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Solid-phase synthesis of linear and cyclic peptides containing a calix[4]arene amino acid

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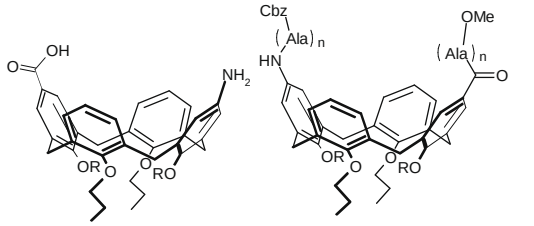
ABSTRACT

Solid-phase synthesis is used to obtain new linear and cyclic N,C-linked peptidocalixarenes. The synthetic strategy allows, for the first time, the condensation of a calix[4]arene amino acid during the stepwise elongation sequence of the peptide. An important role is played by the lower rim functionalization of the calixarene since it modulates the flexibility of the calixarene scaffold and the conformational properties of the resulting non-natural peptide.

N-Linked[1] and C-linked[2] peptidocalix[4]arenes are two families of receptors obtained by functionalizing the upper rim of the calixarene scaffold with multiple units (usually two or four) of amino acids or peptides, which can be linked through the terminal amino or carboxylic acid group, respectively. Their structure, which combines the presence of an aromatic cavity with spatially preorganized polar groups, makes them versatile receptors for polar organic molecules and peptides[3,4] or for the recognition of protein surfaces.[5] Recently, we synthesized the first examples of calix[4]arene amino acids **1** and **2**, which allowed us to obtain the new class of N,C-linked peptidocalixarenes (e.g., **3**-**6**).[6] In contrast to the N-linked and C-linked derivatives, these new compounds can indeed be considered as peptides, characterized by the presence of a non-natural amino acid which is potentially able to transfer new properties to the resulting oligomers. For example, we showed that the different flexibilities of the 1,3-dipropoxycalix[4]arene amino acid **1** and of the tetrapropoxy derivative **2** (two hydrogen bonds between the phenolic OH and O propoxy groups make the aromatic macrocycle of **1** rigid, while **2**, lacking these interactions, retains residual flexibility) are reflected in the conformational and assembly properties of the corresponding peptides **3**-**5** and **6**. The tri-, penta- and heptapeptides **3**-**5**, based on rigid **1**, spontaneously dimerize in apolar solutions through the formation of an antiparallel β-sheet; in the dimer, the calixarene cavities face each other and form a capsule which can accommodate a guest. In contrast, the flexible tetrapropoxycalix[4]arene amino acid **2** behaves as a loop region and the two amino acids at the upper rim of **6** are linked by intramolecular hydrogen bonds.



1 R = H
2 R = *n*-propyl

3 R = H, n = 1
4 R = H, n = 2
5 R = H, n = 3
6 R = *n*-propyl, n = 1

Based on these preliminary results, we believe that the incorporation of a calix[4]arene in a peptide sequence may represent a promising approach for the design of synthetic peptides with tailored properties. The calixarene scaffold, for example, may be used to preorganize the peptide eliminating the need of designing loop regions,[7] while the aromatic cavity can be exploited to encapsulate a biologically active molecule and realize a site-directed molecular delivery system.[8] In this context, the possibility to adopt solid-phase protocols for the synthesis of N,C-linked peptidocalixarenes represents a fundamental requirement, especially in view of a combinatorial approach. We report herein a solid-phase strategy for the synthesis of two new peptides where a calixarene amino acid is introduced, for the first time, during the stepwise elongation sequence of the peptide.[9] In particular, we synthesized two linear endecapeptides **7a**,**b** to verify the extent of dimerization in

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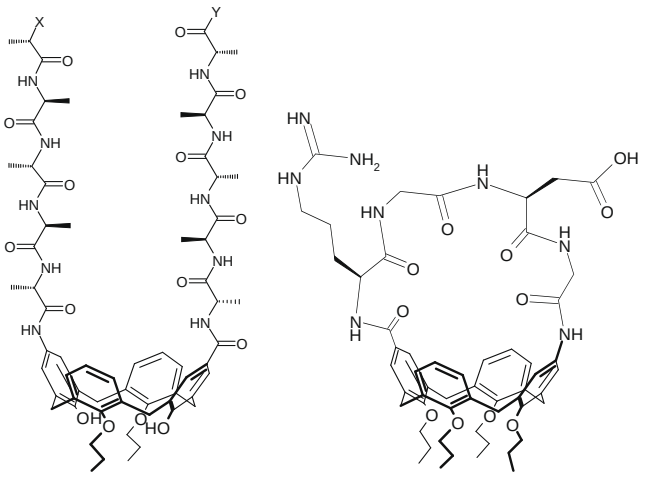
solution, and a cyclic pentapeptide **8**, containing the biologically relevant sequence Arg-Gly-Asp (RGD) which is potentially useful as an integrin antagonist.

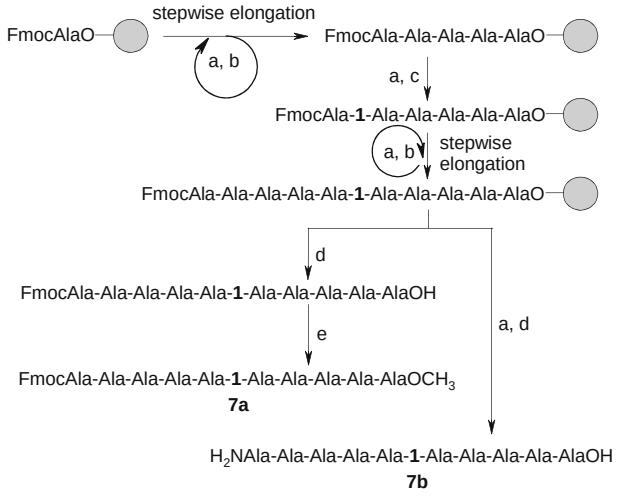


7a X = NHFmoc, Y = OCH_3
7b X = NH_2, Y = OH

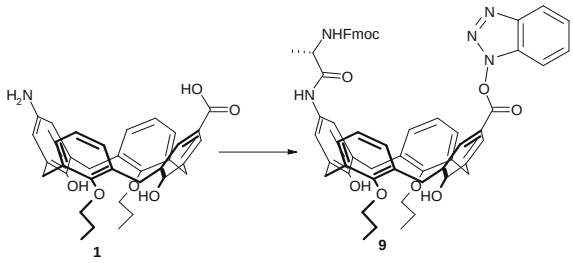
8

The plan adopted for the synthesis of peptidocalixarenes **7a**,**b** is outlined in Scheme 1. It is based on the 9-fluorenylmethoxycarbonyl (Fmoc) protection strategy, which is compatible with calixarene chemistry, and on the use of Wang resin loaded with the first Fmoc-amino acid as the solid support.

Deprotection of the amino groups was achieved under standard conditions, with 20% piperidine solution in DMF, and condensation of the Fmoc-Ala units was performed using PyBop and DIPEA in NMP. The calix[4]arene amino acid **1** was functionalized with an Fmoc-Ala moiety prior to insertion in the peptide sequence since we had observed that a small amount of degradation of calixarene **1** can take place in basic solutions due to oxidation of the *p*-aminophenol ring to a benzoquinone imine species, promoted by deprotonation of the phenolic OH.[6] The reaction of **1** with Fmoc-Ala in the presence of PyBOP (Scheme 2) afforded intermediate **9**,[10]



Scheme 1. Synthesis of the linear peptides **7a**,**b**. Reagents and conditions: (a) 20% piperidine, DMF, 2 × 10 min; (b) Fmoc-Ala (15 equiv), PyBOP (20 equiv), DIPEA (20 equiv), NMP, 2 h; (c) **9** (7 equiv), PyBOP (10 equiv), DIPEA (10 equiv), NMP, 2 d; (d) 1% TFA, 5% TIS, CH_2Cl_2, 2 × 20 min; (e) HCl(g), MeOH, 15 min. *Abbreviations*: PyBOP = benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate, NMP = *N*-methyl pyrrolidinone, TIS = triisopropylsilane.



1 **9**

Scheme 2. Synthesis of intermediate **9**. Reagents and conditions: Fmoc-Ala (2 equiv), PyBOP (4 equiv), DIPEA (8 equiv), DMF, 2 h.

which is characterized by the presence of a benzotriazolyl active ester on the calixarene carboxylic acid. This is a peculiar reaction of dipropoxy calixarene amino acid **1**, since the rigid structure of the calixarene cone prevents the intramolecular reaction and the low reactivity of the aromatic amine hinders intermolecular calixarene–calixarene condensation.[6]

Coupling of the intermediate **9** to the immobilized peptide was subsequently achieved using the same conditions (PyBOP is not strictly necessary, but it proved to be useful to improve the yield in case a small amount of hydrolysis of the active ester takes place). After condensation of the final amino acid, the resin was subdivided into two portions. The first portion was submitted to cleavage of the peptide from the support by treatment with a 1% TFA and 5% TIS solution in dichloromethane. The isolated peptide was then esterified on the free carboxylic group with methanol and HCl(g) to obtain **7a**. The second portion was submitted to deprotection of the terminal amino group, followed by cleavage of the peptide **7b** from the resin. The endecapeptide **7b** was completely insoluble both in water and in organic solvents, but the success of the synthesis was indirectly confirmed by characterization by ESI-MS and ^{1}H NMR of the protected derivative **7a**, which, albeit scarcely soluble in water and organic solvents showed minimal solubility in DMSO.[11] The poor solubility, however, prevented a study of the self-assembly properties in solution and suggests that larger aggregates form instead of discrete dimers. Apparently, taken together, these data indicate that a larger number of hydrogen bonding groups may result in additional interactions, either intra- or intermolecular, which compete with the dimerization process. Subsequently, a water suspension of **7b** was analyzed by atomic force microscopy (AFM). The images show the presence of helical ribbons up to 4 μm long and roughly 50 nm thick (Fig. 1).

Further experiments are needed to clarify the exact nature of these aggregates. Nevertheless, we think that these ribbons may form by coiling of a large number of linear chains of calixarene-containing peptides held together by hydrogen bonds, and, possibly, by hydrophobic interactions between the calixarene scaffolds.

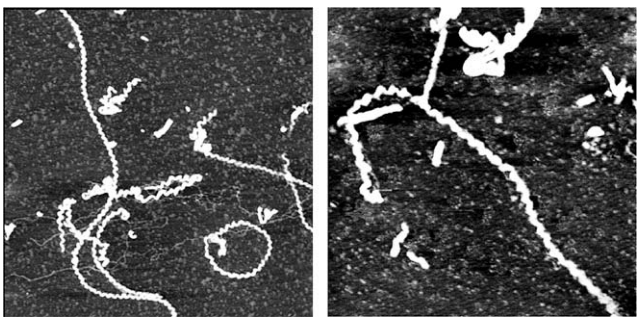


Figure 1. AFM images (left, 4 × 4 μm scan; right, 2 × 2 μm scan) of a sonicated water suspension of **7b** deposited onto mica, obtained with the microscope operating in tapping mode in air.

We also report the solid-phase synthesis of the cyclic non-natural peptide **8**, which contains the calix[4]arene amino acid **2**, the RGD triad and an additional Gly unit to facilitate the cyclization reaction and could be potentially useful in integrin targeting. The integrins, which selectively recognize the RGD sequence, are promising therapeutic targets, since they play a crucial role in tumor-induced angiogenesis and metastasis and acute renal failure.[12] Moreover, since it was found that the conformation of the RGD tripeptide plays a key role in the interaction with the protein,[13–16] we reasoned that our calix[4]arene amino acids could provide an interesting scaffold for constraining the RGD motif in a particularly active structure. We designed the strategy for the synthesis of **8** with the aim of performing the cyclization reaction on the resin, in order to minimize any intermolecular condensation. The two carboxylic groups of Asp allowed us to exploit this amino acid both as an anchoring site to the Wang resin and as one of the cyclization units, with the further advantage that a cyclization reaction between the Asp and Gly amino acids implied minimal steric hindrance. We used Fmoc protection for the amino groups, the allyl group for the main carboxylic functionality of Asp, and the 4-methoxy-2,3,6-trimethylbenzenesulfonyl (Mtr) group for the Arg side chain. The three protecting groups are orthogonal and Mtr is removed under the same acidic conditions that are used to cleave the final product from the resin. At first, we attempted the synthesis using dipropoxycalix[4]arene **1**, but the cyclization reaction failed. The rigidity of the calixarene scaffold, which constrains the amino and carboxylic groups of **1** in divergent orientations, was thought to be the reason for this failure. Molecular modeling calculations revealed that the use of the more flexible tetrapropoxycalix[4]arene amino acid **2** would facilitate the cyclization.

Moreover, calixarene **2**, lacking free OH phenolic groups, does not present the oxidation problems which were observed for **1** and could be introduced as the Fmoc-protected derivative. Fmoc-**2** was synthesized following standard protocols for the protection of amino acids.[17] The ^{1}H NMR spectrum and the X-ray structure of Fmoc-**2** (Fig. 2) show that both in solution and in the crystal, Fmoc-**2** adopts an open-flattened cone conformation. This structure minimizes steric hindrance between the upper rim substituents and allows the formation of intermolecular hydrogen bonds between the NH and the C=O of the carboxylic acid in the solid state (Supplementary data). The solid-phase strategy that led to the synthesis of **8** is reported in Scheme 3. Anchoring of the first amino acid Fmoc-Asp(OAll) to the resin and all the elongation steps were carried out with the PyBOP/DIPEA/DMAP protocol in NMP–CH_2Cl_2 (1:1 v/v).

The anchoring step was followed by capping with Ac_2O in dichloromethane. Unmasking of the Fmoc group was performed with 20% piperidine in DMF. The linear immobilized pentapeptide

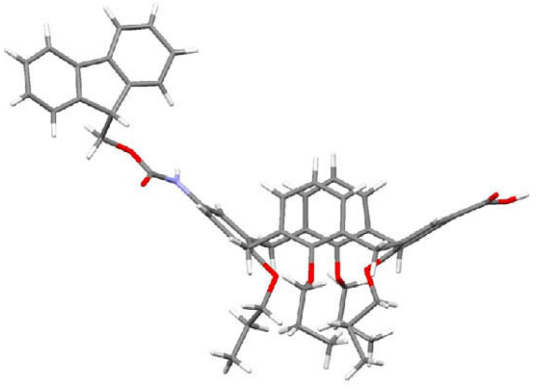
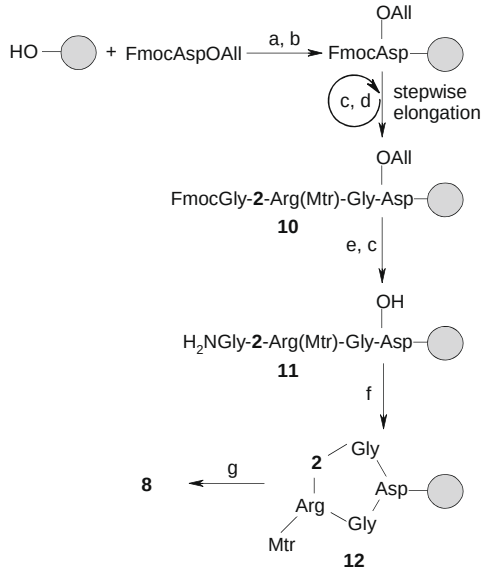


Figure 2. Stick representation of the X-ray structure of Fmoc-**2**.



Scheme 3. Synthesis of the cyclic peptide **8**. Reagents and conditions: (a) Fmoc-AspOAll (3 equiv with respect to the resin active groups), PyBOP (2.8 equiv), DIPEA (4 equiv), DMAP (0.1 equiv), CH_2Cl_2/NMP (1:1), 12 h; (b) Ac_2O (10 equiv), DIPEA (10 equiv), DMAP (0.1 equiv), CH_2Cl_2, 3 h; (c) piperidine, DMF, 3 × 10 min; (d) Fmoc-amino acid or Fmoc-**2** (3 equiv), PyBOP (2.8 equiv), DIPEA (4 equiv), DMAP (0.1 equiv), CH_2Cl_2/NMP (1:1), 45 min (10 h for Fmoc-**2**); (e) $Pd^0(PPh_3)_4$ (3 equiv), $CHCl_3$/AcOH/NMM (37:2:1), 3 h; (f) PyBOP (2 equiv), DIPEA (3 equiv), DMAP (0.1 equiv), CH_2Cl_2/NMP (1:1), 12 h; (g) TFA/H_2O/TIS (95:2.5:2.5), 2 h. *Abbreviation*: NMM = *N*-methylmorpholine.

10 was prepared for cyclization by first deprotecting the allyl group with Pd(0) tetrakis(triphenylphosphine) followed by removal of the Fmoc group. Cyclization of **11** was accomplished with PyBOP/DIPEA/DMAP. Finally, treatment with TFA/H_2O/TIS (95:2.5:2.5) resulted simultaneously in cleavage of the product from the resin and deprotection of the Mtr group. The product was purified by trituration in MeOH and characterized by ESI-MS.[18] Studies to test its efficacy as an integrin antagonist are currently underway.

In conclusion, by synthesizing the linear and cyclic peptides **7a**,**b** and **8**, we have shown that a calix[4]arene amino acid can be easily introduced via solid-phase synthetic protocols as a non-natural amino acid. The lower rim functionalization of the calixarene plays an important role since it modulates the flexibility of the calixarene scaffold and therefore the conformational properties of the resulting peptide. We believe that these results open the way to the synthesis of new non-natural peptides with interesting properties as therapeutic agents,[19,20] molecular receptors,[21] self-assembled nano-materials,[22] and template-based de novo proteins.[23]

Acknowledgements

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tetlet.2009.02.198.

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10. *Characterization data for* **9**: mp = 186–187 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 9.55 (s, 1H, OH), 8.25 (s, 1H, OH), 8.08 (d, 1H, J = 8.2 Hz, Ar), 8.03 (s, 2H, Ar), 7.73 (d, 2H, J = 7.2 Hz, Ar), 7.64–7.20 (m, 9H, Ar), 7.27 (s, 2H, Ar), 6.91 (d, 4H, J = 7.6 Hz, Ar), 6.67 (br s, 2H, Ar), 5.45 (br s, 1H, NH), 4.43 (d, 2H, J = 6 Hz, CH_2), 4.35–4.29 (m, 5H, ArC*H*HAr and CH), 4.01–3.97 (m, 5H, CH_2 and CH), 3.51 (d, 2H, J = 13.2 Hz, ArCH*H*Ar), 3.30 (d, 2H, J = 13.2 Hz, ArCH*H*Ar), 2.10–2.00 (m, 4H, CH_2), 1.44 (d, 3H, J = 6 Hz, CH_3), 1.33 (t, 6H, J = 7.3 Hz, CH_3); ^{13}C NMR (75 MHz, $CDCl_3$): δ 170.15, 162.68, 160.67, 156.26, 151.61, 150.26, 143.61, 143.51, 141.14, 133.13, 132.85, 131.76, 129.50, 129.13, 198.93, 128.57, 128.45, 128.08, 127.68, 127.05, 125.52, 125.02, 124.97, 124.73, 120.67, 120.38, 119.90, 114.27, 108.58, 78.44, 67.31, 51.12, 46.98, 31.26, 23.43, 18.59, 10.88; ESI-MS ($C_{59}H_{55}N_5O_9$): m/z 1000.5 [100%, $(M+Na)^+$].
11. *Characterization data for* **7a**: ^{1}H NMR (600 MHz, DMSO-d_6, selected data): δ 9.49 (s, 1H, OH), 8.95 (s, 1H, OH), 8.32 (s, 2H, Ar), 8.30 (s, 1H, NH), 8.20 (d, J = 7.2 Hz, 1H, NH), 8.17 (d, J = 6.6 Hz, 1H, NH), 8.06 (d, J = 7.2 Hz, 1H, NH), 8.00 (d, J = 6.6 Hz, 1H, NH), 7.96 (d, J = 7.8 Hz, 1H, NH), 7.92 (br s, 1H, NH), 7.87 (d, J = 7.8 Hz, 2H, Ar), 7.85 (d, J = 7.8 Hz, 2H, Ar), 7.76 (s, 1H, Ar), 7.74 (s, 1H, Ar), 7.71 (d, J = 8.4 Hz, 1H, Ar), 7.69 (d, J = 8.4 Hz, 1H, Ar), 7.52 (d, J = 7.2 Hz, 1H, NH), 7.40 (t, J = 7.8 Hz, 2H, Ar), 7.32 (t, J = 7.8 Hz, 2H, Ar), 7.09 (d, J = 7.2 Hz, 1H, NH), 7.07 (d, J = 8.4 Hz, 1H, NH), 6.96 (d, J = 7.8 Hz, 2H, Ar), 6.80 (t, J = 7.8 Hz, 1H, Ar), 6.78 (t, J = 8.4 Hz, 1H, Ar), 6.53 (s, 1H, NH); ESI-MS ($C_{81}H_{99}N_{11}O_{18}$): m/z 1515.5 [100%, $(M+H)^+$], 1537.5 [40%, $(M+Na)^+$]. For **7b**: ESI-MS ($C_{65}H_{87}N_{11}O_{16}$): m/z 661.8 [100%, $(M+2Na)^{2+}$].
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17. Preparation of the Fmoc-protected calix[4]arene amino acid **2** (Fmoc-**2**): an ice-cold solution of Fmoc-*O*-succinimide (0.26 mmol) in THF (2 mL) was added in three portions to an ice-cold solution of **2** (0.13 mmol) and Na_2CO_3 (0.32 mmol) in THF (8 mL). The ice bath was then removed and the reaction mixture was stirred for 3 h. 1 M HCl was then added, the organic solvent evaporated, and the aqueous phase extracted with CH_2Cl_2 (2 × 15 mL). The pure product (41% yield) was obtained after removal of the solvent and trituration of the solid residue with MeOH. Mp = 192 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.77 (d, 2H, J = 7.5 Hz, Ar), 7.60 (br s, 2H, Ar), 7.40 (t, 2H, J = 7.5 Hz, Ar), 7.31 (t, 2H, J = 6.8 Hz, Ar), 6.93 (s, 2H, Ar), 6.90 (br s, 4H, Ar), 6.80 (t, 2H, J = 7.2 Hz, Ar), 6.14 (s, 2H, Ar), 5.97 (s, 1H, NH), 4.43-4.37 (m, 6H, ArC*H*HAr and CH_2), 4.15 (br s, 1H, CH), 3.94-3.86 (m, 4H, CH_2), 3.75–3.65 (m, 4H, CH_2), 3.11 (d, 4H, J = 13.5 Hz, ArCH*H*Ar), 1.92–1.81 (m, 8H, CH_2), 1.08–1.01 (m, 6H, CH_3), 0.89 (t, 6H, J = 7.4 Hz, CH_3); ^{13}C NMR (75 MHz, CD_3OD): δ 170.7, 157.7, 154.4, 145.7, 142.9, 137.1, 136.0, 135.5, 131.6, 129.8, 129.5, 129.0, 128.5, 126.6, 125.5, 123.6, 121.2, 78.3, 78.2, 68.1, 48.7, 32.3, 32.2, 24.8, 24.7, 11.2, 11.1, 11.0; ESI-MS ($C_{56}H_{59}NO_8$): m/z 897.2 [100%, $(M+Na+H)^+$]. Crystallographic data (excluding structure factors) for the structure of Fmoc-**2** have been deposited with the Cambridge Crystallographic Data Centre as supplementary publication no. CCDC-714083.
18. ESI-MS ($C_{55}H_{70}N_8O_{11}$): m/z 1019.6 [100%, $(M+H)^+$]. The very poor solubility of **8** in aqueous and organic phases prevented its NMR characterization.
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