83. Chemical Synthesis, Proton NMR. Parameters, Hydrogen- and Calcium-Ion Complexation of L-γ-Carboxyglutamyl-L-γ-carboxyglutamic Acid and D-γ-Carboxyglutamyl-L-leucine

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Summary

The purpose of this communication is to describe the preparation and some properties of the first two synthetic peptides containing D- and L-γ-carboxyglutamic acid. Use was made of *N*-protected γ,γ'-di-*t*-butyl-γ-carboxyglutamic acids (D, L, and DL) described earlier [1a]. Preliminary ^{1}H-NMR. data (360 MHz) indicate a restricted rotation of the Gla side chain in the free amino acid as well as in the C-terminal Gla of Gla-Gla in H_2O solution at acid pH. The proton dissociation from Gla and Gla-Gla was studied by potentiometric titration and NMR. methods. The pH titration in the presence of Ca^{2+} ions shows that Gla-Gla has a much higher association constant for this cation than Gla. It is almost as great as that of prothrombin ($pCa^{2+} = 3.2$ *vs.* 3.5).

Introduction. – γ-Carboxyglutamic acid (Gla) is found in various vitally important, calcium-dependent proteins involved in blood-clotting and the structure of calcified tissue (lit. see [1]). It has been speculated that Gla-Gla dipeptide units ('tandems') might be responsible for the Ca^{2+} complexation displayed by such proteins [2].

The chemical synthesis of Gla peptides may offer one of the best approaches to the study of the exact molecular basis of the Ca^{2+} complexation process. Since we have been able to prepare for the first time derivatives of DL-, D-, and L-Gla that are suitable for peptide synthesis [1], we are actively persuing this line of research.

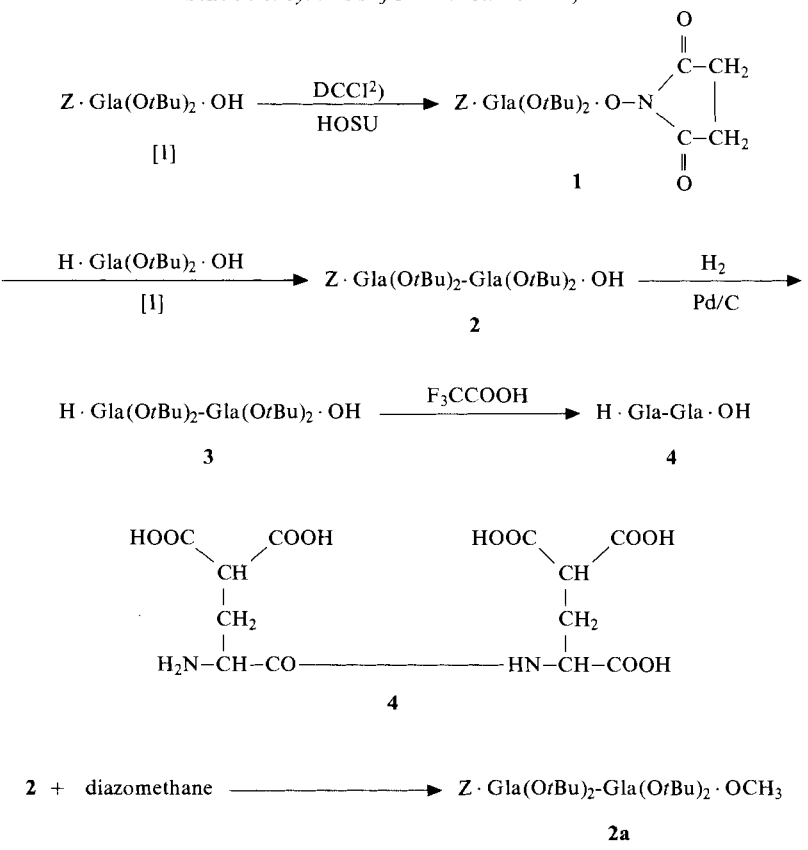
We wish to describe here the first syntheses of L- and D-Gla peptides, namely H · Gla-Gla · OH (L-L) and H · gla-Leu · OH (D-L)[1]). A preliminary account of investigations into the solution conformation of such peptides (NMR.- in collaboration with our biophysis group, Prof. Dr. *K. Wüthrich*) and into their behaviour towards cations, especially Ca^{2+}, is also given.

[1]) As in early reports [3], we abbreviate D-amino acids with three-letter symbols starting with a lower-case letter (L-amino acids with capital letters).

Syntheses of H · Gla-Gla · OH and H · gla-Leu · OH. The idea was, in both cases, to produce a protected dipeptide from which the α-amino- and α-carboxyl-protecting groups could be removed in one step without affecting side-chain protection. The strategy was that of *Schwyzer & Kappeler* and *Schwyzer & Dietrich* [4], who had introduced side-chain carboxyl protection as *t*-butylesters. In *Schemes 1* and *2*, these groups are stable against hydrogenolytic cleavage of *N*-benzyloxycarbonyl and *O*-benzyl groups **(2→3; 5→6).** Additionally, in *Scheme 2*, *O-t*-butyl is stable against alcaline β-elimination of the 2-(methylsulfonyl)-ethoxycarbonyl group and hydrolysis of the methylester group **(8→9).** From the side-chain-protected dipeptides **3, 6,** and **10,** the *t*-butyl groups were cleaved by either trifluoroacetic or hydrochloric acid **(3→4; 6→7; 9→10).**

Scheme 1 shows the path from γ,γ'-di-*t*-butyl L-*N*-benzyloxycarbonyl-γ-carboxyglutamate to L-γ-carboxyglutamyl-L-γ-carboxyglutamic acid **(4).** The first step was a conventional preparation (see [5]) of the active ester **1,** followed by a

Scheme 1. *Synthesis of H · Gla-Gla · OH* **4**[1])

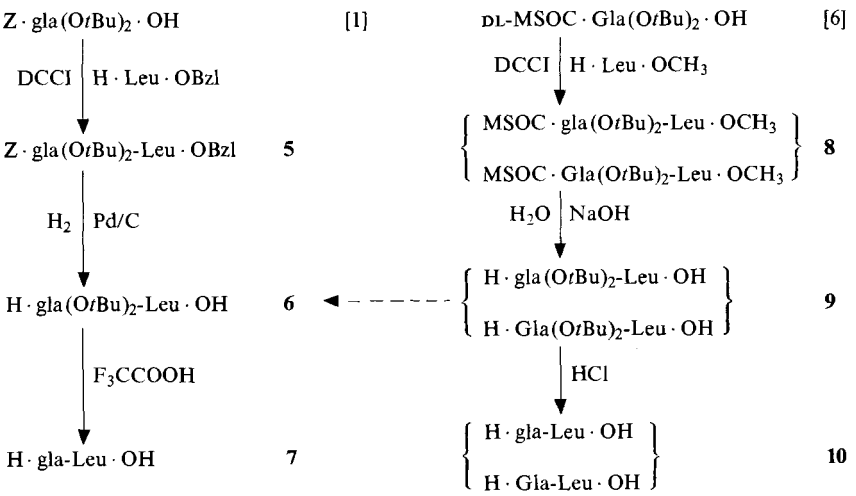


[2]) Abbreviations and general methods, see [5]. Here: DCCI = dicyclohexylcarbodiimide; HOSU = *N*-hydroxysuccinimide; MSOC = 2-(methylsulfonyl)-ethoxycarbonyl; Z = benzyloxycarbonyl.

condensation with γ,γ'-di-*t*-butyl L-γ-carboxyglutamate. The next two steps were described above. In addition to **2,** the methylester **2a** was also prepared for use in further syntheses. All educts and products were crystalline except **3** and **4** that were obtained as amorphous solids.

Scheme 2 shows the two paths adopted for the synthesis of D-γ-carboxyglutamyl-L-leucine **(7)** and its mixture **(10)** with the diastereomeric L-L dipeptide. One involves the condensation of γ,γ'-di-*t*-butyl D-*N*-benzyloxycarbonyl-γ-carboxyglutamate with benzyl L-leucinate by means of dicyclohexylcarbodiimide (see [5]). The amorphous, fully protected dipeptide **5** is further treated as described above to produce crystalline γ,γ'-di-*t*-butyl D-γ-carboxyglutamyl-L-leucinate **6** and pure, amorphous, solid **7.**

Scheme 2. *Synthesis of H · gla-Leu · OH* **7**[1][2])

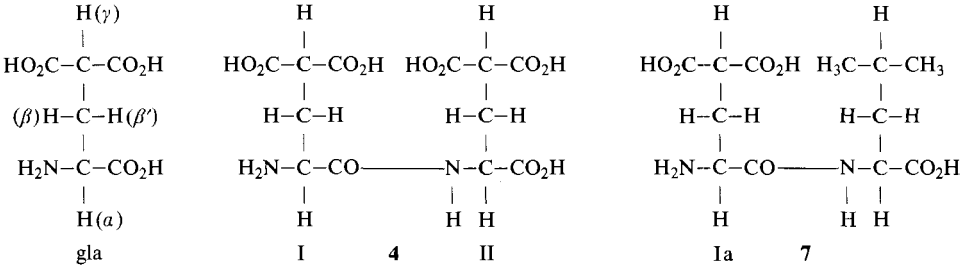


The other path proceeded from γ,γ'-di-*t*-butyl DL-*N*-[2-(methylsulfonyl)-ethoxycarbonyl]-γ-carboxyglutamate [6], *via* the mixtures of diastereomers **8** and **9** to **10.**

Wieland et al. (*e.g.* [7]) had already studied the separation of diastereomeric dipeptides. They found that, quite generally, the diastereomer with identical chirality of the two amino acid residues has the greater Rf value. We found the following Rf's with silica gel thin-layer chromatography using *sec*-butanol/acetic acid/water 100:15:35 *(v/v):* 0.57 for L-L- and 0.35 for D-L- Gla(O*t*Bu)$_2$-Leu, 0.66 for L-L- and 0.44 for D-L- Gla(O*t*Bu)$_2$-Gla(O*t*Bu)$_2$. The broken line of *Scheme* **2** indicates the so achieved, analytical-scale separation of gla(O*t*Bu)$_2$-Leu from Gla(O*t*Bu)$_2$-Leu.

^{1}*H-NMR. Studies.* Our preliminary results are summarized in *Table 1.* The substituted malonic acid γ-proton is easily, but not immediately [1] exchanged against a deuteron: its signal at about 3.8 ppm and its spin-spin coupling with the β-protons in water disappears slowly, but completely in deuterium oxide.

Table 1. *^{1}H-NMR. Parameters at 360 MHz of gla, Gla-Gla* **4**, *and gla-Leu* **7** *in H_2O and D_2O at 25° with tetramethylammonium chloride as internal standard (3.3 ppm).* The chemical shifts, δ, are in ppm from tetramethylsilane, the spin-spin coupling constants, J, in Hz (± 0.3 Hz). The parameters of gla and **4** were refined by iteration (LAOCOON II computer program) and spectral simulation (XNMR 8P). Ionization is not indicated in the chemical formulae, pH values are uncorrected readings. The assignments were made with decoupling experiments. *Additional data.* **4** (H_2O): 9.02 *d*, NH; J(NH,H_a) 7.4; **7** (D_2O): Leu*(ABCX)* 1.03 *d*, CH_3; 1.07 *d*, CH'_3; 1.82 complex signal $H_\beta H_\gamma$; 4.48 *t*, H_a.



	H_2O	pH 1.70	D_2O	pH 1.72	pH 2.64	pH 3.50
	Gla-Gla **4**		Gla-Gla **4**		gla-Leu **7**	gla
	I	II	I	II	Ia	
$\delta(\beta)$	2.573 *qa*[a]	2.432 *sept*	2.573 *d*	2.427 *qa*	2.53 *d*	2.432 *qa*
(β')		2.676 *sept*		2.676 *qa*		2.541 *qa*
(γ)	3.834 *t*	3.721 *q*	–	–	–	–
(a)	4.299 *t*	4.679 *q*	4.299 *t*	4.679 *qa*	4.26 *t*	3.925 *t*
$J(a\beta)$	5.6	9.4	6.6	9.4	7.32	7.5
$(a\beta')$		5.3		5.3		5.86
$(\gamma\beta)$	8.0	6.2	–	–	–	–
$(\gamma\beta')$		8.7	–	–	–	–
$(\beta\beta')$	–	−14.5	–	−14.5	–	−14.65

[a]) *qa* = Quartett.

In the acid pH range the β-protons of the free amino acid (gla) and of one of the Gla residues of Gla-Gla are unequivalent, displaying different chemical shifts and different coupling constants with the neighbouring α- and γ-protons. The other Gla of Gla-Gla and the gla of gla-Leu have equivalent β-protons. We interpret these findings as indicating restricted rotation of the Gla side-chain in the free amino acid and in the C-terminal position of the dipeptide, but 'free' rotation in the dipeptide *N*-terminal positions.

Ca^{2+} complexation and proton dissociation. The two phenomena were studied in aqueous solution with four types of experiment: 1) electrophoresis on cellulose layers (Gla), 2) acid-base titration (Gla and Gla-Gla), 3) ^{1}H-NMR. at different pH values (Gla), and 4) acid-base titration in the presence of Ca^{2+} (Gla and Gla-Gla).

1) The electrophoretic experiments with Gla [8] lead to the following conclusions: (i) the apparent isoelectric point is around ~2.4 (one positive charge on

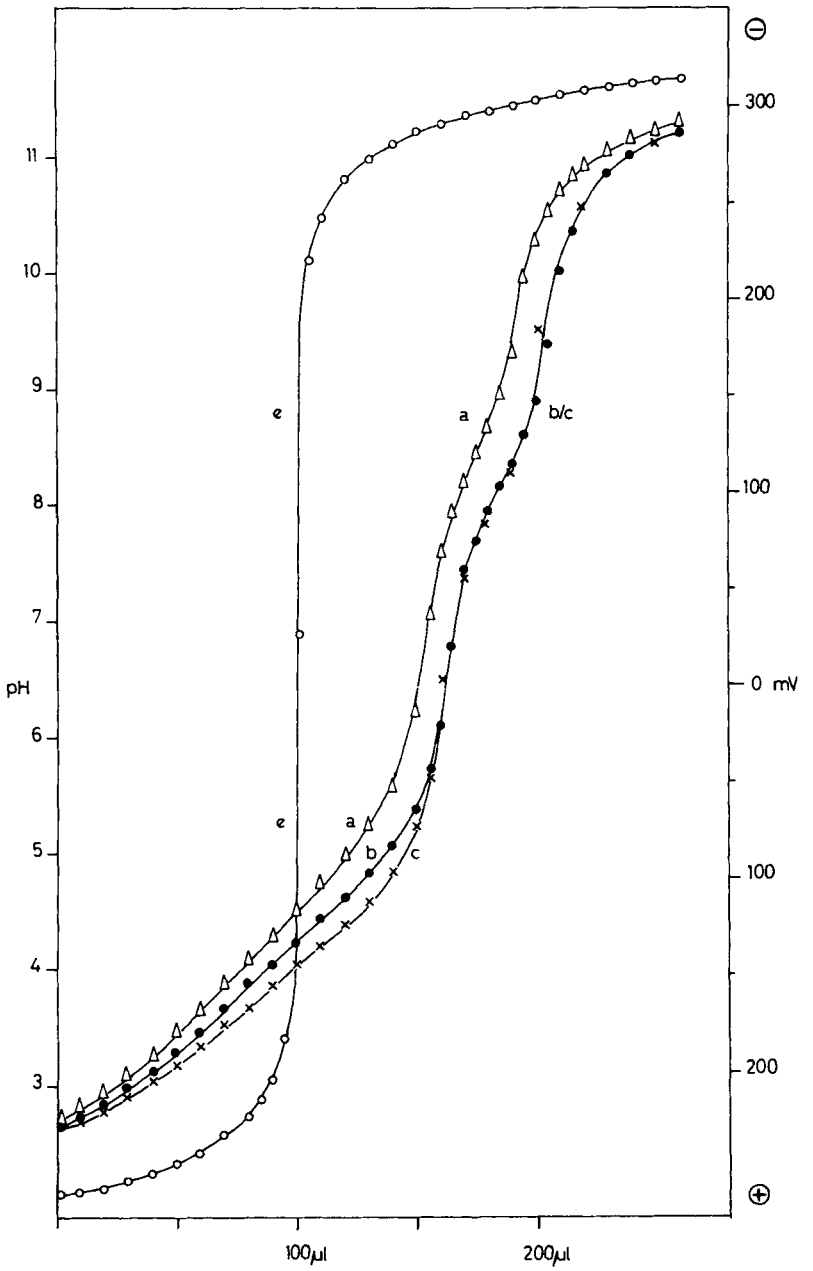


Fig. 1. *Potentiometric titration of Gla-Gla.* Starting volume of 2 ml at 25°. Ionic strength of 0.1 (NaCl). The potential was measured with a glass electrode and a *Metrohm* Digital Voltmeter Mod. E 500 with an accuracy of ±0.1 mV. Standardization by titration of HCl with NaOH ($I = 0.1$; curve e). The so obtained values of $pK_W = 13.75 \pm 0.02$ and 13.59 ± 0.05 for the two electrodes were taken into consideration during the calculations. Titration with a *Metrohm* microburette (0.5 ml) and increments of 10 µl NaOH ($I = 0.1$) of 4 mM Gla-Gla without (a) and in the presence of 4 mM (b) or 40 mM (c) calcium (II) chloride.

Table 2. *Protonation constants of Gla and Gla-Gla in 0.1N NaCl at 25°.* The constants were determined by potentiometric titration (see for example *Fig. 1*) and computation according to [9]. p*I* is the electrophoretically determined isoelectric point [8]

Ligand	$\log K_1$	$\log K_2$	$\log K_3$	$\log K_4$	$\log K_5$	$\log K_6$	p*I*
Gla	1.7	3.2±0.1	4.75±0.1	9.9±0.1			2.45
Gla-Gla	?	3.0±0.2	3.8±0.1	4.7±0.1	5.5±0.1	8.9	

the α-amino group and one negative charge distributed over the three carboxyl groups); (ii) the electrophoretic mobility of Gla with respect to Asp and Glu at pH 6.4 is ~1.4 and ~1.6 respectively, indicating approximately one extra negative charge on Gla; (iii) at pH 1.6, Glu and Asp are completely protonated (charge = +1); however, the relative mobility of Gla indicates only about 60% of a positive charge. This means that there must be a buffer region near pH 1.6.

2) Acid-base titration curves of HCl and Gla-Gla are shown in *Fig. 1;* Gla was titrated similarly. The individual protonation constants, $K_p = [H_pL]/[H_{p-1}L] \cdot [H]$, were defined and computed according to [9]. Their values are assembled in *Table 2.* More exact values could have been obtained with larger volumes (>50 ml) or, for the lower constants, with higher total ligand concentrations, $[L]_t$. The $\log K_1$ was

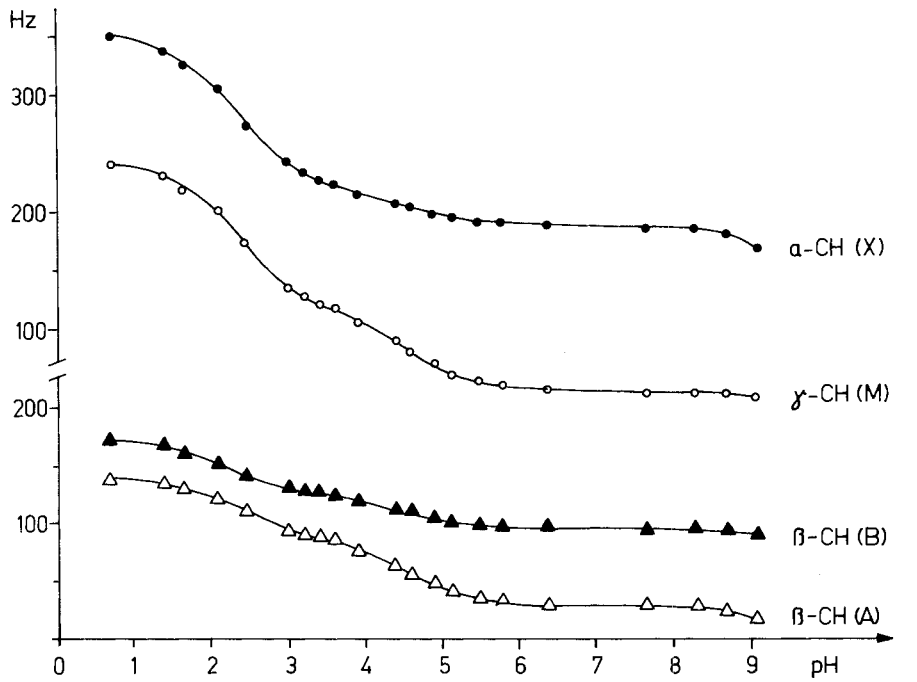


Fig. 2. *^{1}H-NMR.-monitored titration of Gla.* Solutions: 10^{-2}M Gla in aqueous NaCl (I = 0.1) with tetramethylammonium chloride as internal standard. The pH was adjusted with 4N NaOH and determined with a *Philips* Digital pH-Meter Mod. PW 9414. The spectra were measured with a *Bruker* HXS-360 MHz instrument with *sine bell* resolution enhancement [14] and *Fourier* transform techniques. The ordinate shows the chemical shift in Hz with respect to arbitrary, fixed points (lower Hz values indicate higher fields).

not determined by titration; the value indicated for Gla is a rough estimation from $\log K_2$ and the electrophoretic data and might well contain a large error (± 0.2?). Nevertheless, it appears that Gla is the most acidic naturally occurring amino acid, a property that helped lead to its discovery [10].

The constants 1, 2, and 3 are ascribed to carboxylate group protonations, constant 4 to the α-amino group of Gla. For Gla-Gla, $\log K_1$ remains unestimated. The 4 constants 2, 3, 4, and 5 lie in the range of 3–5.5, compatible with the behaviour of malonic acid carboxylate groups. As expected, the α-ammonium group, $K(6)$, of the peptide dissociates at a lower pH than that of the amino acid.

3) The 360-MHz-NMR. spectra of Gla are well resolved and readily interpreted (see, for example, [8]). The resonances A and B(β-H), M(γ-H), and X(α-H) are shifted towards higher fields with increasing pH. *Fig. 2* depicts the changes between pH 0.5 and 9 (higher pH values could not be investigated because of strong precipitations in the solutions). The significant chemical shift parameters and the calculated apparent pK values are summarized in *Table 3*. The α-proton and the γ-proton both 'see' a pK(app.)$=2.4$ as the lowest protonation step. The γ-proton then experiences another change in its surroundings with pK(app.)$=4.6$; this transition has much less influence on the α-proton and is attributed to a γ-carboxylate group. The β-protons 'see' both dissociation steps, but to a much lesser degree. The situation could be interpreted as meaning that the pK's of the α-carboxylate and one of the γ-carboxylate groups are close together and that both groups contribute to the unit negative charge observed around pH 2.4, the electrophoretic isoelectric point. Microscopic constants were not determined.

4) Ca^{2+} complexation was measured by acid-base titration in the presence of two different concentrations of the inorganic ion. The titration curves of *Fig. 1* indicate that a mononuclear complex, $\{Ca^{2\oplus}, \text{Gla-Gla}^{n\ominus}\}^{(n-2)\ominus}$ is very predominant above pH 4 (the titration curves of Gla were determined similarly, but are not included in

Table 3. *Apparent p*K *values and NMR. chemical shift parameters of Gla* (see *Fig. 2*). δ_a and δ_b are the chemical shifts (ppm) of the completely protonated and completely deprotonated states, respectively. The apparent pK's, pK(app), were determined by plotting $\log(\delta_a-\delta)/(\delta-\delta_b)$ against pH

Resonance	pK(app)	δ_a	δ_b	$\delta_a-\delta_b$
X(α-H)	2.4	4.29	3.82	0.47
M(γ-H)	2.4	3.98	3.42	0.56
	4.6	3.98	3.42	0.56
B(β-H′)		2.67	2.46	0.21
A(β-H″)		2.58	2.28	0.30

Table 4. *Over-all calcium(II) complex stability constants, β_n, of Gla, Gla-Gla, and various other carboxylic and aminocarboxylic acids.* Gla and Gla-Gla were titrated at ionic strength 0.1 (NaCl), see *Fig. 1;* the values of the other compounds are taken from the literature [13]

Ligand	$\log\beta_n$	Ligand	$\log\beta_n$
Gla	1.3 ± 0.2	Gly-Gly	1.24
Gla-Gla	3.2 ± 0.2	Acetic acid	0.7
Glu	1.43	Malonic acid	1.46
Gly	1.38		

the figure). The over-all complexation constants, $\beta_n = [ML_n]/[M]\,[L]^n$, were computed according to [9]. They are assembled in *Table 4* and compared with those for simpler carboxylic and aminocarboxylic acids. The amino acid Gla behaves like other, simpler compounds and shows no special affinity for Ca^{2+}. Gla-Gla, however, binds Ca^{2+} more strongly. Inspection of molecular models of the dipeptide shows that intramolecular chelate effects must be excluded. This explains the relatively low values of the complexation constants. The pK_1 value found for Gla-Gla, 3.2, is very close to that observed for the calcium-binding site in prothrombin fragments, 3.5 [11]. This might be taken as support for the 'tandem hypothesis' [2] (see introduction); however, the unknown effect of the α-carboxylate group in Gla-Gla (which is not present in longer peptides) has not yet been accounted for.

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Experimental Part

General methods, apparatus, and materials, see [1]. The experiments are not described in detail whenever the general procedures are sufficiently documented in *Houben-Weyl* [5] or other publications [1].

L-*Z* · *Gla(OtBu)*$_2$ · *OSU*, **1.** 160 mg (0.37 mmol) L-Z · Gla(O*t*Bu)$_2$ · OH [1] [8] were converted into the mixed anhydride, Z · Gla(O*t*Bu)$_2$ · $OCOOCH_2CH(CH_3)_2$, using 1.5 ml ethyl acetate and 0.37 mmol of isobutyl chloroformate at −18°. After 2 min reaction time, 0.44 mmol of *N*-hydroxysuccinimide were added, and the reaction completed at 0° and 40° (2 min). The usual isolation procedure yielded 204 mg (0.37 mmol; 100%) needle-like crystals from pentane, m.p. 64–68°; $[\alpha]_D^{20} = -20°$ ($c = 1.1$, methanol); Rf 0.49 CME (with decomposition).

Z · *Gla(OtBu)*$_2$-*Gla(OtBu)*$_2$ · *OH*, (L-L), **2.** 200 mg (0.36 mmol) **1** and 62 mg (0.20 mmol) H · Gla(O*t*Bu)$_2$ · OH [1] were condensed in 2 ml dimethylformamide in the presence of 39 mg *N*-methylmorpholine. The usual isolation procedure with ethyl acetate yielded a crude product that was chromatographed on 80 times its weight of silica gel. Elution with chloroform/methanol 9:1 *(v/v)*. Yield 119 mg (0.17 mmol; 83%) amorphous solid that was crystallized from diisopropyl ether/pentane: m.p. 112–113°; $[\alpha]_D^{20} = -11.7°$ ($c = 1.2$, methanol), −0.6° ($c = 1.2$, chloroform); Rf 0.30 CME, 0.41 ethyl acetate/methanol 9:1, 0.84 BEW1. – NMR. and IR. spectra agree with the assigned structure.

$C_{36}H_{54}N_2O_{13}$ (722.84) Calc. C 59.82 H 7.53 N 3.88% Found C 59.73 H 7.44 N 3.84%

Z · *Gla(OtBu)*$_2$-*Gla(OtBu)*$_2$ · *OCH*$_3$, (L-L), **2a.** 989 mg (1.37 mmol) **2** were dissolved in 20 ml ether and treated with diazomethane. The product was chromatographed on silica gel with a mixture of hexane/ethyl acetate 8:2 and crystallized from ether/pentane. Yield 705 mg (70%) colourless needles, m.p. 101–102°; $[\alpha]_D^{20} = +16.1°$, $[\alpha]_{546}^{20} = +18.3°$ ($c = 1.1$, chloroform). – The routine NMR. agrees with the assigned structure.

$C_{37}H_{56}N_2O_{13}$ (736.86) Calc. C 60.31 H 7.66 N 3.81% Found C 60.58 H 7.75 N 4.16%

H · *Gla(OtBu)*$_2$-*Gla(OtBu)*$_2$ · *OH*, (L-L), **3.** 108 mg (0.15 mmol) **2** were hydrogenated according to [1]. Yield 82 mg (0.14 mmol; 93%) of an amorphous, colourless solid that did not crystallize from warm water; m.p. above 220° (decomposition); Rf 0.66 BEW1; electrophoresis: one spot, M(Arg) 0.32. $[\alpha]_D^{20} = +11.2°$ ($c = 1.1$, methanol).

H · *Gla-Gla* · *OH* (L-L), **4.** The *t*-butyl groups were removed from **3** with cold, 90% trifluoroacetic acid in the usual manner. Yield 90% of lyophilized, electrophoretically pure product, M(Asp) 1.7 pH 6.4; m.p. above 151° (dec.); Rf 0.15 I; $[\alpha]_D^{20} = +17.6°$ ($c = 0.7$, 6N HCl). – The NMR. spectra agree completely with the assigned structure and the analytical purity *(Table 1)*.

Z · *gla(OtBu)*$_2$-*Leu* · *OBzl* (D-L), **5.** 232 mg (0.53 mmol) D-Z · Gla(O*t*Bu)$_2$ · OH and 153 mg (0.59 mmol) HCl, H · Leu · OBzl [5] were dissolved in 5 ml pure, dry dimethylformamide and treated

with 0.53 mmol *N*-methylmorpholine, 1 mmol *N*(1)-hydroxybenzotriazole, and 0.6 mmol dicyclohexylcarbodiimide. The usual reaction and isolation procedures resulted in an oil that was chromatographed on 50 times its weight of silica gel. Eluants: chloroform/methanol 19:1 and hexane/ethyl acetate 7:3. The combined eluates gave 252 mg (0.39 mmol; 74%) of a colourless oil, $[\alpha]_D^{20} = -7.9°$ ($c = 1.1$, methanol); Rf 0.27 HE1 (double development), 0.65 CM1.

$C_{35}H_{48}N_2O_9$ (640.75) Calc. C 65.60 H 7.55 N 4.37% Found C 65.60 H 7.69 N 4.44%

H · gla(OtBu)$_2$-Leu · OH, (D-L), **6.** Catalytic hydrogenation of **5** in the usual manner [5] [1] cleaved off both the benzyloxy carbonyl and the benzyl groups. The product crystallized from warm (50°) isopropyl alcohol. Yield 85%, m.p. 175–176° (dec.); $[\alpha]_D^{20} = -44.1°$ ($c = 0.94$, methanol); Rf 0.45 BEW1.

$C_{20}H_{36}N_2O_7$ (417.0) Calc. C 57.67 H 8.71 N 6.73% Found C 57.60 H 8.69 N 6.54%

H · gla-Leu · OH, (D-L), **7.** The *t*-butyl groups were removed from **6** in the usual manner [5] [1] with cold, 90% trifluoroacetic acid. Yield 63% of lyophilized, electrophoretically pure product, M(Asp) 1.0 pH 6.4; m.p. above 122° (dec.); $[\alpha]_D^{20} = -58.7°$ ($c = 1.1$, H_2O). – The NMR. spectra agree completely with the assigned structure and the analytical purity *(Table 1)*.

MSOC · Gla(OtBu)$_2$-Leu · OCH$_3$ (D-L + L-L), **8.** Equimolar amounts (0.38 mmol) DL-MSOC · Gla(O*t*Bu)$_2$ · OH (crystal prisms from CCl_4, m.p. 101–106, Rf 0.54 BEW1, 0.73 EMA) [6], HCl, H · Leu · OCH_3, and *N*-methylmorpholine in 3 ml pure, dry dimethylformamide were treated at 0° with 2 equivalents *N*(1)-hydroxybenzotriazole and 1.15 equiv. of dicyclohexylcarbodiimide. After the usual reaction and isolation procedures the product was obtained as a colourless solid from carbon tetrachloride; m.p. 78–80°; Rf 0.63 ethyl acetate/methanol 9:1.

H · Gla(OtBu)$_2$-Leu · OH, (D-L + L-L), **9.** 71 mg (0.12 mmol) **8** were dissolved in 1 ml dioxane and treated dropwise during 3 min with 3 ml 0.1N NaOH. After 15 min at 20° the mixture was neutralized with 1N HCl and lyophilized. The product was extracted from the residue with dry chloroform. Yield 47 mg (0.11 mmol; 92%) of colourless, solid **9.** TLC. gave two spots: Rf 0.57 (L-L) and 0.35 (D-L) BEW1. – Routine NMR. spectra agreed with the assigned structure.

H · Gla-Leu · OH, (D-L + L-L), **10.** The *t*-butyl groups were removed from **9** by dissolving the product (40 mg) in 0.5 ml conc. hydrochloric acid at 0°. The mixture was diluted with water after 20 min and lyophilized. A brown coloration was removed with charcoal and *Celite*. Colourless solid **10.** Yield 90%, m.p. above 85° (decomposition). Rf 0.11 BEW1, 0.63 I. Amino acid analysis after hydrolysis for 24 h at 110° in 2M KOH according to [12] gave Gla emerging at 20 min [1] and Leu at 145 min.

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