Hindered phenolic aminothiazoles – Synthesis, \(\alpha\)-glucosidase inhibitory and antioxidant activities

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Abstract: Base-catalysed heterocyclization of either \(N\)-aryl-\(N'\)-[imino(nitroamino)methyl]thioureas or \(N\)-aryl-\(N'\)-cyanothioureas by reaction with 2-bromo-1-(2,6-di-\(t\)-butyl-4-hydroxyphenyl)ethanone afforded 4-amino-2-(arylamino)-5-(3,5-di-\(t\)-butyl-4-hydroxybenzoyl)thiazoles, designed as molecular hybrids of hindered phenolic and 2-aminothiazole moieties. These compounds were screened for their inhibition activity on carbohydrate hydrolyzing enzymes. Thus, [4-amino-2-(phenylamino)-5-thiazolyl](3,5-di-\(t\)-butyl-4-hydroxyphenyl)methanone exhibited \(\alpha\)-glucosidase inhibition activity with an \(IC_{50}\) value of 117 \(\mu\)M while the standard compound acarbose showed an \(IC_{50}\) value of 48.3 \(\mu\)M and [4-amino-2-{(4-methylphenyl)amino}-5-thiazolyl](3,5-di-\(t\)-butyl-4-hydroxyphenyl)methanone showed good \(\alpha\)-amylase inhibition activity with an \(IC_{50}\) value of 283 \(\mu\)M compared to acarbose (\(IC_{50}\) 532 \(\mu\)M). The antioxidant activities of the hindered phenolic thiazoles were also investigated and the 2-[(4-methoxyphenyl)amino] derivative showed an antioxidant activity better than that of butylated hydroxyanisole in the 2,2-diphenyl-1-picrylhydrazyl radical scavenging assay, better than that of either vitamin C or curcumin in the ferric ion-reducing antioxidant potential assay and comparable with that of butylated hydroxyanisole in the \(\beta\)-carotene bleaching assay.

Keywords: 2,4-diaminothiazolyl; 3,5-di-\(t\)-butyl-4-hydroxyphenyl; hindered phenol; enzyme inhibition.

INTRODUCTION

Diabetes mellitus is a chronic endocrine disease that affects the metabolism of carbohydrates. The goal of diabetes treatment is to maintain a nearly normal level of glycemic control subsequent to food intake so as to maintain the postprandial hyperglycaemia.\(^{1}\) This could be achieved by inhibiting the carbo-
hydrate hydrolyzing enzymes involved in the breakdown of carbohydrates, such as α-glucosidase and α-amylase. Hence, carbohydrate digestive enzyme inhibitors are widely investigated in the identification of lead compounds for the treatment of diabetes.2 In addition, the role oxidative stress and inflammation play in the development of diabetes mellitus has now been recognized and the significance of antioxidants in the control of diabetes mellitus was studied.3–5

Hindered phenols in which the phenolic hydroxyl group is juxtaposed with a sterically demanding group, such as a t-butyl group, have found wide application as antioxidants and permissible food preservatives.6 Typical examples of hindered phenols used in food preservation are 2-t-butyl-4-methylphenol (butylated hydroxytoluene, BHT), 2-t-butyl-4-methoxyphenol (butylated hydroxyanisole, BHA) and t-butylhydroquinone (TBHQ). A recent report highlighted the importance of free phenolic groups in flavone, isoflavone and chalcone derivatives on their α-glucosidase inhibitory activity.7 The incorporation of a 2,6-di-t-butylphenolic unit to improve the bioactivities of flavonoids by designing hindered phenol–flavonoid hybrids and the antioxidant activity of hydrazones bearing a 2,6-di-t-butylphenolic unit have also been reported recently.8 The 2-aminothiazole moiety is isoteric with a phenolic unit and devoid of the acidity of the latter and hence, it finds much use in drug design.9 In connection with our interest in the anticancer10,11 and neuroprotective12 activities of 2,4-diaminothiazoles, it was noted that only a few reports exist on the antioxidant activity of aminothiazoles.13,14 With this background, it was hypothesized that 2,4-diaminothiazoles bearing a hindered phenol moiety could show promising antioxidant activities. Accordingly, the design and synthesis of hitherto unreported 4-amino-2-(aryl-amino)-5-(3,5-di-t-butyl-4-hydroxybenzoyl)thiazoles as molecular hybrids incorporating di-t-butylphenol and 2,4-diaminothiazole moieties, along with their α-glucosidase and α-amylase inhibitory and antioxidant activities are reported herein.

EXPERIMENTAL

Chemistry

Melting points are uncorrected and were determined by the open capillary method. The thin layer chromatographic analyses were performed using silica gel 60 F254 TLC aluminium sheets purchased from Merck, Mumbai, India. The elemental analyses were performed on a Vario EL III elemental analyzer. The IR spectra were recorded on JASCO, Bomem MB and Shimadzu FTIR spectrophotometers. The NMR spectra were recorded on Bruker DPX-400 and 500 MHz spectrometers and FAB mass spectra were recorded on Jeol SX-102 FAB mass spectrometer. HRMS-ESI spectrum was performed at a resolution of 61800 using a Thermo Scientific Exactive mass spectrometer. All chemicals were from Sigma–Aldrich and Merck. The required N-aryl-N′-[imino(nitroamino)methyl]thioureas 1a–e were obtained from nitroguanidine and aryl isothiocyanates 2a–e as reported earlier.15 Reported procedures with slight modifications were used to prepare 1-(2,6-di-t-butyl-4-hydroxyphenyl)ethanone from 2,6-di-t-butylphenol16 and its bromination17 to obtain 2-bromo-1-(2,6-di-t-butyl-4-hydroxyphenyl)ethanone 4.
Analytical and spectral data of the synthesized compounds are given in Supplementary material to this paper.

**General procedure for synthesis of 4-amino-2-(arylamino)-5-(3,5-di-t-butyl-4-hydroxybenzoyl)thiazoles 5a–e**

**Method A:** from N-aryl-N'-(iminonitroamino)methyl]thioureas 1a–e. To a stirred solution of N-aryl-N'-(iminonitroamino)methyl]thioureas (1a–e, 1 mmol) in N,N-dimethylformamide (DMF, 3mL) at room temperature, 2-bromo-1-(2,6-di-t-butyl-4-hydroxyphenyl)-ethanone (4, 1 mmol) was added and stirred. After 15 min, triethylamine (3 mmol) was added and the mixture was further stirred at room temperature for 75 min. The resulting deep brown reaction mixture was poured slowly with stirring into ice cold water. The brownish yellow precipitate of 4-amino-2-(arylamino)-5-(3,5-di-t-butyl-4-hydroxybenzoyl)thiazoles 5a–e obtained was collected by filtration, washed with water and dried.

**Method B:** from N-aryl-N'-cyanothioureas (3a–e) prepared in situ. Cyanamide (1 mmol) and powdered potassium hydroxide (1.1 mmol) were stirred in DMF (2 mL) for 5 min at room temperature and to this mixture, aryl isothiocyanate (2a–e, 1 mmol) in DMF was added drop-wise with stirring over 5 min. After further stirring at room temperature for 90 min, the mixture containing N-aryl-N'-cyanothioureas (3a–e) was treated with 2-bromo-1-(2,6-di-t-butyl-4-hydroxyphenyl)ethanone (4, 1 mmol) and the stirring was continued for a further 30 min. Triethylamine (1.2 mmol) was then added followed by stirring for 30 min. The so-obtained reddish brown mixture was poured into ice cold water and the crude 4-amino-2-(arylamino)-5-(3,5-di-t-butyl-4-hydroxybenzoyl)thiazoles 5a–e were collected, washed with water and dried.

The crude products obtained by methods A and B were purified either by crystallization from ethanol or by dry column flash chromatography on thin layer chromatography grade silica gel eluted with hexane-ethyl acetate.

**α-Glucosidase inhibition activity**

The mode of α-glucosidase inhibition was studied according to Apostolidis et al. Briefly, about 50 µL of homogenized sample solutions of varying concentrations (5–250 µM) and 100 µL of 0.1 M phosphate buffer (pH 6.9) containing α-glucosidase solution (1.0 U mL⁻¹) was incubated in 96 well plates at 25 °C for 10 min. After pre-incubation, 50 µL of p-nitrophenyl α-D-glucopyranoside solution (7.5 mg in 5 ml; 5 mM) in 0.1 M phosphate buffer (pH 6.9) was added to each well at timed intervals. Before and after incubation at 25 °C for 5 min, the absorbance at 405 nm was measured using an Enspire multimode reader (Perkin Elmer). Acarbose was used as the positive control and the results are expressed as percent inhibition, calculated as:

\[
(1-A_{\text{sample}}/A_{\text{control}}) \times 100
\]

where \( A \) is the absorbance.

**α-Amylase inhibition activity**

The inhibitory activity of α-amylase enzymes (from Aspergillus oryzae) was performed using a reported procedure with a slight modification. Briefly, different concentrations of the stock solutions of the samples (100–600 µM) were incubated with an α-amylase solution (0.5 mg/ml) in 0.02 M phosphate buffer (pH 6.9 with 0.006 M NaCl, 500 µL) at 25 °C for 10 min. After pre-incubation, 500 µL of a 1% starch solution in 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M sodium chloride) was added to each tube at timed intervals. The reaction was stopped with 500 µL of 3,5-dinitrosalicylic acid (1%) as a colour reagent. The tubes were then incubated in a boiling water bath for around 5 min, cooled to room temperature and
diluted to 10 mL with distilled water. The absorbance was measured at 540 nm using acarbose as the standard. The percentage of inhibition was calculated using the formula (1).

**Antioxidant capacity assays**

2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity. The DPPH radical scavenging efficacy of the compounds was evaluated based on a reported procedure. Briefly, aliquots of the test samples leading to a concentration range of 100–600 µM were mixed with a methanolic solution of DPPH (1.5 mL; 25 mg L⁻¹), kept in the dark for 30 min and the absorbance was measured at 517 nm against the control. BHA and curcumin served as standards. The percentage radical scavenging activity, calculated as the scavenging effect (SE) from formula (1), was plotted against concentration to obtain the concentration values resulting in 50 % inhibition (IC₅₀).

Ferric ion reducing potential (FRAP) assay The FRAP activity was measured according to the method of Benzie and Strain. Acetate buffer (300 mM; pH 3.6), 2,4,6-tripyridyl-s-triazine (TPTZ; 10 mM in 40 mM aq. hydrochloric acid) and FeCl₃·6H₂O (20 mM) were mixed in the ratio of 10:1:1 to obtain the working FRAP reagent. Test samples (500 µM) in methanol (10 mL) were mixed with 3 mL of working FRAP reagent and absorbance was measured at 593 nm after vortexing. Methanolic solutions of FeSO₄·7H₂O ranging from 100 to 2000 µM were used for the preparation of the calibration curve of known Fe²⁺ concentration. The parameter equivalent concentration (EC) was defined as the concentration of antioxidant having a ferric–TPTZ reducing ability equivalent to that of 1 mM FeSO₄·7H₂O. BHA and curcumin were used as standards.

β-Carotene bleaching assay. The inhibition of the oxidative bleaching of β-carotene in a β-carotene/linoleic acid emulsion is measured in the β-carotene bleaching assay. It was realised using the method of Hidalgo et al. by using an emulsion obtained by mixing β-carotene (0.2 mg), linoleic acid (20 mg) and Tween 20 (200 mg, 0.180 mL) in chloroform (0.5 mL), evaporating off the chloroform and suspending in distilled water (50 mL). The thus obtained emulsion (4 mL) was treated with the test samples in methanol (180 µL) at a concentration 10⁻³ M. BHA was used as the standard together with a control without sample and the absorbance was measured at 470 nm. Antioxidant activity was expressed as the percentage inhibition relative to the control using the equation:

\[ AA = 100(\text{DR}_C - \text{DR}_S)/\text{DR}_C \]

where DR₂ is the degradation rate of the control (\(\text{DR}_C = \ln (a/b)/60\), where \(a\) is initial absorbance of control and \(b\) is the final absorbance of control after 60 min) and DR₃ is the degradation rate of the thiazole sample (\(\text{DR}_S = \ln (a/b)/60\), where \(a\) is the initial absorbance of the sample and \(b\) is the final absorbance of sample after 60 min).

**RESULTS AND DISCUSSION**

**Chemistry**

A retro-synthetic analysis indicated that a [4+1] heterocyclization of the type ((C⁴–N³–C²–S¹)+C⁵), leading to the thiazole ring formation, could be adopted for the synthesis of the hitherto unreported 4-amino-2-(arylamino)-5-(3,5-di-t-butyl-4-hydroxybenzoyl)thiazoles 5. The four ring atoms [(C⁴–N³–C²–S¹)] could be sourced from either N-aryl-N'[imino(nitroamino)methyl]thioureas (1, method A; Scheme 1), obtainable from nitroguanidine and aryl isothiocyanates (2a–e), as reported earlier, or from N-aryl-N'[cyanothioureas (3a–e), accessible from
cyanamide and aryl isothiocyanates (2a–e, method B; Scheme 2).23 The remaining C⁵ carbon required to assemble the thiazole core could arise from 2-bromo-1-(2,6-di-t-butyl-4-hydroxyphenyl)ethanone (4). It was found that both the above two methods afforded the target diaminothiazoles in good yields (see Supplementary material).

Scheme 1. Synthesis of [4-amino-2-(phenylamino)-5-thiazolyl](3,5-di-t-butyl-4-hydroxyphenyl)methanone – Method A.

Scheme 2. Synthesis of [4-amino-2-(phenylamino)-5-thiazolyl](3,5-di-t-butyl-4-hydroxyphenyl)methanone – Method B.
**α-Glucosidase and α-amylase inhibitory activity determination**

The α-glucosidase enzyme inhibition activity was measured using the enzyme from *Saccharomyces cerevisiae*. The different concentrations of 4-amino-2-(arylamino)-5-(3,5-di-t-butyl-4-hydroxybenzoyl)thiazoles (5a–e) showed dose dependent inhibition compared with the standard acarbose. The results showed that [4-amino-2-(phenylamino)-5-thiazolyl][3,5-di-t-butyl-4-hydroxyphenyl]methanone (5a) showed good α-glucosidase enzyme inhibition activity ($IC_{50} = 117.02 \, \mu\text{M}$), compared with the standard compound acarbose that showed an $IC_{50}$ value of 48.26 $\mu\text{M}$ (Table I). The study of inhibition of the α-amylase enzyme revealed that all the compounds showed good inhibition activity compared to that of acarbose. As a typical example, {4-amino-2-[(4-methylphenyl)amino]-5-thiazolyl}(3,5-di-t-butyl-4-hydroxyphenyl)methanone (5d) showed an $IC_{50}$ value of 283.19 $\mu\text{M}$ in comparison with acarbose which showed an $IC_{50}$ value of 531.91 $\mu\text{M}$ (Table I). The results indicated that the phenolic aminothiazole unit could be a potential structural platform for the development of compounds with antidiabetic activity.

**TABLE I. α-Glucosidase and α-amylase enzyme inhibition activity of compounds 5a–e**

<table>
<thead>
<tr>
<th>Compound</th>
<th>$IC_{50}$ / $\mu\text{M}$</th>
<th>α-Glucosidase</th>
<th>α-Amylase</th>
</tr>
</thead>
<tbody>
<tr>
<td>5a</td>
<td>117</td>
<td>350</td>
<td></td>
</tr>
<tr>
<td>5b</td>
<td>146</td>
<td>566</td>
<td></td>
</tr>
<tr>
<td>5c</td>
<td>180</td>
<td>293</td>
<td></td>
</tr>
<tr>
<td>5d</td>
<td>214</td>
<td>283</td>
<td></td>
</tr>
<tr>
<td>5e</td>
<td>239</td>
<td>325</td>
<td></td>
</tr>
<tr>
<td>Acarbose</td>
<td>48.3</td>
<td>532</td>
<td></td>
</tr>
</tbody>
</table>

**Antioxidant activity**

The antioxidant activity of 4-amino-2-(arylamino)-5-(3,5-di-t-butyl-4-hydroxybenzoyl)thiazoles (5a–e) were assessed based on the DPPH radical scavenging assay. These were selected as representative examples of aminothiazoles (5) with a 2-(arylamino) substituent bearing electron withdrawing, neutral or electron donating substituent on the phenyl ring. The 2-[(4-methoxyphenyl)amino] derivative (5b, $EC_{50} = 250 \, \mu\text{M}$) and 2-[(4-ethoxyphenyl)amino] derivative (5e, $EC_{50} = 265 \, \mu\text{M}$) showed better radical scavenging activities than the 2-phenylamino derivative (5a, $EC_{50} = 340 \, \mu\text{M}$), 2-[(4-methylphenyl)amino] derivative (5d, $EC_{50} = 365 \, \mu\text{M}$) and the 2-(4-chlorophenylamino) derivative (5c, $EC_{50} = 590 \, \mu\text{M}$), in comparison with the standards curcumin ($EC_{50} = 200 \, \mu\text{M}$) and BHA ($EC_{50} = 280 \, \mu\text{M}$). The results of the DPPH assay showed that {4-amino-2-[(4-methylphenyl)amino]-5-thiazolyl}(3,5-di-t-butyl-4-hydroxyphenyl)methanone (5b) possessed an antioxidant activity that was better than that of BHA, but lower than that of curcumin.
In the case of FRAP assay, the 2-[(4-methoxyphenyl)amino]thiazole derivative (5b) showed a better activity, with a FeSO₄ equivalence of 1700 µM, compared with those of the 2-[(4-ethoxyphenyl)amino]thiazole (5e, 1500 µM), 2-[(phenylamino)thiazole (5a, 1100 µM), 2-[(4-methylphenyl)amino]thiazole (5d, 900 µM) and the 2-[(4-chlorophenyl)amino]thiazole (5c, 800 µM) derivatives, in comparison with those of vitamin C (1400 µM) and of curcumin (1700 µM).

The data of the β-carotene bleaching assay indicated that {4-amino-2-[(4-methylphenyl)amino]-5-thiazolyl}(3,5-di-t-butyl-4-hydroxyphenyl)methane (5b) exhibited 56 % antioxidant activity whereas the 2-[(4-ethoxyphenyl)amino]thiazole (5e), 2-(phenylamino)- (5a), 2-[(4-chlorophenyl)amino]- (5c) and 2-[(4-methylphenyl)amino]thiazole (5d) derivatives showed 52, 30, 27 and 24 % antioxidant activity, respectively, in comparison with the 58 % activity of BHA (Table II).

<table>
<thead>
<tr>
<th>Compound</th>
<th>DPPH radical scavenging activity, µM</th>
<th>FRAP µM</th>
<th>β-carotene bleaching method, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>5a</td>
<td>340</td>
<td>1100</td>
<td>30</td>
</tr>
<tr>
<td>5b</td>
<td>250</td>
<td>1700</td>
<td>56</td>
</tr>
<tr>
<td>5c</td>
<td>590</td>
<td>800</td>
<td>27</td>
</tr>
<tr>
<td>5d</td>
<td>365</td>
<td>900</td>
<td>24</td>
</tr>
<tr>
<td>5e</td>
<td>265</td>
<td>1500</td>
<td>52</td>
</tr>
<tr>
<td>BHA</td>
<td>280</td>
<td>–</td>
<td>58</td>
</tr>
<tr>
<td>Curcumin</td>
<td>200</td>
<td>1700</td>
<td>–</td>
</tr>
<tr>
<td>Vitamin C</td>
<td>–</td>
<td>1400</td>
<td>–</td>
</tr>
</tbody>
</table>

It appears that the presence of an electron donating substituent on the 2-arylamino group of 4-amino-2-(arylamino)-5-(3,5-di-t-butyl-4-hydroxybenzoyl)thiazoles promotes antioxidant activity. The free radical scavenging activity largely depends on the hydrogen donating ability of phenolic compounds and the phenoxy radicals thus formed are stabilized by resonance or intramolecular hydrogen bonding. The free radical scavenging activity was suggested to be enhanced by the presence of electron donating groups in the aromatic substituents. In the present case, the presence of a hindered phenolic group and the aminothiazole unit together could be responsible for the observed antioxidant potential of the studied 4-amino-(2-arylamino)-5-(3,5-di-t-butyl-4-hydroxybenzoyl)thiazoles.

CONCLUSIONS

In conclusion, hitherto unreported 4-amino-2-(arylamino)-5-(3,5-di-t-butyl-4-hydroxybenzoyl)thiazoles (5a–e) were prepared as molecular hybrids of hindered phenol and 2-aminothiazole moieties, leading to new antioxidants. Among the compounds (5a–e), [4-amino-2-(phenylamino)-5-thiazolyl](3,5-di-t-butyl-4-hyd-
roxophenyl)methanone (5a) exhibited α-glucosidase inhibition activity (IC$_{50}$ = 117.02 µM) and {4-amino-2-[(4-methylphenyl)amino]-5-thiazolyl}(3,5-di-t-butyl-4-hydroxyphenyl)methanone (5d) showed α-amylase inhibition activity (IC$_{50}$ = 283.19 µM). It was further found that {4-amino-2-[(4-methoxyphenyl)amino]-5-thiazolyl}(3,5-di-t-butyl-4-hydroxyphenyl)methanone (5b) exhibited better antioxidant activity in comparison with the activity of BHA or vitamin C. The present results suggest that the antidiabetic potential of these newly synthesized hindered phenolic aminothiazoles, which exhibit a combination of α-glucosidase and α-amylase inhibition activities, as well as antioxidant properties, warrants further investigation.

SUPPLEMENTARY MATERIAL

Analytical and spectral data of the synthesised compounds are available electronically at the pages of the journal website: http://www.shd.org.rs/JSCS/, or from the corresponding author on request.

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ИЗВОД

СТЕРНО ЗАКЛОЊЕНИ АМИНОТИАЗОЛИ – СИНТЕЗА, ИНХИБИЦИЈА АКТИВНОСТИ α-ГЛУКОЗИДАЗЕ И α-АМИЛАЗЕ И АНТИОКСИДАТИВНА АКТИВНОСТ

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У реакцији базно катализовано хетерокциклације N-арил-N’-[амино(нитроамино)-метил]тиоуреа са 2-бром-1-(2,6-ди-t-бутил-4-хидроксифенил)етаноном као производи су добијени 4-амино-2-(ариламино)-5-(3,5-ди-t-бутил-4-хидроксибензоил)тиазоли, једињења која су дизајнирана као хибридни молекули стерио захтевних фенола и 2-аминотиазола. Испитана је инхибиторна активност добијених једињења према хидролитичким ензимима угљених ћиригата. Утврђено је да [4-амино-2-(фениламино)-5-тиазолил](3,5-ди-t-бутил-4-хидроксифенил)етанон показује инхибицију активности α-глукоząдае са IC$_{50}$ = 117,02 µM, док акарбоза као стандарт показује IC$_{50}$ од 48,26 µM, а дериват [4-амино-2-[(4-метилфениламино)-5-тиазолил](3,5-ди-t-бутил-4-хидроксифенил)етанон показује добру инхибицију активности α-амилазе са IC$_{50}$ = 283,19 µM у поређењу са акарбозом (IC$_{50}$ = 531,91 µM). Такође, испитана је антиоксидативна активност стерио заклоњених фенолних тиазола, и 2-[(4-метоксифениламино)дериват показује бољу антиоксидативну активност од бутилованог хидроксинаизола у тесту са 2,2-дифенил-1-пиррилхидразилом као хватачем радикала, затим од витамина це или курумника у тесту са фери-јонима, и сличну активност као бутиловани хидроксинаизол у тесту са β-каротеном.

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REFERENCES