CCA-1439

The Synthesis and Angiotensin Converting Enzyme Inhibitory Activities of *N*-[(3-Substituted)aminocarbonyl]propanoyl-L-prolines

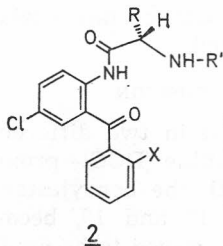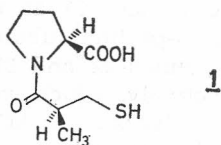*Vitomir Šunjić, Šumski Šimaga, and Ljubinka Vitale*

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Preparations of *N*-[3-(hetero)aryl-aminocarbonyl]propanoyl--L-proline derivatives *12, 13, 22* and *23* are described. A novel approach consists of fusing (80—100 ⁰C) *N*-(hetero)aryl-succinimides (*7, 8, 20, 21*) with unprotected L-proline and imidazole in the presence of dimethylformamide. *In vitro* tests for angiotensin converting enzyme (ACE) inhibition showed that all *N*-(arylaminocarbonyl)-propanoyl-L-prolines (*12, 13, 22* and *23*) exhibit lower activity than captopril (*1*), their IC_{50}'s ranging from 2.5×10^{-4} to 3.3×10^{-3} M.

INTRODUCTION

Angiotensin converting enzyme (ACE) inhibitors show promise in the treatment of hypertension.[1-3] A very potent ACE inhibitor captopril (*1*; *N*-(3--mercapto-2-methylpropanoyl)-L-proline; SQ 14225) has been introduced into the therapy of various forms of renovascular hypertension.[4-6] The first clinical use of captopril generated widespread interest.

R = H ; Me ; CH₂Ph
R' = H ; Ala ; Phe ; Gly ; Ala-Ala ;
      Gly-Ala ; Ala-Phe ; etc.
X = H ; F ; Cl

However, chronic oral administration of this inhibitor caused a number of side effects.[7-9] Some of them most probably were due to the well documented

ability of captopril to reduce S—S bridges and thus alter some peptides and proteins *in vitro* and *in vivo*.[10,11] This stimulated the search for ACE inhibitors without free SH-groups.[12,13]

On the other hand, it was recently argued[14] that the renin-angiotensin system (RAS) in the brain participates in the control of blood pressure. It was found[15] that prolonged oral treatment with captopril was followed by an increase in ACE activity in blood-pressure-controlling brain regions. Also, when captopril was injected into the cerebral ventricles in spontaneously hypertensive rats, it produced a decrease in blood pressure. The decrease was greater than that produced by the same dose of captopril given i. v.[16] The hypothesis was put forward that captopril crosses the blood-brain barrier and reaches brain sites of high RAS activity as part of its blood pressure lowering effect.

All the above data prompted us to develop a working hypothesis, that certain substances which can easily pass the blood-brain barrier, and which possess a certain intrinsic ACE inhibitory activity, would exert a higher anti-hypertensive effect than captopril (or other peptide-like derivatives devoid of lipophilic properties), by reaching brain sites of high RAS activity. The absence of a thiol group in such compounds might be an additional advantage in eliminating the possible *in vivo* side effects mentioned earlier.

Two pairs of compounds, *12* and *13,* and *22* and *23,* respectively, were designated as potential ACE inhibitors on the basis of the following rationales It is know that 2-amino-benzophenone derivatives *2,* bearing one to three α-aminoacid residues on the 2-amino group, exhibit strong regulatory effects on the central nervous system.[17–19] Since the benzophenone moiety in *2* (R′ = H) exists *in vivo* only temporarily cyclising into 1,4-benzodiazepines,[17] the *N*-peptidyl moiety in *2* (R′ = one or two α-aminoacyl residues) acts as a lipophilic carrier for dipeptides and tripeptides. A benzophenone derivative of γ-aminobutyramide has recently been designated as the carrier of the latter into the CNS.[20]

Having these results in mind, along with the documented activity of *N*-succinyl proline,[5] we chose *12* and *13* as the target molecules. In these compounds benzophenone and L-proline subunits are linked by the succinic bridge. The second pair (compounds *22* and *23*) contains the 2-aminopyridyl moiety and its *N*-oxide, respectively, which were expected to act as ligands for Zn(II) ions present at the active site of ACE.[21] 7-Membered chelates with divalent metal ions are well known models for the active sites of urease[22] and hydrolases.[23] Although no direct extrapolation of the results with similar ligands and different metal ions can be made, certain chelating properties of the *N*-oxide subunit in *23* may be expected.
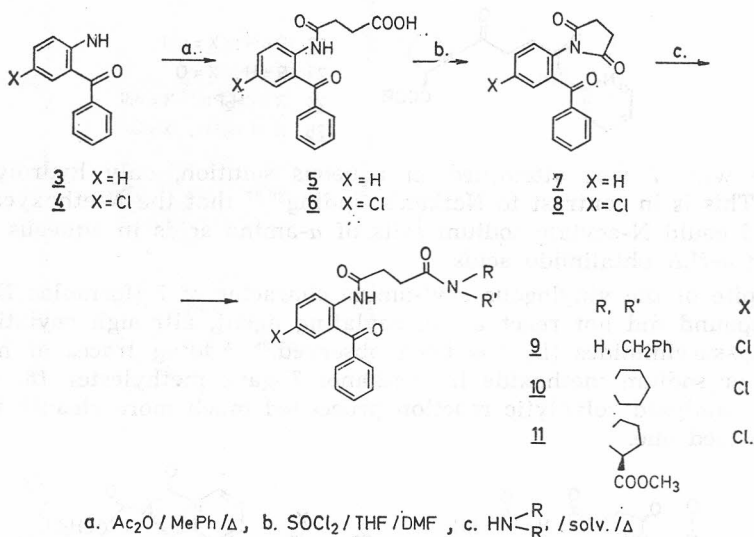
RESULTS AND DISCUSSION

All target compounds were approached in two different ways. The first made use of classical, dicyclohexylcarbodiimide (DCC)- promoted condensation of carboxy derivatives *5, 6, 18* and *19* with the benzylester of L-proline. The reaction rendered some difficulties with *18* and *19,* because of their poor solubility. Only dimethylformamide (DMF) proved to be applicable, but limited solubility even in this solvent required reaction times of up to several days. This caused a known side reaction, i. e. the formation of *N*-acyl-*N,N′*-dicy-clohexyl-urea derivatives *26* and *27* which were separated and characterized.
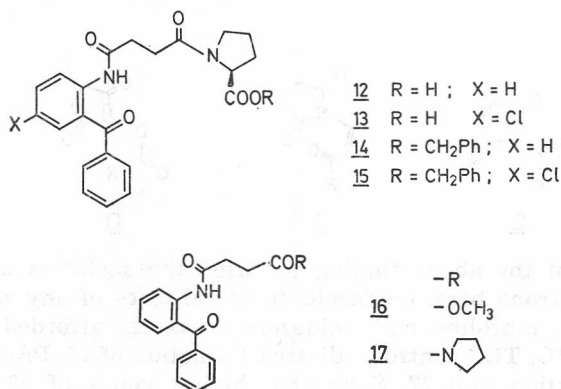
Hydrogenolysis of the intermediary benzyl-esters (*14, 15, 24* and *25*) afforded the free carboxylic acid derivatives in rather low yields, the loss of material being mainly due to adsorption onto the catalyst.
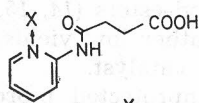
A method of direct acylation of unprotected L-proline was elaborated during this work (Scheme 1.). An attempt to prepare acyl chlorides of *5*
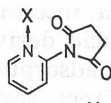
SCHEME I

	R, R'	X
9	H, CH₂Ph	Cl
10		Cl
11		Cl

a. Ac₂O / MePh /Δ , b. SOCl₂ / THF /DMF , c. HN$\stackrel{R}{\diagdown}$R' / solv. /Δ

and *6* using a slight molar excess of thionyl chloride in tetrahydrofuran (THF) at ambient temperature led to quantitative cyclisation to give *7* and *8*. A precedent to this reaction was described by Rudinger,[24] *i. e.* cyclisation of N-tosyl-glutamic acid into N-tosyl-pyroglutamic acid, the latter behaving as a nucleophile.[24] This observation prompted us to examine the acylating properties of *7*. On heating with benzylamine and piperidine in an inert solvent, *7* afforded *9* and *10* in 62% and 79% yield, respectively. It is interesting to note that the methylene congeners of *7*, i. e. compounds *A*, were claimed[25] to provide Michael-type addition products only. When acylation of carboxyl-unprotected
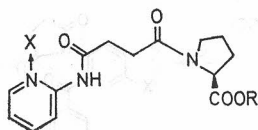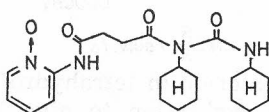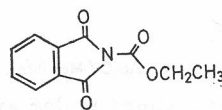

12	R = H ;	X = H
13	R = H	X = Cl
14	R = CH₂Ph ;	X = H
15	R = CH₂Ph ;	X = Cl

	−R
16	−OCH₃
17	−N

18	nill
19	O

20	nill
21	O

22	R = H ; X = nill
23	R = H ; X = O
24	R = CH₂Ph ; X = nill
25	R = CH₂Ph ; X = O

L-proline with 7 was attempted in aqueous solution, only hydrolysis was noticed. This is in contrast to Nefken's finding[26,27] that the N-ethoxycarbonyl--imide 28 could N-acylate sodium salts of α-amino acids in aqueous solution affording α-N,N-phtalimido acids.
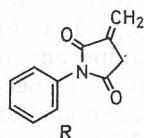
In spite of the vinylogous acyl-amide character of 7 (formulae B and C) this compound did not react as an acylating agent, although acylation with N-acyloxy-succinimides (D) has been observed.[28] Adding traces of hydrogen chloride or sodium methoxide in methanol 7 gave methylester 16, whereby the base catalysed solvolytic reaction proceeded much more cleanly than the acid-catalysed one.

26	nill
27	O

28

A

B　　　　　　**C**　　　　　　**D**
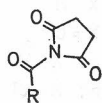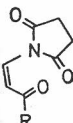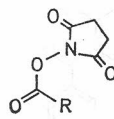
On the basis of the above finding we tried the acylation of L-proline in the presence of a strong base, imidazole, in the absence of any solvent. Fusion of a mixture of 7, L-proline and imidazole in excess afforded a clear melt already at 90—100 °C. TLC control indicated formation of 15. Prolonged heating led to decarboxylation into 17. Somewhat higher yeilds of 22 and 23 were

obtained when DMF was added to assure homogenization at 80—90 °C. Limited racemization, if at all, occurred under the above conditions, as unequivocally proved by comparison of $[\alpha]_D$'s with those of compounds *12, 13, 22* and *23* prepared *via* the DCC/imidazole method (Table I).

TABLE I

Optical Rotations of Compounds 12, 13, 22, and 23, Depending on the Method of Preparation

Compound	$[\alpha]_D{}^c$	
	DCC-method	Fusion with imidazole
12[a]	—49.7°	—55.8°
13[a]	—47.6°	—48.5°
22[b]	—69.0°	—86.3°
23[b]	—64.5°	—69.0°

[a] Determined in $CHCl_3$;   [b] Determined in MeOH;   [c] c = 0.98 — 1.05 for both solvents.

With regard to the possible mechanism of acylation of L-proline with aryl--succinimides in the presence of imidazole, some control experiments were performed. Thus, heating of *20* and imidazole (ca. 20% total concentration in DMF-d_7) in the NMR instrument up to 140 °C did not result in the formation of N-acyl-imidazole. NMR spectra of the reaction components remained unchanged, while a TLC control confirmed their presence as the only compounds in the solution. Thus, N-acylimidazole could safely be excluded as an activated intermediate. On the other hand, heating of *20* and L-proline in DMF-d_7, in the absence of imidazole, resulted in slow dissappearance of *20* above 125—130 °C, and formation of *22* and another product with higher R_f. The NMR spectra revealed accumulation of the multiplet between 1.7—2.7 ppm.

The second product was concluded to be decarboxylated *22*, analogous characteristics were exhibited by its congener *17*, (see Experimental). However, when *20*, imidazole, and L-proline (mol. ratio 1 : 2 : 1, total concn. ca. 25%) were heated in DMF-d_7 at 100—110 °C for 30 min to 1 h, NMR spectra revealed predominant formation of *22*, while traces of its decarboxylation product were visible on TLC. These experiments proved that imidazole did not form an active intermediate, but rather acted as a strong base, as well as a strong hydrogen donor, hindering protonation of the proline amino group and activating N-aryl succinimide by hydrogen bonding to its carbonyl group. A similar role of the imidazole ring is well established in various biological and non--biological systems.[29]

Examination of *in vitro* ACE inhibitory activities of compounds *12, 13, 22* and *23* revealed that they are inferior to captopril (Table II). At the same time they were also shown to be poor inhibitors of leucine aminopeptidase (LAP) and to inhibit no other examined enzymes (Table III).

TABLE II

Inhibition of Porcine Plasma Angiotensin Converting Enzyme

Inhibitor	IC_{50} (M)*
12	2.45×10^{-4}
13	5.70×10^{-4}
22	2.47×10^{-3}
23	3.33×10^{-3}
Captopril	2.57×10^{-8}

* All values are the average results obtained in three or more experiments.

TABLE III

Inhibition of Some Other Proteolytic Enzymes

Enzyme	Inhibition (%)*			
	12	13	22	23
Leucine aminopeptidase	42	46	11	9
Cathepsin B	0	0	0	0
Cathepsin H	—	13	—	—
α-Chymotrypsin	0	0	0	0
Trypsin	0	0	0	0
Elastase	0	0	0	0

* Inhibitor concentration: 10^{-3} M

The presented results indicate that the lipophilic benzophenone group has an unfavorable fit for binding on the enzyme. Newly introduced potential bidentate ligands have a lower ability to complex Zn ion at the active site than the thiol or carboxylic group. Steric hindrance, however, does not seem to be a dominant drawback since compounds 12 and 13 with more bulky residues are better inhibitors than compounds 22 and 23 with the pyridine ring at that position. The same difference in inhibitory activity between the two pairs has been observed with LAP, which is also a metallo-protease with Zn at the active site.

EXPERIMENTAL

Melting points were determined on electrothermal melting point apparatus and have not been corrected. IR spectra (KBr pellets, if not stated otherwise) were obtained with a Perkin Elmer M 297 spectrophotometer (only strong bands are indicated). ¹H NMR spectra were run on a JEOL FX-90Q (90 MHz) spectrophotometer with TMS as internal standard; shifts are given in ppm values downfield from TMS. Optical rotations were measured on a Zeiss-Winkel instrument at ambient temperature. Thin layer chromatography (TLC) was performed on aluminium plates precoated with Merck silica gel 60 F_{254}. Column chromatography was run over granular silica gel 0.05—0.20 mm (Merck). Organic extracts were regularly dried over Na_2SO_4 and evaporated *in vacuo*.

Benzylester of L-proline was prepared in 78% yield using a modified procedure of Patel,[29,30] that mainly consists in performing the reaction at 0 °C for 4 h and overnight at ambient temperature, isolating a product by dilution with ether (1 : 3) and slow crystallization on ice. Compounds 3 and 4 were purchased from Fluka, compounds 18 and 20 were prepared according to ref. 31.

General Procedure for Preparation of N-3'-carboxypropanoyl-arylamines 5 and 6

Amine 3 or 4 (0.05 mole) and succinic anhydride (0.055 mole) were heated in toluene (60 ml) under reflux for 1 h. The reaction mixture was allowed to cool to

ambient temperature, then it was chilled on ice overnight. The crude product was collected on a filter, washed with light petroleum and dried, yield quantitative.

2-N-[(3'-Carboxyl)propanoyl]-aminobenzophenone (7)

On recrystallization from toluene mp. was 126—127 °C.IR: 3300, 2920, 1708, 1630, 1595, 1576, 1510, 1445, 1412, 1318, 1265, 1178, 1160, 695 cm^{-1}, NMR (CDCl$_3$): 2.75/s, 4H), 7.1—7.8 (m, 8H), 8.72 (d, J=8 Hz), 1H), 10.1 (broad s, 1H, NH), 11.15 (S, 1H, COOH).

Anal: C$_{17}$H$_{15}$NO$_4$ (297.32) calc'd.: C 68.67; H 5.08; N 4.72%.
found: C 68.74; H 5.28; N 4.73%.

2-N-[(3'-Carboxyl)propanoyl]-amino-5-chloro-benzophenone (6).

On recrystallization from toluene mp. was 136—138 °C. IR: 3300, 2920, 1688, 1630, 1595, 1575, 1505, 1395, 1320, 1245, 1160, 850, 755, 700 cm^{-1}. NMR (CDCl$_3$), 2.74 (s, 4H), 7.4—7.6 (m, 7H), 8.61 (d, J=8 Hz, 1H), 10.5 (road s, 1H, NH), 10.68 (s, 1H, COOH).

Anal: C$_{17}$H$_{14}$NO$_4$Cl (331.77) calc'd.: C 61.65; H 4.25; N 4.22%.
found: C 61.54; H 4.50; N 4.10%.

General Procedure for Preparation of N-aryl-succinimides 7 and 8.

To the ice-cooled solution of compounds 5 or 6 (0.10 mole) in THF (250 ml), thionylchloride (13.0 g, 0.11 mole) was added dropwise, followed by 5 drops of DMF. After stirring for 24 hrs at ambient temperature, the solvent was evaporated and the residual oil treated with ether (50 ml), until crystallization was initiated. After chilling on ice, the crystals were collected on a filter, washed with ether (3 × 10 ml), and dried to afford 95—96% of the crude product (one spot on TLC, R_f ~ 0.25 with dichlormethane-acetone 9.0 : 1.0 as eluant).

N-(2'-Benzoyl)-phenyl-succinimide (7)

On recrystallization from methanol mp. was 137—138 °C. IR: 1778, 1725, 1710, 1665, 1600, 1485, 1465, 1380, 1315, 1300, 1288, 1175, 705 cm^{-1}. NMR (CDCl$_3$): 2.48 (s, 4H), 7.0—7.9 (m, 9H).

Anal: C$_7$H$_{13}$NO$_3$ (279.30) calc'd.: C 73.11; H 4.69; N 5.01%.
found: C 73.16; H 4.68; N 4.96%.

N-(2'-Benzoyl-4'-chloro)phenyl-succinimide (8)

On recrystallization from methanol mp. was 110—111 °C. IR: 1780, 1725, 1670, 1600, 1485, 1405, 1378, 1290, 1180, 710 cm^{-1}. NMR (CDCl$_3$): 2.59 (s, 4H), 7.2—7.9 (m, 8H).

Anal: C$_7$H$_{12}$NO$_3$Cl (313.75) calc'd.: C 65.08; H 3.86; N 4.46%.
found: C 65.03; H 4.00; N 4.72%.

General Procedure for Preparation of Compounds 9—11

Compound 7 (837 mg, 3.0 mmol) and the corresponding amine (3.3 mmol; L-proline methylester hydrochloride[32] was transformed into free base immediately before use) were dissolved in 5.0 ml of toluene (for condensation with piperidine, xylene was used), and heated under reflux for 4 h.

2-N(3'-Benzylaminocarbonyl)-propanoyl-aminobenzophenone (9)

On chilling on ice overnight crude 9 deposited as prismatic crystals, it was collected on a filter, washed with ether (2 × 2 ml) and dried, affording 647 mg (62%). On recrystallization from methanol it had mp. 105—106 °C. IR: 3260, 3065, 2900, 1720, 1650, 1635, 1600, 1585, 1550, 1518, 1450, 1250, 1165, 755, 735, 700, 640 cm^{-1}. NMR (CDCl$_3$), 2.62 (doublet t, 4H), 4.30 (d, J=5 Hz, 2H), 6.9—7.7 (m, 8H), 8.41 (d, 1H), 10.5 (s, 1H, NH).

Anal. C$_{24}$H$_{22}$N$_2$O$_3$ (386.46) calc'd.: C 74.61; H 5.73; N 7.25%.
found: C 74.36; H 5.73; N 7.00%.

2-N(3'-Piperidylcarbonyl)propanoyl-aminobenzophenone (10)

On evaporation of the solvent crude product was isolated by chromatography on a silica gel column (25 g) using methylene chloride-acetone (9.0 : 1.0) as eluant. Fractions 9—10 (10 ml/fraction) contained 79% of product *10*, which on crystallization from methanol melted at 130—131 °C. IR: 3310, 2980, 1680, 1668, 1620, 1530, 1485, 1450, 1380, 1315, 1280, 1255, 1225, 1180, 925, 762, 710, 700 cm^{-1}. NMR (CDCl$_3$): 1.48 (broad, 6H), 2.66 (s, 4H), 3.32 (broad s, 4H), 6.7—7.6 (m, 8H), 8.42 (d, 1H), 10.5 (s, 1H, NH).

Anal. C$_{22}$H$_{24}$N$_2$O$_3$ (364.45) calc'd.: C 72.51; H 6.64; N 7.68%.
found: C 72.40; H 6.42; N 7.66%.

N-3-(2'-Benzoyl)phenyl-aminocarbonyl-propanoyl-L-proline-methylester (11)

After evaporation of the solvent, the product mixture was separated on a silica gel column (30 g 60 cm × 1 cm) using methylene chloride-acetone (9.0 : 1.0) as eluent. In fractions 43—84 (5 ml/fraction) 51.4% of *11* (oil) was isolated. An analytical sample was obtained by repeated chromatography. IR (film): 3300 (broad), 2950, 1725, 1695, 1645, (multiplet), 1605, 1585, 1520, 1440, 1320, 1290, 1260, 1200, 1175, 755, 700, 690 cm^{-1}. NMR (CDCl$_3$): 2.0—2.15 (m, 4H), 2.77 (m, 4H), 3.69 (s, 3H), 3.6—3.8 (m, 2H), 4.51 (t, 1H), 7.0—7.8 (m, 8H), 8.60 (dd, 1H), 10.8 (s, 1H, NH).

Anal: C$_{23}$H$_{24}$N$_2$O$_5$ (408.43) calc'd.: C 67.63; H 5.92; N 6.86%.
found: C 67.44; H 6.07; N 6.76%.

By further elution with ethylacetate 75 mg of the cyclic dimer of L-proline, mp. 173—175 °C (lit. 33 mp. 174—175 °C) was separated.

N-3-(2'-Benzoyl)phenyl-aminocarbonyl-propanoyl-L-proline-benzylester (14)

Method A. Starting from 7 (1.40 g, 5.0 mmole) and 2.66 g (11.0 mmole) of L-proline benzylester hydrochloride preparation of *14* was performed as described for *13*. The crude product was separated on a silica gel column (120 g) using chloroform--acetone (9.2 : 0.8) a eluant (1.66 g, 68.8%)-glassy oil, [a]$_D$ = —14.0° (c = 0.88 in CHCl$_3$).

Method B. Compound 5 (2.97 g, 10.0 mmole) was dissolved in chloroform (15 ml) and added dropwise to a solution of L-proline benzylester (free base from 2.66 g, 11.0 mmole of hydrochloride), DCC (2.06 g 10.0 mmole), and imidazole (0.2 g), over 15 min at 0° C. Stirring was continued for 1 h at 0 °C, 4 h at ambient temperature, and then the reaction mixture was deposited on ice overnight. Dicyclohexylurea (DCU) that separated was filtered off (2.07 g, 92,5%), the filtrate was evaporated and a sample (400 mg) was purified on a colum (30 g silica gel) using methylene chloride-acetone (9.2—0.8) as eluant. In the fractions 15—34 (10 ml/fraction) 317 mg of pure *14* was obtained, glassy oil, [a]$_D$ = —15.5 (c = 0.94 in CHCl$_3$). IR (film): 2930, 1748, 1700, 1645, 1585, 1525, 1450, 1265, 1170, 755, 702 cm^{-1}. NMR (CDCl$_3$): 2.0 (m, 4H), 2.77 (m, 4H), 3.61 (m, 2H), 4.57 (t, 1H), 5.16 (dd, 2H), 7.1—7.7 (m, 14H), 8.58 (d, 1H), 10.8 (s, 1H, NH).

Anal: C$_{29}$H$_{28}$N$_2$O$_5$ (484.53) calc'd.: C 71.88; H 5.83; N 5.78%.
found: C 71.96; H 5.57; N 5.66%.

N-3-(2'-Benzoyl-4'-chloro)phenyl-aminocarbonyl-propanoyl-L-proline-
-benzylster (15)

This compound was prepared according to method B, described for *14*, starting from 3.31 g (10.0 mmole) of *6*. An analytical sample was obtained on repeated chromatography using methylene chloride-acetone (9.2 : 0.8, R_f ~0.35) as eluant. The pure product transformed into a glassy solid on prolonged drying *in vacuo*, over P$_2$O$_5$. [a]$_D$ = —12.6° (c = 1.4 in CHCl$_3$). IR (film): 3310, 2950, 1745, 1695, 1600, 1580, 1515, 1440, 1400, 1238, 1200, 1160—1180 (multiplet), 735, 705 cm^{-1}. NMR (CDCl$_3$):

1.95—2.08 (m, 4H), 2.7—2.72 (m, 4H), 3.6 (m, 2H), 4.65 (m, 1H), 5.74 (dd, 2H), 7.2—7.7 (m, 13H), 8.55 (d, 1H), 10.6 (s, 1H, NH).

Anal: $C_{29}H_{27}N_2O_5Cl$ (518.98) calc'd.: C 67.11; H 5.24; N 5.40%.
found: C 66.98; H 5.04; N 5.61%.

2-N-(3'-Methoxycarbonyl)-propanoylamino-benzophenone (16)

Compound *5* (838 mg, 3.0 mmole) was dissolved in abs. methanol (10.0 ml) and sodium methoxide (20 mg) was added. The reaction mixture was stirred for 3 hrs with exclusion of moisture. After adjusting the pH to 6.5 the solvent was evaporated, water (30 ml) was added, and the resulting slurry was extracted with chloroform (3 × 20 ml). Organic extracts were washed with water (2 × 20 ml), dried and evaporated to give 898 mg (96%) of crude *16*. After chromatography (30 g silica gel), using chloroform as eluant, pure *16* (610 mg) was obtained, glassy oil. IR: 3320, 2960, 1740, 1705, 1640, 1610, 1588, 1525, 1450, 1320, 1295, 1160, 755, 640 cm^{-1}. NMR (CDCl$_3$): 2.76 (s, 4H), 3.69 (s, 3H), 7.0—7.7 (m, 8H), 8.62 (dd, 1H), 10.0 (broad s, 1H, NH).

Anal: $C_{18}H_{17}NO_4$ (311.32) calc'd.: C 69.44; H 5.50; N 4.50%.
found: C 69.63; H 5.56; N 4.32%.

N-3-(2-Benzoyl)phenyl-aminocarbonyl-propanoyl-L-proline (12)

Method A. Compound *7* (279 mg, 1.0 mmole), imidazole (201 mg, 3.0 mmole), and L-proline (173 mg, 1.5 mmole) were homogenized and then heated with exclusion of moisture and with stirring at 90—95 °C. After 1 h the reaction mixture was allowed to cool to ambient temperature, water (10 ml), and chloroform (10 ml) were added, and the pH adjusted with hydrochloric acid (1 : 1) to 1.5—2.0. The organic phase was separated, the aqueous phase was extracted with chloroform (3 × 10 ml) and the combined organic extracts were washed with water (2 × 20 ml), dried and evaporated. The crude reaction mixture was purified on a silica gel column (25 g) using ethylacetate-methanol (7.0 : 3.0) as eluant. In fractions 10—38 (10 ml per fraction) 214 mg (54.5%) of pure *14* was obtained, which resisted attempts of recrystallization. Due to the extreme hygroscopicity correct results could not be obtained for elemental analysis. $[a]_D = -55.8^0$ (c = 1.02 in CHCl$_3$). IR: 3100—3500 (broad), 1645, 1605, 1520, 1445, 1260, 700 cm^{-1}. NMR (MeOH-d_4); 1.98 (m, 4H), 2.52 (dd, 4H), 3.51 (m, 2H), 4.27 (t, 1H), 7.1—7.9 (m, 9H).

Method B. Compound *14* (5.0 g, 10.3 mmole) was dissolved in 96% ethanol (50 ml), 800 mg of Pd/C was added and hydrogenolysis was performed in a closed system, until consumption of hydrogen ceased (ca. 5 hrs). The catalyst was filtered off, the organic solvent was evaporated to dryness and crude *12* was purified on a silica gel colum (110 g), eluted first with methylenechloride-acetone (9.2 : 0.8) and then with ethylacetate-methanol (1 : 1). 2.88 g (71%) of pure *12* was obtained as a fine powder on addition of ether, $[a]_D = -49.7^0$ (c = 1.1 in CHCl$_3$). NMR and IR identical as for the sample obtained by the method A.

N-3-(2'-Benzoyl-4'-chloro)phenyl-aminocarbonyl-propanoyl-L-proline (13)

Method A. Starting from 1.57 g (5.0 mmole) of compound *8*, 1.70 g (25.0 mmole) of imidazol, and 1.15 g (10.0 mmole) of L-proline, the reaction was performed at 110—125 °C for 1 h. Two products formed (R_f ~0.75 and ~0.05, methylene chloride--acetone 9.2 : 0.8). After elaboration as described for *12*, the crude product mixture was separated on a silica gel colum (100 g) using first methylene chloride-acetone (9.2 : 0.8). In fractions 11—22 (10 ml/fraction) 343 mg of

N-3-(2'-Benzoyl-4'-chloro)phenyl-aminocarbonyl-propanoyl-pyrolidine (17)

were obtained. On crystallization from methanol it had mp. 148—151 °C IR: 3040—3300 (multiplet), 2970, 1698, 1670, 1627, 1605, 1570, 1532, 1470. 1455, 850, 740, 700, 675 cm^{-1}. NMR (CDCl$_3$): 1.75—2.0 (m, 4H), 2.71 (double q, 4H), 3.37—3.52 (m, 4H), 7.4—7.8 (m, 7H), 8.53 (d, 1H), 10.65 (s, 1H, NH).

Anal: C$_{21}$H$_{21}$N$_2$O$_3$Cl (384.85) calc'd.: C 65.53; H 5.50; N 7.28%.
found: C 65.72; H 5.71; N 7.14%.

Further elution was performed with ethylacetate-methanol (6.0 : 4.0), affording 1.046 g (49%) of crude *17*. A sample was purified by repeated chromatography with the same solvent system, dissolving in chloroform, washing with water, drying and evaporation. The amorphous powder that remained resisted attempts at recrystallization; correct results for elemental analysis could not be obtained. $[a]_D = -48.5^0$ (c = 1.05 in CHCl$_3$). IR: 3300 (broad), 2980, 1630, 1600, 1570, 1510, 1480, 1450, 1285, 1245, 700 cm^{-1}. NMR (CDCl$_3$): 1.89 (m, 4H), 2.58 (broad s, 4H), 3.4 (m, 2H), 4.37 (m, 1H), 7.2—7.7 (m, 7H), 8.18 (d, 1H), 10.48 (s, 1H, NH).

Method B. Using identical conditions to those described for hydrogenolysis of *14* into *12*, compound *15* (729 mg, 2.0 mmole) was debenzylated into *13* (787 mg, 92%). $[a]_D = -47.6^0$ (c = 0.97 in CHCl$_3$). IR and NMR spectra were identical to those of a sample obtained by method A.

2-(3'-Carboxyl)propanoylamino-pyridin-N-oxide (18)

Compound *18* (5.45 g, 40 mmole) was slurried in a mixture of 60 ml acetic acid, and 30 ml acetanhydride and 30% aqueous hydrogen peroxide (35 ml) was added dropwise, with stirring at such a rate as to maintain the reaction temperature between 45—50 °C. Stirring at 50 °C was continued for 6 hrs, then the reaction mixture was deposited overnight on ice. The crude product (3.81 g, 64.5%) was collected on a filter and recrystallized from methanol-acetic acid (1 : 1), affording pure *18,* mp. 204—206 °C. IR: 3260, 2500 and 1900 (two broad bands), 1710, 1580, 1505, 1240, 1200, 1158, 1140, 810, 780, 725 cm^{-1}. NMR (DMSO-d$_3$): 3.36 (broad s, 4H), 7.02—7.5 (m, 2H), 8.3—8.4 (double t, 2H), 10.56 (s, 1H, NH).

Anal: C$_9$H$_{10}$N$_2$O$_4$ (210.18) calc'd.: C 51.43; H 4.80; N 13.33%.
found: C 51.14; H 5.00; N 13.26%.

2-Succinimido-pyridin-N-oxide (21)

Compound *19* (1.05 g, 5.0 mmole) was slurried in acetanhydride (15.0 ml) and heated at 60 °C with stirring. After 30 min all solid went into solution. The bright-yellow solution was evaporated at 40 °C, leaving a red, oily residue. Ethylacetate (5 ml) was added, and the solution deposited on ice for crystallization overnight. The crude product (396 mg) was collected on a filter, mother liquors were dilluted with ether (10 ml), affording an additional 160 mg of *21* (total yield 556 mg, 59%). On recrystallization from ethylacetate the pure product had mp. 149—151 °C. IR: 3060, 1798, 1725, 1715, 1505, 1435, 1395, 1260, 1190, 935, 835, 775, 660 cm^{-1}, NMR (CDCl$_3$): 2.8—3.2 (16 lines pattern of an AA'BB' system (CH$_2$CH$_2$), a similar pattern was recently observed for *N*-(2'-phenyl)phenylsuccinimide, and was investigated for diastereotopy recognition[34]); 7.4 (m, 3H), 8.3 (m, 1H).

Anal: C$_9$H$_8$N$_2$O$_3$ (192.17) calc'd.: C 56.25; H 4.20; N 14.58%.
found: C 56.27; H 4.30; N 14.31%.

N-3-(2'-Pyridyl)aminocarbonyl Propanoyl-L-proline-benzylester (24)

Compound *18* (388.5 mg, 2.0 mmole) L-proline benzylester (532 mg, 2.2 mmole — free base), DCC (433 mg, 2.1 mmole), and imidazol (1.0 g, 15 mmole) were stirred in dry DMF (7 ml) at 0 °C, and then for 3 days at ambient temperature. DCU which separated was filtered off, solvent evaporated, and the residual oil partitioned between water (30 ml), and methylene chloride (4 × 20 ml). Dry organic extracts were evaporated and the residue (837 mg) treated with warm ether. This brought to crystallization compound *26,* i. e.

N-N'-Dicyclohexyl-N-3-(2'-pyridyl)-aminocarbonyl-propanoyl-urea

90 mg of crude product were obtained which on crystallization from methanol had mp. 176—178 °C. IR: 3320, 3290, 2960, 2715, 1675, 1648, 1545, 1530 cm^{-1}. NMR (CDCl$_3$): 1.26 and 1.79 (two broad m, 22H), 2.81 (s, 4H), 6.9—8.3 (m, 4H), 9.13 (s, 1H, NH).

Anal: $C_{22}H_{32}N_4O_3$ (400.51) calc'd.: C 65.97; H 8.05; N 13.99%.
found: C 65.77; H 8.22; N 14.05%.

Mother liquors were evaporated to dryness and eluted with chloroform-acetone (7.0 : 3.0) from a silica gel column (35 g). In fractions 12—19 (10 ml/fraction) 228 mg (30%) of pure *24* were obtained, glassy oil. $[a]_D = -71.6^0$ (c = 0.95 in CHCl₃). IR (film): 3270, 2930—2960 (m), 1745, 1695, 1640, 1580, 1530, 1435, 1305, 1170, 770, 740, 700 cm⁻¹. NMR (CDCl₃): 2.0—2.2 (m, 4H), 2.73, 2.76 (dd, 4H), 3.62 (m, 2H), 4.64 (m, 1H), 5.12 (dd, J = 12 Hz, 2H), 6.9—7.0 (m, 1H), 7.30 (s, 5H), 7.5—7.7 (m, 1H), 8.1—8.3 (m, 2H), 9.58 (s, 1H, NH).

Anal. $C_{21}H_{23}N_3O_4$ (381.42) calc'd.: C 66.12; H 6.08; N 11.02%.
found: C 65.84; H 6.13; N 10.79%.

N-3-(1'-Oxy-2'-pyridyl)-aminocarbonyl-propanoyl-L-proline-benzylester (25)

This compound was prepared from *23* (420.5 mg, 2.0 mmole) using the same molar ratios and reaction conditions as described for *24*. The crude product mixture that was obtained on evaporation of extract was separated on a silica gel column (35 g), using ethylacetate-methanol (7.0 : 3.0) as eluant. In fractions 3—6 (10 ml/fraction) compound *27* emerged.

N,N-Dicyclohexyl-N-2-(1'-oxy-2'-pyridyl)aminocarbonyl-propanoyl-urea (27)

245 mg of the crude product was obtained, which on crystallization from methanol had mp. 162—164 °C. IR: 3350, 3230, 2940, 2860, 1700, 1682, 1655, 1620, 1575, 1518, 1435, 1345, 1268, 1210, 765 cm⁻¹. NMR (CDCl₃): 0.9—1.22 (m, 22H), 2.76 (dd, 4H), 3.51 (broad s, 1H, NH), 6.75—6.85 (m, 1H), 7.1—7.3 (m, 1H), 8.05—8.30 (m, 1H), 10.05 (broad s, 1H, NH).

Anal: $C_{22}H_{32}N_4O_4$ (416.51) calc'd.: C 63.44; H 7.74; N 13.45%.
found: C 63.66; H 7.83; N 13.74%.

In fractions 7—11, 167 mg of the mixture *25/27* emerged, while fractions 12—36 contained 347 mg of the pure *25*. An analytical sample was purified by repeated chromatography using chloroform-acetone (7.0 : 3.0) as the eluant (R_f ~0.25 for the pure product). $[a]_D = -82^0$ (c = 1.05 in CHCl₃). IR (film): 3230, 2960, 1742, 1705, 1645, 1568, 1506, 1425, 1270, 1205, 1165, 755, 698 cm⁻¹. NMR (CDCl₃): 2.0—2.2 (m, 4H), 2.75—2.95 (m, 4H), 3.6 (t, 1H), 4.6 (m, 1H), 5.15 (dd, 2H, J = 12 Hz), 6.9 (m, 1H), 7.1—7.3 (m, 6H), 8.1—8.45 (m, 2H), 10.2 (broad s, 1H, NH).

Anal. $C_{21}H_{23}N_3O_5$ (397.42) calc'd.: C 63.46; H 5.83%.
found: C 63.21; H 6.04%.

N-3-(2'-Pyridyl)aminocarbonyl-propanoyl-L.-proline (22)

Method A. Compound *20* (485 mg, 2.53 mmole), L-proline (460 mg, 4.0 mmole), imidazole (340 mg, 5.0 mmole), and DMF (0.5 ml) were mixed and placed in an oil-bath at 90—95 °C. After the mixture melted, stirring and heating were continued for 30 min. The reaction mixture was allowed to cool to ambient temperature, 5 ml of water were added, pH adjusted to 1.5—2.0, saturated with sodium chloride, and extracted with 5 × 10 ml of chloroform. Dried organic extracts were evaporated and the crude product (708 mg) was purified by chromatography (35 g silica gel), ethylacetate-methanol (6.0 : 4.0). In fractions 12—26 (10 ml/fraction) 592 mg (68%) of *22* were obtained, glassy residue, which was crystallized from methanol-ether, mp. 161—163 °C. $[a]_D = -86.3^0$ (c = 1.2 in MeOH). IR: 3250 (multiplet), 3150 (multiplet) 2970, 1720, 1645, 1615, 1580, 1550, 1475, 1312, 1295, 1210, 1180, 1145, 1010, 785, 705 cm⁻¹. NMR (CDCl₃): 2.14 (m, 4H), 2.7—2.8 (m, 4H), 3.65 (dd, 2H), 4.48 (d, 1H), 7.03 (m, 1H), 7.6—7.8 (dt, 1H), 8.08 (d, 1H), 8.29 (t, 1H), 10.6 (broad s, 1H, NH).

Anal: $C_{14}H_{17}N_3O_4$ (291.30) calc'd.: C 57.72; H 5.88; N 14.43%.
found: C 57.66; H 6.05; N 14.24%.

Method B. Hydrogenolysis of compound *24* was performed as described for conversion of *14* into *12*. The chromatographically pure product had mp. 160—163 °C, $[\alpha]_D = -59.0^0$ (c = 0.98 in MeOH).

N-3-(1'-Oxy-2'-pyridyl)aminocarbonyl-propanoyl-L-proline (23)

Method A. Starting from *21* (576 mg, 3.0 mmole) acylation of L-proline was performed using the same conditions as described for *22*. After evaporation of the organic extracts the crude product was crystallized from 2-propanol (608 mg, 66%), mp. 106—108 °C. $[\alpha]_D = -69.0^0$ (c = 1.0 in MeOH). IR: 3570, 3450, 3290, 1725, 1648, 1615, 1510, 1455 (m), 1420, 1180, 810, 785 cm^{-1}. NMR (DMSO-d_6): 1.9 (m, 4H) 2.7 (m, 2H), 3.5 (m, 4H), 4.25 (t, 1H), 7.03—7.50 (two double t, 2H), 8.31 (double t, J = 2 Hz, 2H), 10.4 (s, 1H, NH).

Anal: $C_{14}H_{17}N_3O_5 \times CH_3OH$ (339.35) calc'd.: C 53.09; H 6.23; N 12.38%.
found: C 52.99; H 6.00; N 12.27%.

Method B. Starting from *25* compound *23* was obtained, as described for *12*. $[\alpha]_D = -64.5^0$ (c = 0.98 in MeOH).

Determination of Inhibitory Activity

Inhibitory activities of synthetized compounds were determined *in vitro* with porcine plasma angiotensin-converting enzyme (ACE, B grade, Calbiochem, Lucerne) using a fluorometric method and *N*-carbobenzoxy-L-phenylalanyl-L-histidyl-L-leucine (Serva, Heidelberg) as the enzyme substrate.[35] Reaction mixtures contained borax--phosphate buffer, pH 8.0, (prepared by titration of 0.05 M borax with 0.1 M KH$_2$PO$_4$), 1% Na Cl, 1×10^{-4} M substrate, 35 μg/ml of the enzyme, inhibitors in different concentrations and up to 2.5% methanol, which served as the primary solvent for the substrate and inhibitors. The enzyme was preincubated with inhibitors or buffer alone for 15 min at 37 °C. The reaction was started by the addition of substrate, carried out at 37 °C for different time intervals up to 4 hours, and stopped by placing test tubes in a boiling water-batn for 5 min. To the cooled mixture 0.1 ml/ml of 2.56 M NaOH, 0.05 ml/ml of 0.064% o-phthaldialdehyde, and after 4 minutes 0.05 ml/ml of 7.7 M HCl were added. The developed fluorescence was read after 30 min and after Millipore filtration on an Aminco-Bowman spectrofluorimeter.

Reaction rates were linear during the indicated time interval. IC_{50} were calculated from the reaction rates obtained with inhibitor concentrations giving inhibition in the range of 30—65%. With other enzymes only the effects of inhibitors at a concentration of 10^{-3} M were determined.

For the determination of *leucine aminopeptidase* (hog kidney, 200 U/mg, Serva) hydrolysis of 2×10^{-3} M leucine-p-nitroanilide (Serva) in 0.1 M Tris-HCl buffer, pH 8.6, supplemented with 5×10^{-3} M MgCl$_2$, was followed spectrophotometrically at 410 nm.

Activity of *cathepsin B* (bovine spleen, kind gift of P. Ločnikar, »Jožef Štefan« Institute, Ljubljana) was measured with 4×10^{-4} M *N*-CBZ-L-arginyl-L--arginine-4-methoxy-2-naphthylamide (Serva) as substrate in 0.1 M Na,K-phosphate buffer, pH 6.0, with 10^{-3} M dithiothreitol (DTT) and 10^{-3} M EDTA.[36]

For *cathepsin H* (bovine spleen, kind gift of P. Ločnikar, »Jožef Štefan« Institute) and for *α-chymotrypsin* (bovine pancreas, 45 U/mg, Sigma, Saint Louis) 1% azocasein (Serva) was used as substrate following the procedure described for *cathepsin L*.[35] In the first case the buffer was 0.1 M Na-acetate, pH 6.0, with 10^{-3} M DTT and EDTA, and in the second case 0.1 M Tris-HCl, pH 8.0, with 10^{-3} M CaCl$_2$.

Trypsin (bovine pancreas, Pliva, Zagreb) activity was measured photometrically at 410 nm with 9.2×10^{-4} M benzoyl-D,L-arginine-p-nitroanilide (Boehringer, Mannheim) in 0.1 M Tris-HCl buffer, pH 8.2, with 10^{-3} M CaCl$_2$.[37]

Elastase (porcine pancreas, 120 U/mg, Serva) activity was determined by radial diffusion in agar[38] with 0.08% elastin powder (Sigma) and 0.2 M Tris-HCl buffer, pH 8.8.

The enzymes, except leucine aminopeptidase, were incubated 10 min with inhibitor prior to the start of the reaction.

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SAŽETAK

Sinteza derivata N-[(3-supstituiranih)aminokarbonil]propanoil-L-prolina i njihovo djelovanje na enzim za pretvorbu angiotenzina

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Opisana je priprava derivata N-[3-(hetero)aril-aminokarbonil]propanoil-L-prolina, *12, 13, 22* i *23*. Novi postupak sastoji se od taljenja (80—100 °C) N-(hetero)aril-sukcinimida (*7, 8, 20* i *21*) sa nezaštićenim L-prolinom i imidazolom u prisustvu dimetilformamida. *In vitro* određivanje inhibicije enzima za pretvorbu angiotenzina (ACE) pokazalo je, da priređeni derivati N-(arilaminokarbonil)-propanoil-L-prolina imaju nižu aktivnost od captoprila (*1*). Koncentracije potrebne za 50%-tnu inhibiciju kretale su se između 2.5×10^{-4} i 3.3×10^{-3} M.