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Short communication

Design and synthesis of α,β-unsaturated carbonyl compounds as potential ACE inhibitors

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Abstract – The α,β-unsaturated amide that is incorporated into the basic structural frame of a simple substrate molecule of angiotensin converting enzyme was found to serve as a Michael acceptor for the catalytic carboxylate of Glu-127, inhibiting the enzyme irreversibly.

α,β-unsaturated amides / angiotensin converting enzyme inhibitor / irreversible inhibitors

1. Introduction

The renin-angiotensin system plays an important role in the regulation of blood pressure. Angiotensin II, an octapeptide that is generated from angiotensin I by enzymatic removal of dipeptide from its C-terminal is a potent vasoconstrictor and is responsible for the elevation of blood pressure [1]. Thus, prevention of its formation is expected to bring about a hypotensive effect. In fact, inhibitors of angiotensin converting enzyme (ACE) such as captopril [2] and enalapril [3] are widely used in the clinic for the treatment of hypertension. ACE catalyses the dipeptide removal.

Recently, we have reported that epoxyalkanoyls are effective for ACE [4]. In the present study we explored the use of α,β-unsaturated amides as pseudo-mechanism based inactivators for the enzyme [5]. It is well known that α,β-unsaturated esters and amides can react with the catalytic nucleophile at the active site of enzyme to give a Michael type addition product, thus modifying the nucleophile covalently [6, 7]. Once the nucleophile forms the Michael addition product, the enzyme remains inactive until new copies of the enzyme are synthesized. Accordingly, this type of inhibitor is expected to show a long duration of action.

The active site of ACE is known to consist of three parts [8]; a carboxylate binding functionality such as the guanidinium group of Arg, a pocket that accommodates a hydrophobic side chain of the C-terminal amino acid residue [9] and a zinc ion. The zinc ion coordinates to the carbonyl of the penultimate peptide bond of the substrate, whereby the carbonyl group becomes polarized and is subjected to a nucleophilic attack. The carboxylate of Glu-127 is known to be the nucleophile. It was therefore thought that if the α,β-unsaturated amide moiety is incorporated into the basic structural frame of a simple substrate in such a way that it would bind the enzyme with its carbonyl group being coordinated to the zinc ion, then there may occur a Michael type addition reaction on the α,β-unsaturated amide by the catalytic carboxylate, leading to irreversible inhibition of the enzyme (*figure 1*).

2. Results and discussion

The synthesis of the target compounds were carried out as outlined in *figure 2*. Protected hydroxyacetone was subjected to the Wittig-Horner reaction with triethylphosphonoacetate. After deprotection the resulting alkylalcohol was oxidized using the Jones reagent to give the corresponding carboxylic acid, which was then con-

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Abbreviations: Arg: arginine / Glu: glutamine / Phe: phenylalanine

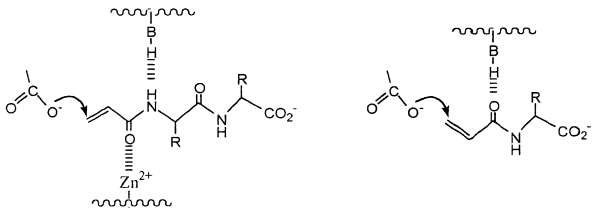


Figure 1. Cartoon representation of ACE inactivation by α,β-unsaturated amides of dipeptide (**a**) and of amino acid (**b**).

densed with amino acid or dipeptide under the Schotten-Baumann conditions.

The inhibition of ACE in the presence of each of the synthesized compounds was measured by the literature method [10] which uses the HPLC quantitation of hippuric acid formed after enzymatic cleavage of tripeptide and the results are summarized in *tables I–III*. As can be seen from the tables, most of the α,β-unsaturated amides studied show weak but distinctive inhibitory activity for ACE with IC_{50} values of 0.1–3 mM. Compound **1** was found to be the most potent in this series with an IC_{50} value of 0.23 mM.

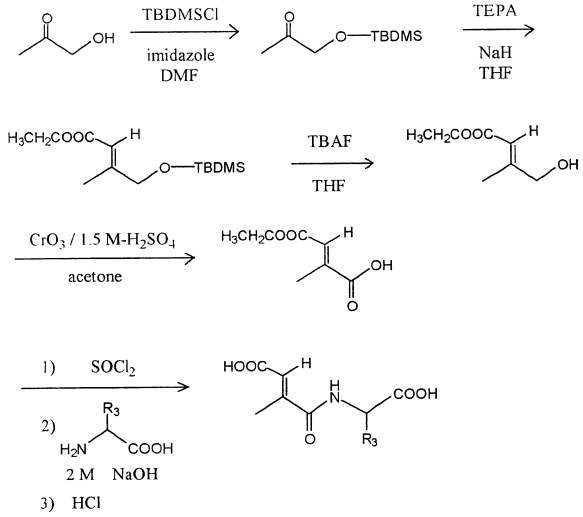
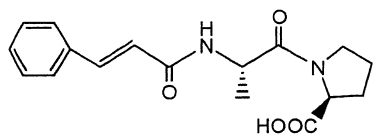
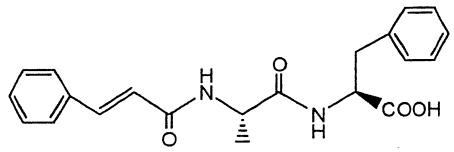
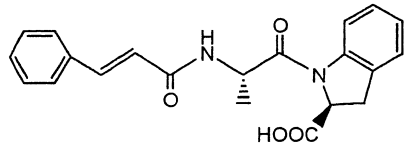
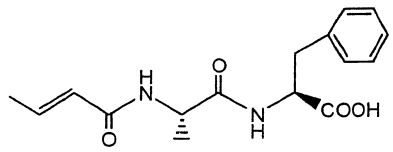


Figure 2. General synthesis of fumaric acid derivatives.

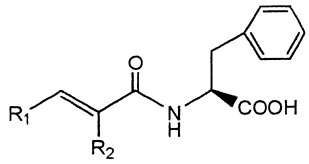
Upon binding substrate to the enzyme, the carbonyl of the scissile amide bond coordinates to the zinc ion at the active site, which polarizes the carbonyl to make the

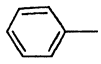
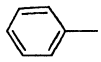
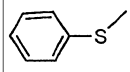
Table I. ACE inhibition of dipeptide analogues.*

		IC_{50}(mM)
1		0.23
2		3.19
3		2.0
4		NE

* NE : No inhibitory activity at 5 mM.

Table II. ACE inhibition of Phe derivatives.

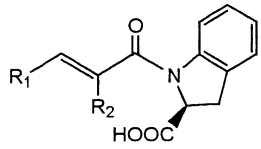


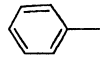
No	R_1	R_2	IC_{50}(mM)
5		H	1.68
6		CH_3	5.76
7		H	NE
8	—COOH	H	2.33
9	—COOH	CH_3	2.28

carbon an electrophilic centre [11]. As anticipated from rational design, the carbonyl of the α,β-unsaturated amide such as compound **1** is expected to coordinate to the zinc ion, rendering it to serve as a Michael acceptor for the carboxylate of Glu-127 (*figure 1*). Apparently, the nucleophilic carboxylate appears to prefer the Michael type addition to the nucleophilic attack on the carbonyl carbon, resulting in tethering the inhibitor to the carboxylate with formation of a covalent bond (*figure 1*). The irreversible nature of the inhibition was experimentally supported by the time-dependent loss of the enzymic activity as shown in *figure 3*.

The failure of the recovery of the enzymic activity of the inactivated ACE upon dialysis is consistent with the conclusion made on the basis of the kinetic analysis that the enzyme is covalently modified. It is worth noting that

Table III. ACE inhibition of indoline derivatives.



No	R_1	R_2	IC_{50}(mM)
10		H	1.2
11	—COOH	H	NE
12	—COOH	CH_3	0.13

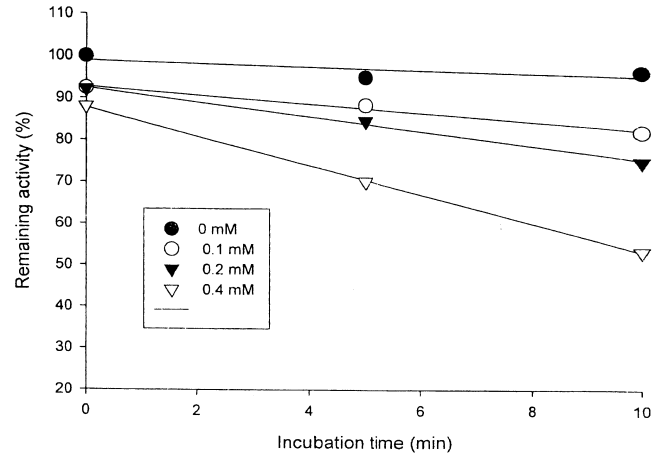


Figure 3. A time dependant inactivation of ACE by dipeptide **1**.

compounds **7–12** are α,β-unsaturated amides of Phe or indolinecarboxylic acid, and still show inactivating properties towards ACE. It appears therefore that in these cases the α,β-unsaturated amide functionality is sufficiently activated as Michael acceptor by the hydrogen bonding of the amide carbonyl oxygen bonding of the amide carbonyl oxygen to a hydrogen of peptide bond (*figure 1b*). The double-reciprocal plot of substrate concentration versus initial velocity (*figure 4*) for the data obtained in the inhibition of ACE with compound **1** suggests that the inhibitions of ACE by the present inhibitors are mixed type.

3. Experimental

3.1. Chemistry

Melting points were determined on a Thomas-Hoover melting point apparatus and are uncorrected. Infrared spectra were obtained with a Perkin-Elmer 1420 infrared spectrometer. Proton nuclear magnetic resonance spectra were obtained with a Bruker AMX 500 NMR spectrometer. Mass spectra were taken on a Hewlett Packard HP5988 (electron-impact), an HP5989A (electrospray ionization), or a JEOL DX-300 (fast atom bombardment). Elemental analyses for carbon, hydrogen, nitrogen and sulfur were determined on a CE instruments EA 1110 CHNS-O elemental analyzer and are within 0.4% of theory. Most of the reagents used were obtained from Aldrich Chemical Co. (USA).

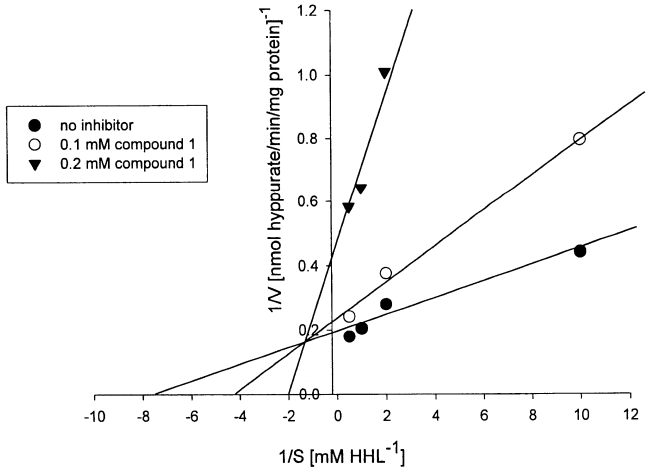


Figure 4. Double reciprocal plot of the inactivation rate versus dipeptide **1** concentration.

3.1.1. General procedure for the preparation of α,β-unsaturated carbonyl compounds [12]

Thionyl chloride (1.79 g, 15 mmol) was added to α,β-unsaturated alkenoic acid (10 mmol) in a round bottom flask and the reaction mixture was stirred at 80 °C for 2 h. After the remaining thionyl choride was removed by rotary evaporation, L-amino acid or dipeptide (6.5 mmol) was dissolved in 1 N sodium hydroxide (6.5 mL) and the solution was chilled in an ice-water bath. Sodium hydroxide (2 N, 3.5 mL) and the acid chlorides just prepared (6.5 mmol) were added and the mixture was stirred vigorously at room temperature for 3 h. The reaction mixture was extracted with ether and the water layer was acidified to pH 1–2 with concentrated hydrochloric acid and extracted with ethyl acetate. The ethyl acetate layer was washed with water and dried over magnesium sulfate. The solvent was removed in vacuo and the residue was recrystallized from ether and chloroform.

3.1.2. Synthesis of dicarboxylic acid compounds **8**, **9**, **11** *and* **12** *[13]*

Acetol (0.74 g, 10 mmol) was added dropwise to *t*-butyldimethylsilyl chloride (2.26 g, 15 mmol) and imidazole (1.63 g, 24 mmol) in 15 mL of DMF under nitrogen. After stirring at room temperature for 2 h, the mixture was diluted with 30 mL of ether and the organic layer was separated. The organic solution was washed sequentially with water, saturated sodium bicarbonate solution and brine. The solution was dried over magnesium sulfate, and solvent evaporated in vacuo. The residue was purified by column chromatography (silicagel, EtOAc: *n*-hexane = 1:5) to give 1-*t*-butyldimethylsilyloxy-2-propanone (1.32 g, yield 70%) as a colourless liquid.

Under nitrogen, triethylphosphonoacetate (2.36 g, 11 mmol) was added to sodium hydride (0.5 g, 21 mmol) in 18 mL of THF. After 30 min of stirring 1-*t*-butyldimethylsilyloxy-2-propanone (1.32 g, 7 mmol) was added and the mixture was stirred for an additional 2 h. The mixture was diluted with ether and the organic layer was separated. The organic solution was washed sequentially with water and brine, and dried over magnesium sulfate. After evaporation, the residue was purified by column chromatography (silicagel, EtOAc: *n*-hexane = 1:10) giving ethyl-4-*t*-butyldimethylsilyloxy-3-methyl-2-propenoate (1.08 g, yield 60%) as a colourless liquid.

Tetrahydrofuran (50 mL) was added to ethyl-4-*t*-butyldimethylsilyloxy-3-methyl-2-propenoate (1.08 g, 4.2 mmol) under nitrogen, then tetrabutylammonium fluoride 1 M THF solution (8.4 mL) was added and the mixture was stirred for 30 min at room temperature. The mixture was diluted with ethyl acetate. The organic layer was washed sequentially with water and brine. The mixture was dried over magnesium sulfate, and solvent evaporated. The residue was purified by column chromatography (silicagel, EtOAc: *n*-hexane = 1:1), and ethyl-4-hydroxy-3-methyl-2-butanoate (0.31 g, yield 51%) was obtained.

Ethyl-4-hydroxy-3-methyl-2-butanoate (0.31 g, 2.2 mmol) in acetone (4.4 mL) was slowly added to chromium trioxide (0.22 g, 2.2 mmol) in 1.5 M sulfuric acid solution (4.4 mL) in 10 min. The mixture was stirred for 30 min at room temperature, diluted with ether, and the organic layer was washed with brine. The organic layer was separated and evaporated in vacuo. The residue was diluted with ether and washed with saturated sodium bicarbonate solution. After the water layer was separated, it was acidified with 6 M sulfuric acid and extracted with ether. The ether layer was washed sequentially with water and brine. The organic layer was dried over magnesium sulfate and solvent evaporated in vacuo to give a white solid (2-methyl-2-butenedioic acid monoethylester, 0.17 g, yield 50%).

1: N-[2-(3-Phenyl-2-propenoylamino)propanoyl]proline: yield 66%. M.p. 119–120 °C. IR (KBr): ν = 3 300 cm^{-1} (CONH), 3 500–2 500 (COOH), 1 760 (CO), 1 670, 1 660, 1 620; ^{1}H-NMR (DMSO-d_6): δ = 1.3 (d, 3H, CH_3), 1.8–2.0 (m, 3H, NCH_2CH_2), 2.0–2.2 (m, 1H, NCH_2CH_2), 3.6 (d, 2H, NCHCH_2), 4.3 (q, 1H, NC*H*-COOH), 6.7 (d, 1H, f-CH=C*H*), 7.3–7.6 (m, 6H, f, f-C*H*=CH), 8.4 (d, 1H, NH), 12.4 (s, 1H, COOH).; MS

(EI): m/z (%) = 198 (55), 131 (41), 103 (40). Anal. $C_{17}H_{20}N_2O_4$ (C, H, N).

2: 2-[2-(3-Phenyl-2-propenoylamino)propanoylamino]-3-phenylpropionic acid: yield 58%. M.p. 190–191 °C. IR (KBr): ν = 3 400 cm^{-1} (CONH), 3 600–2 600 (COOH), 1 760 (CO), 1 670 (CO), 1 600, 1 560; ^{1}H-NMR (DMSO-d_6): δ = 1.2 (d, 3H, NCHCH_3,), 2.8–3.2 (m, 2H, f-CH_2), 4.5 (m, 2H, NHC*H*CO), 6.8 (d, 1H, f-CH=C*H*), 7.2–7.6 (m, 11H, f, f-C*H*=CH), 8.2 (t, 2H, NH), 12.7 (s, 1H, COOH); MS (FAB): m/z (%) = 367 (23) [(M + H)$^+$]. Anal. $C_{21}H_{22}N_2O_4$ (C, H, N).

3: N-[2-(3-Phenyl-2-propenoylamino)propanoyl]indoline-2-carboxylic acid: yield 86%. ^{1}H-NMR (DMSO-d_6): δ = 1.8 (d, 3H, CH_3), 3.0 (d, 1H,), 3.4 (m, 1H,), 3.7 (t, 1H, CO–CH-), 4.8 (q, 1H, CH_3–C*H*-), 6.5 (d, 1H, -CH=CH-), 7.8–6.9 (m, 9H, aromatic H), 7.9 (d, 1H, CH=CH); MS (EI): m/z (%) = 147 (100), 103 (42), 77 (28). Anal. $C_{21}H_{20}N_2O_4$ (C, H, N).

4: 2-[2-(2-Butenoylamino)propanoylamino]-3-phenylpropionic acid: yield 54%. M.p. 157–159 °C. IR (NaCl cell): ν = 3 300 cm^{-1} (CONH), 3 500–2 500 (COOH), 1 720 (CO), 1 670 (CO), 1 640 (CH=CH), 970 (CH=CH); ^{1}H-NMR (DMSO-d_6): δ = 1.2 (d, 3H, NCHCH_3,), 1.8 (d, 3H, CH_3–CH=CH), 2.9–3.2 (m, 2H, f-CH_2), 4.4 (t, 2H, NHC*H*CO), 6.0 (d, 1H, CH_3–CH=C*H*), 6.6 (q, 1H, CH_3–C*H*=CH), 7.2 (s, 5H, aromatic H), 8.0 (q, 1H, NH), 12.6 (s, 1H, COOH); MS (EI): m/z (%) = 139 (6), 111 (2), 95 (6), 69 (28). Anal. $C_{16}H_{20}N_2O_4$ (C, H, N).

5: 2-(3-Phenyl-2-propenoylamino)-3-phenylpropionic acid: yield 82%. M.p. 158–160 °C. IR (KBr): ν = 3 300 cm^{-1} (CONH), 3 400–2 500 (COOH), 1 720 (CO), 1 660 (CO), 1 630 (CH=CH), 980 (CH=CH); ^{1}H-NMR (DMSO-d_6): δ = 2.8–3.4 (m, 2H, f-CH_2), 4.5–4.7 (q, 1H, NHC*H*COOH), 6.6 (d, 1H, f-CH=C*H*), 7.1–7.6 (m, 11H, f, f-C*H*=CH), 8.4 (d, 1H, NH), 12.6 (s, 1H, COOH); MS (ESI): m/z (%) = 294 (100) [(M – H)$^-$]. Anal. $C_{18}H_{17}NO_3$ (C, H, N).

6: 2-(2-Methyl-3-phenyl-2-propenoylamino)-3-phenylpropionic acid: yield 90%. M.p. 140–143.5 °C. IR (NaCl cell): ν = 3 400 cm^{-1} (CONH), 3 500–2 500 (COOH), 1 750 (CO), 1 670 (CO), 1 630 (CH=CH); ^{1}H-NMR ($CDCl_3$); δ 2.0 (s, 3H, CH_3), 3.2 (t, 2H, f-CH_2), 4.9 (q, 1H, NHC*H*C), 6.4 (d, 1H, f-C*H*=C), 7.0–7.5 (d, 10H, aromatic H), 8.0 (s, 2H, NH, COOH); MS (EI): m/z (%) = 289 (28), 145 (100), 117 (85). Anal. $C_{19}H_{19}NO_3$ (C, H, N).

7: 2-(3-Phenylthio-2-propenoylamino)-3-phenylpropionic acid: yield 98%. IR (NaCl cell): ν = 3 330 cm^{-1} (CONH), 3 500–2 500 (COOH), 1 750 (CO), 1 660 (CO), 1 600 (CH=CH); ^{1}H-NMR ($CDCl_3$): δ = 2.9–3.1 (d, 2H, f-CH_2), 4.9 (t, 1H, NHC*H*COOH), 5.6–5.8 (dd, 1H, f-S–CH=C*H*), 6.3–6.5 (dd, 1H, f-S–C*H*=CH), 7.0–7.5 (s, 10H, aromatic H), 7.7 (d, 1H, NH), 11.1 (s, 1H, COOH); MS (EI): m/z (%) = 289 (37), 145 (100), 117 (79). Anal. $C_{18}H_{17}NO_3S$ (C, H, N, S).

8: 2-(3-Carboxy-2-propenoylamino)-3-phenylpropionic acid: yield 72%. M.p. 179–181 °C. IR (KBr): ν = 3 500–2 500 cm^{-1} (COOH), 1 740 (CO), 1 680 (CO), 1 650 (CH=CH); ^{1}H-NMR (DMSO-d_6): δ = 2.7–3.2 (m, 2H, f-CH_2), 4.5 (m, 1H, NHC*H*COOH), 6.4 (d, 1H, HOOC–CH=C*H*), 6.9 (d, 1H, HOOC–C*H*=CH), 7.2 (s, 5H, aromatic H), 8.9 (d, 1H, NH); MS (ESI): m/z (%) = 262 (100) [(M – H)$^-$]. Anal. $C_{13}H_{13}NO_5$ (C, H, N).

9: 2-(3-Carboxy-2-methyl-2-propenoylamino)-3-phenylpropionic acid: yield 72%. M.p. 200–201.5 °C. IR (KBr): ν = 3 500–2 500 cm^{-1} (COOH), 1 730 (CO), 1 670 (CO), 1 450, 1 300; ^{1}H-NMR ($CDCl_3$): δ = 2.1 (s, 3H, CH_3), 2.9–3.2 (m, H, f-CH_2), 4.5 (m, 1H, NHC*H*-COOH), 6.13 (s, 1H, C*H*=C), 7.3 (s, 5H, aromatic H), 8.6 (d, 1H, NH), 12.9 (s, 2H, COOH); MS (EI): m/z (%) = 218 (12), 213 (28), 185 (13). Anal. $C_{14}H_{15}NO_5$ (C, H, N).

10: N-(3-Phenyl-2-propenoyl)indoline-2-carboxylic acid: yield 90%. M.p. 223–225 °C. IR (KBr): ν = 3 300–2 700 cm^{-1} (COOH), 1 750 (CO), 1 650 (CO), 1 630 (CH=CH); ^{1}H-NMR (DMSO-d_6): δ = 3.3–3.6 (q, 2H, NCHCH_2), 5.5 (d, 1H, NC*H*COOH), 6.9–7.4 (m, 7H, f-CH=CH), 7.5–7.8 (m, 3H, aromatic H), 8.2 (d, 1H, aromatic H); MS (ESI): m/z (%) = 292 (100) [(M – H)$^-$]. Anal. $C_{18}H_{15}NO_3$ (C, H, N).

11: N-(3-Carboxy-2-propenoyl)indoline-2-carboxylic acid: yield 99%. M.p. 194–197 °C. IR (KBr): ν = 3 500–2 500 cm^{-1} (COOH), 1 740 (CO), 1 690 (CO); ^{1}H-NMR (DMSO-d_6): δ = 3.2–3.6 (m, 2H, NCHCH_2), 5.4 (dd, 1H, NC*H*COOH), 6.7 (d, 1H, HOOC–CH=C*H*–CO), 7.1–7.3 (m, 4H, f, HOOC–C*H*=CH–CO), 8.1 (d, 1H, aromatic H); MS (ESI): m/z (%) = 260 (100) [(M – H)$^-$]. Anal. $C_{13}H_{11}NO_5$ (C, H, N).

12: N-(3-Carboxy-2-methyl-2-propenoyl)indoline-2-carboxylic acid: yield 72%. M.p. 88–90 °C. IR (KBr): ν = 3 500–2 500 cm^{-1} (COOH), 1 720 (CO), 1 650, 1 410; ^{1}H-NMR (DMSO-d_6): δ = 2.13 (s, 3H, CH_3) 3.1–3.6 (m, 1H, NCHCH_2), 5.1 (dd, 1H, NC*H*COOH), 5.95 (s, 1H, C=C*H*), 7.0–7.3, 8.0 (m, 4H, aromatic H), 12.9 (s, 2H, COOH); MS (EI): m/z (%) = 117 (100), 90 (40). Anal. $C_{14}H_{13}NO_5$ (C, H, N).

3.2. Biology

3.2.1. Inhibition assay

Incubation mixtures contained 40 μg rabbit liver homogenate, 0.3 M sodium chloride, 1 mM hippuryl–histidyl–leucine (HHL) and 0.1 M potassium phosphate buffer (pH 8.4) in a total volume of 0.2 mL. Inhibitors dissolved in methanol were added at various concentra-

tions. The reaction was started by adding substrate and were incubated for 10 min at 37 °C. The reaction was quenched by adding 15 µL of 6 N hydrochloric acid and 40 µL of 0.1 mM phthalic acid was added as an internal standard. Reaction mixtures were then extracted with 400 µL of ethylacetate and the organic phase was evaporated under nitrogen stream. The residue was dissolved in HPLC buffer. Chromatographic separation was achieved on a Partisil ODS column (4.6 mm × 15 cm, 5 µm, Beckman) using isocratic elution of 30% trifluoroacetic acid at a flow rate of 1.0 mL/min. The peak was monitored at 230 nm.

3.2.2. Inactivation experiment

Incubation mixtures contained 2.0 mg of rabbit lung homogenate, 0.1–0.4 mM inhibitor in methanol, and 0.1 M potassium phosphate buffer (pH 8.4) in a total volume of 1 mL. The reaction was started by the addition of inhibitors. Aliquots (100 µL) were removed at 0, 5, 10, 30, 40 and 60 min after incubation and added to the reaction mixture containing 0.3 M sodium chloride, 1 mM hippuryl–histidyl–leucine (HHL) and 0.1 M potassium phosphate buffer (pH 8.4) in a total volume of 0.9 mL. The enzyme reaction was done for 10 min and quenched by adding 0.2 mL of 6 N hydrochloric acid. Quantitation of hippurate was done as described above.

Acknowledgements

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