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Synthesis of tris-tertiary amine CycloTriVeratrilene (TACTV) derivatives as water soluble pre-organized three aromatic ring containing molecular scaffolds for the construction of protein mimics

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ABSTRACT

The synthesis of water soluble highly pre-organized Tris-tertiary Amine CycloTriVeratrilene (TACTV) derivatives was developed. A semi-orthogonally protected derivative allowed one pot sequential introduction of different peptide loops toward the molecular construction of synthetic antibody protein mimics.

One of the great challenges faced in the development of protein mimics is obtaining molecular constructs with sufficient water solubility. This is not different from trying to steer water solubility and resulting absorption and permeation properties of lead compounds in the small molecule drug discovery process. A poor water-solubility of lead compounds is often responsible for the large attrition rate in this process [1].

To a large extent the water solubility of protein mimics is determined by the cyclic peptides or peptide loops attached to the molecular scaffold which are necessary for proper orientation and positioning in space aiming at an adequate mimicry of the protein. Unfortunately, in many cases the amino acids present in these peptide loops do not contain side chains, which contribute to a good water-solubility. Evidently, the possibilities to change amino acids in these peptide loops in order to increase water-solubility are very limited, as their peptide sequences have to resemble the mimicked peptide segments of the protein as closely as possible.

Recently, we have addressed the issue of poor solubility of bicyclic peptides by development of polar hinges, which upon incorporation led to a significant increase of polarity and therefore water solubility of the resulting bicyclic peptides [2].

These polar hinges were also beneficial for increasing the water-solubility of peptide loops for attachment to our molecular scaffolds [3,4].

However, it was increasingly realized that the molecular scaffold to which the peptide loops are attached plays a dominant role in determining the water solubility of the final protein mimics.

Therefore, we have recently developed mono- and diethylene glycol spacer containing CTV-analogues, which already led to a considerable improvement of water solubility albeit still not sufficient [4]. A major step forward towards a CTV derivative with the structurally best possible aqueous solubility characteristics is presented herein by the development of a Tris-tertiary Amine CycloTriVeratrilene derivative (TACTV) of which the TFA-salt is very soluble in water. In addition, the synthesis of a semi-orthogonally protected TACTV derivative is described, which enabled the incorporation of different peptide loops onto this scaffold.

Probably, the cup or basket shape of the CTV structure in which three aromatic rings are linked in a circle provides one of the most pre-organized molecular scaffolds presently available. As a result this scaffold is highly suitable for spatial mimicry of discontinuous epitopes in proteins. Despite the fact that the molecular scaffold forms only a small part of the entire protein mimic it often contributes to a poor solubility of the molecular construct. With the presented TACTV scaffolds in this work outstanding pre-organisation is combined with a good water-solubility.

In order to increase the water solubility of the CTV scaffold considerably we thought it was best to connect a tertiary

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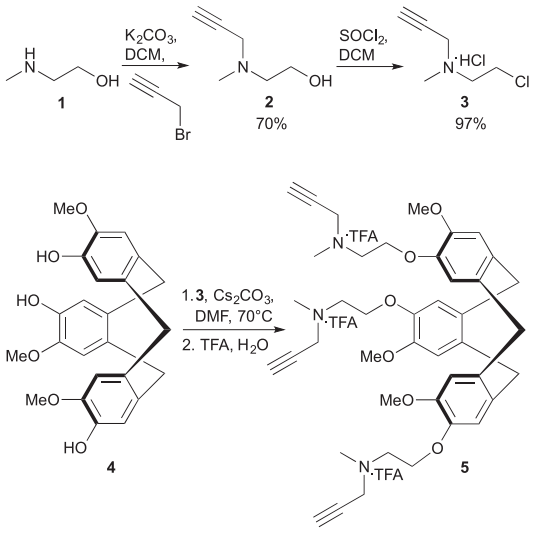
amine-capable of salt-formation-containing linker to each of the aromatic rings. In addition, the linker had to contain a propargyl moiety for future attachment of a peptide loop via copper catalyzed azide-alkyne cycloaddition (CuAAC, see below). For this purpose a versatile linker **3** was conveniently prepared in two steps from *N*-methylethanolamine (**1**, Scheme 1). Alkylation of CTV (**4**) [4], with **3** in the presence of cesium carbonate gave Tris-tertiary Amine CycloTriVeratrilene (TACTV) derivative **5** in good yield (61%), which was readily converted to its TFA salt. This salt was very soluble in water (>300 mg/mL).

This set the stage for the synthesis of a Tris-tertiary Amine CycloTriVeratrilene (TACTV) derivative **6** to enable the incorporation of different peptide loops onto this scaffold (Fig. 1).

Thus TES and TIPS containing linkers **9** and **12** were prepared from *N*-methylethanolamine **1** and previously synthesized silyl protected propargyl bromide derivatives **7** and **10** (Scheme 2) [3]. Next, *N*-methylethanolamine **2** was alkylated with alkyne bromides **7** or **10** in the presence of K_2CO_3 to give alkyne alcohols **8** and **11**, respectively. Finally, the hydroxyl group of the alkyne alcohols **8** and **11** were converted into the corresponding alkyne chlorides **9** and **12**, using thionyl chloride and easily isolated as hydrochloride salts.

For the synthesis of semi-orthogonally protected TACTV derivative **6** (Fig. 1), CTV scaffold **4** [5] was protected first as a ditetrahydropyranyl (diTHP) derivative **13** as was previously described [2].



Scheme 1. Synthesis of solubilizing linker 3 and the TFA-salt of the Tris-tertiary Amine CycloTriVeratrilene (TACTV) scaffold 5.

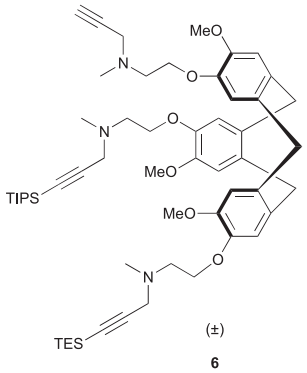
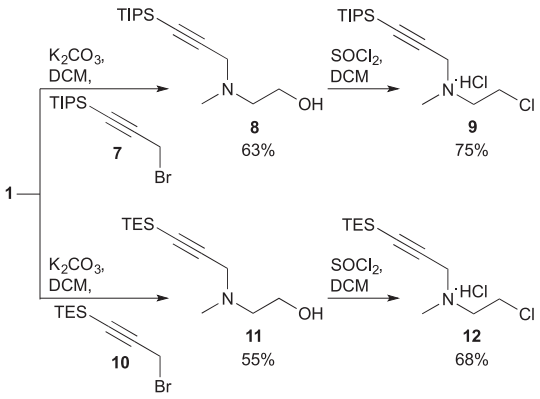
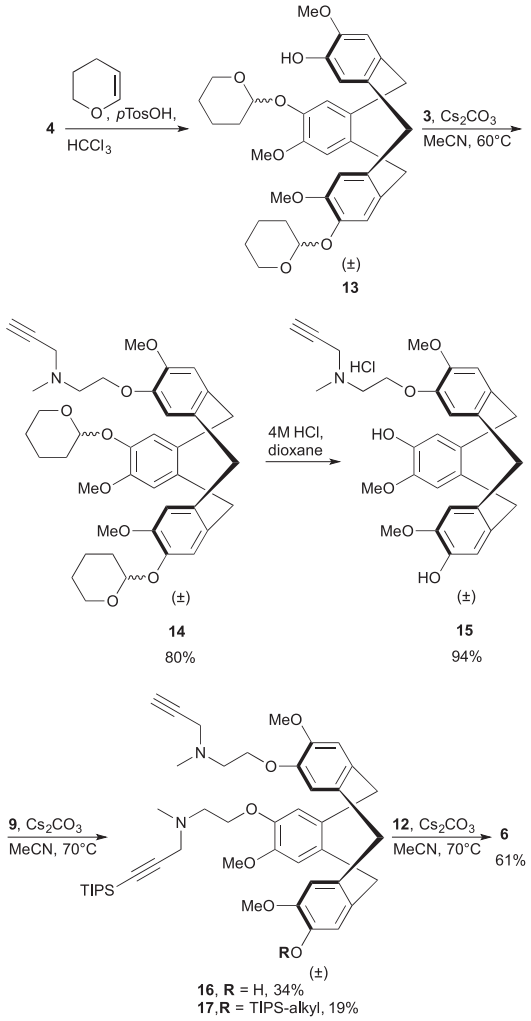


Fig. 1. Semi-orthogonally protected TACTV derivative **6** for sequential introduction of peptide loops.

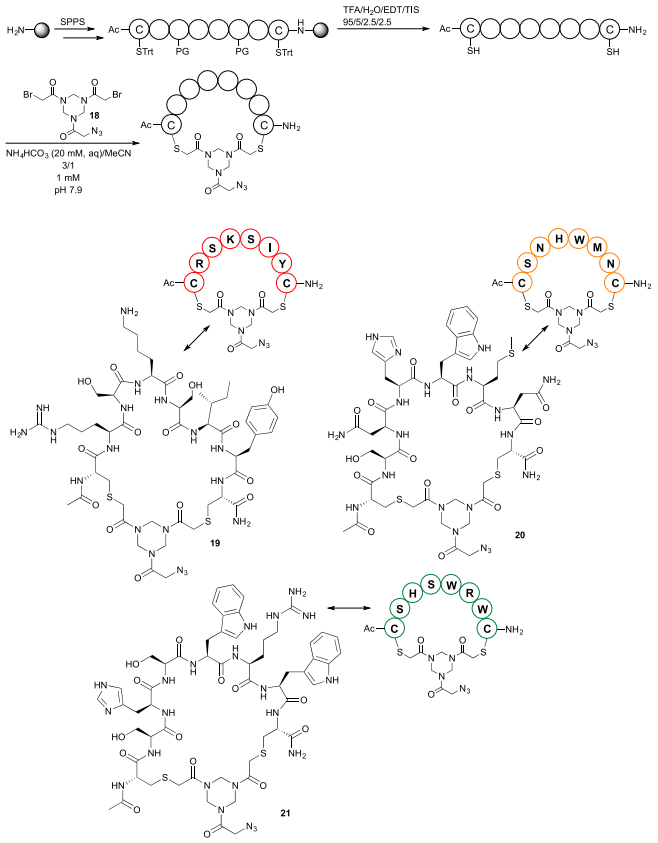


Scheme 2. Synthesis of TIPS and TES protected tertiary amine containing linkers 9 and 12.

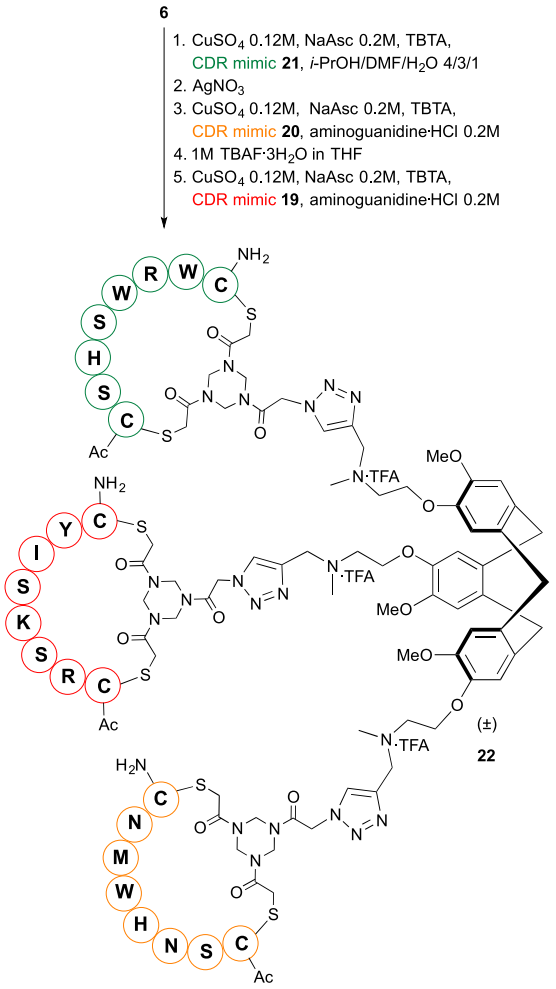
Next, diTHP protected CTV scaffold **13** was alkylated with alkyne tertiary amine linker **3** in the presence of Cs_2CO_3 to afford monoalkylated diTHP protected CTV scaffold derivative **14** in a good (80%) yield (Scheme 3). Next, the THP protecting groups of CTV scaffold derivative **14** were easily removed with 4 M HCl in dioxane to yield the hydrochloride salt of monoalkylated dihydroxyl CTV scaffold derivative **15.** Then, the dihydroxyl CTV scaffold derivative **15** was alkylated with TIPS protected linker **9**



Scheme 3. Synthesis of semi-orthogonally protected TACTV derivative 6.



Scheme 4. Solid phase peptide synthesis of infliximab (remicade®) CDR-peptide sequences followed by CDR-loop construction by alkylation using polar hinge 18[2].



Scheme 5. One pot – five step synthesis of synthetic antibody protein mimic 22 incorporating the tris amino CycloTriVeratrilene (TACTV) scaffold.

providing a rather modest yield of the desired monoTIPS derivative **16** along with undesired diTIPS side product **17** and unreacted dihydroxyl CTV scaffold **15**. Finally, the TES protected linker **12** was introduced to monoTIPS CTV scaffold derivative **16** in dry acetonitrile using dry Cs_2CO_3 as a base to yield the required tris-tertiary amine spacer-containing semi-orthogonally protected CTV scaffold derivatives **6** (Scheme 3).[1]

To illustrate the versatility of the water-soluble semi-orthogonally protected TACTV derivative **6** we have applied it in the preparation of a potential synthetic antibody mimic using a one pot, five step synthesis procedure. To achieve this, assembly of the previously synthesised CDR peptide loop mimics **19**, **20**, and **21** (Scheme 4) onto the CTV scaffold derivative **1** was carried out according to the recently described one-pot method (Scheme 5) [4]. Thus, one equivalent of CDR loop mimic **21** was introduced *via* CuAAC onto one equivalent of CTV scaffold derivative **6** to give a one CDR loop containing protein mimic. Immediately thereafter, silver nitrate was added to the reaction mixture to selectively remove the TES protecting group and without intermediate purification the next CDR-mimicking peptide loop **20** was introduced *via* CuAAC leading to the two CDR loop mimic containing CTV derivative. During this reaction aminoguanidine hydrochloride was added as a scavenger of the formed dehydroascorbic acid [6], to prevent formation of ascorbic acid adducts with arginine residues in the peptide loops. Next, TBAF·$3H_2O$ was added to remove the TIPS protecting group to afford the two loop containing protein mimic. Finally, CDR loop mimic **19** was introduced *via* CuAAC to complete molecular construction of synthetic antibody **22**. After preparative HPLC purification synthetic antibody **22** was obtained as '8-TFA' salt (Scheme 5).

Surface Plasmon Resonance was carried out in order to determine the affinity of synthetic antibody **22** for hTNFα. Although synthetic antibody **22** did exhibit affinity for hTNFα, this affinity could not be reliably quantified due to fast kinetics (k_{off} greater than 1×10^{-1} s^{-1}) of the interaction. Although the water-solubility was improved, the linker length may be not sufficiently optimal compared to the previously described protein mimics [4]. This was somewhat unexpected, since the linker length was identical to the length of the recently described bio-active protein mimics based on the ethylene glycol CTV molecular scaffold [4]. Perhaps the positive charges in the linkers play a too dominant role in reducing the bio-activity as monitored by SPR and studied in tissue culture (see below).

Finally, synthetic antibody **22** was tested for its ability to block TNFα-induced cytotoxicity in L929 cell lines using the Alamar blue assay (see ESI) [7]. Testing was carried out in serum free medium to prevent loss of available protein mimic due to non-specific binding to serum components. In addition, phosphate of the PBS buffer tended to precipitate the protein mimic [8]. Unfortunately, we were unable to show a significant protective effect of our synthetic antibody against TNFα-induced cytotoxicity.

[1] Progressive formation of a side product in which the TES-group is removed in the end product presumably resulting from hydrolysis, occurred when the reaction time was extended beyond *ca.* 7 h. Thus, to minimise the formation of this side product the reaction time was limited to 6 h, which however was not sufficient for full conversion. Nevertheless a better yield was obtained than when the reaction time was prolonged and thus TES hydrolysis increased.

Nevertheless, the development of a very water soluble three-aromatic ring containing molecular scaffold gave us the first opportunity to apply its pre-organized nature for obtaining a water soluble protein mimic suitable for testing under appropriate assay conditions used in the evaluation of biologics. It is expected that by varying and optimizing the peptide loops – which are mimicking the CDR-loops of an antibody – on this new molecular scaffold, we should be able to ultimately develop truly effective synthetic antibodies. Although antibodies are the outstanding examples of proteins in which discontinuous epitopes play key roles in determining their biological activities, there are several other categories of proteins where discontinuous epitope loops plays similar important roles such as toxins [9], peptidase inhibitors [10] and viral proteins interacting with cellular receptors [11]. We are confident that the above described water soluble molecular scaffold can be also applied in the design and molecular construction of versatile protein mimics of these proteins.

Acknowledgment

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Appendix A. Supplementary data

Supplementary data (description of the syntheses, SPR-experiments, description of the Alamar Blue assay, NMR-spectra and Mass spectra) to this article can be found online at https://doi.org/10.1016/j.tetlet.2019.151245.

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