Nonpeptidic $\alpha_v\beta_3$ Integrin Antagonist Libraries: On-Bead Screening and Mass Spectrometric Identification without Tagging**

Christoph Gibson, Gábor A. G. Sulyok, Diane Hahn, Simon L. Goodman, Günter Hölzemann, and Horst Kessler*

Over the past decade, combinatorial synthesis has progressed from being a little-known fringe area to an important technique in drug research.[1] The split synthesis in particular enables comprehensive substance libraries to be produced rapidly.[2] Such libraries also enable those resin particles with attached active compounds to be characterized by biological on-bead evaluation.[3] However, the coding technique required for the identification of a particular selected substance usually reduces the efficiency of this approach.[1] Herein, we describe the combinatorial solid-phase synthesis of uncoded diacylhydrazine libraries and their biological on-bead evaluation using a soluble $\alpha_v\beta_3$ integrin receptor. Orthogonal anchoring with a photolabile linker made it possible to photolytically cleave the compounds from selected beads and to identify them reliably using mass-spectrometric analysis (MS^n) on the basis of fragmentation patterns. The degree of affinity of the selected diacylhydrazines to the $\alpha_v\beta_3$ receptor was confirmed through receptor-binding studies carried out on the isolated compounds, which underlines the success of our strategy. To our knowledge, this is the first successful application of the "one bead–one compound" concept as applied to uncoded, nonpeptidic compound libraries.[3]

The inhibition of the $\alpha_v\beta_3$ integrin receptor is regarded as a promising goal in the therapy of various pathophysiological processes such as tumor-induced angiogenesis, restenosis, osteoporosis, and acute renal failure.[4] The amino acid sequence Arg-Gly-Asp (RGD), which can be recognized by the $\alpha_v\beta_3$ receptor and by at least ten additional integrins,[5] has been used as a lead structure in the development of $\alpha_v\beta_3$ inhibitors.[4]

In previous work, we have been able to show that the substitution of the glycine in cyclic $\alpha_v\beta_3$-selective RGD peptides can take place with an aza-glycine whilst maintaining both affinity and selectivity.[6] Based on these results, we developed a diacylhydrazine library that meets all requirements of the "one bead–one compound" concept.[3] To achieve this, the lead structure Arg-Gly-Asp was transformed into a RGD mimetic, which could be assembled step-by-step on a solid support according to the Fmoc strategy[7] with the building blocks **A**–**D** (see Figure 1). The RGD mimetics were anchored to the resin with a photolinker[8] to achieve maximum orthogonality to the various reaction conditions of the solid-phase synthesis.

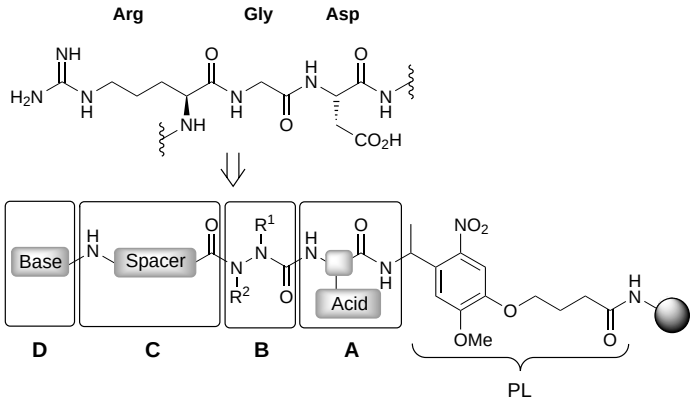

Figure 1. Transformation of the RGD sequence into a modularly assembled RGD mimetic.

The building blocks used for the synthesis of the library are shown in Figure 2.[9] The synthesis of activated aza-substituted building blocks $\mathbf{B^{1-3}}$ and their incorporation onto the solid support has recently been described by us.[10] The building blocks of groups **A** and **C** were selected in such a way that each molecular mass occurred only once within one group: This enabled the structure assignment of a selected RGD mimetic using MS^n (see below). The sequence for the synthesis of the RGD mimetics by applying the split method[2] is shown in Scheme 1. Firstly, the building blocks $\mathbf{A^{1-5}}$ were attached to TentaGel Macrobeads under standard conditions. Building block $\mathbf{A^6}$ could be synthesized on the solid support using a submonomeric approach. To achieve this, TentaGel Macrobeads were acylated with 3-(chloromethyl)benzoyl chloride and the resulting resin-bound benzyl chloride was treated with *tert*-butyl-3-aminopropionate to produce the resin-bound building block $\mathbf{A^6}$. After removal of the Fmoc protecting group, the activated aza-building blocks $\mathbf{B^{1-3}}$ were introduced.[10] Basic deprotection, coupling of the spacer building blocks $\mathbf{C^{1-10}}$, and subsequent removal of the temporary and permanent protecting groups resulted in ten amino-RGD mimetic libraries, each comprising 33 compounds.[11] Through guanylation with $\mathbf{D^1}$ or pyrimidylation with $\mathbf{D^2}$ and subsequent deprotection, two additional libraries each comprising 330 compounds were obtained.[12]

For the quality control of the RGD mimetic libraries and after the biological on-bead evaluation, the compounds were photolytically cleaved from individual beads and characterized subsequently with LC–MS and/or ESI-MS^n. In order to assess the suitability of this strategy, several isolated synthesized aza-RGD mimetics were investigated by MS^n. Figure 3 illustrates the results of the MS^2 analysis of the aza-RGD mimetics **1**–**3**. The MS^2 spectra of the $[M+H]^+$ ions (Figure 3,

[*] Prof. Dr. H. Kessler, Dr. C. Gibson, G. A. G. Sulyok
Institut für Organische Chemie und Biochemie
Technische Universität München
Lichtenbergstrasse 4, 85747 Garching (Germany)
Fax: (+49) 89-289-13210
E-mail: kessler@ch.tum.de
D. Hahn, Dr. S. L. Goodman, Dr. G. Hölzemann
Merck KGaA, Präklinische Forschung
Frankfurter Strasse 250, 64271 Darmstadt (Germany)

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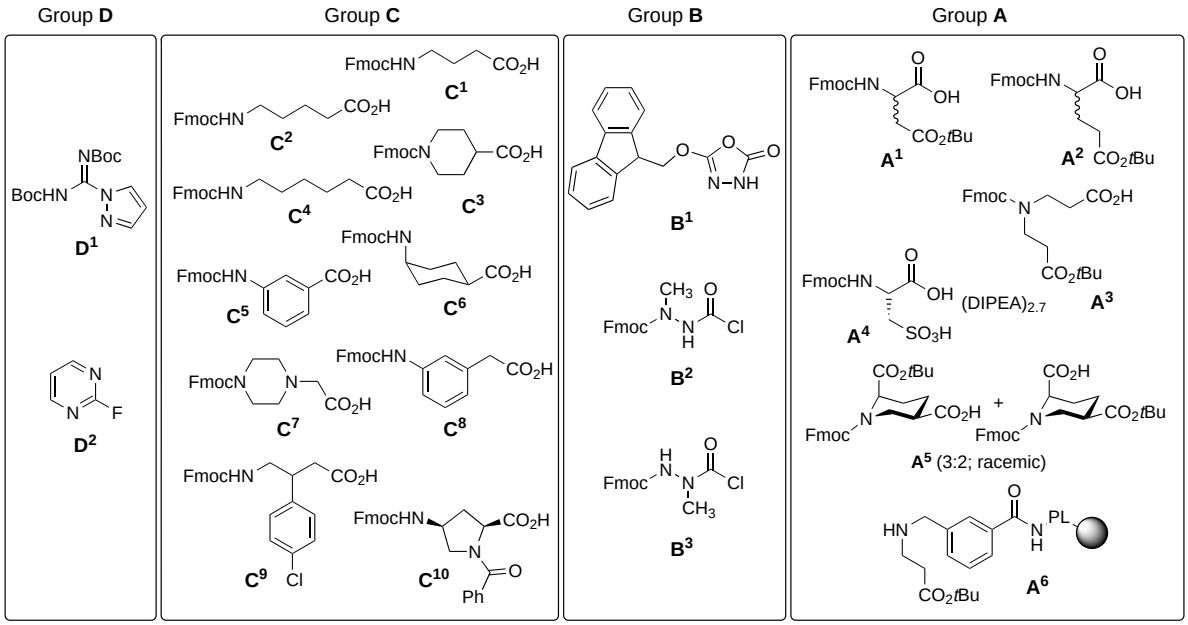
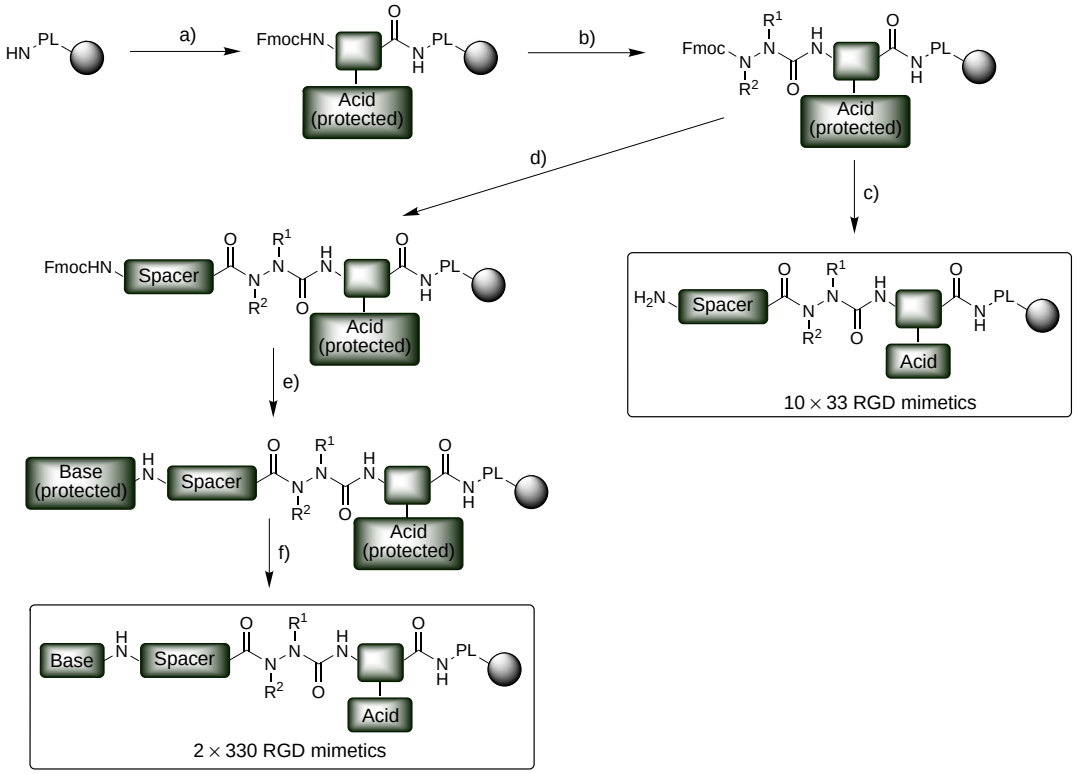


Figure 2. Building blocks used for the synthesis of the RGD mimetic libraries.



Scheme 1. a) **A**[1–5] (3.0 equiv), HATU (2.8 equiv), collidine (30 equiv), DMF, RT, 3 h; **A**[6] 3-(chloromethyl)benzoyl chloride (6.8 equiv), DIEA (14 equiv), CH_2Cl_2, RT, 75 min; *tert*-butyl-3-aminopropionate (150 equiv), DMF, 45 °C, 12 h; b) 20 % piperidine in DMF; **B**[1] (3.1 equiv), CH_2Cl_2, RT, 3 h; **B**[2] (5.1 equiv), DIEA (6.0 equiv), CH_2Cl_2, RT, 2.5 h; **B**[3] (5.1 equiv), DIEA (5.5 equiv), DMF, RT, 15 h; c) 20 % piperidine in DMF; **C**[1–10] (3.0 equiv), HATU (2.8 equiv), collidine (30 equiv), DMF, RT, 12 h; 20 % piperidine in DMF; 50:50:5 CH_2Cl_2:TFA:TIPS, RT, 1.5 h; 20 % DIEA in CH_2Cl_2; d) 20 % piperidine in DMF; **C**[1–10] (3.0 equiv), HATU (2.8 equiv), collidine (30 equiv), DMF, RT, 12 h; e) 20 % piperidine in DMF; **D**[1] (19 equiv), $CHCl_3$, 50 °C, 20 h or **D**[2] (15 equiv), DIEA (15 equiv), DMF, RT, 1 d, then **D**[2] (30 equiv), 5 % $BF_3 \cdot Et_2O$ in DMF, RT, 7 d; f) 50:50:5 CH_2Cl_2:TFA:TIPS, RT, 1.5 h; 20 % DIEA in CH_2Cl_2. Fmoc = fluoren-9-ylmethoxycarbonyl, DMF = *N*,*N*-dimethylformamide, HATU = O-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate, DIEA = diisopropylethylamine, TIPS = triisopropylsilane.

left) and the $[M+Na]^+$ ions (Figure 3, right) show clearly defined signals of the fragment ions of B_B and/or A_B types. This information enables building block **A** to be identified. In particular, the MS^2 spectra of the $[M+Na]^+$ ions of isomeric compounds **2** and **3** show clearly different intensities of the B_B and A_B fragment ions: Compound **2** containing aza-Sar

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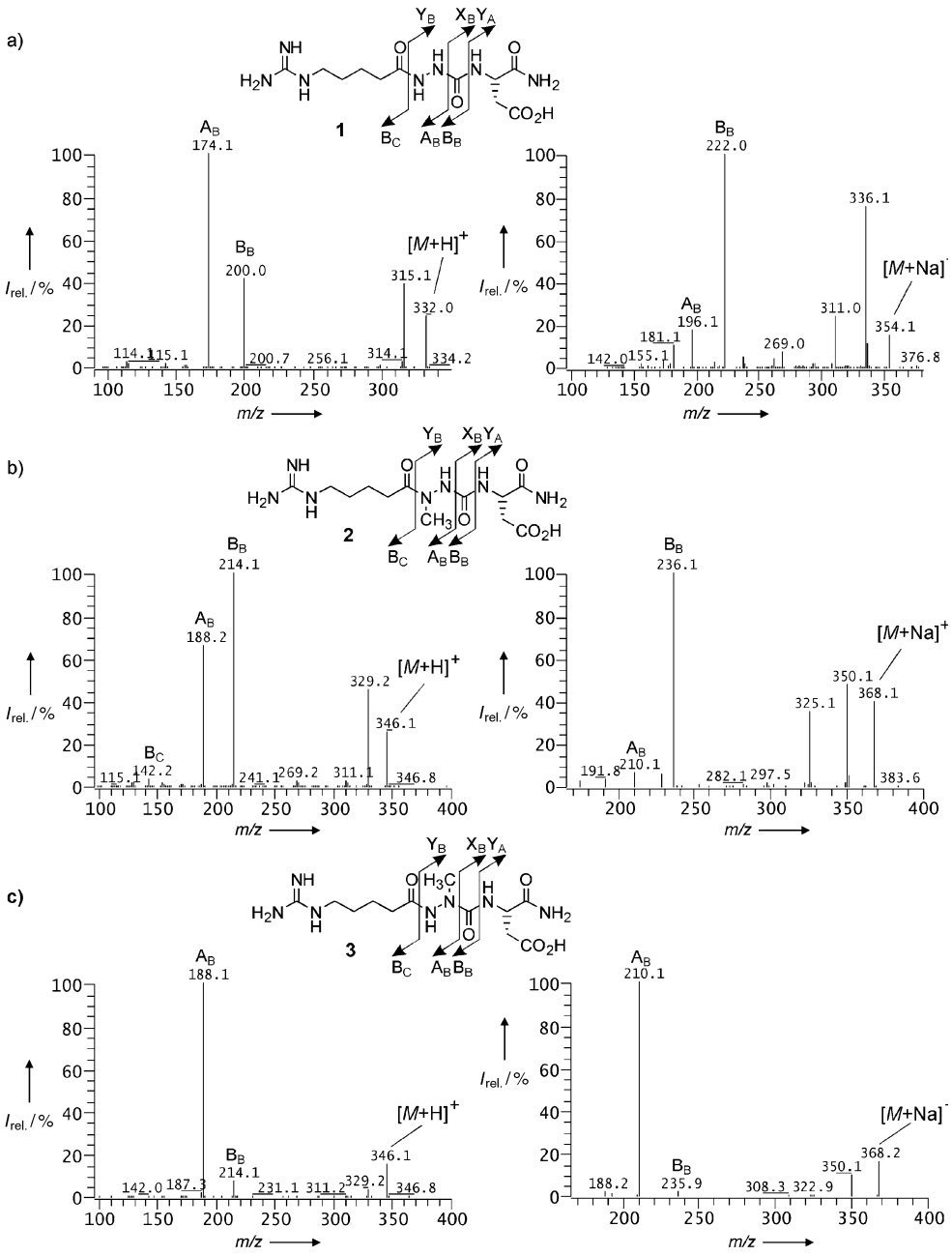

Figure 3. MS^2 analysis of aza-RGD mimetics containing aza-Gly (a), aza-Sar (b), and aza-Ala (c). Note that the $[M+Na]^+$ ion of the aza-Sar-containing compound **2** predominantly yields the fragment ion of the type B_B, whereas the $[M+Na]^+$ ion of the aza-Ala-containing compound **3** predominantly yields the fragment ion of the type A_B. In order to define the fragment ions unequivocally, they were subscripted with the building block, which is adjacent to the fragmentation position in the fragment ion. *RA* = relative abundance.

decomposed with 10:1 selectivity to the B_B fragment ion and compound **3** containing aza-Ala decomposed with approximately the same selectivity to the A_B fragment ion. Thus, one can differentiate clearly between regioisomeric building blocks **B** aza-Sar (**B²**) and aza-Ala (**B³**). In addition, the weak signal of the B_C fragment ion can be recognized in the MS^2 spectrum of the $[M+H]^+$ ion of **2**. On the other hand, this fragment ion appears in all the MS^3 spectra of the B_B and A_B fragment ions of protonated species **1**–**3** in noticeably higher intensities and, hence, enables building blocks **B** and **C** to be assigned. Consequently, our diacyl hydrazines may be fully sequenced using mass spectrometry and unequivocally identified. The reliability of this method was confirmed by applying MS^n analysis to additional aza-RGD mimetics. In this context, the fragment ions of the types X and Y, that correspond to the fragment ions of the types A and B, were also observed.

The ionization properties of the amino- as well as the guanyl-RGD mimetics were considerably improved by introduction of an "N-terminal" Boc protective group. Most of the aza-RGD mimetics investigated exhibited a high degree of purity; about one third of the LC-MS-spectra additionally contained a compound lighter by *m*/*z* 18 with a relative proportion of 30–70%.

For biological on-bead evaluation, the resin-bound RGD mimetics were first incubated with the soluble, biotinylated $\alpha_v\beta_3$ receptor and subsequently with a monoclonal antibiotin alkaline phosphatase conjugate. If a compound was recognized by the receptor, the respective bead could be stained with the well known alkyl phosphatase substrate 5-bromo-4-chloro-3-indolyl phosphate disodium salt (BCIP).[13] No positive beads were found in the amino-libraries. Figure 4 depicts the beads of the guanylated and pyrimidylated libraries after on-bead evaluation with the $\alpha_v\beta_3$ receptor. In the guanylated library, there were four intensely stained and ten weakly stained beads; in the pyrimidylated library, two weakly stained beads were observed.

Using LC-MS and MS^n, all RGD mimetics bound to the beads and designated positive could be characterized unequivocally. The four intensely stained beads all carried aromatic compound **5** (Table 1). Of the ten weakly stained beads from the same library, compound **5** was detected once, compound **6** five times, compound **7** three times, and compound **8** once. Compound **9**—analogous to RGD mimetic **5**—was detected on the two beads selected from the pyrimidylated library. For the determination of the inhibition

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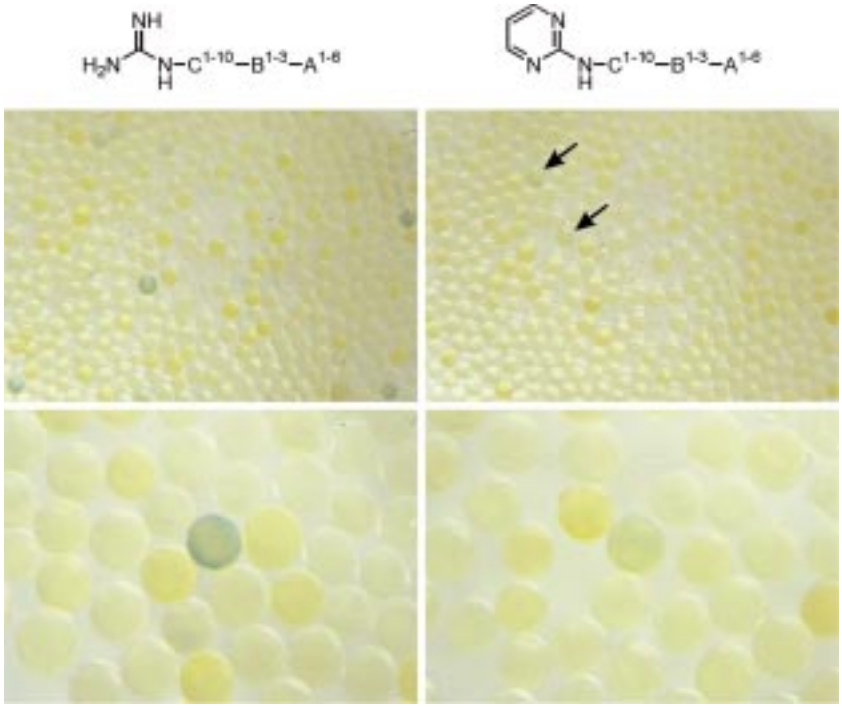
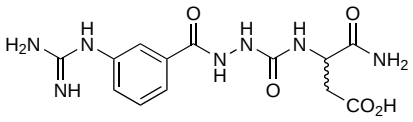
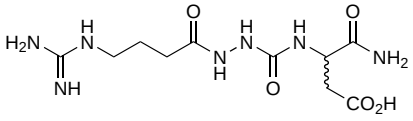
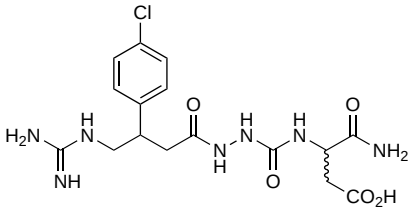
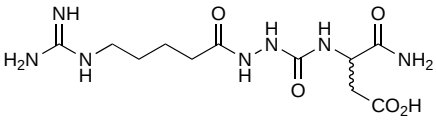
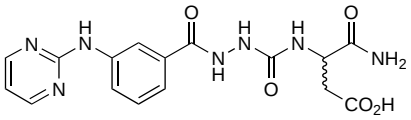
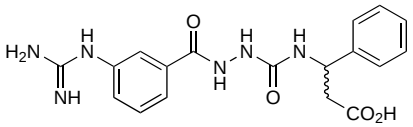


Figure 4. TentaGel Macrobeads after the on-bead receptor assay with the $\alpha_v\beta_3$ integrin. Above: fivefold enlargement, below: fifteenfold enlargement.

constants with respect to the isolated $\alpha_v\beta_3$, $\alpha_v\beta_5$,[4b] and $\alpha_{IIb}\beta_3$[14] receptors, the selected RGD mimetics were synthesized on Rink amide 4-methyl-benzhydrylamine (MBHA) resin as L- and D-enantiomers, respectively. All RGD mimetics showed a measurable activity on the $\alpha_v\beta_3$ receptor but the L-enantiomers, however, exhibited throughout a higher affinity to the $\alpha_v\beta_3$ receptor than the respective D-enantiomers. With an IC_{50} value of 150 nM, compound **5** showed, in contrast to the other selected aza-RGD mimetics, the highest affinity to the $\alpha_v\beta_3$ receptor; thus, the color intensity of the positive beads clearly correlates with the affinity of the resin-bound RGD mimetics to the receptor.

Only the L-isomers of **5** and **6** showed a weak affinity to the $\alpha_v\beta_5$ receptor, both being less pronounced when compared to the $\alpha_v\beta_3$ receptor. All selected aza-RGD mimetics inhibited the binding of the platelet receptor $\alpha_{IIb}\beta_3$ to fibrinogen with IC_{50} values of $>100\ \mu M$. Thus, all the selected

Table 1. Inhibition behavior of the different RGD mimetics with regard to binding of vitronectin to the isolated $\alpha_v\beta_3$ or $\alpha_v\beta_5$ receptor, as well as the binding of fibrinogen to the isolated $\alpha_{IIb}\beta_3$ receptor. The linear peptide GRGDSPK **4** was chosen as standard.

No.	Structure and frequency		Isomer of A	IC_{50} [μM] $\alpha_v\beta_3$	$\alpha_v\beta_5$	$\alpha_{IIb}\beta_3$
4	GRGDSPK			0.40	42	1.1
5		4 of 4; 1 of 10	L-Asp-NH_2	0.15	7.2	>100
			D-Asp-NH_2	7.2	>100	>100
6		5 of 10	L-Asp-NH_2	3.1	57	>100
			D-Asp-NH_2	50	>100	>100
7		3 of 10	L-Asp-NH_2	5.0	>100	>100
			D-Asp-NH_2	41	>100	>100
8		1 of 10	L-Asp-NH_2	6.8	>100	>100
			D-Asp-NH_2	47	>100	>100
9		2 of 2	L-Asp-NH_2	15	>100	>100
			D-Asp-NH_2	53	>100	>100
10				0.0026	0.280	8.3

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aza-RGD mimetics showed a clear preference for the $\alpha_v\beta_3$ receptor.

Lead structure **5** was found to exhibit a relatively high degree of polarity; an unfavorable pharmacokinetic profile can thus be expected for this compound. However, it has been shown in the past that a hydrophobic residue in the β-position to the carboxy group is tolerated by the $\alpha_v\beta_3$ receptor.[4d] For this reason, we replaced the terminal carboxamide group with a phenyl residue. The less polar RGD mimetic **10** exhibited similar selectivity and considerably increased activity on the $\alpha_v\beta_3$ receptor compared to the polar mimetic **5**.

The results obtained would appear to support our concept for identifying new, low molecular weight integrin ligands through application of combinatorial solid-phase synthesis, biological on-bead evaluation, and mass spectrometry to the selected compounds.

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[9] For the synthesis of building blocks **A**$^{3-6}$, see the Supporting Information. Although the regioisomers of compound **A**^{5} could be separated by HPLC, they were used as a mixture of isomers for the library synthesis.

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[11] Six different building blocks in group **A** were used for the library synthesis, whereas building blocks **A**^{1} and **A**^{2} were used as a racemate and **A**^{5} as a racemate and a mixture of regioisomers. In consideration of the isomers in group **A**, 11 different building blocks have been employed.

[12] The guanylation was performed as per: Y. Wu, G. R. Matsueda, M. Bernatowicz, *Synth. Commun.* **1993**, *23*, 2055 – 3060; the pyrimidylation was performed as per: C. Gibson, H. Kessler, *Tetrahedron Lett.* **2000**, *41*, 1825 – 1728.

[13] The substrate BCIP is usually used together with the oxidation agent 4-nitro-blue-tetrazolium (NBT). However, NBT proved to be incompatible with our libraries because it reacted with some of the resin-bound compounds and led to false-positive results.

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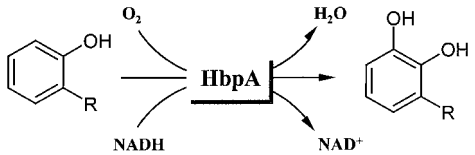
The First Synthetic Application of a Monooxygenase Employing Indirect Electrochemical NADH Regeneration**

Frank Hollmann, Andreas Schmid,* and Eberhard Steckhan†

In memory of Eberhard Steckhan

One of the most important challenges in applying monooxygenase reactions in vitro is to find an effective regeneration system for the necessary co-enzyme (mostly NAD(P)H). The well-established methods for the regeneration of the nicotinamide co-enzyme mainly consist of an enzyme-coupled approach utilizing formate dehydrogenase[1, 4c] (for NAD(P)H) or glucose-6-phosphate dehydrogenase (for NADPH).[2] Additionally, non-enzymatic redox catalysts have been developed and successfully applied to NAD(P)H-dependent dehydrogenases.[3] Thus only the producing enzyme and a mediator together with the electrode, as a source of reducing equivalents, are needed.

Here we report on the first application of an isolated monooxygenase with an indirect electrochemical regeneration of NADH. The enzyme employed is the 2-hydroxybiphenyl-3-monooxygenase (HbpA, E.C. 1.14.13.44), a member the class of flavine-dependent monooxygenases, from *P. azelaica*.[4] The homotetramer with a total mass of 256 kDa catalyzes the specific *ortho*-hydroxylation of several α-substituted phenol derivatives (Scheme 1). To the best of our knowledge no chemical counterpart with comparable specificity is known.



Scheme 1. Specific *ortho*-hydroxylation of α-substituted phenols catalyzed by 2-hydroxybiphenyl-3-monooxygenase. R = alkyl (Et, Pr, *i*Pr), aryl (Ph, 2-HOC_6H_4), Hal (F, Cl, Br).

For the regeneration of NADH we applied the [Cp*Rh(bpy)Cl]Cl complex which had been developed in our group (Cp* = C_5Me_5; bpy = 2,2′-bipyridine). The corresponding hydridorhodium complexes, which can be generated either electrochemically by cathodic reduction at -750 mV (versus $Ag/AgCl_{sat.}$) or chemically with formate, transform $NAD(P)^+$ efficiently into the enzymatically active 1,4-NAD(P)H form[3, 5] (Scheme 2). The conversion rates

[*] Dr. A. Schmid, Dipl.-Chem. F. Hollmann
Institut für Biotechnologie
ETH Hönggerberg
8093 Zurich (Switzerland)
Fax: (+41) 1-633-1051
E-mail: andreas.schmid@biotech.biol.ethz.ch
Prof. Dr. E. Steckhan†
Institut für Organische Chemie und Biochemie
der Universität Bonn (Germany)

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