Ketomethylene Analogues of Phosphoryl Dipeptides Related to Phosphoramidon: Synthesis and Inhibition of Proteases

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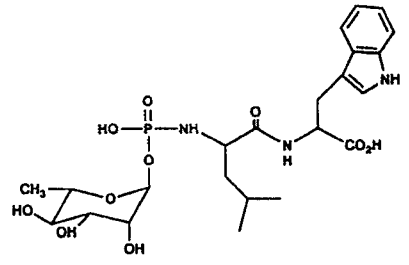
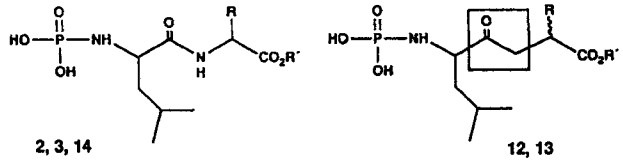
Non-rhamnose-containing phosphoramidon analogues, in which the amide bond was replaced by the isosteric ketomethylene group, have been synthesized in order to stabilize these compounds to peptidase degradation. The key step in this synthesis was suitable alkylation of a 4-ketodiester, prepared from Z-Leu chloromethyl ketone and dimethyl malonate. The ketomethylene dipeptide derivatives P-Leu ψ(COCH$_2$)(*RS*)Xaa-OMe (Xaa = Trp, Phe) are good inhibitors of thermolysin, ACE and specially enkephalinase.

Synthese und Proteasehemmung ketomethylen-analoger vom Phosphoramidon abgeleiteter Phosphoryldipeptide

Nicht-Rhamnose-enthaltende Phosphoramidon-Analoge, bei denen die Amidbindung durch eine isostere Ketomethylengruppe ersetzt ist, sind mit der Absicht hergestellt worden, diese Vebindungen gegen den Abbau durch Peptidasen zu stabilisieren. Der Schlüsselpunkt der Synthese war die entspr. Alkylierung eines 4-Ketodiesters, hergestellt aus Z-Leu-chloromethylketon und Dimethylmalonsäureester. Die Ketomethylen-Dipeptid-Verbindungen P-Leu ψ(COCH$_2$)(*RS*)Xaa-OMe (Xaa = Trp, Phe) zeigten gegenüber Thermolysin, ACE und Enkephalinase einen guten Hemmeffekt.



Phosphoramidon (1)



2, 3, 14 12, 13

Comp.	R	R'
2	CH_2-Indole	H
12	"	CH_3
3	CH_2Ph	H
13	"	CH_3
14	"	CH_3

Scheme 1

Most of the inhibitors of metalloenzymes are peptide analogues having a chelating group (mercapto, phosphoryl, carboxyl, hydroxamic acid) able to interact with the Zn atom present in the active site of the enzyme[1]. Following the isolation, from *Actinomycetes* culture filtrates, of phosphoramidon (**1**)[2], a powerful competitive inhibitor of thermolysin[3], phosphorous derivatives, in particular phosphoramidates, have been widely studied as inhibitors of Zn metalloproteases, such as thermolysin[4], angiotensin converting enzyme (ACE)[5-7] and the neutral endopeptidase (EC 3.4.24.11, NEP) usually termed enkephalinase[8-10]. Analogues of phosphoramidon indicate that the key structural features required to interact with thermolysin are the phosphoryl group and an aromatic or hydrophobic P'$_1$ residue[3]. Thus, the non-rhamnose-containing analogues, P-Leu-Trp-OH (**2**) and P-Leu-Phe-OH (**3**) were also good inhibitors of this enzyme[4]. Moreover, compound **3** is a highly potent selective inhibitor of enkephalins degradation by enkephalinase[8]. However, the development of peptides as potential therapeutic agents is clearly limited by their rapid degradation by peptidases. As a method for increasing the stability of peptides towards proteolytic enzymes, synthesis of analogues with amide bond surrogates have been devised and selectively incorporated into host molecules to yield pseudopeptides[11]. Among these isosteric bonds, replacement of the scissile amide linkage (-CONH-) by a ketomethylene function (-COCH$_2$-) has been successfully applied to the preparation of ACE inhibitors[12,13].

Now, in order to stabilize the peptide bond of phosphoryldipeptide derivatives **2** and **3** to peptidase degradation, the ketomethylene pseudopeptide analogues P-Leu ψ(COCH$_2$)(*RS*)Xaa-OMe (Xaa = Trp, Phe) (**12**) and (**13**)[14], have been synthesized. The inhibitory potency of these compounds towards thermolysin, ACE and enkephalinase is compared to those of P-Leu-Trp-OH (**2**), P-Leu-Phe-OH (**3**), P-Leu-Phe-OMe (**14**), and phosphoramidon **1** as a preliminary study to *in vivo* assays.

Chemistry

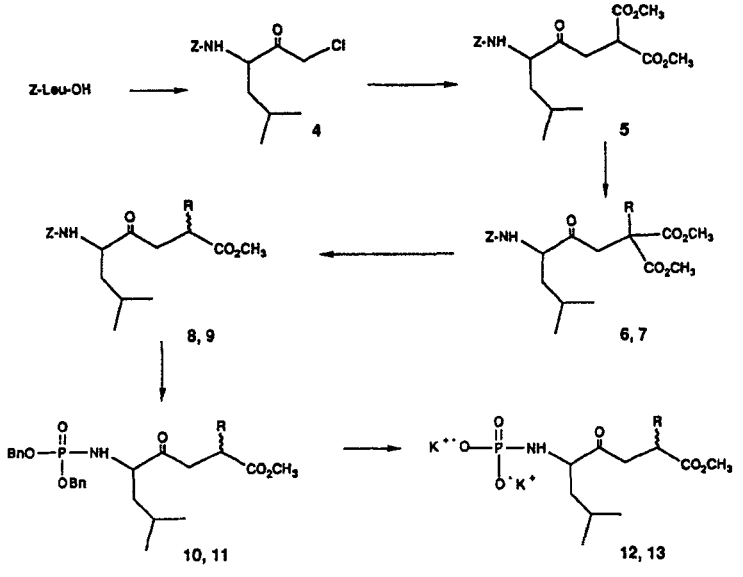
The target ketomethylene pseudodipeptides **12** and **13** were prepared following a general synthetic route recently described by us (Scheme 2)[15,16]. The Leu-containing ketodiester **5** was prepared by conversion of Z-Leu chloromethyl ketone (**4**) to the corresponding iodomethyl ketone *in situ*, followed by reaction with the sodium salt of dimethyl malonate. In order to introduce the C-2 substituent in ketomethylene dipeptides **8** and **9**, the sodium derivative of 4-ketodiester **5** was treated with methiodide of gramine (3-(dimethylamino-methyl)indole) or benzyl bromide to give

  0365-6233/92/0505-0261 $ 3.50 + .25/0

compounds **6** and **7**, respectively. Saponification and decarboxylation of the latter compounds afforded the corresponding 2-substituted-4-ketoacids, which were transformed into the methyl esters **8** and **9** by treatment with diazomethane. In this synthetic route the asymmetric centre of the starting amino acid is not affected, but, the decarboxylation step is not stereoselective affording pseudodipeptides in which the C-terminal amino acid is fully racemic. Separation of *SR* and *SS* diastereoisomers was not observed in our chromatography experiments and, therefore, biological studies were done using mixtures of both diastereomeric derivatives. In the course of our work, H-Leu ψ($COCH_2$) Phe-OH was prepared from Z- or Boc-Leu bromomethyl ketone and diethyl or dibenzyl benzylmalonate[17]. In contrast to our results, poor yields were reported in the alkylation of Z-protected bromomethyl ketones with malonyl derivatives.

(Table 1). *N*-Phosphoryl dipeptide methyl ester **14** and phosphoramidon were included as model compounds. For further comparative purposes, lit. data of **2** and **3** are also indicated in the table.

As shown in Table 1, the inhibitory potencies of the ketomethylene derivatives **12** and **13** against thermolysin, ACE and NEP were, in general, of the same order of magnitude as those of the natural model **1**. A similarity in the IC_{50} values on these three metalloproteases was also found for the *N*-phosphoryl dipeptide methyl ester **14** and its ketomethylene analogue **13**. This fact indicates that the replacement of peptide amide bond by the ketomethylene group did not significantly modify the inhibition on any of these enzymes. However, 100-, 50- and 30-fold decreases in NEP inhibition were respectively observed when the pseudopeptide and dipeptide methyl esters **12**, **13**, and **14** were compared with the dipeptide **3**. These results are in agreement



Scheme 2

The *N*-di-*O*-benzyl phosphoryl pseudodipeptide derivatives **10** and **11** were prepared by removal of the Z group by hydrogenolysis in HCl/MeOH, using 10% Pd-C as catalyst, and subsequent treatment of the resulting deprotected ketomethylene derivatives with dibenzylphosphoryl chloride in the presence of triethylamine[10]. Hydrogenolysis of **10** and **11** at atmospheric pressure by using Pd/C, followed by treatment with N KOH afforded *N*-phosphorylated derivatives P-Leu ψ($COCH_2$)(*RS*)Xaa-OMe (Xaa = Trp, Phe) **12** and **13** as dipotassium salts. In a similar way, H-Leu-Phe-OMe was phosphorylated to provide **14**.

Results and Discussion

N-Phosphoryl pseudodipeptide derivatives **12** and **13** were evaluated as inhibitors of thermolysin, ACE and NEP with the preference of NEP for substrates with a free carboxylic acid terminus[18]. Although compounds **12** and **13** are, in each case, 1:1 diastereomeric mixtures, while compounds **3** and **14** are pure stereoisomers, studies on various dipeptides and ketomethylene pseudodipeptides have shown that the affinity for NEP is independent of the absolute configuration of the amino acid residues[19,20].

Similarly to model compounds **1** and **3**[8,21], the *N*-phosphoryl pseudodipeptide esters **12** and **13** were highly selective inhibitors of enkephalin degradation by NEP, since they did not affect the aminopeptidase N ($IC_{50} > 10^{-5}$, data not shown).

In conclusion, we think that studies on the effects of the *N*-phosphoryl ketomethylene dipeptide derivatives **12** and **13** on nociception deserve attention for the following reasons. Firstly, the inhibitory potencies of **12** and **13** against

Table 1: Inhibitory potency of phosphoryl pseudodipeptide derivatives **12** and **13**

Compound	Thermolysin IC_{50}(M)	ACE IC_{50}(M)	NEP IC_{50}(M)
P-Leuψ($COCH_2$)(RS)Trp-OMe (**12**)	3.18×10^{-7}	1.48×10^{-6}	3.0×10^{-8}
P-Leuψ($COCH_2$)(RS)Phe-OMe (**13**)	1.34×10^{-6}	6.70×10^{-6}	1.5×10^{-8}
Phosphoramidon (**1**)	7.36×10^{-7}	3.68×10^{-6}	3.7×10^{-8}
P-Leu-Trp-OH[a] (**2**)	3.30×10^{-8}	-	-
P-Leu-Phe-OH[a] (**3**)	-	-	0.3×10^{-9}
P-Leu-Phe-OMe (**14**)	1.01×10^{-6}	4.98×10^{-6}	0.9×10^{-8}

[a] From T. Komiyama et al., Arch. Biochem. Biophys. *171*, 727 (1975).

Table 2: Analytical data of compounds **6-14**

Compound	Yield %	Calcd. Found	C	H	N
6	52	$C_{29}H_{34}N_2O_7$	66.65 66.45	6.56 6.63	5.36 5.41
7	89	$C_{27}H_{33}NO_7$	67.06 67.23	6.88 6.98	2.90 2.67
8	52	$C_{27}H_{32}N_2O_5$	69.81 69.95	6.94 7.03	6.03 5.91
9	57	$C_{25}H_{31}NO_5$	70.57 70.38	7.34 7.42	3.29 3.08
10	50	$C_{33}H_{39}N_2O_6P$	67.11 67.33	6.66 6.82	4.74 4.56
11	61	$C_{31}H_{36}NO_6P$	67.50 67.80	6.94 7.01	2.54 2.27
12	88	$C_{19}H_{25}N_2O_6PK_2 . 2H_2O$	43.63 43.41	5.55 5.72	5.36 5.28
13	94	$C_{17}H_{24}NO_6PK_2 . 2H_2O$	42.19 41.97	5.79 5.93	2.89 2.74
14	90	$C_{16}H_{23}N_2O_6PK_2 . 2H_2O$	42.46 42.18	6.01 6.23	6.19 6.05

NEP are of the same order of magnitude as other well known potent inhibitors, such as the model phosphoramidon (**1**) or thiorphan, showing antinociceptive action and/or potentiation of the analgesic effect of other drugs[22,23]. Secondly, it is reasonable to assume that the methyl ester of these compounds, responsible for a decreased affinity for NEP, will be hydrolyzed *in vivo*. Thirdly, as initially mentioned, the replacement of the scissile amide bond with a hydrolytically stable ketomethylene group could lead to a prolonged effect. The *in vivo* assays of **12** and **13** as antinociceptive compounds are now in progress.

Experimental Part

Elemental analyses: Heraeus CHN-O-RAPID analyses instruments.- ^{1}H-NMR spectra: Varian EM-390 and Bruker AM-200 spectrometers, TMS int. stand.- Analytical TLC: Aluminium sheets, 0.2 mm silica gel 60 F_{254} (Merck).- Column chromatography: silica gel 60 (230-400 mesh) (Merck). Detection: UV light (254 nm) and ninhydrin.- Z-Leu-OH: Bachem (Switzerland).- Phosphoramidon, Thermolysin, and ACE: Sigma (USA).- ^{3}H-Leu-enkephalin: Radiochemical Centre (UK).- DBPCl: prepared as described[10].- Enzymatic bioassays: performed as described (thermolysin[3], ACE[24], enkephalinase[25]).

Z-Leu-CH_2-Cl (**4**)

Z-Leu-OH (10 g, 38 mmol) in dry THF (50 ml) was treated at -20°C with *N*-methylmorpholine (4.18 ml, 38 mmol) and isobutyl chloroformate (4.96 ml, 38 mmol). The resulting mixture was stirred at this temp. for 20 min and then filtered. An ethereal solution of diazomethane from *N*-nitroso-methylurea (4.6 g, 45 mmol) was added to the filtrate and the reaction mixture was stirred for 15 min at 0°C, concentrated to a small volume and then 2.5 N methanolic HCl was added at room temp. *until* N_2 *evolution* ceased. Solvents were removed by evaporation and the residue purified by column chromatography eluting with 15% EtOAc in hexane to provide 4 (7.85 g, 70%): ^{1}H-NMR (90 MHz, $CDCl_3$): δ (ppm) = 1.00 (6H, m, δ-Leu

Table 3: ^{1}H-NMR data of derivatives **6-14**

Compound	Solvent	CH_α-Leu	CO_2CH_3	CH_α-Xaa	$COCH_2$	$CH_{2\beta}$-Xaa	Others
6[a]	$CDCl_3$	4.20(m)	3.63(s)	-	3.13(s)	-	7.30-7.00 (10H, m, indole and Z C_6H_5), 3.60 (2H, s, CH_2 indole), 1.60-1.34 (3H, m, ß-Leu CH_2 and Y-Leu CH), 0.82 (6H, d, δ-Leu CH_3)
7[a]	$CDCl_3$	4.30(m)	3.65(s)	-	3.15(s)	-	7.35-7.20 (10H, m, C_6H_5 and Z C_6H_5), 3.40 (2H, s, CH_2-Ph), 1.60-1.40 (3H, m, ß-Leu CH_2 and Y-Leu CH), 0.90 (6H, d, δ-Leu CH_3)
8[a]	$CDCl_3$	4.21(m)	3.60(s)	———3.10-2.49(m)———			7.20-6.90 (10H, m, indole and Z C_6H_5), 1.50-1.20 (3H, m, ß-Leu CH_2 and Y-Leu CH), 0.85 (6H, d, δ-Leu CH_3)
9[a]	$CDCl_3$	4.32(m)	3.59(s)	———3.10-2.50(m)———			7.30-7.10 (10H, m, C_6H_5 and Z C_6H_5), 1.50-1.30 (3H, m, ß-Leu CH_2 and Y-Leu CH), 0.80 (6H, d, δ-Leu CH_3)
10[b]	$CDCl_3$	3.75(m)	3.60(s) 3.65(s)	———3.30-2.70(m)———			7.30-7.00 (15H, m, 2 Bn C_6H_5 and indole), 1.72-1.30 (3H, m, ß-Leu CH_2 and Y-Leu CH), 0.90 (6H, m, δ-Leu CH_3)
11[b]	$CDCl_3$	3.83(m)	3.59(s) 3.61(s)	———3.15-2.50(m)———			7.30-7.10 (15H, m, 2 Bn C_6H_5 and C_6H_5), 1.60-1.20 (3H, m, ß-Leu CH_2 and γ-Leu CH), 0.80 (6H, m, δ-Leu CH_3)
12[b]	CD_3OD	4.00(m)	3.50(s) 3.56(s)	———3.40-3.10(m)———			7.70-7.23 (5H, m, indole), 1.80-1.32 (3H, m, ß-Leu CH_2 and Y-Leu CH), 1.00 (6H, m, δ-Leu CH_3)
13[b]	CD_3OD	3.96(m)	3.48(s) 3.55(s)	3.05(m)	—2.81-2.58(m)—		7.25-7.10 (5H, m, C_6H_5), 1.70-1.30 (3H, m, ß-Leu CH_2 and γ-Leu CH), 0.90 (6H, m, δ-Leu CH_3)
14[b]	D_2O	3.38(m)	3.51(s)	4.47(m)	-	3.02(dd) 2.88(dd)	7.20-7.05 (5H, m, C_6H_5), 2.35 (1H, Y-Leu-CH_2), 1.08 (2H, m, ß-Leu CH_2), 0.64 (6H, m, δ-Leu CH_3)

[a] Spectrum recorded at 90 MHz. [b] Spectrum recorded at 200 MHz.- Doublets of δ-Leu CH_3 show J = 7.0-7.7 Hz.

CH_3), 1.36-1.66 (3H, m, β-Leu CH_2 and γ-Leu CH), 4.0 (2H, s, $COCH_2$), 4.60 (1H, m, α-Leu CH), 5.13 (2H, s, Z CH_2), 5.36 (1H, m, NH), 7.40 (5H, s, Z C_6H_5).- $C_{15}H_{20}ClNO_3$ (297.8) Calcd. C 60.5 H 6.77 Cl 11.9 N 4.7 Found C 60.4 H 6.98 Cl 11.7 N 4.5.

Methyl5-(S)-N-(benzyloxycarbonyl)amino-2-methoxycarbonyl-7-methyl-4-oxooctanoate (**5**)

Chloromethyl ketone **4** (6 g, 20 mmol) and NaI (3 g, 20 mmol) in 1,2-dimethoxyethane (60 ml) were added, at room temp., to a solution of dimethyl malonate (2.9 g, 22 mmol) and $NaOCH_3$ (1.08 g, 20 mmol) in 1,2-dimethoxyethane (20 ml). Stirring was continued at that temp. for 1 h, the solvent was removed and the residue was extracted with chloroform and washed with H_2O. The org. extract was dried over Na_2SO_4 and evaporated leaving a residue which was purified on a silica gel column with 15% EtOAc in hexane to provide the title compound (6.1 g, 77%) as a syrup.- ^{1}H-NMR (90 MHz, $CDCl_3$): δ (ppm) = 1.00 (6H, m, δ-Leu CH_3), 1.20-1.60 (3H, m, β-Leu CH_2, γ-Leu CH), 3.70 (6H, s, 2 CO_2CH_3), 5.06 (2H, s, Z CH_2), 5.26 (1H, m, NH), 7.33 (5H, s, Z C_6H_5).- $C_{20}H_{27}NO_7$ (393.4) Calcd. C 61.1 H 6.92 N 3.6 Found C 61.1 H 7.08 N 3.4.

Alkylation of compound **5** *(General procedure for* **6** *and* **7**)

Compound **5** (10 mmol) and $NaOCH_3$ (12 mmol) in 1,2-dimethoxyethane (50 ml) were treated at room temp. with gramine (11 mmol) and CH_3I (22 mmol) for the synthesis of **6**, or benzyl bromide (13 mmol) for the preparation of **7**. After stirring for 1.5 h, the solvents were removed and the residue was extracted with EtOAc and washed with water. The org. layer was dried over Na_2SO_4, evaporated to dryness and the residue was purified by column chromatography using the following eluents: 20% EtOAc in hexane for compound **6** and 10% EtOAc in hexane for **7**. Analytical and spectral data of these compounds are listed in tables 2 and 3.

Z-Leu ψ(COCH$_2$)(RS)Xaa-OMe (Xaa = Trp, Phe) (**8, 9**)

2-Substituted diesters **6** or **7** (8.2 mmol) in methanol (50 ml) were treated with 6 N NaOH (18 mmol) and the mixture was stirred at room temp. for 3 h. After evaporation of the methanol, the remaining aqueous mixture was diluted with H_2O (30 ml), acidified with conc. HCl to pH = 3 and extracted with EtOAc. The extract was dried over Na_2SO_4 and evaporated. The resulting residue was dissolved in dioxane (30 ml) and heated under reflux for 3 h. After cooling to room temp., the solution was treated with an ethereal solution of diazomethane in order to prepare the corresponding methyl ester. Removal of the solvents and purification by silica gel column chromatography (25% EtOAc in hexane) yielded the title compounds. Analytical and spectral data are recorded in tables 2 and 3.

(BnO)$_2$OP-Leu ψ(COCH$_2$)(RS)Xaa-OMe (Xaa = Trp, Phe) (**10, 11**)

Z-Protected pseudodipeptides **8** or **9** (2 mmol) in 2.5 N methanolic HCl (50 ml) were hydrogenated, at room temp. and atmospheric pressure, in the presence of 10% Pd/C for 1.5 h. The catalyst was removed by filtration and the filtrate was evaporated to dryness. A solution of the resulting crude deprotected compound in chloroform (50 ml) was treated at 0°C with triethylamine (4 mmol) and freshly prepared dibenzylphosphoryl chloride[10] (2 mmol). After 6 h of reaction at room temp., the solution was washed with H_2O, 10% $NaHCO_3$ (w/v) and H_2O, and the org. layer dried over Na_2SO_4. After evaporation, the resulting residue was chromatographed on a silica gel column with 20% EtOAc in hexane, as eluent for both compounds. Analytical and spectral data of **10** and **11** are listed in tables 2 and 3.

(BnO)$_2$OP-Leu-Phe-OMe

A solution of dipeptide derivative H-Leu-Phe-OCH_3 (0.77 g, 2.34 mmol) in chloroform (50 ml) was treated at 0°C with triethylamine (0.65 ml, 4.68 mmol) and dibenzylphosphorylchloride (0.46 g, 2.34 mmol). After stirring

overnight the solution was washed with H_2O, 10% $NaHCO_3$ (w/v) and H_2O, and the org. layer dried over Na_2SO_4. After evaporation the residue was chromatographed on silica gel with 50% EtOAc in hexane, to give 0.9 g (75%) of the title compound.- ^{1}H-NMR (90 MHz, $CDCl_3$): δ (ppm) = 7.40-7.00 (15 H, m, 3 C_6H_5), 5.00 (4H, m, 2 CH_2, Bn), 4.81 (1H, m, α-Phe CH), 3.60 (3H, s, CO_2CH_3), 3.53 (1H, m, α-Leu CH), 3.00 (2H, dd, J_1 = 15 Hz, J_2 = 4.5 Hz, β-Phe CH_2), 1.70-1.20 (3H, m, β-Leu CH_2 and γ-Leu CH), 0.85 (6H, d, J = 7.5 Hz, δ-Leu CH_3).

P-Leu ψ(COCH$_2$)(RS)Xaa-OMe (Xaa = Trp, Phe) (**12**, **13**) *and P-Leu-Phe-OCH$_3$* (**14**)

N-Dibenzylphosphorylated compounds (0.5 mmol) in isopropanol (20 ml) were hydrogenated, at atmospheric pressure and room temp., for 30 min over 10% Pd/C. The catalyst was filtered off and the resulting solution was treated with N KOH (1 mmol). Evaporation of the solvents and precipitation with isopropanol-ether gave compounds **12**, **13**, and **14** as very hygroscopic solids which were used without further purification (stored at low temp.). Analytical and spectral data: tables 2 and 3.

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