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Synthesis and biological evaluation of new conformationally biased integrin ligands based on a tetrahydroazoninone scaffold

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Abstract—The synthesis of new conformationally biased cyclic pentapeptides, incorporating the RGD sequence, and built around a tetrahydroazoninone scaffold, is reported. They exhibit interesting activity towards integrin $\alpha_V\beta_3$ and a remarkable selectivity in comparison with integrin $\alpha_V\beta_5$.

A successful strategy for the design of small molecule inhibitors of protein–protein interactions[1,2] relies in the rational transformation of a biologically important peptide ligand into a peptidomimetic. Since the spatial position of the amino acid side chains involved in the interaction is of paramount importance, peptidomimetics should be best constructed around a rigid or semirigid scaffold that displays these side chains in the correct orientation.[3] Modification of the scaffold nature may be valuable as a chemical tool for better understanding the right topology that leads to potent and selective binders.

Integrins are large heterodimeric surface receptors, which regulate cell–cell and cell–matrix interactions. They are classified according to the nature of their α and β subunits. In the course of an inter-university national project, we are currently interested especially in integrins $\alpha_V\beta_3$ and $\alpha_V\beta_5$, which are involved in important physiological processes, including tumour induced angiogenesis.[4,5] These two receptors are known to interact with the recognition motif RGD, formed by the three aminoacids arginine, glycine and aspartic acid. Cyclic pentapeptides containing this motif have been synthesized by the Kessler's group as constrained highly active ligands for integrin $\alpha_V\beta_3$.[6] In particular c[f(*N*-MeV)RGD] (Cilengitide) is currently under clinical evaluation.

Later, various groups have substituted the two additional aminoacid units with a rigid monocyclic[7,8] or bicyclic scaffold,[9,10] finding, in some cases, novel very efficient ligands.

Z-Tetrahydroazoninones **1** (Fig. 1) are a new family of mesocyclic scaffolds corresponding to a constrained dipeptide.[11,12] We have previously shown that this structure can be accessed in just two synthetic steps, by coupling an Ugi multicomponent reaction with a subsequent ring closing metathesis.[11] An advantage implied in this synthetic approach is that diverse

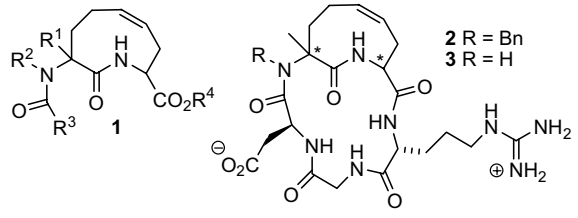

Figure 1.

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substituents can be placed at will as R^1 and R^2. Therefore, a 'fine tuning' of the structural and conformational properties of **1** can be achieved in a straightforward way, without the need to design each time a de novo synthesis. Moreover, the multicomponent reaction allows the introduction of a first aminoacid (R^3CO) making the overall cyclopeptide synthesis more convergent.

We have already described the application of this chemistry to the synthesis of a first cyclic pentapeptide where a GGG triad was anchored to the scaffold.[13] We now report the synthesis of both *cis* diastereoisomers of pentapeptides **2** and **3**, containing the RGD recognition sequence (Fig. 1).

The synthetic routes are depicted in Scheme 1. For the preparation of the *N*-benzylated scaffolds **12a**,**b**, we reacted isocyanide **4**[13] with commercially available protected L-aspartic acid **5** and with the preformed imine of 5-hexen-2-one and benzylamine.[13] When the reaction was carried out under the typical conditions used before (1 M in EtOH at rt), it was rather sluggish and substantial deprotection of the Fmoc group was observed. After a thorough investigation, we found out that the use of a higher dilution and of CF_3CH_2OH/EtOH 2:1 as solvent completely suppressed Fmoc cleavage, and allowed the obtainment of the diastereoisomeric mixture of **6a** and **6b** in good (77%) yield. As expected, the Ugi reaction gave nearly no stereoselection at all. However, this fact did not represent a problem for our purposes (we were interested to test both stereoisomers). Moreover, we found that the two diastereoisomers could be very easily separated through a crystallization from Et_2O/petroleum ether. While the crystalline isomer had a mp of 148.3 °C, the other one was a foam that resisted all crystallization attempts.[14]

The ensuing ring closing metathesis, carried out separately on the two isomers with Grubbs 1st generation catalyst,[15] turned out to be exceptionally efficient. No intermolecular side-products were detected and the isolated yields approached 90% (that became 95% taking into account the recovered starting diene). We think that the combined bulkiness of the malonate and of the Fmoc group prevents complexation of the metal by the amide groups, that is known to be deleterious for these reactions.[13,16] As usual in this series, the double bond was formed only in the *Z* configuration.

Monosaponification of the malonate in **8a**,**b** caused also Fmoc deblocking. So we preferred to change at this stage the amine protecting group shifting to the allyloxycarbonyl (Alloc). This was performed in two simple and high yielding steps. Monosaponification of **10a**,**b**, followed by decarboxylation, afforded the key intermediates **12a**,**b** together with their *trans* counterparts **13a**,**b**.[17] To our pleasure this reaction was somehow selective favouring the desired *cis* compounds **12**.[18]

In the case of **12a** separation from **13a** was easy, either via chromatography or crystallization. On the contrary, we were not able to separate efficiently **12b** from **13b**. This turned out not to be a problem, since only **12b** cyclized efficiently to the final cyclopeptide (vide infra).

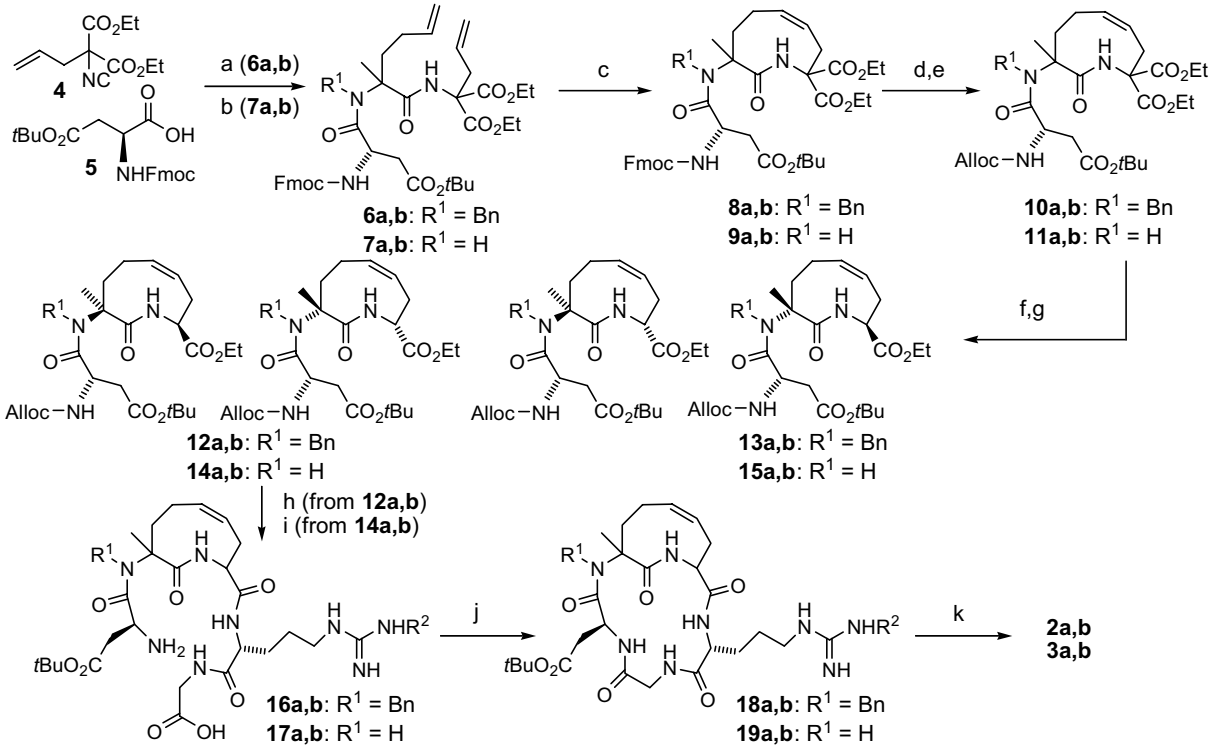
A particular feature of scaffolds **12a**,**b** is the presence of the *N*-benzyl group which of course prevents one of the hydrogen bonds possible in Kessler fVRGD and f(*N*-MeV)RGD cyclopentapeptides. In order to check the importance of the free NH group in this position, we synthesized also the related scaffolds **14a**,**b**, where a free NH group is bound to aspartic acid. For this purpose, benzylamine was substituted with ammonia in the Ugi reaction.

Although examples of Ugi reaction with ketones and ammonia are very rare in the literature,[19,20] we were able, after some optimization, to obtain adducts **7a**,**b** in satisfactory yield. In this case separation of the two diastereoisomers turned out to be impossible neither at this stage nor later and therefore we carried out the synthesis up to the final product **3** on this 50:50 mixture. The route from **7a**,**b** to **14a**,**b** was similar to the one already developed for **12a**,**b**, but was in general less efficient. For example, the RCM was in this case less clean, giving also by-products derived from intermolecular reactions. As a result, the yield of **9a**,**b** was lower. Moreover, the stereochemical course of the decarboxylation reaction was in this case unfavourable, affording preferentially the unwanted *trans* isomers in a 58:42 ratio. However, chromatographic separation of *cis* adducts **14a**,**b** from their *trans* isomers **15a**,**b** was very easy.[17]

Having obtained in good or satisfactory overall yields and in just four steps the enantiomerically pure key scaffolds **12a**,**b** and **14a**,**b**, we then converted them into the RGD containing cyclic peptides **2** and **3** (Scheme 1).[21] In the case of **12a** and **12b** we used a solution-phase protocol and the Mtr group for protection of the arginine side chain. Saponification and coupling with the protected Arg-Gly dipeptide **20**,[22] followed by sequential removal of the Alloc group and of the methyl ester, gave the open pentapeptides **16a** and **16b**. On the contrary, for the preparation of **17a**,**b**, we chose a solid-phase approach, employing, as linker, the acid-labile 2-chlorotrityl group.[23] We also used in this case the more easily removable Pbf group as the guanidine protection.

The stage was now set for the cyclization, that turned out to be relatively efficient for all the *cis* compounds **16a**, **16b** and **17a**,**b**. On the contrary, the *trans* epimer of **16b** gave the cyclic pentapeptide in very low yield, facilitating separation of the *cis* isomer **18b** from its *trans* counterpart.[24]

Removal of the two side-chain protection afforded the final RGD containing peptidomimetics **2a**, **2b** and **3a**,**b**, which were purified through preparative reverse-phase HPLC and evaluated in vitro for their ability to compete with [^{125}I]-echistatin for binding to $\alpha_V\beta_3$ and $\alpha_V\beta_5$ integrins.[25] The results are shown in Table 1. The two diastereoisomers of **2** showed a very similar activity at 1 μM and so we decided to study more deeply just one of them (**2b**). It was shown to inhibit $\alpha_V\beta_3$ with IC_{50} of about 100 nM. Surprisingly, at concentrations between 100 nM and 10 μM, this compound and its isomer **2a** did not show the usual dose-dependent activity,



Scheme 1. Reagents and conditions: (a) N-(Hex-5-en-2-ylidene)benzylamine, CF_3CH_2OH/EtOH 2:1 (0.17 M), 55 °C, 7 days, 77% (**6a**:**6b** ratio = 47:53); (b) 5-hexen-2-one, NH_3 (1.5 equiv), NH_4Cl (0.5 equiv), EtOH–CF_3CH_2OH 1:3, 50 °C, 48 h, 47% (**7a**:**7b** ratio = 50:50); (c) Grubbs 1st gen. cat., CH_2Cl_2 [6 mM (**6a**,**b**) or 3 mM (**7a**,**b**)], reflux, 96 h, 88% (**8a**), 85% (**8b**), 59% (**9a**,**b**); (d) Et_2NH, CH_2Cl_2; (e) Allyl chloroformate, $NaHCO_3$, dioxane-H_2O 84% (**10a**), 84% (**10b**), 83% (**11a**,**b**); (f) NaOH, EtOH; (g) dioxane, reflux, 83% (**12**–**13a**, **12**:**13** ratio = 70:30), 78% (**12**–**13b**, **12**:**13** ratio = 66:34), 79% (**14**–**15a**,**b**, **14**:**15** ratio = 42:58); (h) 1—NaOH, EtOH; 2—Mtr-L-Arg(NH_2)Gly-OMe (**20**), Bop, Et_3N, CH_2Cl_2, 85% (diast. **a**), 88% (diast. **b**); 3—$Pd(PPh_3)_4$, dimedone, THF; 4—LiOH, THF-H_2O; (i) 1—NaOH, EtOH; 2—Pbf-L-Arg(NH_2)Gly-*O*-linker-resin (see text), TBTU, HOBT, EtN(*i*Pr)$_2$; 3—$Pd(PPh_3)_4$, dimedone, THF; 4—AcOH, CF_3CO_2H, CH_2Cl_2 1:1:3; (j) HATU, EtN*i*Pr$_2$, DMF, 10 mM, 53% (**16a**) 58% (**16b**), 32% (**17a**,**b**) (from **14a**,**b**); (k) CF_3CO_2H, tioanisole, 43% (**2a**), 50% (**2b**), 43% (**3a**,**b**).

Table 1. Inhibition of the binding of integrins with echistatin by compounds **2**,**3**[a]

Compound	Integrin	Concentration (μM)	% inhibition
ST-1646[b]	$\alpha_V\beta_3$	0.001	85
2a	$\alpha_V\beta_3$	10	48
2a	$\alpha_V\beta_3$	1	49
2b	$\alpha_V\beta_3$	10	52
2b	$\alpha_V\beta_3$	1	50
2b	$\alpha_V\beta_3$	0.1	53
2b	$\alpha_V\beta_3$	0.01	15
2b	$\alpha_V\beta_3$	0.001	5
3a,**b**	$\alpha_V\beta_3$	10	62
3a,**b**	$\alpha_V\beta_3$	1	30
2a	$\alpha_V\beta_5$	10000	17
2a	$\alpha_V\beta_5$	1000	13
2b	$\alpha_V\beta_5$	10000	15
2b	$\alpha_V\beta_5$	1000	13

[a] Values are means of six experiments. Echistatin concentration was 0.04–0.08 nM.
[b] For the formula of ST-1646 see Refs. 7, 10b.

reaching a plateau at about 50% displacement of echistatin, instead of about 100%. We do not know yet the reason for this anomalous behaviour. An hypothesis could be a strong allosteric binding to the receptor that decreases the affinity towards the natural ligand. The debenzylated adducts **3a**,**b** were found to be less active, and did not show a plateau. **2b** and **2a** were also examined as potential ligands for integrin $\alpha_V\beta_5$. The binding was in this case weaker indicating a certain degree of selectivity among the two receptors.

In conclusion, compound **2b** shows a potency as $\alpha_V\beta_3$ ligand (IC_{50} = 100 nM) slightly higher than that of the first developed Kessler pentapeptide [fVRGD][26] (195 nM when assayed under the same conditions)[7,27] and of recently reported compounds where the RGD

sequence was bonded to an eight-membered lactam.[8] However, the potency of **2a**,**b** is inferior to that of Cilengitide (18.9 nM)[6,7,10a,27] and, most of all, of other recently developed integrin $\alpha_V\beta_3$ ligands,[7,10] such as ST-1646,[10] that have IC_{50} values in the lower nanomolar range. The higher activity of c[f(*N*-MeV)RGD] as well as of the bicyclic bonded RGD compounds[10] has been explained by a shift from a conformation characterized by a β-turn formed by H-bond between the C=O of Asp and the NH of Arg, to one possessing two inverse γ-turns instead, leading to a less 'kinked' RGD arrangement. One of these γ-turns is characterized by H-bond between the NH of D-Phe (or that pointing out from the bicyclic scaffold) and the C=O of Gly. In compounds **2a**,**b** the presence of the *N*-benzyl group in the position corresponding to D-Phe obviously precludes this γ-turn. Actually, we have previously demonstrated, in related system,[13] that the scaffold favours the β-turn and a strong H-bond involving NH of R. In **3a**,**b** the NH pointing out from the scaffold is free, and H-bond with glycine C=O is now possible. However, activity towards $\alpha_V\beta_3$ is even diminished. This result suggests that the scaffold itself tends to favour a β-turn and a more 'kinked' arrangement.

Relatively new integrins are now appearing on the scene[28] and are expected to become in the near future very promising targets for cancer therapy. Therefore, also semirigid scaffolds like ours may be precious in assessing the best conditions for high activity and selectivity. The synthetic strategy employed implies that slight modifications of the scaffold may be realized by simply changing the inputs of the initial Ugi MCR. For example, the *N*-benzyl group in **2a**,**b** represents a diversity element that can be easily varied, also including handles for conjugation with cytotoxic drugs.

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14. We have not been able to determine the relative configurations of these two isomers. In this paper, we will designate with a the isomers derived from the crystalline Ugi adduct.
15. Abbreviations used in this paper: Grubbs 1st gen. cat.: benzylidene-bis(tricyclohexylphosphine)ruthenium dichloride. Bop = (benzotriazol-1-yloxy)*tris*(dimeth-ylamino) phosphonium hexafluorophosphate. HOBT = *N*-Hydroxybenzotriazole. TBTU = *O*-(Benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate. HATU = *O*-(7-Azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate. Mtr = 4-methoxy-2,3,6-trimethylbenzene-1-sulfonyl. Pbf = 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl.
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17. The *cis* compounds were clearly recognized as such on the basis of NOE experiments.[11,13]
18. We had indeed already established that the *trans* isomers were not well suited for the assembly of a pentapeptide due to the excessively high distance between the two handles (Ref. 13).
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21. In the case of **12b** we carried out the synthesis up to **18b** on the 66:34 *cis*:*trans* mixture of **12b** and **13b**. During the final cyclization, the *trans* isomer gave the cyclopeptide in less than 10% yield and it could be separated at this stage by chromatography. The yield reported in the Scheme is calculated on the *cis* isomer only. In the case of **14a**,**b** we carried out the synthesis up to **3a**,**b** without separating the two diastereoisomers.
22. **20** was obtained from L-Fmoc-Arg(Mtr)OH by: (a) coupling with glycine methyl ester hydrochloride with EDCI (*N*-ethyl-*N*′-dimethylaminopropyl carbodiimide), HOBt (*N*-hydroxybenzotriazole), *N*-methylmorpholine, DMF, 91%; (b) Et_2NH, CH_2Cl_2; (c) Cbz-Cl, $NaHCO_3$, dioxane-H_2O, 52%; (d) H_2, Pd–C, MeOH, 85%. Direct use of **20** derived from step b was also possible, but the presence of small amounts of Et_2NH lowered the yields of coupling with the scaffold.
23. In this series, the conditions used for methyl ester saponification led to partial decomposition. Thus, we preferred the mild conditions of deblocking of the 2-chlorotrityl linker.
24. While **18b** and each isomer of **19a**,**b** gave a single set of signals at NMR at room temperature, in **18a** the presence of three discrete conformations was evident. We think that they may arise from interconversion of the two possible rotamers at the tertiary amide as well as of the two conformations of the ring.[13]
25. These tests were performed by Dr. Nicoletta Cini and Prof. Alberto Pupi at the Department of Clinical Physiopathology of the University of Florence. We also thank

Prof. Gloria Menchi for her collaboration in these assays. As a standard in these tests we used ST-1646 (compound **30** of Ref. 10a), which has $IC_{50} = 1.4$ nM under these conditions. The assays were done essentially as described in Refs. 7 or 10a.

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