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Transglutaminase surface recognition by peptidocalix[4]arene diversomers

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Abstract—A series of *N*-linked tetrakis(tetrapeptido)calix[4]arene diversomers, **3A**–**P**, has been synthesized by coupling of a cone calix[4]arene tetracarboxylic acid chloride with tetrapeptides **1A**–**P** obtained in a parallel fashion. The inhibition activity of **3A**–**P** towards tissue and microbial transglutaminase was evaluated by in vitro assays with a labeled substrate. Kinetic analysis using one of the most active derivatives (**3A**) showed a noncompetitive inhibition with respect to the amino acceptor substrate and an uncompetitive inhibition with respect to amino donor substrate. Experimental results are in accordance with an inhibition due to a protein specific surface recognition on a region noncomprising the enzyme active site.

Transglutaminases (TGs; EC 2.3.2.13) form a large family of enzymes that catalyze an acyl transfer reaction between the γ-carboxamide group of the protein-bound glutamine residue and the primary amino group of the protein-bound lysine residue or biogenic polyamines.[1] Due to the TG-catalyzed post-translational modification of selected protein substrates, these enzymes play important roles in several biological functions.[2] Recent findings suggest that some TGs, normally expressed at low levels in many tissue types, are activated and/or overexpressed in a variety of disorders, which include celiac sprue and Huntington disease.[3] Consequently, there is a strong interest in the search of TG inhibitors as potential therapeutic agents.[4]

In this regard, a potential alternative to conventional small-molecule inhibitor approach, could be the exploitation of the principles of protein surface recognition, successfully applied, in the last few years, by Hamilton and co-workers.[5] Following these lines, here we report the synthesis of a series of peptidocalix[4]arene diversomers[6] and their evaluation as inhibitors of two TG isoforms.

In the design of peptidocalixarenes, we first decided to use a calix[4]arene scaffold blocked in the *cone* conformation and bearing tetrapeptide chains *N*-linked at the upper rim.[7] In this way, a convergent presentation to the protein surface is favoured by the fixed conformation of the scaffold, while the intramolecular interchain interaction, observed for some *C*-linked derivatives, should be disfavoured.[8] In addition, glycine was chosen as the first amino acid of the peptide chain because of the minimal steric bulkiness of the α-substituent, which would allow its appropriate folding over the protein surface.

In order to have a more general unbiased approach, we decided to initially test peptidocalix[4]arenes bearing an apolar, anionic or cationic ending residue. Thus, tetrapeptides **1A**–**C** ending with tyrosine, aspartic acid and lysine, respectively, were synthesized by using the solution phase methodology reported by Carpino and coworkers,[9] which relies on the successive coupling of Fmoc-protected amino acid chlorides (Scheme 1). The tetrapeptides were then coupled with the known tetrapropoxycalix[4]arene tetracarboxylic acid chloride **2**,[7] blocked in the cone conformation, in the presence of

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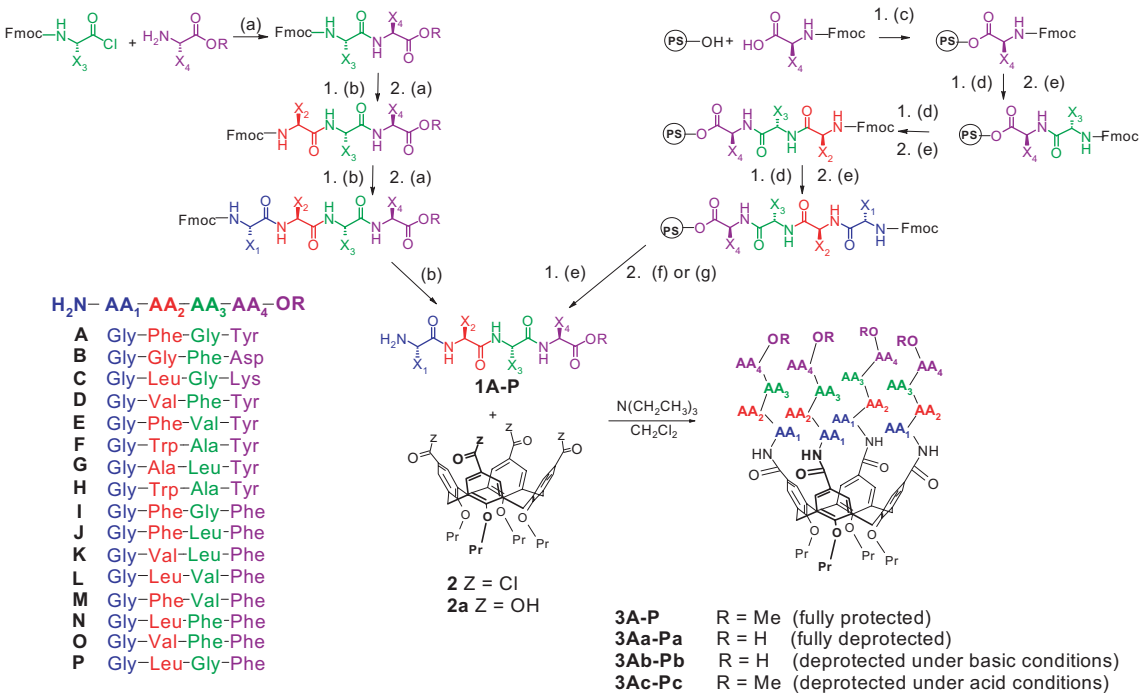

Scheme 1. Reagents and conditions: (a) $NaHCO_3$, 5%, Fmoc-AA-Cl, CH_2Cl_2; (b) TAEA, CH_2Cl_2; (c) CH_2Cl_2/DMF (150/3), DIC, 0 °C, DMAP; (d) 20% piperidine, DMF, 30 min; (e) HBTU, Fmoc-AA-OH, DIPEA, DMF; (f) MeOH, $N(Et)_3$, DMF, 2 d; (g) (1) CF_3COOH, $(Et)_3SiH$, CH_2Cl_2, 1 h; (2) (trimethylsilyl)diazomethane. Side chain of Tyr and Asp amino acids were protected by *tert*-butyl group; ε-amino group of Lys was protected by Boc group. Left- and right-side routes were used to prepare tetrapeptides **1A**–**C** or **1D**–**P**, respectively.

NEt_3, to give tetrakis(tetrapeptido)calix[4]arenes **3A**–**C**, as methyl ester derivatives. These compounds, as well as all the other diversomers successively described, were structurally characterized by NMR, ESI/MS and elemental analysis. In all instances no significant racemization was evidenced by NMR and $[\alpha]_D$ measurements.

The synthesized tetrapeptidocalix[4]arenes were in vitro screened (by using a concentration ranging between 0.01 and 1 mM) for their inhibition activity towards tissue TG (tTG) (Fig. 1) and microbial TG (mTG) (Fig. 2). The assays were carried out by measuring the incorporation of ^{3}H-labeled spermidine (Spd) into *N*,*N*-dimethylated casein (DMC).[10] As shown in panels A (Figs. 1 and 2) the tetrakis(tetrapeptido)calix[4]arene **3A**, ending with tyrosine was found to be more effective than **3B** and **3C** towards both TG isoforms.

Successive deprotection treatments of **3A**–**C** led to derivatives **3Aa**–**Ca**, fully deprotected at both the side chain and the terminal residue. In addition, partially deprotected compounds **3Ab**–**Cb** and **3Ac**–**Cc** were also obtained by a single treatment under basic or acid conditions, respectively (Scheme 1).[11] However, all these derivatives showed a lower inhibition activity with respect to the parent compounds **3A**–**C** (Figs. 1 and 2, panels A).

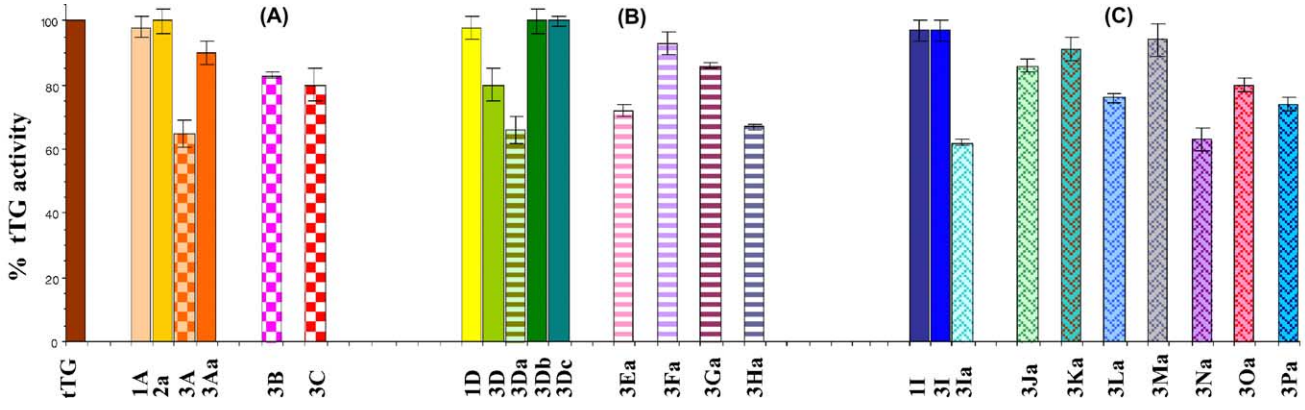

Figure 1. Tissue transglutaminase (tTG) activity in the presence of the synthesized tetrapeptidocalix[4]arene diversomers (see Scheme 1). Inhibitors were used in a concentration ranging between 0.01 and 1 mM (only data for the highest concentration are shown). Columns represent an average of three independent sets of experiments with two replicates and the bars represent the standard deviations. Comparison of inhibition activity of protected, fully and partially deprotected derivatives is shown only for **3A**,**D** and **3I** and their corresponding free tetrapeptides (**1A**,**D** and **1I**), whereas only the datum for the most active form is shown in the other instances.

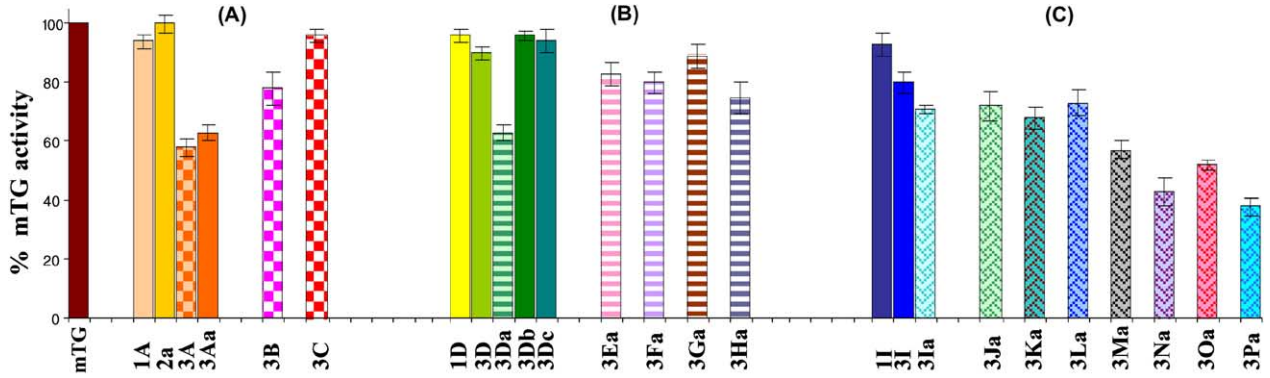

Figure 2. Microbial transglutaminase (mTG) activity in the presence of the synthesized tetrapeptidocalix[4]arene diversomers (see Scheme 1). Inhibitors were used in a concentration ranging between 0.01 and 1 mM (only data for the highest concentration are shown). Columns represent an average of three independent sets of experiments with two replicates and the bars represent the standard deviations. Comparison of inhibition activity of protected, fully and partially deprotected derivatives is shown only for **3A**,**D** and **3I** and their corresponding free tetrapeptides (**1A**,**D** and **1I**), whereas only the datum for the most active form is shown in the other instances.

Interestingly, no significant inhibition was found for free tetrapeptides **1A**–**C** and tetracarboxylic acid **2a**, suggesting a specific, multipoint surface binding interaction of tetrapeptidocalix[4]arenes **3** in enzyme inhibition.

On the basis of the above results, we directed our attention to the synthesis of further apolar peptidocalixarenes ending with tyrosine residue and having a combination of different residues at intermediate positions of the peptide chain. The synthesis of such tetrapeptides, namely **1D**–**H**, was performed in a parallel fashion, in the solid phase by using an automated synthesis workstation (Chemspeed ASW1000 synthesizer).[12] A polystyrene Wang resin was used as solid support on which Fmoc-protected amino acid were then coupled with HBTU as condensing agent (Scheme 1).

The tetrapeptides were detached from the solid support[13] and then coupled with **2** to give tetrapeptidocalix[4]arenes **3D**–**H** that were tested for their inhibition activity towards tTG and mTG (Figs. 1 and 2, panels B, respectively). These compounds exhibited a very low inhibition activity towards both the TG isoforms, thus evidencing the relevance of the internal sequence on inhibition activity.

Compounds **3D**–**H** were subsequently deprotected[11] to give **3Da**–**Ha**, **3Db**–**Hb** and **3Dc**–**Hc** derivatives. Among them **3Da** was found to exhibit the best inhibitory effect towards tTG (34% activity reduction) and mTG (35% activity reduction) (Figs. 1 and 2, panels B, respectively).

The solid phase synthesis was then extended to tetrapeptides **1I**–**P**,[11–13] with phenylalanine ending residue, to give tetrapeptidocalix[4]arenes **3I**–**P** (Scheme 1). These compounds generally showed a low level of inhibition activity, whilst the corresponding deprotected compounds, **3Ia**–**Pa**, were more effective (Figs. 1 and 2, panels C, respectively). In particular, **3Ia** was found to be the best inhibitor for tTG (≈40% activity reduction), while **3Na** and **3Pa** were more effective on mTG (≈58% and 62% activity reduction, respectively).

The proposed TG inhibition by tetrapeptidocalix[4]arenes **3** through a specific complex formation was confirmed by nondenaturing gel electrophoresis on acrylamide/agarose gel (Fig. 3, panel A).[14] In fact, the effective formation of a tTG/**3A** complex, which migrated towards the cathode faster than the uncomplexed enzyme, was observed in the lane corresponding to their mixture.

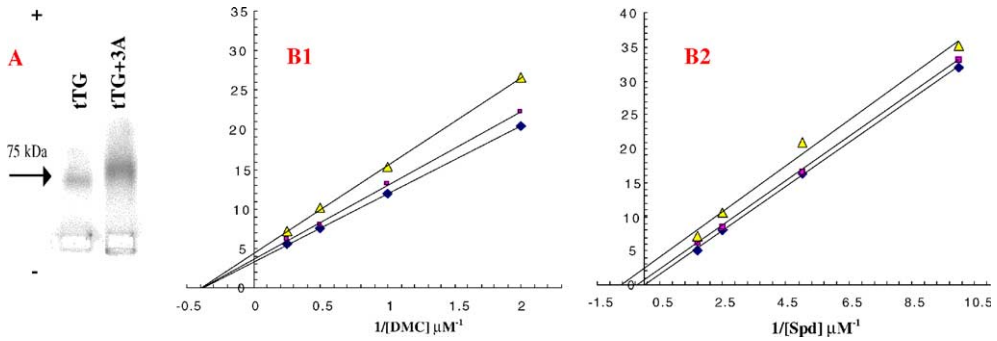

Figure 3. Study on tTG inhibition by **3A**. *Panel A*: nondenaturing gel electrophoresis on acrylamide/agarose gel of free tTG (left lane) and its mixture with **3A** (right lane). *Panel B*: kinetic analysis of tTG inhibition by **3A**. Lineweaver–Burk plots were made by using *N*,*N*-dimethylated casein (DMC) (**B1**) and spermidine (Spd) (**B2**) as variable substrates. tTG from guinea pig liver was incubated in the absence (rhombuses) or in the presence of **3A** (0.1 mM, squares; 1 mM, triangles).

Further information on the type of inhibition (competitive, noncompetitive, and uncompetitive) of **3A** on tTG activity were obtained through a kinetic study.[15] As shown in Figure 3, **3A** exhibited a noncompetitive inhibition with respect to the amino acceptor substrate DMC (Fig. 3, panel B1) and uncompetitive with respect to amino donor substrate Spd (Fig. 3, panel B2). These results lend support to a tTG/**3A** complex formation promoted by a specific surface recognition on a region noncomprising the enzyme active site (hot spot).[5c] Consequently, TG inhibition could be due to a conformational rearrangement of the active form produced by the enzyme/inhibitor recognition interaction.

On the basis of the above results, we concluded that the sequences Gly-Phe-Gly-Tyr (**3A**) and Gly-Phe-Gly-Phe (**3Ia**) are the most effective tTG inhibitors in the frame of our tetrapeptidocalix[4]arene library. Interestingly, both compounds have the same internal sequence (Phe-Gly), which appears to be important for a specific surface recognition of tTG. Furthermore, the presence of an apolar aromatic moiety in the side chain of the terminal amino acid appears to be also necessary. In fact, a comparable activity was observed for **3Ia** and **3A** ending with phenylalanine and Bu^t-*O*-protected tyrosine, respectively, whereas the corresponding *O*-deprotected derivative **3Aa** showed a lower efficiency (Fig. 1). On the other hand, the best inhibitory effect on mTG was observed with derivatives **3Na** and **3Pa** (Fig. 2), having a very similar sequence (Gly-Leu-Phe-Phe and Gly-Leu-Gly-Phe, respectively), again supporting the specificity of surface recognition by different diversomers on the two TG isoforms.

In conclusion, this study demonstrates that TGs can be inhibited by peptidocalix[4]arene diversomers by means of unconventional protein surface recognition, but further efforts are required to improve their limited efficiency. The extension of this strategy to the elaboration of a second generation of TGs inhibitors may include the less symmetrical attachment of diverse peptide chains on the same scaffold or the use of a calix[4]arene skeleton bearing both NH_2 and COOH groups to give peptidomimetic *N*,*C*-linked derivatives.[16] Both approaches are currently under study in our laboratory.

Acknowledgements

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10. *TGs inhibition assay*: TGs activity was assayed according to a modified reported procedure (Achyuthan, K. E.; Greenberg, C. S. *J. Biol. Chem.* **1987**, *262*, 1901) Briefly, mTG or tTG (1.28×10^{-7} M) were incubated at 37 °C for 1 h in 125 mM Tris–HCl, pH 7.5, 70 nM [^{3}H]Spd, 10 mM dithiothreitol, 2.5 mM $CaCl_2$ (for tTG only) and 0.2 mg DMC (0.1 mL of final volume), in the absence or in the presence of each chemical compound (0.01, 0.1 or 1×10^{-3} M). Data have been reported as the mean ± the standard error (SE) obtained from three separate determinations in which each point was performed in duplicate. Statistical analysis was performed with Student's *t*-test.
11. The complete deprotection of compounds **3A**–**P** to give **3Aa**–**Pa**, was obtained by consecutive treatments, when required, to remove the OMe protection at C-terminus carboxylic group (LiOH, EtOH, 12 h, rt), the Bu^t-*O*-protection at the side chain of Tyr and Asp (3 N HCl, MeOH, 16 h, rt), or the Boc protection of ε-amino group of Lys (TFA, CH_2Cl_2, 2 h, rt). Partially deprotected derivatives **3Ab**–**Pb** and **3Ac**–**Pc**, were obtained, when applicable, by a single treatment under basic or acid conditions, respectively.
12. The compounds **1D**–**P** were synthesized by general Fmoc solid phase synthesis, see: Chan, W.; White, P. D. *Fmoc Solid Phase Peptide Synthesis: A Practical Approach*; Oxford University Press: New York, 2000. The computer-controlled automated synthesizer ASW100 was manufactured by CHEMSPEED Ltd–Rheinstrasse 32, CH 4302 Augst, Switzerland.
13. Tetrapeptides **1D**–**H** were detached form the solid support by treatment with MeOH, $N(Et)_3$, in DMF for 2 d [conditions (f) in Scheme 1], while for derivatives **1I**–**P** a treatment with CF_3COOH, $(Et)_3SiH$ in CH_2Cl_2, for 1 h, was used [conditions (g), Scheme 1].

14. *Gel electrophoresis*: tTG (5.12×10^{-7} M) and **3A** (5 mM) were incubated for 1 h at 37 °C in 125 mM Tris–HCl, pH 7.5 (10 μL of final volume), mixed with the buffer sample and loaded onto a nondenaturing acrylamide/agarose (2.5%/0.4%) gel.
15. *Kinetic assay*: tTG activity was measured using DMC or Spd as variable substrates in the absence or presence of **3A** (0.1 and 1 mM). The reaction mixture contained different concentrations of DMC (0.5, 1, 2, 4 μM) with a fixed concentration of Spd (0.6 μM) or different concentrations of Spd (0.1, 0.2, 0.4, 0.6 μM) with a fixed concentration of DMC (4 μM).
16. Similar *N*,*C*-linked peptidocalix[4]arene derivatives were very recently reported by Ungaro group: Sansone, F.; Baldini, L.; Casnati, A.; Chierici, E.; Faimani, G.; Ugozzoli, F.; Ungaro, R. *J. Am. Chem. Soc.* **2004**, *126*, 6204.