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# Scaffolded multiple cyclic peptide libraries for protein mimics by native chemical ligation†

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The accessibility to collections, libraries and arrays of cyclic peptides is increasingly important since cyclic peptides may provide better mimics of the loop-like structures ubiquitously present in and – especially – on the surface of proteins. The next important step is the preparation of libraries of ensembles of scaffolded cyclic peptides, which upon screening may lead to promising protein mimics. Here we describe the synthesis of a tri-cysteine containing scaffold as well as the simultaneous native chemical ligation of three cyclic peptides thereby affording a clean library of multiple cyclic peptides on this scaffold, representing potential mimics of gp120. Members of this collection of protein mimics showed a decent inhibition of the gp120-CD4 interaction.

## Introduction

In the quest for protein mimics possessing desirable properties, the generation of collections or even large libraries is instrumental in screening approaches to uncover "hits", which represent first steps towards these protein mimics. It remains important to realize that "generation of collections or libraries" and "screening or evaluation of many possibilities" are very reminiscent of processes in nature where many possibilities are available or generated and scanned to select the compound members with optimal properties.

Although the availability of arrays or libraries of *linear* peptides has provided excellent starting points for development of new (peptide) ligands and identification of peptide segments in peptides and proteins which are crucial determinants of their biological activities, it is expected that these starting points might be considerably improved by using as a starting point arrays of *cyclic* peptides, which in principle provide better mimics of the loop-like structures ubiquitously present in, and especially, on the surface of proteins.[1] Here the (large) phage display libraries of cyclic disulfide bridged[2] and, more recently, the bicyclic phage display libraries obtained by alkylation of cysteine containing linear peptide phage display libraries[3] represented tremendous progress. However, the number of (bio)organic chemical approaches for the preparation of cyclic peptide libraries, let alone combined in ensembles, for mimicry of discontinuous epitopes is still pretty limited. So far most examples in the literature, which have used chemoselective ligation methods to conjugate peptides to a scaffold molecule, mainly have focussed on attachment of multiple *identical* peptides.[4–6]

We have been increasingly interested in the preparation of cyclic peptides especially as ensembles of different combinations. Recently, we reported the first strides towards modest libraries of ensembles of scaffolded cyclic peptides using the bio-orthogonal Cu(I)-catalyzed azide–alkyne-cycloaddition (CuAAC).[7,8] However, probably the best biocompatible/bio-orthogonal reaction to date is native chemical ligation (NCL),[9,10] so next to CuAAC, we have great interest in applying native chemical ligation for the construction of protein mimics. The recently described convenient synthesis of cyclic peptides containing a thioester handle for native chemical ligation[11] enables now their attachment onto a suitably functionalized scaffold for the preparation of libraries, which will be described in this manuscript. The clean preparation of a library allowed us to carry out a facile screening and evaluation of the resulting cyclic peptide ensembles as potential protein mimics of gp120 in a gp120-CD4 receptor ELISA binding assay. The cyclic peptides in the gp120 mimics correspond to the sequences of the peptide segments of loops in gp120 together forming the discontinuous epitope interacting with CD4 (Fig. 1).[12–14]

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† Electronic supplementary information (ESI) available: NMR data of compound **4**, HPLC and MALDI-TOF MS data of loop 1 (**11a**), loop 2 (**11b**), loop 3 (**11c**) and compound **12**, HPLC data of **5**, mass data of mixture **14**, and HPLC and MS data of **13** and all purified protein mimics. See DOI: 10.1039/c4ob00190g

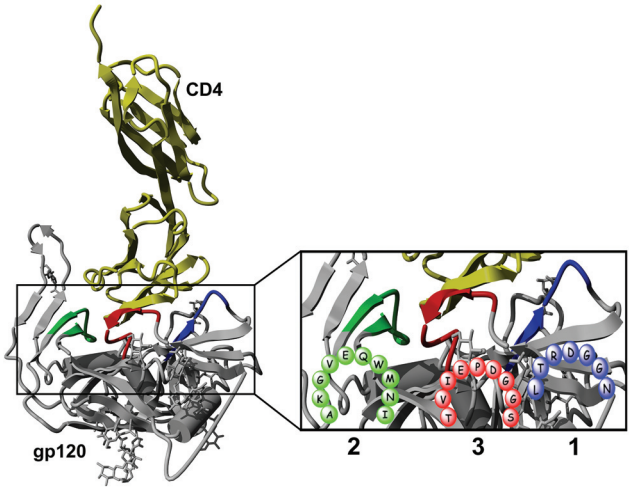



Fig. 1 Conserved discontinuous epitope of the CD4 binding site on gp120.[12–14]

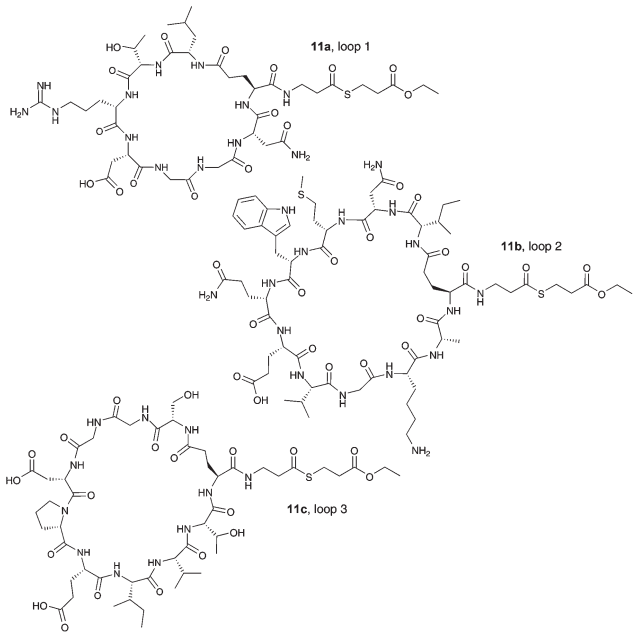


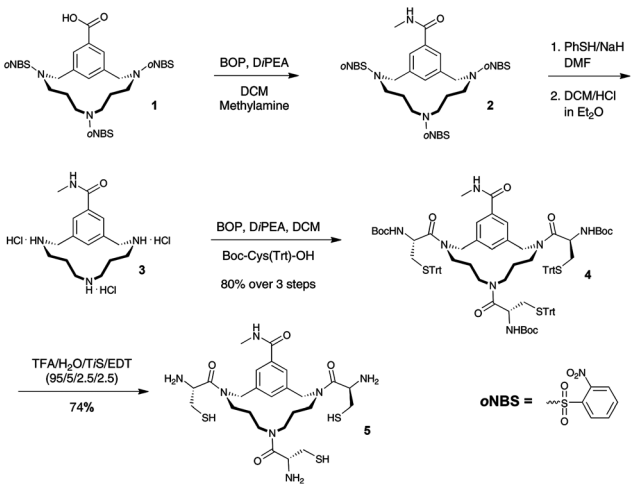

Fig. 2 Structures of the LTRDGGN (**11a**, loop 1), INMWQEVGKA (**11b**, loop 2) and SGGDPEIVT (**11c**, loop 3) gp120 peptide thioester loops.[11]

## Results and discussion

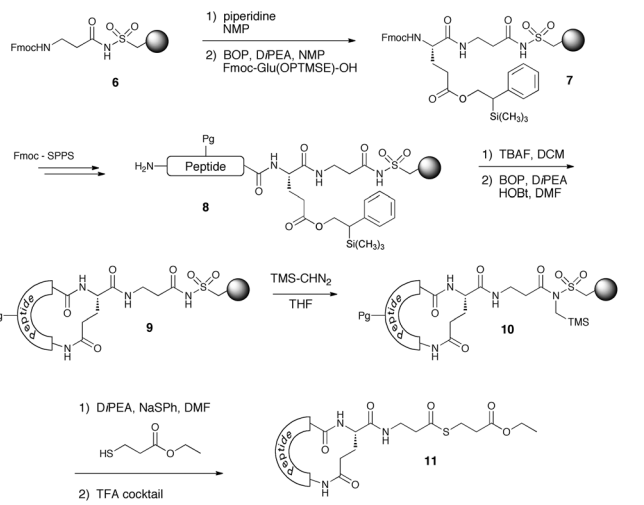
### Synthesis of the tri-cysteine TAC-scaffold and cyclic peptide loops containing a thioester handle

The required triazacyclophane (TAC) scaffold, capable of accepting cyclic peptide thioesters in a NCL reaction, was prepared by attachment of (three) cysteine residues to the deprotected TAC-scaffold **3**. This was easily accessible starting from the tri-*o*NBS protected TAC-scaffold (**1**).[15] Amidation of the carboxylic acid moiety and removal of the *o*NBS groups from scaffold **2**, using *in situ* prepared sodium thiophenolate, was followed by the simultaneous coupling of three protected cysteine (Boc-Cys(Trt)-OH) residues to afford scaffold **4**. Finally, the acid-labile protecting groups were removed and the tri-cysteine TAC-scaffold (**5**) was purified by preparative HPLC and obtained in a yield of 74% (Scheme 1).

Cyclic peptide thioesters for native chemical ligation to scaffold **4** were prepared by a solid phase peptide synthesis




Scheme 1 Synthesis of the tri-cysteine TAC-scaffold **5**.




Scheme 2 General SPPS procedure for obtaining cyclic peptides, containing a thioester handle.[11]

method involving the use of a *N*-acyl sulfonamide linker, which is conveniently accessible by a sulfo-click reaction (Fig. 2).[11,16–20] Briefly, our general procedure (Scheme 2) for the preparation of cyclic peptide thioesters encompassed first attachment of an orthogonally protected glutamic acid residue required for peptide cyclization followed by assembly of the linear peptide. Cyclization was performed on the solid phase after removal of the (2-phenyl-2-trimethylsilyl)ethyl (PTMSE) protecting group with fluoride, using (benzotriazol-1-yloxy)tris-(dimethylamine)phosphonium hexafluorophosphate (BOP) as a coupling reagent. After alkylation the *N*-acyl sulfonamide linker can be cleaved using nucleophilic reagents, such as

β-mercaptopropionic acid ethyl ester, leading to the desired thioester. Finally, the acid-labile side-chain protecting groups were cleaved and the resulting unprotected cyclic peptide, containing a thioester handle, was purified by preparative HPLC (Scheme 2). In this way, the gp120 cyclic peptide thioesters with amino acid sequences LTRDGGN (**11a**), INMWQEVGKA (**11b**) and SGGDPEIVT (**11c**) were obtained (Fig. 2) in 5.6–7.0% overall yield, corresponding to 87–90% average yield per reaction step.[11]

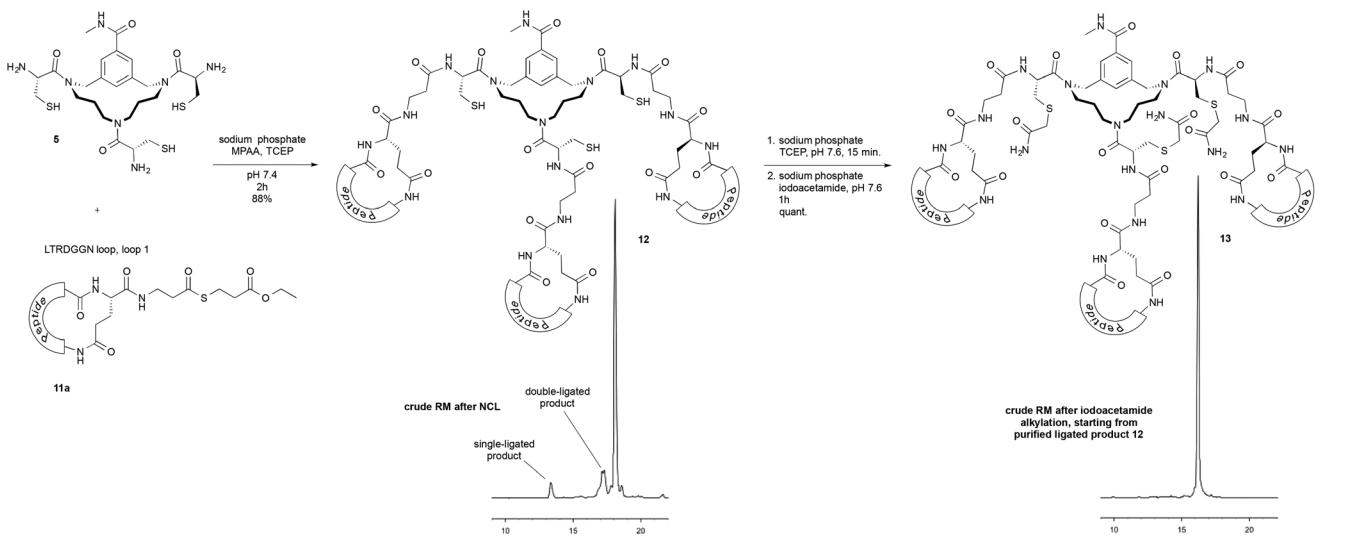
### Library synthesis of the gp120 discontinuous epitope mimics

A library, but especially a library of protein mimics, should not contain impurities, which for example can give rise to false positives or negatives in the screening assay. We have previously described the preparation of smart libraries of discontinuous epitopes.[7,8] Crucial characteristics of these libraries, which have been obtained through CuAAC, are that the prepared collections of library members were very clean, virtually free of (unknown) impurities and obtained in a very reproducible manner. The alternative method using NCL might be even more attractive since it is devoid of any traces of Cu(I) or Cu(II).

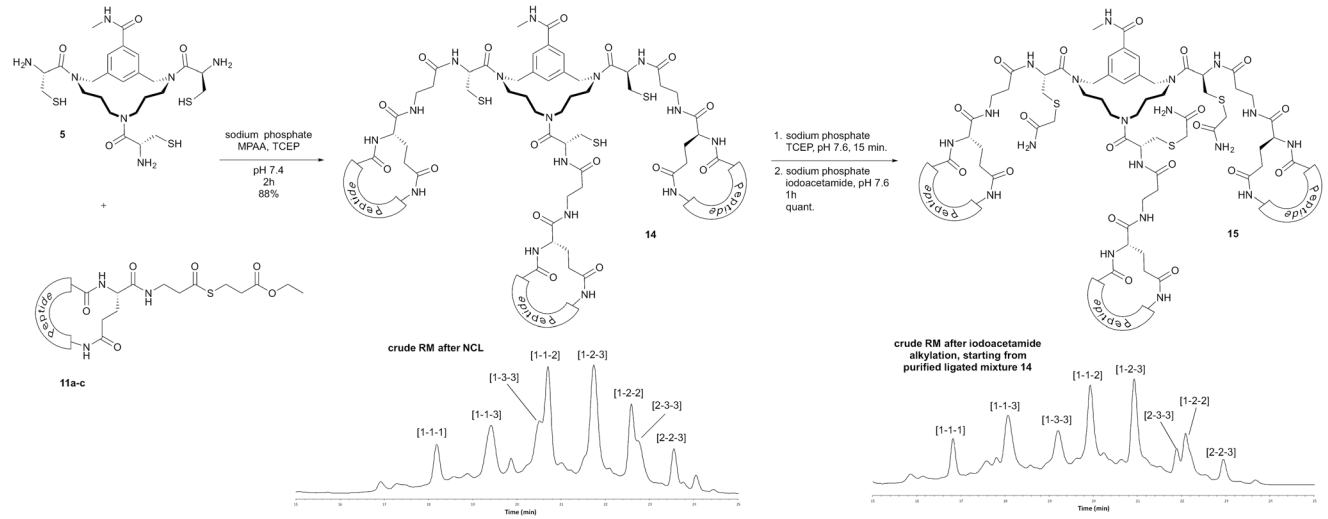
However, in a NCL strategy, normally a buffer system is used with a large excess of a thiol to increase the reactivity of the peptide thioester. Nowadays, 4-mercaptophenylacetic acid (MPAA) is the most popular thiol additive in NCL.[21] A drawback of MPAA is that the HPLC retention time of this thiol is in the same range as that of most peptidic compounds. Therefore, when performing HPLC analysis or purification, it is possible that MPAA co-elutes with the ligated product. This co-elution is an even bigger risk when a library of compounds is prepared. Fortunately, we found that it was possible to remove MPAA quickly and completely by washing the TFA-acidified NCL-reaction mixture with ether.[11] First a test ligation reaction of cyclic peptide thioester **11a** and tri-cysteine scaffold **5** was carried out in ligation buffer (200 mM sodium phosphate, 200 mM MPAA, 40 mM tris(2-carboxyethyl)-phosphine hydrochloride (TCEP), pH 7.4) with 1 mM scaffold and 3 mM cyclic peptide thioester followed by TFA-acidification and washing with ether to remove MPAA. Within two hours almost complete conversion to the ligated product **12** was observed. Only small amounts of the double- and single-ligated products were found upon LC-MS of the reaction mixture. After preparative HPLC, the triple ligated product **12** was obtained in an excellent yield of 88% (Scheme 3). To avoid interference with biological assays, the sulfhydryl group of the scaffolded cysteine residues was capped. To achieve complete capping and to avoid formation of any by-products, **12** was first treated for 20 minutes with a TCEP buffer to reduce any possible disulfides, followed by addition of a sodium phosphate buffer containing iodoacetamide. After one hour, complete and clean conversion to the triple-capped product **13** was observed. Preparative HPLC purification afforded **13** in a quantitative yield (Scheme 3).

These initial results suggested that it would be possible to obtain a clean collection of cyclic peptides ligated to the three cysteine residue-containing scaffold **5**. Thus, an equimolar mixture of three different cyclic peptide thioesters **11a–c**, corresponding to the discontinuous epitope of gp120, was subjected to a NCL-reaction with TAC-scaffold **5** as was described above. After acidification and washing with ether, LC-MS analysis showed that almost all combinations of peptide loops ligated to the TAC-scaffold had been obtained (Scheme 4). After alkylation with iodoacetamide a relatively clean reaction mixture of the capped protein mimics was formed, with an improved separation of the peaks of the individual mimics as compared to the starting mixture (Scheme 4).

Although the LC-MS chromatogram of the iodoacetamide capped reaction mixture showed only one peak in the chromatogram for the mimic with the three different ligated loops, it was expected that our NCL approach has yielded three




**Scheme 3** Synthesis of the alkylated triple ligated mimic **13**. Analytical HPLC profiles ($\lambda$ = 214 nm) of the crude NCL (bottom-left) and the crude iodoacetamide alkylation reaction (bottom-right).




**Scheme 4** Synthesis of the TAC-scaffolded library of gp120 mimics. Analytical HPLC profiles ($\lambda$ = 214 nm) of the crude NCL (middle-bottom) and crude iodoacetamide capping reaction (right-bottom).

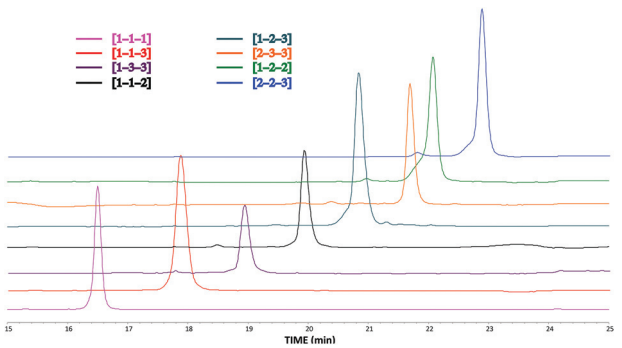



**Fig. 3** Analytical HPLC profiles ($\lambda$ = 214 nm) of the purified [1-1-1], [1-1-3], [1-3-3], [1-1-2], [1-2-3], [2-3-3], [1-2-2] and [2-2-3] mimics.

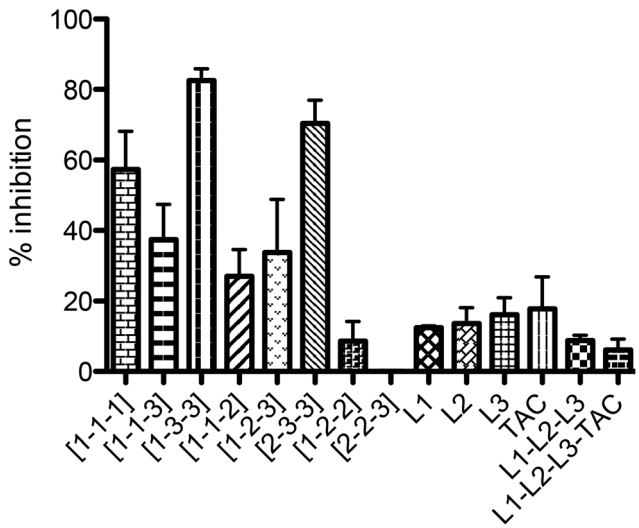



**Fig. 4** Results of the competitive gp120(IIIB)-CD4 ELISA for all purified protein mimics, different cyclic peptide thioesters loops, TAC-scaffold, equimolar mixture of the cyclic peptide thioesters and equimolar mixture of the cyclic peptide thioesters as well as the TAC-scaffold. The concentration of the mimics was 125 μg $mL^{-1}$, which corresponds to 31 μM for the [1-2-3] mimic. The concentration of the thioester loops (L1, L2 and L3) was 31 μM. In the mixtures each individual component (L1, L2, L3 and TAC-scaffold) was present in a concentration of 31 μM.

different isomers of the [1-2-3] construct: one with peptide loop 1 in the middle, one with peptide loop 2 in the middle and one with peptide loop 3 in the middle. Due to the ring-flipping ability of the TAC-scaffold for example isomer [1-2-3] is identical to isomer [3-2-1]. It is expected that the different isomers have similar physical properties and therefore it is likely that the three [1-2-3] mimics also have similar HPLC retention times. Analogously, for example the [1-1-2] purified construct probably consists of two different compounds (Fig. 3).

### Activity evaluation of the gp120 discontinuous epitope mimics

Binding of the resulting gp120 discontinuous epitope mimics to CD4 was studied in a gp120-capture ELISA experiment. Most of the gp120 mimics were able to compete with the recombinant monomeric gp120(IIIB) for binding to CD4, whereas the individual non-ligated loop thioesters, the TAC-scaffold, an equimolar mixture of loop thioesters or an equimolar mixture of TAC-scaffolds together with cyclic peptide loops showed hardly any activity (Fig. 4). This experiment clearly showed a synergistic effect of ligating the gp120 discontinuous epitope loops to the TAC-scaffold. When comparing the different gp120 mimics, it becomes evident that the protein mimics that contain peptide loop 3 are stronger binders of CD4 than the other protein mimics. The binding affinity of these molecular constructs was in the same range (low micromolar) as the TAC-scaffolded gp120 mimics, which have been obtained using the CuAAC reaction.[7] Using the CuAAC method, protein mimics containing loop 2 and loop 3 were the best inhibitors of binding of recombinant monomeric

gp120(IIIB) to CD4. In contrast, the [1-2-2] and [2-2-3] gp120 mimics obtained in this work showed hardly any binding to the CD4 receptor. This may indicate that the cyclic peptide **11b** representing loop 2 in this work is a less ideal mimic of the natural $^{424}$INMWQEVGKA$^{433}$ loop, as compared to the cyclic peptide used for representing loop 2 in the CuAAC method. The observation that the [1-2-3] protein mimic containing all three loops of the discontinuous epitope of gp120 did not show the highest inhibition in this ELISA assay might be indicative of a non-optimal positioning of the different peptide loops on the scaffold. Moreover, the molecular construct [1-2-3] likely consists of three isomers. An approach for synthesising the individual isomers thereby enabling the synthesis of the different isomers of the most active molecular construct that is the [1-3-3] mimic, and of course of the [1-2-3] mimic will be reported soon and should enable evaluation of the importance of the relative positioning of the peptide loops on the scaffold.

## Conclusions

In conclusion, we have developed a native ligation method for obtaining molecular constructs containing multiple cyclic peptides on a scaffold; these may act as protein mimics capable of mimicry of discontinuous epitopes. The required tri-cysteine containing the TAC-scaffold was conveniently synthesized in a high yield. Native chemical ligation using cyclic peptide thioesters followed by capping was both a highly efficient and very clean reaction, by which to obtain these relatively complex molecular constructs of intermediate size in a both straightforward and reproducible manner. Several of the obtained protein gp120 discontinuous epitope mimics showed an inhibition of the recombinant monomeric gp120(IIIB) – CD4 binding. These library members may be used as promising starting points for optimization studies of these protein mimics. Finally, the library approach developed for the construction of protein mimics might represent a promising tool for constructing mimics of other discontinuous epitopes.

## Experimental

### General information

All reagents were obtained from commercial sources and used without further purification. Peptide grade DiPEA and TFA were purchased from Biosolve B.V. (Valkenswaard, The Netherlands) and $CH_2Cl_2$, NMP and HPLC grade solvents were purchased from Actu-All (Oss, The Netherlands). Lyophilizations were performed on Christ Alpha 1–2 apparatus. Reactions were carried out at ambient temperature unless stated otherwise. Solvents were evaporated under reduced pressure at 40 °C. Reactions in solution were monitored by TLC analysis and $R_f$ values were determined on Merck pre-coated silica gel 60 F-254 (0.25 mm) plates. Spots were visualized by UV-light and by heating plates after dipping in a ninhydrin solution. $^1$H NMR spectra were recorded on a Varian VNMRS400 400 MHz spectrometer in DMSO-$d_6$ as the solvent at 373 K because at room temperature very broad signals were observed, most likely due to the *cis*/*trans* isomerization of the tertiary amides. Chemical shifts ($\delta$) are reported in parts per million (ppm) relative to DMSO-$d_6$ (2.50 ppm). Splitting patterns are designated as singlets (s) and multiplets (m). $^{13}$C NMR spectra were recorded on a Varian VNMRS400 400 MHz spectrometer at 100 MHz in DMSO-$d_6$ as the solvent. Chemical shifts ($\delta$) are reported in parts per million (ppm) relative to the solvent residual signal, DMSO-$d_6$ (39.52 ppm). 2-D NMR data (HSQC and COSY) were acquired on a Varian VNMRS400 400 MHz spectrometer. Analytical HPLC was accomplished on a Shimadzu-10Avp (Class VP) with a UV-detector operating at 214 and 254 nm using a Alltech Prosphere C4 column (300 Å, 5 μm, 250 × 4.60 mm, column A) or a Phenomenex Gemini C18 column (110 Å, 5 μm, 250 × 4.60 mm, column B) at a flow rate of 1 mL min$^{-1}$ using a standard protocol: 100% buffer A for 5 min, followed by a linear gradient of buffer B (0–100% in 20 min, method A) or 100% buffer A for 2 min, next a linear gradient of buffer B (0–100% in 48 min, method B). The mobile phase was $H_2O$–$CH_3CN$–TFA (95 : 5 : 0.1, v/v/v, buffer A) and $H_2O$–$CH_3CN$–TFA (5 : 95 : 0.1, v/v/v, buffer B). Purification of the peptidic compounds was performed on a Prep LCMS-QP8000α HPLC system (Shimadzu) using a Alltech Prosphere C4 column (300 Å, 10 μm, 250 × 22 mm, column A) or a Phenomenex Gemini C18 column (110 Å, 10 μm, 250 × 20 mm, column B) at a flow rate of 12.5 mL min$^{-1}$ using a standard protocol: 100% buffer A for 5 min, followed by a linear gradient of buffer B (0–60% in 100 min, method A) or 100% buffer A for 5 min, followed by a linear gradient of buffer B (0–100% in 20 min, method B) using the same buffers as described for analytical HPLC. Analytical LC-MS was performed on a Thermo-Finnigan LCQ Deca XP Max ion trap mass spectrometer using the same buffers and protocols as described for analytical HPLC. Routine electrospray ionization mass spectrometry (ESI-MS) was performed on a Shimadzu LCMS-QP8000 single quadrupole bench-top mass spectrometer operating in a positive ionization mode or on a Thermo-Finnigan LCQ Deca XP Max ion trap mass spectrometer. The MALDI-TOF-MS spectrum was recorded on a Kratos Analytical (Shimadzu) AXIMA CFR mass spectrometer using sinapic acid as a matrix and bovine insulin oxidized B chain (monoisotopic $[M + H]^+$ 3494.6513) as a reference. High-resolution electrospray ionization (ESI) mass spectra were measured on a Bruker micrOTOF-Q II in positive mode and calibrated with ESI tuning mix from Agilent Technologies. The pH was measured using a PHM210 standard pH meter from Radiometer Analytical equipped with a micro combined pH electrode pHC3359-8 from Radiometer Analytical. The microtiter plate reader used in the ELISA experiments was a BioTEK μQuant (Beun de Ronde, Abcoude, The Netherlands). Software used for data analysis was the Full Mode-KC4 version 3.4.

**(*o*NBS)$_3$-TAC(N(H)Me) 2.** Tri-*o*NBS protected TAC-scaffold **1**[15] (3.11 g, 3.74 mmol, 1 eq.) and BOP (1.82 g, 4.11 mmol, 1.1 eq.) were dissolved in DCM (25 mL) at room temperature. Next,

DiPEA (2.61 mL, 15.0 mmol, 4 eq.) and methylamine (2 M in THF, 9.35 mL, 18.7 mmol, 5 eq.) were added and the reaction mixture was stirred overnight. After the evaporation of the solvents *in vacuo*, the crude product was dissolved in EtOAc (100 mL) and washed twice with 1 N $KHSO_4$ (both 100 mL) and twice with 5% $NaHCO_3$ (both 100 mL) and brine (100 mL). Finally, the organic layer was dried over $Na_2SO_4$ and evaporated *in vacuo*, yielding the relatively clean amidated scaffold **2**, which was used as such for the preparation of **3**.

**TAC(N(H)Me) 3.** NaH (60% dispersion in mineral oil, 1.35 g, 33.7 mmol, 9 eq. (3 eq. per *o*NBS group)) was added slowly to a solution of thiophenol (3.82 mL, 37.4 mmol, 10 eq. (3.33 eq. per *o*NBS group)) in DMF (45 mL) at room temperature. After *ca.* 30 min stirring at room temperature, bubbling of $H_2$ gas has subsided and (*o*NBS)$_3$-TAC(N(H)Me) **2** (3.74 mmol, 1 eq.), dissolved in DMF (5 mL), was added to the above freshly prepared sodium thiophenolate solution. Upon this addition, the homogeneous, yellow reaction mixture became a brown-red suspension. After stirring overnight at room temperature, the solvents were evaporated *in vacuo* and DCM (90 mL) and $Et_2O$ saturated with HCl (75 mL) were added to the crude product, yielding a yellowish suspension, which was filtered over a glass filter. The residue was washed with DCM and dried *in vacuo*, yielding the crude unprotected TAC-scaffold amide **3** as a white solid, which was directly used in the next reaction. Although the crude product contained approximately 9 eq. of NaCl, it was not expected that this would give rise to problems in the next reaction step.

**(Boc-Cys(Trt))$_3$-TAC(N(H)Me) 4.** To a solution of Boc-Cys-(Trt)-OH (5.20 g, 11.2 mmol, 3 eq. (1 eq. per amine of the TAC-scaffold)) and BOP (4.96 g, 11.2 mmol, 3 eq. (1 eq. per amine of the TAC-scaffold)) in DCM (40 mL), DiPEA (7.8 mL, 45 mmol, 12 eq. (4 eq. per amine of the TAC-scaffold)) was added. Next, crude TAC(N(H)Me) **3** (3.74 mmol, 1 eq.) was added to the reaction mixture, yielding a white suspension. The reaction mixture was stirred overnight at room temperature, followed by the evaporation of the solvents *in vacuo*. Next, the crude product was dissolved in EtOAc (100 mL) and washed twice with 1 N $KHSO_4$ (both 100 mL) and twice with 5% $NaHCO_3$ (both 100 mL) and brine (100 mL). Finally, the organic layer was dried over $Na_2SO_4$, evaporated *in vacuo* and the product was purified by column chromatography (hexanes–EtOAc, 1 : 1, v/v) to afford the TAC-scaffold (Boc-Cys-(Trt))$_3$-TAC(N(H)Me) **4** as a white foam (4.85 g, 80% over 3 steps). $R_f$ = 0.26 (Hexanes–EtOAc, 1 : 1, v/v); HRMS *m*/*z* calcd for $C_{97}H_{108}N_7O_{10}S_3$ $[M + H]^+$ 1626.7314, found 1626.7358; $^1$H-NMR (400 MHz, DMSO-d$_6$) $\delta$: 0.86, 1.22 (2 m, 4H, 2 × $CH_2CH_2CH_2$), 1.33, 1.34 (2 s, 27H, 3 × $C(CH_3)_3$), 2.34, 2.55 (2 m, 6H, 3 × $CHCH_2S$), 2.72, 3.02, 3.24 (3 m, 8H, 4 × $NCH_2CH_2$), 2.78 (s, 3H, C═ONHC$H_3$), 4.11–4.67 (2 m, 7H, 3 × $CHCH_2S$ and 2 × $PhCH_2$), 6.37, 6.73 (2 m, 3H, 3 × NHBoc), 7.20–7.33, 7.69 (2 m, 48H, Ar–CH), 7.87 (m, 1H, C═ONHCH$_3$). $^{13}$C-NMR (100 MHz, DMSO-d$_6$): 25.7 (C═ONHC$H_3$), 26.9 (3 × $CH_2CH_2CH_2$), 27.7 (3 × $C(CH_3)_3$), 33.7, 33.8 (3 × $CHCH_2S$), 44.5 (4 × $NCH_2CH_2$), 49.7, 50.5 (3 × $CHCH_2S$), 50.7 (2 × $PhCH_2$), 66.0, 66.3 (3 × $C(Ph)_3$), 78.3, 78.4 ($C(CH_3)_3$), 126.2, 126.3, 126.9, 127.4, 127.4, 128.0, 128.2, 128.7, 128.8, 129.5, 130.1, 135.6, 137.8, 144.1 (Ar–C), 154.1, 154.5 (3 × C═O Boc), 165.9 (C═O TAC), 169.2, 169.9 (3 × C═O Cys).

**(H-Cys)$_3$-TAC(N(H)Me) 5.** The TAC-scaffold **4** (150 mg, 92.2 μmol) was dissolved in a solution of TFA–$H_2O$–TIPS–EDT (10 mL, 90 : 5 : 2.5 : 2.5, v/v/v/v) for 2 h at room temperature. The volume of the reaction mixture was reduced to approximately 2 mL by evaporation. Next, this mixture was added drop wise to a cold (4 °C) solution of MTBE–hexanes (1 : 1, v/v). After centrifugation (3500 rpm, 5 min) the supernatant was decanted and the pellet was resuspended in MTBE–hexanes (1 : 1, v/v) and centrifuged again. This was repeated twice after which the pellet was dissolved in water containing $H_2O$–TFA (100 : 0.01, v/v) and purified by preparative HPLC (column A, method A, using $H_2O$–TFA (100 : 0.01, v/v) as buffer A). Fractions corresponding to **5** were pooled and lyophilized to yield the TFA salt of the tri-cysteine TAC-scaffold **5** as a white fluffy solid (64.6 mg, 74%, 3 TFA molecules were added to the molecular weight of the scaffold). $t_R$ = 14.1 min (column A, method A, using $H_2O$–TFA (100 : 0.01, v/v) as buffer A)); HRMS *m*/*z* calcd for $C_{25}H_{42}N_7O_4S_3$ $[M + H]^+$ 600.2455, found 600.2493.

### Triple-ligated product 12

$N_2$ was bubbled through a freshly prepared solution of $NaH_2PO_4{\cdot}2H_2O$ (200 mM, 62.4 mg), MPAA (200 mM, 67.3 mg), and TCEP (40 mM, 22.9 mg), pH 7.4 in 2 mL of $H_2O$, for 5 minutes. Next, this solution (2 mL) was added to solid cyclic peptide thioester **1**[11] (compound **11a**, 7.55 mg, 6 μmol, final conc.: 3 mM, the molecular weight of **11a** was assumed to include 2 molecules of TFA) and triple-cysteine TAC-scaffold **5** (1.88 mg, 2 μmol, final conc.:1 mM, the molecular weight of **5** was assumed to include 3 molecules of TFA) and the reaction mixture was stirred at room temperature. After 2 hours the reaction mixture was diluted with $H_2O$–TFA (9 : 1, v/v, 2 mL) to precipitate MPAA. After two washing steps with $Et_2O$ (4 mL) to remove MPAA, TCEP (*ca.* 10 mg) was added and the solution was stirred for 5 min. After two additional washing steps with $Et_2O$ (4 mL), purification, using preparative HPLC (column B, method A), was performed. Fractions corresponding to the ligated product **12** were pooled and lyophilized to yield the triple-ligated product **12** as a white fluffy solid (7.00 mg, 88%, its molecular weight was assumed to include 6 TFA molecules). $t_R$ = 17.9 min (column B, method B); MALDI-TOF MS *m*/*z* calcd for $C_{133}H_{213}N_{46}O_{46}S_3$ $[M + H]^+$ 3286.49, found 3286.49.

### Triple-capped product 13

The triple-ligated product **12** (1.00 mg, 0.252 μmol, MW.6TFA) was dissolved in 252 μL of a freshly prepared solution of $NaH_2PO_4{\cdot}2H_2O$ (200 mM, 62.4 mg) and TCEP (40 mM, 22.9 mg), pH 7.6 in 2 mL of $H_2O$ and the mixture was stirred for 20 minutes at room temperature. Next, 252 μL of a freshly prepared solution of $NaH_2PO_4{\cdot}2H_2O$ (200 mM, 62.4 mg) and iodoacetamide (60 mM, 22.2 mg), pH 7.6 in 2 mL of $H_2O$ was added to the reaction mixture. After stirring for 1 hour in the dark, the mixture was directly transferred and purified by preparative HPLC (column B, method A). Fractions corresponding

to **13** were pooled and lyophilized to yield the alkylated triple-loop 1 mimic **13** as a white fluffy solid (1.15 mg, quant, MW.6TFA). $t_R$ = 16.5 min (column B, method B); HRMS *m*/*z* calcd for $C_{139}H_{224}N_{49}O_{49}S_3$ $[M + 3H]^{3+}$ 1153.1902, found 1153.1889.

### General native chemical ligation procedure of (H-Cys)$_3$-TAC(N(H)Me) 5 and cyclic peptide thioesters 11a–c

$N_2$ was bubbled through a freshly prepared solution of $NaH_2PO_4{\cdot}2H_2O$ (200 mM, 124.8 mg), MPAA (200 mM, 134.6 mg) and TCEP (40 mM, 45.9 mg), pH 7.4 in 4 mL of $H_2O$, for 5 minutes. Next, this solution (4 mL) was added to a falcon tube, containing cyclic peptide thioester **1**[11] (compound **11a**, 5.03 mg, 4 μmol, 1 mM, MW.2TFA), cyclic peptide thioester **2**[11] (compound **11b**, 6.35 mg, 4 μmol, 1 mM, MW.1TFA), cyclic peptide thioester **3**[11] (compound **11c**, 4.69 mg, 4 μmol, 1 mM) and tri-cysteine TAC-scaffold **5** (3.76 mg, 4 μmol, 1 mM, MW.3TFA) and the resulting reaction mixture was stirred at room temperature. After 3 hours TFA (500 μL) was added to the reaction mixture to precipitate MPAA. After two wash steps with $Et_2O$ (each 5 mL), TCEP (*ca.* 10 mg) was added and the solution was stirred for 5 min. After two additional wash steps with $Et_2O$ (each 5 mL), a sample was taken from the reaction mixture for LC-MS analysis (column B, method B) to verify the absence of MPAA. Finally, the reaction mixture was directly purified by a fast preparative HPLC run (column B, method B) and all peptide fractions were pooled and lyophilized to yield collection **14** as a white fluffy solid, which was directly used in the next reaction.

### General procedure for the alkylation of the sulfhydryl group of the cysteine residues in protein mimics with iodoacetamide

Collection **14** (4 μmol, 3 cysteine residues per molecule) was dissolved in a freshly prepared solution of $NaH_2PO_4{\cdot}2H_2O$ (200 mM, 124.8 mg) and TCEP (40 mM, 45.9 mg), pH 7.6 in 4 mL of $H_2O$ and was stirred for 20 minutes at room temperature. Next, a freshly prepared solution of $NaH_2PO_4{\cdot}2H_2O$ (200 mM, 124.8 mg) and iodoacetamide (60 mM, 44.4 mg), pH 7.6 in 4 mL of $H_2O$ was added to the reaction mixture. After stirring for 1 hour in the dark, a sample was taken from the reaction mixture for LC-MS analysis (column B, method B). Finally, the reaction mixture was lyophilized, dissolved in $H_2O$–$CH_3CN$–TFA (75 : 25 : 0.01, v/v/v) and the individual components were purified by preparative HPLC (column B, method A).

[**1-1-1**] **mimic**: $t_R$ = 16.5 min (column B, method B); HRMS *m*/*z* calcd for $C_{139}H_{224}N_{49}O_{49}S_3$ $[M + 3H]^{3+}$ 1153.1902, found 1153.1889.

[**1-1-3**] **mimic**: $t_R$ = 17.9 min (column B, method B); HRMS *m*/*z* calcd for $C_{147}H_{234}N_{47}O_{53}S_3$ $[M + 3H]^{3+}$ 1200.5407, found 1200.5335.

[**1-3-3**] **mimic**: $t_R$ = 18.9 min (column B, method B); HRMS *m*/*z* calcd for $C_{155}H_{244}N_{45}O_{57}S_3$ $[M + 3H]^{3+}$ 1247.8913, found 1247.8859.

[**1-1-2**] **mimic**: $t_R$ = 19.9 min (column B, method B); HRMS *m*/*z* calcd for $C_{163}H_{257}N_{52}O_{52}S_4$ $[M + 3H]^{3+}$ 1300.9316, found 1300.9133.

[**1-2-3**] **mimic**: $t_R$ = 20.8 min (column B, method B); HRMS *m*/*z* calcd for $C_{171}H_{267}N_{50}O_{56}S_4$ $[M + 3H]^{3+}$ 1348.2822, found 1348.2861.

[**2-3-3**] **mimic**: $t_R$ = 21.7 min (column B, method B); HRMS *m*/*z* calcd for $C_{179}H_{276}N_{48}O_{60}S_4$ $[M + 3H]^{3+}$ 1395.2968, found 1395.6229.

[**1-2-2**] **mimic**: $t_R$ = 22.1 min (column B, method B); HRMS *m*/*z* calcd for $C_{187}H_{290}N_{55}O_{55}S_5$ $[M + 3H]^{3+}$ 1448.6730, found 1448.6644.

[**2-2-3**] **mimic**: $t_R$ = 22.9 min (column B, method B); HRMS *m*/*z* calcd for $C_{195}H_{300}N_{53}O_{59}S_5$ $[M + 3H]^{3+}$ 1496.0236, found 1496.0155.

### HIV-1 gp120 capture ELISA

The HIV-1 gp120 capture ELISA experiments were performed according to literature procedures.[7,8] Before binding of the TAC-scaffold was studied in this ELISA experiment, the template was first alkylated with iodoacetamide according to the above described method. $t_R$ = 13.3 min (column A, method A, using $H_2O$–TFA (100 : 0.01, v/v) as buffer A). MS (ESI) *m*/*z* calcd for $C_{31}H_{51}N_{10}O_7S_3$ $[M + H]^+$ 771.31, found 771.25; calcd for $C_{31}H_{50}N_{10}NaO_7S_3$ $[M + Na]^+$ 793.30, found 793.40.

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