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New Imidazoles as Probes of the Active Site Topology and Potent Inhibitors of β-Glucosidase[1]

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Series of 4-arylimidazoles, ω-N-acylhistamines and 4-(ω-phenylalkyl)imidazoles were synthesized in order to probe the active site topology of sweet almond β-glucosidase. These imidazole derivatives were shown to be very powerful competitive inhibitors. Among the 20 tested compounds, ω-N-benzoylhistamine and 4-(3′-phenylpropyl)imidazole are the most potent inhibitors of the enzyme, with pH-independent K_i values of 0.06 and 0.07 μM, respectively. The inhibition of 4-(ω-phenylalkyl)imidazoles exhibited an interesting trend as to K_i values: 4-phenylimidazole (6.6 μM) > 4-benzylimidazole (1.4 μM) > 4-(2′-phenylethyl)imidazole (0.82 μM) > 4-(3′-phenylpropyl)imidazole (0.07 μM) < 4-(4′-phenylbutyl)imidazole (0.13 μM) < 4-(5′-phenylpentyl)imidazole (0.3 μM). This revealed that the imidazole and aryl binding sites (which result from favorable interactions within the corresponding glycone and aglycone binding subsites) are separated by the optimal distance equivalent to the length of a -CH_2-CH_2-CH_2- group. Substitutions of the phenyl moieties of 4-phenylimidazole and 4-benzoylhistamine result in weaker inhibition. These classes of imidazoles are particularly powerful inhibitors of sweet almond β-glucosidase.

Key words: β-glucosidase, inhibition, 4-arylimidazoles, ω-N-acylhistamines, 4-(ω-phenylalkyl)imidazoles.

Inhibitors are powerful probes of the active-site topology and tools for investigating the catalytic mechanisms of enzymes. The design and synthesis of inhibitors have therefore become two of the essential goals for academic research and in the pharmaceutical industry. Powerful inhibitors are potential chemotherapeutic agents. For instance, angiotensin-converting enzyme (ACE) has proved to be a useful target for treating cardiovascular diseases. Several ACE inhibitors, such as captopril, and more recently benazepril, quinapril, and ramipril, have been developed and used as drugs for treating essential hypertension. Specific inhibitors of glycosidases are also of great interest from a number of different aspects. These include mechanistic studies on the enzymes themselves (*1, 2*), studies on glycoprotein processing (*3*), the development of animal models for studying glycosyl ceramide lipidoses (*4*), and the inhibition of HIV replication *in vitro* (*5*). An irreversible inhibitor has already been shown to be useful for differentiating the properties of the normal and Gaucher's disease enzymes (*4*). Powerful inhibitors of β-glucosidase were screened from the available chemical sources in previous studies (*6-9*). Some glucose derivatives, such as δ-gluconolactone, δ-gluconolactam, and 1-deoxynorjirimycin, and imidazole derivatives were found to be very potent reversible inhibitors of the sweet almond enzyme. Trehazolin (*10*), trehazoloid (*11*), benzylamidine (*12-14*), and deoxynorjirimycin analogs containing an imidazole moiety (*15*) constitute additional new classes of glycosidases inhibitors. The syntheses of all of these compounds are complicated. Their K_i values for sweet almond β-glucosidase were shown to be in the range of 10 to 100 μM. More recently, isofagomine and its analogs were reported to be very strong glycosidase inhibitors. The K_i value for isofagomine interacting with sweet almond β-glucosidase is 0.1 μM (*16*).

4-Phenylimidazole is one of the most potent reversible inhibitors of sweet almond β-glucosidase (*9*). It is also fairly specific for β-glucosidase, binding at least three orders of magnitude less tightly to other glycosidases such as β-N-acetylhexosaminidase, α-glucosidase, β-galactosidase, and β-glucoronidase. This inhibitor combines, as a mono-molecular species, the binding affinities of benzene, which binds at the hydrophobic aglycone binding site, and imidazole, which binds at the sugar binding site of β-glucosidase. The binding energy of 4-phenylimidazole can be attributed to the sum of the intrinsic binding energies of the phenyl and imidazole moieties. Thus, there is no significant entropic advantage of combining the component parts of phenylimidazole in a single species. ω-N-Benzoylhistamine is also a very potent inhibitor of sweet almond β-glucosidase, with a pH-independent $K_i = 0.11$ μM, reported by Field *et al.* (*17*). Unfortunately, the data in Field's paper were insufficient to explain the enigma of the binding of 4-phenylimidazole to the enzyme. However, they do support the prediction of Li and Byers that a minor structural modification of 4-phenylimidazole can result in an even more powerful inhibitor. In order to elucidate the intrinsic binding properties of the imidazole and phenyl

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moieties of inhibitors in enzyme, a series of imidazole derivatives were designed and synthesized. Here, we report the inhibition of sweet almond β-glucosidase by these newly designed imidazoles.

MATERIALS AND METHODS

^{1}H and ^{13}C NMR spectra were taken in appropriate *d*-solvents with a Varian UNITY-300FT spectrometer. GC mass spectra were recorded with a Trio-2000 mass spectrometer. High resolution mass (HRMS) values were obtained with a JEOL JMS-D300 mass spectrometer and measured by the DIP (directed inlet probe) method to ensure the purity of the tested samples. Elemental analyses were performed with a Heraeus CHN-O-RAPID combustion apparatus.

Materials—Crude almond β-glucosidase (Sigma, G-0395, Lot 70H4069) and all glycosides used as substrates were obtained from Sigma Chemical. The buffers were purchased from either Sigma or E. Merck. 4-Phenylimidazole, Diazald, acyl chlorides, formamidine, histamine, and all other chemicals for the synthesis were obtained from Aldrich Chemical. The buffer system usually consisted of 100 mM NaCl and 50 mM buffer: sodium acetate (3.5-5.6); 2-(*N*-morpholino)ethanesulfonate (MES) (5.6-7.0); or 3-(*N*-morpholino)propanesulfonate (MOPS) (7.0-7.9).

Enzyme Purification—The purification procedures consisted of chromatographies on a Protein-Pak Q 15HR (Waters) anion exchange column (Waters AP-1) and a Superdex 75 HR 10/30 gel filtration column (Pharmacia Biotech.). All column chromatographies, including determination of column buffer pH values, were performed at room temperature. The purity of the active fraction from each column was determined by SDS-PAGE. In general, 20 mg of crude β-glucosidase was resuspended in 2.5 ml sodium phosphate buffer, 20 mM, pH 5.5. The solution was then loaded onto a PD-10 column to remove excess salts. The filtrate (3.5 ml) was then applied to an anion exchange Q column (1 cm×10 cm) with a linear gradient of 0 to 200 mM NaCl in 20 mM sodium phosphate buffer, pH 5.5. The flow rate was 1 ml/min. The fractions exhibiting β-glucosidase activity were collected and concentrated using Amicon Centriprep 10 centrifugation ultra filtration devices, and then further purified on a Superdex 75 HR (10/30) gel filtration column equilibrated with 0.1 M, pH 7.0, phosphate buffer, at the flow rate of 0.5 ml/min.

Enzyme Assays—β-Glucosidase activity was assayed with *p*-nitrophenyl-β-D-glucopyranoside as the substrate by determining the amount of *p*-nitrophenol released. In all assays, one enzyme unit was defined as the amount of enzyme required to release 1 μmol of *p*-nitrophenol from the substrate in 1 min. In the activity screening assay, 0.01-0.05 μg of the purified protein was added to 0.5 ml MES buffer (50 mM, pH 6.2) containing 1 mM *p*-nitrophenyl-β-D-glucopyranoside.

Kinetics—The β-glucosidase-catalyzed hydrolysis of *p*-nitrophenyl-β-D-glucopyranoside was monitored spectrophotometrically. In general, kinetics were followed with a Hewlett Packard Model 8452A Diode Array Spectrophotometer. When the pH effect was studied, the reaction was always monitored at the *p*-nitrophenol/*p*-nitrophenolate isosbestic point (348 nm). The reactions were initiated by the addition of the enzyme to a solution of the substrate in buffer which had been thermally equilibrated at 25°C. The hydrolysis of the glucoside by the β-glucosidase follows Michaelis-Menten kinetics. The reactions were carried out with a low substrate concentration, which allowed the Michaelis-Menten equation to be simplified as $v = V_{max}[S]/K_m$. Thus, the reaction is first-order in [S]. The apparent first-order rate constant, V_{max}/K_m, can be determined by means of a plot of $\ln(A_\infty - A_t)$ *vs.* t or by non-linear regression. The dissociation constant of the enzyme-inhibitor complex, K_i, was calculated from the effect of the inhibitor on the ratio of the two steady-state parameters:

$$(K/V)_+ = (K/V)_o(1+[I]/K_i)$$

$(K/V)_+$ is the ratio of K_m to V_{max} in the presence of the inhibitor. The parameter, K/V, was determined under first order conditions ($[S] \ll K_m$).

Synthesis of Inhibitors—*(a) Synthesis of 4-arylimidazoles:* 4-(*p*-Methylphenyl)imidazole was synthesized according to Bredereck's method (*18*). 4-(*p*-Nitrophenyl)-imidazole was synthesized by nitration of 4-phenylimidazole in concentrated nitric acid at room temperature for 24 h. The reaction mixture was poured into ice water to obtain the crude product, which was then crystallized from the ethanol with a yield of 75%. 4-(*p*-Aminophenyl)imidazole was synthesized by the photochemical reduction of 4-(*p*-nitrophenyl)imidazole in the presence of TiO_2 (*19*).

4-(*p*-Methylphenyl)imidazole: mp 114-115°C; ^{1}H NMR (d_6-acetone): δ 2.31 (s, 3H), 7.16 (d, J=8.1 Hz, 2H), 7.47 (s, 1H), 7.69 (s, 1H), 7.71 (d, J=8.1 Hz, 2H).

4-(*p*-Nitrophenyl)imidazole: mp 227-228°C; ^{1}H NMR (d_6-DMSO): δ 7.85 (s, 1H), 7.95 (s, 1H), 8.04 (d, J=9 Hz, 2H), 8.23 (d, J=8.7 Hz, 2H); ^{13}C NMR (d_6-DMSO): 145.18 (CH), 141.30 (C), 137.20 (CH), 127.58 (C), 124.70 (CH), 124.14 (CH), 117.0 (C); MS (EI, 70 eV), m/z (rel. int.): 189 (M^+, 100), 116 (45), 89 (38).

4-(*p*-Aminophenyl)imidazole dihydrochloride salt: ^{1}H NMR (d_6-DMSO): 7.37 (d, J=9 Hz, 2H), 7.67 (s, 1H), 7.68 (d, J=8.1 Hz, 2H), 8.66 (s, 1H). MS for free base (EI, 70 eV), m/z (rel. int.): 159 (M^+, 100), 119 (35), 104 (31).

(b) Synthesis of ω-N-acylhistamines: ω-*N*-Acylhistamines were synthesized by the condensation of an acyl chloride with histamine in the presence of triethylamine. The general procedure is outlined below.

Histamine [0.2 g (1.8 mmol)] and 1.34 ml (5.4 mmol) of triethylamine were added to a sealed 100 ml flask containing 15 ml dry dichloromethane pre-cooled to −20°C. An acyl chloride (2 mmol) in dry dichloromethane (10 ml) was then added dropwise by means of a syringe. The solution was stored at room temperature for 24 h and then evaporated. The resulting residue was washed with 100 ml Tris buffer (75 mM, pH 8.0), followed by with 100 ml water. The crude product was obtained either as a solid powder or in an oil form. Solid products were crystallized from methanol/chloroform (1:1). The products in an oil form were purified further by silica gel column (2.5 cm×25 cm) chromatography with an eluent gradient from 100% ethyl acetate to 1:1 ethyl acetate/methanol. The acyl chlorides used for the syntheses were: benzoyl chloride, 4-cyanobenzoyl chloride, 4-nitrobenzoyl chloride, *p*-toluoyl chloride, *p*-methoxybenzoyl chloride, phenylacetyl chloride, phenylpropionyl chloride, *p*-nitrophenylacetyl chloride, phenylbutyryl chloride, and 1-naphthylacetyl chloride. The last three acyl chlorides were synthesized by

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treating the corresponding acids with thionyl chloride.

The melting points (mp) and NMR data for ω-*N*-(benzoyl)histamine and ω-*N*-(phenylacetyl)histamine are consistent with those in the literature (*17*).

ω-*N*-(Cyanobenzoyl)histamine: mp 194-195°C; ^1H NMR (CD_3OD): δ 2.89 (t, $J=7$ Hz, 2H), 3.62 (t, $J=7$ Hz, 2H), 6.87 (s, 1H), 7.59 (s, 1H), 7.80 (d, $J=8.1$ Hz, 2H), 7.91 (d, $J=8.7$ Hz, 2H); MS (EI, 70 eV), m/z (rel. int.): 240 (M^+, 9), 130 (26), 110 (5), 94 (100).

ω-*N*-(Nitrobenzoyl)histamine: mp 209-210°C; ^1H NMR (CD_3OD): δ 2.92 (t, $J=7.4$ Hz, 2H), 3.66 (t, $J=7.1$ Hz, 2H), 6.86 (s, 1H), 7.49 (s, 1H), 8.01 (d, $J=8.7$ Hz, 2H), 8.29 (d, $J=7.2$ Hz, 2H); MS (EI, 70 eV), m/z (rel. int.): 260 (M^+, 7), 150 (15), 110 (6), 94 (100).

ω-*N*-(Methoxybenzoyl)histamine: mp 161-162°C; ^1H NMR (CD_3OD): δ 2.88 (t, $J=7.1$ Hz, 2H), 3.60 (t, $J=7.2$ Hz, 2H), 3.82 (s, 3H), 6.83 (s, 1H), 6.92 (d, $J=7.8$ Hz, 2H), 7.55 (s, 1H), 7.75 (d, $J=8.1$ Hz, 2H); MS (EI, 70 eV), m/z (rel. int.): 245 (M^+, 3), 135 (83), 94 (100).

ω-*N*-(Methylbenzoyl)histamine: mp 188-189°C; ^1H NMR (CD_3OD): δ 2.35 (s, 3H), 2.90 (t, $J=7.2$ Hz, 2H), 3.61 (t, $J=7.2$ Hz, 2H), 6.88 (s, 1H), 7.22 (d, $J=8.1$ Hz, 2H), 7.63 (s, 1H), 7.69 (d, $J=8.1$ Hz, 2H); MS (EI, 70 eV), m/z (rel. int.): 229 (M^+, 10), 119 (83), 110 (5), 94 (100).

ω-*N*-(Phenylpropionyl)histamine: mp 135-136°C; ^1H NMR (CD_3OD): δ 2.44 (t, $J=7.6$ Hz, 2H), 2.70 (t, $J=7.2$ Hz, 2H), 2.88 (t, $J=7.5$ Hz, 2H) , 3.37 (t, $J=7.2$ Hz, 2H), 6.73 (s, 1H), 7.16-7.55 (m, 5H), 7.5 (s, 1H); MS (EI, 70 eV), m/z (rel. int.): 243 (M^+, 29), 133 (9), 110 (8), 94 (100), 77 (7).

ω-*N*-(Phenylbutyryl)histamine: mp 166-168°C; ^1H NMR (CD_3OD): δ 1.88 (m, 2H), 2.16 (t, $J=7.4$ Hz, 2H), 2.57 (t, $J=7.7$ Hz, 2H), 2.76 (t, $J=7.4$ Hz, 2H), 3.4 (t, $J=7.4$ Hz, 2H), 6.83 (s, 1H), 7.13-7.23 (m, 5H), 7.57 (s, 1H); MS (EI, 70 eV), m/z (rel. int.): 257 (M^+, 13), 147 (12), 110 (10), 94 (100), 77 (3).

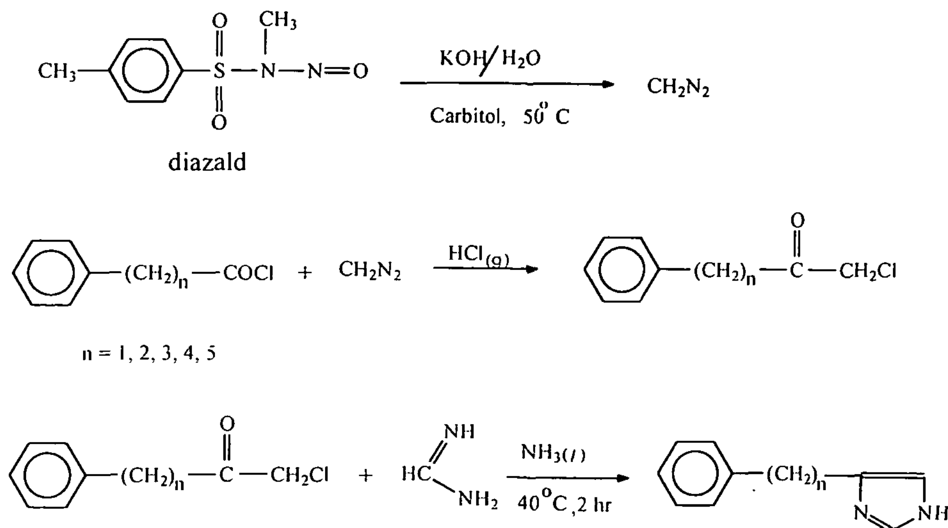
ω-*N*-(*p*-Nitrophenylacetyl)histamine: mp 161-163°C; ^1H NMR (CD_3OD): δ 2.79 (t, $J=7.4$ Hz, 2H), 3.46 (t, $J=6.9$ Hz, 2H), 3.62 (s, 2H), 6.75 (s, 1H), 7.47 (d, $J=8.7$ Hz, 2H), 7.57 (s, 1H), 8.17 (d, $J=8.7$ Hz, 2H); MS (EI, 70 eV), m/z (rel. int.): 274 (M^+, 8), 110 (4), 94 (100).

ω-*N*-(2-Naphthylacetyl)histamine: mp 164-165°C; ^1H NMR (CD_3OD): δ 2.71 (t, $J=6.6$ Hz, 2H), 3.42 (t, $J=6.6$ Hz, 2H), 3.95 (s, 2H), 6.63 (s, 1H), 7.38-7.50 (m, 5H), 7.5-7.88 (m, 3H); MS (EI, 70 eV), m/z (rel. int.): 297 (M^+, 36), 185 (47), 141 (100), 138 (72), 95 (72).

(c) Synthesis of 4-(ω-phenylalkyl) imidazoles: In general, imidazoles were synthesized according to the method of Schunack (*20*) (shown in Scheme 1) with a few modifications. The general procedure was as follows: 0.05 mol of acyl chloride was added to 100 ml of a pre-distilled ether-diazomethane solution to obtain the corresponding chloromethyl ketone. Diazomethane was distilled very carefully at 50°C by slowly adding 70 ml of an ether solution containing 11.8 g Diazald to 50 ml of pre-mixed alkaline ethanol. For safety, scratch-free glassware is required for diazomethane distillation. For the synthesis of 4-benzylimidazole, for example, benzyl chloromethyl ketone was mixed with one equivalent of formamidine acetate and liquid ammonia (~100 ml, trapped with dry ice) in a 1 liter pressure reactor (Parr Instruments). The reaction was carried out at 25-35°C for 2 h. After releasing the ammonia, the residue in the reactor was washed out with acetone. The solution was evaporated to dryness, and then extracted with ethyl acetate (3×50 ml). The combined organic extracts were dried over $MgSO_4$ and then concentrated to obtained crude products. Pure products were often obtained by silica gel column separation with ethyl acetate/methanol gradient elution.

4-Benzylimidazole: mp 84-85°C; ^1H NMR ($CDCl_3$): δ 3.91 (s, 2H), 6.70 (s, 1H), 7.10-7.40 (m, 5H), 7.38 (s, 1H), 9.95 (b, 1H); ^{13}C NMR ($CDCl_3$): δ 33.24 (CH_2), 117.59 (CH), 126.15 (CH), 128.37 (CH), 128.63 (CH), 134.84 (CH), 136.41 (C), 139.73 (C); MS (EI, 75 eV), m/z (rel. int.): 159 (M^++1, 8.01), 158 (M^+, 90.32), 130 (100), 103 (36.09), 81 (26.01), 77 (28.43); HRMS (EI) m/z calcd for $C_{10}H_{10}N_2$: 158.0845; found: 158.0844.

4-(2′-Phenylethyl)imidazole: mp 84-85°C; ^1H NMR ($CDCl_3$): δ 2.8-3.1 (m, 4H), 6.77 (s, 1H), 7.1-7.3 (m, 5H), 7.57 (s, 1H), 12.14 (b, 1H); ^{13}C NMR ($CDCl_3$): δ 28.53 (CH_2), 35.83 (CH_2), 117.53 (CH), 125.89 (CH), 128.25 (CH), 134.40 (CH), 136.26 (C), 141.48 (C); MS (EI, 75 eV), m/z (rel. int.): 173 (M^++1, 4.77), 172 (M^+, 28.18), 158 (12.50), 130 (13.77), 81 (100), 77 (10.96); HRMS (EI)



Scheme 1. **The synthetic pathway for 4-(ω-phenylalkyl) imidazole.**

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m/z calcd for $C_{11}H_{12}N_2$: 172.1002; found: 172.1003; Anal. calcd for $C_{11}H_{12}N_2$: N 16.27%, C 76.70%, H 7.03%; found: N 16.20%, C 76.62%, H 7.10%.

4-(3′-Phenylpropyl)imidazole: ^{1}H NMR ($CDCl_3$): δ 1.99-2.10 (m, 2H), 2.60-2.80 (m, 4H), 6.85 (s, 1H), 7.20-7.40 (m, 5H), 7.61 (s, 1H), 10.13 (b, 1H); ^{13}C NMR ($CDCl_3$): δ 25.66 (CH_2), 30.70 (CH_2), 35.19 (CH_2), 117.00 (CH), 125.75 (CH), 128.25 (CH), 128.34 (CH), 133.88 (CH), 135.94 (C), 141.74 (C); MS (EI, 75 eV), *m/z* (rel. int.): 187 (M^++1, 1.48), 186 (M^+, 8.84), 131 (18.79), 110 (100), 95 (64.97), 77 (3.46); HRMS calcd for $C_{12}H_{14}N_2$: 186.1158; found: 186.1145.

4-(4′-Phenylbutyl)imidazole: ^{1}H NMR ($CDCl_3$): δ 1.5-1.8 (m, 4H), 2.4-2.7 (m, 4H), 6.71 (s, 1H), 7.0-7.3 (m, 5H), 7.53 (s, 1H), 10.4-10.8 (b, 1H); ^{13}C NMR ($CDCl_3$): δ 26.10 (CH_2), 26.81 (CH_2), 30.88 (CH_2), 35.54 (CH_2), 117.24 (CH), 125.20 (CH), 128.19 (CH), 128.28 (CH), 133.93 (CH), 136.15 (C), 142.27 (C); MS (EI, 75 eV), *m/z* (rel. int.): 201 (M^++1, 9.04), 200 (M^+, 50.00), 104 (4.92), 95 (100), 91 (42.02), 81 (63.03), 77 (5.78); HRMS calcd for $C_{13}H_{16}N_2$: 200.1315; found: 200.1320.

4-(5′-Phenylpentyl)imidazole: ^{1}H NMR ($CDCl_3$): δ 1.3-1.4 (m, 2H), 1.5-1.7 (m, 4H), 2.5-2.7 (m, 4H), 6.76 (s, 1H), 7.1-7.3 (m, 5H), 7.77 (s, 1H); ^{13}C NMR ($CDCl_3$): δ 25.69 (CH_2), 28.69 (CH_2), 28.84 (CH_2), 31.08 (CH_2), 35.71 (CH_2), 116.57 (CH), 125.63 (CH), 128.22 (CH), 128.31 (CH), 133.61 (CH), 135.77 (C), 142.47 (C); MS (EI, 75 eV), *m/z* (rel. int.): 215 (M^++1, 6.83), 214 (M^+, 57.56), 95 (91.71), 91 (55.12), 81 (100), 77 (6.10); HRMS calcd for $C_{14}H_{18}N_2$: 214.1471; found: 214.1475.

4-(1-Naphthylmethyl)imidazole: ^{1}H NMR ($CDCl_3$): δ 4.34 (s, 2H), 6.57 (s, 1H), 7.2-7.5 (m, 4H), 7.57 (s, 1H), 7.71 (d, J=8.1 Hz, 1H), 7.75-7.90 (m, 1H), 7.9-8.1 (m, 1H); ^{13}C NMR ($CDCl_3$): δ 31.29 (CH_2), 118.29 (CH), 124.73 (CH), 126.27 (CH), 126.56 (CH), 127.41 (CH), 127.90 (CH), 129.33 (CH), 132.94 (C), 135.10 (C), 135.59 (CH), 136.59 (C), 137.40 (C); MS (EI, 75 eV), *m/z* (rel. int.): 209 (M^++1, 21.93), 208 (M^+, 100), 207 (M^+−1, 78.61), 180 (79.68), 127 (14.30), 81 (17.38); HRMS calcd for $C_{14}H_{12}N_2$: 208.1002; found: 208.0998.

RESULTS AND DISCUSSION

Enzyme Purification and Characterization—The enzyme used in this study was purified from the crude product of Sigma (G-0395, 25,000 units, Lot 70H4069) (*21*). The purification process was simplified and optimized by passing the crude sample through a Protein-Pak Q column, following by gel filtration chromatography. After these purification steps, the enzyme was found to be electrophoretically homogeneous on SDS-PAGE. The purified enzyme is a monomeric glycoprotein with a molecular weight of 58 kDa and a pI=4.55, and thus is distinguishable from the reported isozymes, isozyme A (135 kDa, pI=7.3) (*22*), isozyme B (190-200 kDa, pI=4.8), and isozyme AB (155 kDa, pI=5.5) (*23*).

Synthesis and Identification of 4-(ω-Phenylalkyl) Imidazoles—4-Benzylimidazole (*a*), 4-(2′-phenylethyl)imidazole (*b*), 4-(3′-phenylpropyl)imidazole (*c*), 4-(4′-phenylbutyl)imidazole (*d*), 4-(5′-phenylpentyl)imidazole (*e*), and 4-(1-naphthylmethyl)imidazole (*f*) were synthesized, purified and identified by NMR, and GC-Mass. All of these compounds were recrystallized twice and further confirmed by elemental analysis or DIP (directed inlet probe) High Resolution Mass Spectroscopy (HRMS). Compounds *b, c, d, e,* and *f* are compounds have never previously been reported in the literature. The synthesis, shown in Scheme 1, was performed by mixing the corresponding chloromethyl ketone with formamidine in liquid ammonia. Although the synthesis of 4-benzylimidazole has been reported (*18*), it could not be repeated unless the reaction conditions were modified (shown in synthesis section). Due to the harsh reaction conditions, the yields, from the corresponding chloromethyl ketones, were about 20-38%. All the synthetic compounds were recrystallized or column chromatographically purified to assure sufficient purity for the inhibition study.

Inhibitory Nature of Imidazole Derivatives—Several imidazole derivatives have been synthesized to probe the topography of the active site of sweet almond β-glucosidase. Seven of them, comprising arylimidazoles 4-phenylimidazole and 4-(*p*-nitrophenyl)imidazole, ω-*N*-acylhist amines ω-*N*-benzoylhistamine, ω-*N*-(*p*-methoxybenzoyl)-histamine and ω-*N*-phenylpropionylhistamine, and 4-(ω-phenylalkyl) imidazoles 4-benzylimidazole and 4-(3′-phenylpropyl)imidazole, were shown to be competitive inhibitors of β-glucosidase (data not shown). This is consistent with the findings reported by Li and Byers (*9*).

Inhibition by 4-Arylimidazoles—4-Phenylimidazole was shown to be one of the most potent competitive inhibitors of sweet almond β-glucosidase with a pH-independent K_i= 0.83 μM (for sweet almond isozyme A). The binding to the enzyme has been studied extensively. The results showed that the phenyl moiety binds to the aglycone binding subsite and the imidazole moiety binds to the glycone binding subsite (*9*). In order to further examine the aglycone binding pocket, a group of 4-arylimidazoles with either an electron withdrawing or an electron donating group at the *para*-position of the phenyl moiety were examined as inhibitors of the purified isozyme. The results are shown in Table I. With either a nitro group or a methyl group at the *para*-position of the benzene ring in 4-phenylimidazole, the binding affinity was attenuated by a factor of 2-9-fold (based on K_i values). However, an amino group increased the binding affinity about 3-fold. This might indicate that the aglycone binding site is somewhat restricted in size, and perhaps a hydrogen binding group on the enzyme, which is responsible for the tighter binding of 4-aminophenylimidazole, is present. Unfortunately, 4-(*p*-hydroxyphenyl)imidazole, which might clarify this, could not be obtained even with a great deal of effort.

Inhibition by ω-N-Acyl Histamines—In Li and Byers' paper (*9*), the binding energy of 4-phenylimidazole as to β-glucosidase was shown to be comparable to the sum of the intrinsic binding energies of the phenyl group and the imidazole moiety. One simple explanation for this is that in 4-phenylimidazole the benzene moiety and the imidazole moiety can not optimally interact with their binding subsites on the enzyme unless a conformational change occurs. The entropic advantage derived from connecting benzene and imidazole to form a single molecule is possibly compensated by this unfavorable structural situation. Therefore, it is likely that only minor structural modification will lead to an even more powerful inhibitor. Encouragingly, ω-*N*-benzoylhistamine was shown to strongly inhibit sweet almond β-glucosidase. The pH-independent K_i value is 0.11 μM,

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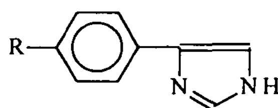
which is 8-hold lower than that of 4-phenylimidazole (0.83 μM for isozyme A). This finding might indicate that the interactions with the two binding subsites (glycone and aglycone) on the enzyme are optimized when the former inhibitor binds. A series of ω-N-acylhistamines, with various inserts between the aryl group and the imidazole moiety, were therefore synthesized and examined as inhibitors of the purified isozyme. The inhibition constants are summarized in Table II. As shown, when 1-3 methylene groups were inserted between the phenyl group and the carbonyl group of ω-N-benzoylhistamine, the binding affinity of the inhibitor decreased as the number of the inserted methylene groups increased. The data exhibited an interesting trend which suggests an optimal distance between the two binding subsites corresponding to that of a -C(O)-NH-CH_2-CH_2- chain or possibly even shorter. Furthermore, ω-N-(2-napthoyl)histamine and ω-N-(2-napthylacetyl)histamine, inhibitors with a larger hydrophobic group, are weaker inhibitors than the corresponding phenyl substituted acylhistamines. Also, substitution with either an electron donating group (*e.g.* methyl or methoxy) or an electron withdrawing group (nitro or cyano) on the phenyl ring of ω-N-benzoylhistamine (shown in Table III) resulted in a decrease in the binding affinity of the inhibitor by a factor of 5-62-fold (see Table III). This observation is consistent with the results obtained with 4-arylimidazoles, which indicates the hydrophobic binding subsite is most complementary to the phenyl moiety. Modifications (except for HN_2) of the phenyl group of 4-phenylimidazole or ω-N-benzoylhistamine result in weaker binding.

Inhibition by 4-(ω-Phenylalkyl) Imidazoles—To probe the size of the active cavity of sweet almond β-glucosidase, 4-(ω-phenylalkyl) imidazole derivatives were synthesized, which were 4-benzylimidazole, 4-(2′-phenylethyl)imidazole, 4-(3′-phenylpropyl)imidazole, 4-(4′-phenylbutyl)imidazole, 4-(5′-phenylpentyl)imidazole, and 4-(1′-naphthylmethyl)-imidazole. These new imidazoles were designed to determine the optimal distance between the two binding subsites. The dissociation constants (K_i) of the enzyme-inhibi-

TABLE I. **Inhibition of β-glucosidase by 4-arylimidazoles.**

Inhibitor	pK_a [a]	K_i (μM) [b]	K_i^{lim} (μM) [c]
4-Phenylimidazole	6.1	26.6	6.6
4-(p-Methylphenyl)imidazole	6.2	73	13
4-(p-Nitrophenyl)imidazole	4.7	69	61
4-(p-Aminophenyl)imidazole	6.7	31	2.3

[a]The pK_a values were determined by potentiometric titration at 25°C in 100 mM NaCl. [b]Measured at pH 5.6 and 25°C. The uncertainties in K_i are within 5%. [c]Based on the inhibition study on 4-phenylimidazole in Ref. *9*, we assumed that the deprotonated forms of inhibitors were the active species. Corrections were made with the following equations: $r = 1/(1+10^{pK_a-pH}) = [I]/[I]_{total}$, $K_i^{lim} = r \cdot K_i$.

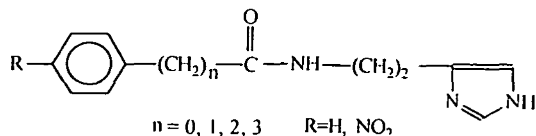


R= H, CH_3, NO_2, NH_2

TABLE II. **K_i values of β-glucosidase with ω-N-acylhistamines.**

Inhibitor[a]	K_i (μM) [b]	K_i^{lim} (μM) [c]
ω-N-Benzoylhistamine	1.28	0.06, (0.11)[d]
ω-N-(2-Naphthoyl)histamine	12.6	0.6, (0.86)[d]
ω-N-Phenylacetylhistamine	21	1.0, (2.2)[d]
ω-N-(p-Nitrophenylacetyl)histamine	72	3.4
ω-N-(2-Napthylacetyl)histamine	37	1.8
ω-N-Phenylpropionylhistamine	82	3.9
ω-N-Phenylbutyrylhistamine	87	4.2

[a]All of the pK_a values were determined to be 6.9±0.1 by potentiometric titration at 25°C in 100 mM NaCl. [b]Measured at pH 5.6, 25°C. The standard deviation in K_i is less than 5%. [c]The calculation of K_i^{lim} was described in the legend to Table I. [d]Data from Ref. *17*.



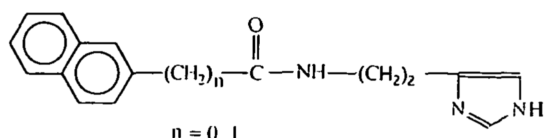
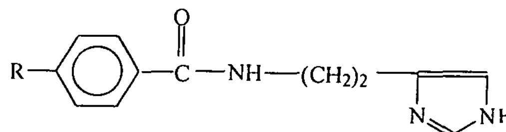


TABLE III. **K_i values of substituted ω-N-benzoylhistamine.**

Inhibitor	pK_a [a]	K_i (μM) [b]	K_i^{lim} (μM) [c]
ω-N-Benzoylhistamine	6.9	1.3	0.06
ω-N-(p-Methylbenzoyl)histamine	7.0	8.0	0.3
ω-N-(p-Methoxybenzoyl)histamine	7.0	26	1.0
ω-N-(p-Nitrobenzoyl)histamine	6.8	62	3.7
ω-N-(p-Cyanobenzoyl)histamine	6.9	69	3.3

[a]The pK_a values were determined by potentiometric titration at 25°C in 100 mM NaCl. [b]Measured at pH 5.6, 25°C. The standard deviation in K_i is within 5%. [c]The calculation of K_i^{lim} was described in the legend to Table I.

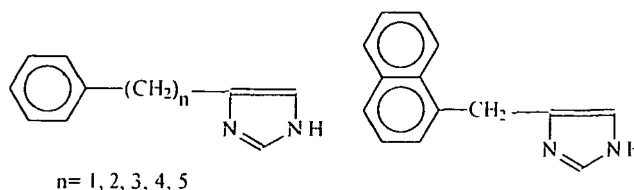


R= CH_3, CH_3O, NO_2, CN

TABLE IV. **Inhibition of β-glucosidase by 4-(ω-phenylalkyl) imidazoles.**

Inhibitor[a]	K_i (μM) [b]	K_i^{lim} (μM) [c]
4-Benzylimidazole	38.4	1.4
4-(2′-Phenylethyl)imidazole	26.6	0.82
4-(3′-Phenylpropyl)imidazole	2.13	0.07
4-(4′-Phenylbutyl)imidazole	4.1	0.13
4-(5′-Phenylpentyl)imidazole	9.9	0.30
4-(1′-Naphthylmethyl)imidazole	48.9	1.54

[a]All of the pK_a values were determined to be 7.1±0.1 by potentiometric titration at 25°C in 100 mM NaCl. [b]Measured at pH 5.6, 25°C. The standard deviation in K_i is less than 5%. [c]The calculation of K_i^{lim} was described in the legend to Table I.



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tor complexes are listed in Table IV. This study showed that this group of new imidazoles constitutes very powerful inhibitors of β-glucosidase from sweet almond. 4-(3′-Phenylpropyl)imidazole, with a pH-independent $K_i = 0.07$ μM (comparable to the K_i value of ω-*N*-benzoylhistamine), is the best inhibitor for this enzyme reported to date. The K_i value of 4-(3′-phenylpropyl)imidazole is 110 times lower than that of 4-phenylimidazole. The binding affinities of these reversible inhibitors exhibit an interesting trend: 4-phenylimidazole < 4-benzylimidazole < 4-(2′-phenylethyl)imidazole < 4-(3′-phenylpropyl)imidazole > 4-(4′-phenylbutyl)imidazole > 4-(5′-phenylpentyl)imidazole. This strongly indicates that the two binding subsites (glycone and aglycone) of the enzyme are separated by an optimal distance equivalent to that of a $-CH_2-CH_2-CH_2-$ group. The inhibition of 4-(1′-naphthylmethyl)imidazole was found to be stronger than that of 4-phenylimidazole but somewhat weaker than that of 4-benzylimidazole. This could be due to, at least partially, a "distance effect."

Specificity and pH-Dependence—4-(3′-Phenylpropyl)-imidazole and ω-*N*-benzoylimidazole have been shown to be the most potent inhibitors of sweet almond β-glucosidases. These two compounds were examined as inhibitors of several other glycohydrolases, including β-glucosidases from *Calocellum saccharolyticum* (Sigma) and *Flavobacterium meningosepticum*, β-xylosidase from *Trichoderma koningii* (Li, Y.-K., unpublished data), and α-glucosidase from yeast (Sigma). No significant inhibition was observed up to 2 mM inhibitor. For a better understanding of the interactions of the two inhibitors with this almond β-glucosidase, the pH-dependence of their inhibition was investigated. The catalytic activity of this isozyme, with *p*-nitrophenyl-β-D-glucopyranoside as the substrate, was reported in our previous paper (*21*). The two apparent pK_as which mediate the enzyme function are $pK_1 = 4.7$ and $pK_2 = 6.4$. The data for pH-dependent inhibition of 4-(3′-phenylpropyl)imidazole, shown in Fig. 1, exhibited a bell-shaped curve character. This bell-shaped curve can be explained by either the unprotonated inhibitor (I) binding to the monodeprotonated enzyme (EH^-) or, less likely, the protonated inhibitor (HI^+) binding to the deprotonated enzyme (E^{2-}) (*9*). The bell-shaped curve was analyzed (*24*) and fitted with the following equation:

$$1/K_i^{obs} = 1 \Big/ \left\{ K_i^{lim} \left(\frac{[H^+]}{K_1} + 1 + \frac{K_2}{[H^+]} \right) \left(\frac{[H^+]}{K_a} + 1 \right) \right\}$$

where K_a is the acid dissociation constant of the inhibitor, and K_1 and K_2 are the dissociation constants of the enzyme which affect the binding of the inhibitor. K_i^{lim} is the pH-independent K_i value. As shown in Fig. 1, the solid line is the theoretical fit assuming unprotonated 4-(3′-phenylpropyl)imidazole binding only to the catalytically active species (EH). For both 4-(3′-phenylpropyl)imidazole (Fig. 1) and ω-*N*-benzoylimidazole (data not shown), the two apparent pK_as, 4.7 and 6.3, of the enzyme were obtained

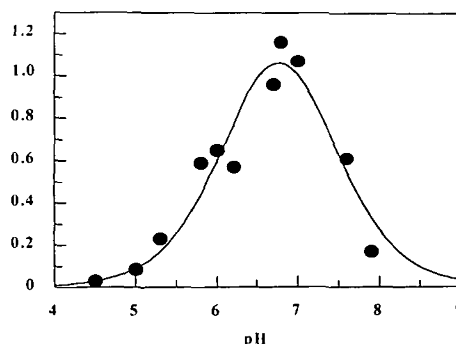
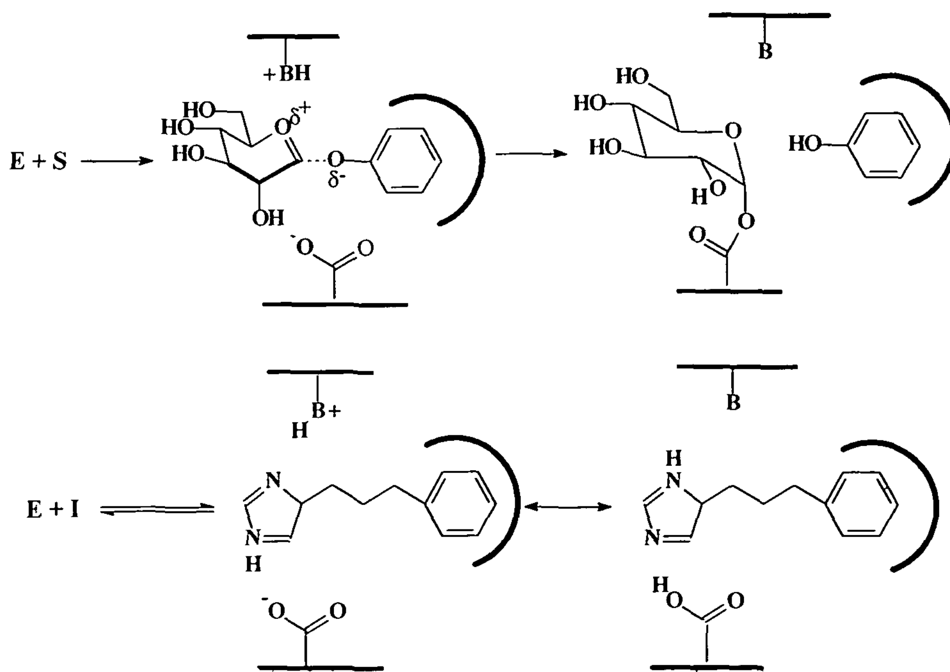


Fig. 1. **pH dependence of the inhibition of β-glucosidase by 4-(3′-phenylpropyl)imidazole.** The solid line is the theoretical fit assuming unprotonated 4-(3′-phenylpropyl)imidazole binding only to the catalytically active species (EH) with a pH-independent dissociation constant of 0.07 μM, and two apparent pK_a values of 4.7 and 6.3.



Scheme 2. **The proposed binding models for 4-(3′-phenylpropyl)imidazole and a substrate with almond β-glucosidase.**

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when the pK_as of the inhibitors were set at 7.1 and 6.9 for 4-(3′-phenylpropyl)imidazole and ω-N-benzoylimidazole, respectively. The pH-independent K_i values of the two inhibitors are similar (around 0.06 μM). Based on the catalytic activity and inhibition of the enzyme, the highly superimposable pH behavior indicates that the catalytically active form of the enzyme might interact with an unprotonated inhibitor in a manner analogous to its interaction with the reactants in the transition state. Proposed binding models for 4-(3′-phenylpropyl)imidazole and a substrate with the enzyme are illustrated in Scheme 2.

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