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Research Article

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Synthesis and Evaluation of Alkyl 4-(2-Cyanoacetamido) Benzoates for Antioxidant and Analgesic Activities

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ABSTRACT

1-Cyanoacetyl-3,5-dimethylpyrazole is a versatile cyanoacetylating agent used for N-cyanoacetylation of aromatic amines and it is proved to be superior to other cyanoacetylating agents. Hence, in the present study 1-cyanoacetyl-3,5-dimethylpyrazole was used for N-cyanoacetylation of alkyl 4-aminobenzoates to give alkyl 4-(2-cyanoacetamido)benzoates. Four compounds were synthesized and characterized by their physical and spectral data. 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 analgesic activity in mice by acetic acid induced writhing test. The activity data revealed that the synthesized compounds were found to possess low antioxidant activity in DPPH free radical scavenging and moderate activity in nitric oxide free radical scavenging. Further, the evaluated compounds exhibited good analgesic activity comparable to the standard drug paracetamol.

Keywords: Cyanoacetylation; 1-Cyanoacetyl-3,5-dimethylpyrazole; Alkyl 4-(2-cyanoacetamido)benzoates; Antioxidant activity; Analgesic activity

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INTRODUCTION

N-Cyanoacetylation of aromatic amines can be performed by various reagents such as cyanoacetic ester, cyanoacetyl chloride, cyanoacetyl azide, mixed anhydride of cyanoacetic and acetic acids, N-(cyanoacetyl)imidazole and 1-cyanoacetyl-3,5-dimethylpyrazole [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 the reaction time for the formation of N-cyanoacetylation products (N-substituted cyanoacetamide) is less and they can be isolated in higher yields from reaction mixture in crystalline form, while the 3,5-dimethylpyrazole by-product remains in the mother liquor [2]. In the literature various N-substituted cyanoacetamide derivatives and related heterocyclic moieties were reported to possess diverse biological and pharmaceutical activities [3,4]. Further, the commercially available drugs such as Tofacitinib and Cephacetrile have N-cyanoacetyl group in their structure and are in use for a variety of medical treatments [5,6]. The alkyl 4-aminobenzoates such as benzocaine (ethyl 4-aminobenzoate) and butamben (butyl 4-aminobenzoate) are known to have valuable therapeutic properties, especially as local anesthetics. Derivatives of alkyl 4-aminobezoates are also reported to possess various biological and pharmacological activities [7,8]. In view of diverse pharmacological activities of alkyl 4-aminobenzoates and N- substituted cyanoacetamide, it has been considered worthwhile to hybridize these two pharmacophores to give alkyl 4-(2-cyanoacetamido)benzoates. In the present work it has been planned to synthesize alkyl 4-(2-cyanoacetamido)benzoates by cyanoacetylation of alkyl 4-aminobenzoates with 1-cyanoacetyl-3,5-dimethylpyrazole. Further, to evaluate the synthesized compounds for *in vitro* antioxidant activity

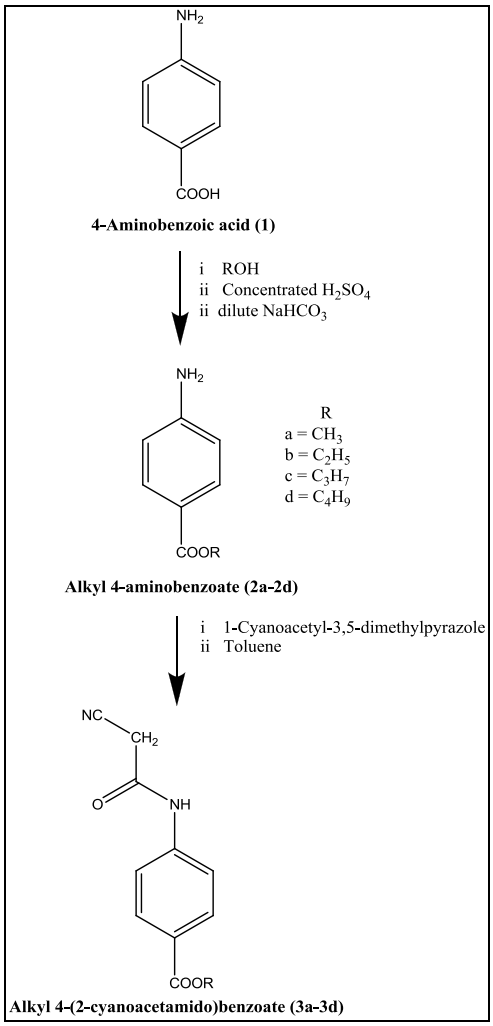
by scavenging 1,1-diphenyl-2-picrylhydrazyl (DPPH) and nitric oxide free radicals and *in vivo* analgesic activity in mice by acetic acid induced writhing test.

EXPERIMENTAL SECTION

All the chemicals were procured from Sigma Aldrich and SD fine chemicals. Melting points were determined in open capillaries on a digital Stuart melting point apparatus and are uncorrected. Purity of the compounds was checked by using the glass plates coated with Silicagel-G, and spots are detected by iodine vapour. The IR spectra were recorded using KBr Pellets on a BRUKER Infrared spectrophotometer (cm^{-1}). The ^1H NMR spectra were recorded on Avance-400 MHz spectrometer using TMS as internal standard (chemical shifts in δ ppm) and mass spectra were recorded in negative ion mode on Agilent 6120 Quadrupole LC-MS system. Animal experiments were carried out in accordance with the guidelines of CPCSEA and by the approval of institutional animal ethical committee [Regd. No. 1677/PO/Re/2012/CPCSEA/26].

General procedure for synthesis of alkyl 4-(2-cyanoacetamido)benzoates (Scheme-I, 3a-3d):

To a solution of 10 mM of alkyl 4-aminobenzoate in toluene (20 ml), an equimolar amount of 1-cyanoacetyl-3,5-dimethylpyrazole (1.63 gm, 10 mM) was added and mixed thoroughly. The reaction mixture was refluxed for about 30 minutes at 110 to 120ºC. 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 with ethanol. Using the above procedure, four compounds were prepared and characterized by IR, ^1H NMR and Mass spectra.



Scheme 1: Synthesis of Alkyl 4-(2-cyanoacetamido)benzoates (3a-3d)

Methyl 4-(2-cyanoacetamido)benzoate: mp. 162 °C; yield 90%; IR (KBr) υ_{max}: 3428 (N-H str), 2260 (C≡N str), 1722 (C=O str), 1669 (N-H def) cm^{-1} ; ^1H NMR (DMSO) δ: 3.83 (s, 3H, CH_3), 3.96 (s, 2H, CH_2CN), 7.68-7.70 & 7.94-7.96 (2d, 4H, Ar), 10.63 (s, br, 1H, NH) ppm; Mass m/z: 217 (M-1).

Ethyl 4-(2-cyanoacetamido)benzoate: mp. 162 °C; yield 92%; IR (KBr) υ_{max}: 3338 (N-H str), 2256 (C≡N str), 1690 (C=O str), 1604 (N-H def) cm^{-1} ; ^1H NMR ($CDCl_3$) δ: 1.38-1.41 (t, 3H, CH_2CH_3), 3.59 (s, 2H, CH_2CN), 4.35-4.40 (q, 2H, CH_2CH_3), 7.59-7.62 & 8.04-8.07 (2d, 4H, Ar), 7.85 (s, br, 1H, NH) ppm; Mass m/z: 231 (M-1).

Propyl 4-(2-cyanoacetamido)benzoate: mp. 138 °C; yield 84%; IR (KBr) υ_{max}: 3299 (N-H str), 2268 (C≡N str), 1697 (C=O str), 1604 (N-H def) cm^{-1} ; ^1H NMR ($CDCl_3$) δ: 1.01-1.05 (t, 3H, $CH_2CH_2CH_3$), 1.75-1.83 (sextet, 2H, $CH_2CH_2CH_3$), 3.60 (s, 2H, CH_2CN), 4.26-4.30 (t, 2H, $CH_2CH_2CH_3$), 7.59-7.61 & 8.03-8.05 (2d, 4H, Ar), 8.07 (s, br, 1H, NH) ppm; Mass m/z: 245 (M-1).

Butyl 4-(2-cyanoacetamido)benzoate: mp. 131 °C; yield 80%; IR (KBr) υ_{max}: 3303 (N-H str), 2268 (C≡N str), 1689 (C=O str), 1604 (N-H def) cm^{-1} ; ^1H NMR ($CDCl_3$) δ: 0.96-0.99 (t, 3H, $CH_2CH_2CH_2CH_3$), 1.42-1.51 (sextet, 2H, $CH_2CH_2CH_2CH_3$), 1.71-1.78 (quintet, 2H, $CH_2CH_2CH_2CH_3$) 3.62 (s, 2H, CH_2CN), 4.29-4.32 (t, 2H, $CH_2CH_2CH_2CH_3$), 7.58-7.61 & 8.00-8.02 (2d, 4H, Ar), 8.35 (s, br, 1H, NH) ppm; Mass m/z: 259 (M-1).

***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 [9,10].

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 concentrations of test compounds dissolved in alcohol, at 25 °C for 150 minutes. Then 2 ml of incubation solution was removed and added to 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 546nm. Control experiment was conducted in an identical manner without test compound but with equal amount of solvent. The percentage of scavenging activity was calculated using the above formula.

Analgesic activity: acetic acid induced writhing test

Mice of either sex weighing approximately 20-25 gm are used. An aliquot of 0.2 ml of 0.6% acetic acid was injected intraperitoneally in each animal. The animal reacts with a characteristic stretching behavior, i.e. a series of constructions occur that travel along the abdominal wall, sometimes accompanied by turning movements of the body abdominal extension of the hind limbs. This response is called writhing.

The mice were divided into six groups each of four animals. One group consisting of four served as control, while the other groups of four animals received the test compounds and standard drug. The mice dosed (100 mg/ kg) orally with test compounds one hour before injection of 0.2 ml of 0.6 % acetic acid intraperitoneally in each animal. The total number of writhings following intraperitoneal administration of acetic acid solution was recorded over a period of 10minutes, starting 5minutes after acetic acid injection [11].

% writhing inhibition = [(No. of writhes in control group - No. of writhes in test group) / No. of writhes in control group] × 100

RESULTS AND DISCUSSION

N-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 cyanoacetate, cyanoacetic acid, anhydride or acid chloride of cyanoacetic acid.

In the present study, the cyanoacetylating agent, 1-cyanoacetyl-3,5-dimethylpyrazole was prepared according to the procedure available in literature. It is prepared by condensation and subsequent cyclization of acetyl acetone with cyanoacetic acid hydrazide in water containing catalytic amount of concentrated hydrochloric acid [12]. Using this cyanoacetylating agent, four alkyl 4-(2-cyanoacetamido)benzoates (3a-3d) were prepared by refluxing 1-cyanoacetyl-3,5-dimethylpyrazole with equimolar quantities of four different alkyl 4-aminobenzoates in toluene for 30 minutes at 120 ºC. These compounds were obtained in good yields ranging from 80-92%. The alkyl 4-aminobenzoates were prepared by esterification of 4-aminobenzoic acid with methanol, ethanol, n-propanol and n-butanol in presence of catalytic amount of concentrated sulphuric acid. The physical data of all synthesized compounds have been given in table 1.

Table 1: Physical data of alkyl 4-(2-cyanoacetamido)benzoates

Compound	R	Mol. Formula	M.P (^{0}C)	Yield (%)
3a	-CH_3	$C_{11}H_{10}O_3N_2$	162	90
3b	-C_2H_5	$C_{12}H_{12}O_3N_2$	162	92
3c	-C_3H_7	$C_{13}H_{14}O_3N_2$	138	84
3d	-C_4H_9	$C_{14}H_{16}O_3N_2$	131	80

The IR spectra of compounds showed an absorption band in the region of 3428-3299 cm^{-1} indicative of N-H stretching. The spectra revealed the presence of absorption band due to C≡N stretching in the region 2368-2256 cm^{-1}. The absorption bands in the region of 1722-1689 and 1669-1604 cm^{-1} were assignable to amide I and II bands. The ^{1}H NMR spectra of all compounds showed singlet in the region of δ 3.59 - 3.96 due to two CH_2 protons of cyanoacetamido group and two doublets in the region of δ 7.58-7.61 and 8.04-8.07 due to four aromatic protons. The spectra also exhibited a broad singlet in the region of δ 8.07 – 10.63 due to NH proton of cyanoacetamido group. The ^{1}H NMR spectrum of compound 3a (methyl 4-(2-cyanoacetamido)benzoate) showed singlet at δ 3.83 due to three protons of methyl ester group. The spectrum of compound 3b (ethyl 4-(2-cyanoacetamido)benzoate) showed triplet at a region δ 1.38-1.41 due to three methyl protons and a quartet at δ 4.35-4.40 due to two methylene protons of ethyl ester. The spectrum of compound 3c (propyl 4-(2-cyanoacetamido)benzoate) showed triplet at δ 1.01-1.05 due to three methyl protons, a sextet at δ 1.75-1.83 due to two methylene protons and a triplet at δ 4.26-4.30 due to two methylene protons of propyl ester. The spectrum of compound 3d (ethyl 4-(2-cyanoacetamido)benzoate) showed triplet at δ 0.96-0.99 due to three methyl protons, a sextet at δ 1.42-1.51 due to two methylene protons, a quintet at δ 1.71-1.78 and a triplet at δ 4.29-4.32 due to two methylene protons of butyl ester. The Mass spectra of all compounds showed their characteristic molecular ion peak. Thus, the structures of the compounds were confirmed by IR, ^{1}H NMR and Mass spectral data. All the synthesized compounds (alkyl 4-(2-cyanoacetamido)benzoates, 3a-3d) were evaluated for *in vitro* antioxidant activity by scavenging 1,1-diphenyl-2-picrylhydrazyl (DPPH) and nitric oxide free radicals at 100 µM concentration. Ascorbic acid was used as reference standard. The results were presented in table 2. The activity data revealed that compound 3d (butyl 4-(2-cyanoacetamido)benzoate) exhibited highest antioxidant activity in both free radical scavenging models. Further, it is observed that the antioxidant activity of synthesized compounds increased with increase in alkyl chain length of ester group. This may be due to increase in order of solubility of methyl through butyl ester in alcohol, as evidenced by previous literature [13]. However, the activity data revealed that the synthesized compounds were found to possess low antioxidant activity in DPPH free radical scavenging and moderate activity in nitric oxide free radical scavenging.

Table 2: *In vitro* antioxidant activity of alkyl 4-(2-cyanoacetamido)benzoates

Compound	R	% Scavenging of DPPH	% Scavenging of Nitric oxide
3a	-CH_3	11.0	14.4
3b	-C_2H_5	15.9	21.1
3c	-C_3H_7	16.0	38.6
3d	-C_4H_9	18.2	39.9
Ascorbic acid		48.9	51.8

Analgesic activity of synthesized compounds was screened by acetic acid induced writhing test in mice and the data was represented in table 3. On observation of results obtained, the compounds with increase in the chain length of alkyl ester increase the analgesic activity. Thus, butyl 4-(2-cyanoacetamindo)benzoate 3d and propyl 4-(2-cyanoacetamido)benzoate 3c showed highest analgesic activity and equipotent to the standard drug paracetamol.

The highest activity of these compounds may be due to the high lipophilic nature of ester group. The other evaluated compounds, methyl 4-(2-cyanoacetamindo)benzoate 3a and ethyl 4-(2-cyanoacetamido)benzoate 3b showed moderate analgesic activity.

Table 3: *In vivo* analgesic activity of alkyl 4-(2-cyanoacetamido)benzoates

S.No	Compounds	Mean±SD (number of wriths)	Writhing inhibition (%)
1	Control	34.5±3.50	-
2	3a	18.0±1.87	47.82
3	3b	17.0±3.80	50.72
4	3c	9.5±1.80	72.46
5	3d	8.25±4.60	76.08
6	Paracetamol	8.0±2.54	76.81

CONCLUSION

In conclusion, a set of new alkyl 4-(2-cyanoacetamido)benzoates were prepared by N-cyanoacetylation of respective alkyl 4-aminobenzoates with the versatile cyanoacetylating agent 1-cyanoacetyl-3,5-dimethylpyrazole. All the synthesized compounds were evaluated for *in vitro* antioxidant activity and found to possess low anti-oxidant activity in DPPH free radical scavenging and moderate activity in nitric oxide free radical scavenging. Analgesic activity of synthesized compounds was screened by acetic acid induced writhing test in mice and found that butyl 4-(2-cyanoacetamindo)benzoate **3d** and propyl 4-(2-cyanoacetamido)benzoate **3c** were equipotent to the standard drug paracetamol.

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