Synthesis of 6-fluoro-3-(4-piperidinyl)-1,2-benzisoxazole amino acid Derivatives and Anti-DNA Damaging Activity

S. R. Ranganatha, K. Vinaya, S. Chandrappa, C. S. Ananda Kumar, S. B. Benaka Prasad, D. S. Prasanna, K. S. Rangappa

Department of Studies in Chemistry, University of Mysore, Manasagangotri, Mysore -570 006, India

ABSTRACT: Series of novel 6-fluoro-3-(4-piperidinyl)-1,2-benzisoxazole L-amino acid derivatives 5(a-f) were synthesized by varying substitution at N-position of the 6-fluoro-3-(4-piperidinyl)-1,2-benzisoxazole. All the compounds 5(a-f) were characterized by 1H-NMR, IR and LC/MS spectroscopy. The compounds were tested for anti-DNA damaging property by using biophysical techniques such as agarose gel, thermal melting temperature (Tm) and ethidium bromide binding to DNA by using fluorescence spectrophotometer. The results revealed that 5c, 5b and 5d showed high activity. These observations can be related to the presence of the lysine, tryptophan and proline amino acid residues with basic amine, aromatic and heterocyclic groups respectively.

KEYWORDS: 1,2-benzisoxazole; Amino acids; Alzheimer's disease; Anti-DNA damage.

Introduction

Alzheimer’s disease (AD) is an irreversible, progressive brain disorder that occurs gradually and results in memory loss, unusual behavior, personality changes and a decline in thinking abilities. These are related to the death of brain cells and the breakdown of connection between them. Reactive oxygen species (ROS) such as super oxide, hydrogen peroxide, and hydroxyl radicals are continuously produced in aerobic organisms. Under physiological condition the level of ROS formation is in equilibrium with the antioxidant capacity. However, when the production of ROS overwhelms the cellular antioxidant capacity, oxidative stress (OS) and subsequent damages occur [1]. The OS causes oxygen radical formation with resultant neurodegeneration and possibly plaque formation in the central nervous system [2]. OS has been implicated in the pathogenesis of AD and it is characterized by enhanced lipid peroxidation in specific areas of the brain in postmortem studies [3]. The central nervous system is particularly sensitive to OS, owing to a high oxygen consumption and enrichment in polyunsaturated fatty acids, making it particularly vulnerable to lipid peroxidation. OS leading to injury has been implicated in the pathogenesis of neurodegenerative disorders including AD [4]. Oxidative damage to nucleotides causes modification of pyrimidine and purine bases [5]. The mitochondrial DNA (mtDNA) is highly susceptible to ROS induced damage because it is located in close proximity to the production site of ROS and the repair mechanisms of mtDNA are limited [6].

The chemistry of substituted 1,2-benzisoxazole amides plays an extremely important role in the field of pharmaceuticals and in medicinal fields. Compounds containing amide bond, benzisoxazoles, chromans and fluorine atom substitution can alter the chemical properties, disposition, and biological activities of drugs [7]. Many fluorinated compounds, 1,2-benzisoxazole derivatives and various amides are currently used in the treatment of diseases. There is a large amount of literature dealing with the physiological significance of free amino acids and their effects on a variety of metabolic and physiological systems. Amino acids and their metabolic and physiological ramifications are among the most investigated topics in biomedical science. The synthesis of substituted amino acids has attracted the attention of chemists due to their biological activities and the interesting structural properties of their molecules [8,9]. For example, it has been inserted into NS5B polymerase inhibitors against the hepatitis C virus [10], anti-inflammatory bradykinin B1 receptor antagonists [11], anticancer matrix metalloproteinase (MMP-12) inhibitors [12] or analgesic endomorphin-1 analogue tetrapeptides [13]. (S)-β-Phenylalanine has been applied in the synthesis of novel antibiotics [14]. Some amino acids are reported to have strong antioxidant activity in linoleic acid and methyl linolate model systems [15]. Therefore developments of new substituted amino acids are important in medicinal chemistry.
In view of above, we planned to synthesize a system of two biolabile component to give a compact structure like title compounds 5(a-f) and study their anti-DNA damaging property by using biophysical techniques such as agarose gel, thermal melting temperature (Tm) and ethidium bromide (EtBr) binding to DNA by using fluorescence spectrophotometer.

**Results and Discussion**

The anti-DNA damaging activity was analysed by carrying out the reaction of 6-fluoro-3-(4-piperidinyl)-1,2-benzisoxazole (4) with different L-amino acids. The synthesized 6-fluoro-3-(4-piperidinyl)-1,2-benzisoxazole analogues 5(a-f) were evaluated for their anti-DNA damaging activity by using biophysical techniques such as agarose gel, thermal melting temperature (Tm) and ethidium bromide binding to DNA by using fluorescence spectrophotometer.

All the synthesized compounds 5(a-f) protected DNA damage induced by 1 mM FeSO₄ and 10 mM ascorbic acid in agarose gel (Fig 2). The thermal melting temperature study (Table 1 and Fig 1) showed increase in stability of the DNA in the order 5c (83 °C) > 5b (82 °C) > 5d (81 °C) > 5a (78 °C) > 5e (76 °C) > 5f (75 °C) > control (74 °C) < DNA alone (84 °C). The ethidium bromide binding studies showed that 5c treated DNA showed less number of ethidium bromide /base pair (EtBr/bp) value almost nearing to DNA alone followed by 5b and 5d treated. Number of EtBr/bp value is negatively correlated with the extent of DNA damage. The range of EtBr/bp is in the order DNA alone < 5c < 5b < 5d < 5a < 5e < 5f < Control.

The results showed that 5c protected better, the DNA damage induced by free radicals generated by the 1 mM FeSO₄ and 10 mM ascorbic acid and this is followed by 5b, 5d, 5a, 5e and 5f. This data gives an insight into the understanding of DNA damage and its implications in the degenerative disorders, which generate free radicals as a consequence of OS and that could be prevented by the use of 5(a-f).

**Table 1.** Chemical structures, sample codes, temperature values of calf thymus DNA (CDNA) interacted with ascorbic acid + FeSO₄ and scatchard plot values of the compounds 5(a-f). (Legend is listed in the figure 3)

<table>
<thead>
<tr>
<th>Compound</th>
<th>R</th>
<th>Sample</th>
<th>Tm in °C</th>
<th>No.ethidium bromide/base pair</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>-</td>
<td>(A) CDNA+AA+FeSO₄</td>
<td>74</td>
<td>0.017</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>(B) CDNA alone</td>
<td>84</td>
<td>0.004</td>
</tr>
<tr>
<td>5a</td>
<td></td>
<td>(F)CDNA+AA+FeSO₄+5a</td>
<td>78</td>
<td>0.0148</td>
</tr>
<tr>
<td>5b</td>
<td></td>
<td>(C)CDNA+AA+FeSO₄+5b</td>
<td>82</td>
<td>0.0095</td>
</tr>
<tr>
<td>5c</td>
<td></td>
<td>(D)CDNA+AA+FeSO₄+5c</td>
<td>83</td>
<td>0.007</td>
</tr>
<tr>
<td>5d</td>
<td></td>
<td>(E)CDNA+AA+FeSO₄+5d</td>
<td>81</td>
<td>0.0125</td>
</tr>
<tr>
<td>5e</td>
<td></td>
<td>(G)CDNA+AA+FeSO₄+5e</td>
<td>76</td>
<td>0.015</td>
</tr>
<tr>
<td>5f</td>
<td></td>
<td>(H)CDNA+AA+FeSO₄+5f</td>
<td>75</td>
<td>0.015</td>
</tr>
</tbody>
</table>
The brain is the most aerobically active organ in the body due to its high metabolic requirements. The brain accounts for 2% of total body mass yet consumes 20% of total oxygen in a resting individual. Therefore, it is imperative to maintain oxidative balance and control in the brain, and this is tightly regulated by antioxidants that are present in vastly higher amounts in brain than in any other organ. Therefore alterations in normal oxidative metabolism as observed in AD brain provide strong evidence that oxidative stress plays an important role in AD pathogenesis.

As a general principle, the chemical origin of the majority of ROS is the reaction of molecular oxygen with the redox active metals Cu and Fe. The ability of these metal ions to occupy multiple valence states and undertake facile redox cycling, thereby activating molecular oxygen, has been utilized by a variety of enzymes including ceruloplasmin, cytochrome C oxidase [16] and amine oxidases [17]. However, unregulated redox-active metals react inappropriately with oxygen to generate ROS. Antioxidant defense mechanisms include removal of O2, scavenging reactive oxygen/nitrogen species or their precursors, inhibition of ROS formation, binding of metal ions needed for the catalysis of ROS generation and up-regulation of endogenous antioxidant defenses. The protective efficacy of antioxidants depends on the type of ROS generated, the place of generation (body barriers such as the blood brain barrier reduce the permeability of most antioxidants) and the severity of the damage [18].

OS has been implicated in the pathogenesis of AD by the finding of several characteristics, such as enhanced lipid peroxidation in specific areas of the brain in postmortem studies [19]. Moreover evidence has been provided [20] for the hypothesis that β-amyloid protein, the major constituent of the senile plaque, is neurotoxic and that such toxicity is mediated by ROS in vitro in a transgenic mouse model of AD. Hence, the use of antioxidants has proven to be a promising approach for slowing progression of AD by inhibiting oxidative stress damage linked to cognitive and functional decline.

All amino acid residues are susceptible to oxidation by *OH. However, the products formed in the oxidation of some residues have not been fully characterized [21]. The potent anti-DNA damaging activity exhibited by 5c may be due to the presence of lysine residue. Oxidation of the side-chains of lysine, residues yield carbonyl derivatives. In this case the lysine residue is assumed to be the target for Fe(II) catalyzed oxidation. According to this mechanism, the chelate complex formed by the binding of Fe(II) to the amino group of lysine can react with hydrogen peroxide to generate a hydroxyl radical that will preferentially attack the lysine moiety leading to its conversion to a 2-amino-adipic-semialdehyde residue [22]. This may lead to potent anti-DNA damaging activity as shown by the compound. The inhibitory activity of 5b might be due to the presence of tryptophan residue. These aromatic amino acid residues are prime targets for oxidation by various forms of ROS. Tryptophan residues are converted to the 2-, 4-, 5-, 6-, or 7-hydroxy derivatives, and also to N-formylkynurenine and kynurenine [23]. Potent inhibition was shown by 5d due to...
the presence of proline residue. This contains heterocyclic moiety, which is susceptible to react with ROS leading to 2-pyrrolidone derivatives [24]. The remaining 5a, 5e and 5f have shown less anti-DNA damaging activity as they contain aliphatic side chains when compared to those of cyclic and aryl amino acid residues.

Fig 2. Agarose gel showing calf thymus DNA incubated with DNA damage protection compounds 5(a-f) against free radical induced DNA damage. Legend: M 100 bp marker. 3 µg of calf thymus DNA was pre-incubated with 5 µg compounds 5(a-f) for 15 min at 37 °C. Then the above mixture was incubated with 1 mM FeSO4 and 10 mM ascorbic acid in 10 mM Tris-cl (pH 7.4) for 30 min and the sample was loaded on to 1.5 % agarose gel. The run was at 50 volts for 4 hr and then the gel was stained with ethidium bromide for 30 min in cold water and then the gel was destained using water and observed under UV gel documentation system.
Conclusion

In summary, we synthesized novel N-substituted amino acid derivatives of the compound (4) and tested their in vitro anti-DNA damaging agent. Compounds 5b, 5c and 5d are found more potent than 5a, 5e and 5f. These observations can be related to the presence of tryptophan, lysine and proline amino acid residues with aromatic, basic amine and heterocyclic groups respectively. These results provide promising information for further development of potent anti-DNA damaging agents, which in turn can reduce the cause of AD. Hence, there is a need for further investigations to understand the features underlying the anti-DNA damaging activities of these new derivatives. Further work is under progress.

Experimental

Infrared (IR) spectra were recorded using a Jasco FTIR-4100 series instrument. Nuclear magnetic resonance (1H NMR) spectra were recorded on a Bruker AM-400 and chemical shifts are expressed in parts per million (ppm, for δ) relative to tetramethyl silane as an internal standard and DMSO-d6 as solvent. Spin multiplets are given as s (singlet), d (doublet), t (triplet), q (quartet) and m (multiplet). Mass and purity were recorded on a LC-MSD-Trap-XCT. Silica gel column chromatography was done using Merck 7734 silica gel (60-120 mesh) and Merck made TLC plates. All the reagents and chemicals used were purchased from Sigma Aldrich Chemicals Pvt Ltd.

Chemistry

The reaction of isonipecotic acid (1) with mixed anhydride at 0-5 °C for 14 h resulted the intermediate product N-formylisonipecotic acid. This reaction was found to be novel and easy deprotectable group for (1). The second step was Friedel-Crafts acylation reaction. We stabilized this route to obtain good yield consistently in large amount. Since the lot wise addition of aluminium chloride to the reaction mass at 0-5 °C as formed a sticky mass with poor yield. So used Friedel-Crafts acylation process by adding acid chloride to the mixture of 1,3-difluorobenzene and aluminium chloride in N,N-dimethyl foramide as a solvent at 0-5 °C, which is a feasible process for the large-scale synthesis of (3). 6-fluoro-3-(4-piperidinyl)-1,2-benzisoxazole hydrochloride (4) was obtained in one step from the key intermediate 2,4-difluorobenzoyl-4-piperidine (3), which involved hydroxylamine sulfate/powdered potassium hydroxide mediated, in situ generated oxime formation and its subsequent internal cyclisation followed by alkaline hydrolysis of the protected simultaneously, piperidinyl group which was reported for the first time from our laboratory [25].

This is a simple and efficient method for large-scale synthesis of 6-fluoro-3-(4-piperidinyl)-1,2-benzisoxazole. The progress of the reaction was monitored by TLC. After completion of the reaction, the residue was taken in H2O and extracted with ethyl acetate and dried over anhydrous Na2SO4. Pure compounds were obtained by column chromatography using hexane/ethyl acetate (8:2) as an eluent. All the compounds were characterized by 1H-NMR spectroscopy and LC/MS.

General procedure for the synthesis of 6-fluoro-3-(piperidin-4-yl)benzisoxazole L-amino acid derivatives 5(a-f)

A solution of different N-Boc-amino acids (1.2 eq) in absolute tetrahydrofuran (THF) (20 ml) was cooled to 0 °C and isobutyl chloroformate (1.5 eq) was added, stirred for 10 min. N- methyl morpholine (5.0 eq) was added to the cool reaction mixture then added 6-fluoro-3-(4-piperidinyl)-1,2-benzisoxazole (4) (1.0 eq) in dry THF. The reaction mixture was allowed at room temperature with stirring for 8-10 hr. The progress of the reaction
mixture was monitored by TLC (Hexane:Ethyl acetate 8:2). Upon completion, the solvent was removed under reduced pressure and residue was taken in water and extracted with ethyl acetate. The organic layer was washed with saturated solution of sodium bicarbonate and 1% hydrochloric acid and finally water wash was given to organic layer and dried with anhydrous sodium sulphate. The solvent was evaporated to get crude product which was purified by column chromatography over silica gel (60-120 mesh) using hexane: ethyl acetate (8:2) as an eluent. All the synthesized compounds 5(a-f) were obtained in good yield and the schematic representation of the synthesized compounds is shown in Scheme 1. The yield and chemical structures of the derivatives are shown in Table 1. A cooled solution of 6-fluoro-3-(piperidin-4-yl)benzisoxazole L-amino acid (2 mmol) in dry dichloromethane (5 ml) was treated with a cooled (0 °C) dry HCl in ether solution (3 M, 10 ml). The reaction mixture was stirred for 0.5-1 h (completion of the reaction was determined by TLC), treated with absolute ether (100 ml), and held at 0 °C for 1 h. The precipitate was filtered off and dried under vacuum to yield hydrochlorides. Hydrochlorides when neutralized yielded free amine derivative compounds.

**Fig 1.** Reagents and conditions: (i). Ac₂O, HCOOH, iso-propylalcohol. (ii). SOCl₂, methylene dichloride, N,N-dimethylformamide, AlCl₃, 1,3-difluorobenzene. (iii). (NH₂OH)₂.H₂SO₄, KOH, MeOH/H₂O. (iv). L-Boc-amino acids, isobutyl chloroformate, N-methyl morpholine, terahedrofuran.

**Synthesis of 2-aminol-(4-(6-fluorobenzisoxazol-3-yl)piperidin-1-yl)propan-1-one (5a)**

The product obtained from 6-fluoro-3-(4-piperidinyl)-1,2-benzisoxazole (4) (0.2 g, 0.9081 mmol), isobutyl chloroformate (0.1852 g, 1.3622 mmol), N-methyl morpholine (0.4585 g 4.54 mmol), N-Boc-alanine (0.205 g, 1.089 mmol) and dry HCl in ether was white solid. Yield: 79%. ¹H-NMR (DMSO-d₆, 400 MHz) δ: 8.05 (q, 1H, Ar-H), 7.69 (dd, 1H, Ar-H), 7.27 (t, 1H, Ar-H), 4.09 (dd, 2H, -CH₂), 3.80 (m, 1H, -CH), 3.01 (br s, 2H, -NH₂), 2.60 (m, 1H, -CH), 1.89 (m, 2H, -CH₂), 1.70 (m, 2H, -CH₂), 1.35-1.14 (m, 2H, -CH₂), 1.09 (br s, 3H, -CH₃). IR (KBr, cm⁻¹): 3541, 2860, 1671, 1430, 873, 615 cm⁻¹. MS (ESI) m/z: 306.35 (M+H⁺).

**Synthesis of 2,6-diamino-1-(4-(6-fluorobenzisoxazol-3-yl)piperidin-1-yl)hexan-1-one (5c)**

The product obtained from 6-fluoro-3-(4-piperidinyl)-1,2-benzisoxazole (4) (0.2 g, 0.9081 mmol), isobutyl chloroformate (0.1852 g, 1.3622 mmol), N-methyl morpholine (0.4585 g, 4.54 mmol), N-Boc-tryptophan (0.3316 g, 2.43 mmol) and dry HCl in ether was white solid. Yield: 84%. ¹H-NMR (DMSO-d₆, 400 MHz) δ: 10.83 (br s, 1H, -NH), 8.03 (m, 1H, Ar-H), 7.68 (dd, 1H, Ar-H), 7.51 (d, 1H, Ar-H), 7.26-6.95 (m, 5H Ar-H), 4.68 (d, 2H, -CH₂), 4.40 (t, 1H, -CH), 4.09 (dd, 2H, -CH₂), 3.80 (m, 1H, -NH₂), 3.04 (br s, 2H, -NH₂), 3.04-2.94 (m, 2H, -CH₂), 2.79 (m, 2H, -CH₂), 1.87-1.65 (m, 2H, -CH₂), IR (KBr, cm⁻¹): 3550, 2144, 1650, 1460, 869, 612 cm⁻¹. MS (ESI) m/z: 421.48 (M+H⁺).

**Synthesis of 2,6-diamino-1-(4-(6-fluorobenzisoxazol-3-yl)piperidin-1-yl)hexan-1-one (5e)**

The product obtained from 6-fluoro-3-(4-piperidinyl)-1,2-benzisoxazole (4) (0.2 g, 0.9081 mmol), isobutyl chloroformate (0.1852 g, 1.3622 mmol), N-methyl morpholine (0.4585 g, 4.54 mmol), N-Boc-Lysine (0.427 g, 1.0897 mmol) and dry HCl in ether was white solid. Yield: 80%. ¹H-NMR (DMSO-d₆, 400 MHz) δ: 8.02(d,
Synthesis of (4-(6-fluorobenzisoxazol-3-yl)piperidin-1-yl)benzaldehyde (5e)
The product obtained from 6-fluoro-3-(4-piperidinyl)-1,2-benzisoxazole amino acid (4) (0.2 g, 0.9081 mmol), isobutyl chloroformate (0.1852 g, 1.3622 mmol), N-methyl morpholine (0.4585 g, 4.54 mmol), N-Boc-proline (0.2345 g, 1.091 mmol) and dry HCl in ether was white solid. Yield: 79%. \(^{1}\)H-NMR (DMSO-d\(_6\), 400 MHz) \(\delta\): 7.96 (m, 1H, Ar-H), 7.58 (d, 1H, Ar-H), 7.45 (d, 1H, Ar-H), 4.10 (d, 2H, -CH-CH\(_2\)), 3.80 (m, 1H, -CH), 3.01 (br s, 2H, -NH\(_2\)), 2.25 (t, 1H, -CH), 2.20-2.10 (m, 4H, -CH\(_2\)), 1.80-1.95 (m, 2H, -CH\(_2\)), 1.75 (m, 1H, -CH\(_3\)), 1.10 (d, 3H, -CH\(_3\)), 1.09 (d, 3H, -CH\(_3\)). IR (KBr, cm\(^{-1}\): 3389, 2143, 1462, 1376, 845, 723 MS (ESI) m/z: 363.34 (M+H\(^+\)).

Synthesis of 2-aminoo-1-(4-(6-fluorobenzisoxazol-3-yl)piperidin-1-yl)-3-methylbutan-1-one (5d)
The product obtained from 6-fluoro-3-(4-piperidinyl)-1,2-benzisoxazole amino acid (4) (0.2 g, 0.9081 mmol), isobutyl chloroformate (0.1852 g, 1.3622 mmol), N-methyl morpholine (0.4585 g, 4.54 mmol), N-Boc-valine (0.2155 g, 1.091 mmol) and dry HCl in ether was white solid. Yield: 79%. \(^{1}\)H-NMR (DMSO-d\(_6\), 400 MHz) \(\delta\): 7.96 (m, 1H, Ar-H), 7.58 (m, 1H, Ar-H), 7.45 (m, 1H, Ar-H), 4.10 (d, 2H, -CH-CH\(_2\)), 3.80 (m, 1H, -CH), 3.01 (br s, 2H, -NH\(_2\)), 2.25 (t, 1H, -CH), 2.20-2.10 (m, 4H, -CH\(_2\)), 1.80-1.95 (m, 2H, -CH\(_2\)), 1.75 (m, 1H, -CH\(_3\)), 1.10 (d, 3H, -CH\(_3\)), 1.09 (d, 3H, -CH\(_3\)). IR (KBr, cm\(^{-1}\): 3389, 2143, 1462, 1376, 845, 723 MS (ESI) m/z: 320.37 (M+H\(^+\)).

Ethidium bromide binding studies: Ethidium bromide (EtBr) bound in moles per base pair of calf thymus DNA was measured with 3 µg of calf thymus DNA was pre-incubated with 5 µm compounds 5(a-f) for 15 min at 37 °C. The compounds 5(a-f) were pre-incubated with calf thymus DNA for 15 min before adding FeSO\(_4\) and ascorbic acid. Then the incubated sample was used to record the Tm starting from 45 °C to 95 °C with temperature rise of 1 °C every minute using spectrophotometer fitted with thermo regulator. The temperature at which, there was a 50% hyperchromic shift was calculated as melting temperature.

Agarose gel studies: calf thymus DNA (3 µg) was pre-incubated with 5 µg compounds 5(a-f) for 15 min at 37 °C. Then above mixture was incubated with 1 mM FeSO\(_4\) and 10 mM ascorbic acid in 10 mM Tris-cl (pH 7.4) for 30 min and the sample was loaded on to 1.5 % agarose gel. The run was at 50 volts for 4 h and then the gel was stained with ethidium bromide for 30 min in cold water and then the gel was destined using water and observed under UV gel documentation system.

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Ethidium bromide, Tris-Cl, glacial acetic acid, EDTA and HEPES were purchased from SRL, India.
The concentration of free dye (Cf) was then calculated by using the formula

\[ Cf = Co - Cb \]

Where, Cf, Co and Cb are in pmoles. The amount of bound ethidium bromide per base pair was calculated by \( r = Cb \) (pmoles)/DNA concentration (pmoles) of base pair.

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References