the molecules containing 2-aminothiophenes are available in the market. They are Olanzapine, an antipsychotic drug and Tinoridine, a potent nonsteroidal anti-inflammatory drug with antiperoxidative properties. Few other compounds containing 2-aminothiophene moiety were under clinical and preclinical development, they includes T-62, PD81723, ATL525, AX20017 and TPCA-1 [10].

In view of diverse pharmacological activities of N-substituted cyanoacetamide derivatives and multi-substituted 2-aminothiophenes, it has been considered worthwhile to prepare N-cyanoacetylation products from multi-substituted 2-aminothiophenes and to evaluate the synthesized compounds for *in vitro* antioxidant and antibacterial activities.

EXPERIMENTAL

All the chemicals obtained from Merck, Sd-fine, Sigma-Aldrich and they are of AR grade. Analytical TLC was performed using glass plates coated with silica gel G and the spots were detected by iodine vapour. The melting points reported were determined in open capillaries, using digital Stuart melting point apparatus and are uncorrected. IR spectra (KBr, υ_{max},cm^{-1}) were run on Bruker FTIR Spectrophotometer. ^{1}H NMR spectra were recorded on Bruker Avance-400 MHz Spectrometer and the chemical shifts are expressed as δ values (ppm) downfield from tetramethylsilane (TMS as internal standard) using either $CDCl_3$ or DMSO as solvent. Mass spectra were recorded on Shimadzu QP 2010PLUS GC-MS system.

Chemistry

General procedure for cyanoacetylation of substituted 2-aminothiophenes (Scheme-I, 3a-3f):

1-Cyanoacetyl-3,5-dimethylpyrazole (1.63 gm, 10 mM) was added to a solution of 1.0 equivalent amount of substituted 2-aminothiophene in toluene (20 ml). The mixture was refluxed for about 1 hr at 100 to 110ºC. The completion of reaction was monitored by TLC. Then the reaction mixture was cooled to room temperature. The crude product formed was filtered and recrystallized from ethanol.

3a: 2-Cyano-N-(3-cyano-4,5-dimethylthiophen-2-yl)acetamide: mp. 258-259 °C; yield 92%; IR (KBr) υ_{max}: 3237 (N-H), 2216 (C≡N),1696 (C=O amide) cm^{-1} ; ^{1}H NMR (DMSO) δ: 2.1-2.5 (2s, 6H, CH_3), 4.1 (s, 2H, CH_2CN), 11.8 (s, br, 1H, NH) ppm; Mass m/z: 218 (M-1).

3b: 2-Cyano-N-(3-cyano-4,5,6,7-tetrahydrobenzo[b]thiophen-2-yl)acetamide: mp. 234-235 °C; yield 91%; IR (KBr) υ_{max}: 3216 (N-H), 2220 (C≡N), 1696 (C=O amide) cm^{-1} ; ^{1}H NMR (DMSO) δ: 1.7-2.6 (m, 8H, -CH_2-), 4.1 (s, 2H, CH_2CN), 11.9 (s, br, 1H, NH) ppm; Mass m/z: 244 (M-1).

3c: 2-(2-Cyanoacetamido)-4,5-dimethylthiophene-3-carboxamide: mp. 235-236 °C; yield 92%; IR (KBr) υ_{max}: 3514, 3303 (NH_2), 3246 (N-H), 2253 (C≡N), 1682 (C=O amide) cm^{-1}; ^{1}H NMR ($CDCl_3$) δ: 2.2-2.3 (2s, 6H, CH_3), 3.5 (s, 2H, CH_2CN), 6.0 (s, br, 2H, -$CONH_2$), 12.8 (s, br, 1H, NH) ppm; Mass m/z: 236 (M-1).

3d: 2-(2-Cyanoacetamido)-4,5,6,7-tetrahydrobenzo[b]thiophene-3-carboxamide: mp. 182 – 184 °C; yield 89%; IR (KBr) υ_{max}: 3414, 3333 (NH_2), 3225 (N-H), 2261 (C≡N), 1686 (C=O amide) cm^{-1}; ^1H NMR ($CDCl_3$) δ: 1.6-2.8 (m, 8H, -CH_2-), 3.6 (s, 2H, -CH_2CN), 6.0 (s, br, 2H, -$CONH_2$), 13.0 (s, br, H, NH) ppm; Mass m/z: 263 (M^+).

3e: Ethyl 2-(2-cyanoacetamido)-4,5-dimethylthiophene-3-carboxylate [11]**:** mp. 128-129 °C; yield 80%; IR (KBr) υ_{max}: 3181 (N-H), 2256 (C≡N), 1662 (C=O amide) cm^{-1} ; ^1H NMR ($CDCl_3$) δ: 1.4 (t, 3H,-CH_2CH_3), 2.2-2.3 (2s, 6H,-CH_3), 3.6 (s, 2H, -CH_2CN), 4.3-4.4 (q, 2H,-CH_2CH_3), 11.9 (s, br, 1H, NH) ppm; Mass m/z: 266 (M^+).

3f: Ethyl 2-(2-cyanoacetamido)-4,5,6,7-tetrahydrobenzo[b]thiophene-3-carboxylate [12]**:** mp. 111-113 °C; yield 81%; IR (KBr) υ_{max}: 3256cm^{-1} (N-H), 2259cm^{-1}(C≡N), 1649 (C=O amide) cm^{-1}; ^1H NMR ($CDCl_3$) δ: 1.4 (t, 3H,-CH_2CH_3), 1.8-2.8 (m, 8H,-CH_2-), 3.7 (s, 2H, -CH_2CN), 4.3-4.4 (q, 2H,-CH_2CH_3), 11.9 (s, br, 1H, NH) ppm; Mass m/z: 292 (M^+).

Biological Activities

***In vitro* Antioxidant Activity:**

The synthesized compounds were evaluated for *in vitro* antioxidant activity by scavenging 1,1-diphenyl-2-picrylhydrazyl (DPPH) and nitric oxide free radicals at 100 µM concentration [13, 14].

Assay of DPPH free radical scavenging:

The solutions containing 100 µM test compounds in 95% alcohol were added to 100 µM DPPH in 95% ethanol. The samples were kept at ambient temperature for 20 minutes and the decrease in absorbance was measured at 517 nm. Control experiment was carried out with solvent only and ascorbic acid was used as reference standard. All the measurements were performed in triplicate. The percentage of scavenging activity was calculated as follows:

Percentage of Scavenging = [Control-Test]/Control × 100

Assay of nitric oxide free radical scavenging:

Sodium nitroprusside (10 µM) in phosphate buffer pH 7.4 was incubated with 100 µM concentration of test compounds dissolved in alcohol and tubes were incubated at 25 °C for 150 minutes. Then 2 ml of incubation solution was removed and added 2 ml of Griess reagent. The absorbance of chromophore formed during diazotization of nitrite with sulphanilamide and subsequent coupling with N-naphthylethylenediamine was read at 546 nm. Control experiment was conducted in an identical manner without test compound but with equal amount of solvent. The percentage of scavenging was calculated using the above formula.

Antibacterial Activity:

The antibacterial activity of the synthesized compounds was conducted against one gram negative bacteria (*E. coli*) and one gram positive bacteria (*S. aureus*) by Agar well diffusion method [12]. Streptomycin was employed as reference standard to compare the results. Solutions of the test compounds were prepared by dissolving 10 mg each in 10 ml of dimethyl formamide (AR grade). The reference compound, streptomycin was also prepared similarly.

The bacterial subcultures of *E. coli* and *S. aureus* were inoculated aseptically into nutrient agar media taken in two different conical flasks (6 ml of inoculum to 300 ml of nutrient agar media). The nutrient agar media was poured 40 ml each into sterile petriplates and was allowed to solidify at room temperature. Three cups of 6 mm diameter were made in each petriplate using a sterile borer. Then 0.2 ml of the test solutions containing 200 µg was added to the cups aseptically. Simultaneously, 0.2 ml of standard solution containing 200 µg Streptomycin was added in another petriplate and the petriplates were labeled accordingly. All the plates were incubated at 37 ± 1°C for 24 hrs. After incubation, diameters of the circular inhibition zones (mm) were measured.

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RESULTS AND DISCUSSION

Cyanoacetylation of aromatic amines with 1-cyanoacetyl-3,5-dimethylpyrazole is a versatile preparative method for the synthesis of difficultly available N-substituted cyanoacetamides. This method is appeared to be more convenient and economical, as cyanoacetylation with 1-cyanoacetyl-3,5-dimethylpyrazole occurs at much faster rate and gives high yield of the product when compared with other cyanoacetylating agents such as ethyl cynanoacetate, cyanoacetic acid, anhydride or acid chloride of cyanoacetic acid.

Normally, cyanoacetylation of aromatic amines were prepared by direct condensation of ethyl/methyl ester of cyanoacetic acid and aromatic amine with several hours of reaction time. However, the direct condensation of ethyl/methyl ester of cyanoacetic acid with some heterocyclic amines such as substituted 2-aminothiophenes failed to give respective amide even after stirring for longer period of time (24-36 hrs). Hence, cyanoacetylation of substituted 2-aminothiophenes was performed by refluxing substituted 2-aminothiophenes with 1-cyanoacetyl-3,5-dimethylpyrazole, a versatile cyanoacetylating agent, in sufficient quantities of toluene.

In the present study, the cyanoacetylating agent, 1-cyanoacetyl-3,5-dimethylpyrazole was prepared by condensing cyanoacetic acid hydrazide with acetyl acetone in presence of catalytic amount of concentrated hydrochloric acid [15]. Using this cyanoacetylating agent, six cyanoacetylated derivatives (**3a-3f**) were prepared by addition of equimolar quantities of six different substituted 2-aminothiophenes in toluene (Scheme-I). These compounds were obtained in good yields ranging from 80-92%. Among the six compounds, the synthesis of two compounds (**3e-3f**) was reported previously in our laboratory and four compounds (**3a-3d**) are newly synthesized by this method. The physical data of all synthesized compounds have been given in Table-1. The IR spectra of synthesized compounds showed an absorption band in the region of 3514-3181 cm^{-1} indicative of N-H stretching. The absorption band in the region of 1696-1649 cm^{-1} indicates the presence of carbonyl group of amide in the structure. The spectra also revealed the presence of absorption band in the region of 2361-2216 cm^{-1} indicative of C≡N group.

The ^1H NMR spectra of all compounds showed singlet at δ 3.6 - 4.11 due to two CH_2 protons of cyanoacetamido group. The spectra also exhibited a broad singlet peak in the region δ 13.0 – 11.89 due to NH proton of cyanoacetamido group. The ^1H NMR spectra of compound **3b, 3d** and **3f** (4,5,6,7-tetrahydrobenzo[b]thiophene derivatives) showed multiplets in the region of δ 1.76-2.61, δ 1.6-2.8 and δ 1.8-2.8 respectively, due to eight protons of tetrahydrobenzene ring. The ^1H NMR spectra of the compounds **3a, 3c** and **3e** (4,5-dimethylthiophene derivatives) exhibited two singlets in the region of δ 2.1-2.5, δ 2.2-2.3 and δ 2.2-2.3 respectively, due to six protons of two methyl groups. The ^1H NMR spectra of compounds **3e** and **3f** showed a triplet at δ 1.4 due to three protons and a quartet at δ 4.3-4.4 due to two protons of ethyl ester.

The Mass spectra of all compounds showed their characteristic molecular ion peak. Thus, the structure of synthesized compounds confirmed by IR, ^1H NMR and Mass spectral data.

TABLE-1: PHYSICAL DATA OF SUBSTITUTED 2-(2-CYANOACETAMIDO) THIOPHENES

Compound	R_1, R_2	R_3	Mol. Formula	M.P (^0C)	Yield (%)	M.W
3a	$-CH_3$	-CN	$C_{10}H_9N_3OS$	258-259	92	219
3b	$-(CH_2)_4-$	-CN	$C_{12}H_{11}N_3OS$	234-235	91	245
3c	$-CH_3$	$-CONH_2$	$C_{10}H_{11}N_3O_2S$	235-236	92	237
3d	$-(CH_2)_4-$	$-CONH_2$	$C_{12}H_{13}N_3O_2S$	182-184	89	263
3e	$-CH_3$	$-COOC_2H_5$	$C_{12}H_{14}N_2O_3S$	128-129	80	266
3f	$-(CH_2)_4-$	$-COOC_2H_5$	$C_{14}H_{16}N_2O_3S$	111-113	80	292

***In vitro* Antioxidant Activity:**

Assay of DPPH free radical scavenging:

The nitrogen centered stable free radical 1,1-diphenyl-2-picrylhydarzyl (DPPH) has been used to characterize antioxidants. It is reversibly reduced and due to its unpaired electron, densely colored. This property

makes it suitable for spectrophotometric studies. A radical scavenging antioxidant reacts with DPPH stable free radical and converts to 1,1-diphenyl-2-picryl hydrazine (DPPH-H). The change in the absorbance produced in this reaction has been used to measure antioxidant properties.

All the synthesized compounds were tested for their ability to reduce DPPH free radical at 100 µM concentration using 95% ethanol as solvent. The activity data has been presented in Table-2. Among the evaluated compounds, 2-(2-cyanoacetamido)-4,5-dimethylthiophene-3-carboxamide (Compound **3c**) exhibited highest activity (52.4%). The other compound, 2-cyano-N-(3-cyano-4,5-dimethylthiophen-2-yl)acetamide (Compound **3a**) exhibited 50.3% of antioxidant activity. The activities of these two compounds were comparable to that of standard employed (Ascorbic acid 64.7%). The greater activity of these compounds may be attributed to the polar nature of carboxamide or nitrile group at 3rd position on thiophene ring. The activity data revealed that introduction of ethyl ester group at 3rd position on thiophene ring system causes the reduction in activity.

Among the 4,5,6,7-tetrahydrobenzo[b]thiophene derivatives (Compounds **3b, 3d** and **3f**) and 4,5-dimethylthiophene derivatives (Compounds **3a, 3c** and **3e**), the later compounds showed greater activity than the former compounds. This may be due to greater non polar nature of tetramethylene group at 4th and 5th positions of thiophene ring and thus indicates the fusion of tetrahydrobenzene to thiophene ring does not enhance the activity.

TABLE- 2: REDUCTION OF DPPH BY SUBSTITUTED 2-(2-CYANOACETAMIDO) THIOPHENES

Compound	R_1, R_2	R_3	%Inhibition at 100 µM
3a	$-CH_3$	-CN	50.3
3b	$-(CH_2)_4-$	-CN	37.3
3c	$-CH_3$	$-CONH_2$	52.4
3d	$-(CH_2)_4-$	$-CONH_2$	33.4
3e	$-CH_3$	$-COOC_2H_5$	36.6
3f	$-(CH_2)_4-$	$-COOC_2H_5$	24.2
Ascorbic acid			64.7

Assay of nitric oxide free radical scavenging:

Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide. This nitric oxide reacts with oxygen to produce nitrite ions which can be estimated using griess reagent. Scavengers of nitric oxide compete with oxygen leading to a reduced production of nitric oxide.

All the compounds were tested for their ability to scavenge nitric oxide free radical at 100 µM concentration. The activity data was given in Table-3. Among the evaluated compounds, the highest activity was exhibited by 2-(2-cyanoacetamido)-4,5-dimethylthiophene-3-carboxamide (Compound **3c**, 56.9%) and 2-(2-cyanoacetamido)-4,5,6,7-tetrahydrobenzo[b]thiophene-3-carboxamide (Compound **3d**, 55.5%). The greater activity of these compounds may be due to the carboxamide group at 3rd position on thiophene ring structure.

On replacement of carboxamide group with nitrile causes slight reduction in the activity (compound **3a**, 52.7% and compound **3b**, 47.6%). Further, introduction of ethyl ester group at 3rd position on thiophene ring system causes the reduction in activity (compound **3e**, 48.9% and compound **3f**, 34.2%).

TABLE-3: EFFECT OF SUBSTITUTED 2-(2-CYANOACETAMIDO)THIOPHENES ON SCAVENGING OF NITRIC OXIDE

Compound	R_1, R_2	R_3	%Inhibition at 100 µM
3a	$-CH_3$	-CN	52.7
3b	$-(CH_2)_4-$	-CN	47.6
3c	$-CH_3$	$-CONH_2$	56.9
3d	$-(CH_2)_4-$	$-CONH_2$	55.5
3e	$-CH_3$	$-COOC_2H_5$	48.9
3f	$-(CH_2)_4-$	$-COOC_2H_5$	34.2

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ANTIBACTERIAL ACTIVITY:

The antibacterial activity of synthesized compounds was conducted against gram negative bacteria (*E.coli*) and gram positive bacteria (*S.aureus*) by Agar well diffustion method at a concentration 200 µg. Streptomycin was employed as standard to compare the results. The data was presented in Table-4.

The activity data revealed that, among the evaluated compounds, 2-(2-cyanoacetamido)-4,5-dimethylthiophene-3-carboxamide (Compound **3c**) exhibited maximum zone of inhibition against *E.coli* and 2-(2-cyanoacetamido)-4,5,6,7-tetrahydrobenzo[b]thiophene-3-carboxamide (Compound **3d**) exhibited maximum zone of inhibition against *S.aureus*. The antibacterial activity of compound **3c** against *E.coli* was almost nearer to the activity exhibited by standard drug Streptomycin. The highest activity of compound **3c** and compound **3d** may be due to the carboxamide group at 3rd position of thiophene ring. From the activity data it is evident that on changing the amide group to nitrile or ethyl ester at 3rdposition of thiophene causes reduction in the activity.

TABLE-4: ANTIBACTERIAL ACTIVITY OF SUBSTITUTED 2-(2-CYANOACETAMIDO)THIOPHENES

Sl.No.	NAME OF THE SAMPLE	DIAMETERS OF THE INHIBITION ZONES (IN MILLIMETERS)					
		E.coli			*S.aureus*		
		1	2	3	1	2	3
3a	2-cyano-N-(3-cyano-4,5-dimethylthiophen-2-yl)acetamide	15	17	17	16	17	15
3b	2-cyano-N-(3-cyano-4,5,6,7-tetrahydrobenzo[b]thiophen-2-yl)acetamide	16	15	16	16	15	15
3c	2-(2-cyanoacetamido)-4,5-dimethylthiophene-3-carboxamide	21	24	22	17	15	15
3d	2-(2-cyanoacetamido)-4,5,6,7-tetrahydrobenzo[b]thiophene-3-carboxamide	13	12	15	17	18	17
3e	Ethyl 2-(2-cyanoacetamido)-4,5-dimethylthiophene-3-carboxylate	15	13	14	15	15	15
3f	Ethyl 2-(2-cyanoacetamido)-4,5,6,7-tetrahydrobenzo[b]thiophene-3-carboxylate	16	18	15	17	15	15
	Streptomycin	27	27	27	22	23	25

CONCLUSION

An eco-friendly and easy method has been used for the synthesis of substituted 2-(2-cyanoacetamido)thiophenes by cyanoacetylation of substituted 2-aminothiophenes. The method includes mild conditions, requires less reaction time and gives high yield of the product. The compounds synthesized were screened for *in vitro* antioxidant activity by scavenging DPPH and nitric oxide free radicals at 100 µM concentrations. Among the evaluated compounds, highest activity was exhibited by 2-(2-cyanoacetamido)-4,5-dimethylthiophene-3-carboxamide (Compound **3c**) and the remaining compounds exhibited moderate to good antioxidant activity. Hence, further investigations are needed to be performed to know their toxicity and they may be evaluated for diseases associated with oxidative stress such as Cancer, Inflammation, Parkinsonism, Alzheimer's disease. Further, the substituted 2-(2-cyanoacetamido)thiophenes could be used as a template for the future development of more potent therapeutic agents, as several evaluated compounds also possess moderate to good antibacterial activity.

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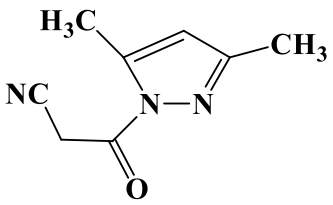

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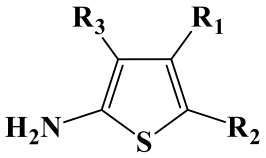


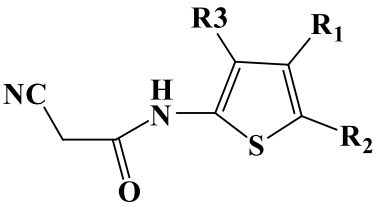
1-Cyanoacetyl-3,5-dimethylpyrzole

1

Substituted 2-aminothiophene

2a-2f

Toluene
reflux at100 ^{0}C



Substituted 2-(2-cyanoacetamido)thiophene

3a-3f

2/3	R_1	R_2	R_3
a	$-CH_3$	$-CH_3$	-CN
b		$-CH_2-$	-CN
c	$-CH_3$	$-CH_3$	$-CONH_2$
d		$-CH_2-$	$-CONH_2$
e	$-CH_3$	$-CH_3$	$-COOC_2H_5$
f		$-CH_2-$	$-COOC_2H_5$

Scheme-I

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