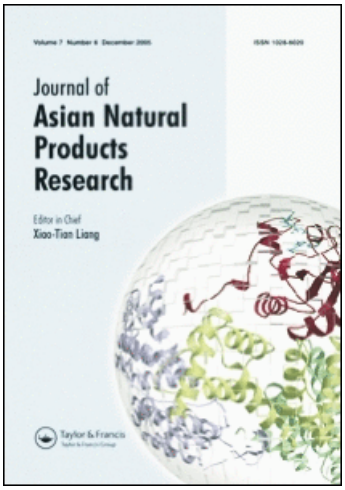

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Kinetics studies on the lignan class of natural compounds that inhibits α-chymotrypsin

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The mechanism of inhibition of the α-chymotrypsin enzyme by two lignans of the fused bistetrahydrofuran series, epiexcelsin (**1**) and 5′-demethoxyepiexcelsin (**2**), which were isolated from the *Commiphora mukul* Engl., was investigated. Lineweaver–Burk and Dixon plots and their secondary replots showed that these compounds were noncompetitive inhibitors of the enzyme. K_i values for **1** and **2** were found to be 22.29 ± 0.015 and 336.30 ± 0.053 μM, respectively.

Keywords: *Commiphora mukul* Engl.; lignans; α-chymotrypsin inhibition

1. Introduction

Commiphora mukul Engl. (Guggul) is a medicinal plant, and the pharmacological studies on the crude drug as well as on some of its fractions and pure constituents have revealed significant anti-inflammatory, anti-rheumatic, and hypocholestremic activities [1].

In all metazoan species, proteases play a prominent role in a wide array of physiological processes such as food digestion, blood clotting, embryogenesis, tissue reorganization (e.g. wound healing, regeneration, molting, metamorphosis, etc.), defense mechanisms, and immune responses. Many of these processes are proteolytic cascades, which, once set in action, lead very rapidly and irreversibly to a specific cellular response. Activation and inactivation of protease cascades have to be closely controlled at different regulatory levels such as protease gene transcription, mRNA translation, zymogen activation, substrate specificity, enzyme kinetics, and by means of enzyme inhibitors. Most animal species synthesize a variety of protease inhibitors with different specificities, whose function is to prevent unwanted proteolysis. It follows that – and evidence for this is accumulating – proteases are involved in various disease states. For instance, the destruction of the extracellular matrix of articular cartilage and bone in arthritic joints is thought to be mediated by excessive proteolitic activity [2]. In emphysema, gingivitis, tumor invasion, and inflammatory infections, it is suggested that tissue destruction is caused by proteases [2]. Among the enzymes involved in extracellular matrix degradation, a few serine proteases (elastase, collagenase, cathepsin

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G, chymotrypsin) are able to solubilize fibrous proteins such as elastin and collagen [3,4]. Given the specific recognition by proteases of defined amino acid sequences, it may be possible to inhibit these enzymes when they are involved in pathological processes. Potent inhibitors have the potential to be developed as new therapeutic agents. In vertebrates, serine protease inhibitors have been studied for many years, and they are known to be involved in phagocytosis, coagulation, complement activation, fibrinolysis, blood pressure regulation, etc. In the last decade, it has become obvious that, in invertebrates, serine proteases and their inhibitors are also involved in parallel physiological processes (e.g. blood clotting cascade in *Limulus* [5] and the innate immune response [6]). Moreover, some of the protease inhibitors isolated from invertebrate sources are quite specific towards individual mammalian serine proteases. This also offers huge opportunities for medicine. Thus, the development of nontoxic protease inhibitors extracted from natural sources for *in vivo* application may be quite important [2]. In future, it is likely that numerous specific protease inhibitors will be tested clinically for the treatment of human diseases, such as emphysema, inflammation, dermitis, and cancer. Since the current serine protease inhibitors (such as α-chymotrypsin) are still far from perfection, the interests and efforts in the discovery of novel serine protease inhibitors are expected to continue in future. As part of our ongoing investigations on the α-chymotrypsin inhibition by natural products, we now describe the detailed α-chymotrypsin inhibitory activity and inhibition kinetics of these potential compounds.

2. Results and discussion

Epiexcelsin (**1**) and 5′-demethoxyepiexcelsin (**2**), which are lignans of the bistetrahydrofuran series, were isolated from the chloroform-soluble fraction of *Commiphora mukul* Engl., and their structures were determined through extensive spectral studies (Figure 1) [7–9]. Both lignans **1** and **2** exhibited inhibitory potential against the chymotrypsin enzyme with the IC_{50} values of 110 ± 0.025 and 649 ± 0.013 μM, respectively [8], and in continuation of our previous work, here we report the detailed α-chymotrypsin inhibitory activity and inhibition kinetics of these potential lignans. Chymotrypsin catalyzes the hydrolysis of peptide bonds of proteins in the small intestine. It is selective for peptide bonds with aromatic or large hydrophobic side chains (Tyr, Trp, Phe, Met) on the carboxyl side of this bond. Chymotrypsin also catalyzes the hydrolysis of ester bonds. X-ray studies have revealed a 'charge relay system' of Asp-102, His-57, and Ser-195. This grouping has been found in a whole group of enzymes called the 'serine proteases.' Neutron diffraction studies on trypsin show that His-57 acts as a base in the catalytic process. The hydrolysis of peptide bonds occurs by general base-catalyzed nucleophilic attack on the carbonyl carbon of the substrate by the hydroxyl oxygen of Ser-195. At the same time, the hydroxyl proton of serine is transferred to the imidazole of His-57, the chemical base in the hydrolysis reaction. The hydroxyl group of Ser-195 attacks the carbonyl carbon atom of the peptide bond to give a

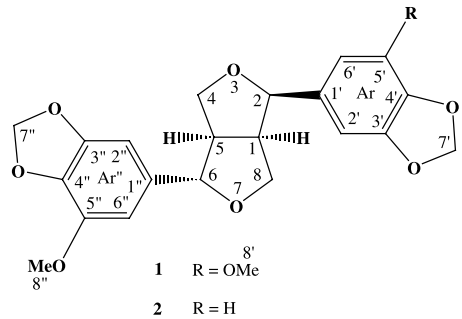


Figure 1. Structures of compounds **1** and **2**.

tetrahedral intermediate. His-57 donates a proton to the nitrogen atom of the peptide bond, leading to cleavage and acylation of the enzyme. Then, deacylation occurs with water taking the place of the amine group of the substrate [10].

Lignans **1** and **2** inhibited the chymotrypsin enzyme in a concentration-dependent manner with the K_i values of 22.29 ± 0.015 and 336.30 ± 0.053 μM, respectively. The K_i values were calculated in three ways: first, the slopes of each line in the Lineweaver–Burk plot were plotted against different concentrations of epiexcelsin (**1**) and 5′-demethoxyepiexcelsin (**2**); second, the $1/V_{max\,app}$ was calculated by plotting different fixed concentrations of the substrate vs. ΔV in the presence of different fixed concentrations of these lignans in the respective assays of chymotrypsin. Third, K_i was calculated by plotting different concentrations of **1** and **2** vs. $1/V_{max\,app}$. K_i was the intercept on the *x*-axis, in the third method; K_i was directly measured from the Dixon plot as an intercept on the *x*-axis. Determination of the inhibition type is critical for the identification of the mechanism of inhibition and the sites of inhibitor binding. Lineweaver–Burk, Dixon plots, and their replots indicated a pure noncompetitive type of inhibition for both the lignans against the chymotrypsin enzyme, as in both cases there was a decrease in V_{max} without affecting the affinity (K_m values) of the chymotrypsin towards the *N*-succinyl-phenylalanine-*p*-nitroanilide. In other words, we can say that both the compounds and *N*-succinyl-phenylalanine-*p*-nitroanilide bind randomly and independently at different sites of chymotrypsin. It indicates that inhibition depends only on the concentration of compounds and dissociation constant (K_i). The K_i and the type of inhibition are listed in Table 1. The graphical analysis of steady-state inhibition data for both the compounds against chymotrypsin has been presented in Figure 2. The structure–activity relationship reveals that when R = OMe (in **1**), the activity increases as compared to when R = H (in **2**). This indicates that the polar oxygen could possibly make hydrogen bonding with the receptor or OMe, which is a very bulky moiety, and could be better accommodated in the protein when compared with its other counterpart. Clearly, there are many questions regarding the mode of action of these ligands that need to be answered, and the answer to these questions will play important role in the development of future generations of these inhibitors; for this reason, we are synthesizing several derivatives of **1** and **2** to be evaluated against chymotrypsin *in vitro* through STD NMR and molecular dynamics simulation and molecular docking to establish a detailed mechanism of inhibition of these lignans.

3. Experimental

3.1 General experimental procedures

For column chromatography (CC) and flash chromatography (FC), silica gel (70–230 mesh and 230–400 mesh, respectively) was used. They were obtained from

Table 1. *In vitro* inhibition of chymotrypsin by lignans **1** and **2**.

No.	Compounds	Type of inhibition	K_i[a] ± SEM[b] (μM)
1	Epiexcelsin (**1**)	Noncompetitive	22.29 ± 0.015
2	5′-Demethoxyepiexcelsin (**2**)	Noncompetitive	336.30 ± 0.053
3	Chymostatin (standard)		8.24 ± 0.11

[a] K_i is the mean of three values calculated using the Dixon plot and Lineweaver–Burk secondary plots.
[b] Standard mean error of 3–5 assays.

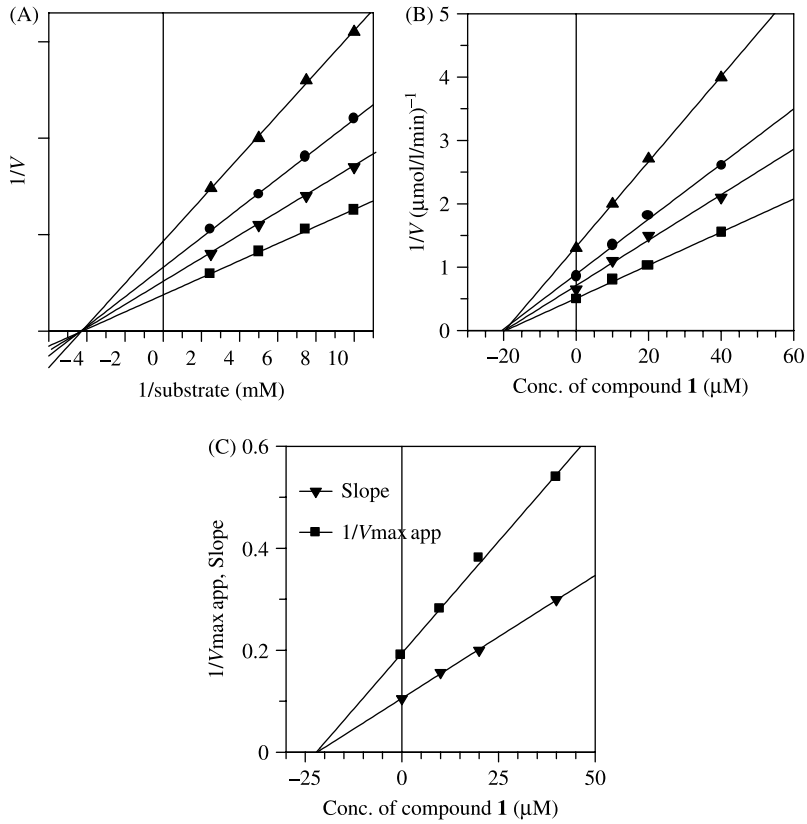


Figure 2. Steady-state inhibition of chymotrypsin by **1**. (A) The Lineweaver–Burk plot of the reciprocal of the initial velocities vs. the reciprocal of four fixed substrate concentrations in the absence (■) and presence of 10.0 μM (▼), 20.0 μM (●), and 40 μM (▲) of **1**. (B) The Dixon plot of the reciprocal of the initial velocities vs. various concentrations of **1** at fixed substrate concentrations, 0.01 mM (■), 0.03 mM (▼), 0.045 mM (●), and 0.06 mM (▲). (C) The secondary replot of the Lineweaver–Burk plot, $1/V_{\text{max app}}$ or slope vs. various concentrations of compound **1**.

Merck (Pvt.) Ltd (Darmstadt, Germany). TLC was performed on precoated silica gel G-25-UV_{254} plates. Detection was carried out at 254 nm, and by ceric sulfate. Purity was checked on TLC with different solvent systems using acetone, *n*-hexane, and $CHCl_3$ giving single spot. Purity was 99%. Optical rotations were measured on a Jasco-DIP-360 digital polarimeter. The UV and IR spectra were recorded on Hitachi-UV-3200 and Jasco-320-A spectrophotometers, respectively. ^{1}H NMR, ^{13}C NMR, COSY, HMQC, and HMBC spectra were run on Bruker spectrometers operating at 500, 400, and 300 MHz. The chemical shifts (δ) are given in ppm and coupling constants (J) in Hertz. EI-MS and FAB-MS spectra were recorded on a JMS-HX-110 spectrometer, with a data system.

3.2 Plant material

Commiphora mukul Engl. was purchased in March 2003 from Liaquatabad supermarket, Karachi and identified by Dr Surayya Khatoon, Department of Botany, University of Karachi. The voucher specimen of the plant has been deposited in the herbarium of the Botany Department of the same university.

3.3 Extraction and isolation

The shade-dried ground whole plant (20 kg) was exhaustively extracted with methanol at room temperature. The extract was evaporated to yield the residue (285 g). The whole residue was extracted with *n*-hexane, chloroform, ethyl acetate, and *n*-butanol. The chloroform-soluble fraction (135 g) was subjected to column chromatography over a silica gel column using *n*-hexane with a gradient of acetone up to 100% and followed by methanol as the eluent. Eight fractions (1–8) were collected. Fraction 4 was loaded on silica gel (flash silica 230–400 mesh) and eluted with acetone–*n*-hexane (1.5:8.5) to purify **2** and **1**, respectively.

3.3.1 *Epiexcelsin* (**1**)

White powder (26.9 mg): $C_{22}H_{22}O_8$; $[\alpha]_D^{23}$ + 88 (c = 0.14, $CHCl_3$); UV λ_{max} (nm) ($\log \varepsilon$) (MeOH): 389 (1.21), 280 (1.57), 265 (1.82), 213 (3.03), 199 (4.12); IR ν_{max} ($CHCl_3$): 2959 (C—H Ar), 1365, 1137 (C—O—C), 812, 756 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): 6.56 (1H, d, J = 1.5 Hz, H-6″), 6.54 (1H, d, J = 1.4 Hz, H-6′), 6.51 (1H, d, J = 1.4 Hz, H-2′), 6.48 (1H, d, J = 1.5 Hz, H-2″), 5.94 (2H, s, H-7″), 5.93 (2H, s, H-7′), 4.78 (1H, d, J = 5.4 Hz, H-6), 4.34 (1H, d, J = 7.1 Hz, H-2), 4.07 (1H, m, H_b-4), 3.89 (6H, s, H-8″ and H-8′), 3.79 (2H, m, H_b-8, H_a-4), 3.28 (1H, m, H_a-8), 3.25 (1H, m, H-5), 2.82 (1H, m, H-1); ^{13}C NMR (100 MHz, $CDCl_3$): 149.0 (C-3′), 148.9 (C-3″), 143.6 (C-5′), 143.5 (C-5″), 135.9 (C-1′), 134.7 (C-4′), 134.2 (C-4″), 132.9 (C-1″), 105.7 (C-6″), 105.2 (C-6′), 101.4 (C-7″), 101.3 (C-7′), 100.1 (C-2′), 99.9 (C-2″), 87.6 (C-2), 82.0 (C-6), 70.9 (C-4), 69.6 (C-8), 56.6 (C-8′ and C-8″), 54.5 (C-1), 50.0 (C-5); EI-MS *m/z* (rel. int.): 414 $[M]^+$(79), 234 (15), 233 (20), 208 $[ArCH{:}CHCH_2OH]^{\cdot+}$(37), 203 (17), 191 $[ArCH{:}CHCH_2]^+$(53), 181 $[ArCHOH]^{\cdot+}$(50), 180 $[ArCOH]^{\cdot+}$(68), 179 $[ArCO]^{\cdot+}$(100), 165 $[ArCH_2]^+$(94), 161 (26), 153 $[ArH_2]^+$(44), 152 $[ArH]^+$ (44), 151 $[Ar]^+$(31).

3.3.2 *5′-Demethoxyepiexcelsin* (**2**)

Gummy solid (15.4 mg): $C_{21}H_{20}O_7$; $[\alpha]_D^{23}$ +116.3 (c = 1.35, $CDCl_3$); UV λ_{max} (nm) ($\log \varepsilon$) (MeOH): 278 (1.94), 262 (1.51), 210 (4.27), 198 (2.83), 196 (4.90); IR ν_{max} ($CHCl_3$): 2964 (C—H, Ar), 2863 (C—H, Aliphat), 1634, 1450 (C=C, Ar), 1322, 1080 (C—O—C), 832, 736, 670 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): 6.84 (1H, d, J = 1.6 Hz, H-2′), 6.78 (1H, dd, J = 7.8, 1.6 Hz, H-6′), 6.74 (1H, d, J = 7.8 Hz, H-5′), 6.56 (1H, d, J = 1.5 Hz, H-6″), 6.48 (1H, d, J = 1.5 Hz, H-2″), 5.95 (2H, s, H-7″), 5.92 (2H, s, H-7′), 4.78 (1H, d, J = 5.4 Hz, H-6), 4.36 (1H, d, J = 7.1 Hz, H-2), 4.06 (1H, m, H_b-4), 3.89 (3H, s, H-8″), 3.81 (2H, m, H_b-8, H_a-4), 3.29 (1H, m, H_a-8), 3.25 (1H, m, H-5), 2.82 (1H, m, H-1); ^{13}C NMR (100 MHz, $CDCl_3$): 148.8 (C-3″), 147.9 (C-4′), 147.2 (C-3′), 143.5 (C-5″), 135.0 (C-1′), 134.1 (C-4″), 132.9 (C-1″), 119.5 (C-6′), 108.1 (C-5′), 106.5 (C-2′), 104.8 (C-6″), 101.4 (C-7″), 101.0 (C-7′), 99.8 (C-2″), 87.6 (C-2), 82.0 (C-6), 70.9 (C-4), 69.6 (C-8), 56.6 (C-8″), 54.5 (C-1), 50.1 (C-5); EI-MS *m/z* (rel. int.): 384 $[M]^+$(46.9), 208 $[ArCH{:}CHCH_2OH]^{\cdot+}$ (11.6), 179 [Ar″ CH:$CHCH_2OH$] (36.1), 151 $[Ar^+]$ (16.8), 149 [Ar″ CO^+] (100), 135 [Ar″ CH_2^+] (70.9) and 122 [Ar″ H^+] (12.9).

3.4 Chymotrypsin assay

Chymotrypsin inhibitory activities of the compounds were performed by the method of Cannell *et al.* [11]. Chymotrypsin (9 units/ml of 50 mM Tris–HCl buffer, pH 7.6; Sigma Chemical Co., St Louis, MO, USA) was pre-incubated with the compounds for 20 min at 25°C. One hundred microliters of substrate solution (0.01–0.06 mM *N*-succinyl-phenyl-alanine-*p*-nitroanilide of 50 mM Tris–HCl buffer, pH 7.6) were added to start the enzyme reaction. The absorbance of released *p*-nitroaniline was continuously

monitored at 410 nm until a significant color change had been achieved. The final DMSO concentration in the reaction mixture was 7%.

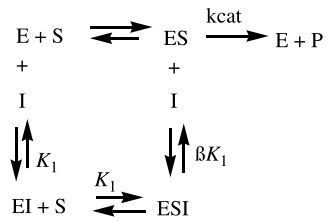
3.5 Estimation of the kinetic parameter

Two different methods were applied to monitor the effect of the inhibitor (test sample) on both K_m and V_{max} values. This was done first by plotting the reciprocal of the rate of the reactions against the reciprocal of the substrate concentration as the Lineweaver–Burk plot, and, second, by the Dixon plot in which the reciprocal of the rate of the reactions was plotted against the inhibitor concentrations [12]. In the case of the noncompetitive and linear mixed-type inhibitions, the slope of each line of the inhibitor concentration on the Lineweaver–Burk plot was plotted against the inhibitor concentrations. The secondary replot of the Dixon plot was constructed as the slope of each line of the substrate concentration in the original Dixon plot against the reciprocals of the substrate concentrations. The K_i values (the dissociation constant of the dissociation of the enzyme–inhibitor complex into free enzyme and inhibitor) were determined by the interpretation of the Dixon plot, Lineweaver–Burk plot, and the secondary replots using initial velocities. The dependency of V_{max}/K_m and V_{max} on the inhibitor [I] is given by

$$\frac{V_{max}}{K_m} = \left(\frac{(V_{max}/K_m)K_i}{K_i + [I]}\right) \Rightarrow I = \frac{K_i}{K_i + [I]}$$

$$\Rightarrow K_i = K_i + [I].$$

These velocities were obtained over a range of the substrate concentrations between 0.01 and 0.06 mM. The assay conditions for the measurement of the residual activities of all inhibitors were identical to a mentioned spectrophotometric assay procedure except that fixed concentrations of inhibiting compounds were used in the assay medium. The types of inhibition were determined by the graphical views of the Dixon plots, Lineweaver–Burk plots, and their secondary plots. The interaction of lignans **1** and **2** with chymotrypsin is described in Scheme 1.



Scheme 1. Interaction of lignans with chymotrypsin.

ES is the chymotrypsin–substrate complex and P is the product. K_1 and βK_1 are the inhibition constants reflecting the interaction of **1** and **2** with the free enzyme and enzyme–substrate complex (Scheme 1).

3.6 Statistical analysis

All assays were conducted in triplicate. Graphs were plotted using the GraFit program [13]. Values of the correlation coefficient, slope, intercept, and their standard errors were obtained by the linear regression analysis using the same software. The correlation coefficients for all the lines of all graphs were >0.99, and each point in the constructed graphs represents the mean of three experiments.

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