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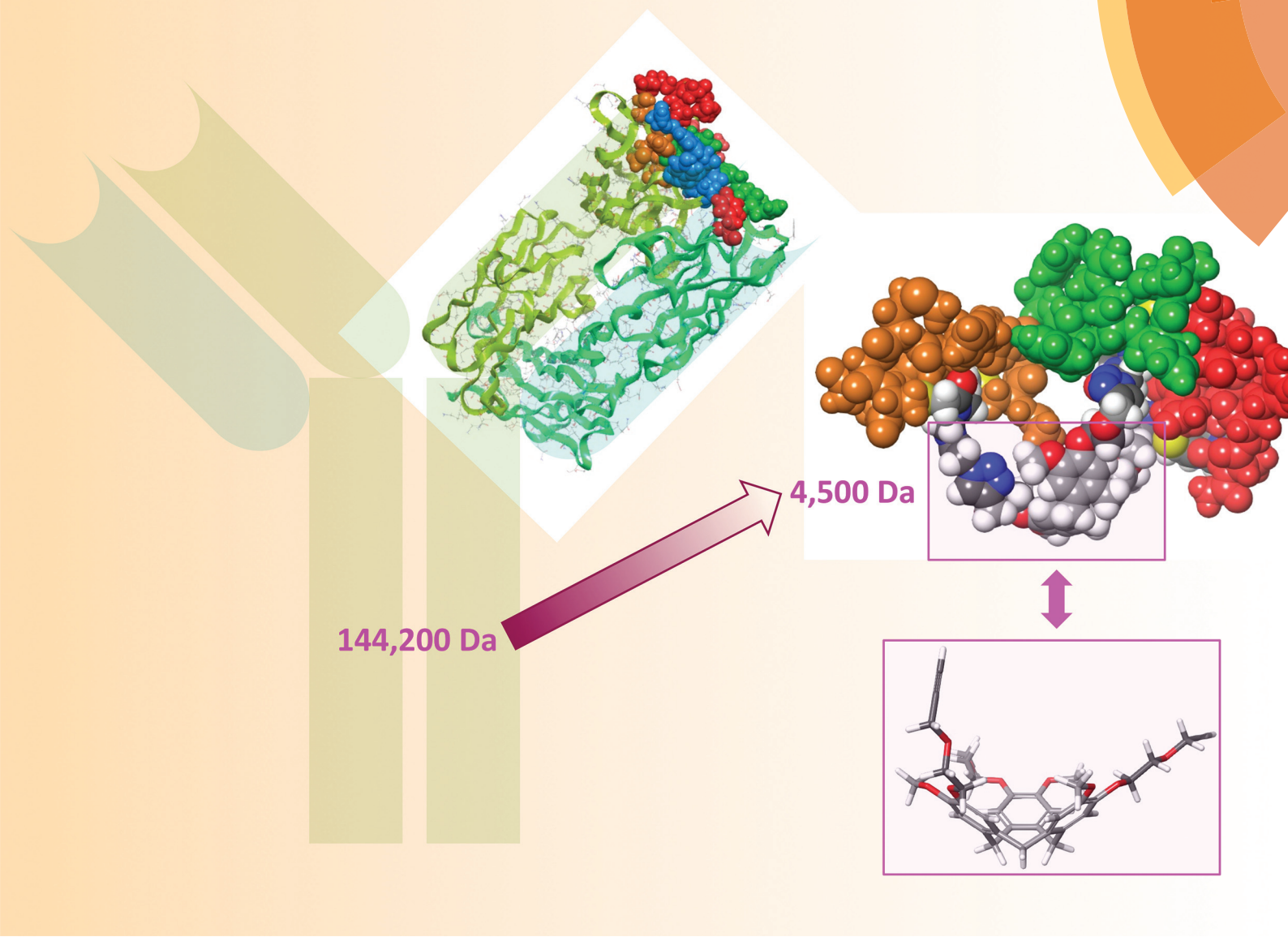

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Synthetic antibody protein mimics of infliximab by molecular scaffolding on novel CycloTriVeratrilene (CTV) derivatives†

Ondřej Longin, Mohammed Hezwani, Helmus van de Langemheen and Rob M. J. Liskamp *

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Syntheses of novel semi-orthogonally protected CycloTriVeratrilene (CTV) analogues with enhanced water solubility, that is **3** and **4**, derived from the previously described CTV scaffold derivative **2** are described here. These scaffolds **2–4** enabled a sequential introduction of three different complementarity determining region (CDR) mimics *via* Cu(I)-catalysed azide–alkyne cycloaddition towards medium-sized protein mimics denoted as "synthetic antibodies". The highly optimised sequential introduction enabled selective attachment of three different CDR mimics in a one-pot fashion. This approach of obtaining synthetic antibodies, demonstrated by the synthesis of paratope mimics of monoclonal antibody infliximab (Remicade®), provided a facile access to a range of (highly) pre-organised molecules bearing three different (cyclic) peptide segments and may find a wide range of applications in the field of protein–protein interaction disruptors as well as in the development of synthetic vaccines or lectin mimics. The prepared synthetic antibodies were tested for their affinity towards tumour necrosis factor alpha using surface plasmon resonance and synthetic antibodies with micromolar affinities were uncovered.

Introduction

For several years we have been interested in the molecular construction and further development of protein mimics toward medium-sized molecule alternatives of biologics such as antibodies and vaccines.[1–4] In this area, we are facing several important challenges, which may determine a successful outcome of this quest. One challenge of our continuous attention is the development of pre-organized molecular scaffolds as the core molecular unit for attaching complementarity determining region (CDR)-loops mimics. This goes hand-in-hand with our desire to develop scalable syntheses. In these endeavours we are increasingly faced with a limited or even poor solubility of our molecular constructs. With respect to this, it is of course realised that an important condition for the use of any bio-active compound is an adequate solubility in an aqueous environment. In fact a (very)poor water solubility or insolubility is responsible for the largest attrition rate of molecule candidates in the drug discovery process.[5] If it turns out to be impossible to improve water solubility by chemical modification, then depending on the drug candidate, more sophisticated drug delivery approaches are called for. Thus, the solubility issue is a crucial challenge in our approaches and if it can be dealt with in an early stage of molecular construction of our protein mimics, this is a preferred route. Concomitantly, convenience and scalability of synthesis remain very important issues in the assembly of these medium-size molecules.

Although paratope mimics consisting of a single CDR loop have recently been reported to be successful as synthetic antibody mimics,[6] molecular construction of mimics having more than one cyclic peptide mimicking the individual CDR loops may still be very beneficial for their affinity and selectivity.[1,2]

Recently, we described[4] the synthesis of the first semi-orthogonally protected derivative of a highly pre-organized CTV scaffold **1** (Fig. 1), the CTV scaffold derivative **2** (Fig. 2) onto which three different peptide segments representing CDR loops can be sequentially introduced. Furthermore, a convenient "click and cleavage" approach was presented for the sequential introduction of the three different CDR loop mimics onto this scaffold derivative towards the synthesis of synthetic antibodies.

Although synthesis of relatively complex synthetic antibodies could be achieved quite conveniently, the water solubility of the resulting protein mimic was rather low. Hence, in order to improve water solubility and thus facilitate subsequent evaluation of synthetic antibodies, the semi-orthogonally protected CTV scaffold derivatives **3** and **4** (Fig. 2) with improved water solubility were developed. In addition, the

School of Chemistry, Joseph Black Building, University of Glasgow, University Avenue, Glasgow G12 8QQ, UK and Department of Pharmaceutical Sciences, Faculty of Science, Utrecht University, P.O. Box 80082, NL-3508 TB Utrecht, The Netherlands. E-mail: Robert.liskamp@glasgow.ac.uk

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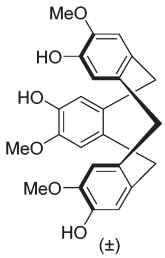

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Fig. 1 CTV scaffold enantiomers.

incorporation of a mono or diethylene glycol spacer (MEG or DEG) will indicate whether it is necessary to allow more space for accommodation of the CDR loop mimics, since they are significantly larger than the CTV scaffold itself and an elongated spacer may provide additional room. To obtain some insight into the structure of these CTV derivatives, molecular models were constructed using the Maestro (http://www.schrödinger.com) modelling suite in which MacroModel software was incorporated (Fig. 3). These molecular models (without silyl protecting groups) clearly show the pre-organized bowl shape of all three CTV derivatives. In addition the mono ethylene glycol moieties containing CTV derivative **3** may, next to increasing water solubility, provide some room for the relatively large peptide loops to be attached. In the model of the diethylene glycol moieties containing CTV derivative **4**, likely increased water solubility due to the diethylene glycol moieties, may reduce the pre-organization of the molecular construct, because of their increased flexibility.

Furthermore, the previously described "click and cleavage" protocol for synthesis of synthetic antibodies, which required purification and lyophilisation after each step of synthesis, has been optimised in such degree that purification and lyophilisation is only required at the very end of the synthesis. Moreover, the optimised one-pot protocol includes splitting of the reaction mixture towards the end of synthesis allowing for two different synthetic antibodies to be prepared simultaneously. These changes significantly reduced time required for synthesis of synthetic antibodies and greatly improved the overall yield of synthesis. Thus, two synthetic antibodies have now been prepared within only 28 hours with overall yield of 12% to 21%, corresponding to 65% to 73% per step of the five-step synthesis.

This optimised protocol was applied in the synthesis of synthetic antibodies, which mimic the paratope of monoclonal antibody (mAb) infliximab (Remicade®). Infliximab is a clinically used biologic for treatment of human tumour necrosis factor alpha (hTNFα) mediated autoimmune diseases and directly binds to hTNFα. Cyclised versions of CDRs of the paratope, *i.e.* CDR mimics **28–31**, were introduced on the CTV scaffold derivatives **2**, **3** and **4** to yield a collection of synthetic antibodies comprising of 12 compounds. The ability of the prepared synthetic antibodies to bind to hTNFα was tested by Surface Plasmon Resonance (SPR) using expressed hTNFα immobilised on a CM5 chip. The best hTNFα-binder, antibody mimic **36**, exhibited a K_D of 11 μM.

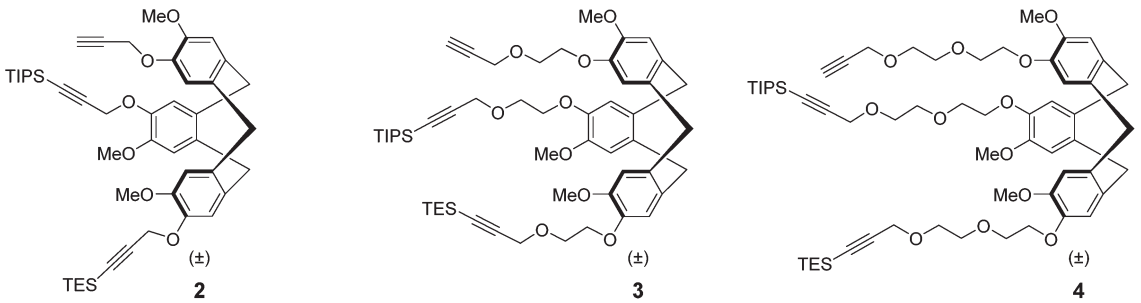


Fig. 2 Previously prepared semi-orthogonally protected CTV scaffold derivative **2** and the newly synthesised mono and diethylene glycol spacer containing analogues **3** and **4**.

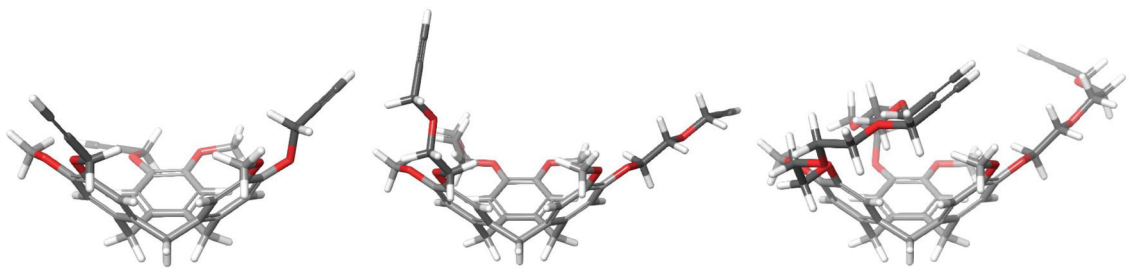
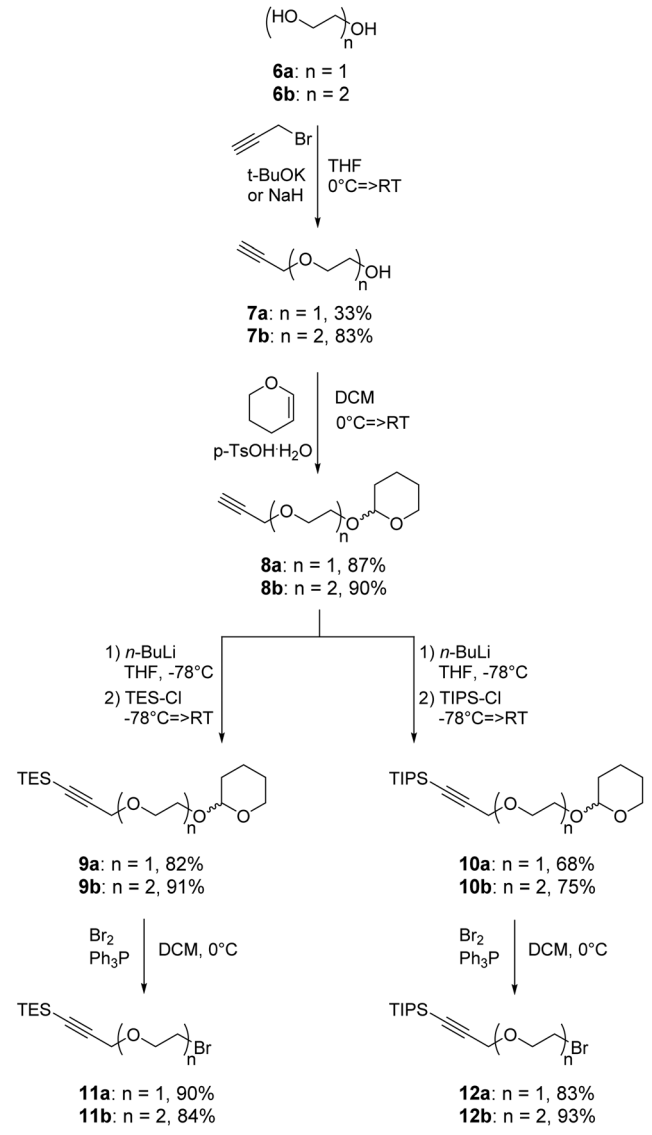


Fig. 3 Molecular models of CTV derivatives **2–4** (without TES and TIPS protecting groups) showing their pre-organization and possible influence of the mono or diethylene glycol moieties.

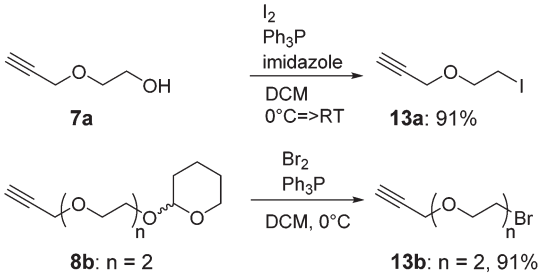
Results and discussion

The recent development of a semi-orthogonally protected CycloTriVeratrylene (CTV) scaffold derivative **2** opened up the way towards design and synthesis of other semi-orthogonally protected CTV scaffold derivatives with altered physico-chemical properties; that is mono and diethylene glycol spacer containing CTV scaffold derivatives **3** and **4**.

The required (oligo)ethylene glycol linkers for alkylation of the CTV scaffold, *i.e.* linkers of **a** and **b** series, were prepared by similar synthetic pathways (Schemes 1 and 2). Firstly, mono ethylene glycol **6a** and diethylene glycol **6b** were treated with sodium hydride and potassium *tert*-butoxide, respectively, followed by a treatment with propargyl bromide to yield the corresponding propargyl ether derivatives **7a** and **7b**. Next, the hydroxyl group of propargyl containing derivatives **7a,b** was protected with dihydropyran to provide tetrahydropyranyl (THP) protected derivatives **8a,b**. Then, the free alkyne was silylated with triethylsilyl (TES) chloride or triisopropylsilyl (TIPS) chloride using *n*-butyl lithium as a base to yield TES and TIPS protected derivatives **9a,b** and **10a,b**, respectively. Finally, the THP protected hydroxyl groups of the derivatives **9a,b** and **10a,b** were converted into bromides using bromine and triphenylphospine to give the desired TES **11a,b** and TIPS **12a,b** protected alkylation linkers. The linker derivatives **13a** and **13b** bearing unprotected alkyne moieties were prepared from derivatives **7a** and **8b** by conversion of free or the THP protected hydroxyl groups into iodide and bromide, respectively.



Scheme 1 Synthesis of silyl protected alkylation linkers **11a,b** and **12a,b**.



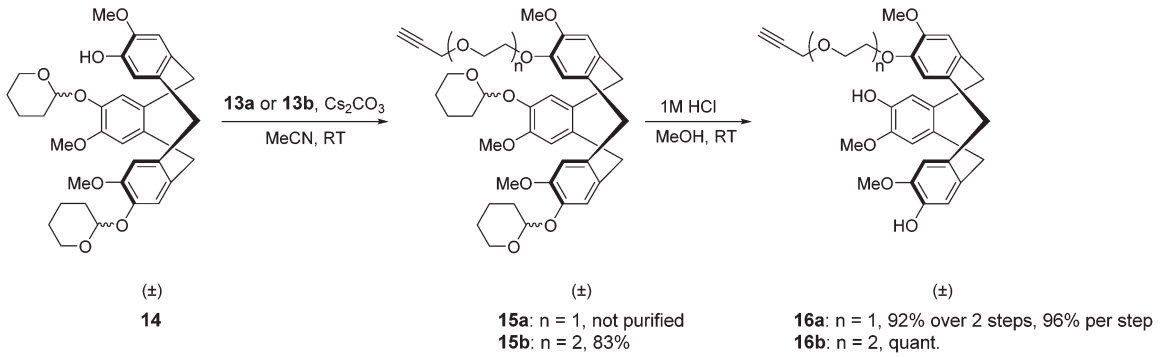
Scheme 2 Synthesis of free alkyne bearing alkylation linkers **13a** and **13b**.

After this CTV scaffold **1** was prepared according to the procedure of Canceill *et al.*[7] Protection of CTV scaffold **1** as a ditetrahydropyranyl (diTHP) derivative **14** as well as the recovery of CTV scaffold **1** from side products of the protection was performed as previously described.[4] Alkylation of diTHP protected scaffold **14** with (oligo)ethylene glycol linkers **13a,b** was performed in acetonitrile in the presence of Cs_2CO_3 to yield monoalkylated diTHP protected CTV scaffold derivative **15a,b** (Scheme 3) in high yield. Following the removal of THP protecting groups with 1 M HCl in methanol (Scheme 3), the dihydroxyl CTV scaffold derivatives **16a,b** were alkylated with TIPS protected linkers **12a,b** (Scheme 4) providing rather modest yields of the desired monoTIPS derivatives **17a,b** along with unwanted diTIPS side products **18a,b** (Scheme 4) while leaving some of the dihydroxyl CTV scaffold **16a,b** unreacted. Finally, the TES protected linkers **11a,b** were introduced to monoTIPS CTV scaffold derivatives **17a,b** in DMF using Cs_2CO_3 as a base to yield the semi-orthogonally protected CTV scaffold derivatives **3** and **4** (Scheme 4). In case of the CTV scaffold derivative **3** a hydrolysis of TES protecting group and therefore formation of a side product **5** lacking the TES protecting group was observed.

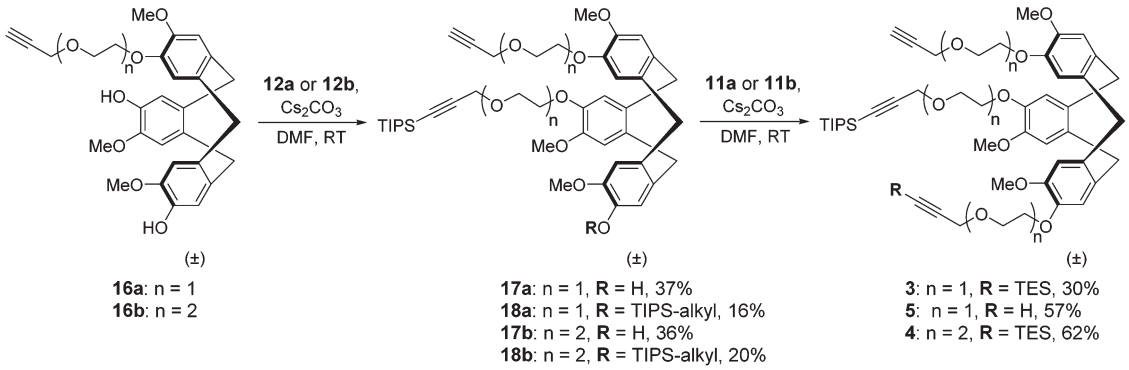
Improvement of aqueous solubility by incorporation of ethylene glycol spacers

Since silyl groups are not present in the final products, *i.e.* synthetic antibodies, aqueous solubility of the desilylated analogues **20**, **21**, and **22** (Scheme 5) of CTV scaffold derivatives **2**, **3**, and **4**, respectively, were determined, by measurement of dissolved quantities using reverse phase HPLC.

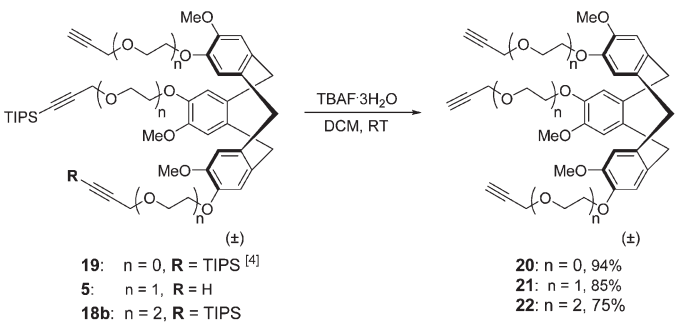
Indeed, incorporation of the (oligo)ethylene glycol linkers improved water solubility as the mono-ethylene glycol derivative **21** and diethylene glycol derivative **22** were found to be 5 and 14 times more soluble in deionised water (supplemented



Scheme 3 Indirect monoalkylation of CTV scaffold.



Scheme 4 Introduction of TIPS/TES protected linkers onto monoalkylated CTV scaffold.



Scheme 5 Synthesis of desilylated analogues **20**, **21**, **22** of CTV scaffold derivatives **2**, **3**, and **4**.

with 2% of ethanol) than CTV scaffold derivative **20** without a solubilizing linker.

Unfortunately, improvement of the solubility of the synthetic antibodies containing these CTV derivatives in PBS buffer pH 7.4 was not sufficient to allow for their biological activity evaluation on cell lines. The prepared synthetic antibodies were reasonably soluble in 2-(*N*-morpholino)ethanesulfonic acid hydrate (MES) buffer pH 6.0 containing 5% DMSO.‡

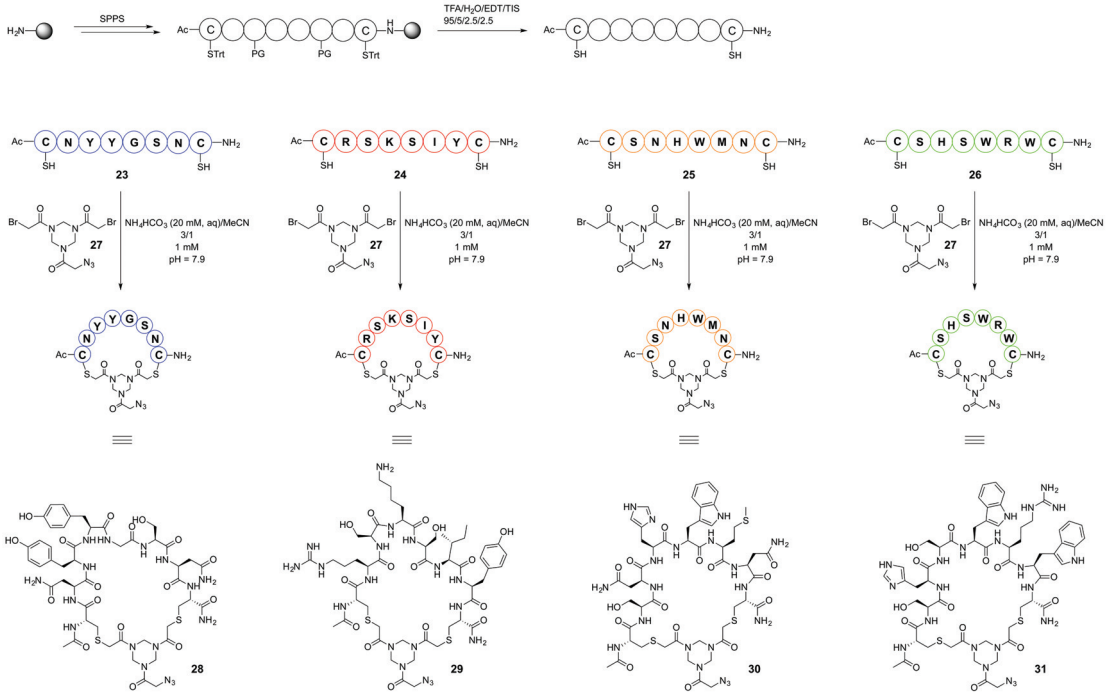
‡A few of the synthetic antibody constructs derived from CTV scaffold derivative **2** tended to precipitate from the solution within several hours of solution preparation. Nevertheless, this time frame still allowed for carrying out the SPR experiments.

Log *P* determination

Log *P* values of the desilylated analogues **20**, **21**, and **22** were also determined using an Organisation for Economic Co-operation and Development (OECD) approved reverse phase HPLC method.[8] Log *P* values of 4.1, 4.3, and 4.6 were determined for compounds **20**, **22** and **21**, respectively. The similar log *P* values indicate that the resulting protein mimics will have similar cell permeability properties and ensuing biological activity properties, which are not influenced by variation of the scaffold.

CDRs selection and peptide cyclisation

As was described previously[4] selection of peptide sequences mimicking CDRs of the paratope of mAb infliximab



Scheme 6 Synthesis and cyclisation of linear CDR mimics.

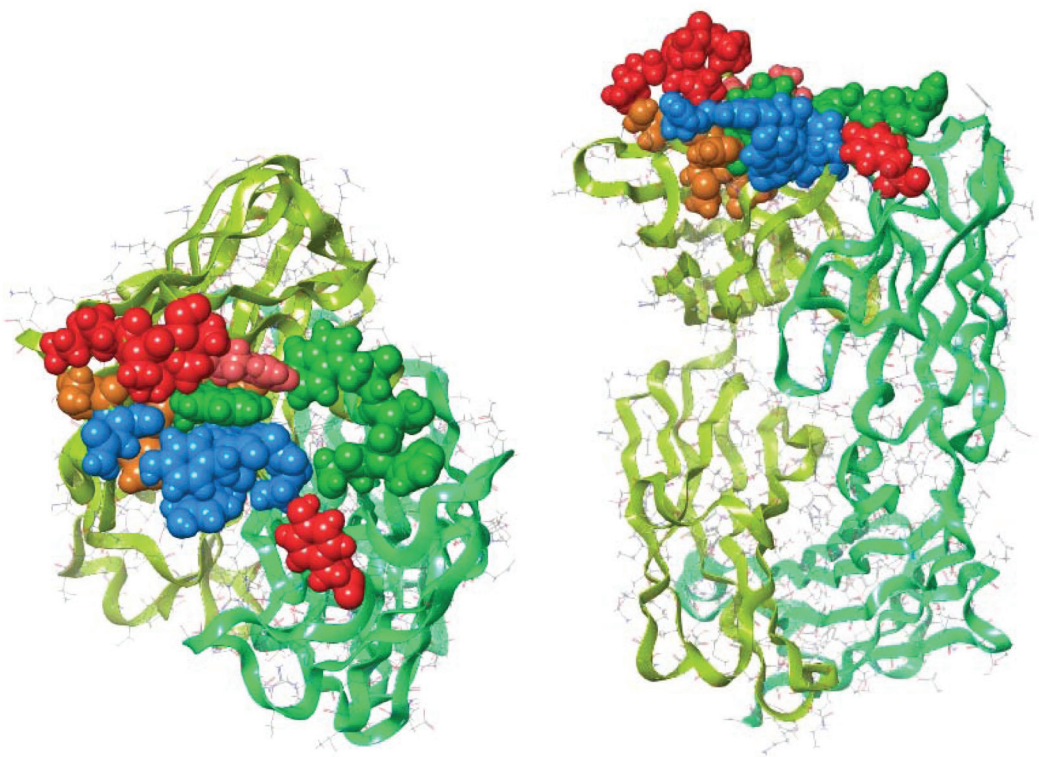


Fig. 4 CDR loops in infliximab based on reported interactions between hTNFα and infliximab Fab.[9] The colour coding corresponds to the peptides in the CDR mimics and toward the molecular synthetic antibody constructs. A top view (interaction site with hTNFα) and a side view are shown. The residue (Arg-52, Heavy chain), which was part of the red and green loop, is salmon-coloured.

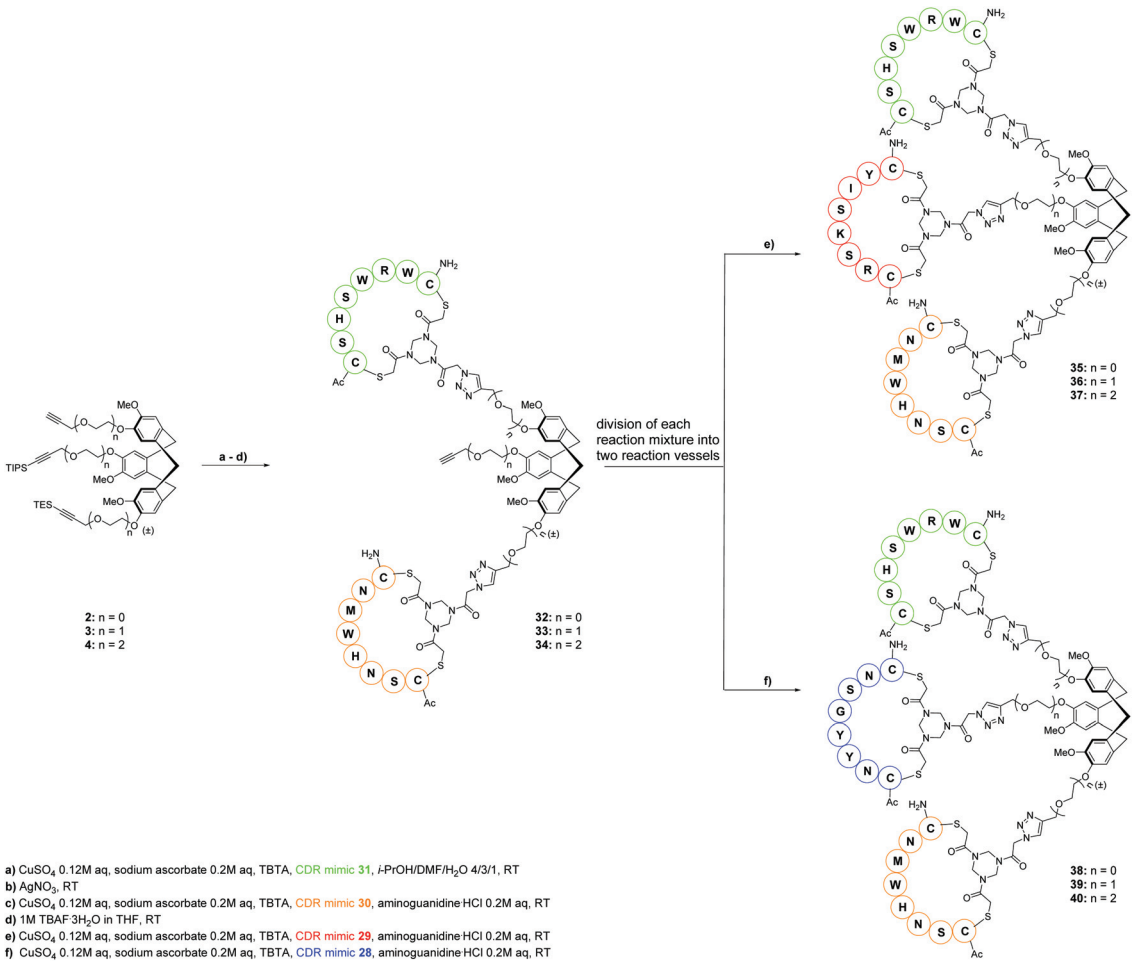
(Remicade®) was based on the X-ray crystal structure[9] of a complex of infliximab with hTNFα trimer. In this research, selection of CDR mimics was extended by including peptide segment **23** (Scheme 6) as its cyclised derivative **28**. This particular sequence has been shown to be crucial for binding to human hTNFα as was demonstrated by a mutation study.[9] Thus, a total of four peptide segments which mimic the selected CDRs were prepared. Three sequences, **23**, **24**, **26**, correspond to the sequences present in the entire peptide segment of the CDR loops of infliximab. Peptide sequence **25** mainly consists of distinct amino acid residues present in the antibody which are important for binding to hTNFα as is apparent from the X-ray structure of the complex and which are in close spatial proximity to target amino acids in hTNFα. These peptide segments of the CDRs of infliximab are shown in colour (Fig. 4), which corresponds to the colours of the CDR loop mimics **28–31** used in the construction of the synthetic antibodies.

The selected peptide sequences were furnished with both an N and a C terminal cysteine residue to allow their chemoselective cyclisation using our recently developed polar hinge **27**[10] (Scheme 5) which should enhance water solubility of the cyclised peptides. The resulting azide handle containing cyclized peptides **28–31** were subsequently introduced *via* Cu(I)-catalyzed azide alkyne cycloaddition (CuAAC) onto the CTV scaffold derivatives **2**, **3** and **4** as will be described below.

One-pot synthesis of synthetic antibodies

From the very start of this research, it was our desire to ultimately develop synthesis protocols for these medium-size molecules, which could be conveniently reproduced and applied also by researchers not directly working in the peptide field. Our recent CTV scaffold based protein mimic was assembled in a "click and cleavage" approach with several intermediate purifications.[4] Here, this approach was extended to a one-pot procedure where the CDR loop mimics were subsequently introduced on each of the CTV scaffolds **2**, **3** or **4**. For clarity: only one CTV scaffold derivative was used in each reaction and not a mixture of **2–4**. After introduction of the first and second CDR loop mimic the silyl protecting groups were removed and only after complete assembly the antibody mimics were purified.

Thus, one equivalent of CDR loop mimic **31** was introduced *via* CuAAC onto one equivalent of either CTV scaffold derivative **2**, **3**, or **4** (Scheme 7) to give a one CDR loop containing protein mimic. Immediately thereafter, silver nitrate was



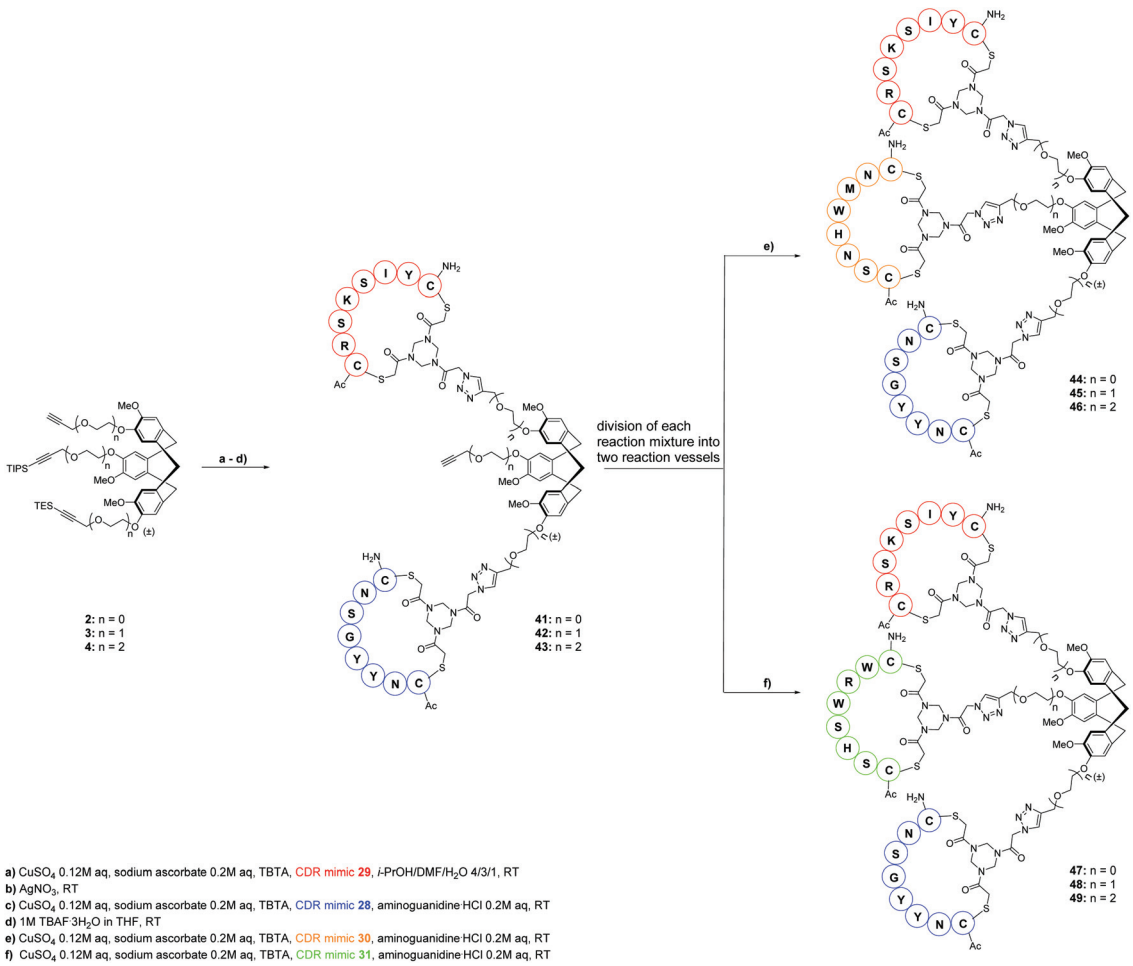
Scheme 7 One-pot synthetic approach towards synthetic antibodies **35–40**.

added to the reaction mixture to selectively remove the TES protecting group[11] and without intermediate purification the next CDR-mimicking peptide loop **30** was introduced *via* CuAAC leading to the two CDR loop mimic containing CTV derivatives. During this reaction aminoguanidine hydrochloride was added as a scavenger of the formed dehydroascorbic acid *i.e.*[12] 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 giving two loop containing protein mimic intermediates **32**, **33**, **34** and the reaction mixture was split into two equal portions. To one portion, 1.2 equivalents of CDR loop mimic **29** were added while to the other, 1.2 equivalents of CDR loop mimic **28** were added. Thereby in a convenient manner two different synthetic antibodies were prepared. Upon completion of this one-pot procedure, the reaction mixtures were concentrated and the antibody mimics were purified by semi-preparative HPLC.

By using this same one-pot procedure albeit changing the sequences of addition of the various CDR-mimicking peptide loops to CTV scaffold derivative **2**, **3**, or **4** the other antibody mimics **44–49** were prepared (Scheme 8). Thus, CDR loop mimic **29** was introduced onto either scaffold **2**, **3**, or **4** *via* CuAAC, followed by removal of TES group with silver nitrate and subsequent introduction of the CDR mimic **28** before TIPS protecting group was removed with TBAF·3H_2O to give intermediates **41**, **42**, **43**. After splitting of the reaction mixture in two equal parts, CDR loop mimic **30** was added to one part of the mixture while CDR loop mimic **31** was added to the other part.

Hence, the described approach led to a collection of twelve protein mimics **35–40** and **44–49** designated as "synthetic antibodies" (Fig. 5). This collection comprised all possible combinations of the four CDR loop mimics **28–31** onto each of the CTV scaffold derivatives **2**, **3** or **4**.

Progress of conversion of the CTV derivatives by subsequent peptide loop introduction was conveniently monitored by analytical reversed phase HPLC as the retention times of the intermediate products were sufficiently different (Fig. 6). As was apparent from the chromatograms, the reaction mixture remained very clean until the introduction of the third CDR loop mimic when side products, undesired combinations of previously unreacted CDR mimics on CTV scaffold derivative, with retention times close to that of the desired synthetic antibody mimic started to appear. Nevertheless, these side products could be largely separated with an optimised gradient



Scheme 8 One-pot synthetic approach towards synthetic antibodies **44–49**.

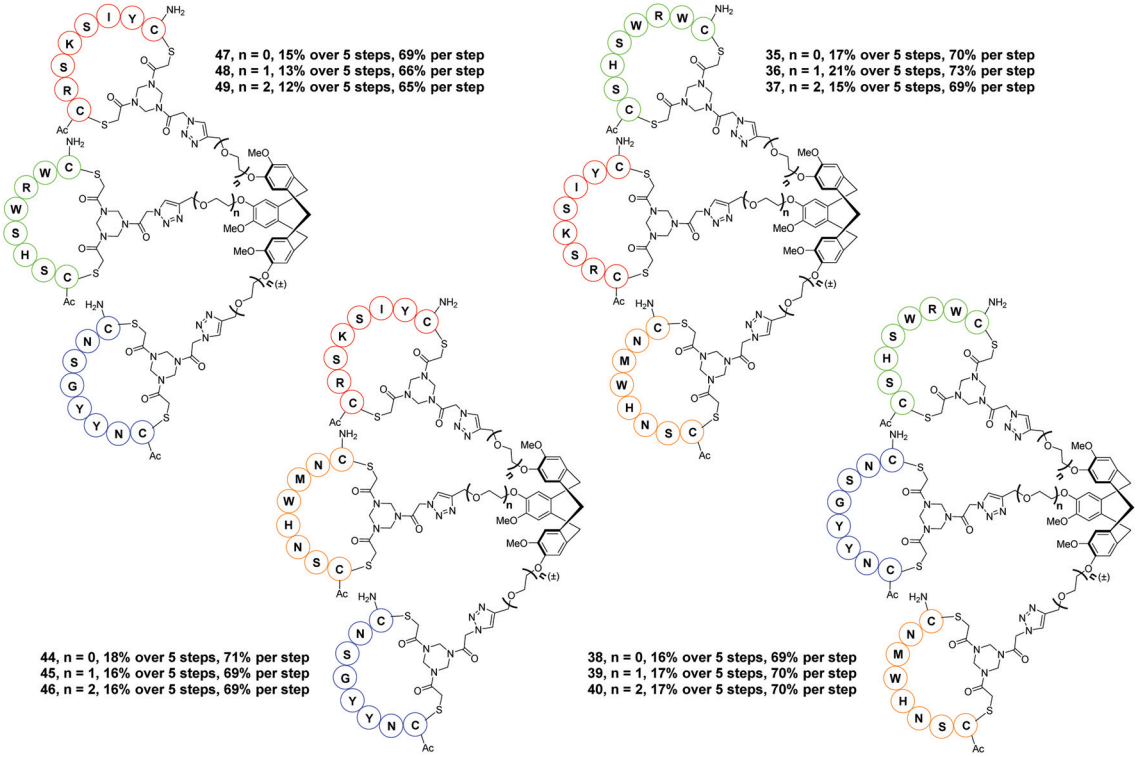


Fig. 5 Prepared collection of antibody mimics toward synthetic antibodies.

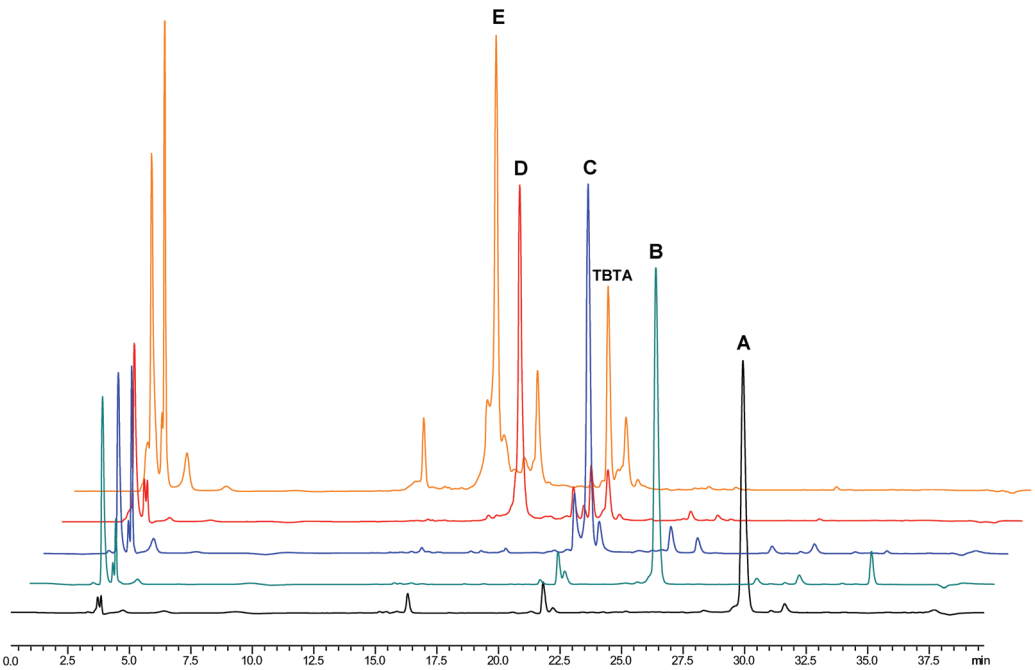


Fig. 6 Representative analytical HPLC chromatogram of the one-pot synthesis of synthetic antibodies – overlay of the entire reaction sequence for synthetic antibody **37**. Black (A) – product after introduction of CDR mimic **31** onto CTV scaffold derivative **4**. Green (B) – product after TES protecting group removal. Blue (C) – product after introduction of CDR mimic **30**. Red (D) – product after TIPS protecting group removal. Orange (E) – product after introduction of CDR mimic **29**. Peak at t_R = 21.8 min represents TBTA.

on a preparative C8 reversed phase column. Thus, the desired protein mimics were obtained in purities of 91%–96%.

Surface plasmon resonance

Initially, binding studies of protein mimics were attempted using Isothermal Titration Calorimetry (ITC). However, these were unsuccessful because of the need to dialyse hTNFα and synthetic antibodies to exactly match DMSO concentration (2% of final volume of appropriate buffer) in separately prepared samples and thus to avoid an artificial DMSO signal. Unfortunately, it was found that the synthetic antibodies stick to the cellulose dialysis membrane and as a consequence disappear from solution. Therefore binding studies of the prepared synthetic antibodies **35–40** and **44–49** were carried out by Surface Plasmon Resonance (SPR) on immobilised hTNFα. The integrity of immobilised hTNFα was first verified by flowing an anti-hTNFα specific monoclonal antibody (Life Technologies, Thermo Fisher Scientific, clone 68B 2B3) over the chip surface and indeed was capable of interacting with immobilised hTNFα (K_D = 32 nM). After an initial screening of the prepared protein mimics at 2 μM (runs in duplicate) positive hits, *i.e.* compounds exhibiting binding, were selected and their binding characteristics were further determined. A representative sensogram of the best binding synthetic antibody **36** (Fig. 7) as well as kinetic parameters of synthetic antibodies **35**, **36**, **44**, **47**, and **48** (Table 1) are shown below.

The common crucial feature of synthetic antibodies **35**, **36**, **37**, **44**, **47**, **48** and **49**, capable of binding hTNFα, was the presence of CDR (red loop) mimic **29**. CDR (green loop) mimic **31** proved to be important too as it was present in all synthetic antibodies capable of successfully binding to hTNFα except for synthetic antibody **44** which contained CDR (orange loop) **30** instead next to (red loop) mimic **29**. In general, incorporation of combinations of CDR mimics (red loop) **29** and (green loop) **31** together with either CDR mimic (blue loop) **28** or (orange loop) **30** in a synthetic antibody led to antibody mimics with micromolar range affinity for hTNFα. Based on the obtained K_D values (Table 1), the combination of CDR mimic (red loop) **29**, (orange loop) **30**, (green loop) **31** has slightly better affinity for hTNFα than combination of CDR mimics (blue loop) **28**, (red loop) **29**, (green loop) **31**. Incorporation of the combination of CDR mimics (blue loop) **28** and (orange loop) **30** into a synthetic antibody resulted generally in lack of binding of the corresponding synthetic antibodies to hTNFα, *i.e.* **38**, **39**, **40**, **45** and **46**, with the exception of construct **44**, which still displayed a decent affinity (K_D = 66 μM). Upon closer examination of this phenomenon supported by modelling (Fig. 8) it was concluded that CDR mimic (blue loop) **28** and (orange loop) **30** originate from an overlapping region of infliximab and thus may interfere with each other resulting in the lack of binding for hTNFα. This however is not the case for synthetic antibody **44** where the absence of an ethylene glycol linker might have prevented this interference.

Synthetic antibodies derived from CTV scaffolds **2** or **3** capable of binding hTNFα, had a kinetic profile suitable for fitting of the measured curves. Synthetic antibodies derived from CTV scaffold derivative **4** had too fast kinetics (k_{off} greater than 1×10^{-1} s^{-1}) to reliably determine their dissociation constant. This might be due to a higher flexibility, of the diethylene glycol spacer, resulting in an increase of flexibility, possibly leading to a decrease in binding affinity. This is also apparent from Fig. 3 showing that the diethylene glycol spacer may lead to less directionality, so that the loops are gathered more around the CTV scaffold, rather than pointing in the same direction.

In view of the above results it was not entirely surprising that CDR (green loop) mimic **31** by itself showed affinity for hTNFα nevertheless the measured kinetics was too fast to reliably obtain a dissociation constant. However, the other three CDR mimics including the CDR (red loop) **29**, had no affinity for hTNFα whatsoever. As anticipated, a multivalent effect induced by attachment of the appropriate CDR mimics on suitable CTV scaffold derivatives, resulting in increased affinities was observed. This also clearly demonstrated the crucial role of CTV scaffold derivatives in the molecular construction of antibody mimics by covalent assembly of individual CDR loop mimics.

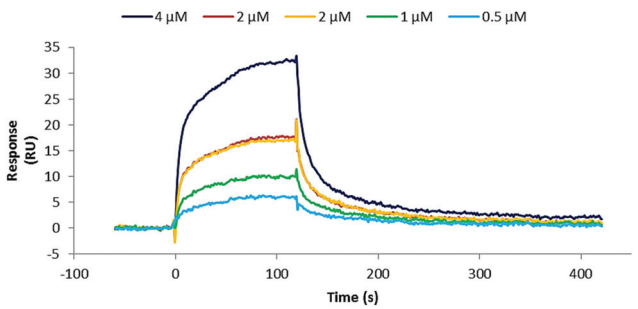


Fig. 7 Sensogram representing binding of synthetic antibody **36** to immobilised hTNFα. Raw data presented.§

Conclusion

A new one-pot approach allowing for rapid synthesis of synthetic antibodies has been described. The approach was applied in the construction of a collection of synthetic antibody candidates mimicking the paratope of mAb infliximab (Remicade®). The ability of the synthetic antibodies to mimic the original monoclonal antibody infliximab was determined by measuring their binding to hTNFα, using SPR. SPR measurements showed that 7 out of 12 prepared synthetic antibody candidates were capable of binding hTNFα with 5 of them displaying dissociation constants ranging from 11 μM to 66 μM. The relatively strong binding with a K_D of 11 μM clearly supported the validity of the concept of molecular scaffolding of CDR mimics towards possible synthetic antibodies.

§ Selected data, cleaned and fitted with heterogeneous ligand binding model, are included in the ESI.†

Table 1 Obtained kinetic parameters for interaction of synthetic antibodies with immobilized hTNFα

Synthetic antibody	k_{on} M^{-1} s^{-1}	k_{off} s^{-1}	K_D (M)
36 (scaffold **3**:CDRs **29**-**31**-**30**)	$7.76 \pm 1.23 \times 10^{3}$	$7.68 \pm 0.44 \times 10^{-2}$	$1.10 \pm 0.28 \times 10^{-5}$
35 (scaffold **2**:CDRs **29**-**31**-**30**)	$7.86 \pm 5.50 \times 10^{3}$	$6.99 \pm 2.40 \times 10^{-2}$	$1.35 \pm 0.60 \times 10^{-5}$
48 (scaffold **3**:CDRs **29**-**31**-**28**)	$4.32 \pm 0.96 \times 10^{3}$	$8.40 \pm 0.30 \times 10^{-2}$	$2.07 \pm 0.52 \times 10^{-5}$
47 (scaffold **2**:CDRs **29**-**31**-**28**)	$1.51 \pm 0.04 \times 10^{3}$	$4.83 \pm 0.51 \times 10^{-2}$	$3.23 \pm 0.29 \times 10^{-5}$
44 (scaffold **2**:CDRs **29**-**28**-**30**)	$1.04 \pm 0.11 \times 10^{3}$	$6.83 \pm 0.52 \times 10^{-2}$	$6.60 \pm 0.22 \times 10^{-5}$

Average values of 2 experiments performed in duplicate ($n = 2$) reported.

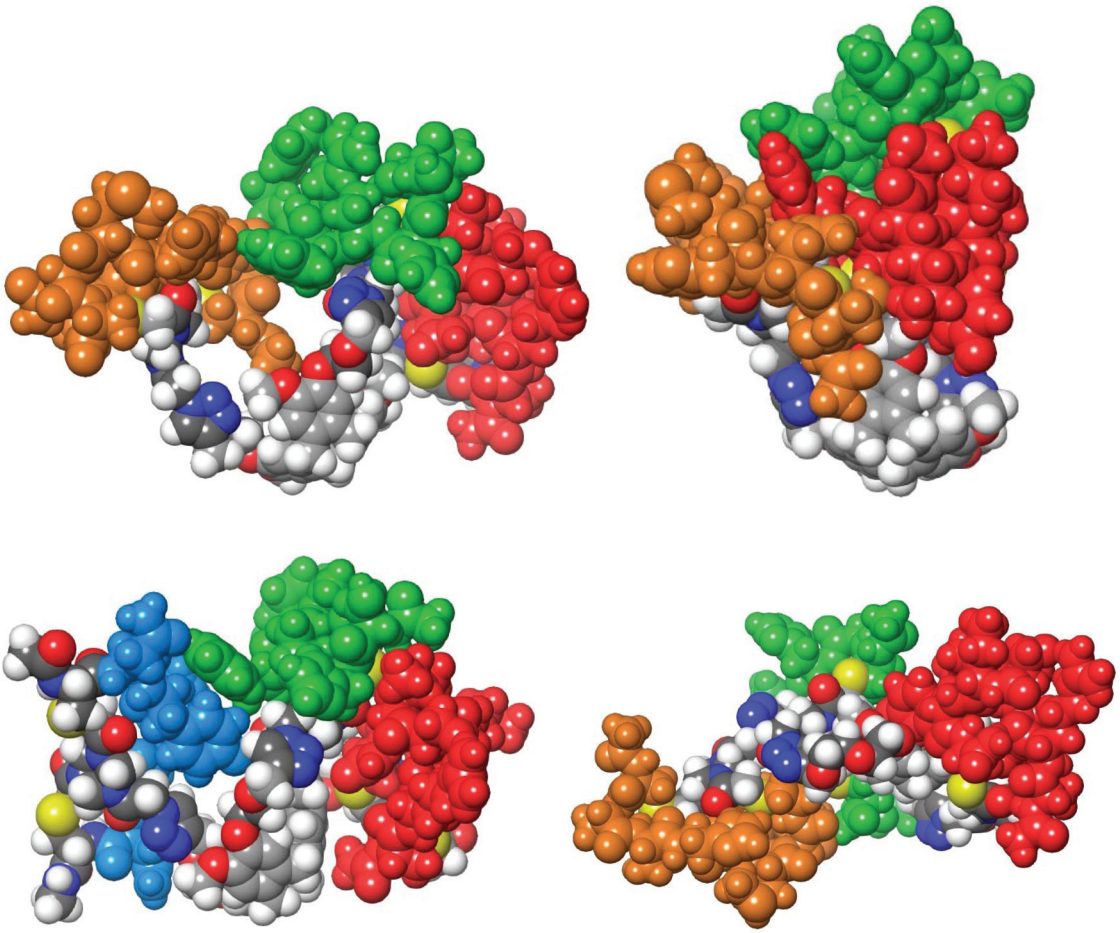


Fig. 8 Molecular models of the synthetic antibody protein mimics **36** (top-left), **35** (top-right), **48** (bottom-left) and **37** (bottom-right). The first three are the best binding candidates. Molecular construct **37** is included to compare the influence of the diethylene glycol moiety with the constructs **36** and **35** having a mono ethylene glycol moiety or none, respectively. The weak affinity for immobilized hTNFα by **37** might be explained by the increased flexibility of the diethylene glycol moieties not allowing an optimal multivalent interaction.

Since the synthetic antibodies are prepared from racemic CTV, diastereomers of the synthetic antibodies have been obtained. However, it is likely that the size the attached CDR loop mimics will mask the difference between the enantiomeric CTV moieties.

Moreover, in this approach the rather low water solubility of the previously reported[4] semi-orthogonally protected CTV scaffold derivative **2** was addressed by synthesising its monoethylene and diethylene glycol spacer containing analogues **3** and **4** respectively. Although incorporation of the glycol spacers did improve water solubility 5 times and 14 times respectively, these improvements were not sufficient for dissolution of these synthetic antibodies in PBS buffer pH 7.4 to allow bio-activity evaluation on cell lines. Therefore, under present investigation is further improvement of the water solubility of CTV derivatives.

In conclusion, the concept of replacing the majority of the structure of a monoclonal antibody by a molecular scaffold capable of performing a similar structural functional role as in the parent antibody leading to a significantly smaller protein

mimic has proven to be valid. The obtained protein mimics denoted as a "synthetic antibodies" have less than 4% of the molecular mass of the mimicked monoclonal antibody infliximab. Nevertheless, the affinity of the prepared protein mimics is still considerably lower than that of the original monoclonal antibody, but the obtained micromolar range affinity might indicate that in addition to room for improvement, that this is a promising approach. It is realised, however that synthesis of these fairly complex molecular constructs has to be continuously improved if not simplified. In this work, considerable strides have been made toward significantly improving the synthesis of these protein mimics in terms of time and efficiency.

Two synthetic antibodies have been conveniently prepared in less than 30 hours while only requiring semi-preparative reverse phase HPLC purification at the very end of the synthesis. Thus, this synthetic protocol is a great improvement compared to our previously described methods for CTV scaffold derivatives as well as other TAC and related scaffolds,[3,4,13] which required semi-preparative HPLC purification and isolation after practically each step of the synthesis of corresponding protein mimics, in total 4 times or 5 times. Furthermore, this protocol enabled attachment of three different ligands/substituents to one single molecule *via* Cu(I)-mediated azide–alkyne cycloaddition in a one pot reaction. Application of this protocol can however go beyond the introduction of peptide loops leading to synthetic antibodies as virtually any azide handle containing small or larger ligand can be introduced onto the CTV scaffold derivatives **2**, **3**, and **4** for example carbohydrates, nucleic acids and small molecules or combinations of these, giving rise to synthetic vaccines, molecular constructs interacting with carbohydrate binding proteins (lectins), covalent multi-enzyme complexes, enzyme mimics *etc*.

Experimental part

General information

All analytical or HPLC grade chemicals and solvents were purchased from commercial sources and were used as received unless stated otherwise. ^{1}H NMR and the ^{13}C NMR spectra were recorded on a Bruker AVIII 400 MHz or 500 MHz Spectrospin spectrometer in $CDCl_3$. Chemical shifts (δ) are reported in parts per million (ppm) relative to trimethylsilane (TMS, 0.00 ppm) or $CDCl_3$ (7.26 ppm). TLC was carried out on silica gel plates (Merck $60F_{254}$) and visualization was carried out by both UV detection (254 nm) and staining (cerium molybdenate, potassium permanganate) followed by heating. For column chromatography, silica gel Geduran® Si 60 (40–63 μm) was used. Dry solvents (THF, CH_2Cl_2) were dispensed from Pure Solv™ 500 Solvent Purification System and other dry solvents (acetonitrile, DMF) were obtained from freshly opened commercially available HPLC grade solvents by removal of residual water with activated 4 Å molecular sieves overnight. HRMS-ESI was recorded on Bruker microTOFq High Resolution Mass Spectrometer in the positive mode. EI-MS was recorded on Jeol MSTATION JMS-700 in the positive mode. Commercial solutions of *n*-butyl lithium were titrated using diphenyl acetic acid to determine their actual concentration prior to use. Degassed solvents for synthesis of synthetic antibodies were obtained upon sonication of the solvents in degas mode for 30 minutes while purging the headspace with nitrogen stream trough septum. Petroleum ether used refers to a mixture with boiling point of 40 °C–60 °C.

Fmoc-amino acids were obtained from Activotec (Cambridge, United Kingdom) and *N*,*N*,*N*′,*N*′-tetramethyl-*O*-(6-chloro-1*H*-benzotriazol-1-yl)uranium hexafluorophosphate (HCTU) was obtained from Matrix Innovation (Quebec, Canada). Tentagel S RAM resin (particle size 90 μm, capacity 0.25 mmol g^{-1}) was obtained from IRIS Biotech (Marktredwitz, Germany). Methyl *tert*-butyl ether (MTBE), *n*-hexane (HPLC grade) and TFA were obtained from Aldrich (Milwaukee, USA). DMF (Peptide grade) was obtained from VWR (Lutterworth, United Kingdom). Piperidine and DiPEA were obtained from AGTC Bioproducts (Hessle, United Kingdom), and 1,2-ethanedithiol (EDT) was obtained from Merck (Darmstadt, Germany). HPLC grade CH_2Cl_2 and acetonitrile were obtained from Fischer Scientific (Loughborough, United Kingdom). Solid phase peptide synthesis was performed on a PTI Tribute-UV peptide synthesizer. Lyophilisations were performed on a Christ Alpha 2–4 LD*plus* apparatus. Analytical high pressure liquid chromatography (HPLC) was carried out on a Shimadzu instrument comprising a communication module (CBM-20A), autosampler (SIL-20HT), pump modules (LC-20AT), UV/Vis detector (SPD-20A) and system controller (Labsolutions V5.54 SP), with a Phenomenex Gemini C18 column (110 Å, 5 μm, 250 × 4.60 mm). UV measurements were recorded at 214 and 254 nm, using a standard protocol: 100% buffer A (acetonitrile/H_2O 5 : 95 with 0.1% TFA) for 2 min followed by a linear gradient of buffer B (acetonitrile/H_2O 95 : 5 with 0.1% TFA) into buffer A (0–100%) over 30 min at a flow rate of 1.0 mL min^{-1}. Purification of synthetic antibodies was performed on an Agilent Technologies 1260 infinity preparative system using UV detector with a Dr Maisch C8 column (110 Å, 10 μm, 250 × 20 mm) starting with 100% buffer A for 10 min followed by a linear gradient of buffer B into buffer A (27–37%) over 40 min at a flow rate of 10.0 mL min^{-1}. CDR mimics were purified on the same system using Phenomenex Gemini C18 column (110 Å, 10 μm, 250 × 20 mm) starting with 100% buffer A for 5 min followed by and linear gradient of buffer B into buffer A (gradient specified for each compound) over 60 min at a flow rate of 12.5 mL min^{-1}. The buffers had same composition as described for analytical HPLC. Auto-collection of fractions was based on the UV measurements at 214 nm. One-pot reaction mixtures were analysed by taking 10 μL of the reaction mixture, removing solvent under stream of nitrogen, dissolving the dried residue in buffer A/buffer B (1/1 v/v, 500 μL) and injecting of 50 μL of the obtained solution on analytical HPLC. Liquid chromatography mass spectrometry (LCMS) was carried out on a Thermo Scientific LCQ Fleet quadrupole mass spectrometer with a Dionex Ultimate 3000 LC using a

Dr Maisch Reprosil Gold 120 C18 column (110 Å, 3 μm, 150 × 4.0 mm), using a 0–100% linear gradient of buffer B into buffer A and the same flow rate and buffers as described for analytical HPLC. Deionised water was obtained on Milli-Q® station (Merck Millipore).

Alkylation linker synthesis and CTV scaffold derivatisation

Propargyl-MEG-OH 7a. Compound **7a** was prepared according to a modified literature procedure.[14] To a solution of Anhydrous ethylene glycol **6a** (15.0 mL, 271 mmol) in dry THF (20 mL), NaH (2.71 g, 67.8 mmol, 60% dispersion in mineral oil) was added portion wise under nitrogen stream. Once hydrogen formation ceased, propargyl bromide (7.31 mL (67.8 mmol) of a 80 wt% solution in toluene) was slowly added and the resulting reaction mixture was stirred for 2 h at 45 °C. Then the reaction mixture was diluted with CH_2Cl_2 (250 mL) and washed with water (250 mL). The aqueous layer was extracted with CH_2Cl_2 (125 mL) and combined organic layers were dried over $MgSO_4$, filtered, and CH_2Cl_2 was removed under reduced pressure. The crude product was purified by column chromatography (*n*-hexane/Et_2O 6/4 to Et_2O). Compound **7a** (2.0 g, 33%) was obtained as a yellow oil. R_f = 0.43 (*n*-hexane/EtOAc 1/1). **^{1}H-NMR** (400 MHz, $CDCl_3$): δ = 2.44 (t, J = 2.4 Hz, 1H), 2.47 (t, J = 5.2 Hz, 1H), 3.61–3.63 (m, 2H), 3.72–3.75 (m, 2H), 4.18 (d, J = 2.4 Hz, 2H). **^{13}C-NMR** (100 MHz, $CDCl_3$): δ = 58.5, 61.7, 71.3, 74.8, 79.6. The characterisation of this compound has also been previously reported.[15]

***O*-THP-propargyl-MEG 8a.** Compound **8a** was prepared according to a literature procedure.[16] To a solution of compound **7a** (2.03 g, 20.3 mmol) in dry CH_2Cl_2 (20 mL), DHP (1.94 mL, 21.3 mmol) was added and the reaction mixture was cooled to 0 °C. Next, p-TsOH·H_2O (0.04 g, 0.20 mmol) was added to the reaction mixture which was then allowed to warm to RT and stirred for 4 h. Then, the reaction mixture was washed with aqueous saturated $NaHCO_3$ (30 mL), the aqueous phase was extracted with CH_2Cl_2 (10 mL) and the combined organic layers were dried over $MgSO_4$, filtered, and CH_2Cl_2 was removed under reduced pressure with the water bath kept at RT (19 °C–21 °C). The crude product was purified by column chromatography (*n*-hexane/Et_2O 8/2). Compound **8a** (3.3 g, 87%) was obtained as a yellowish oil. R_f = 0.72 (*n*-hexane/EtOAc 7/3). **^{1}H-NMR** (400 MHz, $CDCl_3$): δ = 1.48–1.63 (m, 4H), 1.68–1.86 (m, 2H), 2.42 (t, J = 2.4 Hz, 1H), 3.46–3.52 (m, 1H), 3.59–3.64 (m, 1H), 3.70–3.72 (m, 2H), 3.83–3.90 (m, 2H), 4.23 (t, J = 2.3 Hz, 2H), 4.61–4.63 (m, 1H). **^{13}C-NMR** (100 MHz, $CDCl_3$): δ = 19.6, 25.5, 30.6, 58.5, 62.4, 66.6, 69.2, 74.5, 79.9, 99.0. **HRMS-ESI:** m/z calcd for $C_{10}H_{16}NaO_3$ $[M + Na]^+$, 207.0992; found, 207.0993. The characterisation of this compound has also been previously reported.[17]

***O*-THP-(TES)propargyl-MEG 9a.** Compound **9a** was prepared according to a modified literature procedure.[18] A solution of compound **8a** (2.30 g, 12.5 mmol) in dry THF (35 mL) was cooled to −78 °C (solid CO_2/acetone). Next, *n*-BuLi (5.50 mL, 13.1 mmol, 2.4 M in hexanes) was added slowly dropwise and the reaction mixture was stirred under nitrogen atmosphere at −78 °C for 1 h before TES-Cl (2.31 mL, 13.8 mmol) was added. Dropwise addition of *n*-butyl lithium had to very slow (at least over 10 min) otherwise also silylation of the propargyl methylene occurred. The reaction mixture was then allowed to warm up to RT and stirred under a nitrogen atmosphere for an additional 4 h before it was quenched by addition of an aqueous saturated NH_4Cl solution (30 mL). The reaction mixture was extracted with Et_2O (50 mL and 25 mL) and the combined organic layers were dried over $MgSO_4$, filtered, and Et_2O was removed under reduced pressure with the water bath kept at RT (19 °C–21 °C). The crude product was purified by column chromatography (*n*-hexane/Et_2O 98/2 to *n*-hexane/Et_2O 9/1). Compound **9a** (3.1 g, 82%) was obtained as yellowish oil. R_f = 0.44 (*n*-hexane/EtOAc 9/1). **^{1}H-NMR** (400 MHz, $CDCl_3$): δ = 0.58 (q, J = 8.1 Hz, 6H), 0.97 (t, J = 8.1 Hz, 9H), 1.46–1.64 (m, 4H), 1.66–1.85 (m, 2H), 3.45–3.51 (m, 1H), 3.59–3.64 (m, 1H), 3.69–3.72 (m, 2H), 3.83–3.85 (m, 2H), 4.21 (d, J = 1.5 Hz, 2H), 4.63 (t, J = 3.5 Hz, 1H). **^{13}C-NMR** (100 MHz, $CDCl_3$): δ = 4.4, 7.5, 19.5, 25.5, 30.6, 59.2, 62.3, 66.5, 68.9, 88.8, 98.9, 102.8. **HRMS-ESI:** m/z calcd for $C_{16}H_{30}NaO_3Si$ $[M + Na]^+$, 321.1856; found, 321.1842.

***O*-THP-(TIPS)propargyl-MEG 10a.** Compound **10a** was prepared according to a modified literature procedure.[18] A solution of compound **8a** (2.30 g, 12.5 mmol) in dry THF (35 mL) was cooled to −78 °C (solid CO_2/acetone). Next, *n*-BuLi (6.05 mL, 13.1 mmol, 2.2 M in hexanes) was added slowly dropwise and the reaction mixture was stirred under nitrogen atmosphere at −78 °C for 1 h before TIPS-Cl (3.07 mL, 14.4 mmol) was added. Dropwise addition of *n*-butyl lithium had to very slow (at least over 10 min) otherwise also silylation of the propargyl methylene occurred. Then the procedure described for compound **9a** was followed. The crude product was purified by column chromatography (*n*-hexane/Et_2O 95/5 to *n*-hexane/Et_2O 8/2). Compound **10a** (2.9 g, 68%) was obtained as a yellowish oil. R_f = 0.46 (*n*-hexane/EtOAc 9/1). **^{1}H-NMR** (400 MHz, $CDCl_3$): δ = 1.04 (m, 21H), 1.46–1.62 (m, 4H), 1.66–1.86 (m, 2H), 3.45–3.51 (m, 1H), 3.59–3.63 (m, 1H), 3.71–3.74 (m, 2H), 3.81–3.89 (m, 2H), 4.23 (d, J = 1.1 Hz, 2H), 4.62 (t, J = 3.6 Hz, 1H). **^{13}C-NMR** (100 MHz, $CDCl_3$): δ = 11.2, 18.7, 19.5, 25.6, 30.6, 59.2, 62.2, 66.5, 68.7, 87.6, 98.9, 103.5. **HRMS-ESI:** m/z calcd for $C_{19}H_{36}NaO_3Si$ $[M + Na]^+$, 363.2326; found, 363.2313.

(TES)propargyl-MEG bromide 11a. Compound **11a** was prepared according to a modified literature procedure.[18] Br_2 (370 μL, 7.04 mmol) was added dropwise at 0 °C to a solution of Ph_3P (1.94 g, 7.39 mmol) in dry CH_2Cl_2 (28 mL) and the solution was stirred under nitrogen atmosphere for 30 min during which a white precipitate was formed. Next, compound **9a** (2.10 g, 7.04 mmol) was added dropwise and the reaction mixture was stirred under nitrogen atmosphere at 0 °C for additional 4.5 h during which the white precipitate dissolved. The reaction mixture was then diluted with water (30 mL) and extracted with CH_2Cl_2 (30 mL and 15 mL). The combined organic layers were washed with aqueous saturated $NaHCO_3$ (30 mL), dried over $MgSO_4$, filtered, and CH_2Cl_2 was removed under reduced pressure with the water bath kept at RT (19 °C–21 °C). The crude product was purified by column chromato-

graphy (*n*-hexane/Et_2O 98/2). Compound **11a** (1.75 g, 90%) was obtained as a colourless oil. R_f = 0.57 (*n*-hexane/Et_2O 95/5). **^{1}H-NMR** (400 MHz, $CDCl_3$): δ = 0.61 (q, J = 7.9 Hz, 6H), 0.99 (t, J = 7.9 Hz, 9H), 3.51 (t, J = 6.2 Hz, 2H), 3.87 (t, J = 6.2 Hz, 2H), 4.25 (s, 2H). **^{13}C-NMR** (100 MHz, $CDCl_3$): δ = 4.3, 7.5, 30.2, 59.1, 69.5, 89.7, 102.0. **HRMS-ESI:** *m*/*z* calcd for $C_{11}H_{21}NaOSi^{79}Br$ $[M + Na]^+$, 299.0437; found, 299.0425.

(TIPS)propargyl-MEG bromide 12a. Compound **12a** was prepared according to a modified literature procedure.[18] Br_2 (380 μL, 7.34 mmol) was added dropwise at 0 °C to a solution of Ph_3P (2.02 g, 7.71 mmol) in dry CH_2Cl_2 (29 mL) and the solution was stirred under nitrogen atmosphere for 30 min during which a white precipitate was formed. Next, compound **10a** (2.50 g, 7.34 mmol) was added dropwise and the reaction mixture was stirred under nitrogen atmosphere at 0 °C for additional 4.5 h during which the white precipitate dissolved. Then the procedure described for compound **11a** was followed. The crude product was purified by column chromatography (*n*-hexane to *n*-hexane/Et_2O 99/1). Compound **12a** (1.9 g, 83%) was obtained as a colourless oil. R_f = 0.61 (*n*-hexane/Et_2O 95/5). **^{1}H-NMR** (400 MHz, $CDCl_3$): δ = 1.08 (m, 21H), 3.51 (t, J = 6.1 Hz, 2H), 3.89 (t, J = 6.1 Hz, 2H), 4.27 (s, 2H). **^{13}C-NMR** (100 MHz, $CDCl_3$): δ = 11.1, 18.6, 30.1, 59.0, 69.3, 88.4, 102.6. **HRMS-ESI:** *m*/*z* calcd for $C_{14}H_{27}NaOSi^{79}Br$ $[M + Na]^+$, 341.0907; found, 341.0900.

Propargyl-MEG iodide 13a. Compound **13a** was prepared according to a literature procedure.[19] I_2 (2.74 g, 10.8 mmol) was added to a solution of Ph_3P (2.83 g, 10.8 mmol) and imidazole (740 mg, 10.8 mmol) in dry CH_2Cl_2 (6.4 mL) at 0 °C. Next, a solution of compound **7a** (830 mg, 8.30 mmol) in CH_2Cl_2 (0.6 mL) was added to the reaction mixture and the resulting orange suspension was allowed to warm up to RT and stirred for 2 h. Then, the formed precipitate was removed by filtering over Celite®, washed with CH_2Cl_2 and CH_2Cl_2 was removed from the filtrate under reduced pressure. The crude product was purified by column chromatography (pentane to pentane/Et_2O 99/1). Compound **13a** (1.6 g, 91%) was obtained as a colourless oil, which slowly crystallised upon storage in a fridge. R_f = 0.56 (*n*-hexane/Et_2O 9/1). **^{1}H-NMR** (400 MHz, $CDCl_3$): δ = 2.46 (t, J = 2.4 Hz, 1H), 3.27 (t, J = 6.8 Hz, 2H), 3.80 (t, J = 6.8 Hz, 2H), 4.21 (d, J = 2.4 Hz, 2H). **^{13}C-NMR** (100 MHz, $CDCl_3$): δ = 2.3, 58.1, 70.5, 75.1, 79.3. **EI-MS**: *m*/*z* calcd for C_5H_7OI $[M]^+$, 210; found, 210. The characterisation of this compound has also been previously reported.[20]

Di(*O*-THP)-*O*-MEG-propargyl CTV 15a. To a solution of diTHP protected CTV scaffold **14** (3.20 g, 5.55 mmol) in acetonitrile (28 mL), Cs_2CO_3 (2.17 g, 6.66 mmol) was added, followed by addition of a solution of alkyne bromide **13a** (1.51 g, 7.21 mmol) in acetonitrile (1 mL) and the resulting reaction mixture was stirred for 27 h. Next, the formed solid was removed by filtering over Celite®, washed with CH_2Cl_2 and the solvents were removed under reduced pressure. Crude compound **15a** (3.8 g) was obtained as a yellow foam. An aliquot was purified for analysis by column chromatography (*n*-hexane/EtOAc 7/3 to *n*-hexane/EtOAc 1/1) giving compound **15a** as a white foam. R_f = 0.56 (*n*-hexane/EtOAc 1/1). **^{1}H-NMR** (500 MHz, $CDCl_3$): δ = 1.56–1.70 (m, 6H), 1.83–1.95 (m, 4H), 1.99–2.07 (m, 2H), 2.46 (m, 1H), 3.49–3.58 (m, 5H), 3.81–3.85 (m, 9H), 3.87–3.89 (m, 3H), 4.00–4.05 (m, 1H), 4.14–4.20 (m, 2H), 4.26 (m, 2H), 4.70–4.73 (m, 3H), 5.18–5.23 (m, 1H), 5.41–5.44 (m, 1H), 6.84–6.86 (m, 3H), 6.91–6.93 (m, 1H), 7.13–7.17 (m, 2H). **^{13}C-NMR** (125 MHz, $CDCl_3$): δ = 18.7, 19.0, 25.3, 30.4, 30.5, 36.3, 36.4, 36.5, 55.9, 56.0, 56.2, 56.4, 56.5, 56.6, 56.7, 58.5, 61.9, 62.2, 68.3, 69.0, 74.7, 79.7, 97.1, 97.2, 98.2, 98.3, 113.6, 113.7, 113.8, 114.0, 114.3, 114.4, 114.6, 116.5, 116.6, 116.7, 116.8, 118.6, 118.7, 118.9, 119.0, 119.6, 119.9, 120.0, 131.4, 131.6, 131.7, 131.8, 131.9, 132.1, 132.2, 132.3, 133.1, 133.2, 133.3, 133.4, 133.8, 133.9, 134.0, 144.9, 145.0, 145.1, 145.2, 146.6, 146.7, 148.6, 149.0. **HRMS-ESI:** *m*/*z* calcd for $C_{39}H_{46}NaO_9$ $[M + Na]^+$, 681.3034; found, 681.3008.

***O*-MEG-propargyl CTV-diOH 16a.** To a solution of crude compound **15a** (3.8 g) in MeOH (100 mL), 1 M HCl (5 mL) was added dropwise resulting in a yellow solution. The reaction mixture was stirred for 40 min after which TLC showed complete deprotection of **15a**. Next, MeOH was removed under reduced pressure and the remaining slurry was dissolved in $CHCl_3$ (150 mL) and washed with water (125 mL). Afterwards, the aqueous phase was extracted with $CHCl_3$ (75 mL and 50 mL) and the combined organic layers were dried over $MgSO_4$, filtered, and $CHCl_3$ was removed under reduced pressure. The crude product was purified by column chromatography (*n*-hexane/EtOAc 6/4 to *n*-hexane/EtOAc 4/6). Compound **16a** (2.5 g, 92% over 2 steps, 96% per step) was obtained as a brownish foam. R_f = 0.22 (*n*-hexane/EtOAc 1/1). **^{1}H-NMR** (400 MHz, $CDCl_3$): δ = 2.46 (t, J = 2.4 Hz, 1H), 3.43–3.52 (m, 3H), 3.77–3.84 (m, 9H), 3.88 (t, J = 5.0 Hz, 2H), 4.10–4.20 (m, 2H), 4.25 (d, J = 2.3 Hz, 2H), 4.61–4.72 (m, 3H), 5.58 (s, 1H), 5.60 (s, 1H), 6.77 (s, 1H), 6.79 (s, 1H), 6.82 (s, 1H), 6.90 (s, 3H). **^{13}C-NMR** (100 MHz, $CDCl_3$): δ = 36.3, 36.4, 56.0, 56.2, 58.5, 68.4, 69.1, 74.8, 79.7, 112.3, 113.9, 115.6, 116.7, 131.2, 131.3, 132.0, 132.6, 133.1, 144.2, 145.3, 146.7, 148.6. **HRMS-ESI:** *m*/*z* calcd for $C_{29}H_{30}NaO_7$ $[M + Na]^+$, 513.1884; found, 513.1872.

***O*-MEG-propargyl-*O*-MEG-(TIPS)propargyl CTV-OH 17a.** To a solution of compound **16a** (2.47 g, 5.04 mmol) in DMF (66 mL), Cs_2CO_3 (2.13 g, 6.55 mmol) was added, followed by addition of a solution of TIPS-protected alkyne bromide **12a** (1.77 g, 5.54 mmol) in DMF (10 mL) and the resulting reaction mixture was stirred for 22 h until the majority of compound **12a** was consumed (based on TLC – cerium molybdenate stain). Next, DMF was removed under reduced pressure and the viscous residue was dissolved in EtOAc (400 mL) and washed with aqueous 1 M $KHSO_4$ (400 mL). Next, the aqueous phase was extracted with EtOAc (100 mL) and the combined organic layers were washed with aqueous 1 M LiCl (2 × 100 mL) for efficient DMF removal, dried over $MgSO_4$, filtered, and EtOAc was removed under reduced pressure. The crude product was purified by column chromatography (*n*-hexane/EtOAc 7/3 to *n*-hexane/EtOAc 3/7). Compound **17a** (1.4 g, 37%) was obtained as a yellowish foam. R_f = 0.28 (*n*-hexane/EtOAc 6/4). **^{1}H-NMR** (400 MHz, $CDCl_3$): δ = 1.06 (m, 21H), 2.43 (m, 1H), 3.49 (d, J = 13.8 Hz, 3H), 3.81 (s, 6H), 3.83 (s, 3H),

3.85–3.92 (m, 4H), 4.11–4.21 (m, 4H), 4.23 (s, 2H), 4.28 (d, J = 1.6 Hz, 2H), 4.63–4.75 (m, 3H), 5.48 (s, 1H), 6.78 (s, 1H), 6.81 (m, 2H), 6.88–6.91 (m, 3H). **^{13}C-NMR** (100 MHz, $CDCl_3$): δ = 11.2, 18.6, 36.3, 36.5, 56.2, 56.3, 56.4, 58.6, 59.3, 68.1, 68.4, 69.1, 74.7, 79.6, 87.8, 103.2, 112.3, 113.8, 113.9, 114.0, 114.1, 115.7, 116.7, 116.9, 131.2, 131.7, 132.1, 132.4, 132.9, 133.0, 133.2, 144.2, 145.3, 146.8, 146.9, 147.0, 148.7. **HRMS-ESI:** m/z calcd for $C_{43}H_{56}NaO_8Si$ $[M + Na]^+$, 751.3637; found, 751.3606. Compound **18a**, a side product, (760 mg, 16%) was obtained as a colourless amorphous solid. R_f = 0.61 (*n*-hexane/EtOAc 6/4). **^{1}H-NMR** (400 MHz, $CDCl_3$): δ = 1.06 (m, 42H), 2.43 (t, J = 2.4 Hz, 1H), 3.51 (d, J = 13.7 Hz, 3H), 3.82 (s, 9H), 3.85–3.91 (m, 6H), 4.11–4.20 (m, 6H), 4.24 (d, J = 2.4 Hz, 2H), 4.28 (s, 4H), 4.72 (d, J = 13.6 Hz, 3H), 6.82 (s, 3H), 6.91 (s, 3H). **^{13}C-NMR** (100 MHz, $CDCl_3$): δ = 11.2, 18.6, 36.5, 56.3, 56.4, 58.5, 59.3, 68.1, 69.0, 74.7, 79.6, 87.8, 103.2, 114.0, 114.1, 116.7, 131.9, 132.9, 133.0, 146.9, 147.0, 148.6. **HRMS-ESI:** m/z calcd for $C_{57}H_{82}NaO_9Si_2$ $[M + Na]^+$, 989.5390; found, 989.5367. The starting material **16a** (520 mg, 21%) was recovered.

***O*-MEG-propargyl-*O*-MEG-(TIPS)propargyl-*O*-MEG-(TES)propargyl CTV 3.** To a solution of compound **17a** (1.34 g, 1.84 mmol) in dry DMF (18 mL) 10 beads of activated 4 Å sieves were added and the resulting solution was stirred under nitrogen atmosphere. After 1 h Cs_2CO_3 (660 mg, 2.02 mmol) was added, followed by the addition of TES-protected alkyne bromide **11a** (610 mg, 2.21 mmol) and the resulting reaction mixture was stirred under nitrogen atmosphere for a week during which additional Cs_2CO_3 (587 mg, 1.80 mmol) and compound **11a** (500 mg, 1.80 mmol) were added in order to increase conversion. Then, DMF was removed under reduced pressure and the viscous residue was dissolved in Et_2O (200 mL) and washed with aqueous 1 M $KHSO_4$ (200 mL). The aqueous layer was extracted with Et_2O (70 mL) and combined organic layers were washed with aqueous 1 M LiCl (2 × 50 mL) for efficient DMF removal, dried over $MgSO_4$, filtered, and Et_2O was removed under reduced pressure. The crude product was purified by column chromatography (*n*-hexane/EtOAc 8/2 to *n*-hexane/EtOAc 1/1). Compound **3** (0.5 g, 30%) was obtained as a yellowish amorphous solid. R_f = 0.55 (*n*-hexane/EtOAc 6/4). **^{1}H-NMR** (400 MHz, $CDCl_3$): δ = 0.61 (q, J = 7.9 Hz, 6H), 1.00 (t, J = 7.9 Hz, 9H), 1.08 (m, 21H), 2.44 (t, J = 2.4 Hz, 1H), 3.53 (d, J = 13.8 Hz, 3H), 3.82 (s, 9H), 3.85–3.91 (m, 6H), 4.11–4.20 (m, 6H), 4.25 (d, J = 2.4 Hz, 2H), 4.27 (s, 2H), 4.29 (s, 2H), 4.73 (d, J = 13.8 Hz, 3H), 6.83 (s, 3H), 6.93 (s, 3H). **^{13}C-NMR** (100 MHz, $CDCl_3$): δ = 4.3, 7.4, 11.2, 18.6, 36.5, 56.4, 58.5, 59.3, 68.1, 68.2, 68.4, 69.1, 74.7, 79.6, 87.8, 102.5, 103.2, 114.0, 114.1, 114.2, 116.7, 116.8, 131.9, 132.9, 133.0, 146.9, 147.0, 148.6, 148.7. **HRMS-ESI:** m/z calcd for $C_{54}H_{76}NaO_9Si_2$ $[M + Na]^+$, 947.4920; found, 947.4915. Compound **5**, a side product, (0.9 g, 57%) was obtained as a yellow amorphous solid. R_f = 0.32 (*n*-hexane/EtOAc 6/4). **^{1}H-NMR** (400 MHz, $CDCl_3$): δ = 1.07 (s, 21H), 2.44 (t, J = 2.4 Hz, 2H), 3.53 (d, J = 13.9 Hz, 3H), 3.83 (s, 9H), 3.86–3.90 (m, 6H), 4.11–4.20 (m, 6H), 4.24 (d, J = 2.4 Hz, 4H), 4.28 (s, 2H), 4.73 (d, J = 13.8 Hz, 3H), 6.82 (s, 3H), 6.91 (s, 3H). **^{13}C-NMR** (100 MHz, $CDCl_3$): δ = 11.2, 18.6, 36.5, 56.4, 58.5, 59.3, 68.1, 68.4, 69.1, 74.7, 79.6, 87.8, 103.2, 114.0, 114.1, 116.8, 131.9, 132.9, 133.0, 146.9, 147.0, 148.7. **HRMS-ESI:** m/z calcd for $C_{48}H_{62}NaO_9Si$ $[M + Na]^+$, 833.4055; found, 833.4027.

Propargyl-DEG-OH 7b. Compound **7b** was prepared according to a literature procedure.[21] Anhydrous diethylene glycol **6b** (8.54 mL, 90.0 mmol) was added to the solution of *t*-BuOK (5.10 g, 44.5 mmol) in dry THF (125 mL) at 0 °C which led to the formation of a white precipitate. Next, the reaction mixture was allowed to warm to RT and after 30 min of stirring a solution of propargyl bromide (4.85 mL (45.0 mmol) of a 80 wt% solution in toluene) in dry THF (25 mL) was added dropwise and the reaction mixture was stirred for 16 h under nitrogen atmosphere. Next, the precipitate was removed by filtering over Celite®, washed with THF and the filtrate was concentrated under reduced pressure. The obtained residue was purified by column chromatography (*n*-hexane/EtOAc 1/1 to EtOAc). Compound **7b** (5.3 g, 83%) was obtained as a yellow oil. R_f = 0.57 (EtOAc). **^{1}H-NMR** (400 MHz, $CDCl_3$): δ = 2.43 (t, J = 2.3 Hz, 1H), 2.77 (br s, 1H), 3.56 (t, J = 4.6 Hz, 2H), 3.63–3.71 (m, 6H), 4.18 (d, J = 2.3 Hz, 2H). **^{13}C-NMR** (100 MHz, $CDCl_3$): δ = 58.4, 61.7, 69.1, 70.2, 72.6, 74.8, 79.5. **HRMS-ESI:** m/z calcd for $C_7H_{12}NaO_3$ $[M + Na]^+$, 167.0679; found, 167.0678. The characterisation of this compound has also been previously reported.[21]

***O*-THP-propargyl-DEG 8b.** Compound **8b** was prepared according to a literature procedure.[16] To a solution of compound **7b** (3.75 g, 26.0 mmol) in dry CH_2Cl_2 (26 mL), DHP (2.50 mL, 27.3 mmol) was added and the reaction mixture was cooled to 0 °C. Next, *p*-TsOH·H_2O (50.0 mg, 0.30 mmol) was added to the reaction mixture which was then allowed to warm to RT and stirred for 4 h during which additional DHP (0.20 mL, 2.20 mmol) was added. Then, the reaction mixture was washed with aqueous saturated $NaHCO_3$ (40 mL), the aqueous phase was extracted with CH_2Cl_2 (20 mL) and combined organic layers were dried over $MgSO_4$, filtered, and CH_2Cl_2 was removed under reduced pressure. The crude product was purified by column chromatography (*n*-hexane/EtOAc 9/1 to *n*-hexane/EtOAc 7/3). Compound **8b** (5.4 g, 90%) was obtained as a yellow oil. R_f = 0.68 (*n*-hexane/EtOAc 1/1). **^{1}H-NMR** (400 MHz, $CDCl_3$): δ = 1.48–1.63 (m, 4H), 1.68–1.86 (m, 2H), 2.42 (t, J = 2.4 Hz, 1H), 3.47–3.52 (m, 1H), 3.59–3.64 (m, 1H), 3.67–3.70 (m, 6H), 3.84–3.89 (m, 2H), 4.21 (d, J = 2.4 Hz, 2H), 4.62–4.64 (m, 1H). **^{13}C-NMR** (100 MHz, $CDCl_3$): δ = 19.6, 25.6, 30.7, 58.6, 62.4, 66.8, 69.3, 70.6, 70.7, 74.6, 79.8, 99.1. **HRMS-ESI:** m/z calcd for $C_{12}H_{20}NaO_4$ $[M + Na]^+$, 251.1254; found, 251.1248.

***O*-THP-(TES)propargyl-DEG 9b.** Compound **9b** was prepared according to a modified literature procedure.[18] A solution of compound **8b** (1.50 g, 6.57 mmol) in dry THF (18 mL) was cooled to −78 °C (solid CO_2/acetone). Next, *n*-BuLi (4.70 mL, 7.56 mmol, 1.6 M in hexanes) was added dropwise and the reaction mixture was stirred under nitrogen atmosphere at −78 °C for 1 h before TES-Cl (1.38 mL, 8.21 mmol) was added. The reaction mixture was then allowed to warm up to RT and stirred under nitrogen atmosphere for additional 5 h before it was quenched by addition of an aqueous saturated NH_4Cl solution (20 mL). Subsequently, the reaction mixture was

extracted with Et_2O (2 × 25 mL) and the combined organic layers were dried over $MgSO_4$, filtered, and solvents were removed under reduced pressure with the water bath kept at RT (19 °C–21 °C). The crude product was purified by column chromatography using (*n*-hexane/Et_2O 9/1 to *n*-hexane/Et_2O 7/3). Compound **9b** (2.0 g, 91%) was obtained as a yellow oil. R_f = 0.69 (*n*-hexane/EtOAc 7/3). **^{1}H-NMR** (400 MHz, $CDCl_3$): δ = 0.60 (q, J = 8.0 Hz, 6H), 0.97 (t, J = 8.0 Hz, 9H), 1.47–1.63 (m, 4H), 1.67–1.86 (m, 2H), 3.46–3.52 (m, 1H), 3.58–3.63 (m, 1H), 3.66–3.70 (m, 6H), 3.83–3.88 (m, 2H), 4.22 (s, 2H), 4.62 (t, J = 3.7 Hz, 1H). **^{13}C-NMR** (100 MHz, $CDCl_3$): δ = 4.4, 7.5, 19.6, 25.6, 30.7, 59.3, 62.3, 66.8, 69.0, 70.6, 70.7, 88.9, 99.1, 102.7. **HRMS-ESI:** *m*/*z* calcd for $C_{18}H_{34}NaO_4Si$ $[M + Na]^+$, 365.2119; found, 365.2103.

***O*-THP-(TIPS)propargyl-DEG 10b.** Compound **10b** was prepared according to a modified literature procedure.[18] A solution of compound **8b** (1.50 g, 6.57 mmol) in dry THF (18 mL) was cooled to −78 °C (solid CO_2/acetone). Next, *n*-BuLi (2.76 mL, 6.90 mmol, 2.5 M in hexanes) was added dropwise and the reaction mixture was stirred under nitrogen atmosphere at −78 °C for 1 h before TIPS-Cl (1.54 mL, 7.23 mmol) was added. Then the procedure described for compound **9b** was followed. The crude product was purified by column chromatography (*n*-hexane/Et_2O, 95/5 to *n*-hexane/Et_2O 8/2). Compound **10b** (1.9 g, 75%) was obtained as a yellowish oil. R_f = 0.73 (*n*-hexane/EtOAc 7/3). **^{1}H-NMR** (400 MHz, $CDCl_3$): δ = 1.06 (m, 21H), 1.48–1.64 (m, 4H), 1.68–1.86 (m, 2H), 3.46–3.52 (m, 1H), 3.58–3.63 (m, 1H), 3.67–3.73 (m, 6H), 3.84–3.89 (m, 2H), 4.24 (s, 2H), 4.63 (t, J = 3.7 Hz, 1H). **^{13}C-NMR** (100 MHz, $CDCl_3$): δ = 11.3, 18.7, 19.6, 25.6, 30.7, 59.3, 62.3, 66.7, 68.8, 70.6, 70.7, 87.7, 99.1, 103.4. **HRMS-ESI:** *m*/*z* calcd for $C_{21}H_{40}NaO_4Si$ $[M + Na]^+$, 407.2588; found, 407.2569.

(TES)propargyl-DEG bromide 11b. Compound **11b** was prepared according to a modified literature procedure.[18] Br_2 (300 μL, 5.84 mmol) was added dropwise at 0 °C to a solution of Ph_3P (1.61 g, 6.13 mmol) in dry CH_2Cl_2 (23 mL) and the solution was stirred under nitrogen atmosphere for 30 min during which a white precipitate was formed. Next, solution of compound **9b** (2.00 g, 5.84 mmol) in dry CH_2Cl_2 (5 mL) was added dropwise and the reaction mixture was stirred under nitrogen atmosphere at 0 °C for additional 5 h during which the white precipitate dissolved. Next, the reaction mixture was diluted with CH_2Cl_2 (20 mL), water (20 mL) and then extracted with CH_2Cl_2 (2 × 20 mL). The combined organic layers were washed with aqueous saturated $NaHCO_3$ (20 mL), dried over $MgSO_4$, filtered, and CH_2Cl_2 was removed under reduced pressure with water bath kept at RT (19 °C–21 °C). The crude product was purified by column chromatography (*n*-hexane/Et_2O 95/5 to *n*-hexane/Et_2O 9/1). Compound **11b** (1.6 g 84%) was obtained as a pale yellow oil. R_f = 0.27 (*n*-hexane/Et_2O 9/1). **^{1}H-NMR** (400 MHz, $CDCl_3$): δ = 0.60 (q, J = 8.0 Hz, 6H), 0.98 (t, J = 8.0 Hz, 9H), 3.47 (t, J = 6.4 Hz, 2H), 3.68–3.73 (m, 4H), 3.81 (t, J = 6.4 Hz, 2H), 4.23 (s, 2H). **^{13}C-NMR** (100 MHz, $CDCl_3$): δ = 4.4, 7.5, 30.3, 59.4, 68.9, 70.5, 71.3, 89.2, 102.5. **HRMS-ESI:** *m*/*z* calcd for $C_{13}H_{25}NaO_2Si^{79}Br$ $[M + Na]^+$, 343.0699; found, 343.0685.

(TIPS)propargyl-DEG bromide 12b. Compound **12b** was prepared according to a modified literature procedure.[18] Br_2 (250 μL, 4.86 mmol) was added dropwise at 0 °C to a solution of Ph_3P (1.34 g, 5.11 mmol) in dry CH_2Cl_2 (20 mL) and the solution was stirred under nitrogen atmosphere for 30 min during which a white precipitate was formed. Next, solution of compound **10b** (1.87 g, 4.86 mmol) in dry CH_2Cl_2 (8 mL) was added dropwise and then the procedure described for compound **11b** was followed. The crude product was purified by column chromatography (*n*-hexane/Et_2O 95/5 to *n*-hexane/Et_2O 9/1). Compound **12b** (1.7 g, 93%) was obtained as a colourless oil. R_f = 0.29 (*n*-hexane/Et_2O 9/1). **^{1}H-NMR** (400 MHz, $CDCl_3$): δ = 1.06 (m, 21H), 3.47 (t, J = 6.4 Hz, 2H), 3.69–3.74 (m, 4H), 3.81 (t, J = 6.4 Hz, 2H), 4.25 (s, 2H). **^{13}C-NMR** (100 MHz, $CDCl_3$): δ = 11.2, 18.7, 30.3, 59.3, 68.8, 70.5, 71.3, 87.9, 103.2. **HRMS-ESI:** *m*/*z* calcd for $C_{16}H_{31}NaO_2Si^{79}Br$ $[M + Na]^+$, 385.1169; found, 385.1155.

Propargyl-DEG bromide 13b. Compound **13b** was prepared according to a modified literature procedure.[18] Br_2 (260 μL, 5.04 mmol) was added dropwise at 0 °C to a solution of Ph_3P (1.37 g, 5.25 mmol) in dry CH_2Cl_2 (22 mL) and the solution was stirred under nitrogen atmosphere for 30 min during which a white precipitate was formed. Next, solution of compound **8b** (1.14 g, 4.99 mmol) in dry CH_2Cl_2 (2 mL) was added dropwise and then the procedure described for compound **11b** was followed. The crude product was purified by column chromatography (*n*-hexane/Et_2O 1/2). Compound **13b** (1.0 g, 91%) was obtained as a colourless oil. R_f = 0.12 (*n*-hexane/EtOAc 95/5). **^{1}H-NMR** (400 MHz, $CDCl_3$): δ = 2.44 (t, J = 2.4 Hz, 1H), 3.49 (t, J = 6.3 Hz, 2H), 3.69–3.74 (m, 4H), 3.82 (t, J = 6.3 Hz, 2H), 4.22 (d, J = 2.4 Hz, 2H). **^{13}C-NMR** (100 MHz, $CDCl_3$): δ = 30.4, 58.6, 69.2, 70.5, 71.4, 74.8, 79.7. **HRMS-ESI:** *m*/*z* calcd for $C_7H_{11}NaO_2{}^{79}Br$ $[M + Na]^+$, 228.9835; found, 228.9839.

Di(*O*-THP)-*O*-DEG-propargyl CTV 15b. To a solution of diTHP protected CTV scaffold **14** (1.50 g, 2.60 mmol) in acetonitrile (12 mL) Cs_2CO_3 (1.02 g, 3.12 mmol) was added, followed by the addition of a solution of alkyne bromide **13b** (700 mg, 3.38 mmol) in acetonitrile (1 mL) and the resulting reaction mixture was stirred for 15 h. Afterwards, the reaction mixture was concentrated under reduced pressure and the residue was diluted with EtOAc (150 mL) and washed with aqueous 1 M $KHSO_4$ (150 mL). Aqueous layer was extracted with EtOAc (50 mL) and combined organic layers were washed with brine (100 mL), dried over $MgSO_4$, filtered, and EtOAc was removed under reduced pressure. The crude product was purified by column chromatography (*n*-hexane/EtOAc 7/3 to *n*-hexane/EtOAc 1/1). Compound **15b** (1.5 g, 83%) was obtained as a white foam. R_f = 0.29 (*n*-hexane/EtOAc 1/1). **^{1}H-NMR** (500 MHz, $CDCl_3$): δ = 1.51–1.76 (m, 6H), 1.80–2.07 (m, 6H), 2.45 (t, J = 2.4 Hz, 1H), 3.49–3.60 (m, 5H), 3.70–3.76 (m, 4H), 3.81–3.87 (m, 12H), 4.00–4.06 (m, 1H), 4.10–4.22 (m, 4H), 4.70–4.74 (m, 3H), 5.19–5.22 (m, 1H), 5.41–5.43 (m, 1H), 6.84–6.86 (m, 3H), 6.90–6.92 (m, 1H), 7.13–7.17 (m, 2H). **^{13}C-NMR** (125 MHz, $CDCl_3$): δ = 18.7, 19.0, 25.3, 30.4, 30.5, 36.3, 36.4, 36.5, 55.9, 56.0, 56.1, 56.2, 56.4, 56.5, 56.7, 58.4, 61.9, 62.2, 69.0, 69.2, 69.7, 70.6, 74.6, 79.7, 97.1, 98.2, 98.3, 113.5, 113.7, 113.8, 114.0, 114.2,

114.3, 114.5, 116.1, 116.3, 118.5, 118.6, 118.8, 118.9, 119.6, 119.8, 119.9, 120.0, 131.4, 131.6, 131.7, 131.8, 131.9, 132.1, 132.2, 133.0, 133.1, 133.2, 133.8, 133.9, 144.9, 145.0, 145.1, 145.2, 146.6, 146.7, 148.4, 148.5, 149.0. **HRMS-ESI:** *m*/*z* calcd for $C_{41}H_{50}NaO_{10}$ $[M + Na]^+$, 725.3296; found, 725.3273.

***O*-DEG-propargyl CTV-diOH 16b.** To a solution of compound **15b** (1.47 g, 2.09 mmol) in MeOH (40 mL), 1 M HCl (2 mL) was added dropwise which resulted in the formation of a precipitate. The reaction mixture was stirred for 2.5 h during which the suspension turned into a yellow solution. Afterwards, MeOH was removed under reduced pressure and the solid residue was dissolved in $CHCl_3$ (60 mL) and washed with water (50 mL). Next, the aqueous phase was extracted with $CHCl_3$ (2 × 30 mL) and the combined organic layers were dried over $MgSO_4$, filtered, and $CHCl_3$ was removed under reduced pressure. The crude product was purified by column chromatography ($CHCl_3$ to $CHCl_3$/MeOH 25/1). Compound **16b** (1.1 g, 100%) was obtained as a white foam. R_f = 0.33 (*n*-hexane/EtOAc 3/7). **^{1}H-NMR** (500 MHz, $CDCl_3$): δ = 2.45 (t, J = 2.4 Hz, 1H), 3.45–3.49 (m, 3H), 3.69–3.74 (m, 4H), 3.78 (s, 3H), 3.80–3.87 (m, 8H), 4.09–4.13 (m, 1H), 4.15–4.24 (m, 3H), 4.64–4.70 (m, 3H), 5.62 (m, 1H), 5.65 (m, 1H), 6.77 (s, 1H), 6.77 (s, 1H), 6.82 (s, 1H), 6.87–6.91 (m, 3H). **^{13}C-NMR** (125 MHz, $CDCl_3$): δ = 36.2, 36.4, 56.0, 56.2, 58.4, 69.0, 69.1, 69.7, 70.6, 74.7, 79.7, 112.3, 113.8, 115.6, 115.7, 116.3, 131.2, 131.3, 131.9, 132.3, 132.6, 132.9, 144.2, 145.3, 146.8, 148.5. **HRMS-ESI:** *m*/*z* calcd for $C_{31}H_{34}NaO_8$ $[M + Na]^+$, 557.2164; found, 557.2133.

***O*-DEG-propargyl-*O*-DEG-(TIPS)propargyl CTV-OH 17b.** To the solution of compound **16b** (845 mg, 1.58 mmol) in DMF (20 mL) Cs_2CO_3 (567 mg, 1.74 mmol) was added, followed by the addition of the solution of TIPS-protected alkyne bromide **12b** (603 mg, 1.66 mmol) in DMF (4 mL) and the resulting reaction mixture was stirred for 28 h during which additional Cs_2CO_3 (520 mg, 1.60 mmol) was added in order to improve the conversion. Then, DMF was removed under reduced pressure and the viscous residue was dissolved in EtOAc (100 mL) and washed with aqueous 1 M $KHSO_4$ (150 mL). Next, the aqueous phase was extracted with EtOAc (50 mL) and the combined organic layers were dried over $MgSO_4$, filtered, and EtOAc was removed under reduced pressure. The crude product was purified by column chromatography (*n*-hexane/EtOAc 7/3 to *n*-hexane/EtOAc 3/7). Compound **17b** (470 mg, 36%) was obtained as a yellow gum. R_f = 0.59 (*n*-hexane/EtOAc 3/7). **^{1}H-NMR** (500 MHz, $CDCl_3$): δ = 1.07 (m, 21H), 2.43 (dd, J = 2.4 Hz, 4.4 Hz, 1H), 3.49–3.51 (d, J = 13.8 Hz, 3H), 3.67–3.70 (m, 2H), 3.71–3.75 (m, 6H), 3.82 (s, 6H), 3.83–3.87 (m, 7H), 4.09–4.19 (m, 6H), 4.25 (d, J = 1.4 Hz, 2H), 4.66–4.75 (m, 3H), 5.50 (m, 1H), 6.80 (m, 1H), 6.80–6.83 (m, 2H), 6.88–6.91 (m, 3H). **^{13}C-NMR** (125 MHz, $CDCl_3$): δ = 11.2, 18.6, 36.3, 36.5, 56.2, 56.4, 58.5, 59.2, 68.8, 69.0, 69.2, 69.7, 69.8, 70.7, 74.7, 79.7, 87.8, 103.3, 112.3, 113.9, 114.0, 115.7, 116.2, 116.4, 116.5, 131.3, 131.8, 132.1, 132.5, 132.8, 132.9, 144.3, 145.4, 146.9, 147.0, 148.6. **HRMS-ESI:** *m*/*z* calcd for $C_{47}H_{64}NaO_{12}Si$ $[M + Na]^+$, 839.4161; found, 839.4128. Compound **18b**, a side product, (350 mg, 20%) was obtained as a yellow syrup. R_f = 0.74 (*n*-hexane/EtOAc 3/7). **^{1}H-NMR** (500 MHz, $CDCl_3$): δ = 1.06 (m, 42H), 2.41 (t, J = 2.4 Hz, 1H), 3.52 (d, J = 13.8 Hz, 3H), 3.67–3.70 (m, 2H), 3.71–3.75 (m, 10H), 3.79–3.87 (m, 15H), 4.07–4.19 (m, 8H), 4.24 (s, 4H), 4.72 (d, J = 13.8 Hz, 3H), 6.80–6.82 (m, 3H), 6.88–6.90 (m, 3H). **^{13}C-NMR** (125 MHz, $CDCl_3$): δ = 11.2, 18.7, 36.6, 56.4, 58.5, 59.2, 68.8, 69.0, 69.2, 69.7, 69.8, 70.7, 74.7, 79.7, 87.8, 103.3, 114.0, 116.3, 116.4, 131.9, 132.8, 147.0, 147.1, 148.6. **HRMS-ESI:** *m*/*z* calcd for $C_{63}H_{94}NaO_{12}Si_2$ $[M + Na]^+$, 1121.6176; found, 1121.6140. Starting material **16b** (210 mg, 25%) was recovered.

***O*-DEG-propargyl-*O*-DEG-(TIPS)propargyl-*O*-DEG-(TES)propargyl CTV 4.** To the solution of compound **17b** (400 mg, 490 μmol) in dry DMF (7 mL) Cs_2CO_3 (210 mg, 636 μmol) was added, followed by the addition of the solution of TES-protected alkyne bromide **11b** (240 mg, 734 μmol) in dry DMF (1 mL) and the resulting reaction mixture was stirred under nitrogen atmosphere for 7 h during which additional Cs_2CO_3 (160 mg, 491 μmol) and the solution of compound **11b** (160 mg, 498 μmol) in dry DMF (0.8 mL) was added in order to improve the conversion. Afterwards, DMF was removed under reduced pressure and the viscous residue was dissolved in EtOAc (70 mL) and washed with aqueous 1 M $KHSO_4$ (60 mL). The aqueous layer was extracted with EtOAc (50 mL) and combined organic layers were washed with aqueous 1 M LiCl (55 mL) for efficient DMF removal. The aqueous LiCl phase was extracted with EtOAc (20 mL) and the organic layer was combined with the previous one, dried over $MgSO_4$, filtered, and EtOAc was removed under reduced pressure. The crude product was purified by column chromatography (CH_2Cl_2 to CH_2Cl_2/MeOH 100/1). Compound **4** (320 mg, 62%) was obtained as a yellow syrup. R_f = 0.27 (*n*-hexane/EtOAc 1/1). **^{1}H-NMR** (500 MHz, $CDCl_3$): δ = 0.58 (q, J = 7.9 Hz, 6H), 0.98 (t, J = 7.9 Hz, 9H), 1.06 (m, 21H), 2.42 (t, J = 2.4 Hz, 1H), 3.51 (d, J = 13.9 Hz, 3H), 3.66–3.75 (m, 12H), 3.79–3.87 (m, 15H), 4.08–4.19 (m, 8H), 4.22 (s, 2H), 4.24 (s, 2H), 4.72 (d, J = 13.8 Hz, 3H), 6.82 (s, 3H), 6.89 (s, 3H). **^{13}C-NMR** (125 MHz, $CDCl_3$): δ = 3.3, 6.5, 10.2, 17.6, 35.5, 55.4, 57.5, 58.2, 67.8, 67.9, 68.0, 68.2, 68.7, 68.8, 69.7, 73.6, 78.7, 86.7, 88.0, 101.6, 102.3, 113.0, 115.3, 115.4, 130.9, 131.8, 146.0, 147.5. **HRMS-ESI:** *m*/*z* calcd for $C_{60}H_{88}NaO_{12}Si_2$ $[M + Na]^+$, 1079.5707; found, 1079.5671.

Tri-*O*-propargyl CTV 20. TBAF·3H_2O (78.9 mg, 250 μmol) was added to a solution of compound **19** (83.5 mg, 100 μmol) in dry CH_2Cl_2 (0.5 mL) and the resulting solution, which quickly turned into a suspension, was stirred for 4 h. The suspension was then diluted with CH_2Cl_2 (2.5 mL) and $CHCl_3$ (2.0 mL) and gently heated to *ca.* 40 °C to dissolve the precipitate. The organic phase was then washed with brine (5.0 mL) and the resulting aqueous phase was extracted with $CHCl_3$ (3.0 mL). The combined organic layers were dried over $MgSO_4$, filtered, and solvents were removed under reduced pressure. The crude product was purified by column chromatography (CH_2Cl_2 to CH_2Cl_2/MeOH 50/1). Compound **20** (49 mg, 94%) was obtained as a white solid. R_f = 0.82 (petroleum ether/EtOAc 1/1). **^{1}H-NMR** (400 MHz, $CDCl_3$): δ = 2.46 (t, J = 2.4 Hz, 3H), 3.57 (d, J = 13.8 Hz, 3H), 3.85 (s, 9H), 4.72 (d, J = 2.4 Hz, 6H), 4.77 (d, J = 13.8 Hz, 3H), 6.88 (s, 3H), 7.02 (s, 3H). **^{13}C-NMR** (100 MHz, $CDCl_3$): δ = 36.5, 56.2, 57.0, 75.7, 79.0, 113.9, 116.5, 131.6,

133.4, 145.5, 148.4. **HRMS-ESI:** *m*/*z* calcd for $C_{33}H_{30}NaO_6$ $[M + Na]^+$, 545.1935; found, 545.1935. The characterisation of this compound has also been previously reported.[2]

Tri-*O*-MEG-propargyl CTV 21. TBAF·3H_2O (47.3 mg, 150 μmol) was added to a solution of compound **5** (81.1 mg, 100 μmol) in dry CH_2Cl_2 (0.5 mL) and the resulting solution was stirred for 1 h. The solution was then diluted with CH_2Cl_2 (4.5 mL), washed with brine (5.0 mL) and aqueous phase was extracted with CH_2Cl_2 (3.0 mL). The combined organic layers were dried over $MgSO_4$, filtered, and CH_2Cl_2 was removed under reduced pressure. The crude product was purified by column chromatography (CH_2Cl_2 to CH_2Cl_2/MeOH 100/1). Compound **21** (56 mg, 85%) was obtained as a white foam. R_f = 0.61 (petroleum ether/EtOAc 1/1). **^{1}H-NMR** (400 MHz, $CDCl_3$): δ = 2.42–2.46 (m, 3H), 3.51 (d, J = 13.8 Hz, 3H), 3.82 (s, 9H), 3.84–3.88 (m, 6H), 4.10–4.19 (m, 6H), 4.22–4.26 (m, 6H), 4.71 (d, J = 13.8 Hz, 3H), 6.81 (s, 3H), 6.90 (s, 3H). **^{13}C-NMR** (100 MHz, $CDCl_3$): δ = 36.5, 56.3, 58.5, 68.3, 69.0, 74.7, 79.6, 114.0, 116.8, 131.9, 133.0, 146.9, 148.6. **HRMS-ESI:** *m*/*z* calcd for $C_{39}H_{42}NaO_9$ $[M + Na]^+$, 677.2721; found, 677.2701.

Tri-*O*-DEG-propargyl CTV 22. TBAF·3H_2O (78.9 mg, 250 μmol) was added to the solution of compound **18b** (110.0 mg, 100 μmol) in dry CH_2Cl_2 (0.5 mL) and the resulting solution was stirred for 1 h. Then the procedure described for compound **21** was followed. The crude product was purified by column chromatography (CH_2Cl_2 to CH_2Cl_2/MeOH 100/1). Compound **22** (59 mg, 75%) was obtained as a pale yellow amorphous solid. R_f = 0.11 (petroleum ether/EtOAc 1/1). **^{1}H-NMR** (400 MHz, $CDCl_3$): δ = 2.43 (m, 3H), 3.52 (d, J = 13.7 Hz, 3H), 3.65–3.77 (m, 12H), 3.79–3.88 (m, 15H), 4.08–4.21 (m, 12H), 4.73 (d, J = 13.8 Hz, 3H), 6.82 (s, 3H), 6.90 (s, 3H). **^{13}C-NMR** (100 MHz, $CDCl_3$): δ = 36.5, 56.3, 58.4, 69.0, 69.1, 69.7, 70.6, 74.6, 79.7, 114.0, 116.4, 131.9, 132.8, 147.0, 148.5. **HRMS-ESI:** *m*/*z* calcd for $C_{45}H_{54}NaO_{12}$ $[M + Na]^+$, 809.3507; found, 809.3496.

Peptide synthesis and Cu(I)catalyzed azide–alkyne cycloaddition chemistry

Synthesis of linear peptides 23–26. General method for automated peptide synthesis: the peptides were synthesized on a PTI Tribute-UV peptide synthesizer. Tentagel S RAM resin (1.0 g, 0.25 mmol, 1.0 equiv. or 400 mg, 0.1 mmol, 1.0 equiv.) was allowed to swell (3 × 10 min). Removal of the Fmoc group was achieved by treatment of the resin with 20% piperidine in DMF using the RV_top_UV_Xtend protocol from the Tribute-UV peptide synthesizer followed by a DMF washing step (5 × 30 s). The Fmoc-protected amino acids (on a 0.1 mmol scale 5 eq. of Fmoc amino acids were used and on a 0.25 mmol scale 4 eq. of Fmoc amino acids were used) were coupled using HCTU (5 eq. or 4 eq. respectively) and DiPEA (10 eq. or 8 eq. respectively) in DMF, with 2 min pre-activation. The coupling time was 10 min when the peptide was synthesized on a 0.1 mmol scale and 20 min when the 0.25 mmol scale was conducted. After every coupling the resin was washed with DMF (6 × 30 s). After coupling of the last amino acid, the Fmoc group was cleaved using the deprotection conditions described above and the resulting free N-terminus was acetylated by treatment of the resin bound peptide with acetic anhydride (250 μL) and DiPEA (eq. as above) in DMF using the standard coupling times (as above). After the last step the resin was washed with DMF (5 × 30 s), CH_2Cl_2 (5 × 30 s), dried by nitrogen flow for 10 min, followed by cleavage of the resin-bounded peptide. Cleavage and global deprotection was achieved by treatment of the resin with TFA/H_2O/TIS/EDT (10 mL for the 0.25 mmol scale and 5 mL for the 0.1 mmol scale, 90/5/2.5/2.5, v/v/v/v) for 3 hours. Next, the peptide was precipitated by dropwise addition of the TFA mixture to a cold (4 °C) solution of MTBE/*n*-hexane (1/1, 90 mL for the 0.25 mmol scale and 45 mL for the 0.1 mmol scale). After centrifugation (3500 rpm for 5 min) the supernatant was decanted and the pellet was resuspended in MTBE/*n*-hexane (volumes as above) and the above procedure was repeated twice. Finally, the pellet was dissolved in *t*-BuOH/H_2O (1/1, v/v) and lyophilized to yield the crude linear peptides. The following peptides were synthesized:

Linear peptide ***23***, Ac-CNYYGSNC-NH_2, t_R = 13.9 min. **LCMS-ESI**: average mass calcd $[M]^+$: 964.0; found: 964.3.

Analytical data of linear peptides **24–26** were identical to those described previously.[4]

Peptide cyclisation – CDR mimics 28–31. All linear peptides were cyclized at a concentration of 1 mM in the following way. The crude linear peptide and the perhydrotriazine hinge **27** were weighed into a round bottom flask (250 mL or 500 mL). Then, acetonitrile was added followed by the addition of aqueous solution of NH_4HCO_3 (20 mM, pH 7.9) to obtain a 1/3 (acetonitrile/NH_4HCO_3-solution v/v) mixture. Progress of the cyclization was monitored by analytical HPLC after 30 min. For all peptides, 30 min were sufficient for the complete cyclization. Next, acetonitrile was removed under reduced pressure and the remaining aqueous solution/suspension was lyophilized. The crude cyclic peptides were purified using (semi)preparative reverse phase HPLC. The fractions containing product were pooled and lyophilized yielding the desired cyclic peptides as white fluffy solids.

Cyclic peptide ***28***, $_{Ac\text{-}CNYYGSNC\text{-}NH_2}^{\ulcorner hinge \urcorner}$ purified using 0% to 40% of buffer B in buffer A. Overall yield: 14% (19 steps, average yield 90% per step). t_R = 14.5 min. **LCMS-ESI**: average mass calcd $[M]^+$: 1214.3; found: 1214.3.

Purification and analytical data of cyclic peptides (CDR mimics) **29–31** were described previously.[4]

Preparation of synthetic antibodies 35–40 and 44–49

General procedure for the synthesis of synthetic antibodies 35–40. Scaffold **2** or **3** or **4** (5.2 mg or 6.0 mg or 6.9 mg, respectively, 6.50 μmol), CDR mimic **31** (10.3 mg, 6.50 μmol), TBTA (1.0 mg, 1.95 μmol) were dissolved in degassed DMF (975 μL) and the resulting solution was kept under nitrogen atmosphere in a 5 mL round bottom flask. Then the solution was diluted with degassed i-PrOH (1300 μL) and deionised H_2O (290 μL) before freshly prepared 0.2 M aqueous sodium ascorbate (19.5 μL, 3.90 μmol) and 0.12 M aqueous $CuSO_4$ (16.3 μL, 1.95 μmol) were added in the given order. After 1 h and

15 min of stirring, $AgNO_3$ (6.1 mg, 35.75 μmol) was added to the reaction mixture which was stirred for another 2 h. Following this treatment with $AgNO_3$, another portion of TBTA (1.0 mg, 1.95 μmol) and CDR mimic **30** (9.1 mg, 6.50 μmol) were added to the reaction mixture, followed by the addition of 0.2 M aqueous sodium ascorbate (29.0 μL, 5.85 μmol) and 0.12 M aqueous $CuSO_4$ (16.3 μL, 1.95 μmol). The reaction mixture was stirred for 3 h and after two hours of stirring freshly prepared 0.2 M aqueous aminoguanidine hydrochloride (59.0 μL, 11.70 μmol) and additional 0.2 M aqueous sodium ascorbate (10.0 μL, 1.95 μmol) were added. Then, 1 M solution of TBAF·$3H_2O$ in THF (114 μL, 113.75 μmol) was added and the resulting suspension was stirred overnight (*i.e.* 18 h). This was followed by addition of TBTA (1.4 mg, 2.60 μmol) to the reaction mixture which was then stirred for 45 min before being briefly sonicated and split into two halves. Next, a solution of CDR mimic **29** (5.6 mg, 3.90 μmol) in degassed DMF (150 μL) was added to one half of the reaction mixture while a solution of CDR mimic **28** (4.7 mg, 3.90 μmol) in degassed DMF (250 μL) was added to the other half. In addition, freshly prepared 0.2 M aqueous sodium ascorbate (39.0 μL, 7.80 μmol), 0.12 M aqueous $CuSO_4$ (10.9 μL, 1.30 μmol) and freshly prepared 0.2 M aqueous aminoguanidine hydrochloride (39.0 μL, 7.80 μmol) were added to both reaction mixtures which were then stirred for 2.5 h during which, after 1.5 h of stirring, additional 0.2 M aqueous sodium ascorbate (19.5 μL, 3.90 μmol) and 0.2 M aqueous aminoguanidine hydrochloride (39.0 μL, 7.80 μmol) were added. Then, the solvents were removed under stream of nitrogen (for *ca*. 1.5 h) and the resulting viscous residue (usually around 50 μL) was diluted with HPLC buffer A (350 μL) and HPLC buffer B (150 μL), sonicated, centrifuged (13 000*g* for 6 min) and the product in the supernatant was purified by semi-preparative reverse phase HPLC using a gradient of 27% to 37% of buffer B in buffer A. Fractions containing the product were pooled and lyophilised. In the majority of the experiments, significant amounts (89% on average) of desired synthetic antibodies were obtained in purities of 91%–96% along with smaller portion of impure fractions which were re-purified using the same gradient.

General procedure for the synthesis of synthetic antibodies 44–49. The above described procedure was also used with the following order of addition of CDR mimics. CDR mimic **29** (9.6 mg, 6.50 μmol) was introduced *via* CuAAC as the first and the CDR mimic **28** (7.9 mg, 6.50 μmol) as the second onto the either scaffold **2**, **3**, or **4**. After the reaction mixture was split a solution of CDR mimics **31** (6.2 mg, 3.90 μmol) in degassed DMF (150 μL) was added to one half of the reaction mixture while a solution of CDR mimic **30** (5.5 mg, 3.90 μmol) in degassed DMF (150 μL) was added to the other half. Moreover, for this particular sequence of addition of CDR mimics, double amount 1 M solution of TBAF·$3H_2O$ in THF (228 μL, 227.5 μmol) had to be used for successful removal of TIPS protecting group.

Synthetic antibody **35** (2.8 mg, overall yield 17%, average yield 70% per step, 5 steps) was obtained as a white fluffy solid. t_R = 17.1 min. **LCMS-ESI:** average mass calcd $[M + 3H]^{3+}$: 1472.3; found: 1472.0. **MALDI:** exact mass calcd $[M + H]^+$ 4411.7; found: 4411.1.

Synthetic antibody **36** (3.4 mg, overall yield 21%, average yield 73% per step, 5 steps) was obtained as a white fluffy solid. t_R = 17.2 min. **LCMS-ESI:** average mass calcd $[M + 3H]^{3+}$: 1516.4; found: 1516.0. **MALDI:** exact mass calcd $[M + H]^+$ 4543.8; found: 4543.9.

Synthetic antibody **37** (2.6 mg, overall yield 15%, average yield 69% per step, 5 steps) was obtained as a white fluffy solid. t_R = 17.3 min. **LCMS-ESI:** average mass calcd $[M + 3H]^{3+}$: 1560.4; found: 1560.2. **MALDI:** exact mass calcd $[M + H]^+$ 4675.9; found: 4675.6.

Synthetic antibody **38** (2.4 mg, overall yield 16%, average yield 69% per step, 5 steps) was obtained as a white fluffy solid. t_R = 17.7 min. **LCMS-ESI:** average mass calcd $[M + 3H]^{3+}$: 1460.2; found: 1460.0. **MALDI:** exact mass calcd $[M + H]^+$ 4375.6; found: 4376.0.

Synthetic antibody **39** (2.6 mg, overall yield 17%, average yield 70% per step, 5 steps) was obtained as a white fluffy solid. t_R = 17.7 min. **LCMS-ESI:** average mass calcd $[M + 3H]^{3+}$: 1504.3; found: 1504.0. **MALDI:** exact mass calcd $[M + H]^+$ 4507.6; found: 4507.5.

Synthetic antibody **40** (2.8 mg, overall yield 17%, average yield 70% per step, 5 steps) was obtained as a white fluffy solid. t_R = 17.8 min. **LCMS-ESI:** average mass calcd $[M + 3H]^{3+}$: 1548.4; found: 1548.0. **MALDI:** exact mass calcd $[M + H]^+$ 4639.7; found: 4639.8.

Synthetic antibody **44** (2.7 mg, overall yield 18%, average yield 71% per step, 5 steps) was obtained as a white fluffy solid. t_R = 17.2 min. **LCMS-ESI:** average mass calcd $[M + 3H]^{3+}$: 1425.2; found: 1424.9. **MALDI:** exact mass calcd $[M + H]^+$ 4270.6; found: 4270.9.

Synthetic antibody **45** (2.4 mg, overall yield 16%, average yield 69% per step, 5 steps) was obtained as a white fluffy solid. t_R = 17.3 min. **LCMS-ESI:** average mass calcd $[M + 3H]^{3+}$: 1469.3; found: 1469.0. **MALDI:** exact mass calcd $[M + H]^+$ 4402.7; found: 4402.6.

Synthetic antibody **46** (2.5 mg, overall yield 16%, average yield 69% per step, 5 steps) was obtained as a white fluffy solid. t_R = 17.3 min. **LCMS-ESI:** average mass calcd $[M + 3H]^{3+}$: 1513.3; found: 1513.0. **MALDI:** exact mass calcd $[M + H]^+$ 4534.7; found: 4534.5.

Synthetic antibody **47** (2.4 mg, overall yield 15%, average yield 69% per step, 5 steps) was obtained as a white fluffy solid. t_R = 17.2 min. **LCMS-ESI:** average mass calcd $[M + 3H]^{3+}$: 1448.6; found: 1448.3. **MALDI:** exact mass calcd $[M + H]^+$ 4340.7; found: 4340.7.

Synthetic antibody **48** (2.0 mg, overall yield 13%, average yield 66% per step, 5 steps) was obtained as a white fluffy solid. t_R = 17.3 min. **LCMS-ESI:** average mass calcd $[M + 3H]^{3+}$: 1492.6; found: 1492.3. **MALDI:** exact mass calcd $[M + H]^+$ 4472.8; found: 4472.8.

Synthetic antibody **49** (1.9 mg, overall yield 12%, average yield 65% per step, 5 steps) was obtained as a white fluffy solid. t_R = 17.3 min. **LCMS-ESI:** average mass calcd $[M + 3H]^{3+}$:

1536.7; found: 1536.4. **MALDI**: exact mass calcd $[M + H]^+$ 4604.8; found: 4604.7.

Notes on the one-pot protocol. During optimization of the above protocols the following observations turned out to be crucial to the success of the preparation of the synthetic antibodies.

1. Solvents had to be degassed prior to carrying out the reaction sequence otherwise the CuAAC introduction of the CDR mimics proceeded poorly. This was especially important for introduction of the third CDR mimic else almost no conversion was observed.

2. Using less than 5.5 equivalents of $AgNO_3$ led, in some cases, to incomplete TES removal within 2 h. However, using more than 5.5 equivalents of $AgNO_3$ (10 equivalents at the maximum) slowed down the subsequent introduction of the second CDR mimic although it did expedite TES removal.

3. Addition of the least soluble CDR mimic (that is compound **28** which did not fully dissolve in the chosen solvent mixture) to a scaffold already bearing another CDR mimic prevented the need for prolonged reaction times which was not the case when the least soluble CDR mimic **28** was attached as the first mimic to either scaffold **2**, **3** or **4**. In the latter case, the first CuAAC CDR mimic introduction required up to 8 h for completion and subsequent reactions usually required more time than described in the general procedure.

4. In cases when CDR mimics **28** and **29** were attached onto CTV scaffold derivatives **3** or **4**, conversion of TIPS removal decreased to *ca.* 60%–75%. However, close to complete conversion could be achieved by increasing the amount of used TBAF·$3H_2O$ in degassed THF from 17.5 to 35 equivalents.

5. Fresh portions of $CuSO_4$, sodium ascorbate and TBTA had to be added for every CuAAC introduction of CDR mimics to scaffolds **2**, **3** or **4** as well as its CDR mimic(s) containing derivatives in order to secure good conversion. "Recycling" of copper from the first CuAAC or alternatively from the first and the second CuAAC by reducing the initially added $CuSO_4$ with additional sodium ascorbate for the subsequent CuAAC did not lead to satisfactory conversions.

6. Extension of reaction times of CuAAC introduction of the third CDR mimic beyond 2.5 h hardly improved the conversion even when additional $CuSO_4$, sodium ascorbate and TBTA were added during the reaction. The reaction tended to stop and a slow degradation of the product as well as formation of side products were observed after *ca.* 4 h.

7. Formation of dehydroascorbic acid adducts of arginine containing CDR mimics, which was mainly observed at the very end of the synthesis when reaction mixture was concentrated under stream of nitrogen, could be effectively prevented by addition of aminoguanidine hydrochloride as a scavenger of the formed dehydroascorbic acid.[12]

8. After evaluation of several gradients on C4, C8, and C18 reversed-phase columns, a gradient of 27% to 37% of buffer B into buffer A on a C8 column was selected as the best one for purification of all prepared synthetic antibodies.

Surface plasmon resonance experiments

The surface plasmon resonance experiments were performed using Biacore™ X100 SPR biosensor (Biacore AB, Uppsala, Sweden) equipped with CM5 sensor chip (Biacore AB, Uppsala, Sweden). hTNFα (*ca.* 51 kDa = trimer, >97% pure based on SDS-PAGE) was immobilised using amine-coupling chemistry.[22] Surfaces of two flow cells were activated for 7 min with a 1 : 1 mixture of 0.1 M NHS (*N*-hydroxysuccineimide) and 0.4 M EDC [3-(*N*,*N*-dimethylamino)propyl-*N*-ethylcarbodiimide] at a flow rate of 5 μL min^{-1}. hTNFα at a concentration of 30 μg mL^{-1} in 10 mM sodium acetate, pH 4.5, was immobilised at a density of 984 RU and 1024 RU, representing two different CM5 chips, on flow cell 2. Flow cell 1 was left blank to serve as a reference surface. Both surfaces were blocked with a 7 min injection of 1 M ethanolamine, pH 8.5, at a flow rate of 5 μL min^{-1}. The immobilisation was performed at 21 °C with MES running buffer (10 mM MES, 150 mM NaCl, 3 mM EDTA, 0.05% P20, pH 6.0) using Biacore immobilisation wizard with target density of 1600 RU. Following the immobilisation, non-covalently bound oligomeric units of hTNFα were removed by injections of regeneration solution (5 mM NaOH, 150 mM NaCl, *ca.* 6 × 20 s).

The activity of immobilised hTNFα was verified with anti-hTNFα specific monoclonal antibody (Life Technologies, Thermo Fisher Scientific, clone 68B 2B3) in a single-cycle experiment with 3-fold dilutions of the mAb (highest concentration 100 nM) at a flow rate of 30 μL min^{-1} and a temperature of 21 °C. The experiment was performed using two different running buffers; the above mentioned MES buffer as well as its DMSO supplemented version, MES-DMSO, (10 mM MES, 150 mM NaCl, 3 mM EDTA, 0.05% P20, 5% DMSO, pH 6.0). Duration of the association/dissociation phase was set to 180 s and 420 s, respectively. The sensogram data were fitted according to 1 : 1 binding model using Biacore X100 Evaluation software version 2.0.1 (Biacore AB, Uppsala, Sweden). The anti-hTNFα specific mAb bound immobilised hTNFα with K_D of 15 nM and 32 nM in MES and MES-DMSO buffer, respectively. Thus, a 2-fold decrease in affinity for experiments performed in MES-DMSO buffer was observed.

Solutions of synthetic antibodies were prepared at a concentration of 16 μM by dissolution of the synthetic antibody in DMSO (volume corresponding to 5% of the final volume) and subsequent dropwise dilution with 1.05 × MES-DMSO (10.5 mM MES, 157.5 mM NaCl, 3.15 mM EDTA, 0.0525% P20, pH 6.0) aided by vigorous stirring by vortex. The affinity of synthetic antibodies towards hTNFα was screened at 2 μM and the synthetic antibodies which showed binding were further tested in a multi-cycle kinetic experiment at 8 μM with 2-fold dilutions at a flow rate of 30 μL min^{-1} and a temperature of 21 °C in MES-DMSO buffer. Duration of the association/dissociation phase was set to 120 s and 300 s, respectively. Regeneration of the sensor surface was accomplished by a 12 s injection of a regeneration solution (5 mM NaOH, 150 mM NaCl) followed by a 300 s stabilisation period. Experiments were performed in duplicate in two independent experiments (n = 2) on chips with different immobilisation level of hTNFα

(984 RU and 1024 RU). The medium concentration (2 μM) was injected twice as a control of stability of the experimental setup. During data processing the highest tested concentration, *i.e.* 8 μM was excluded from fitting as the obtained curves had irregular shape. The blank subtracted and injection peaks cleaned data were evaluated in Biacore X100 Evaluation software version 2.0.1 (Biacore AB, Uppsala, Sweden) using heterogeneous ligand fitting model. The heterogeneous fitting model was used as three out of six lysine residues of hTNFα, *via* which immobilisation happens, reside near the binding site of infliximab and thus synthetic antibodies. Following data evaluation, K_D representing the binding event contributing the most to total R_{max} was selected as the value best describing the strength of the observed binding.

Modelling of the molecular constructs

Molecular models of CTV-derivatives, peptide loops and synthetic antibody mimics were constructed using the Maestro software (http://www.Schrödinger.com) interfaced with MacroModel[23] minimization and conformational analysis options using the MMFFs and OPLS-2005 force fields, PRCG minimization with water as a solvent. In short CDR loop mimics were generated by construction of the peptides in the grow mode, followed by introduction of the cyclization hinge **27** and minimization of obtained CDR peptide loops **28–31**. Multi conformer analysis options allowed obtaining CDR mimics close to the global minimum energy conformations. The CDR mimics were connected subsequently to the appropriate CTV scaffold derivatives **2**, **3** or **4** and minimized after each connection step. At this stage MD (Molecular Dynamics) studies were omitted, since the primary goal was to obtain a visual insight as a qualitative explanation of the observed results.

Aqueous solubility of CTV scaffold derivatives determination

Excess of scaffold derivatives **20**, **21**, and **22** (*ca.* 1.0 mg) was suspended in EtOH (2% of final volume) and diluted with deionised water (to a final volume of 1 mL). The suspension was briefly sonicated (<10 s), stirred by vortex at 2500 rpm for 5 min, allowed to stand for 25 min and centrifuged (13 000*g* for 10 min) before a sample was taken for analytical HPLC analysis (50 μL injected). HPLC runs were performed using isocratic gradient of acetonitrile/H_2O 50 : 50 at a flow rate of 1 mL min^{-1} using a Phenomenex Gemini C18 column (110 Å, 5 μm, 250 × 4.60 mm). Calibration curves were constructed using 5 concentration points of 2-fold dilutions of an appropriate CTV scaffold derivative in acetonitrile/H_2O 50/50, v/v. The results are a mean of three independent experiments performed in triplicate. The solubilisation had to be aided by addition of 2% of ethanol in order to break clusters of namely compound **21** which would otherwise barely interact with water. Even though such addition alters the final solubility, the ratio of solubility between the three scaffold derivatives should remain the same.

Log *P* determination

Following the OECD method,[8] 6 standards of known log *P* (anisole, toluene, bromobenzene, phenyl benzoate, diphenyl ether, bibenzyl) were used to construct a calibration curve. Log *P* values of compounds **20**, **21**, and **22** were determined from their retention time using regression equation obtained from the calibration curve of standards. Analytical HPLC runs were performed using isocratic gradient of acetonitrile/H_2O 50/50, v/v at a flow rate of 1 mL min^{-1} using a Phenomenex Gemini C18 column (110 Å, 5 μm, 250 × 4.60 mm). Dead volume of the HPLC system was determined to be 2.89 min using uracil.

Recombinant hTNFα expression

Soluble recombinant hTNFα (Val^{77} – Leu^{233}) was obtained by cloning its *E. coli* optimised gene sequence into a pDEST14™ expression vector (Thermo Fisher Scientific). The expression vector was transformed into competent *E. coli* BL21-AI™ One Shot® according to the manufacturer's protocol. A single colony of transformed cells was selected and allowed to expand in 2-YT bacterial growth media (16 g of tryptone, 10 g of yeast extract, 5 g of NaCl per 1 L supplemented with 50 μg mL^{-1} carbenicillin; 20 mL) at 37 °C overnight with 165 RPM shaking. The overnight bacterial culture was expanded into fresh 2-YT media at a ratio of 1/20 and the culture was incubated at 37 °C with 165 RPM shaking until the cells reached log phase (approximately 2 h). The cells were then induced for 5 h with the addition of L-(+)-arabinose (final concentration of 0.2% w/v) to initiate protein expression. After induction, the cells were pelleted by centrifugation at 12 000*g* for 5 min at 4 °C. The pellet was suspended in cold lysis buffer (20 mM Tris-HCl, 1 mM EDTA, pH 8.0, supplemented with EDTA-free protease inhibitor cocktail; 10 mL) and the cells were lysed on ice by sonication (10 s with 25% amplitude followed by 30 s break, repeated 15 times). The lysate was centrifuged at 12 000*g* for 15 min at 4 °C and the supernatant was transferred into a new tube. The remaining pellet was resuspended in fresh ice cold lysis buffer (10 mL) and the above procedure was repeated and the supernatant was pooled. The hTNFα was purified from the supernatant using an optimised procedure from Zhang *et al.*[24] In short, the supernatant containing recombinant hTNFα was applied on to two HiTrap™ DEAE Fast Flow (GE Healthcare) columns with 10 mL of total column volume (CV) using an AKTA Start purification system (GE Healthcare) at a flow rate of 1 mL min^{-1}. The columns were equilibrated with 8 CV of buffer A (20 mM Tris-HCl, pH 8.0) and hTNFα was eluted by applying 15% step elution gradient of buffer B (20 mM Tris-HCl with 1 M NaCl, pH 8.0). The second purification step was carried out on a HiTrap™ CM FF (GE Healthcare) column with 5 mL of CV (equilibrated with 5 CV of buffer C) after 1/5 dilution of hTNFα containing fractions from DEAE column with buffer C (20 mM Na_2HPO_4/KH_2PO_4, pH 6.0) to decrease conductivity below 5 mS cm^{-1}. Elution of hTNFα was achieved by applying gradient (0%–30%) of buffer D (20 mM Na_2HPO_4/KH_2PO_4 with 1 M NaCl, pH 6.0). Fractions containing hTNFα were pooled and dialysed (benzoylated cellulose, MWCO 2 kDa) against PBS pH 7.4 overnight at 4 °C. The dialysed protein was sterile-filtered using a 0.2 μm filter and aliquots were stored at −78 °C. The activity and purity of

hTNFα was assessed by an MTT assay utilising L929 cell line and SDS-PAGE respectively. Typically, 10 mg–14 mg of TNFα with a purity ≥97% and IC_{50} of 0.07 ng mL^{-1} was obtained from a 250 mL of bacterial culture.

Conflicts of interest

There are no conflicts to declare.

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References

1 M. Hijnen, D. J. van Zoelen, C. Chamorro, P. van Gageldonk, F. R. Mooi, G. Berbers and R. M. Liskamp, *Vaccine*, 2007, **25**, 6807.
2 G. E. Mulder, H. L. Quarles van Ufford, J. van Ameijde, A. J. Brouwer, J. A. Kruijtzer and R. M. Liskamp, *Org. Biomol. Chem.*, 2013, **11**, 2676.
3 P. R. Werkhoven, M. Elwakiel, T. J. Meuleman, H. C. Q. van Ufford, J. A. W. Kruijtzer and R. M. J. Liskamp, *Org. Biomol. Chem.*, 2016, **14**, 701.
4 O. Longin, H. van de Langemheen and R. M. J. Liskamp, *Bioorg. Med. Chem.*, 2017, **25**, 5008.
5 S. Kalepu and V. Nekkanti, *Acta Pharm. Sin. B*, 2015, **5**, 442.
6 R. U. Kadam, J. Juraszek, B. Brandenburg, C. Buyck, W. B. G. Schepens, B. Kesteleyn, B. Stoops, R. J. Vreeken, J. Vermond, W. Goutier, C. Tang, R. Vogels, R. H. E. Friesen, J. Goudsmit, M. J. P. van Dongen and I. A. Wilson, *Science*, 2017, **358**, 496.
7 J. Canceill, J. Gabard and A. Collet, *J. Chem. Soc., Chem. Commun.*, 1983, 122.
8 Partition Coefficient (*n*-octanol/water), High Performance Liquid Chromatography (HPLC) Method, OECD Guideline for Testing of Chemicals, adopted on 30.03.1989.
9 S. Liang, J. Dai, S. Hou, L. Su, D. Zhang, H. Guo, S. Hu, H. Wang, Z. Rao, Y. Guo and Z. Lou, *J. Biol. Chem.*, 2013, **288**, 13799.
10 H. van de Langemheen, V. Korotkovs, J. Bijl, C. Wilson, S. S. Kale, C. Heinis and R. M. J. Liskamp, *ChemBioChem*, 2017, **18**, 387.
11 I. E. Valverde, A. F. Delmas and V. Aucagne, *Tetrahedron*, 2009, **65**, 7597.
12 A. C. Conibear, K. Farbiarz, R. L. Mayer, M. Matveenko, H. Kahlig and C. F. W. Becker, *Org. Biomol. Chem.*, 2016, **14**, 6205.
13 P. R. Werkhoven, H. van de Langemheen, S. van der Wal, J. A. Kruijtzer and R. M. Liskamp, *J. Pept. Sci.*, 2014, **20**, 235.
14 J. A. Nieman, S. K. Nair, S. E. Heasley, B. L. Schultz, H. M. Zerth, R. A. Nugent, K. Chen, K. J. Stephanski, T. A. Hopkins, M. L. Knechtel, N. L. Oien, J. L. Wieber and M. W. Wathen, *Bioorg. Med. Chem. Lett.*, 2010, **20**, 3039.
15 T. Mayer and M. E. Maier, *Eur. J. Org. Chem.*, 2007, 4711.
16 P. A. Allegretti and E. M. Ferreira, *Org. Lett.*, 2011, **13**, 5924.
17 M. Inouye, K. Akamatsu and H. Nakazumi, *J. Am. Chem. Soc.*, 1997, **119**, 9160.
18 D. Bom, D. P. Curran, S. Kruszewski, S. G. Zimmer, J. Thompson Strode, G. Kohlhagen, W. Du, A. J. Chavan, K. A. Fraley, A. L. Bingcang, L. J. Latus, Y. Pommier and T. G. Burke, *J. Med. Chem.*, 2000, **43**, 3970.
19 F. Bellotta, M. V. D'Auria, V. Sepe and A. Zampella, *Tetrahedron*, 2009, **65**, 3659.
20 T. Harada, K. Muramatsu, K. Mizunashi, C. Kitano, D. Imaoka, T. Fujiwara and H. Kataoka, *J. Org. Chem.*, 2008, **73**, 249.
21 V. Percec, P. Leowanawat, H. J. Sun, O. Kulikov, C. D. Nusbaum, T. M. Tran, A. Bertin, D. A. Wilson, M. Peterca, S. Zhang, N. P. Kamat, K. Vargo, D. Moock, E. D. Johnston, D. A. Hammer, D. J. Pochan, Y. Chen, Y. M. Chabre, T. C. Shiao, M. Bergeron-Brlek, S. Andre, R. Roy, H. J. Gabius and P. A. Heiney, *J. Am. Chem. Soc.*, 2013, **135**, 9055.
22 C. Poiesi, A. Albertini, S. Ghielmi, G. Cassani and A. Corti, *Cytokine*, 1993, **5**, 539.
23 F. Mohamadi, N. G. J. Richards, W. C. Guida, R. Liskamp, M. Lipton, C. Caufield, G. Chang, T. Hendrickson and W. C. Still, *J. Comput. Chem.*, 1990, **11**, 440.
24 C. Zhang, Y. D. Liu, D. W. Zhao, X. N. Li, R. Yu and Z. G. Su, *Protein Expression Purif.*, 2014, **95**, 195.

