Synthesis, NMR Spectra and Function of Peptides with α-Methylserine attached to the RGD Sequence of Osteopontin

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Mickos, H., Sundberg, K. and Lüning, B., 1992. Synthesis, NMR Spectra and Function of Peptides with α-Methylserine attached to the RGD Sequence of Osteopontin. – Acta Chem. Scand. 46: 989–993.

The three-dimensional structure of the RGD-adhesion sequence has been studied previously by means of linear and cyclic peptides. These peptides show widely differing affinities to integrins, ascribed to a strong dependence on steric factors in the receptor recognition. Insertion of α-methylserine next to the RGD sequence in this investigation resulted in lower affinity for both stereoisomers, and several small changes in chemical shifts and coupling constants relative to the parent serine peptide GRGDSL.

The multifunctional protein fibronectin owes its cell adhesion properties to the RGD (Arg Gly Asp) sequence contained in an 11.5 KD *C*-terminal fragment.[1] This adhesion function has been demonstrated to occur in a number of proteins with suspected or actual adhesive properties.[2] Speculation has followed regarding the non-cross-reactivity between the different receptor proteins called integrins[3] and the various RGD-proteins. One idea is that the RGD sequence is a necessary but insufficient criterion for the reaction between the integrin and the RGD-protein, and that other sequences in the protein modify the reaction.[4] A variation of this idea is that the RGD sequence adopts somewhat differing three-dimensional structures in the various proteins, induced by the neighbouring amino acids.[4] A knowledge of the stereostructure of the RGD-group would therefore help clarify the nature of the interaction between the RGD-sequence and integrins. A structure, consisting of two nested β-bends, has been reported[5–7] in the peptide GRGDSP in H_2O between the amino acids G and D and between R and S, that is believed to be important for cell binding. It has also been inferred that the adhesion property is dependent on the presence of L- or D-serine next to aspartic acid in the RGD sequence.[8] L-Serine in this position gives better inhibition of the binding of cells to fibronectin-coated surfaces than does D-serine.

Cyclization of a peptide restricts its number of conformers and some cyclic peptides containing the RGD sequence show a ten-thousand-fold higher affinity for a vitronectin receptor than for a fibronectin receptor[8] although linear peptides do not. A pre-established firm conformation might be expected to favour binding provided the flexibility of the ligand is not a prerequisite for adaptation to the receptor site.

Another way of restricting the free formation of conformers is to introduce branching at various positions in the peptide backbone. A common example is α-aminoisobutyric acid (Aib),[9] naturally found in the peptaibols, which has been used to induce helix formation in peptides. An analogue to this amino acid is α-methylserine found in amicetin.[10] It has been synthesized[11–14] and used in peptide synthesis in solution.[15] Conformational studies of peptides containing this amino acid employing CD and IR spectroscopy have also been published.[16]

In order to avoid the *cis–trans* isomerism induced by proline in GRGDSP[5,6] we prefer to use the osteopontin-related sequence GRGDSL[17] as the basis for the present and future comparisons. The peptides inhibit equally well cell binding to fibronectin-coated surfaces.

Preliminary simulation experiments with energy minimization and molecular dynamics showed that substitution of the α-hydrogen for a methyl group in serine in GRGDSL would not severely interfere with the conformation postulated for the peptide. It was therefore considered of interest to study such peptides and we present here two peptides, GRGD(L-α-methylserine)L and GRGD(D-α-methylserine)L, their synthesis, adhesion inhibition potency, and NMR shifts and coupling constants.

We also present a suitable way of producing both enantiomers of α-methylserine, based on the Schöllkopf method with alkylation of a bis-lactim ether,[13] protected for use by the Boc-protocol during solid-phase synthesis. The synthesis was straightforward, and the intermediate products found to be sufficiently pure even without the recommended vacuum distillations.

The use of Boc-α-methyl-α-amino acids in an ordinary solid-phase synthesis is not without its problems. The amino group does not give a proper ninhydrin reaction owing to the inability to form an aldehyde of the amino acid. The colour developed is more like that of proline. Acylation with the activated α-alkylamino acid is slow and inefficient, as is also the acylation of the amino group of the α-alkylamino acid. Analysis with FAB MS and ^{1}H NMR

Table 1. Coupling conditions for GRGD(D-α-CH_3S)L and GRGD (L-α-CH_3-S)L.

Amino acid	Excess	No. of couplings	Coupling time /h	
			First	Second
GRGD(D-α-CH_3-S)L				
D-α-Me-Ser (OBz)	3	2	16	2
Asp (OBz)	6	2	18	2
Gly	6	2	3.5	2
Arg (Tos)	4	1	7.5	–
Gly	4	1	2	–
GRGD(L-α-CH_3-S)L				
D-α-Me-Ser (OBz)	3	2	18	2.5
Asp (OBz)	4	1	20	–
Gly	4	1	3	–
Arg (Tos)	4	1	17	–
Gly	4	1	3	–

spectroscopy of aliquots cleaved from the resin indicated that the coupling time should be extended and the remaining amino groups should be acetylated. Long coupling times demand the use of coupling reagents that are both reactive and stable enough for the long coupling period and are usually active esters or mixed anhydrides. We have successfully used HOBT esters to produce the peptides. The coupling times are listed in Table 1. According to FAB MS (*m/z* 617) and NMR spectroscopy the peptides were essentially pure.

After the completion of this work other useful coupling reagents suitable for α-alkylamino acids were published, giving potentially higher yields and shorter coupling times.[18]

^{1}H NMR chemical shifts and spin coupling constants of the peptides are collected in Table 2. The NH signals of glycine and arginine in GRGDS(D-α-CH_3)L had partially collapsed at 37 °C and pH 3.5, and therefore no coupling constants could be detected. The ^{13}C NMR chemical shifts are found in Table 3. The α-carbon signals adjacent to the free carboxy groups were broadened. Except for expected differences in the ^{1}H and ^{13}C chemical shifts of serine and the amino acids surrounding serine, the other amino acids are also affected, in comparison with GRGDSL.[19]

Relative to GRGDSL, the D-α-CH_3-Ser analogue shows a difference in ^{1}H NMR chemical shift of 0.11 ppm for the α-H and 0.17 ppm for the β-H in arginine, and the chemical shifts of the α-protons in glycine are separated. This indicates a change in the distribution of preferred conformations of the peptide. Furthermore, the collapse of the NH proton signals of glycine and arginine indicates a more favourable position of these protons for exchange with the solvent.

In the L-α-CH_3-Ser analogue the only notable change is in the coupling constant for the NH proton in arginine which decreases from 6.2 Hz in GRGDSL to 5.1 in the L-α-CH_3-Ser analogue.

The result of the binding experiments shows that the amino acid adjacent to the GRD sequence influences binding to the receptor. The mechanism of action underlying this effect is not clear. A clearly diminished inhibition of adhesion of both α-methylserine peptides compared with GRGDSL was observed (see Fig. 1). The NMR spectral observations may also account for the lower affinity of these peptides for the receptor.

Studies of these peptides by NMR and simulation methods as well as further syntheses of sterically hindered linear peptides containing the GRD-sequence will be published separately.

Experimental

Throughout this paper the one letter code for amino acids is used. L-Serine and L-α-CH_3-serine both have the *S*-configuration. Silica gel 60F-254 (Merck, Darmstadt, Germany) was used for TLC and spots were visualised with UV, 8 % H_2SO_4 and/or ninhydrin. For column chromatography, silica gel (Matrex Silica Si, 60 Å, 35–70 mm, Amicon) was used.

*(*R*)-O-Benzyl-α-methylserine methyl ester* was synthesized from the Schöllkopf–Hartwig reagent (*bis*-lactim ether of *cyclo*-L-Val-DL-Ala) as previously described[13] but including a separation on a silica column (chloroform–methanol–acetic acid–water 30:9:3:2 by volume) prior to vacuum distillation of the alkylated bis-lactim ether. The synthesis of (*R*)-*O*-benzyl-α-methylserine methyl ester resulted in an acceptably high enantiomeric purity, b.p. 94 °C/0.25 mmHg; $[\alpha]_D^{20} = +4.2$ ($c = 0.05$ in $CHCl_3$). ^{1}H NMR ($CDCl_3$): δ 1.25 (s, CH_3), 2.20 (s, NH_2), 3.4, 3.7 (AB system, CH_2), 3.65 (s, OCH_3), 4.55 (s, CH_2Ph). Except for removal of some small solvent peaks the distillation did not cause any changes in the ^{1}H NMR spectrum.

*(*R*)-Boc-O-Benzyl-α-methylserine.* To a solution of 0.92 g (4.12 mmol) (*R*)-*O*-benzyl-α-methylserine methyl ester and 12 ml dioxane–water 2:1 was added 1 M NaOH dropwise to pH 12. The solution was stirred at 0 °C, and when the solution became clear 1.2 g (5.50 mmol) di-*tert*-butyl dicarbonate was added, and the mixture was stirred for another 24 h at room temperature. After removal of dioxane *in vacuo* the residue was chilled in an ice bath, ethyl acetate was added, and the solution was acidified with saturated $NaHSO_4$ to pH = 2–3. The aqueous layer was extracted three times with ethyl acetate, and the combined ethyl acetate layers were washed once with water and dried over Na_2SO_4. After evaporation the product was obtained as a viscous oil. The reactions were monitored to completion by TLC (ethyl acetate–methanol–acetic acid–water 30:9:3:2 R_f 0.62). Yield: 1.20 g (94 %) $[\alpha]_D^{20} = +4.4°$ ($c = 0.05$), ^{1}H NMR ($CDCl_3$): δ 1.38 (d, CH_3), 1.55 (s, *tert*-butyl CH_3), 3.65, 3.80 (AB system, CH_2), 5.41 (s, CH_2Ph), 7.3 (Ar-H).

Table 2. ^{1}H NMR spectral shifts of the hexapeptides GRGD(L-α-CH_3-S)L,[a] GRGD(D-α-CH_3-S)L[a] and GRGDSL.[a]

Amino acid	N-H	α-H (α-CH_3)	β-H[b]	γ-H	δ-H
GRGD(L-α-CH_3-S)L					
Gly	–	3.91	–	–	–
Arg[c]	8.68 (5.1)	4.33	1.87 (5.7)	1.68 (–)	3.22 (6)
Gly	8.58 (5.2)	3.95	–	–	–
Asp	8.11 (7.0)	4.65	β1 2.88 (6.7) β2 2.81 (8.4) (ββ 13)	–	–
Ser-α-CH_3	7.95	(1.47)	β1 3.97 β2 3.78 (ββ 11.7)	–	–
Leu	7.70 (7.3)	4.32	1.60 (7)	1.53 (–)	δ1 0.88 (5.8) δ2 0.85 (5.4)
GRGD(D-α-CH_3-S)L[a]					
Gly	–	3.91	–	–	–
Arg[c]	8.67 (–)	4.44	1.66 (–)	1.67 (–)	3.22 (6.7)
Gly	8.57 (–)	α1 3.99 α2 3.89 (αα 16)	–	–	–
Asp	8.09 (6.8)	4.74	β1 2.95 (6) β2 2.85 (5) (ββ 15)	–	–
Ser-α-CH_3	7.95	(1.44)	β1 3.83 β2 3.76 (ββ 11.7)	–	–
Leu	7.63 (7.7)	4.38	β1 1.85 (–) β2 1.70 (–) (ββ –)	1.68 (–)	δ1 0.90 (6.2) δ2 0.87 (5.8)
GRGDSL[a]					
Gly	–	3.88	–	–	–
Arg[c]	8.68 (6.2)	4.34	β1 1.83 (7)	1.67 (–)	3.22 (6.7)
Gly	8.61 (6.0)	3.94	–	–	–
Asp	8.15 (7.3)	4.72	β1 2.84 (6) β2 2.77 (7) (ββ 16.5)	–	–
Ser	8.21 (7.3)	4.47	3.87 (5.5)	–	–
Leu	7.99 (7.8)	4.28	1.63 (8.3)	1.61 (–)	δ1 0.91 (5.9) δ2 0.87 (5.9)

[a]The values in parentheses are spin–spin coupling constants in Hz. (–) = not detectable. [b]Coupling constants refer to coupling with α-H. [c]The shift values for the guanidinium protons are 7.22 and 6.65. [d]The shift values for the guanidinium protons are 7.30 and 6.66.

(S)-Boc-O-benzyl-α-methylserine was synthesized in the same way starting from the enantiomeric Schöllkopf–Hartwig reagent (*bis*-lactim ether of *cyclo*-D-Val-DL-Ala). Yield: 0.71 g (77%), $[\alpha]_D^{20} = -4.4°$ ($c = 0.05$). Anal. $C_{16}H_{23}NO_5$. ^{1}H NMR ($CDCl_3$): δ 1.38 (d, CH_3), 1.55 (s, *tert*-butyl CH_3), 3.65, 3.80 (AB system, CH_2), 4.51 (s, CH_2Ph), 7.3 (Ar-H).

Peptide synthesis. The peptides were synthesized manually using Merrifield's solid-phase technique with *tert*-butyloxycarbonyl (Boc) protected amino acids.[20] The first Boc-amino acid was covalently linked to a Biorad® chloromethylated polystyrene resin (1.25 meq g^{-1} substitution) according to the KF method[21] in DMF. Boc-aspartic acid was protected as the benzyl ester, and Boc-arginine was NG-tosylated. Coupling of the Boc-amino acids to the *N*-terminus of the growing peptide was performed for 1-hydroxy-benzotriazole esters, with CH_2Cl_2–DMF 10:2 as the coupling solvent. The acylation was monitored to completion by the ninhydrin test[22] except for the coupling of aspar-

Table 3. ^{13}C NMR spectral shifts for GRGD(L-α-CH_3-S)L, GRGD (D-α-CH_3-S)L and GRGDSL.

Amino acid	α-C	β-C	γ-C	δ-C
GRGD(L-α-CH_3-S)L				
Gly	40.92	–	–	–
Arg	54.44	28.38	24.71	40.92
Gly	43.06	–	–	–
Asp	51.14	27.03	–	–
Ser	61.14	63.73 α-CH_3 20.90	–	–
Leu	54.07	40.38	24.60	δ1 22.79 δ2 20.90
GRGD(D-α-CH_3-S)L				
Gly	41.31	–	–	–
Arg	54.58	28.93	25.15	41.39
Gly	43.44	–	–	–
Asp	51.12	36.40	–	–
Ser	61.86	66.04 α-CH_3 19.62	–	–
Leu	54.42	40.31	25.05	δ1 23.15 δ2 21.29
GRGDSL				
Gly	41.25	–	–	–
Arg	54.77	28.81	25.12	41.38
Gly	43.43	–	–	–
Asp	51.62	37.82	–	–
Ser	56.35	61.93	–	–
Leu	53.82	40.90	25.35	δ1 23.16 δ2 21.61

tic acid where the reaction was monitored by NMR and FAB MS after cleavage of an aliquot from the resin. Arginine was coupled three times in neat DMF. The completed peptides were deprotected and cleaved from the resin with Low-high HF[23] and purified on a Sephadex® G15-column in 0.1 M ammonium hydrogen carbonate solution. Further purification and analysis was achieved by reversed-phase liquid chromatography. A Pharmacia FPLC Gradient Programmer equipped with a Waters 10 μ C18-column in a Z-module and a water–acetonitrile 0–100 % gradient were used. TFA (0.05 %) was added to both components of the gradient. Detection was with UV at 214 nm. Both peptides appeared as single peaks. The qualitative purity was checked by FAB MS. This was carried out on a JEOL double-focusing mass spectrometer with a FAB ion source, and a JMA DA6000 data system. An aliquot of the sample was dissolved in 30% aqeous acetic acid and evaporated on the FAB-target, then a 1:1 (vol/vol) mixture of 70 % aqueous formic acid and glycerol was added as the matrix, and the sample was bombarded with 10 keV Xenon atoms.

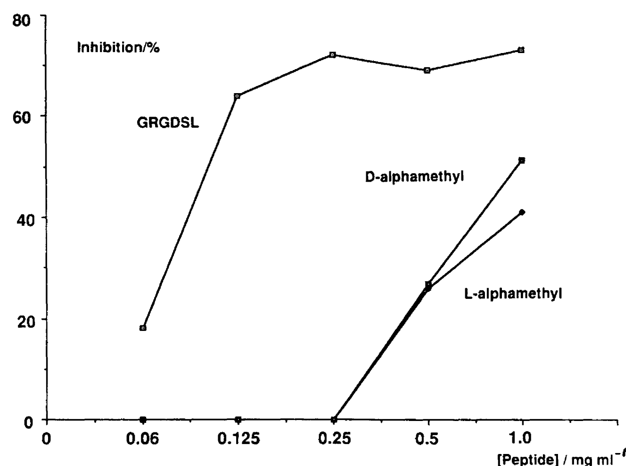

Fig. 1. Inhibition of cell attachment to BSP by the peptides GRGDSL, GRGD(L-α-CH_3-S)L and GRGD(D-α-CH_3-S)L. Inhibition is given as the percentage binding relative to the total binding in the absence of peptide.

Spectra of positive ions were recorded in the range *m/z* 50–2560 with a resolution of 3000. Both peptides gave a molecular ion *m/z* 617. Minor peaks at *m/z* 517 and 561 corresponding to loss of serine or glycine could be detected in the crude material. The yield of crude product was 76 % for GRGD(L-α-CH_3-S)L and 66 % for GRGD(D-α-CH_3-S)L.

NMR spectral experiments. Samples for NMR spectroscopy were prepared at 30–60 mM peptide concentration, at pH 3.5 (0.5) in either 99.9 % ^2H_2O or 90 % ^1H_2O–10 % ^2H_2O. (For ^2H_2O solutions, the 'pH' refers to the pH-meter reading without correction for isotope effect.[24]) The NMR spectra were recorded on a JEOL GSX 270 and a JEOL GSX 400 spectrometer. Assignments were made using two-dimensional COSY,[25] two-dimensional double quantum filter COSY,[26] C–H correlated spectroscopy[27] and *J*-resolved spectroscopy[28] at 37 °C. Chemical shifts are given relative to trimethylsilylpropionic acid (TSP; $\delta_H = 0.00$) for ^1H spectra and dioxane ($\delta_C = 67.4$) for ^{13}C spectra. Prior to the NMR measurements in ^2H_2O, the samples were lyophilized three times in ^2H_2O.

Cell binding assays were performed as reported earlier[19] with BSP[29,30] as the coating protein.

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Received December 23, 1991.