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In vitro effect of novel β -lactam compounds on xanthine oxidase enzyme activity

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Abstract

Carbazole substituted imines (**2a-l**) were prepared from N-methyl-3-amino carbazole with different aldehydes. The imines compounds undergo (2 + 2) cycloaddition reactions with in situ ketenes to produce β -lactam compounds (**3a-l**). The β -lactam compounds were tested as inhibitors of the xanthine oxidase (XO) purified from bovine milk. The results show that these compounds exhibit inhibitory effects on XO at low concentrations with IC_{50} values ranging from 21.65 to 58.04 μ M. The most effective compound for XO was 4-(4-chlorophenyl)-1-(9-ethyl-9H-carbazol-3-yl)-3-phenylazetidin-2-one with IC_{50} of 21.65 μ M. The lactams investigated here showed effective XO inhibitory effects, in the same range as the clinically used allopurinol.

Keywords: Xanthine oxidase, inhibition, purification, β -lactam compounds

Introduction

Enzyme inhibitors can interact with enzymes and block their activity towards natural substrates. The importance of enzyme inhibitors as drugs is enormous since these molecules have been used for treating a number of pathophysiological conditions Banner and Hadvary 1991, Sandstrom 1989). Xanthine oxidase (XO; EC 1.2.3.22) is situated at the end of a catabolic sequence of the purine nucleotide metabolism in humans and a few other uricotelic species. Its major function is to catalyze the oxidation of hypoxanthine to xanthine and that of xanthine to uric acid (Harris et al. 1999). XO is widely distributed throughout various organs including the liver, gut, lung, kidney, heart, and brain, as well as the plasma (Sathisha et al. 2011). Xanthine oxidase inhibitors (XOI) are very useful, since they possess fewer side-effects compared to uricosuric and anti-inflammatory agents. Allopurinol (1H-pyrazolo [3,4-d] pyrimidin-4-ol) is

a substrate and a specific potent inhibitor for XO, and has been used for gout treatment for a number of years. The synthetic analogues of allopurinol, pyrazolopyrimidine, 1-phenylpyrazoles, and cytokines are also used as XO inhibitors (Tamta et al. 2005, Ishibuchi et al. 2001). Inhibition mechanism and molecular modeling studies of these analogues have also been conducted by scientists (Sheu et al. 1997, Tamta et al. 2006). Recently, several studies have indicated that allopurinol may induce hypersensitivity syndrome and Stevens–Johnson syndrome in patients (Hammer et al. 2001, Bashir et al. 2000, Hsieh et al. 2007).

The β -lactam structures known from penicillin and cephalosporin have been well developed as miracle drugs for the therapy of bacterial infectious diseases in clinics (Georg and Ravikumar 1992). Apart from clinical use as antibacterial agents, these compounds serve as synthons in the synthesis of other biological active heterocyclic (Ojima 1995), and in many examples the biological activity can be interpreted as an interaction between the β -lactam and a serine-containing enzyme (serine protease) like elastase (Clemente et al. 2001, Elriati et al. 2008). Therefore, the search for clinically useful β -lactams is a growing field of interest. In this study, we describe a number of carbazole groups containing β -lactams and study their properties as inhibitors of XO purified from bovine milk.

Experimental

Instruments

Melting points of the compounds were measured using an Electro thermal 9100 apparatus. ¹H and ¹³C NMR spectra were recorded using a Varian 300 MHz Mercury Plus instrument using TMS as internal standards.

Materials

Sephacrose 4B, L-tyrosine, benzamidine, protein assay reagents, and chemicals for electrophoresis were obtained

from Sigma Chem. Co. All other chemicals obtained from Sigma and Merck were used without further purification.

Enzyme purification

Fresh bovine milk, without added preservatives, was cooled down to 4 °C overnight. EDTA and toluene were then added to give final concentrations of 2 mM and 3% (v/v), respectively. The milk was churned with a blender at top speed for 30 min at room temperature. During this time, the temperature rose from 4 to 45 °C. After cooling the churned milk to about 4 °C, the curdling process was repeated and the sample was filtered. This sample was brought to 38% saturation by addition of solid ammonium sulphate (Özer et al. 1999). The suspension was centrifuged at 15000 rpm for 30 min and the precipitate formed was discarded. The supernatant was brought to 50% saturation with solid ammonium sulphate. The precipitate formed was collected by centrifugation at 15000 rpm for 60 min and dissolved in 0.1 M Tris-HCl, pH = 7.6. The affinity column containing sepharose-4B-L-tyrosine-p-amino benzamidine was equilibrated in 0.1 M glycine, 0.1 M NaCl, pH = 9.0 (Beyaztaş and Arslan 2011). The sample was applied to the affinity gel, washed with 0.1 M glycine, pH = 9.0, and then eluted with 25 mM benzamidine in 0.1 M glycine, 0.1 M NaCl, pH = 9.0 (McManaman et al. 1996). Fractions of 1.5 mL were collected and their absorbance measured at 280 nm.

Activity measurements

Xanthine oxidase activity was determined at 37 °C by the modified method of Massey et al. (1969). The conversion of xanthine to uric acid was followed by monitoring the change in absorbance at 295 nm, using a CARY 1E, UV-Visible Spectrophotometer-VARIAN spectrometer ($\epsilon_{292} = 9.5 \text{ mM}^{-1} \text{ cm}^{-1}$). The reaction mixture contained 50 mM Tris-HCl, pH = 7.6, and 0.15 mM xanthine, at 37 °C. The assay was initiated by the addition of the enzyme. One unit of enzyme activity was defined as the amount of enzyme that converts one μmol of xanthine to uric acid per min under defined conditions (Özer et al. 1999).

In vitro inhibition kinetic studies

For the inhibition studies of some β -lactams derivatives, different concentrations were added to the enzyme activity. XO enzyme activity with β -lactams derivatives was assayed by following the oxidation of xanthine. Activity % values of xanthine oxidase for six different concentrations of β -lactams derivatives were determined by regression analysis using Microsoft Office 2000 Excel. Xanthine oxidase activity without a β -lactam was accepted as 100% active. The graphs determined that the inhibitor concentration caused up to 50% inhibition (IC_{50} values) on the enzyme (Isik et al. 2009).

Total protein determination

Quantitative protein determination was achieved by absorbance measurements at 595 nm according to the Bradford (1976) method, with bovine serum albumin as a standard.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

Sodium Dodecyl Sulphate (SDS) polyacrylamide gel electrophoresis was performed after having a purified enzyme. It was carried out in 10% and 3% acrylamide-bisacrylamide concentrations for the running and stacking gel, respectively, containing 0.1% SDS according to the Laemmli (1970) method. The sample was applied to the electrophoresis medium. Gel was stained overnight in 0.1% Coomassie Brilliant Blue R-250 in 50% methanol and 10% acetic acid, and then destained by frequently changing the same solvent, without using dye.

General procedure for the synthesis of imines. 14.26 mmol of 3-amino-9-ethylcarbazole were dissolved in ethanol (30 ml) and added to 14.26 mmol Na_2CO_3 and 14.26 mmol aldehydes derivatives. The reaction mixture was then refluxed for 24 hours at 90 °C. The solution was extracted with AcOEt with an aqueous phase. The organic phase was washed three times with water, dried with anhydrous magnesium sulphate, and evaporated under reduced pressure.

N-benzylidene-9-ethyl-9H-carbazol-3-amine (2a, $\text{C}_{21}\text{H}_{18}\text{N}_2$). Yield was 96%, recrystallized from ether/hexane to give light-yellow crystals. M.p.: 96.5 °C; ^1H NMR (300 MHz, CDCl_3): 8.65 (1H,s), 8.12-8.10 (1H,d), 8.10-8.09 (1H,d), 8.03-8.02 (1H,d), 7.97-7.96 (1H,d), 7.95-7.94 (1H,t), 7.50-7.48 (1H,t), 7.47-7.46 (1H,t), 7.46-7.45 (2H,d), 7.41 (1H,s), 7.23-7.21 (2H,t), 4.37-4.32 (2H,q), 1.46-1.38 (3H,t); ^{13}C NMR (75 MHz, CDCl_3): 158.33, 144.03, 140.76, 139.05, 136.94, 131.12, 129.00(2C), 128.81(2C), 126.13, 123.71, 123.31, 120.83, 120.23, 119.12, 112.78, 109.00, 108.91, 37.95, 14.12.

N-(4-methoxybenzylidene)-9-ethyl-9H-carbazol-3-amine (2d, $\text{C}_{22}\text{H}_{20}\text{N}_2\text{O}$). Yield was 92% recrystallized from dichloromethane/hexane to give as dark-yellow crystals. M.p.: 118.4 °C; ^1H NMR (300 MHz, CDCl_3): 8.57 (1H,s); 8.12-8.11 (1H,d), 8.09 (1H,s), 7.91-7.89 (1H,d), 7.46-7.44 (1H,t), 7.43-7.40 (2H,d), 7.38-7.37 (1H,d), 7.25-7.24 (1H,d), 7.22-7.20 (1H,t), 7.01-6.98 (2H,d), 4.39-4.32 (2H,q), 3.86 (3H,s), 1.46-1.43 (3H,t); ^{13}C NMR (75 MHz, CDCl_3): 162.11, 157.87, 144.40, 140.73, 138.81, 130.46 (2C), 129.99, 126.05, 123.70, 123.32, 120.80, 120.22, 119.03, 114.41 (2C), 112.55, 108.96, 108.87, 55.66, 37.93, 14.12.

N-(3-methoxybenzylidene)-9-ethyl-9H-carbazol-3-amine (2e, $\text{C}_{22}\text{H}_{20}\text{N}_2\text{O}$). Yield was 50%, recrystallized from ether/hexane to give light-brown crystals. M.p.: 99.8 °C; ^1H NMR (300 MHz, CDCl_3): 8.66 (1H,s), 8.17-8.15 (1H,d), 8.08 (1H,s), 7.65-7.64 (1H,d), 7.54-7.52 (1H,d), 7.52-7.51 (1H,t), 7.50 (1H,s), 7.49-7.44 (1H,t), 7.44-7.43 (1H,d), 7.42-7.40 (1H,d), 7.31-7.28 (1H,t), 7.09-7.05 (1H,d), 4.39-4.32 (2H,q), 3.93 (3H,s), 1.48-1.43 (3H,t); ^{13}C NMR (75 MHz, CDCl_3): 160.28, 158.16, 143.91, 140.78, 139.10, 138.44, 129.99, 126.19, 123.74, 123.34, 122.40, 120.87, 120.27, 119.19, 118.08, 112.92, 111.86, 109.06, 108.98, 55.71, 37.94, 14.16.

N-(3-chlorobenzylidene)-9-ethyl-9H-carbazol-3-amine (2g, $\text{C}_{21}\text{H}_{17}\text{ClN}_2$). Yield was 99%, recrystallized from ether/

dichloromethane/hexane to give yellow crystals. M.p.: 102.5 °C; ^1H NMR (300 MHz, CDCl_3): 8.62 (1H,s), 8.14-8.13 (1H,d), 8.05-8.04 (1H,d), 8.00-7.79 (1H,d), 7.79-7.78 (1H,d), 7.51-7.50 (1H,t), 7.49-7.48 (1H,t), 7.48-7.47 (1H,d), 7.43 (1H,s), 7.43-7.41 (1H,d), 7.26-7.23 (1H,t), 7.25 (1H,s), 4.39-4.35 (2H,q), 1.48-1.43 (3H,t); ^{13}C NMR (75 MHz, CDCl_3): 156.25, 143.33, 140.77, 139.28, 138.75, 135.18, 130.94, 130.22, 128.30, 127.12, 126.23, 123.73, 123.26, 120.84, 120.21, 119.24, 112.99, 109.06, 108.96, 37.97, 14.12.

N-(3-nitrobenzylidene)-9-ethyl-9H-carbazol-3-amine (2i, $\text{C}_{21}\text{H}_{17}\text{N}_3\text{O}_2$). Yield was 98%, recrystallized from ether/dichloromethane/hexane to give orange crystals. M.p.: 144.2 °C; ^1H NMR (300 MHz, CDCl_3): 8.76 (1H,s), 8.74 (1H,s), 8.27-8.25 (1H,d), 8.25-8.24 (1H,d), 8.09-8.06 (1H,d), 7.64-7.61 (1H,t), 7.53-7.51 (1H,t), 7.51-7.49 (1H,d), 7.48-7.46 (1H,d), 7.42 (1H,s), 7.27-7.25 (1H,d), 7.25-7.22 (1H,t), 4.39-4.32 (2H,q), 1.46-1.40 (3H,t); ^{13}C NMR (75 MHz, CDCl_3): 154.35, 148.91, 142.59, 140.81, 139.57, 138.69, 133.98, 129.90, 126.36, 125.15, 123.76, 123.39, 123.24, 120.84, 120.26, 119.38, 113.29, 109.14, 109.04, 37.98, 14.11.

N-(2-nitrobenzylidene)-9-ethyl-9H-carbazol-3-amine (2j, $\text{C}_{21}\text{H}_{17}\text{N}_3\text{O}_2$). Yield was 90%, recrystallized from ether/dichloromethane/hexane to give red crystals. M.p.: 135.4 °C; ^1H NMR (300 MHz, CDCl_3): 9.13 (1H,s), 8.41-8.39 (1H,d), 8.15-8.12 (1H,d), 8.11 (1H,s), 8.06-8.03 (1H,d), 7.74-7.71 (1H,t), 7.57-7.56 (1H,d), 7.54-7.53 (1H,t), 7.50-7.48 (1H,d), 7.43-7.40 (1H,d), 7.30-7.27 (1H,t), 7.26-7.24 (1H,t), 4.39-4.32 (2H,q), 1.47-1.42 (3H,t); ^{13}C NMR (75 MHz, CDCl_3): 152.75, 149.42, 142.86, 140.80, 139.63, 133.71, 131.81, 130.82, 129.76, 126.33, 124.77, 123.74, 123.27, 120.93, 120.35, 119.39, 113.87, 109.13, 109.02, 37.96, 14.12.

N-(2-bromobenzylidene)-9-ethyl-9H-carbazol-3-amine (2k, $\text{C}_{21}\text{H}_{17}\text{BrN}_2$). Yield was 80%, recrystallized from dichloromethane/hexane to give dark-green crystals. M.p.: 120.2 °C; ^1H NMR (300 MHz, CDCl_3): 9.07 (1H,s), 8.35-8.32 (1H,d), 8.17-8.10 (1H,d), 8.09 (1H,s), 7.66-7.64 (1H,d), 7.56-7.53 (1H,t), 7.51-7.48 (1H,t), 7.48-7.45 (1H,d), 7.44-7.42 (1H,t), 7.31-7.28 (1H,t), 7.27-7.25 (1H,d), 4.39-4.34 (2H,q), 1.46-1.44 (3H,t); ^{13}C NMR (75 MHz, CDCl_3): 156.81, 143.63, 140.80, 139.35, 135.32, 133.44, 132.11, 129.09, 127.96, 126.23, 126.09, 123.77, 123.33, 120.92, 120.32, 119.25, 113.42, 109.06, 108.96, 37.97, 14.12.

N-(4-fluorobenzylidene)-9-ethyl-9H-carbazol-3-amine (2l, $\text{C}_{21}\text{H}_{17}\text{FN}_2$). Yield was 99%, recrystallized from methanol to give yellow-green crystals. M.p.: 120.4 °C; ^1H NMR (300 MHz, CDCl_3): 8.03-8.01 (1H,d), 7.48-7.46 (2H,d), 7.45 (1H,s), 7.45-7.44 (1H,t), 7.43-7.38 (1H,d), 7.37 (1H,s), 7.25-7.22 (1H,t), 7.21-7.19 (2H,d), 7.18-7.16 (1H,d), 6.95-6.92 (1H,d), 4.34-4.27 (2H,q), 1.43-1.38 (3H,t); ^{13}C NMR (75 MHz, CDCl_3): 140.64, 139.08, 134.79, 125.71 (2C), 123.89, 122.68, 120.69 (2C), 118.27 (2C), 115.85 (2C), 109.27 (2C), 108.63 (2C), 106.65 (2C), 37.76, 14.11.

General procedure for the synthesis of β -lactams

A solution of Et_3N in benzene was added drop-wise to a hot solution of imines and phenylacetylchlorid in benzene with

continuous stirring. The reaction mixture was then refluxed at 85 °C for 24 hours. The solution was extracted with benzene with an aqueous phase. Organic phase was washed three times with water, dried with anhydrous magnesium sulphate, and evaporated under reduced pressure. 3a, 3b, 3d, 3e, and 3f were purified by recrystallization from acetone and other β -lactams were purified by column chromatography over silica gel using AcOEt /hexane as eluents followed by recrystallization from acetone.

1-(9-ethyl-9H-carbazol-3-yl)-3,4-diphenylazetididin-2-one (3a, $\text{C}_{29}\text{H}_{24}\text{N}_2\text{O}$). Yield was 54%, giving light-yellow crystals. M.p.: 184 °C; ^1H NMR (300 MHz, CDCl_3): 8.16 (1H,s), 8.03-8.00 (1H,d), 7.50-7.48 (1H,d), 7.49-7.48 (1H,t), 7.47-7.45 (2H,t), 7.44-7.43 (1H,t), 7.41-7.40 (2H,d), 7.40-7.38 (2H,d), 7.37-7.37 (1H,d), 7.37-7.36 (2H,t), 7.36-7.35 (1H,d), 7.34-7.33 (1H,t), 7.21-7.19 (1H,t), 5.157-5.154 (1H,d), 4.346-4.321 (2H,q), 4.297-4.279 (1H,d), 1.607-1.601 (3H,t); ^{13}C NMR (75 MHz, CDCl_3): 165.45, 140.66, 138.11, 137.07, 135.34, 130.19, 129.53 (2C), 129.28 (2C), 128.85 (2C), 128.10 (2C), 127.82, 126.29 (2C), 123.22, 122.77, 121.01, 119.01, 116.17, 109.68, 108.94, 108.80, 65.24, 64.36, 37.82, 14.05.

1-(9-ethyl-9H-carbazol-3-yl)-3-phenyl-4-*p*-tolylazetididin-2-one (3b, $\text{C}_{30}\text{H}_{26}\text{N}_2\text{O}$). Yield was 71%, giving white crystals. M.p.: 190.5 °C; ^1H NMR (300 MHz, CDCl_3): 7.60 (1H,s), 7.46-7.44 (1H,d), 7.16-7.25 (1H,t), 6.95-6.94 (2H,d), 6.93-6.91 (1H,t), 6.9-6.90 (2H,d), 6.90-6.88 (2H,t), 6.87-6.85 (2H,d), 6.84-6.83 (1H,t), 6.81-6.80 (2H,d), 6.71-6.68 (1H,d), 4.60-4.59 (1H,d) ($J = 2.35$), 3.83-3.80 (2H,q), 3.78-3.77 (1H,d), 1.83 (3H,s), 0.87-0.83 (3H,t); ^{13}C NMR (75 MHz, CDCl_3): 164.92, 140.12, 138.16, 136.47, 134.93, 134.47, 129.75 (2C), 129.66, 128.86 (2C), 127.68 (2C), 127.38, 126.04 (2C), 125.94, 122.46, 122.04, 120.35, 118.67, 115.90, 109.07, 108.84, 108.67, 64.70, 63.41, 37.33, 21.05, 13.69.

1-(9-ethyl-9H-carbazol-3-yl)-3-phenyl-4-*m*-tolylazetididin-2-one (3c, $\text{C}_{30}\text{H}_{26}\text{N}_2\text{O}$). Yield was 82%, giving light-brown crystals. M.p.: 196.8 °C; ^1H NMR (300 MHz, CDCl_3): 8.14 (1H,s), 8.13, 7.95 (1H,d), 7.50-7.48 (2H,t), 7.48-7.46 (1H,d), 7.42-7.41 (1H,t), 7.40-7.39 (2H,d), 7.37 (1H,s), 7.33-7.32 (1H,t), 7.30-7.28 (1H,t), 7.28-7.26 (1H,t), 7.24-7.21 (1H,d), 7.15-7.12 (1H,d), 7.11-7.10 (1H,d), 7.10-7.09 (1H,d), 5.29-5.28 (1H,d) ($J = 2.34$), 4.37-4.36 (1H,d), 4.33-4.31 (2H,q), 2.26 (3H,s), 1.26-1.23 (3H,t); ^{13}C NMR (75 MHz, CDCl_3): 165.47, 140.66, 138.97, 138.49, 137.00, 135.77 (2C), 130.10, 129.81, 129.57, 129.52 (2C), 128.26 (2C), 127.49, 126.75, 124.20, 122.73, 122.35, 120.98, 119.34, 116.80, 110.09, 109.80, 109.76, 64.71, 63.14, 37.66, 21.74, 14.32.

1-(9-ethyl-9H-carbazol-3-yl)-4-(4-methoxyphenyl)-3-phenylazetididin-2-one (3d, $\text{C}_{30}\text{H}_{26}\text{N}_2\text{O}_2$). Yield was 36%, giving beige crystals. M.p.: 154.2 °C; ^1H NMR (300 MHz, CDCl_3): 8.15-8.15 (1H,s), 8.03-8.00 (1H,d), 7.51-7.50 (1H,d), 7.50-7.48 (1H,d), 7.48-7.47 (1H,t), 7.46-7.43 (2H,d), 7.41-7.39 (1H,t), 7.35-7.32 (2H,d), 7.31-7.26 (1H,t), 7.225-7.22

(1H,d), 7.19-7.17 (2H,t), 6.94-6.19 (2H,d), 5.03-5.02 (1H,d) ($^3J = 2.35$), 4.31-4.30 (1H,d), 4.34-4.27 (2H,q), 3.80 (3H,s), 1.41-1.36 (3H,t); ^{13}C NMR (75 MHz, CDCl_3): 165.64, 160.02, 140.62, 137.02, 135.42, 130.18, 129.90, 129.28 (2C), 128.05, 127.82 (2C), 127.60 (2C), 126.29, 123.17, 122.76, 121.03, 118.99, 116.20, 114.88 (2C), 109.72, 108.92, 108.81, 63.34, 64.10, 55.57, 37.82, 14.08.

1-(9-ethyl-9H-carbazol-3-yl)-4-(3-methoxyphenyl)-3-phenylazetididin-2-one (3e, $\text{C}_{30}\text{H}_{26}\text{N}_2\text{O}_2$). Yield was 41%, giving white crystals. M.p.: 197.2 °C; ^1H NMR (300 MHz, CDCl_3): 8.13 (1H,s), 8.12-8.02 (1H,d), 7.58-7.55 (1H,d), 7.46-7.43 (1H,t), 7.42-7.40 (1H,d), 7.40 (1H,s), 7.38-7.36 (1H,t), 7.35-7.34 (1H,d), 7.33-7.32 (1H,d), 7.31-7.28 (2H,t), 7.28-7.17 (1H,t), 7.15-7.13 (1H,t), 7.10-7.08 (2H,d), 6.90-6.87 (1H,d), 5.38-5.41 (1H,d) ($^3J = 1.73$), 4.41-4.43 (1H,d), 4.33-4.32 (2H,q), 3.65 (3H,s), 1.51-1.35 (3H,t); ^{13}C NMR (75 MHz, CDCl_3): 165.57, 160.35, 140.70, 140.24, 137.07, 135.81 (2C), 130.93, 129.98, 129.62 (2C), 128.33 (2C), 126.85, 122.68, 122.29, 121.04, 119.44, 119.27, 116.99, 114.36, 112.99, 110.33, 110.01, 109.91, 64.35, 62.84, 55.77, 37.66, 14.38.

4-(4-chlorophenyl)-1-(9-ethyl-9H-carbazol-3-yl)-3-phenylazetididin-2-one (3f, $\text{C}_{29}\text{H}_{23}\text{ClN}_2\text{O}$). Yield was 66%, giving white crystals. M.p.: 185.2 °C; ^1H NMR (300 MHz, CDCl_3): 8.125 (1H,s), 8.11-8.00 (1H,d), 7.46-7.44 (2H,d), 7.44-7.43 (2H,d), 7.42-7.41 (1H,t), 7.41-7.40 (1H,d), 7.40-7.39 (1H,t), 7.39-7.37 (2H,d), 7.37-7.36 (1H,t), 7.36-7.31 (1H,d), 7.28-7.22 (1H,d), 7.20-7.17 (2H,t), 5.05-5.04 (1H,d), 4.337-4.312 (2H,q), 4.288-4.280 (1H,d), 1.604-1.599 (3H,t); ^{13}C NMR (75 MHz, CDCl_3): 165.15, 140.68, 137.13, 136.64, 135.00, 134.66, 129.90, 129.78(2C), 129.35(2C), 128.24, 127.78(2C), 127.63 (2C), 126.40, 123.27, 122.69, 121.01, 119.09, 116.04, 109.63, 109.02, 208.85, 65.33, 63.70, 37.84, 14.04.

4-(3-chlorophenyl)-1-(9-ethyl-9H-carbazol-3-yl)-3-phenylazetididin-2-one (3g, $\text{C}_{29}\text{H}_{23}\text{ClN}_2\text{O}$). Yield was 52% giving white powder. M.p.: 128.2 °C; ^1H NMR (300 MHz, CDCl_3): 8.12 (1H,s), 8.11-8.12 (1H,d), 7.49-7.48 (2H,d), 7.48-7.46 (1H,t), 7.46-7.45 (1H,t), 7.44 (1H,s), 7.43,7.40 (2H,d), 7.39-7.37 (1H,d), 7.37 (1H,t), 7.35-7.32 (2H,t), 7.32-7.7.28 (1H,d), 7.28-7.26 (1H,d), 7.22-7.19 (1H,t), 5.03-5.02 (1H,d) ($^3J = 3.51$), 4.36-4.29 (2H,q); 4.31-4.25 (1H,d); 1.51-1.35 (3H,t); ^{13}C NMR (75 MHz, CDCl_3): 165.11, 140.65, 140.26, 137.13, 135.48, 134.88, 130.95, 129.83, 129.38(2C), 129.13(2C), 128.30, 127.81, 126.46, 126.39, 124.30, 123.24, 122.67, 121.05, 119.07, 116.01, 109.60, 109.05, 108.85, 65.26, 63.67, 37.85, 14.09.

1-(9-ethyl-9H-carbazol-3-yl)-4-(4-nitrophenyl)-3-phenylazetididin-2-one (3h, $\text{C}_{29}\text{H}_{23}\text{N}_3\text{O}_3$). Yield was 42%, giving orange crystals. M.p.: 125.8 °C; ^1H NMR (300 MHz, CDCl_3): 8.28-8.26 (2H,d), 8.09 (1H,s), 8.02-7.99 (1H,d), 7.64-7.61 (2H,d), 7.47,7.45 (1H,d), 7.44-7.42 (2H,d), 7.41-7.39 (1H,d), 7.39-7.38 (1H,t), 7.38-7.37 (1H,t), 7.36-7.29 (1H,d), 7.26-7.20 (2H,t), 7.19-7.17 (1H,t), 5.19-5.17 (1H,d) ($^3J = 2.1$), 4.39-4.35 (2H,q), 4.33-4.28 (1H,d), 1.54-1.38 (3H,t); ^{13}C NMR (75 MHz, CDCl_3): 164.60, 148.26, 145.50, 140.70,

137.24, 134.42, 129.52 (2C), 128.58 (2C), 127.82 (2C), 127.14 (2C), 126.57 (2C), 124.93 (2C), 123.33, 122.55, 121.01, 119.20, 115.87, 109.53, 108.95, 65.35, 63.39, 37.88, 14.09.

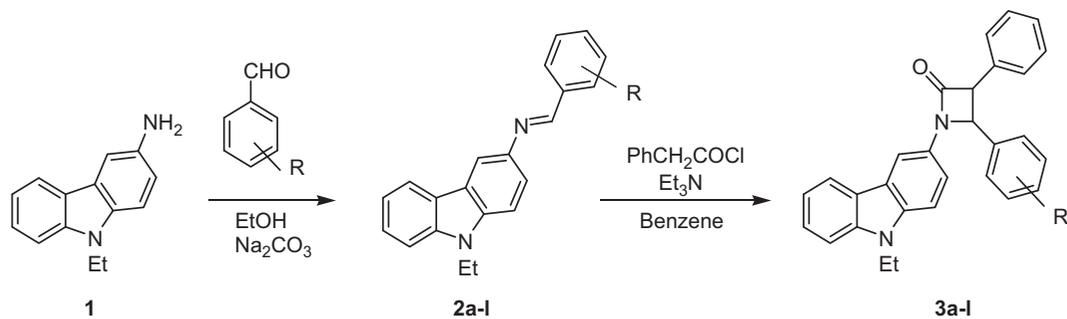
1-(9-ethyl-9H-carbazol-3-yl)-4-(3-nitrophenyl)-3-phenylazetididin-2-one (3i, $\text{C}_{29}\text{H}_{23}\text{N}_3\text{O}_3$). Yield was 79%, giving yellow crystals. M.p.: 113.5 °C; ^1H NMR (300 MHz, CDCl_3): 8.34 (1H,s), 8.24-8.23 (1H,d), 8.07 (1H,s), 8.07-7.99 (1H,d), 7.82-7.79 (1H,d), 7.62-7.57 (1H,t), 7.48-7.47 (1H,d), 7.45-7.44 (1H,d), 7.42-7.40 (1H,t), 7.39-7.37 (1H,t), 7.36-7.30 (1H,d), 7.26-7.22 (2H,d), 7.19-7.17 (2H,t), 5.19-5.19 (1H,d) ($^3J = 2.34$), 4.33-4.32 (1H,d), 4.33-4.31 (2H,q), 4.29-4.28 (1H,d), 1.41-1.39 (3H,t); ^{13}C NMR (75 MHz, CDCl_3): 164.72, 149.12, 140.69, 140.49, 137.26, 134.42, 131.98, 130.82, 129.50(2C), 128.52(2C), 127.78(2C), 126.52, 123.95, 123.33, 121.59(2C), 121.00, 119.17, 116.01, 109.55, 109.20, 108.91, 65.40, 63.34, 37.86, 14.05.

1-(9-ethyl-9H-carbazol-3-yl)-4-(2-nitrophenyl)-3-phenylazetididin-2-one (3j, $\text{C}_{29}\text{H}_{23}\text{N}_3\text{O}_3$). Yield was 36% and intense yellow. M.p.: 214.4 °C; ^1H NMR (300 MHz, CDCl_3): 8.13 (1H,s), 8.00-8.06 (1H,d), 8.00-7.97 (1H,d), 7.59-7.56 (1H,d), 7.56-7.51 (1H,d), 7.51-7.48 (2H,d), 7.48-7.46 (2H,t), 7.46-7.40 (1H,d), 7.42-7.37 (1H,t), 7.34-7.28 (1H,t), 7.27-7.26 (1H,t), 7.26-7.25 (1H,d), 7.25-7.23 (1H,t), 6.30-6.28 (1H,d) ($^3J = 6.11$), 5.30-5.38 (1H,d), 4.33-4.31 (2H,q), 4.29-4.28 (1H,d), 1.41-1.39 (3H,t); ^{13}C NMR (75 MHz, CDCl_3): 165.85, 147.48, 140.70, 137.23, 133.94, 132.38, 131.70, 130.16, 129.72, 129.63 (2C), 128.93(2C), 128.41, 127.88, 126.51, 125.67, 123.30, 122.63, 121.09, 119.16, 115.98, 109.31, 109.17, 108.92, 61.37, 59.00, 37.920, 14.12.

4-(2-bromophenyl)-1-(9-ethyl-9H-carbazol-3-yl)-3-phenylazetididin-2-one (3k, $\text{C}_{29}\text{H}_{23}\text{BrN}_2\text{O}$). Yield was 88% and white crystals. M.p.: 221 °C; ^1H NMR (300 MHz, CDCl_3): 8.13 (1H,s), 8.06-8.03 (1H,d), 7.67-7.64 (1H,d), 7.49-7.48 (1H,d), 7.47-7.46 (2H,d), 7.44-7.42 (1H,d), 7.43-7.41 (2H,t), 7.40-7.38 (1H,t), 7.37-7.35 (1H,t), 7.34-7.33 (1H,t), 7.30-7.29 (2H,d), 7.27-7.25 (1H,t), 7.25-7.13 (1H,t), 5.59-5.61 (1H,d) ($^3J = 2.34$), 4.32-4.40 (2H,q), 4.30-4.25 (1H,d), 1.51-1.35 (3H,t); ^{13}C NMR (75 MHz, CDCl_3): 165.38, 140.66, 137.19, 137.14, 135.23, 133.58, 130.01, 129.81, 129.12 (2C), 128.46 (2C), 128.09 (2C), 127.27, 126.39, 123.27, 123.08, 122.69, 121.08, 119.08 (2C), 116.10, 109.08, 108.84, 64.93, 62.53, 37.86, 14.09.

1-(9-ethyl-9H-carbazol-3-yl)-4-(4-fluorophenyl)-3-phenylazetididin-2-one (3l, $\text{C}_{29}\text{H}_{23}\text{FN}_2\text{O}$). Yield was 63%, giving white crystals. M.p.: 188.2 °C; ^1H NMR (300 MHz, CDCl_3): 8.12 (1H,s), 8.02-8.00 (1H,d), 7.47-7.46 (2H,d), 7.45-7.43 (1H,t), 7.42-7.41 (2H,d), 7.40-7.38 (2H,d), 7.37-7.36 (1H,d), 7.31-7.28 (2H,d), 7.26-7.25 (1H,t), 7.22-7.19 (1H,t), 7.12-7.07 (2H,t), 5.06-5.05 (1H,d) ($J = 2.34$), 4.33-4.31 (2H,q), 4.29-4.28 (1H,d), 1.41-1.39 (3H,t); ^{13}C NMR (75 MHz, CDCl_3): 165.31, 140.65, 137.08, 135.08, 133.82, 133.78, 129.91, 129.35 (2C), 128.22, 128.06, 127.95, 127.80 (2C), 126.38, 123.21, 122.68, 121.01, 119.07, 116.73, 116.44, 116.08, 109.65, 109.00, 108.85, 65.37, 63.72, 37.84, 14.08.

General reaction



- a. R= H
- b. R= 4-CH₃
- c. R= 3-CH₃
- d. R= 4-OCH₃
- e. R= 3-OCH₃
- f. R= 4-Cl
- g. R= 3-Cl
- h. R= 4-NO₂
- i. R= 3-NO₂
- j. R= 2-NO₂
- k. R= 2-Br
- l. R= 4-F

Results and discussion

All β-lactams were synthesized from the appropriate imines (2a-l), which were obtained by reaction between aldehydes and aromatic amine according to literature procedures. β-lactam ring formation was carried out by the addition of imines to ketenes in benzene. The effects of new β-lactam derivatives on the enzyme activity of a purified XO from bovine milk were assayed for enzyme activity in the presence of various concentrations of β-lactam derivatives. IC₅₀ values were determined from the graphs (Figure 1) and are shown in Table I.

XO, a cellular redox enzyme, is highly expressed in mammary epithelial cells (Stevens et al. 2000), but XO was extracted from fresh bovine milk without added preservatives using toluene and EDTA (Elriati et al. 2008). XO from bovine milk was purified using two-step procedures, namely ammonium sulphate precipitation and a Sepharose 4B-L-tyrosine-*p*-aminobenzamidine affinity chromatography (Beyaztaş and Arslan 2011). The purification procedure is described in the Experimental section and the results are summarized in Table II. The purified enzyme was homogeneous by the criteria of denaturing and non-denaturing polyacrylamide gel electrophoresis (Figure 2). Only one protein-staining band was detectable on gels loaded with up to 20 μg of protein.

In this study, we examined the effect of new β-lactams derivatives on xanthine oxidase. The inhibitor molecule was bound in a long, narrow channel leading to the molybdenum-pterin active site of the enzyme. It filled up most of the channel and the immediate environment of the cofactor, very effectively inhibiting the activity of the enzyme through the prevention of substrate binding. Although the inhibitor did not directly coordinate to the molybdenum ion, numerous hydrogen bonds as well as hydrophobic interactions with the protein matrix were observed, most of which are also used in substrate recognition (Okamoto et al. 2003).

An increasing number of researchers during the past decade have also suggested that XO plays an important role in various forms of ischemic and other types of tissue and vascular injuries, inflammatory diseases, and chronic heart failure (Pacher et al. 2006a, Harrison 2002, Harrison 2004, Berry and Hare 2004). Allopurinol and its active metabolite oxypurinol showed beneficial effects in the treatment of these conditions, both in experimental animal models and in small-scale human clinical trials. Although some of the beneficial effects of these compounds go beyond inhibition of XO, these studies generated renewed interest in the development of additional, novel series of XO inhibitors for various therapeutic indications (Borges et al. 2002).

Inhibition of XO was thought to inhibit oxidation of 6-MP and potentiate the antitumor properties. XO was one of the test enzymes in the early experiments in the laboratory; several nontoxic XO inhibitors, including a hypoxanthine analog, allopurinol, were available to directly confirm this suggestion. It was known that XO is involved in the formation of uric acid from xanthine and hypoxanthine (Pacher et al. 2006b). Allopurinol is a drug used as an XO inhibitor in gout treatment (Nguyen et al. 2006). It is a substrate and specific potent inhibitor for XO, but oxypurinol (1H-pyrazolo [3,4-d]-pyrimidine-4,6 diol) is the basic functioning ingredient found in allopurinol. XO could catalyze the allopurinol to oxypurinol, and inhibition occurs mainly through direct substrate competition in the breakdown of purine (Tamta et al. 2006). The reaction has the effect of reducing the amount of concentration of insoluble urates in tissues, plasma, and urine, which leads to the reversal of urate crystal deposits in tissues and uric acid stones (Rang et al. 1999). XO also could catalyze 6-aminopurine to 8-hydroxyadenine and 2,8-dihydroxyadenine (the final product) then inhibited XO (Manfredi and Holmes 1985). Based on the structure of 6-aminopurine, scientists have selected 6-aminopurine and

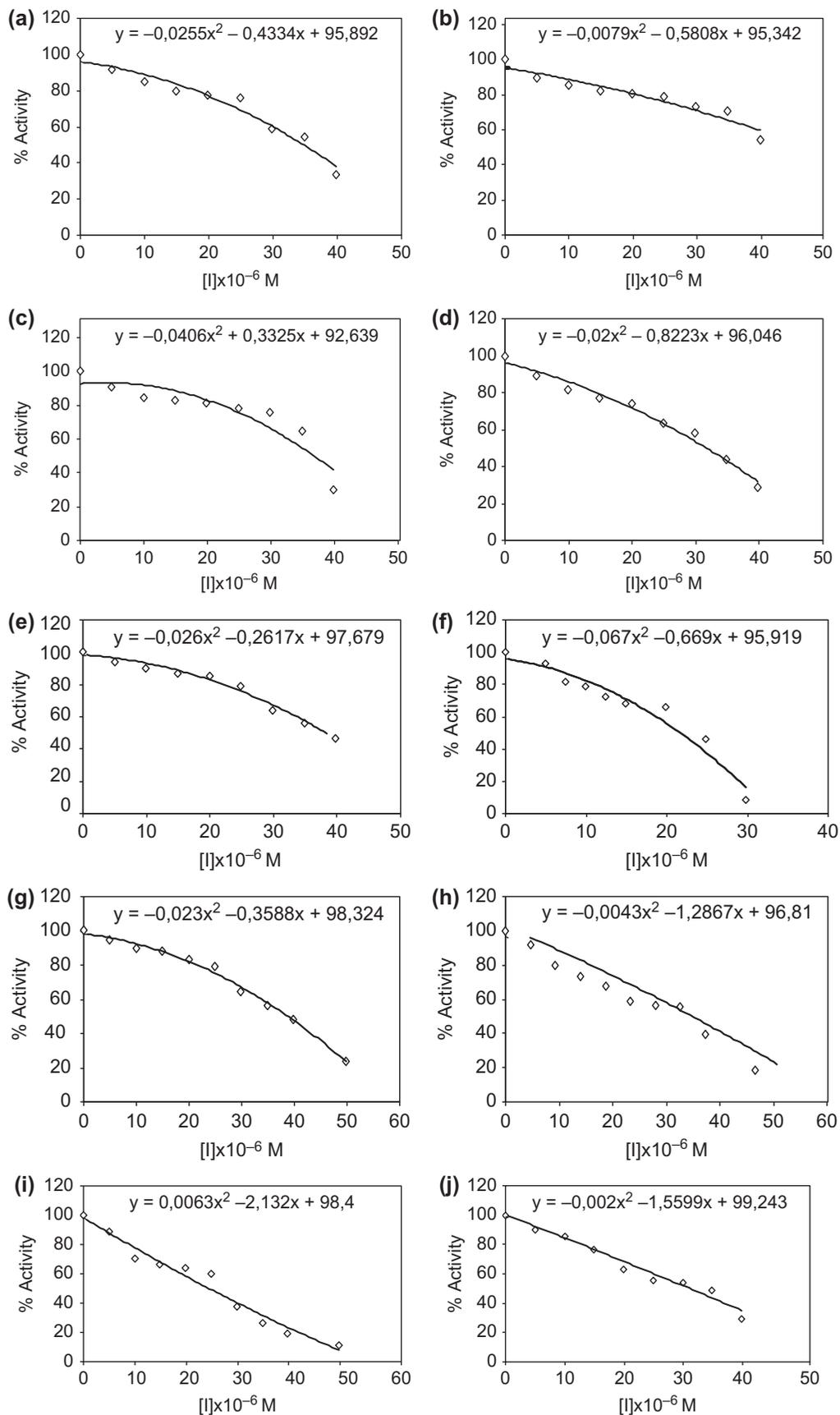


Figure 1. IC_{50} graphics of β -lactam derivatives.

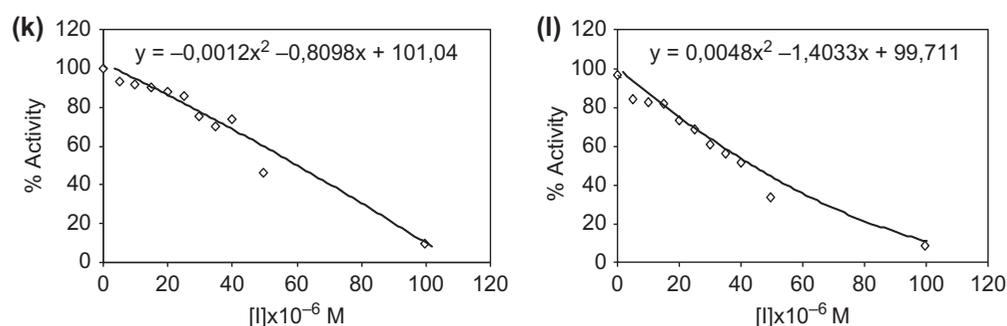


Figure 1. (Continued).

its analogues for XO inhibitory activity assay (Elriati et al. 2008). Allopurinol is a clinically used XO inhibitor, and its IC₅₀ value was 5.43 μM (Fernandes et al. 2002). Allopurinol, 2-chloro-6(methyl amino) purine, 6-aminopurine, and 4-aminopyrazolo [3,4-d]-pyrimidine showed a strong inhibitory effect on XO, and the IC₅₀ of these compounds were 7.82, 10.19, 10.89, and 30.26 μM, respectively. Moreover, 5-nitrobenzimidazole and 6-thioguanine showed a slight inhibitory effect on XO, and the IC₅₀ of these compounds were 86.84 and 92.42, respectively. However, the other 6-aminopurine analogues did not show any significant inhibitory effect on XO. The inhibitory property was of allopurinol, 6-aminopurine, 2-chloro-6(methylamine) purine, and 4-aminopyrazolo [3,4-d] pyrimidine on XO. The inhibitory effects of allopurinol, 6-aminopurine, 2-chloro-6(methylamine) purine, and 4-aminopyrazolo [3,4-d] pyrimidine on XO were tested at different concentrations. XO inhibition increased significantly with the addition of allopurinol. Furthermore, similar trends were also observed in the results of 2-chloro-6(methylamine) purine, 6-aminopurine and 4-aminopyrazolo [3,4-d] pyrimidine. The XO inhibition reached a maximum when 30 μM of inhibitors was added, while the XO inhibition of allopurinol, 6-aminopurine, 2-chloro-6(methylamine) purine, and 4-aminopyrazolo [3,4-d] pyrimidine was 93.8, 61.3, 78.6, and 49.8%, respectively. The results showed that low concentrations (2–5 μM) of 2-chloro-6(methylamine) purine and 6-aminopurine demonstrated a higher XO inhibition than allopurinol and 4-aminopyrazolo [3,4-d] pyrimidine, while high concentrations (20–30 μM) of allopurinol and 2-chloro-6(methylamine) purine demonstrated a higher XO inhibition than 6-aminopurine and 4-aminopyrazolo [3,4-d]

pyrimidine (Elriati et al. 2008). Scientists observed that the metabolism of 4-aminopyrazolo [3,4-d] pyrimidine has been studied in several normal and neoplastic mouse tissues (Henderson and Junga 1961). Moreover, the administration of 4-aminopyrazolo [3, 4-d]-pyrimidine decreased serum cholesterol level in the rat (Andersen and Dietschy 1976).

During the past decade, definite progress has also been achieved in the understanding of the XO enzyme structure, and rational drug development approaches led to the discovery of new, powerful XO inhibitors of various classes, including purine analogs, imidazole and diazole derivatives, and flavonoids, among many others (Borges et al. 2002). Two of these very potent new compounds, febuxostat [2-[3-cyano-4-(2-methylpropoxy) phenyl]-4-methylthiazole-5-carboxylic acid] and Y-700 (1-[3-cyano-4-(2,2-dimethylpropoxy) phenyl]-1H-pyrazole-4-carboxylic acid), were reported to have a favorable toxicology profile, high bioavailability, and more potent and longer-lasting hypouricemic action than allopurinol.

Table I. Effects of new β-lactam derivatives on the enzyme activity of a purified XO from bovine milk.

Lactams	R-	IC ₅₀ (μM)
3a	H	34.77
3b	4-CH ₃	47.42
3c	3-CH ₃	36.73
3d	4-OCH ₃	31.64
3e	3-OCH ₃	38.08
3f	4-Cl	21.65
3g	3-Cl	38.69
3h	4-NO ₂	32.79
3i	3-NO ₂	24.47
3j	2-NO ₂	30.37
3k	2-Br	58.04
3l	4-F	41.24

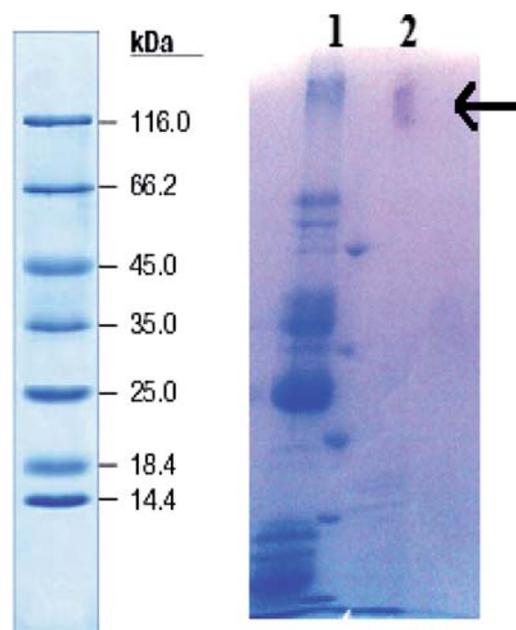


Figure 2. SDS-PAGE of xanthine oxidase. The pooled fractions from affinity chromatography were analyzed by SDS-PAGE (12% and 3%) and revealed by Coomassie Blue staining. Experimental conditions were as described in the method. Lane 1 contained 5 μL of various molecular mass standards: 3-galactosidase, (116.0), bovine serum albumin (66.2), ovalbumin (45.0), lactate dehydrogenase (35.0), restriction endonuclease (25.0), 3-lactoglobulin (18.4), lysozyme (14.4). Only one protein-staining band was detectable on Line 2.

Table II. Summary of the purification procedure of milk xanthine oxidase.

Step	Volume (mL)	Activity (U/mL)	Total Activity (U)	Protein Amount (mg/mL)	Total Protein (mg)	Specific Activity (U/mg)	Overall Yield (%)	Overall Purification (fold)
Milk	100	91.12	9112	4.31	431.25	21.13	100	-
Ammonium sulphate precipitation	12	125.31	1503.72	6.55	78.64	19.12	16.50	0.90
Affinity chromatography	3.0	361.90	1085.7	0.093	0.28	1292.5	11.91	67.6

These new compounds are currently in human clinical trials for the treatment of hyperuricemia and gout (Andersen and Dietschy 1976, Okamoto et al. 2004, Yamamoto 2003, Becker et al. 2004, Becker et al. 2005, Fukumari et al. 2004, Hoshide et al. 2004, Komoriya et al. 2004, Yamada et al. 2004, Hashimoto et al. 2005, Mayer et al. 2005, Takano et al. 2005, Naito et al. 2000). Therefore, the effects of new β -lactams derivatives have initially been studied on the purified xanthine oxidase enzyme activity *in vitro*. New β -lactams derivatives, whose effects we investigated in our studies, showed that different levels affected the xanthine oxidase. For the β -lactams' shown inhibition effect, the IC_{50} values of the chemicals causing inhibition were determined by means of activity percentage-[I] diagrams. In this study, all β -lactams indicated inhibitory effect on the enzyme activity.

Conclusions

Enzyme inhibition is an important issue for drug design and biochemical applications (Aydemir and Kavrayan 2009, Gencer and Arslan 2011, Gencer and Arslan 2009, Sinan et al. 2007, Kiranoglu et al. 2007, Sinan et al. 2006). The results showed that new β -lactams derivatives inhibited the xanthine oxidase enzyme activity. β -lactams derivatives are 3a, 3b, 3c, 3d, 3e, 3f, 3g, 3h, 3i, 3j, 3k, and 3l. The values of IC_{50} were 34.77, 47.42, 36.73, 31.64, 38.08, 21.65, 38.69, 32.79, 24.47, 30.37, 58.04, and 41.24 μ M, respectively. The most effective compound for XO was 3f. In addition, IC_{50} values of β -lactams were similar to each other. Furthermore, the IC_{50} of β -lactams were similar to that of other XO inhibitors, which were well-known XO inhibitors clinically used for gout treatment. Therefore, our results suggested that new β -lactams derivatives are likely to be adopted as candidates to treat gout and may be taken for further evaluation in in-vivo studies.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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