



HAL
open science

Artificial RGD Receptor Molecules

Thomas Schrader, Thomas Gersthagen, Carsten Schmuck

► **To cite this version:**

Thomas Schrader, Thomas Gersthagen, Carsten Schmuck. Artificial RGD Receptor Molecules. *Supramolecular Chemistry*, 2010, 22 (11-12), pp.853-861. 10.1080/10610278.2010.514613 . hal-00640229

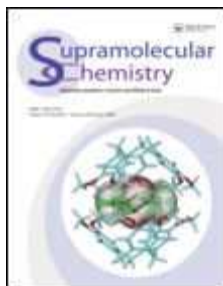
HAL Id: hal-00640229

<https://hal.science/hal-00640229>

Submitted on 11 Nov 2011

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Artificial RGD Receptor Molecules

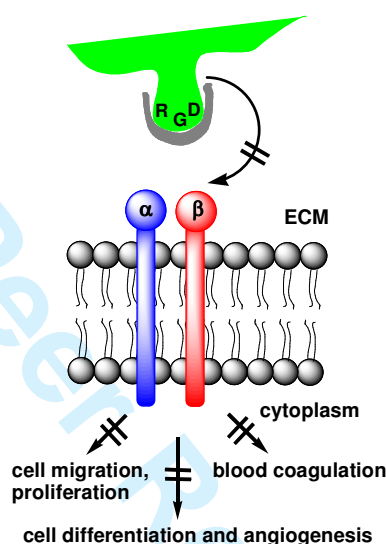
Journal:	<i>Supramolecular Chemistry</i>
Manuscript ID:	GSCH-2010-0130.R1
Manuscript Type:	Special Issue Paper
Date Submitted by the Author:	19-Jul-2010
Complete List of Authors:	Schrader, Thomas; Universitätsstr. 5 Gersthagen, Thomas; University of Duisburg-Essen, Chemistry Schmuck, Carsten; University of Duisburg-Essen, Chemistry
Keywords:	RGD peptide, integrins, molecular recognition, artificial receptors, amino acids

SCHOLARONE™
Manuscripts

Artificial RGD Receptor Molecules

Thomas Gersthagen, Carsten Schmuck, Thomas Schrader*

TOC-Graphic.



Abstract. Integrins play a pivotal role in cell-cell adhesion, signaling and apoptosis. Many extracellular proteins use the RGD sequence (arginine-glycine-aspartate) as a key to dock onto and unlock their respective binding partners at the cell membrane ($\alpha_V\beta_3$ -, $\alpha_{IIb}\beta_3$ - and the $\alpha_5\beta_1$ -integrin). Here, the RGD signal is transduced into the cytoplasm and triggers a variety of biological events like blood coagulation, cell matrix binding, cell differentiation and angiogenesis. A misfunction of this recognition system causes severe diseases, rendering the RGD recognition system an attractive drug target. Inhibition of RGD-integrin interactions can be reached in two different ways, by blocking integrins with RGD-mimetics or by capping RGD-containing proteins by artificial RGD receptors. This review provides an overview over the very young history of artificial RGD receptor development, beginning with early research in arginine recognition, over the discovery of the first primitive RGD-receptor until the current state of research and future prospects.

Key words: RGD peptide, integrins, molecular recognition, artificial receptors, amino acids

Introduction.

Integrins represent the major class of biological receptors which mediate cell-cell and cell-matrix interactions.[1] They are heterodimeric glycoproteins, located inside the **cell membrane** and present the head portions of both subunits to the extracellular space. Cell recognition takes place when specific extracellular proteins dock onto the cell membrane and recognize a specific integrin type. This recognition process hinges on a small key peptide sequence, presented on a

1
2
3 solvent-exposed loop of all approaching extracellular proteins. It contains 3 consecutive critical
4 amino acids: arginine-glycine-aspartate, and is therefore generally coined RGD motif. This
5 tripeptide sequence literally serves as a key which can be inserted into the integrin lock and open
6 it.
7

8 In 1984 it was discovered, that the RGD sequence mediates cell adhesion by Fibronectin; [2]
9 subsequent findings quickly demonstrated that the RGD sequence is a general powerful
10 recognition motif utilized by many other proteins like vitronectin, laminin or the von willebrand
11 factor, which all play a pivotal role in cell adhesion biology. Fundamental biological processes
12 such as cell differentiation, signaling, apoptosis and others depend on the interplay between
13 RGD-containing proteins and their related integrin-receptors.[3,4,5] The integrin family is highly
14 variable because its members differ in their composition through the combination of eight
15 different α - and β -subunits. A single integrin is able to discriminate between the RGD-sequences
16 of different proteins by two main mechanisms; it recognizes not only the specific chemical nature
17 of these three amino acids, but also the specific conformation of each of these individual RGD-
18 loops. The relative orientation of the alkylguanidinium and the alkylcarboxylate side chains can
19 adopt all transitions from a linear, fully extended conformation up to a tightly folded cyclic loop.
20 In some cases, the integrin also interacts with the fourth amino acid residue, e. g., in the RGDS
21 sequence typical for fibronectin.
22

23
24 Dysfunction in this critical recognition system can cause pathogenic effects like tumor-induced
25 angiogenesis, osteoporosis, myocardial infarction or thrombosis.[6,7,8] The resulting severe
26 diseases reveal the medicinal and pharmaceutical importance of the RGD recognition system and
27 render it an attractive drug target. A precise control over pathologic RGD recognition events by
28 externally added small molecules would not only help to better understand this important natural
29 signalling pathway, but also have immediate therapeutic relevance. In principle, inhibition of the
30 RGD- integrin interaction can be reached in two different ways. One has been pursued
31 intensively by pharmaceutical research; the other – more difficult one - has been totally
32 neglected. A great deal of synthetic, computational and biological effort has been devoted to
33 develop so-called *RGD Mimetics* which imitate polar groups and their relative orientations in a
34 specific RGD-sequence, in order to compete with the native ligands of such integrins (Figure 1a
35 and 1b).
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

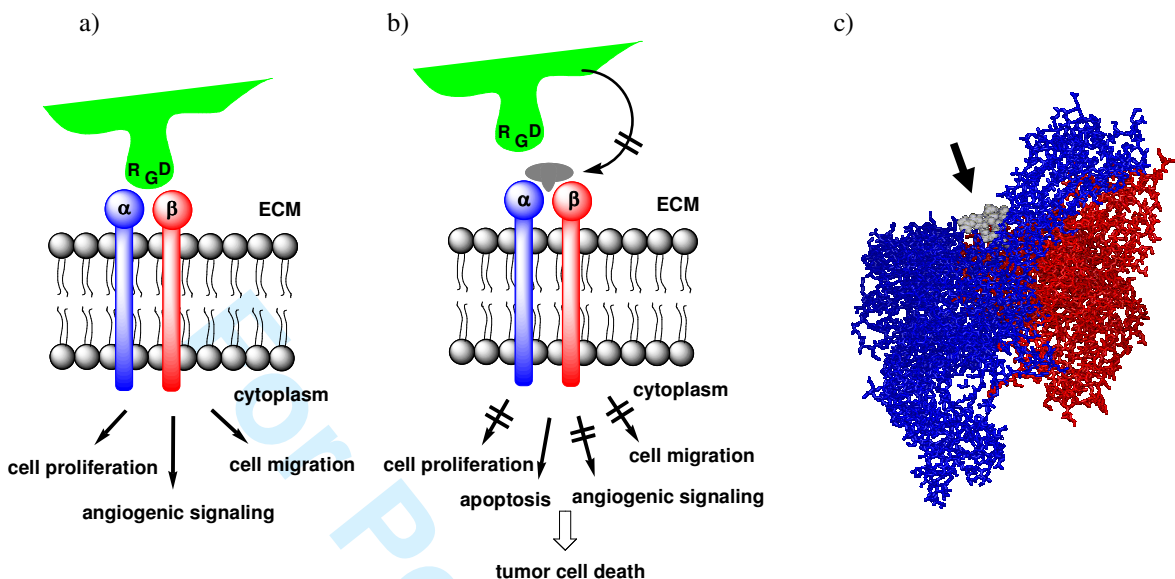


Figure 1. a) Schematic of the integrin/RGD “lock and key” principle: An RGD-containing protein (key) docks onto the membrane-spanning α,β -integrin dimer (lock) and induces a biological effect. b) Conventional interference principle: the ligand binding site on the Integrin is blocked by an RGD mimetic (grey); c) Crystal structure of the cRGDf[NMe]V peptide (grey) in its complex with the α,β -integrin dimer $\alpha V\beta 3$ (blue/red).

Numerous academic and industrial groups have developed peptide-derived or non-peptidic RGD mimetics, most of which showed some biological activity. Most notably, an elegant approach was introduced by *Kessler et al.*, who synthesized a series of RGD-containing cyclopeptides with 5- and 6-membered rings containing single D-amino acid residues. In extended NMR-spectroscopic studies the structural principles governing the formation of preferred fixed conformations were unraveled and used for a conformational design of integrin-specific RGD mimetics, which showed promising *in vitro* biological effects. During the course of these studies, a whole set of cyclic hexapeptides and pentapeptides was synthesized and thermodynamic equilibria as well as dynamics of their three-dimensional structures in solution were analyzed by 2D-NMR techniques.

Finally, systematic tuning of conformational restrictions accompanied by MD simulations and subsequent bioassays culminated in the preparation of the strong lead compound *cyclo*-RGDf[NMe]V (Figure 2), a powerful integrin antagonist, which effectively inhibits tumor-associated angiogenesis.[9,10,11] This very small cyclopeptide, which is currently undergoing clinical trials, imitates the RGD-loop in Vitronectin. In 2002 *Arnaout et al.* gained a crystal structure of this cyclopeptide in its complex with integrin $\alpha V\beta 3$, the only crystal structure of an RGD-integrin complex which is available today. This structure reveals that the peptide is inserted in a cleft between the so called β -propeller from the αV -unit and βA domains from the $\beta 3$ -unit on the integrin head. As expected, almost all peptide-protein contacts are established by the RGD sequence (Figure 1c).[12]

Figure 2 displays some related RGD mimetics, some of which have already been approved as drugs. Eptifibatide, a somewhat more flexible cyclohexapeptide with an internal disulfide bond, inhibits integrin $\alpha_{IIb}\beta_3$. It was developed entirely by pharmaceutical industry, and is used against

myocardial infarction or in heart surgery. Other potent drugs are drastically simplified and often rigidified RGD mimetics which contain only one stereogenic center and replace guanidinium ions by (hydroxy)amidines (Figure 2).

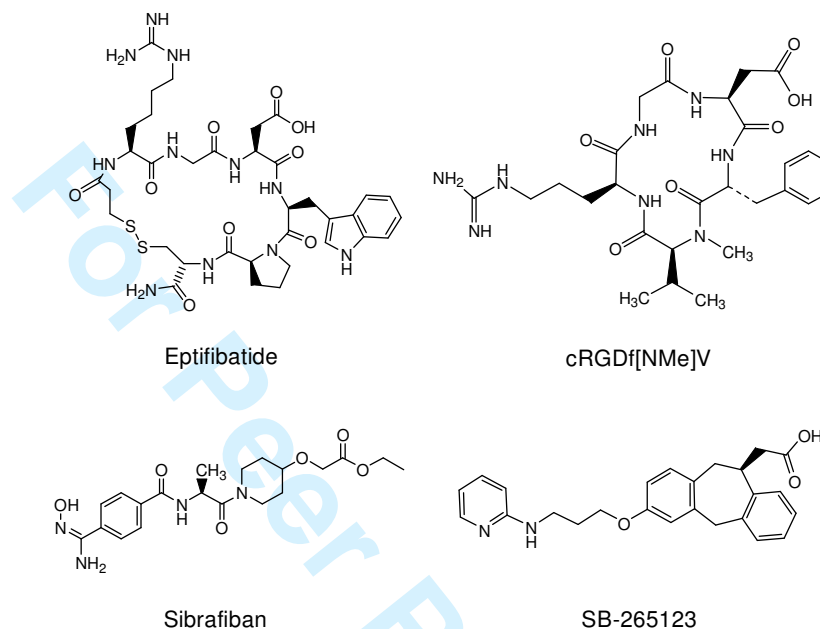


Figure 2. Synthetic RGD-mimetics: cRGDf[NMe]V (Cilengitide, Merck KGaA, specific for $\alpha V\beta 3$ -Integrin), Eptifibatide (Integrilin[®], Millennium Pharmaceuticals/GlaxoSmithKline/ Schering-Plough, specific for $\alpha_{IIb}\beta_3$ -Integrin); Sibrafiban (Hoffmann-LaRoche, specific for $\alpha_{IIb}\beta_3$ -Integrin); SB-265123 (GlaxoSmithKline, specific for $\alpha V\beta 3$ -Integrin).

Artificial RGD Receptor Molecules.

A completely different approach to block pathologic RGD-Integrin interactions is to construct integrin mimetics which recognize RGD-containing proteins. In principle this simplifies the task to developing a small artificial host molecule which can serve as a competitive “lock” for the RGD-sequence in native proteins. Due to the encapsulated RGD-“key” the whole extracellular protein is not able any more to bind to its respective target integrin (Figure 3a). However, since the RGD-sequence exists in a variety of different conformations, depending on the parent protein, it is mandatory to provide synthetic hosts which can distinguish between them (Figure 3 b).

Some years ago, the *Schrader* and *Schmuck* groups embarked on a program to pursue this alternative approach towards conformation-specific RGD host molecules. The starting point was marked by the discovery that *m*-xylylene bisphosphonates **1** bind guanidinium cations in a chelate-type manner reminiscent to the natural diaspertate binding site found in the crystal structure of the cRGDf[NMe]V/ $\alpha V\beta 3$ complex. In both cases a bidentate salt bridge with a network of ion-pair reinforced hydrogen bonds firmly embraces arginine’s **guanidinium** cation and holds it in place (Figure 3 c,d).[13]

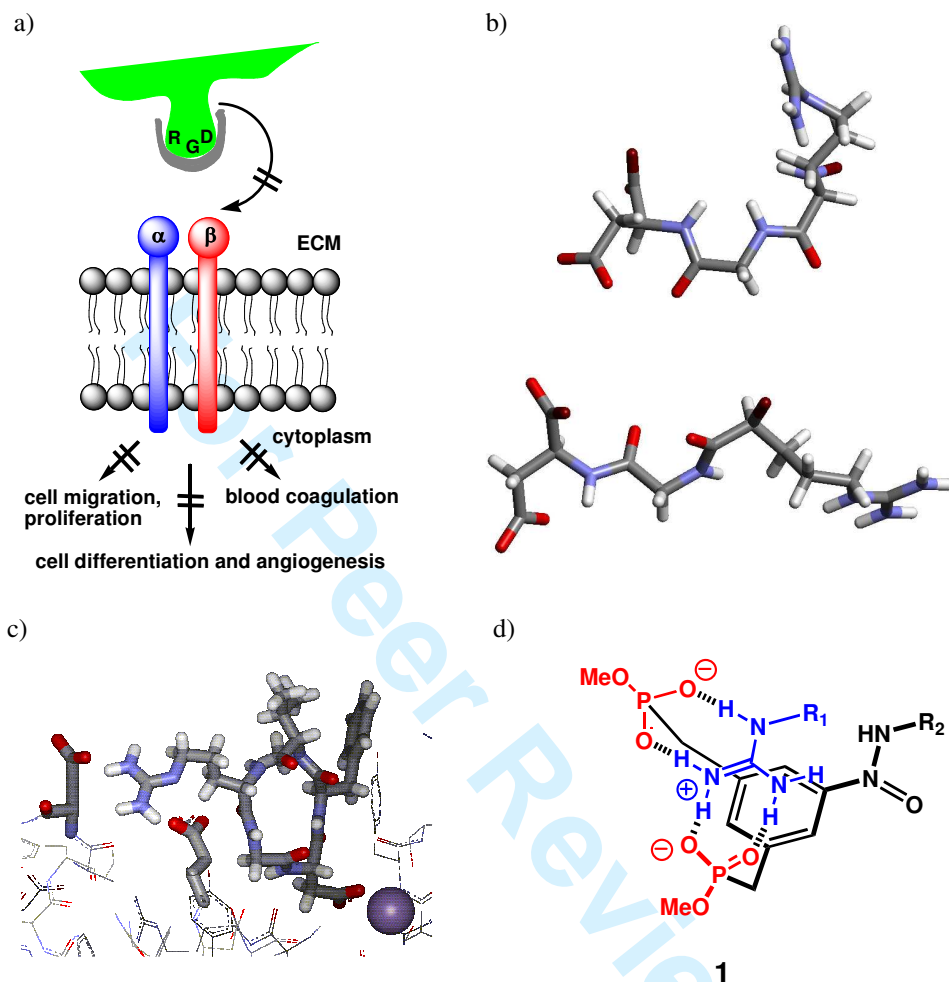


Figure 3. a) Alternative concept for RGD/integrin interruption: RGD-capping by an artificial RGD receptor molecule (grey) prevents docking onto the membrane-bound integrin (blue/red); b) Folded and extended conformation found in the RGD-loops of Fibronectin and Vitronectin (from crystal structures); c) chelate-type binding motif from the crystal structure of the complex between cRGDf[NMe]V and α V β 3; d) chelate-type binding motif in a *m*-xylylene bisphosphonate/arginine complex.

An early screening experiment for RGD-binders was carried out by *Ruoslahti et al.* In 1999 a patent was filed which announced the discovery that cyclic peptides with the consensus sequence (W/P)DD(G/L)(W/L)(W/L/M) bind to fibronectin and related RGD-containing proteins. The best candidate was cyclo-CWDDGWLC **2**, with an internal disulfide bond, which is depicted in Figure 4 a. It was isolated from a library of short cyclic peptides and peptidomimetics in the course of specific assays for RGD-binding activity. The peptides were presented in form of a phage library which was used to isolate minimal receptor sequences that bind to fibronectin in affinity panning. Thus, the cyclic octapeptide c-CWDDGWLC was identified as the most potent inhibitor for RGD dependent cell attachment to fibronectin and vitronectin. Fibronectin itself tightly bound to a c-CWDDGWLC affinity column and could subsequently be reeluted with an RGD-containing peptide. Interestingly, a specific region within the β -subunit of RGD-binding integrins had previously been shown by site-directed mutagenesis to be involved in ligand binding [14], which includes a the short peptide stretch KDDLW – quite similar to the above-

described sequence. Short synthetic peptides corresponding to this integrin region bind to the RGD containing protein, but their affinities were markedly reduced if both aspartate residues are mutated to alanines. In summary, the combination of phage display technology, rational design and mutagenesis has led to structural integrin mimetics which can significantly reduce the cell attachment of an RGD-containing protein [15,16]. However, these are still sensitive to proteolytic degradation.

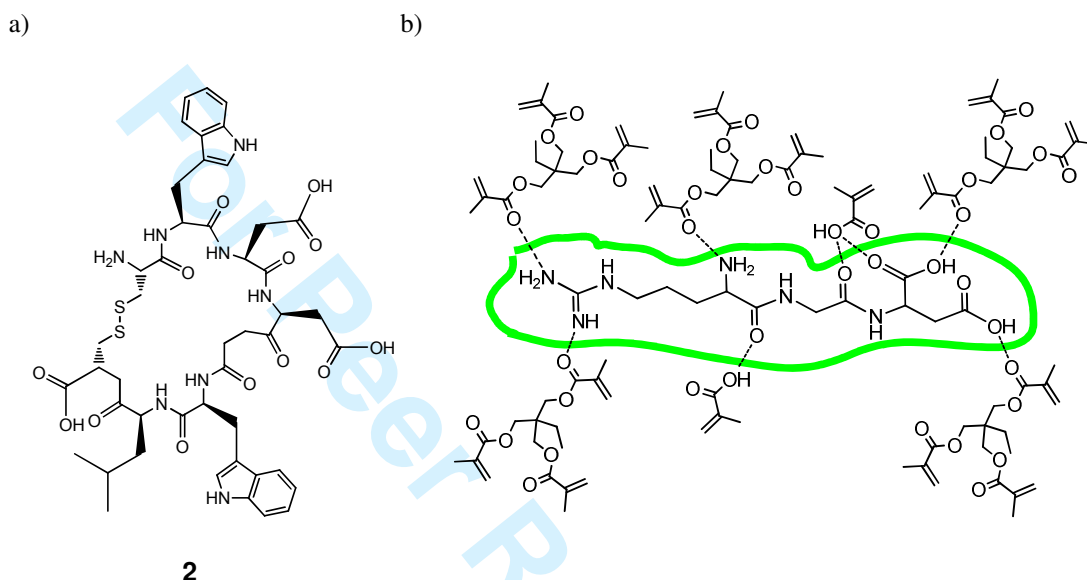


Figure 4. a) Lead candidate cyclo-CWDDGWLC, identified from phage-display assays as a general RGD-protein binder b) Attempted Molecular Imprinting for the free RGD template peptide: proposed noncovalent interactions between functional monomer, crosslinker and template.

In 2009, *Liakopoulou-Kyriakides* et al. tried to overcome this drawback by employing the Molecular Imprinting technology (Figure 4 b) in order to generate a new chromatography stationary phase for the separation of RGD-peptides from mixtures. They used the free RGD peptide as template molecule, and prepared molecularly imprinted polymers (MIPs) from only two functional monomers, i. e., methacrylic acid (MAA) and acrylamide (AA). Three different crosslinking monomers were tried, with trimethylpropane trimethacrylate producing the best results in terms of RGD-selectivity. MAA was later postulated to form interactions with arginine, while AA's amide is both a moderate hydrogen bond donor and acceptor. To establish RGD selectivity, four other peptides were used in rebinding experiments, namely KGD and three other completely different peptides. Selectivity factor values ranged from 1.27 up to 1.98, net rebinding values for RGD and KGD were similar (~ 8-10%), while those for other peptides remained very small (2-4 %). These data indicate low affinity and only moderate selectivity for the template molecule. In addition, the free RGD peptide was examined in methanol solution, where K_d values were in the high micromolar range.[17]

Surprisingly, to the best of our knowledge, apart from our own project, these are the only artificial RGD receptor molecules published to date. We tentatively explain this with a lack of powerful concepts for the generation of water-soluble host molecules for zwitterions in physiological solution. In the aqueous medium, hydrogen bonds must be reinforced with electrostatic interactions and complemented by hydrophobic forces to achieve high affinities. Zwitterions are prone to undergo intramolecular as well as intermolecular self-association, which

often renders the desired host-guest complexes insoluble in buffered aqueous solution – especially in the presence of high salt loads.

For isolated arginine and aspartate as well as for model peptides containing only anionic or cationic residues, some powerful receptor molecules have been developed: *Hamilton et al.* created a rigid concave scaffold **3** with two preorganized guanidinium moieties (Figure 5 a) which specifically bind two aspartate carboxylates in the *i*+3-relation of an α -helix. Small aspartate-containing model peptides are bound with millimolar affinities, albeit only in a 10% water-methanol mixture.[18]

Bell et al. synthesized crescent-shape molecule **4** with multiple annulated pyridine and dihydrobenzene rings, which they coined “the arginine cork” because of its high preference for arginine (Figure 5 b). It prefers diarginine as its guest, and displays K_d values in the low micromolar range for molecular recognition in water. The architectural concept is based on a remarkably rigid host skeleton which leads to a large degree of preorganization of hydrogen bond acceptors, reinforced by two strategically placed carboxylates for additional ionic interactions between the dianionic host and the dicationic guest [19]. Problematic features are limited solubility and difficult synthetic access.

Later the *Dougherty group* constructed a much larger, highly charged cyclophane receptor (**5**), which is selective for arginine and lysine dipeptides (almost micromolar affinities in aqueous solution, Figure 5 c). The clue is a combination of cation- π interactions with electron-rich receptor π -faces and multiple salt bridges, complemented by total inclusion of one amino acid inside the receptor cavity, leading to solvent exclusion; the second basic residue remains solvent-exposed.[20]

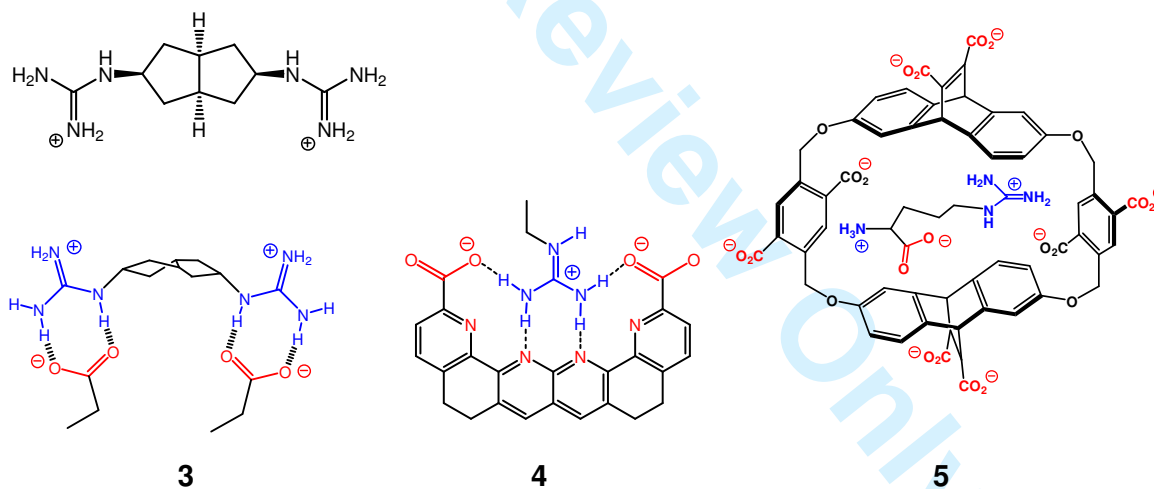


Figure 5. a) Preorganized host molecules for aspartate and arginine: Hamilton’s concave *i*+3-diaspartate binder **3**; b) Bell’s planar “arginine cork” **4**; c) Dougherty’s arginine-cavitand **5** with putative arginine inclusion.

In 1997 *Schrader* discovered that the *m*-xylylene bisphosphonate dianion is a powerful guanidinium binder in dipolar aprotic solution (DMSO) and forms a similar arrangement as the well-known Tat-TAR complex between the AIDS virus’s RNA fragment (two phosphate groups) and a regulatory protein (arginine, Figure 3 d).[14] This recognition motif prefers delocalized, soft guanidinium cations over hard ammonium ions and especially arginine over lysine residues. Systematic investigations were carried out to study the specific interplay of noncovalent attractive forces with modified model compounds. To this end, additional substituents with

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

varying electronic and steric properties were introduced in the 5-position of the central benzene ring (see selection in Figure 6).

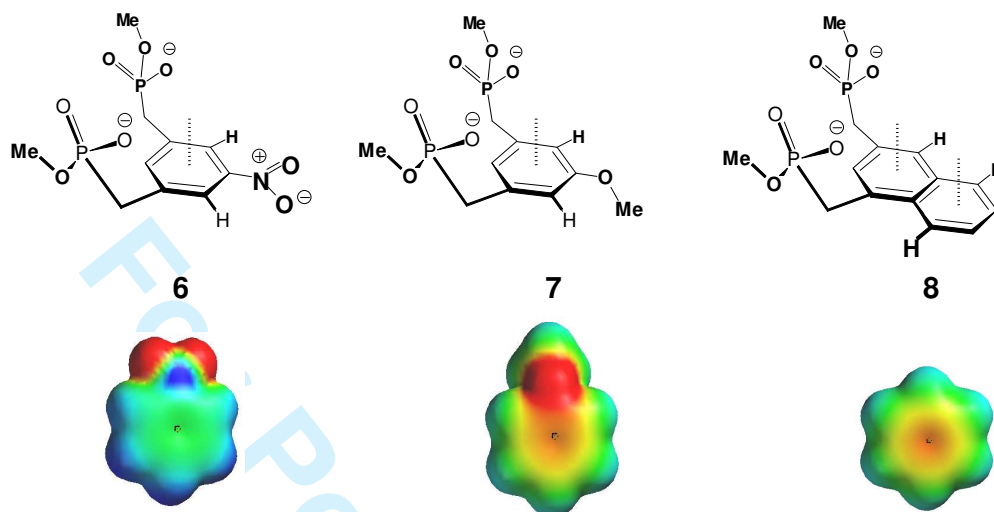


Figure 6. *m*-Xylylene bisphosphonate host molecules **6-8** with additional substituents in the 5-position - probing the π -cation interaction. Below: Electrostatic Surface Potentials (ESP) of nitrobenzene, anisole and benzene (AM1-calculations).

It was assumed that three kinds of noncovalent interactions play a key role in guanidinium recognition: electrostatic and hydrogen bond interactions with both phosphonate moieties and cation- π interactions with the receptor's central aromatic ring, which was placed in direct van-der-Waals contact by force-field calculations. Direct proof of cation- π interactions was provided with small electronic-withdrawing and donating groups in 5-position of this central benzene ring; these substituents could not form any additional hydrogen bonds to the guanidinium cation because they were geometrically locked in the aromatic plane of the receptor molecule. Intriguingly, all electronic-withdrawing groups lowered and all donating groups raised relative affinities towards methylguanidine hydrochloride, reflecting weakened or strengthened interactions between the guanidinium cation and the aromatic system. A close correlation was found between free binding enthalpies and the electrostatic surface potential of the respective central rings. Dissociation constants could also be lowered by increasing the π -face of the receptor, e. g., by exchanging the central benzene for a naphthalene ring (as in **8**).

Even better than all the preceding modifications, however, was the introduction of a third phosphonate anion at a certain distance from the benzylic bisphosphonate (**9**). Monte Carlo simulations and NOESY experiments confirmed the postulated additional hydrogen bond contacts to all five guanidinium NH protons of the model guanidinium salt, which was now surrounded by all three phosphonate arms (Figure 7).

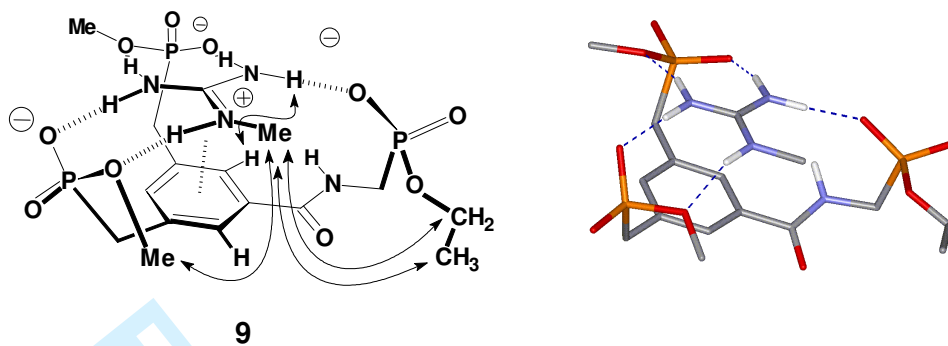


Figure 7. Summarized reciprocal NOE contacts measured between the new arginine host molecule **9** and methylguanidinium cation (500 MHz, 1:1 complex in CD₃OD). Right: Monte Carlo simulation (MacroModel 7.0, water/GBA, Amber*, 1000 steps).

Strong binding and a pronounced selectivity for arginine over lysine and histidine derivatives was now observed in methanol, with K_d values in the low micromolar range ($\sim 30 \mu\text{M}$). Monte Carlo simulations in water showed that the long triphosphonate allows the arginine side chain to form a thermodynamically favoured extended conformation while an electrostatic interaction with the α -ammonium group still occurs. Unfortunately, affinities in aqueous solution remained small.[21]

From the new triphosphonate unit we subsequently developed the first synthetic RGD receptor molecule (Figure 8). Alkylation of the deprotonated benzamide-NH group with phthaloyl-protected *m*-aminobenzyl bromide smoothly yielded **10** after hydrazinolysis and phosphonate ester cleavage with LiBr. Now a simple aspartate binding site is present, while at the same time the rigid benzyl spacer prevents intramolecular dimerization, and the ammonium functionality is a bad guest for the triphosphonate and should thus circumvent an unwanted intermolecular dimerization.

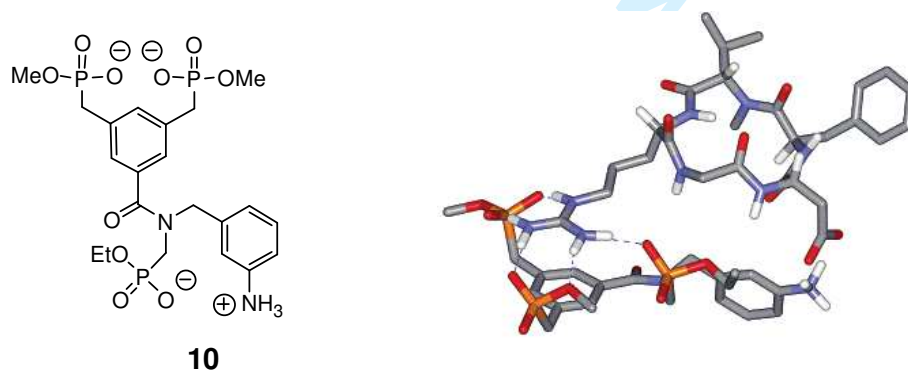


Figure 8. Primitive synthetic RGD-receptor molecule **10** with restricted rotational freedom. Left: Lewis structure; Right: molecular mechanics calculation of its 1:1 complex with cyclo(RGDfV) (MacroModel 7.0, water (GBA), Amber*, 1000 steps).

NMR titrations of the new RGD-receptor molecule with the free RGD peptide as well as the integrin antagonist cRGDfV became now possible in water, and furnished K_d values in the high micromolar regime. Distinct NMR signal shifts were observed for the aspartate CH₂ protons, testifying to its additional interaction with the RGD host; complex stoichiometries were

1
2
3 determined at a precise 1:1 ratio. This showed that the concept of combining an arginine binder
4 with an aspartate binder was valid.[22]
5
6

7 Inspired by this work the *Gilson group* chose our simple ditopic RGD receptor molecules and
8 their complexes with RGD peptides as a biologically relevant model case to study the accuracy of
9 their new program for the calculation of absolute Gibbs energies. Their results were in good
10 agreement with the experimental values that had been independently determined in the *Schrader*
11 *group*. For the interaction between **10** and cRGDfV, e. g., the experimental data showed a ΔG
12 value of -3.91 kcal/mol, which was reproduced *in silico* with -3.11 kcal/mol. A most remarkable
13 feature of the new program is its ability to include solvent and entropic effects in the M2
14 modeling method. In the course of these calculations, the *Gilson group* noticed, that in cRGDfV-
15 complexation by **10**, electrostatic interactions play a much smaller role than for the linear RGD
16 peptide, while van-der-Waals and nonpolar terms became dominating. This is in line with
17 intuition, since cRGDfV has two charged groups less than free RGD, but carries two additional
18 nonpolar groups. The M2 calculations also suggested that much of the receptor did not interact
19 directly with the RGD sequence. Finally new receptor molecules were designed *in silico* and
20 predicted to produce Gibbs energies of up to 5 kcal/mol higher than **10**. One of the calculated
21 structures reached predicted nanomolar affinities with the cyclic RGD model peptide, rivalling
22 protein-ligand interactions. The underlying design concept either strived to extend the
23 hydrophobic contact area between host and guest ligand or to reduce entropic or electrostatic
24 desolvation penalties. The only problem with these optimized virtual RGD receptor molecules
25 was that they were too difficult to synthesize [23].
26
27
28
29

30 In an independent investigation, the influence of the spacer unit was studied with simple model
31 systems. The benzyl group in **10** was replaced by small peptides, capable of hydrogen bonding to
32 the backbone amides of the RGD sequence. These carried on one end a bisphosphonate, and on
33 the other end an anilinium unit. Interestingly, only short dipeptides turned out to constitute
34 suitable spacers for RGD recognition. Obviously, the distance between arginine and aspartate
35 recognition unit is critical for high affinity, and a better aspartate binder is needed.[24]
36
37
38
39

40 In 2007 the next generation of artificial RGD receptor molecules was developed. To this end, the
41 *Schrader* and *Schmuck* groups joined forces and connected the bisphosphonate motif with a
42 guanidiniocarbonylpyrrol, developed earlier by *Schmuck*. This acylguanidinium species with
43 lowered basicity ($pK \sim 6-7$) forms strong H-bond enforced ion pairs to carboxylate anions; these
44 are further strengthened by additional hydrogen bonds from the pyrrole NH and its aromatic
45 carboxamide. *In toto*, these combined hydrogen bond donors comprise the most potent aspartate
46 and glutamate receptors for aqueous media known to date (Figure 9). Through the combination of
47 ion pairing with multiple ionic hydrogen bonds, these receptor molecules effectively bind to
48 single, isolated amino acid carboxylates with millimolar K_d values in aqueous DMSO [25,26].
49
50
51
52
53
54
55
56
57
58
59
60

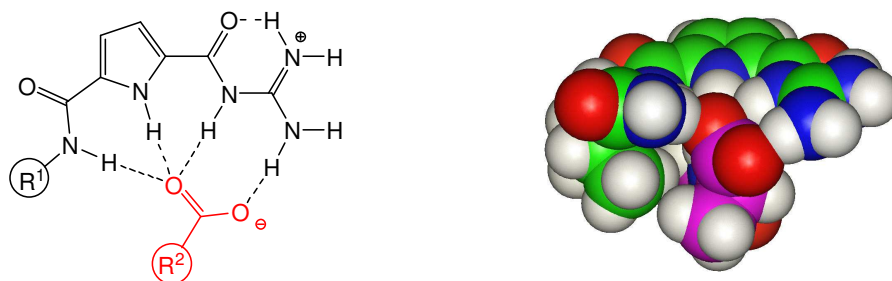


Figure 9. General structure of the guanidiniocarbonylpyrrole recognition motif and the network of ionic hydrogen bonds formed on complexation of carboxylate ions. Right: CPK model showing the calculated complex structure of Ac-RGD-NH₂ peptide (pink) bound by superior receptor molecule **14** with a **guanidiniocarbonylpyrrole** skeleton (green).

In order to investigate the influence of the spacer unit connecting the arginine and aspartate receptor units, four different hosts were synthesized with short peptidic linkers of varying length and rigidity (Figure 10). Compounds **11-13** were very well soluble in water (> 1mM), whereas the solubility of compound **14** with an aromatic spacer was still in the high micromolar range. Detailed binding studies were carried out with NMR titrations for hosts **11-13**, and UV-vis as well as fluorescence titrations for **14**. The pH was fixed at 6.1 (NaH₂PO₄/Na₂HPO₄ buffer), and complexation was monitored and quantitatively evaluated by standard nonlinear regression methods for the free RGD peptide as well as for N/C-protected derivatives.

No binding occurs with receptor **13**, weak complexation is found for hosts **11** and **12**. As a potential explanation, force-field calculations produce stable structures for the isolated host molecules, with inter- or even intramolecular self-association between their bisphosphonate head and guanidiniocarbonylpyrrol tail. These were in part confirmed by dilution studies.

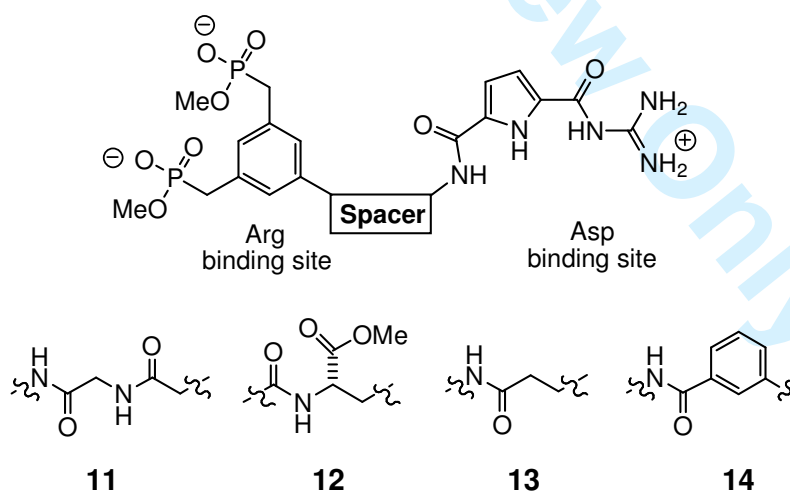


Figure 10. New generation of RGD receptor molecules **11-14** with bisphosphonate and guanidiniocarbonylpyrrole moieties interconnected by various spacers.

Obviously, the peptidic linkers built into hosts **11-13** are too flexible. On the contrary, no intramolecular complexation is possible in receptor molecule **14** with its rigid *m*-aminobenzoyl linker. Consequently, UV-vis as well as fluorescence titrations pointed to a significant attraction of RGD peptides by host **14**, with the N/C-protected Ac-RGD-NH₂ superior to the free RGD peptide. An attempt to probe the contribution of electrostatic interactions between bisphosphonate anion and guanidinium cation furnished a surprising result: Receptor **14** with methyl esters instead of phosphonate anions produced the tightest complex of all. This is counterintuitive, but may well reflect differences in the solvation behaviour of both receptors. It is also in good agreement with observations from *Gilson et al.*, who showed earlier that Coulomb interactions between the highly solvated phosphonate anions and arginine's guanidinium cation are of limited importance, since the structure and complex stability depend more on hydrophobic interactions (vide supra).[27]

Conclusion and Outlook.

Research directed towards powerful RGD receptor molecules has been difficult, and is still in progress. Several steps forward have been taken, demonstrating the need for strong arginine and aspartate binders, as well as for suitable spacers which prevent self-association of the self-recognizing complementary head groups and provide the optimal distance for complex formation of the RGD sidechains without introducing any strain. Mere Coulomb attraction seems to be outperformed by nonpolar interactions and hydrophobic effects. Affinities in buffered aqueous solution are still several orders of magnitude lower than those found for the RGD-protein/integrin pairs. In order to bridge this gap, the *Schrader* and *Schmuck* groups are currently synthesizing a third receptor generation in which the relatively weak binding and highly solvated bisphosphonate will be replaced by a much more powerful arginine binder which was discovered very recently. This is a concave belt like molecular tweezer with phosphonate tips, which binds N/C protected arginine derivatives with low micromolar affinity in water.[28] Interconnection of the tweezer with the guanidiniocarbonylpyrrole by an appropriate and optimized linker unit will hopefully not only lead to powerful RGD receptors, but at the same time provide some selectivity for different RGD conformations, another prerequisite for a potential application as therapeutic agents.

The best candidates will be tested in bioassays for their *in vivo* properties. For major therapeutic target integrins (α V β 3, α IIb β 3 and α 5 β 1), *Humphries et al.* developed (a) solid-phase protein-protein assays and (b) cell-based adhesion assays, which allow determination of IC₅₀ values for cell attachment inhibition.[29,30]

One day, such artificial receptors may serve as lead structures for the development of new therapeutic drugs for the treatment of diseases such as arthritis, heart attack and stroke, viral infections or cancer.

Acknowledgements. Funding of both the Schrader and Schmuck groups by the DFG (Deutsche Forschungsgemeinschaft) is gratefully acknowledged.

References:

- (1) Hynes, R.O. *Cell* **2002**, *110*, 673-687 (Review Integrins).
- (2) Pierschbacher, M. D and Ruoslathi, E. *Nature* **1984**, *309*, 30-33.
- (3) Albeda, S. M & Buck, C. A.. *FASEB J.* **1990**, *4*, 2868-2880.

- 1
2
3 (4) Humphries, M. J. *J. Cell Sci.* **1997**, *110*, 585-595.
4 (5) Yamada, K. M. and Kennedy, D. W. *J. Cell Biochem.* **1985**, *28*, 99-104.
5 (6) Clezardin, P. *Cell Mol. Life Sci.* **1998**, *54*, 541-548.
6 (7) Eliceiri, B. P.; Cheres D.A. *J. Clin. Invest.* **1999**, *103*, 1227-1230.
7 (8) Hynes R. O.; Lander A.D. *Cell* **1992**, *68*, 303-322.
8 (9) Aumailley M.; Gurrath M.; Müller G.; Calvete J.; Timpl R.; Kessler H. *FEBS Lett.* **1991**, *291*, 50-54.
9 (10) Haubner R.; Finsinger D.; Kessler H. *Angew. Chem. Int. Ed.* **1997**, *36*, 1374-1389.
10 (11) Dechantsreiter M. A ; Planker E.; Mathä B.; Lohof E.; Hölemann E.; Jonczyk A.; Goodman S. L.;
11 Kessler, H. *J. Med. Chem.* **1999**, *42*, 3033-3040.
12 (12) Xiong, J.-P.; Stehle, T.; Zhang, R.; Joachimiak, A.; Frech, M.; Goodman, S. L.; Arnaout, M. A.
13 *Science* **2002**, *296*, 151-155.
14 (13) Schrader, T. *Chem. Eur. J.* **1997**, *3*, 1537-1541. Schrader, T. *Tetrahedron Lett.* **1998**, *39*, 517-520.
15 (14) Pasqualini, R.; Koivunen, E.; Ruoslathi, E. *J. Cell Bio.* **1995**, *130*, 1189-1196.
16 (15) Bajt, M. L.; Loftus, J. C. *J. Biol. Chem.* **1994**, *269*, 20913-20919.
17 (16) Ruoslathi, E.; Pasqualini, R. *United States Patent*, Patent Number 5,955,572, **1999**.
18 (17) Papaioannou, E.; Koutsas, C.; Liakopoulou-Kyriakides, M. *Amino Acids* **2009**, *36*, 563-569.
19 (18) Albert, J. S.; Goodman, M. S.; Hamilton, A. D. *J. Am. Chem. Soc.* **1995**, *117*, 1143-1144.
20 (19) Bell, T. W.; Khasanov, A. B. ; Drew, M. G. B. ; Filikov, A.; James, T. L. *Angew. Chem. Int. Ed.*
21 **1999**, *38*, 2543-2547.
22 (20) Ngola, S. M.; Kearney, P. C.; Mecozzi, S.; Russell, K.; Dougherty, D. A. *J. Am. Chem. So.* **1999**,
23 *121*, 1192-1201.
24 (21) Rensing, S. ; Arendt, M. ; Springer, A. ; Grawe, T. ; Schrader, T. *J. Org. Chem.* **2001**, *66*, 5814-5821.
25 (22) Rensing, S. ; Schrader, T. *Org. Lett.* **2002**, *4*, 2161-2164.
26 (23) Chen, W. ; Chang, C.-E.; Gilson, M. K. *J. Am. Chem. Soc.* **2006**, *128*, 4675-4684.
27 (24) Junckers, M. *unpublished results*.
28 (25) Schmuck, C. *Chem. Eur. J.* **2000**, *6*, 709-718.
29 (26) Schmuck, C.; Geiger, L. *J. Am. Chem. Soc.* **2004**, *126*, 8898-8899.
30 (27) Schmuck, C. ; Rupprecht, D. ; Junkers, M. ; Schrader, T. *Chem. Eur. J.* **2007**, *13*, 6864-6873.
31 (28) Fokkens, M. ; Schrader, T. ; Klärner, F.-G. *J. Am. Chem. So.* **2005**, *127*, 14415-14421.
32 (29) Humphries, M. J. *Current Protocols Cell Biol.* **1998**, 9.1.1-9.1.11.
33 (30) Mould, A. P.; Akiyama, S. K. ; Humphries, M. J. *J. Biol. Chem.* **1996**, *271*, 20365-20374.
34
35
36
37
38

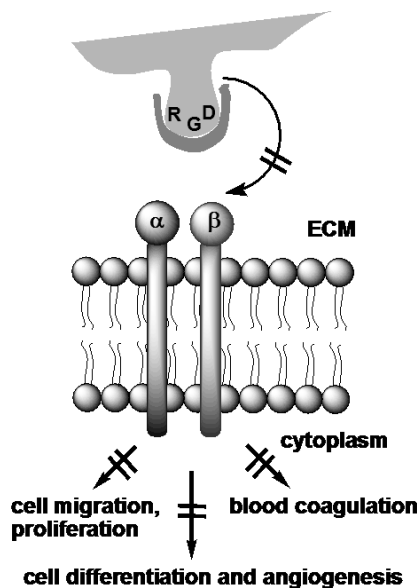
39 ***Correspondence Author Address:**

40 Prof. T. Schrader, Fakultät für Chemie der Universität Duisburg-Essen,
41 Universitätsstr. 5, 45117 Essen (Germany)
42 Phone: (+49) 201-183-3081
43 Fax: (+49) 201-183-4252
44 E-mail: Thomas.Schrader@uni-due.de
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Artificial RGD Receptor Molecules

Thomas Gersthagen, Carsten Schmuck, Thomas Schrader*

TOC-Graphic.



Abstract. Integrins play a pivotal role in cell-cell adhesion, signaling and apoptosis. Many extracellular proteins use the RGD sequence (arginine-glycine-aspartate) as a key to dock onto and unlock their respective binding partners at the cell membrane ($\alpha_V\beta_3$ -, $\alpha_{IIb}\beta_3$ - and the $\alpha_5\beta_1$ -integrin). Here, the RGD signal is transduced into the cytoplasm and triggers a variety of biological events like blood coagulation, cell matrix binding, cell differentiation and angiogenesis. A misfunction of this recognition system causes severe diseases, rendering the RGD recognition system an attractive drug target. Inhibition of RGD-integrin interactions can be reached in two different ways, by blocking integrins with RGD-mimetics or by capping RGD-containing proteins by artificial RGD receptors. This review provides an overview over the very young history of artificial RGD receptor development, beginning with early research in arginine recognition, over the discovery of the first primitive RGD-receptor until the current state of research and future prospects.

Key words: RGD peptide, integrins, molecular recognition, artificial receptors, amino acids

Introduction.

Integrins represent the major class of biological receptors which mediate cell-cell and cell-matrix interactions.[1] They are heterodimeric glycoproteins, located inside the cell membrane and

1
2
3 present the head portions of both subunits to the extracellular space. Cell recognition takes place
4 when specific extracellular proteins dock onto the cell membrane and recognize a specific
5 integrin type. This recognition process hinges on a small key peptide sequence, presented on a
6 solvent-exposed loop of all approaching extracellular proteins. It contains 3 consecutive critical
7 amino acids: arginine-glycine-aspartate, and is therefore generally coined RGD motif. This
8 tripeptide sequence literally serves as a key which can be inserted into the integrin lock and open
9 it.
10

11 In 1984 it was discovered, that the RGD sequence mediates cell adhesion by Fibronectin; [2]
12 subsequent findings quickly demonstrated that the RGD sequence is a general powerful
13 recognition motif utilized by many other proteins like vitronectin, laminin or the von willebrand
14 factor, which all play a pivotal role in cell adhesion biology. Fundamental biological processes
15 such as cell differentiation, signaling, apoptosis and others depend on the interplay between
16 RGD-containing proteins and their related integrin-receptors.[3,4,5] The integrin family is highly
17 variable because its members differ in their composition through the combination of eight
18 different α - and β -subunits. A single integrin is able to discriminate between the RGD-sequences
19 of different proteins by two main mechanisms; it recognizes not only the specific chemical nature
20 of these three amino acids, but also the specific conformation of each of these individual RGD-
21 loops. The relative orientation of the alkylguanidinium and the alkylcarboxylate side chains can
22 adopt all transitions from a linear, fully extended conformation up to a tightly folded cyclic loop.
23 In some cases, the integrin also interacts with the fourth amino acid residue, e. g., in the RGDS
24 sequence typical for fibronectin.
25
26
27

28 Dysfunction in this critical recognition system can cause pathogenic effects like tumor-induced
29 angiogenesis, osteoporosis, myocardial infarction or thrombosis.[6,7,8] The resulting severe
30 diseases reveal the medicinal and pharmaceutical importance of the RGD recognition system and
31 render it an attractive drug target. A precise control over pathologic RGD recognition events by
32 externally added small molecules would not only help to better understand this important natural
33 signalling pathway, but also have immediate therapeutic relevance. In principle, inhibition of the
34 RGD- integrin interaction can be reached in two different ways. One has been pursued
35 intensively by pharmaceutical research; the other – more difficult one - has been totally
36 neglected. A great deal of synthetic, computational and biological effort has been devoted to
37 develop so-called *RGD Mimetics* which imitate polar groups and their relative orientations in a
38 specific RGD-sequence, in order to compete with the native ligands of such integrins (Figure 1a
39 and 1b).
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

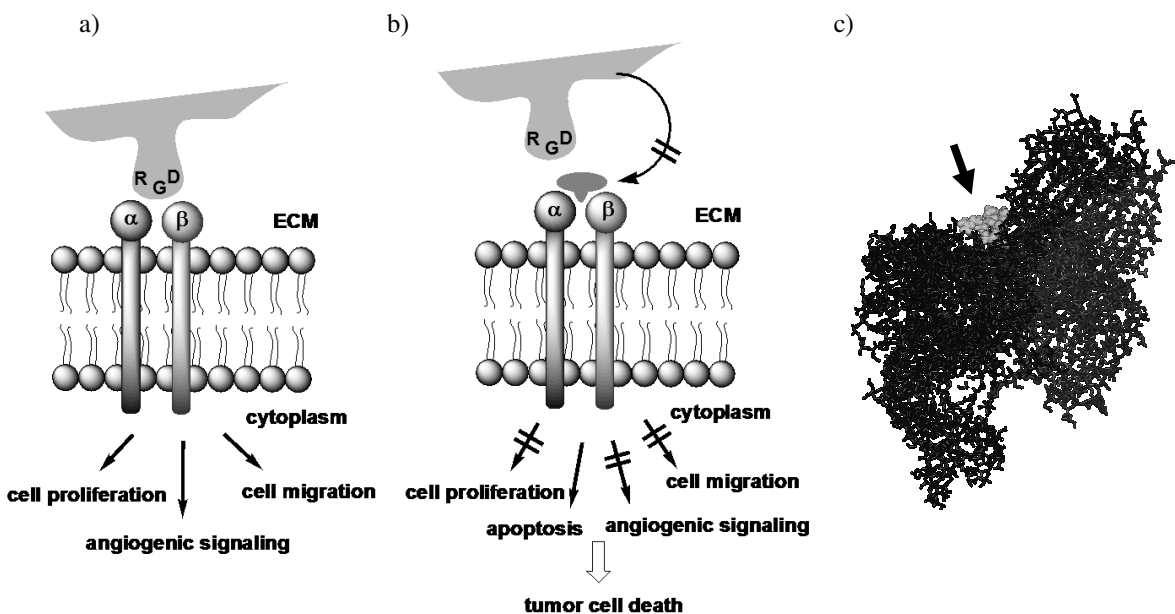


Figure 1. a) Schematic of the integrin/RGD “lock and key” principle: An RGD-containing protein (key) docks onto the membrane-spanning α,β -integrin dimer (lock) and induces a biological effect. b) Conventional interference principle: the ligand binding site on the Integrin is blocked by an RGD mimetic; c) Crystal structure of the cRGDf[NMe]V peptide in its complex with the α,β -integrin dimer $\alpha V\beta 3$.

Numerous academic and industrial groups have developed peptide-derived or non-peptidic RGD mimetics, most of which showed some biological activity. Most notably, an elegant approach was introduced by *Kessler et al.*, who synthesized a series of RGD-containing cyclopeptides with 5- and 6-membered rings containing single D-amino acid residues. In extended NMR-spectroscopic studies the structural principles governing the formation of preferred fixed conformations were unraveled and used for a conformational design of integrin-specific RGD mimetics, which showed promising *in vitro* biological effects. During the course of these studies, a whole set of cyclic hexapeptides and pentapeptides was synthesized and thermodynamic equilibria as well as dynamics of their three-dimensional structures in solution were analyzed by 2D-NMR techniques.

Finally, systematic tuning of conformational restrictions accompanied by MD simulations and subsequent bioassays culminated in the preparation of the strong lead compound *cyclo*-RGDf[NMe]V (Figure 2), a powerful integrin antagonist, which effectively inhibits tumor-associated angiogenesis.[9,10,11] This very small cyclopeptide, which is currently undergoing clinical trials, imitates the RGD-loop in Vitronectin. In 2002 *Arnaout et al.* gained a crystal structure of this cyclopeptide in its complex with integrin $\alpha V\beta 3$, the only crystal structure of an RGD-integrin complex which is available today. This structure reveals that the peptide is inserted in a cleft between the so called β -propeller from the αV -unit and βA domains from the $\beta 3$ -unit on the integrin head. As expected, almost all peptide-protein contacts are established by the RGD sequence (Figure 1c).[12]

Figure 2 displays some related RGD mimetics, some of which have already been approved as drugs. Eptifibatide, a somewhat more flexible cyclohexapeptide with an internal disulfide bond, inhibits integrin $\alpha_{IIb}\beta_3$. It was developed entirely by pharmaceutical industry, and is used against myocardial infarction or in heart surgery. Other potent drugs are drastically simplified and often rigidified RGD mimetics which contain only one stereogenic center and replace guanidinium ions by (hydroxy)amidines (Figure 2).

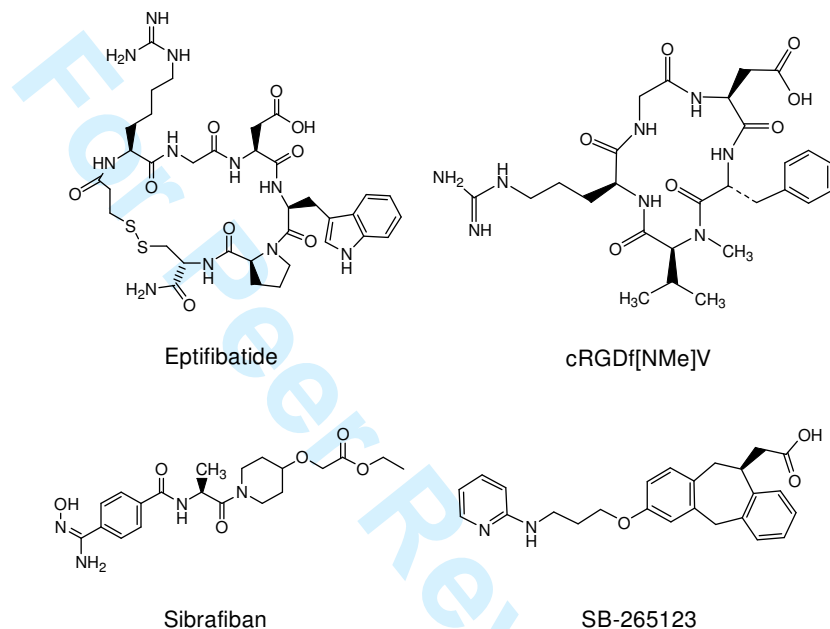


Figure 2. Synthetic RGD-mimetics: cRGDf[NMe]V (Cilengitide, Merck KGaA, specific for $\alpha V\beta_3$ -Integrin), Eptifibatide (Integrilin[®], Millennium Pharmaceuticals/GlaxoSmithKline/ Schering-Plough, specific for $\alpha_{IIb}\beta_3$ -Integrin); Sibrafiban (Hoffmann-LaRoche, specific for $\alpha_{IIb}\beta_3$ -Integrin); SB-265123 (GlaxoSmithKline, specific for $\alpha V\beta_3$ -Integrin).

Artificial RGD Receptor Molecules.

A completely different approach to block pathologic RGD-Integrin interactions is to construct integrin mimetics which recognize RGD-containing proteins. In principle this simplifies the task to developing a small artificial host molecule which can serve as a competitive “lock” for the RGD-sequence in native proteins. Due to the encapsulated RGD-“key” the whole extracellular protein is not able any more to bind to its respective target integrin (Figure 3a). However, since the RGD-sequence exists in a variety of different conformations, depending on the parent protein, it is mandatory to provide synthetic hosts which can distinguish between them (Figure 3 b).

Some years ago, the *Schrader* and *Schmuck* groups embarked on a program to pursue this alternative approach towards conformation-specific RGD host molecules. The starting point was marked by the discovery that *m*-xylylene bisphosphonates **1** bind guanidinium cations in a chelate-type manner reminiscent to the natural diaspertate binding site found in the crystal structure of the cRGDf[NMe]V/ $\alpha V\beta_3$ complex. In both cases a bidentate salt bridge with a network of ion-pair reinforced hydrogen bonds firmly embraces arginine’s guanidinium cation and holds it in place (Figure 3 c,d).[13]

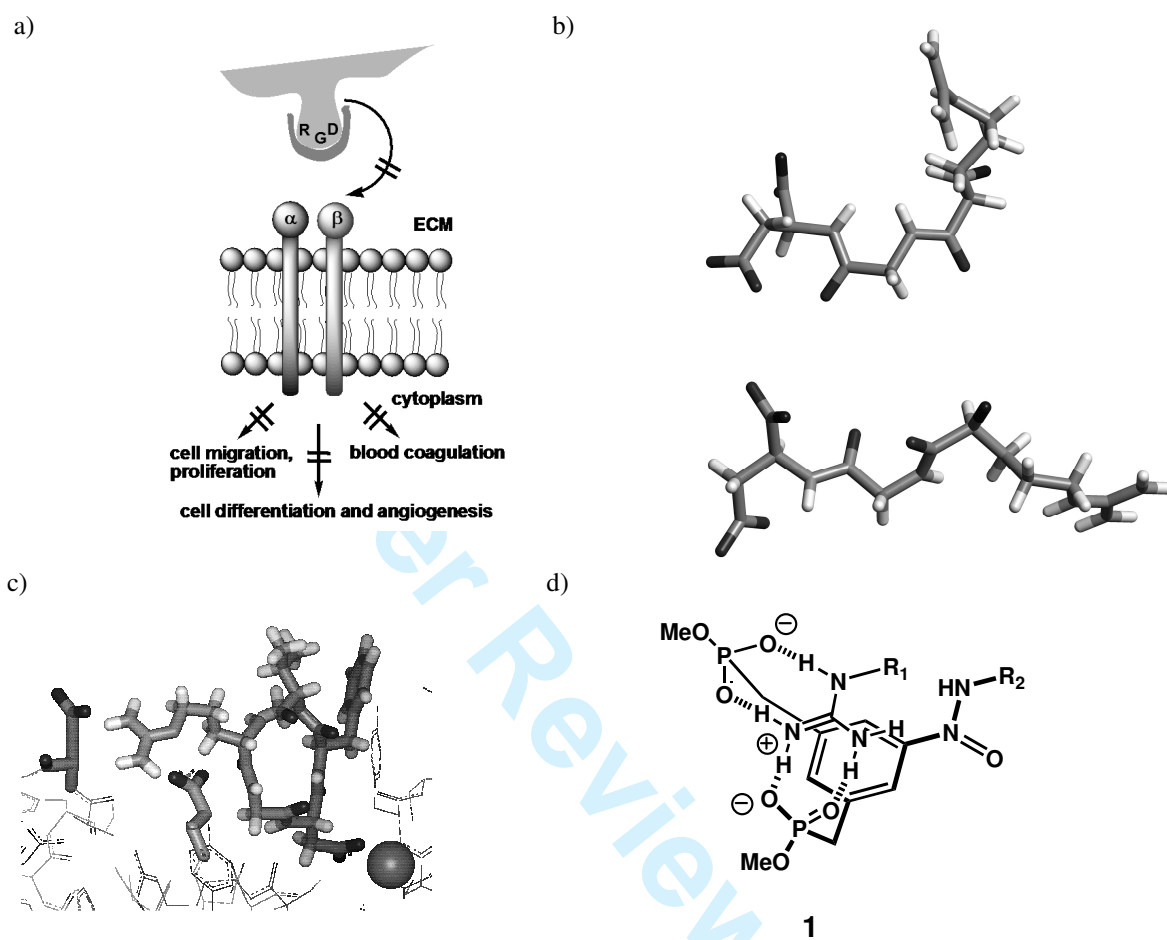


Figure 3. a) Alternative concept for RGD/integrin interruption: RGD-capping by an artificial RGD receptor molecule (grey) prevents docking onto the membrane-bound integrin (blue/red); b) Folded and extended conformation found in the RGD-loops of Fibronectin and Vitronectin (from crystal structures); c) chelate-type binding motif from the crystal structure of the complex between cRGDf[NMe]V and α V β 3; d) chelate-type binding motif in a *m*-xylylene bisphosphonate/arginine complex.

An early screening experiment for RGD-binders was carried out by *Ruoslahti et al.* In 1999 a patent was filed which announced the discovery that cyclic peptides with the consensus sequence (W/P)DD(G/L)(W/L)(W/L/M) bind to fibronectin and related RGD-containing proteins. The best candidate was cyclo-CWDDGWLC **2**, with an internal disulfide bond, which is depicted in Figure 4 a. It was isolated from a library of short cyclic peptides and peptidomimetics in the course of specific assays for RGD-binding activity. The peptides were presented in form of a phage library which was used to isolate minimal receptor sequences that bind to fibronectin in affinity panning. Thus, the cyclic octapeptide c-CWDDGWLC was identified as the most potent inhibitor for RGD dependent cell attachment to fibronectin and vitronectin. Fibronectin itself tightly bound to a c-CWDDGWLC affinity column and could subsequently be reeluted with an RGD-containing peptide. Interestingly, a specific region within the β -subunit of RGD-binding

integrins had previously been shown by site-directed mutagenesis to be involved in ligand binding [14], which includes a the short peptide stretch KDDLW – quite similar to the above-described sequence. Short synthetic peptides corresponding to this integrin region bind to the RGD containing protein, but their affinities were markedly reduced if both aspartate residues are mutated to alanines. In summary, the combination of phage display technology, rational design and mutagenesis has lead to structural integrin mimetics which can significantly reduce the cell attachment of an RGD-containing protein [15,16]. However, these are still sensitive to proteolytic degradation.

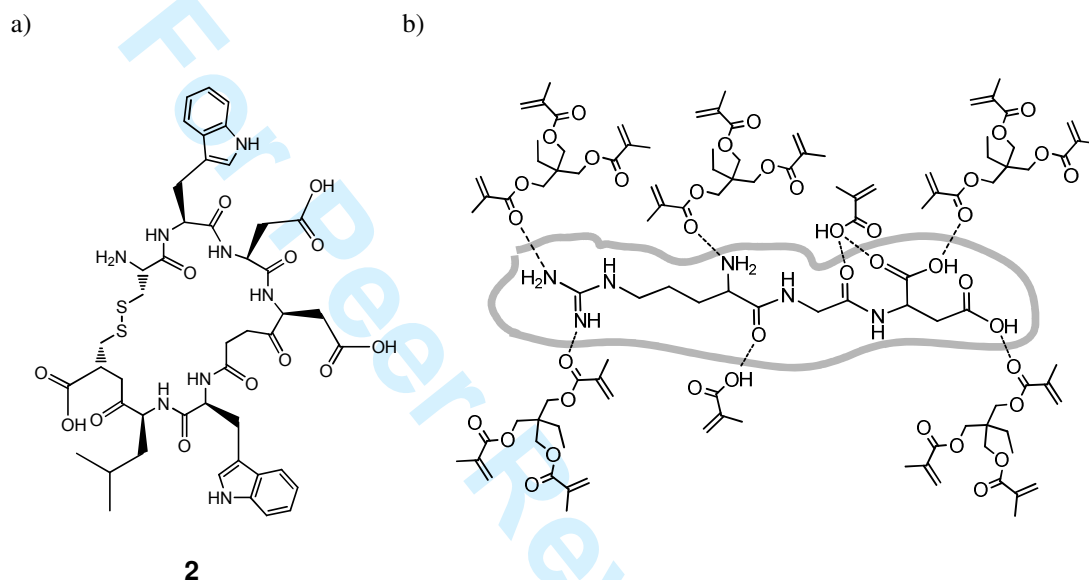


Figure 4. a) Lead candidate cyclo-CWDDGWLC, identified from phage-display assays as a general RGD-protein binder b) Attempted Molecular Imprinting for the free RGD template peptide: proposed noncovalent interactions between functional monomer, crosslinker and template.

In 2009, *Liakopoulou-Kyriakides* et al. tried to overcome this drawback by employing the Molecular Imprinting technology (Figure 4 b) in order to generate a new chromatography stationary phase for the separation of RGD-peptides from mixtures. They used the free RGD peptide as template molecule, and prepared molecularly imprinted polymers (MIPs) from only two functional monomers, i. e., methacrylic acid (MAA) and acrylamide (AA). Three different crosslinking monomers were tried, with trimethylpropane trimethacrylate producing the best results in terms of RGD-selectivity. MAA was later postulated to form interactions with arginine, while AA's amide is both a moderate hydrogen bond donor and acceptor. To establish RGD selectivity, four other peptides were used in rebinding experiments, namely KGD and three other completely different peptides. Selectivity factor values ranged from 1.27 up to 1.98, net rebinding values for RGD and KGD were similar (~ 8-10%), while those for other peptides remained very small (2-4 %). These data indicate low affinity and only moderate selectivity for the template molecule. In addition, the free RGD peptide was examined in methanol solution, where K_d values were in the high micromolar range.[17]

Surprisingly, to the best of our knowledge, apart from our own project, these are the only artificial RGD receptor molecules published to date. We tentatively explain this with a lack of powerful concepts for the generation of water-soluble host molecules for zwitterions in physiological solution. In the aqueous medium, hydrogen bonds must be reinforced with

electrostatic interactions and complemented by hydrophobic forces to achieve high affinities. Zwitterions are prone to undergo intramolecular as well as intermolecular self-association, which often renders the desired host-guest complexes insoluble in buffered aqueous solution – especially in the presence of high salt loads.

For isolated arginine and aspartate as well as for model peptides containing only anionic or cationic residues, some powerful receptor molecules have been developed: *Hamilton et al.* created a rigid concave scaffold **3** with two preorganized guanidinium moieties (Figure 5 a) which specifically bind two aspartate carboxylates in the *i*+3-relation of an α -helix. Small aspartate-containing model peptides are bound with millimolar affinities, albeit only in a 10% water-methanol mixture.[18]

Bell et al. synthesized crescent-shape molecule **4** with multiple annulated pyridine and dihydrobenzene rings, which they coined “the arginine cork” because of its high preference for arginine (Figure 5 b). It prefers diarginine as its guest, and displays K_d values in the low micromolar range for molecular recognition in water. The architectural concept is based on a remarkably rigid host skeleton which leads to a large degree of preorganization of hydrogen bond acceptors, reinforced by two strategically placed carboxylates for additional ionic interactions between the dianionic host and the dicationic guest [19]. Problematic features are limited solubility and difficult synthetic access.

Later the *Dougherty group* constructed a much larger, highly charged cyclophane receptor (**5**), which is selective for arginine and lysine dipeptides (almost micromolar affinities in aqueous solution, Figure 5 c). The clue is a combination of cation- π interactions with electron-rich receptor π -faces and multiple salt bridges, complemented by total inclusion of one amino acid inside the receptor cavity, leading to solvent exclusion; the second basic residue remains solvent-exposed.[20]

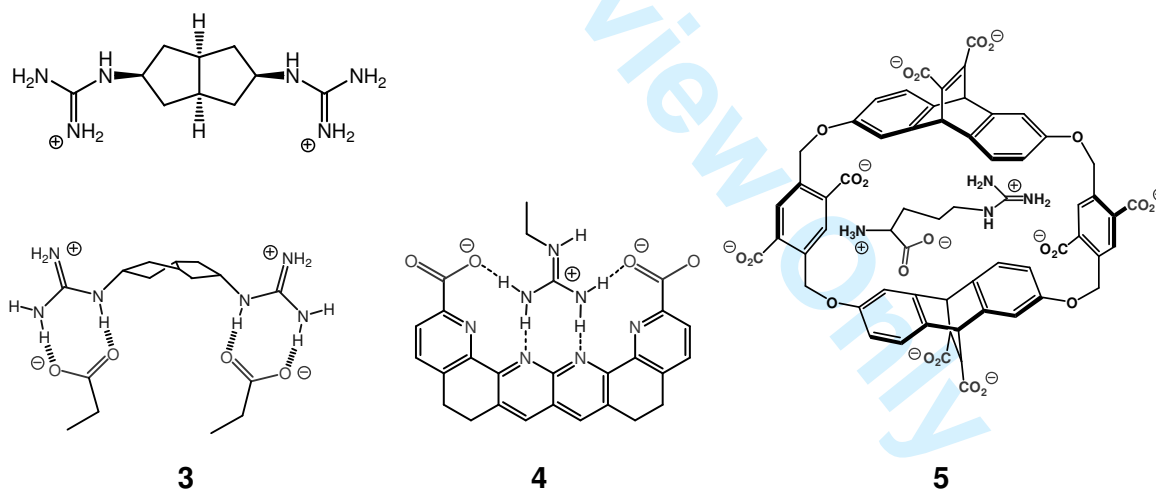


Figure 5. a) Preorganized host molecules for aspartate and arginine: Hamilton’s concave *i*+3-diaspartate binder **3**; b) Bell’s planar “arginine cork” **4**; c) Dougherty’s arginine-cavitand **5** with putative arginine inclusion.

In 1997 *Schrader* discovered that the *m*-xylylene bisphosphonate dianion is a powerful guanidinium binder in dipolar aprotic solution (DMSO) and forms a similar arrangement as the well-known Tat-TAR complex between the AIDS virus’s RNA fragment (two phosphate groups) and a regulatory protein (arginine, Figure 3 d).[14] This recognition motif prefers delocalized, soft guanidinium cations over hard ammonium ions and especially arginine over lysine residues.

Systematic investigations were carried out to study the specific interplay of noncovalent attractive forces with modified model compounds. To this end, additional substituents with varying electronic and steric properties were introduced in the 5-position of the central benzene ring (see selection in Figure 6).

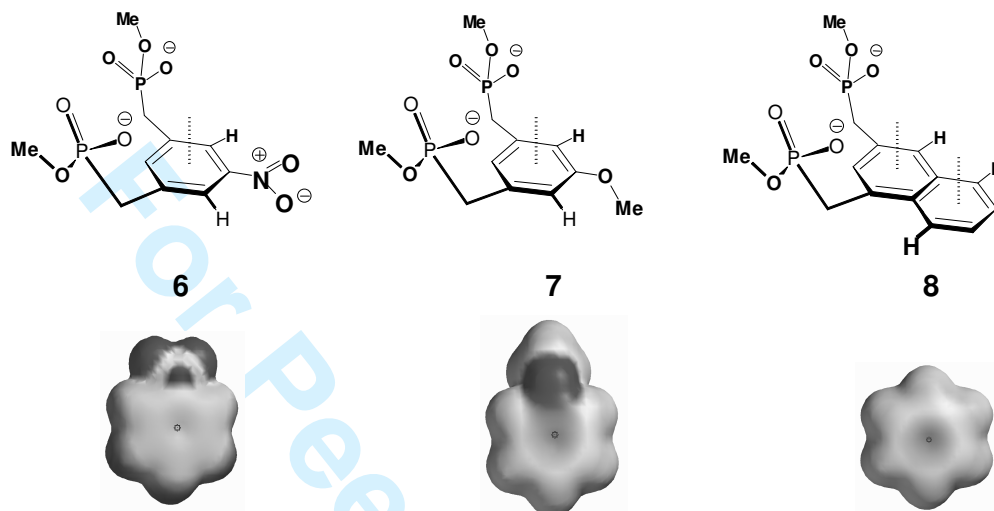


Figure 6. *m*-Xylylene bisphosphonate host molecules **6-8** with additional substituents in the 5-position - probing the π -cation interaction. Below: Electrostatic Surface Potentials (ESP) of nitrobenzene, anisole and benzene (AM1-calculations).

It was assumed that three kinds of noncovalent interactions play a key role in guanidinium recognition: electrostatic and hydrogen bond interactions with both phosphonate moieties and cation- π interactions with the receptor's central aromatic ring, which was placed in direct van-der-Waals contact by force-field calculations. Direct proof of cation- π interactions was provided with small electronic-withdrawing and donating groups in 5-position of this central benzene ring; these substituents could not form any additional hydrogen bonds to the guanidinium cation because they were geometrically locked in the aromatic plane of the receptor molecule. Intriguingly, all electronic-withdrawing groups lowered and all donating groups raised relative affinities towards methylguanidine hydrochloride, reflecting weakened or strengthened interactions between the guanidinium cation and the aromatic system. A close correlation was found between free binding enthalpies and the electrostatic surface potential of the respective central rings. Dissociation constants could also be lowered by increasing the π -face of the receptor, e. g., by exchanging the central benzene for a naphthalene ring (as in **8**).

Even better than all the preceding modifications, however, was the introduction of a third phosphonate anion at a certain distance from the benzylic bisphosphonate (**9**). Monte Carlo simulations and NOESY experiments confirmed the postulated additional hydrogen bond contacts to all five guanidinium NH protons of the model guanidinium salt, which was now surrounded by all three phosphonate arms (Figure 7).

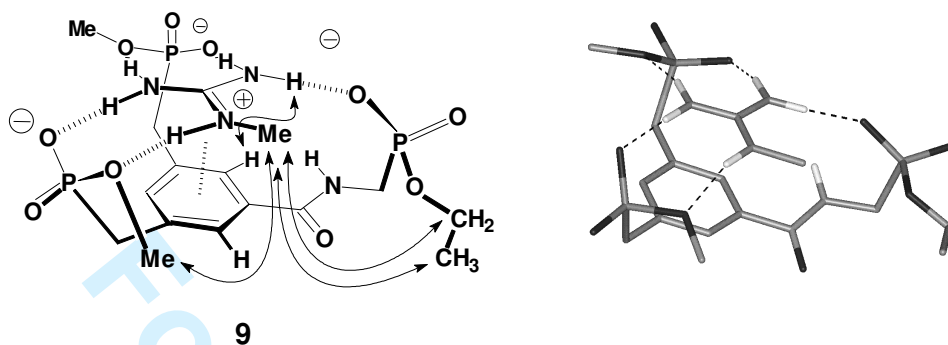


Figure 7. Summarized reciprocal NOE contacts measured between the new arginine host molecule **9** and methylguanidinium cation (500 MHz, 1:1 complex in CD₃OD). Right: Monte Carlo simulation (MacroModel 7.0, water/GBA, Amber*, 1000 steps).

Strong binding and a pronounced selectivity for arginine over lysine and histidine derivatives was now observed in methanol, with K_d values in the low micromolar range ($\sim 30 \mu\text{M}$). Monte Carlo simulations in water showed that the long triphosphonate allows the arginine side chain to form a thermodynamically favoured extended conformation while an electrostatic interaction with the α -ammonium group still occurs. Unfortunately, affinities in aqueous solution remained small.[21]

From the new trisphosphonate unit we subsequently developed the first synthetic RGD receptor molecule (Figure 8). Alkylation of the deprotonated benzamide-NH group with phthaloyl-protected *m*-aminobenzyl bromide smoothly yielded **10** after hydrazinolysis and phosphonate ester cleavage with LiBr. Now a simple aspartate binding site is present, while at the same time the rigid benzyl spacer prevents intramolecular dimerization, and the ammonium functionality is a bad guest for the triphosphonate and should thus circumvent an unwanted intermolecular dimerization.

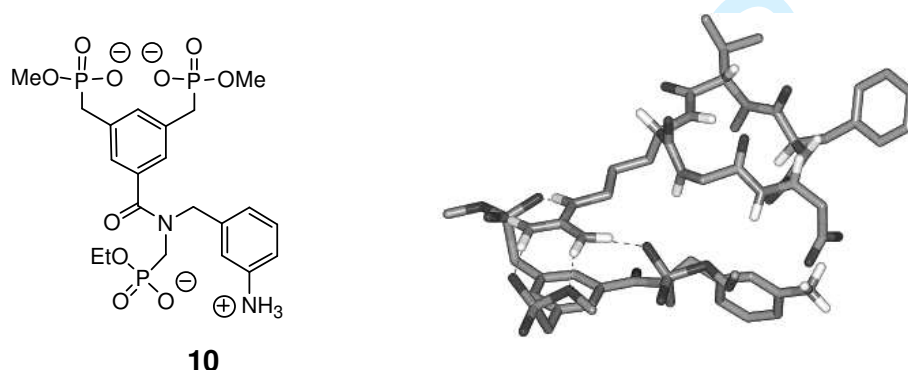


Figure 8. Primitive synthetic RGD-receptor molecule **10** with restricted rotational freedom. Left: Lewis structure; Right: molecular mechanics calculation of its 1:1 complex with cyclo(RGDfV) (MacroModel 7.0, water (GBA), Amber*, 1000 steps).

NMR titrations of the new RGD-receptor molecule with the free RGD peptide as well as the integrin antagonist cRGDfV became now possible in water, and furnished K_d values in the high

1
2
3 micromolar regime. Distinct NMR signal shifts were observed for the aspartate CH₂ protons,
4 testifying to its additional interaction with the RGD host; complex stoichiometries were
5 determined at a precise 1:1 ratio. This showed that the concept of combining an arginine binder
6 with an aspartate binder was valid.[22]
7
8

9 Inspired by this work the *Gilson group* chose our simple ditopic RGD receptor molecules and
10 their complexes with RGD peptides as a biologically relevant model case to study the accuracy of
11 their new program for the calculation of absolute Gibbs energies. Their results were in good
12 agreement with the experimental values that had been independently determined in the *Schrader*
13 *group*. For the interaction between **10** and cRGDfV, e. g., the experimental data showed a ΔG
14 value of -3.91 kcal/mol, which was reproduced *in silico* with -3.11 kcal/mol. A most remarkable
15 feature of the new program is its ability to include solvent and entropic effects in the M2
16 modeling method. In the course of these calculations, the *Gilson group* noticed, that in cRGDfV-
17 complexation by **10**, electrostatic interactions play a much smaller role than for the linear RGD
18 peptide, while van-der-Waals and nonpolar terms became dominating. This is in line with
19 intuition, since cRGDfV has two charged groups less than free RGD, but carries two additional
20 nonpolar groups. The M2 calculations also suggested that much of the receptor did not interact
21 directly with the RGD sequence. Finally new receptor molecules were designed *in silico* and
22 predicted to produce Gibbs energies of up to 5 kcal/mol higher than **10**. One of the calculated
23 structures reached predicted nanomolar affinities with the cyclic RGD model peptide, rivalling
24 protein-ligand interactions. The underlying design concept either strived to extend the
25 hydrophobic contact area between host and guest ligand or to reduce entropic or electrostatic
26 desolvation penalties. The only problem with these optimized virtual RGD receptor molecules
27 was that they were too difficult to synthesize [23].
28
29
30
31
32

33 In an independent investigation, the influence of the spacer unit was studied with simple model
34 systems. The benzyl group in **10** was replaced by small peptides, capable of hydrogen bonding to
35 the backbone amides of the RGD sequence. These carried on one end a bisphosphonate, and on
36 the other end an anilinium unit. Interestingly, only short dipeptides turned out to constitute
37 suitable spacers for RGD recognition. Obviously, the distance between arginine and aspartate
38 recognition unit is critical for high affinity, and a better aspartate binder is needed.[24]
39
40
41

42 In 2007 the next generation of artificial RGD receptor molecules was developed. To this end, the
43 *Schrader* and *Schmuck* groups joined forces and connected the bisphosphonate motif with a
44 guanidiniocarbonylpyrrol, developed earlier by *Schmuck*. This acylguanidinium species with
45 lowered basicity (pK ~ 6-7) forms strong H-bond enforced ion pairs to carboxylate anions; these
46 are further strengthened by additional hydrogen bonds from the pyrrole NH and its aromatic
47 carboxamide. *In toto*, these combined hydrogen bond donors comprise the most potent aspartate
48 and glutamate receptors for aqueous media known to date (Figure 9). Through the combination of
49 ion pairing with multiple ionic hydrogen bonds, these receptor molecules effectively bind to
50 single, isolated amino acid carboxylates with millimolar K_d values in aqueous DMSO [25,26].
51
52
53
54
55
56
57
58
59
60

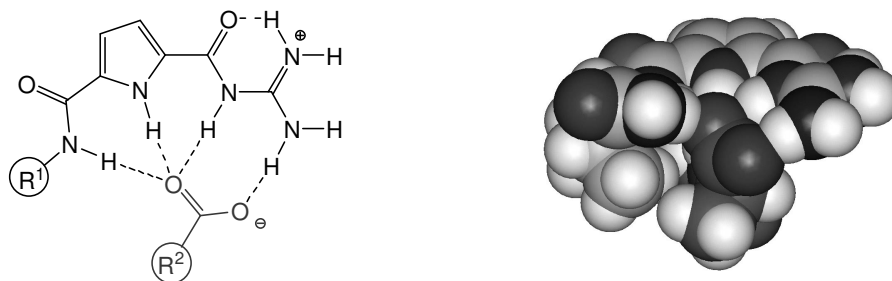


Figure 9. General structure of the guanidiniocarbonylpyrrole recognition motif and the network of ionic hydrogen bonds formed on complexation of carboxylate ions. Right: CPK model showing the calculated complex structure of Ac-RGD-NH₂ peptide (pink) bound by superior receptor molecule **14** with a guanidiniocarbonylpyrrole skeleton (green).

In order to investigate the influence of the spacer unit connecting the arginine and aspartate receptor units, four different hosts were synthesized with short peptidic linkers of varying length and rigidity (Figure 10). Compounds **11-13** were very well soluble in water (> 1mM), whereas the solubility of compound **14** with an aromatic spacer was still in the high micromolar range. Detailed binding studies were carried out with NMR titrations for hosts **11-13**, and UV-vis as well as fluorescence titrations for **14**. The pH was fixed at 6.1 (NaH₂PO₄/Na₂HPO₄ buffer), and complexation was monitored and quantitatively evaluated by standard nonlinear regression methods for the free RGD peptide as well as for N/C-protected derivatives.

No binding occurs with receptor **13**, weak complexation is found for hosts **11** and **12**. As a potential explanation, force-field calculations produce stable structures for the isolated host molecules, with inter- or even intramolecular self-association between their bisphosphonate head and guanidiniocarbonylpyrrol tail. These were in part confirmed by dilution studies.

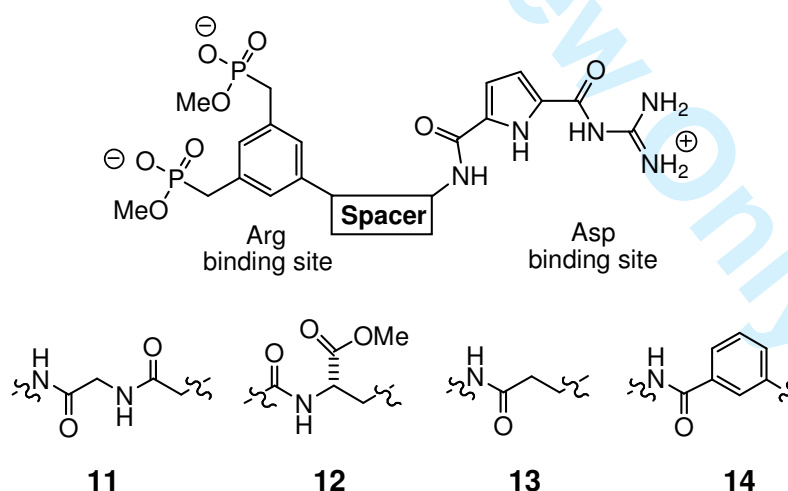


Figure 10. New generation of RGD receptor molecules **11-14** with bisphosphonate and guanidiniocarbonylpyrrole moieties interconnected by various spacers.

Obviously, the peptidic linkers built into hosts **11-13** are too flexible. On the contrary, no intramolecular complexation is possible in receptor molecule **14** with its rigid *m*-aminobenzoyl linker. Consequently, UV-vis as well as fluorescence titrations pointed to a significant attraction of RGD peptides by host **14**, with the N/C-protected Ac-RGD-NH₂ superior to the free RGD peptide. An attempt to probe the contribution of electrostatic interactions between bisphosphonate anion and guanidinium cation furnished a surprising result: Receptor **14** with methyl esters instead of phosphonate anions produced the tightest complex of all. This is counterintuitive, but may well reflect differences in the solvation behaviour of both receptors. It is also in good agreement with observations from *Gilson et al.*, who showed earlier that Coulomb interactions between the highly solvated phosphonate anions and arginine's guanidinium cation are of limited importance, since the structure and complex stability depend more on hydrophobic interactions (vide supra).[27]

Conclusion and Outlook.

Research directed towards powerful RGD receptor molecules has been difficult, and is still in progress. Several steps forward have been taken, demonstrating the need for strong arginine and aspartate binders, as well as for suitable spacers which prevent self-association of the self-recognizing complementary head groups and provide the optimal distance for complex formation of the RGD sidechains without introducing any strain. Mere Coulomb attraction seems to be outperformed by nonpolar interactions and hydrophobic effects. Affinities in buffered aqueous solution are still several orders of magnitude lower than those found for the RGD-protein/integrin pairs. In order to bridge this gap, the *Schrader* and *Schmuck* groups are currently synthesizing a third receptor generation in which the relatively weak binding and highly solvated bisphosphonate will be replaced by a much more powerful arginine binder which was discovered very recently. This is a concave belt like molecular tweezer with phosphonate tips, which binds N/C protected arginine derivatives with low micromolar affinity in water.[28] Interconnection of the tweezer with the guanidiniocarbonylpyrrole by an appropriate and optimized linker unit will hopefully not only lead to powerful RGD receptors, but at the same time provide some selectivity for different RGD conformations, another prerequisite for a potential application as therapeutic agents.

The best candidates will be tested in bioassays for their *in vivo* properties. For major therapeutic target integrins (α V β 3, α IIb β 3 and α 5 β 1), *Humphries et al.* developed (a) solid-phase protein-protein assays and (b) cell-based adhesion assays, which allow determination of IC₅₀ values for cell attachment inhibition.[29,30]

One day, such artificial receptors may serve as lead structures for the development of new therapeutic drugs for the treatment of diseases such as arthritis, heart attack and stroke, viral infections or cancer.

Acknowledgements. Funding of both the Schrader and Schmuck groups by the DFG (Deutsche Forschungsgemeinschaft) is gratefully acknowledged.

References:

- (1) Hynes, R.O. *Cell* **2002**, *110*, 673-687 (Review Integrins).
- (2) Pierschbacher, M. D and Ruoslathi, E. *Nature* **1984**, *309*, 30-33.
- (3) Albeda, S. M & Buck, C. A.. *FASEB J.* **1990**, *4*, 2868-2880.

- 1
2
3 (4) Humphries, M. J. *J. Cell Sci.* **1997**, *110*, 585-595.
4 (5) Yamada, K. M. and Kennedy, D. W. *J. Cell Biochem.* **1985**, *28*, 99-104.
5 (6) Clezardin, P. *Cell Mol. Life Sci.* **1998**, *54*, 541-548.
6 (7) Eliceiri, B. P.; Cheres D.A. *J. Clin. Invest.* **1999**, *103*, 1227-1230.
7 (8) Hynes R. O.; Lander A.D. *Cell* **1992**, *68*, 303-322.
8 (9) Aumailley M.; Gurrath M.; Müller G.; Calvete J.; Timpl R.; Kessler H. *FEBS Lett.* **1991**, *291*, 50-54.
9 (10) Haubner R.; Finsinger D.; Kessler H. *Angew. Chem. Int. Ed.* **1997**, *36*, 1374-1389.
10 (11) Dechantsreiter M. A ; Planker E.; Mathä B.; Lohof E.; Hölemann E.; Jonczyk A.; Goodman S. L.;
11 Kessler, H. *J. Med. Chem.* **1999**, *42*, 3033-3040.
12 (12) Xiong, J.-P.; Stehle, T.; Zhang, R.; Joachimiak, A.; Frech, M.; Goodman, S. L.; Arnaout, M. A.
13 *Science* **2002**, *296*, 151-155.
14 (13) Schrader, T. *Chem. Eur. J.* **1997**, *3*, 1537-1541. Schrader, T. *Tetrahedron Lett.* **1998**, *39*, 517-520.
15 (14) Pasqualini, R.; Koivunen, E.; Ruoslathi, E. *J. Cell Bio.* **1995**, *130*, 1189-1196.
16 (15) Bajt, M. L.; Loftus, J. C. *J. Biol. Chem.* **1994**, *269*, 20913-20919.
17 (16) Ruoslathi, E.; Pasqualini, R. *United States Patent*, Patent Number 5,955,572, **1999**.
18 (17) Papaioannou, E.; Koutsas, C.; Liakopoulou-Kyriakides, M. *Amino Acids* **2009**, *36*, 563-569.
19 (18) Albert, J. S.; Goodman, M. S.; Hamilton, A. D. *J. Am. Chem. Soc.* **1995**, *117*, 1143-1144.
20 (19) Bell, T. W.; Khasanov, A. B. ; Drew, M. G. B. ; Filikov, A.; James, T. L. *Angew. Chem. Int. Ed.*
21 **1999**, *38*, 2543-2547.
22 (20) Ngola, S. M.; Kearney, P. C.; Mecozzi, S.; Russell, K.; Dougherty, D. A. *J. Am. Chem. So.* **1999**,
23 *121*, 1192-1201.
24 (21) Rensing, S. ; Arendt, M. ; Springer, A. ; Grawe, T. ; Schrader, T. *J. Org. Chem.* **2001**, *66*, 5814-5821.
25 (22) Rensing, S. ; Schrader, T. *Org. Lett.* **2002**, *4*, 2161-2164.
26 (23) Chen, W. ; Chang, C.-E.; Gilson, M. K. *J. Am. Chem. Soc.* **2006**, *128*, 4675-4684.
27 (24) Junckers, M. *unpublished results*.
28 (25) Schmuck, C. *Chem. Eur. J.* **2000**, *6*, 709-718.
29 (26) Schmuck, C.; Geiger, L. *J. Am. Chem. Soc.* **2004**, *126*, 8898-8899.
30 (27) Schmuck, C. ; Rupprecht, D. ; Junkers, M. ; Schrader, T. *Chem. Eur. J.* **2007**, *13*, 6864-6873.
31 (28) Fokkens, M. ; Schrader, T. ; Klärner, F.-G. *J. Am. Chem. So.* **2005**, *127*, 14415-14421.
32 (29) Humphries, M. J. *Current Protocols Cell Biol.* **1998**, *9.1.1-9.1.11*.
33 (30) Mould, A. P.; Akiyama, S. K. ; Humphries, M. J. *J. Biol. Chem.* **1996**, *271*, 20365-20374.
34
35
36
37
38

39 ***Correspondence Author Address:**

40 Prof. T. Schrader, Fakultät für Chemie der Universität Duisburg-Essen,
41 Universitätsstr. 5, 45117 Essen (Germany)
42 Phone: (+49) 201-183-3081
43 Fax: (+49) 201-183-4252
44 E-mail: Thomas.Schrader@uni-due.de
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Dear Jeff,

we have now revised the paper according to the reviewers' comments and highlighted all respective changes in yellow.

Page 1 Line 30-Differentiation spelled wrong in TOC Graphical Abstract Page 1 Line **changed**
55-No hyphen for "cell membrane"
Page 2 Line 9- RGD sequence, sequence should not be capitalized Page 2 line 10-I **changed**
don't think protein names should be capitalized, vitronectin, laminin Page 2 line **changed**
24-fibronectin, no caps Page 2, line 26-should be myocardial infarction? Page 3, line 59-myocardial infarction? **changed**
Page 4, line 53-guanidinium spelled wrong Page 5, line 20-differentiation spelled **changed**
wrong in Fig 3a Page 5 line 47-no space in "Figure 4a" **changed**
Page 6 line 37- no space in "Figure 4b" **changed**
Page 7, line 7-no space in Figure 5a **changed**
Page 10, lines 3 and 4-should be "an" arginine and "an" aspartate Page 10, line 42 **changed**
and Figure 9 caption-guanidinocarbonylpyrrole with an "e" on the end?
Page 12, line 23-forward not foreward **changed**

We attached the final manuscript in Word format, there is a black and white version for the printed issue and a coloured version for the online issue. All figures are attached separately as TIFF format, each (except 2, 4a), 10) in black/white and coloured.

Regards

Thomas S. and Thomas G.

List of attached files:

Artificial RGD Receptors finalonlineversion

Artificial RGD Receptors finalprintversion

figure 1a), b) colour

figure 1a), b)

figure 1c) colour

figure 1c)

figure2

TOC graphic,figure 3a) colour

1
2
3 TOC graphic, figure 3a)
4

5 figure 3b) colour
6

7 figure 3b)
8

9 figure 3c) colour
10

11 figure 3c)
12

13 figure 3d) colour
14

15 figure 4a)
16

17 figure 4b) colour
18

19 figure 4b)
20

21 figure 5a) colour
22

23 figure 5a)
24

25 figure 5b) colour
26

27 figure 5b)
28

29 figure 5c) colour
30

31 figure 5c)
32

33 figure 6 colour
34

35 figure 6
36

37 figure 7 colour
38

39 figure 7
40

41 figure 8 colour
42

43 figure 8
44

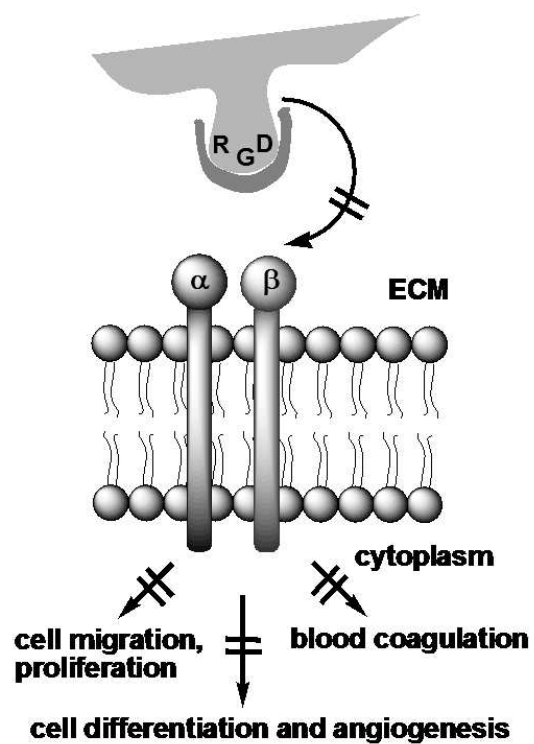
45 figure 9 colour
46

47 figure 9
48

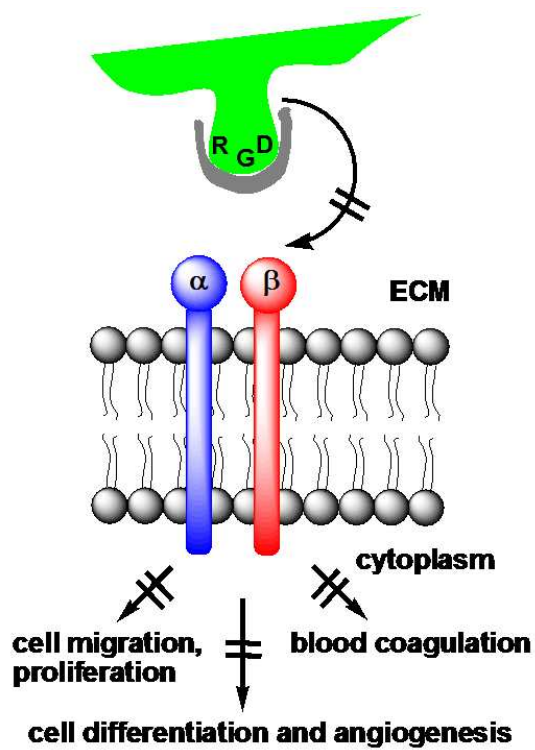
49 figure 10
50
51
52
53
54
55
56
57
58
59
60

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

For Peer Review Only

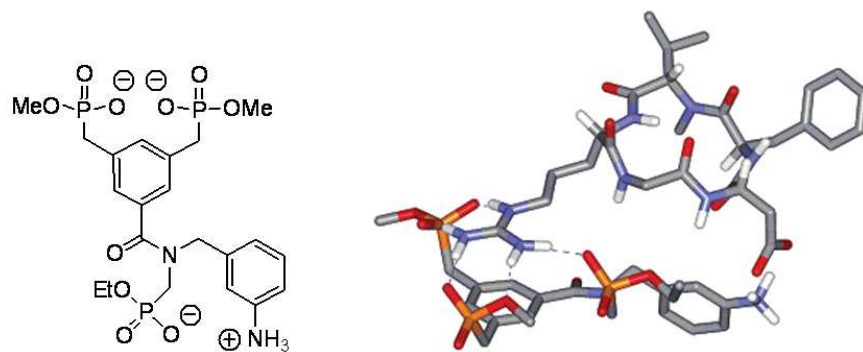


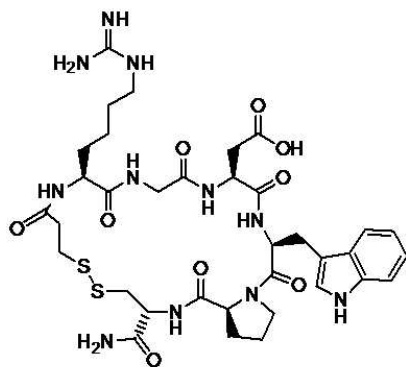
254x190mm (96 x 96 DPI)



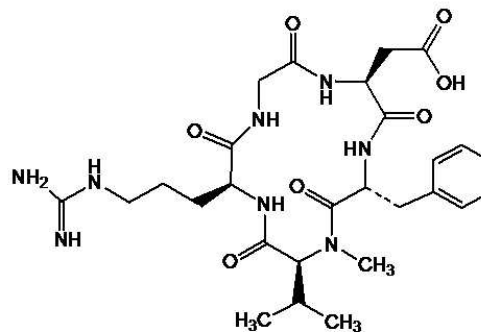
254x190mm (96 x 96 DPI)

new Only

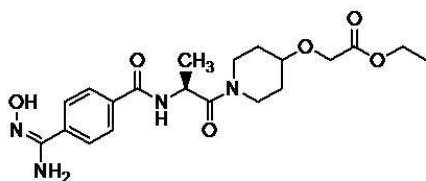




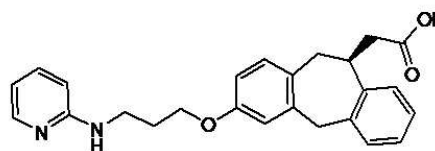
Eptifibatide



cRGDf[NMe]V

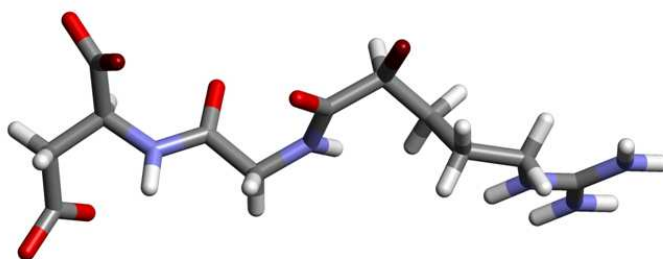
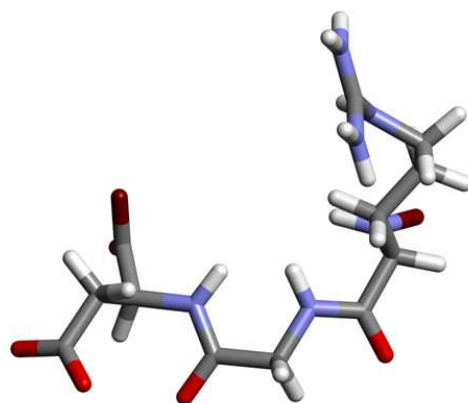


Sibrafiban



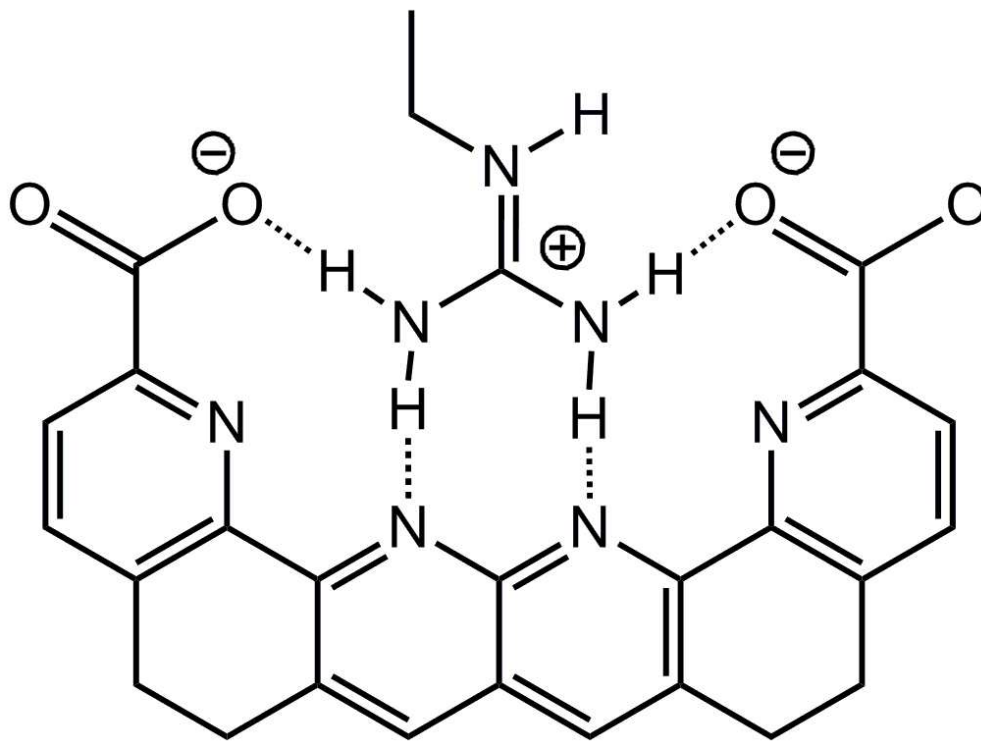
SB-265123

254x190mm (96 x 96 DPI)

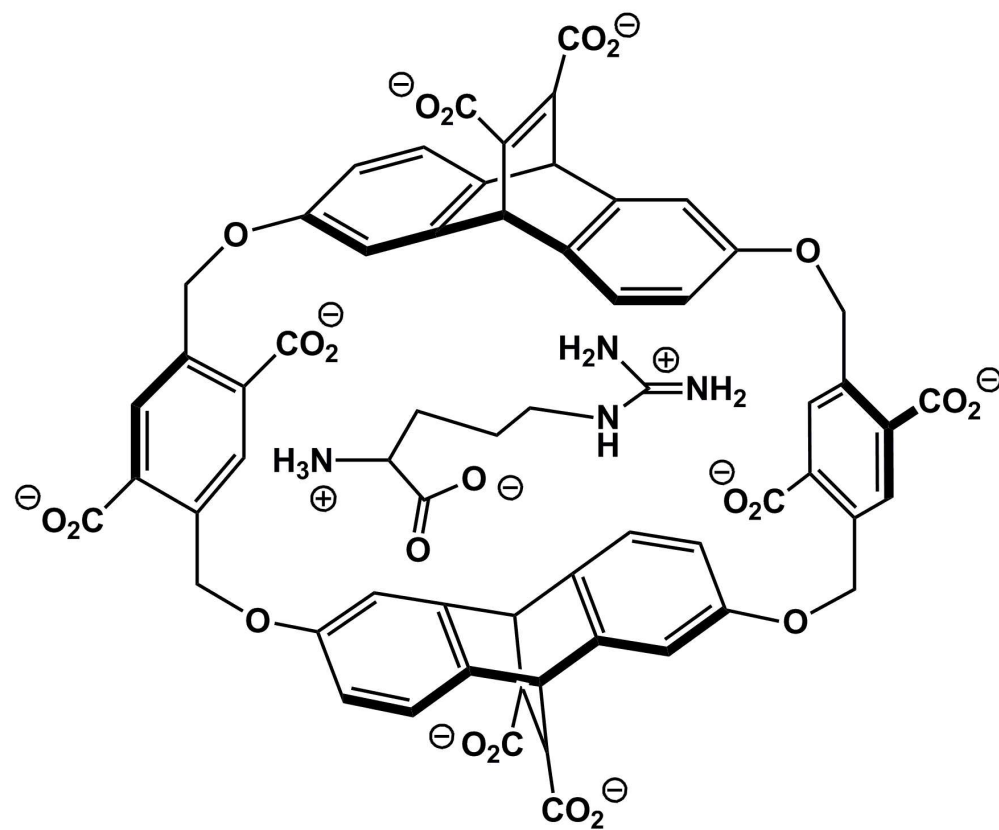


254x190mm (96 x 96 DPI)

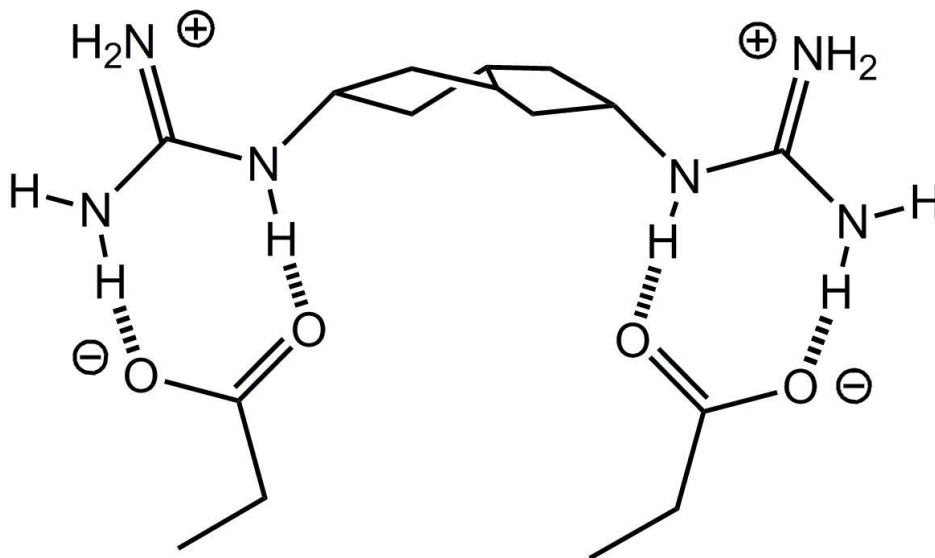
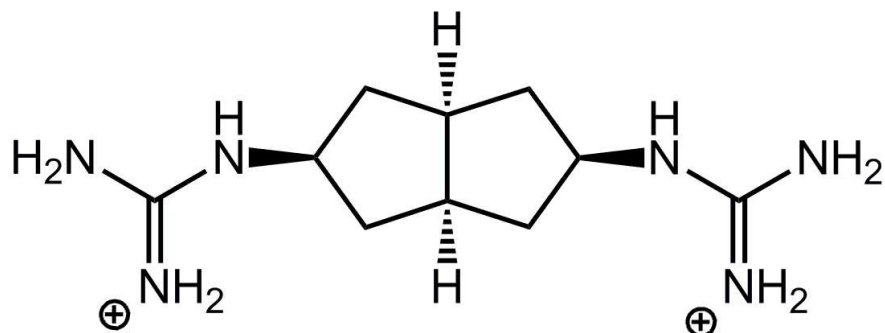
ew Only



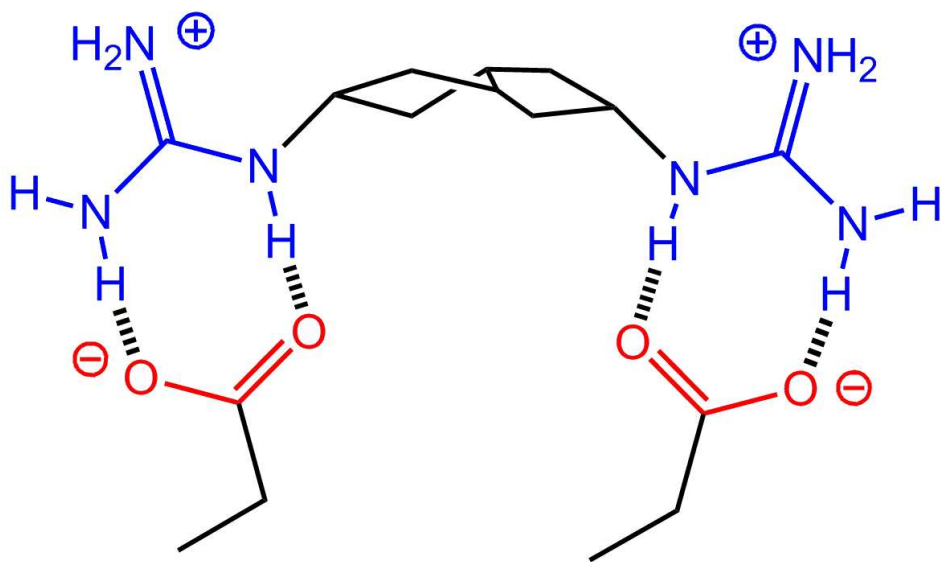
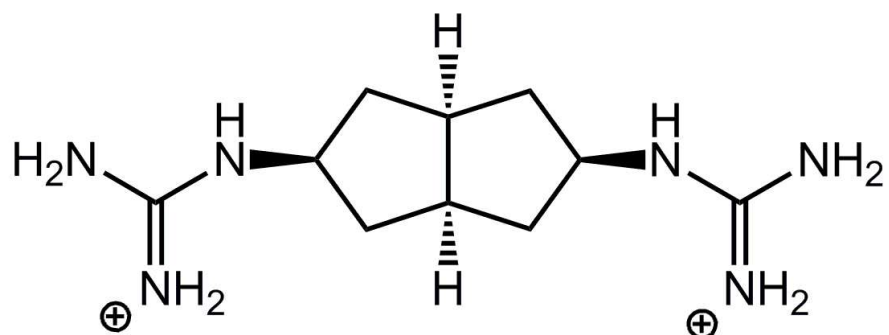
86x64mm (300 x 300 DPI)



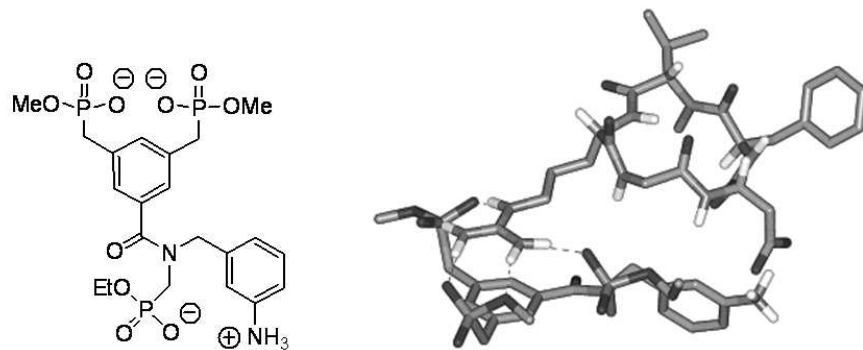
148x123mm (300 x 300 DPI)

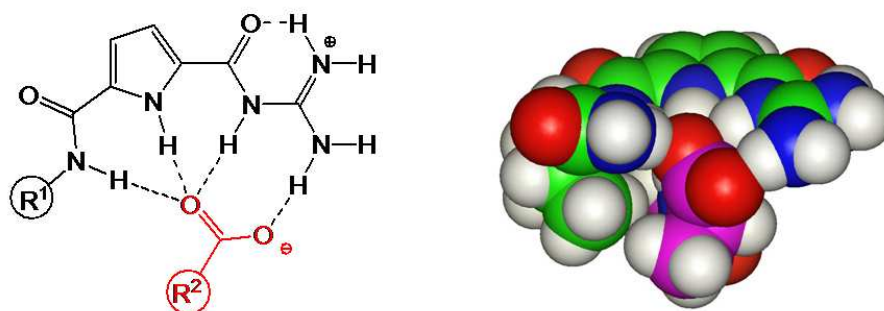


99x126mm (300 x 300 DPI)

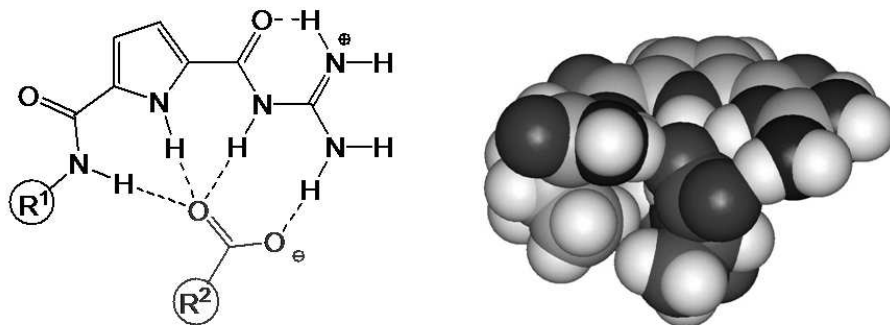


99x126mm (300 x 300 DPI)

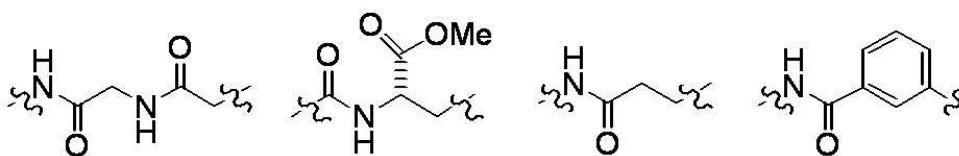
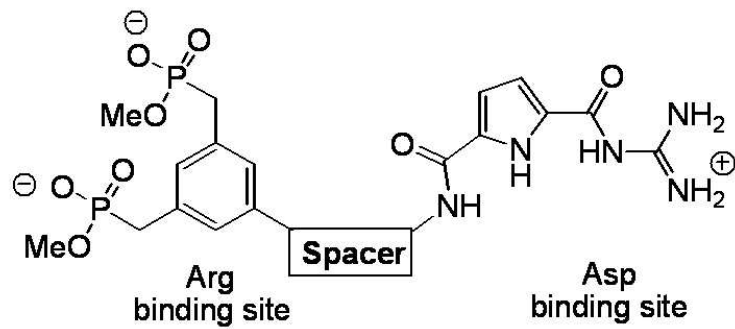




254x190mm (96 x 96 DPI)



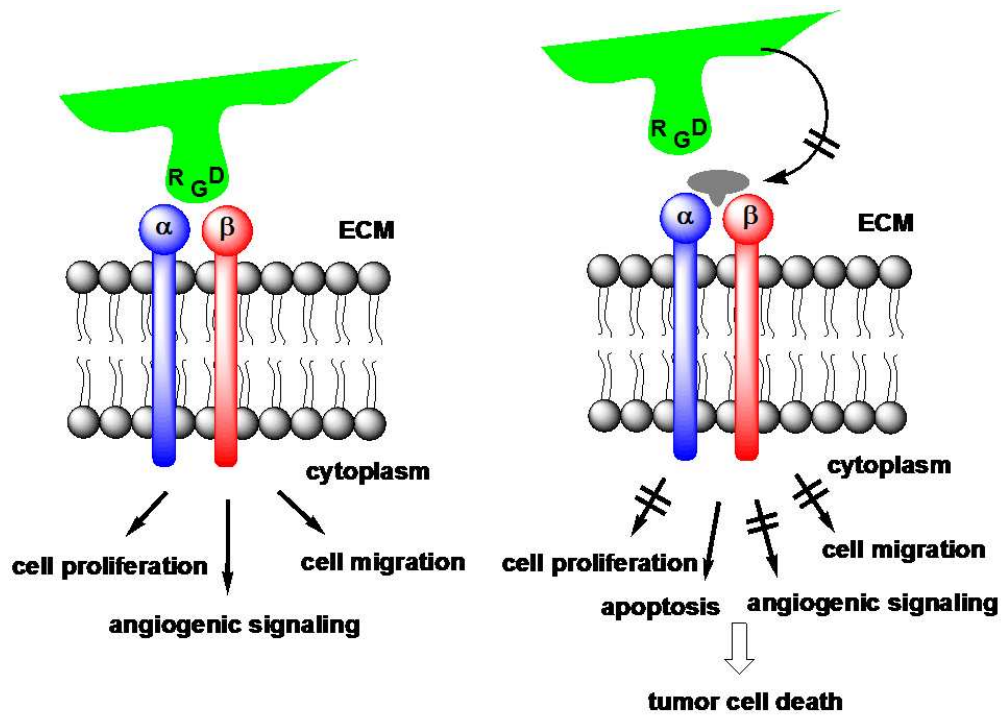
254x190mm (96 x 96 DPI)



34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

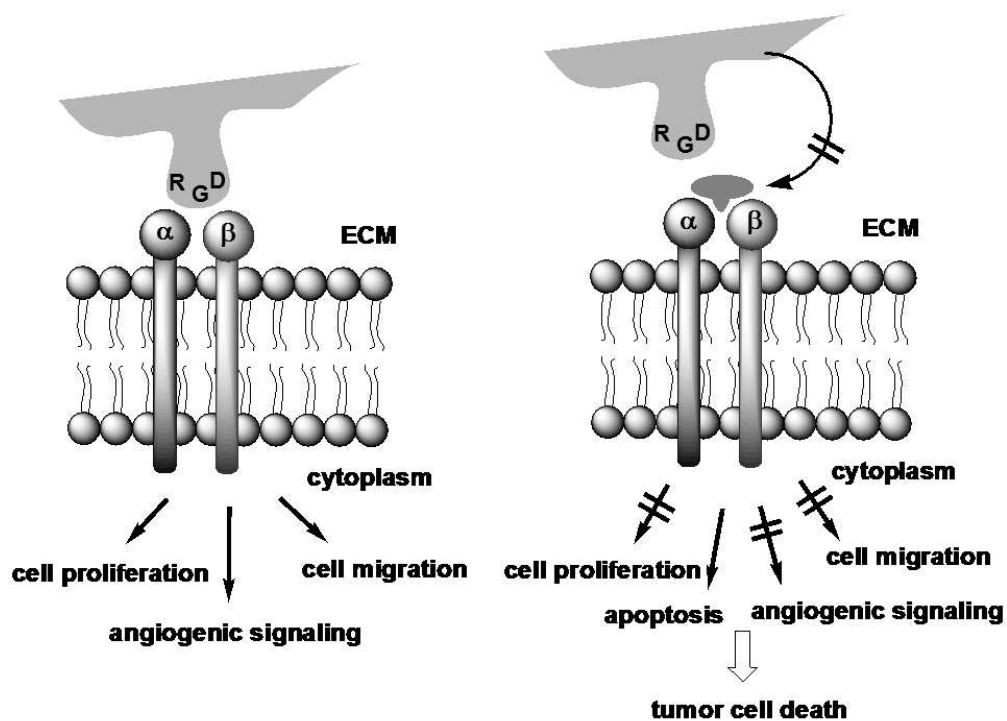
254x190mm (96 x 96 DPI)

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60



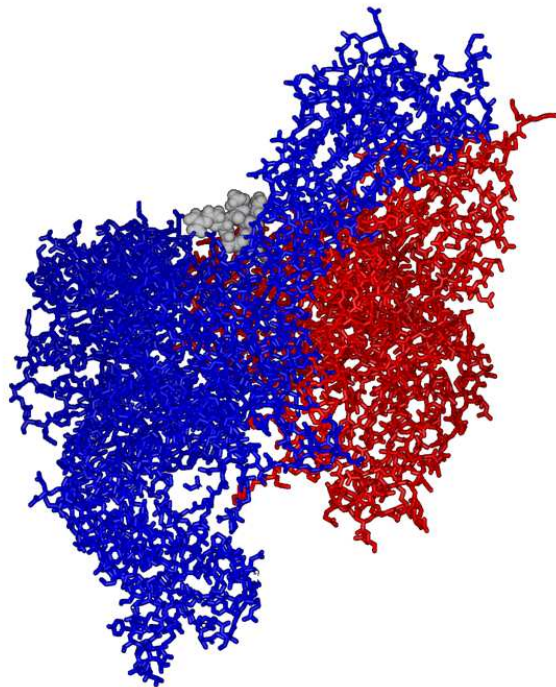
254x190mm (96 x 96 DPI)

new Only



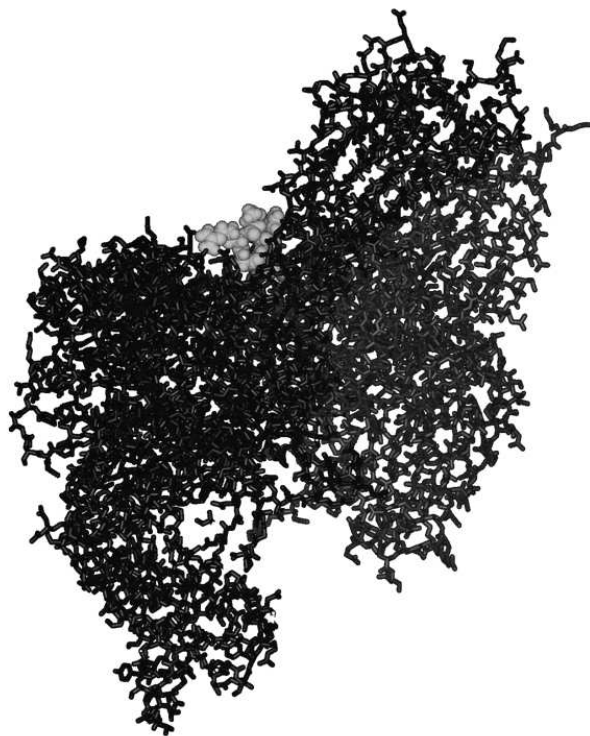
254x190mm (96 x 96 DPI)

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

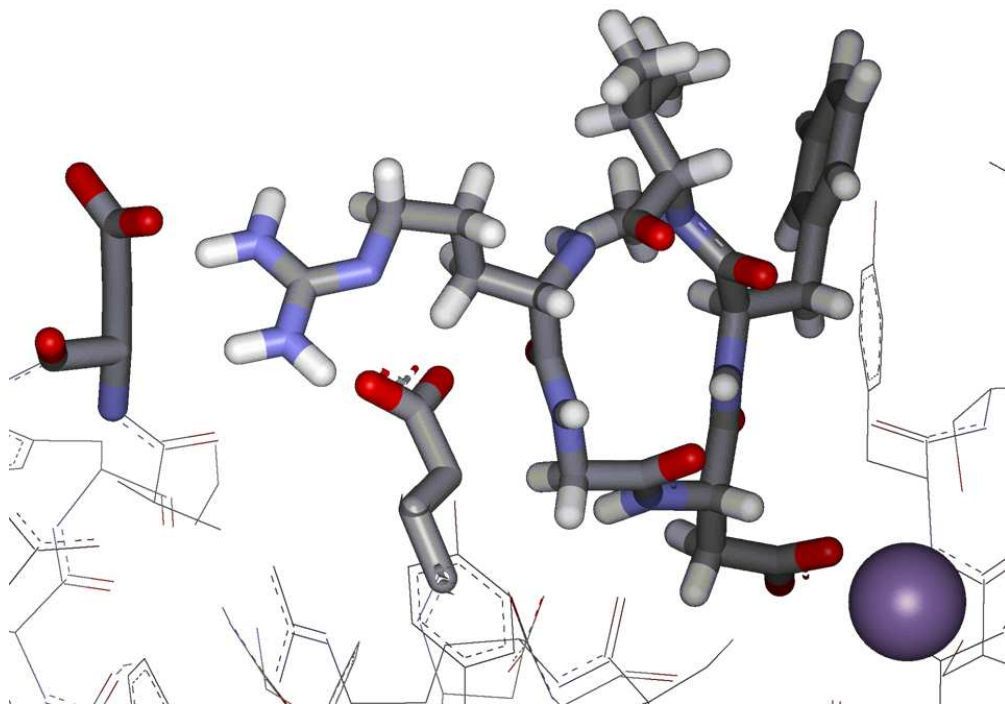


254x190mm (96 x 96 DPI)

Review Only

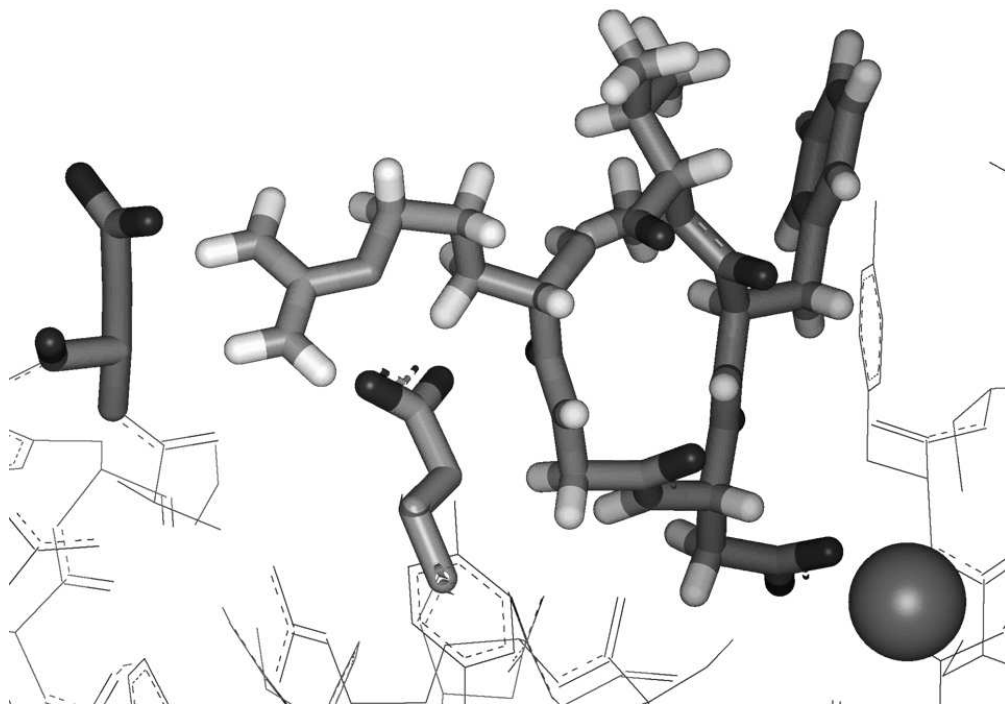


254x190mm (96 x 96 DPI)



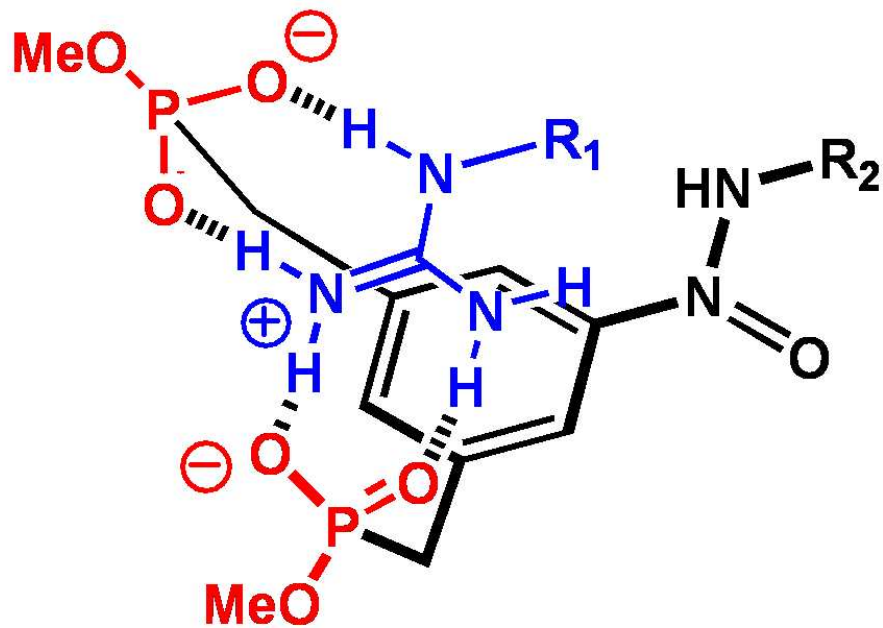
254x190mm (96 x 96 DPI)

Pre-proof Only

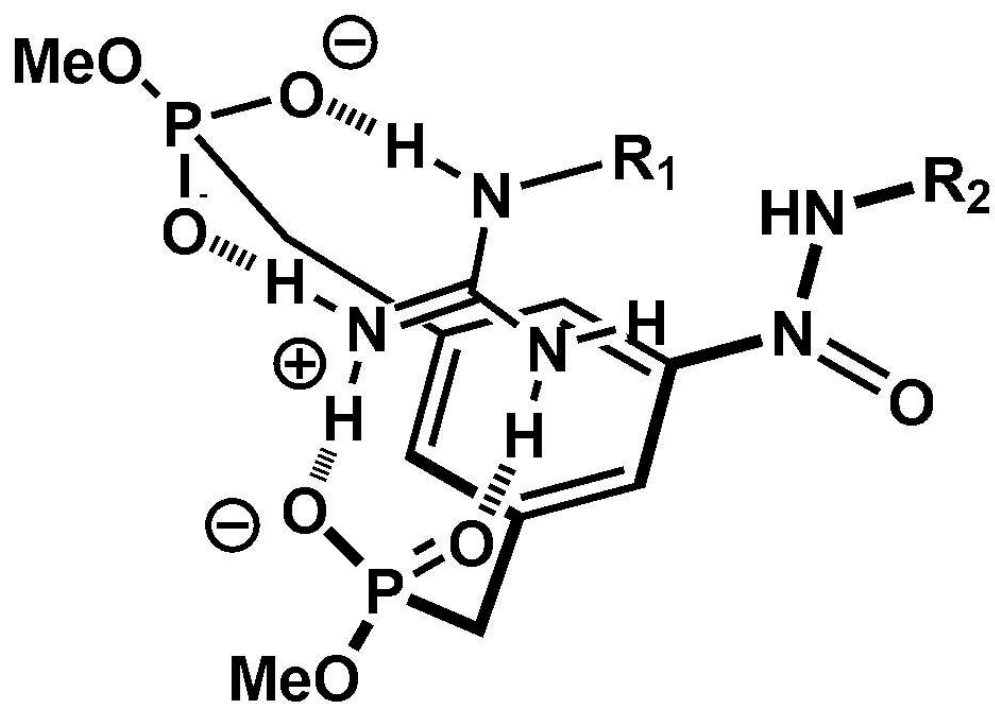


254x190mm (96 x 96 DPI)

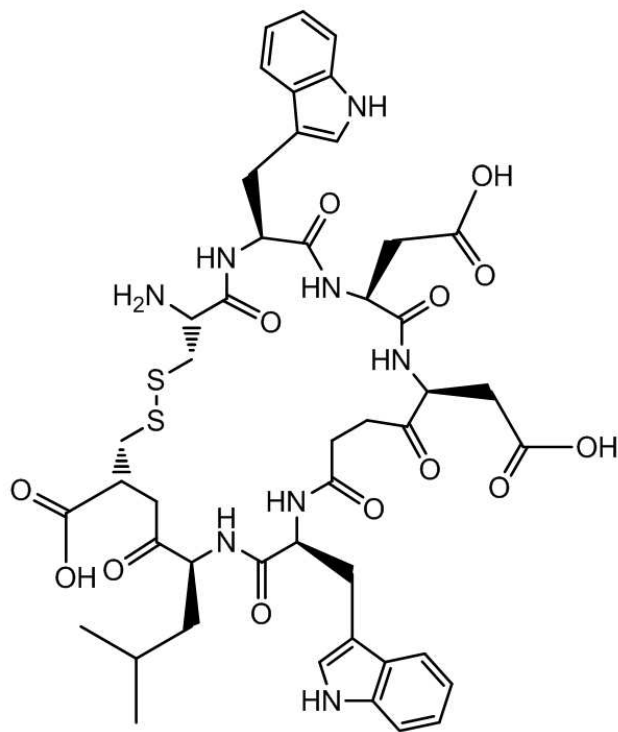
Pre-proof Only



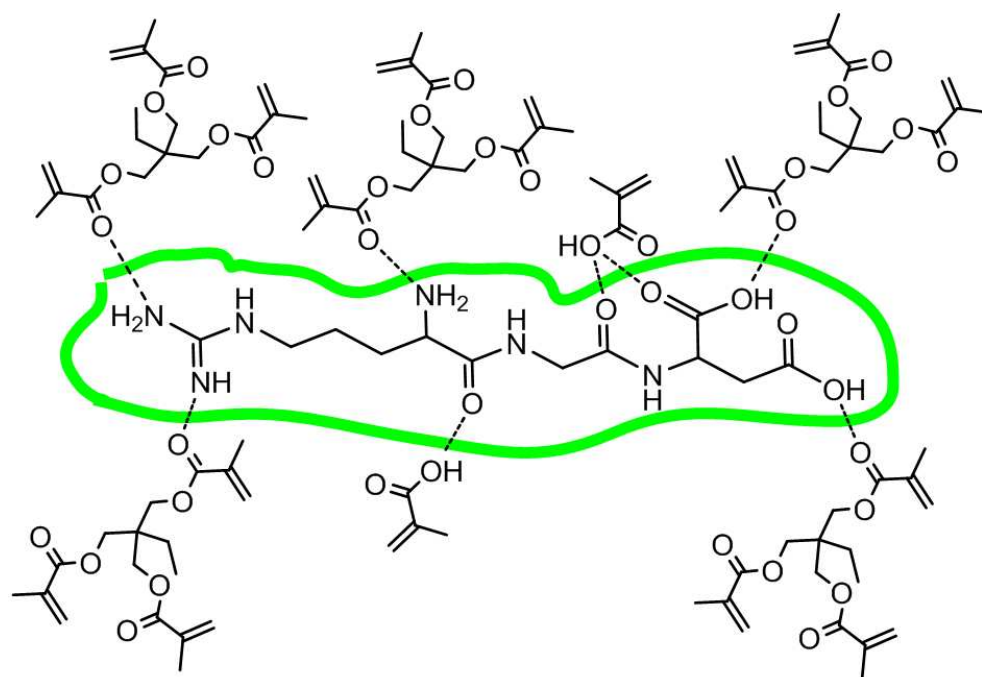
254x190mm (96 x 96 DPI)



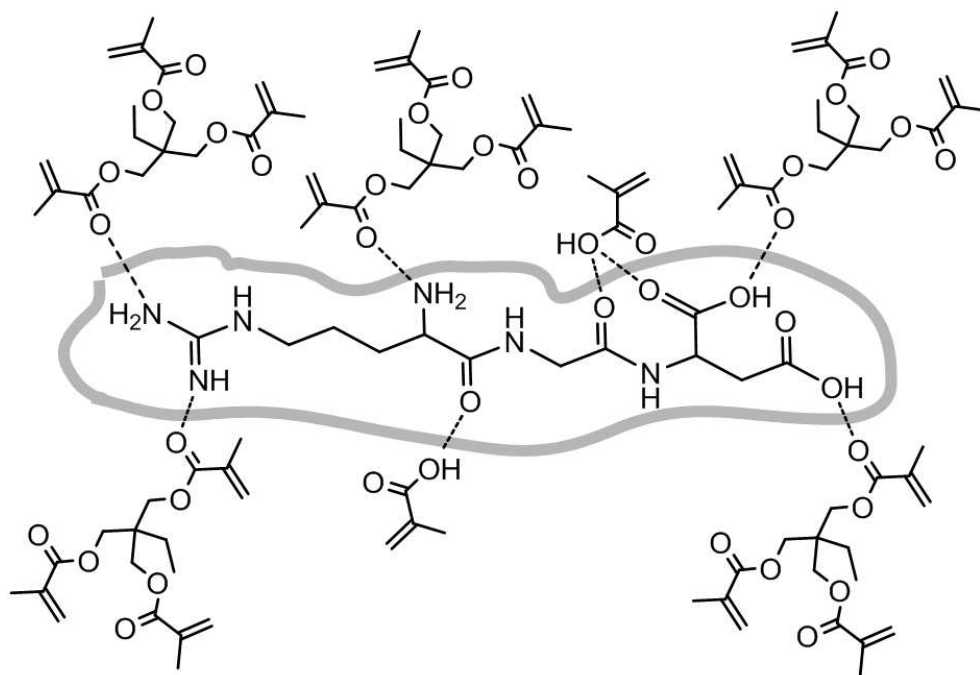
79x56mm (300 x 300 DPI)



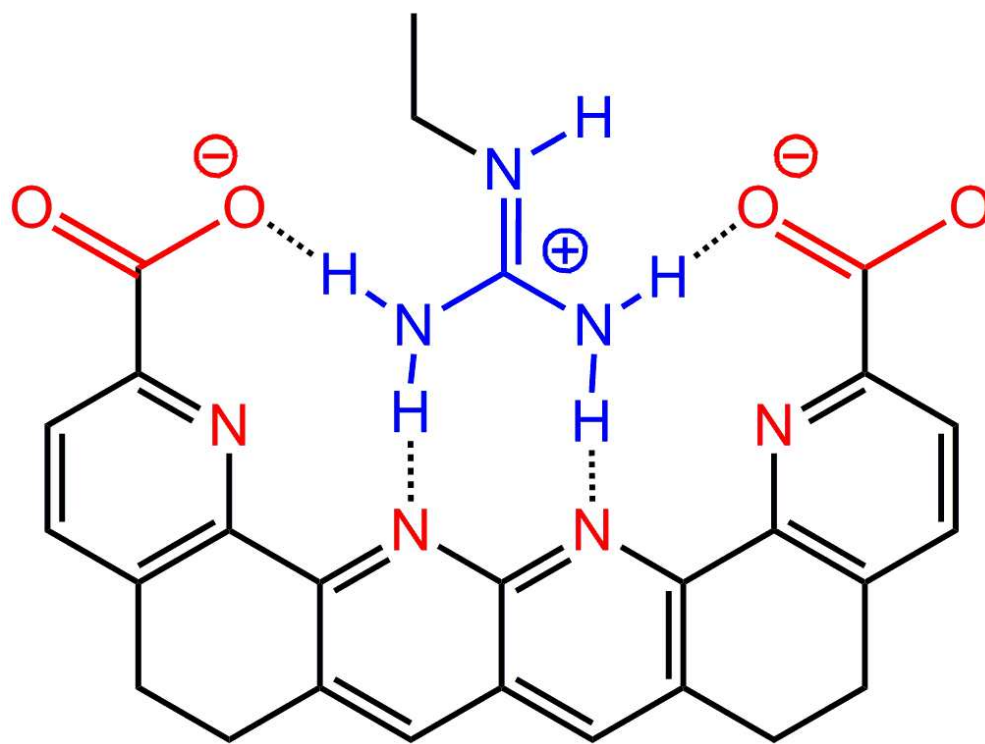
254x190mm (96 x 96 DPI)



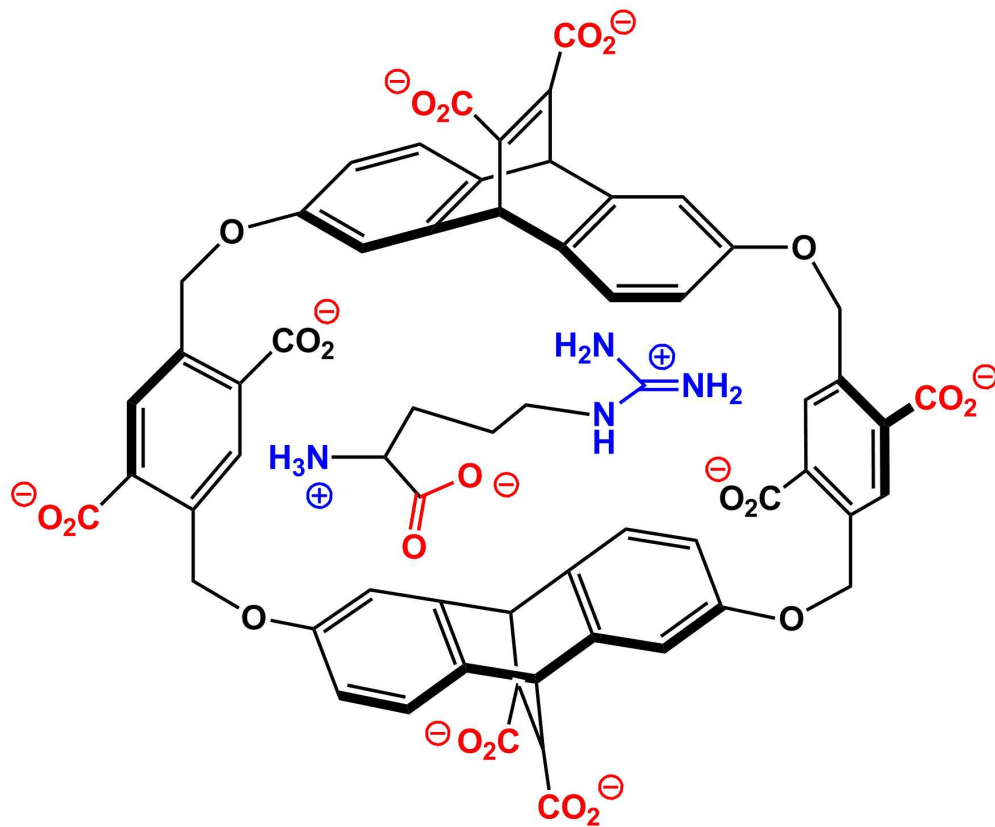
254x190mm (96 x 96 DPI)



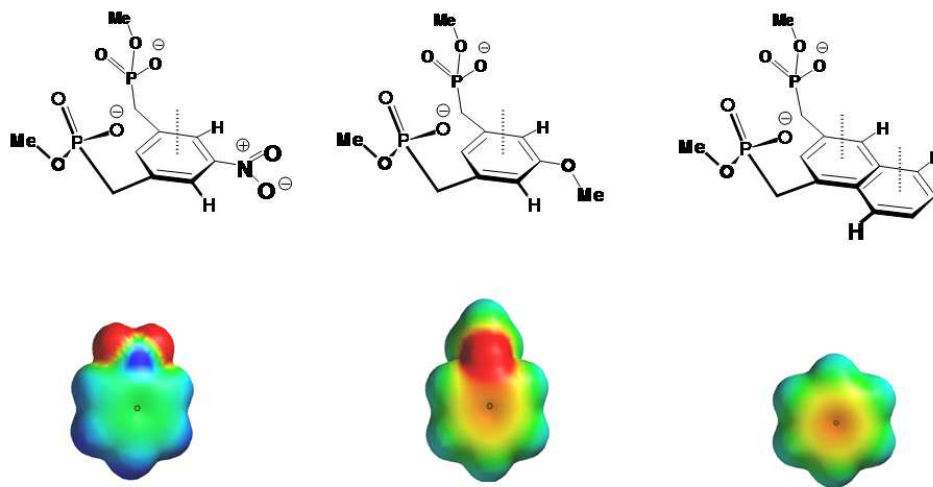
254x190mm (96 x 96 DPI)



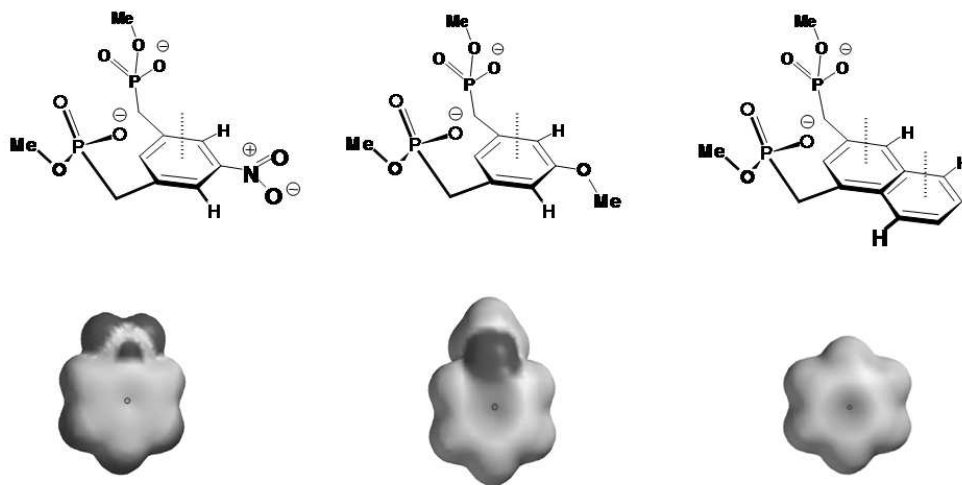
86x64mm (300 x 300 DPI)



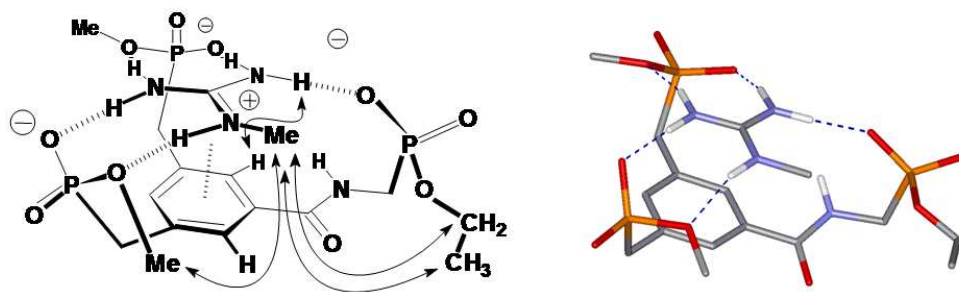
148x123mm (300 x 300 DPI)



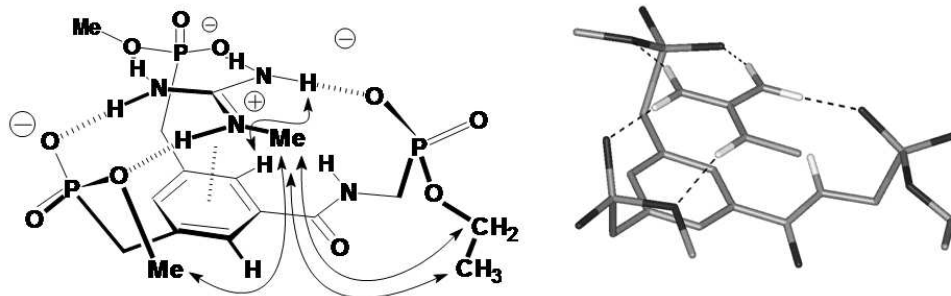
254x190mm (96 x 96 DPI)



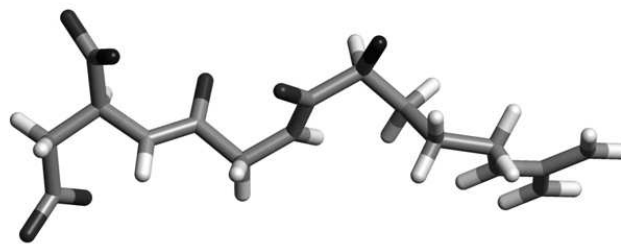
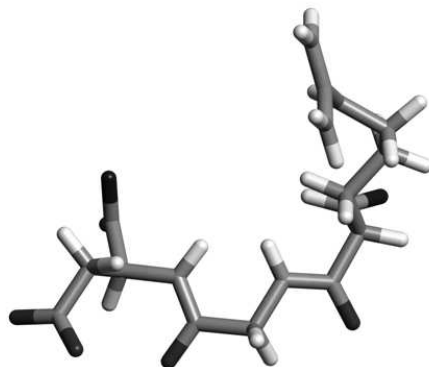
254x190mm (96 x 96 DPI)



254x190mm (96 x 96 DPI)



254x190mm (96 x 96 DPI)



254x190mm (96 x 96 DPI)

Review Only