α-GLUCOSIDASE INHIBITION OF LACTONE INTERMEDIATES OF THE IMINOSUGAR DEOXYNOJIRIMYCN

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Abstract

α-Glucosidase enzymes hydrolyse α-glycosidic linkages and are involved in bodily processes such as the catabolism of glycans, intestinal digestion, and the degradation of glycoproteins. Various types of diseases which are caused by the failure of these enzyme to function properly can be treated through enzyme inhibition. The hydroxyethyl derivative of DNJ (Miglitol) is a clinical drug for the treatment of type 2 diabetes. Although the iminosugar α-deoxynojirimycin (α-DNJ) is an excellent micromolar glycosidase inhibitor, the α-glucosidase inhibition activity of α-DNJ lactone intermediates has yet to be reported. Therefore, the scalable synthesis of the α-DNJ intermediates 1,2-O-isopropylidene-α-D-glucurono-3,6-lactone (2), 1,2-O-isopropylidene-β-L-idurono-3,6-lactone (3) and 5-azido-5-deoxy-1,2-O-isopropylidene-α-D-glucurono-3,6-lactone (4) was carried out using α-glucuronolactone (1) as the starting material based on the method reported by Best et al. 2010 with some modification and subsequently, evaluated for anti-α-glucosidase activity. All products were characterised and identified by HPLC-ELSD, mass spectrometry (DI-ESI-MS) and NMR spectroscopy (via comparison of 1D 1H and 13C data with previously reported values). The inhibitory activity of compounds 1-4 towards α-glucosidase from Saccharomyces cerevisiae was evaluated using the p-nitrophenyl α-D-glucopyranoside substrate. Compound 3 showed 29.5% inhibition followed by 2 (21.4%), 1 (15.8%) and 4 (15.7%) compared to the positive control, quercetin (72.7%).
1.0 INTRODUCTION

Glycosidases are carbohydrate-digesting enzymes that catalyse the hydrolysis of glycosidic bonds in carbohydrates to produce hemiketal and hemiacetal sugars. These enzymes are relevant to various diseases such as type 2 diabetes, lysosomal storage diseases (Gaucher, Fabry, Tay-Sachs, Sandhoff, Niemann-Pick C type II disease), Hepatitis B and C, HIV, cystic fibrosis and cancer. Several classes of glycosidase inhibitors have been identified and developed over the last five decades which include iminosugars, thiosugars, carbasugars and other non-carbohydrate-type compounds [1]. Iminosugars or azasugars are polyhydroxylated secondary and tertiary amines that mimic natural aldose and ketose sugars whereby the ring oxygen is replaced with a nitrogen atom [2]. Previous studies have shown that iminosugars can inhibit oligosaccharide processing enzymes such as glycosidases and glycosyltransferases [3, 4]. Examples of iminosugars used clinically are Miglitol (N-hydroxyethyl deoxynojirimycin) for type 2 diabetes and Zavesca/Miglustat (N-butyl deoxynojirimycin) for Gaucher disease [1, 5]. Iminosugars inhibit glycosidase enzymes via their structural resemblance to the carbocation/oxacarbenium transition intermediate formed during the hydrolysis process of the natural substrates. Therefore, iminosugars may competitively inhibit glycosidases, hence lowering the rate of carbohydrate breakdown [6]. The iminosugar DNJ is an analogue of glucose that has been extensively studied [7, 8, 9]. Besides lacking a hydroxyl (-OH) group at the carbon C1 position, the oxygen atom in the pyranose ring of DNJ is replaced by a nitrogen atom. The high structural similarity between DNJ and glucose results in the competitive inhibition of glycosidase enzymes. DNJ acts as an antihyperglycemic agent by reducing the breakdown of complex carbohydrates to monosaccharides. Thus, glucose absorption is delayed to lower blood glucose levels after food intake [10].

Methods of DNJ preparation have been widely studied to increase yields for large scale production to facilitate commercialisation. Initially, DNJ was obtained from the unstable nojirimycin isolated from Streptomyces spp. after conversion into the more stable bisulfite adduct followed by the removal of the OH group at C1 [11]. Direct isolation of DNJ from...
natural sources such as the white mulberry plant (Morus alba) [12] and microorganisms such as Streptomyces and Bacilli bacteria [13] was subsequently achieved. The large-scale preparation of DNJ has mainly been achieved through chemical synthesis. Behling et al. (1991) synthesised DNJ in five steps using l-sorbose as the starting material [14]. Although this synthesis was performed on a large scale (kilogram) and afforded a high yield, the final deprotection step involved the use of ion-exchange resin (Dowex) that would be expensive for large scale use. More recently, Best et al. (2010) reported the cost-effective and large scale (gram) chemical synthesis of DNJ, producing 12.2 g of DNJ in seven synthetic steps from 250 g of the inexpensive starting material glucuronolactone [15] Besides isolation from natural sources and chemical synthesis, DNJ has also been obtained via chemoenzymatic synthesis using the D-fructose-6-phosphate aldolase enzyme in an aldol reaction [16]. In vitro and in vivo α-glucosidase inhibition by DNJ has been widely reported in the literature [17, 18]. However, the inhibitory activities of the intermediate compounds formed during the chemical synthesis of DNJ have yet to be reported. In this study, the intermediate compounds 1,2-O-isopropylidene-α-D-glucurono-3,6-lactone (2), 1,2-O-isopropylidene-β-L-idurono-3,6-lactone (3) and 5-azido-5-deoxy-1,2-O-isopropylidene-α-D-glucurono-3,6-lactone (4) as well as the starting material glucuronolactone (1) were synthesised as reported by Best et al. (2010) and subsequently evaluated for anti-α-glucosidase activity.

2.0 METHODOLOGY

2.1 Chemical and Biochemical Reagents

All the chemicals (analytical grade) used were purchased from Sigma Aldrich. Analytical thin-layer chromatography (TLC) was carried out on aluminium sheets coated with 60 F254 silica purchased from Merck. The TLC sheets were stained with cerium (IV) sulfate and ammonium molybdate in sulfuric acid. Preparative TLC was carried out on glass plates coated with 60 F254 silica purchased from Merck. Vacuum liquid chromatography (VLC) was performed on silica (0.015-0.040 mm) purchased from Merck. Enzyme inhibition was carried out using α-glucosidase from Saccharomyces cerevisiae (Sigma) and p-nitrophenyl (pNP) α-D-glucopyranoside (Sigma) as the substrate.

2.2 Synthesis of 1,2-O-Isopropylidene-α-D-glucurono-3,6-lactone (2)

d-Glucuronolactone (1) (25.0 g) was added to acetone (600 mL) and stirred until completely dissolved. Concentrated sulfuric acid (20 mL) was then added to the suspension and stirred at room temperature for 5 h. TLC analysis was performed every 30 minutes using ethyl acetate to monitor product formation. The reaction mixture was neutralised with sodium carbonate, filtered and concentrated using a rotary evaporator. The crystalline crude material (yellow) was dissolved in dichloromethane, filtered and concentrated using a rotary evaporator. Recrystallisation of the residue was carried out with hot toluene and decolourised with activated charcoal. The product was analysed by high-performance liquid chromatography coupled to an evaporative light scattering detector (HPLC-ELSD), direct infusion electrospray ionisation mass spectrometry (DI-ESI-MS) and nuclear magnetic resonance spectroscopy (NMR).

2.3 Synthesis of 1,2-O-Isopropylidene-β-L-idurono-3,6-lactone (3)

1,2-O-Isopropylidene-α-D-glucurono-3,6-lactone (2) (7.0 g) was initially dissolved in dichloromethane (57.9 mL) at room temperature followed by the addition of pyridine (7.8 mL). Triflic anhydride (6.1 mL) was added dropwise (every 10 min/mL) into the previous solution at -30 °C for 1 h. The temperature of -30 °C was achieved by the combination of dry ice and acetonitrile. The mixture of reaction was stirred at -30 °C for 1.5 h. TLC analysis (2:1 hexane/ethyl acetate) revealed that the starting material was completely consumed and that a major product was formed. The mixture was washed (3x) with aqueous hydrochloric acid (57.9 mL, 2M) and then the organic phase was concentrated using a rotary evaporator at 30 °C. The product was dissolved in DMF (28.9 mL) and then sodium trifluoroacetate (8.8 g) was added stepwise (2.2 g every 15 min). The reaction mixture was stirred at room temperature for 2 h. Once again, the major product was detected using TLC analysis (2:1 hexane/ethyl acetate) (the starting material was completely consumed). The reaction mixture was diluted with saturated aqueous sodium bicarbonate (130.0 mL) and extracted 3x with ethyl acetate (57.9 mL). The organic layers were combined, concentrated and recrystallised using hot toluene. The solution was cooled in an ice bath to aid crystallisation. This sample (1.0 g) was then subjected to VLC performed on a self-packed silica gel SPE column (40.0 g, 0.015-0.040 mm, 12 x 2.1 Ø cm) connected to an empty SPE cartridge used as the solvent reservoir. Elution was carried out using an isocratic solvent system (2:1 hexane/ethyl acetate) with the aid of a vacuum pump. The collected fractions (each 5 ml) were analysed by TLC (2:1 hexane/ethyl acetate) to determine fractions that contained the product. Combined fractions of the product were further analysed by HPLC-ELSD, DI-ESI-MS and NMR.
2.4 Synthesis of 5-Azido-5-deoxy-1,2-O-isopropylidene-α-D-glucurono-3,6-lactone (4)

Iduronolactone (3), (1.0 g) was dissolved in dichloromethane (10.2 mL) and then pyridine (1.2 mL) was added into the solution. Triflic anhydride (0.9 mL) was added stepwise into the solution at -40 °C over 1 h. The temperature of -40 °C was achieved by the combination of dry ice and acetonitrile. TLC analysis (2:1 hexane/ethyl acetate) was performed to monitor the reaction. The reaction mixture was diluted with dichloromethane (10.2 mL) and washed (3x) with aqueous hydrochloric acid (25 mL). The organic layer was dried over magnesium sulfate for 10 minutes (was a combination of dry ice and acetonitrile). TLC analysis (2:1 hexane/ethyl acetate) was performed before injection through a needle pump. Dried samples (1 mg) were dissolved in 0.5 mL acetonitrile and prepared at a concentration of 0.5 mg/ml. Samples were done in analysis. The mobile phase was acetonitrile: water of 1.0 mL/min (isocratic run) with an injection volume of 10 µL. The reaction mixture was stirred at 40 °C for 4 h. TLC analysis (2:1 hexane/ethyl acetate) was performed to reveal the formation of a major product. The reaction mixture was diluted with 5% sodium chloride and extracted (3x) with ethyl acetate (25 mL). The combined organic layers were dried over magnesium sulfate, filtered and concentrated using a rotary evaporator. The dried sample (300 mg) was purified by VLC as described above for intermediate 3 with slight modification (isocratic, 3:1 hexane/ethyl acetate; 12 x 2.6 Ø cm). The collected fractions (each 2 mL) were analysed by TLC (2:1 hexane/ethyl acetate). The impure sample was further purified by preparative TLC (2:1 hexane/ethyl acetate). The resulting product was analysed by HPLC-ELSD, DI-ESI-MS and NMR.

2.5 High-Performance Liquid Chromatography (HPLC) Analysis

An HPLC instrument coupled to an evaporative light scattering detector (HPLC-ELSD) was used to determine the purity of the samples prior to further enzyme (α-glucosidase) inhibition analysis. The samples were analysed on a Waters XBridge HILIC amide column (3.5 µm, 4.6 x 250 mm) at a flow rate of 1.0 mL/min (isocratic run) with an injection volume of 10 µL. The mobile phase was acetonitrile: water (95:5). All the samples were dissolved in 100 % acetonitrile and prepared at a concentration of 0.5 mg/ml.

2.6 Direct Infusion Electrospray Ionisation Mass Spectrometry (DI-ESI-MS)

Samples (1 mg) were dissolved in 0.5 mL acetonitrile and 0.1% formic acid. The samples were diluted (100x) before injection through a needle pump (Harvard apparatus, 11 plus, USA) at a flow rate of 5 µL/min for electrospray ionisation (Finn, 2009). Mass detection spectrometry was carried out using an ACQUITY® SQD with a single quadrupole detector (Waters Corporation, Milford, MA USA) and acquired in positive mode. Mass spectral data processing was performed using MassLynx V4.1 (Waters Corp., Milford, MA). Assignment of the m/z values from D-ESI-MS were calculated using the MS Adduct Calculator in the following website: http://fiehnlab.ucdavis.edu/staff/kind/Metabolomics/MS-Adduct-Calculator/.

2.7 Fourier Transform NMR Spectroscopy (FT-NMR)

Dried samples (1-10 mg) were dissolved in deuterated chloroform (600 µL). Samples were filtered with 0.45 µm filter prior to loading into NMR tube and analysed with a 1H 600 MHz (13C 150 MHz Bruker/AVANCE III NMR spectrometer. 1H and 13C NMR spectra were processed with the ACD Spectrus Processor software.

2.8 Determination of α-Glucosidase Inhibitory Activity

The starting material and all the intermediates produced were evaluated for α-glucosidase inhibition as described by Ryu et al. (2011) [19] and Simone et al. (2012) [20] with some modification. The test samples were assayed using α-glucosidase from S. cerevisiae and p-nitrophenyl (pNP) α-D-glucopyranoside as the substrate. The reaction mixture contained 10 µL enzyme (0.015U), 10 µL inhibitor (0.33 mM in 25% DMSO) and 130 µL sodium phosphate buffer (pH 6.5). The enzyme reaction was incubated at 37 °C for 5 minutes. Then, 50 µL of pNP-α-glucoside (0.3 mg/mL) was added into the mixture and further incubated at 37 °C for 30 minutes. The absorbance (Abs) at 405 (A405) was measured using a microplate reader. All the reactions were done in triplicate and the averages were taken as the final results. The negative control used did not contain any sample and substrate while quercetin was used as the positive control. Enzyme inhibition was calculated using the formula below:

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\text{Percentage of inhibition (\%) = } \frac{\text{Abs average}}{\text{Abs control}} \times 100\%
\]

Percentage of inhibition (%) = 100% - percentage of activity (%)

3.0 RESULTS AND DISCUSSION

3.1 Synthesis of DNJ Intermediates

The overall scheme of synthesis starting with glucuronolactone (1) to produce the DNJ-related intermediates 1,2-O-isopropylidene-α-D-glucurono-3,6-lactone (2), 1,2-O-isopropylidene-β-D-uridurono-3,6-lactone (3) and 5-azido-5-deoxy-1,2-O-isopropylidene-α-D-glucurono-3,6-lactone (4) in the
synthesis reported by Best et al. (2010) is shown in Figure 1. This strategy encompassed the selective protection of the hydroxyl groups at C1 and C2 so that reactions only occurred at C5, the subsequent formation of the inverted iido-lactone followed by the introduction of an azide at C5 of glucuronolactone (net retention of configuration).

Figure 1 Overall scheme of synthesis. Reagents and conditions (i) Me2CO, conc. H2SO4, RT 5 h; (ii) (CF3SO2)2O, pyridine, CH2Cl2, -30 °C, 1 h; then CF3CO2Na, DMF, RT 1 h; (iii) (CF3SO2)2O, pyridine, CH2Cl2; then NaN3, DMF, -40 °C, 1 h

3.1 Synthesis of 1,2-O-Isopropylidene-α-D-glucanono-3,6-lactone (2)

The widely used and versatile D-glucuronolactone [14] was chosen as the starting material because of its 6-carbon atom structure (avoids the addition of reduction of carbons), chirality in high enantiomeric purity (presence of chiral carbons at positions 3, 4 and 5), and low price. In this study, TLC monitoring showed that the starting material D-glucuronolactone (Ri 0.03) was readily catalytically converted into the major 1,2-acetonide product (Ri 0.75) using concentrated sulfuric acid as reported by Best [2010] [15] (Figure 2B). The starting material 1 is visible under UV, but intermediate 2 cannot be detected directly under UV, thus was detected with cerium ammonium molybdate (CAM) detection system. Based on the HPLC-ELSD analysis, compound 2 [16.9 g, 55.1%] was successfully purified (Figure 2A) and Di-ESI-MS analysis gave adduct [M + Na] = 239 (M = 216 g/mol). FT-NMR spectroscopic analysis: δH (CDCl3, 600 MHz): 1.28, 1.46 (2 x 3H, s, Me), 4.49 (1H, d, H5, J5,6 = 4.2, J5,3/J3,2 = 9.9), 4.75–4.77 (2H, dd, H2, H3, J2,1/J3,4 = 3.3), 4.87–4.89 (1H, dd, H4, Jd,J4 = 2.7, J4,6 = 4.8), 5.93 (1H, d, H1, J1,2 = 3.6); δC (CDCl3, 150 MHz): 25.6, 25.9 (Me), 69.6 (C5), 77.3 (C4), 80.4, 81.9 (C2, C3), 105.7 (C1), 112.6 (CMe2), 173.1 (C6).

Figure 2 A HPLC-ELSD chromatogram compound (2) at the retention time 3.72 minutes. B. TLC analysis of (Ethyl acetate, CAM stain) compound (2) (Ri 0.75)

3.2 Synthesis of 1,2-O-Isopropylidene-β-L-idurono-3,6-lactone (3)

The configuration of the azide introduced at C5 of intermediate 3 (inverted lido-lactone configuration) was the same as intermediate 2. This was done by firstly, esterification of the gluco-alcohol using triflic anhydride and secondly, reaction of the resulting trflate with sodium trifluoroacetate in DMF. Triflic anhydride that has a good leaving group was added slowly to ensure that all of compound 2 reacted. The intermediate 2 was mixed with anhydride and pyridine triphosphate at -40 °C to produce the intermediate 2a (Ri 0.78) that was washed with hydrochloric acid to remove pyridine by converting it to a pyridinium salt (pyridinium chloride). The organic layer was dried and dissolved in NN-dimethylformamide (DMF) and reacted with sodium trifluoroacetate to obtain intermediate 3. The organic fraction that contained intermediate 3 in DMF was extracted with ethyl acetate. Evaporation of DMF requires a high temperature and compound 3 is sensitive to high temperature. Besides that, ethyl acetate extraction allowed solvent exchange and removed excess reactants. Intermediate 3 was then purified by recrystallisation with hot toluene. The wet weight of compound 3 obtained was 8.0 g. The TLC analysis (2:1 hexane/ethyl acetate) showed that most of the starting material of compound 2a was consumed in the reaction and also detected the formation of a major product, compound 3 (Ri 0.45) including the presence of other compounds at Ri 0.20 and 0.95 (data not shown). Therefore, further purification by VLC (2:1 hexane/ethyl acetate), and TLC analysis (2:1 hexane/ ethyl acetate) showed the isolation of the pure intermediate 3 (Ri 0.45) (Figure 3B). Based on the HPLC-ELSD analysis, compound 3 [3.2 g, 46%] showed a single peak indicating a pure sample without the presence of other compounds (Figure 3A). Analysis of Di-ESI-MS; [M + 2IsoProp + 2H] = 338 (M = 216 g/mol). Analysis spectroscopy FT-NMR; δH (CDCl3, 600 MHz): 1.28, 1.45 (2 x 3H, s, Me), 3.15 (1H, s, OH), 4.25 (1H, s, H5), 4.72 (1H, d, H4, J4,3 = 3.0), 4.76 (1H, d, H2, J2,1 = 3.6), 4.98 (1H, d, H3 J3,4 = 3.6), 5.86 (1H, d, H1, J1,2 = 3.6); δC (CDCl3, 150 MHz): 25.6, 26.0 (Me), 70.7 (C5), 81.0 (C2), 81.4 (C4), 84.0 (C3), 105.1 (C1), 112.2 (CMe2), 173.6 (C6).
3.3 Synthesis of 5-Azido-5-deoxy-1,2-O-isopropylidene-α-D-glucurono-3,6-lactone (4)

L-ido-alcohol (3) reacted with triflic anhydride to yield the corresponding trflate, which with sodium azide in DMF produced the α-gluco-azide, intermediate 4. Intermediate 3 easily dissolved when dichloromethane and pyridine were added. Pyridine acted as a base and catalyst in the reaction. Triflic anhydride was added slowly to form an intermediate at OH group of 3. After stirring for 3 hours, the TLC analysis (2:1 hexane/ethyl acetate) showed the formation of product 3a at Rf 0.82 from its starting material 3 (Rf 0.45). The intermediate 3a was washed with hydrochloric acid to remove excess pyridine by converting it to pyridinium chloride. The crude was then dissolved and cooled to -20 C. Sodium azide was then added and the reaction mixture was stirred at -20 C for 4 hours to allow the formation of intermediate 4. The formation of intermediate 4 was observed on a TLC sheet (2:1 hexane/ethyl acetate) at Rf 0.73. The organic fraction was then extracted with ethyl acetate as mentioned before. Intermediate 4 was initially purified by normal-phase liquid chromatography (3:1 hexane/ethyl acetate) and followed by preparative TLC (2:1 hexane/ethyl acetate). Three bands were observed with preparative TLC whereby 4 was present in the topmost band. The compound 4 was recovered from the preparative TLC plate by scraping and dissolved in DCM to form a yellow solution. This solution was then concentrated using a rotary evaporator. TLC analysis (2:1 hexane/ethyl acetate) revealed only one spot at Rf 0.73 (Figure 4B). Based on HPLC-ELSD analysis, compound 4 [0.013 g, 1.2 %] displayed only one peak at retention time 3.52 minutes (Figure 4A). DI-ESI-MS analysis of intermediate 4: [M+MeOH+Na+MeCN+H+] = 338 [M = 241 g/mol]. FT-NMR: δH (CDCl3, 600 MHz): 1.28, 1.44 (2 x 3H, s, Me), 4.16 (1H, s, H5), 4.75–4.79 (2H, m, H2, H3), 4.88 (1H, d, H4, Jα,3 = 3.0), 5.87 (1H, d, H1, J1,2 = 3.0); δC (CDCl3, 150 MHz): 26.5, 27.0 (Me), 61.5 (C5), 79.1 (C4), 81.9, 81.1 (C2, C3), 106.3 (C1), 113.4 (CMe2), 170.7 (C6).

3.4 Anti-α-Glucosidase Inhibition by Glucuronolactone and the DNJ

Anti-α-glucosidase activity was evaluated under previously reported optimised conditions using the starting material 1 and the intermediates 2, 3 and 4 as inhibitors. All the compounds tested showed inhibitory activity towards α-glucosidase. Based on Figure 5, the percentage of inhibition displayed by compounds 1, 2, 3 and 4 were 15.8%, 21.4%, 29.5% and 15.7%, respectively. Therefore, intermediate 3 showed the highest inhibition among compounds 1-4. Intermediates 1 and 4 showed approximately the same level of enzyme inhibition. The positive control quercetin showed the highest enzyme inhibition percentage value of 72.5%. Although the inhibitory activity values of compounds 1-4 were not as high as the positive control, this study showed that compound 3 exhibited 30% inhibition of α-glucosidase. The inhibitory activity of compound 3 can be increased by using a higher concentration of this compound (~2.5x for comparable activity to the positive control, quercetin). The advantage of using intermediate 3 as an α-glucosidase inhibitor is that its synthesis involves only two high-yield steps. Therefore, this makes intermediate 3 more cost-effective and less time consuming to prepare than standard inhibitors such as quercetin and DNJ.

Figure 3 A. HPLC-ELSD chromatogram compound 3 at retention time 3.59 minutes. B. TLC analysis (2:1 hexane/ethyl acetate) compounds 3 (Rf 0.45)

Figure 4 A. HPLC-ELSD chromatogram of intermediate 4 at retention time 3.52 minutes. B. TLC analysis (2:1 hexane/ethyl acetate) for intermediate 4 at Rf 0.73

Figure 5 S. cerevisiae α-glucosidase enzyme inhibition of quercetin, α-glucuronolactone (1), 1,2-O-isopropylidene-α-D-glucurono-3,6-lactone (2), 1,2-O-isopropylidene-β-D-idurono-3,6-lactone (3) and 5-azido-5-deoxy-1,2-O-isopropylidene-α-D-glucurono-3,6-lactone (4)
4.0 CONCLUSIONS

Intermediate 4 was synthesised in three steps starting with D-glucuronolactone that produced 1,2-O-isopropylidene-α-D-glucurono-3,6-lactone (2), followed by 1,2-O-isopropylidene-β-L-idurono-3,6-lactone (3) and finally 5-azido-5-deoxy-1,2-oxysopropylidene-α-D-glucurono-3,6-lactone (4) using the method reported by Best et al. (2010) with some modification. Compounds 1-4 exhibited inhibition towards S. cerevisiae α-glucosidase at 15.8%, 21.4%, 29.5% and 15.7%, respectively. The intermediate compound 3 produced in gram scale showed the highest percentage of inhibition and may be used as a low-cost inhibitor at higher concentrations to achieve inhibition comparable to the known inhibitor quercetin.

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