Cyclotriveratrylene (CTV) as a New Chiral Triacid Scaffold Capable of Inducing Triple Helix Formation of Collagen Peptides Containing either a Native Sequence or Pro-Hyp-Gly Repeats

Erik T. Rump,[a, b, c] Dirk T. S. Rijkers,[b] Hans W. Hilbers,[b] Philip G. de Groot,[a] and Rob M. J. Liskamp*[b]

Abstract: A new triacid scaffold is described based on the cone-shaped cyclotriveratrylene (CTV) molecule that facilitates the triple helical folding of peptides containing either a unique blood platelet binding collagen sequence or collagen peptides composed of Pro-Hyp-Gly repeats. The latter were synthesized by segment condensation using Fmoc-Pro-Hyp-Gly-OH. Peptides were coupled to this CTV scaffold and also coupled to the Kemp's triacid (KTA) scaffold. After assembly of peptide H-Gly-[Pro-Hyp-Gly]$_2$-Phe-Hyp-Gly-Glu(OAll)-Arg-Gly-Val-Glu(OAll)-Gly-[Pro-Hyp-Gly]$_2$-NH_2 (**13**) by an orthogonal synthesis strategy to both triacid scaffolds, followed by deprotection of the allyl groups, the molecular constructs spontaneously folded into a triple helical structure. In contrast, the non-assembled peptides did not. The melting temperature (T_m) of (+/−) CTV[CH_2C(O)N(H)Gly-[Pro-Hyp-Gly]$_2$-Phe-Hyp-Gly-Glu-Arg-Gly-Val-Glu-Gly-[Pro-Hyp-Gly]$_2$-NH_2]$_3$ (**14**) is 19 °C, whereas KTA[Gly-Gly-[Pro-Hyp-Gly]$_2$-Phe-Hyp-Gly-Glu-Arg-Gly-Val-Glu-Gly-[Pro-Hyp-Gly]$_2$-NH_2]$_3$ (**15**) has a T_m of 20 °C. Thus, it was shown for the first time that scaffolds were also effective in stabilizing the triple helix of *native* collagen sequences. The different stabilizing properties of the two CTV enantiomers could be measured after coupling of racemic CTV triacid to the collagen peptide, and subsequent chromatographic separation of the diastereomers. After assembly of the two chiral CTV scaffolds to the model peptide H-Gly-Gly-(Pro-Hyp-Gly)$_5$-NH_2 (**24**), the (+)-enantiomer of CTV **28b** was found to serve as a better triple helix-inducing scaffold than the (−)-enantiomer **28a**. In addition to an effect of the chirality of the CTV scaffold, a certain degree of flexibility between the CTV cone and the folded peptide was also shown to be of importance. Restricting the flexibility from two to one glycine residues resulted in a significant difference between the two collagen mimics **20a** and **20b**, whereas the difference was only slight when two glycine residues were present between the CTV scaffold and the peptide sequence in collagen mimics **30a** and **30b**.

Keywords: circular dichroism · helical structures · protein folding · protein models · solid-phase synthesis

Introduction

Synthetic collagen structures that mimic the structure and conformation of native collagens are of special interest for studying cell-matrix interactions. The triple helical conformation of collagen molecules is of crucial importance since it is thought to be essential for recognition by its ligand.[1] A synthetic collagen mimic that meets this structural feature can therefore serve as a valuable tool to identify the exact binding sites that interact with their receptors. This has been shown for collagen molecules that include recognition sites for for example integrins and metalloproteinases.[2, 3] The exact nature of collagen–protein interactions are now beginning to be elucidated, as was recently shown by the first high-resolution crystal structure of a synthetic collagen peptide interacting with an integrin fragment.[4]

The collagen triple helix is composed of three separate peptide chains, which are staggered by one residue and

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  0947-6539/02/0820-4613 $ 20.00+.50/0

intertwine to form a right-handed triple helix.[5] The primary structure of these peptides is characterized by trimeric repeating units X-Y-G, where X and Y can be any amino acid. One trimer, Pro-Hyp-Gly (Hyp = hydroxyproline) or POG is found most prominently in native collagen, and peptides composed solely of these trimers give rise to the most stable triple helices.

In order to increase the thermal stability of synthetic collagen mimics,[6] different branching protocols with the aim to connect all three peptides of the collagen molecule have been applied. Branching can be achieved by the use of synthetic scaffold molecules. This has been shown for either collagen peptides with Pro-Hyp-Gly repeats or native collagen sequences with a much lower content of imino acids.[6–8] Goodman et al. used the Kemp's triacid (KTA, **1**) as a template to assemble peptide–peptoid structures.[9] This triacid scaffold is currently the most stabilizing collagen triple helix template, since it was shown to induce triple helical folding of the very short nona-peptide H-$(GPO)_3$-NH_2. However this scaffold was only used to study triple helices of *model* peptides.

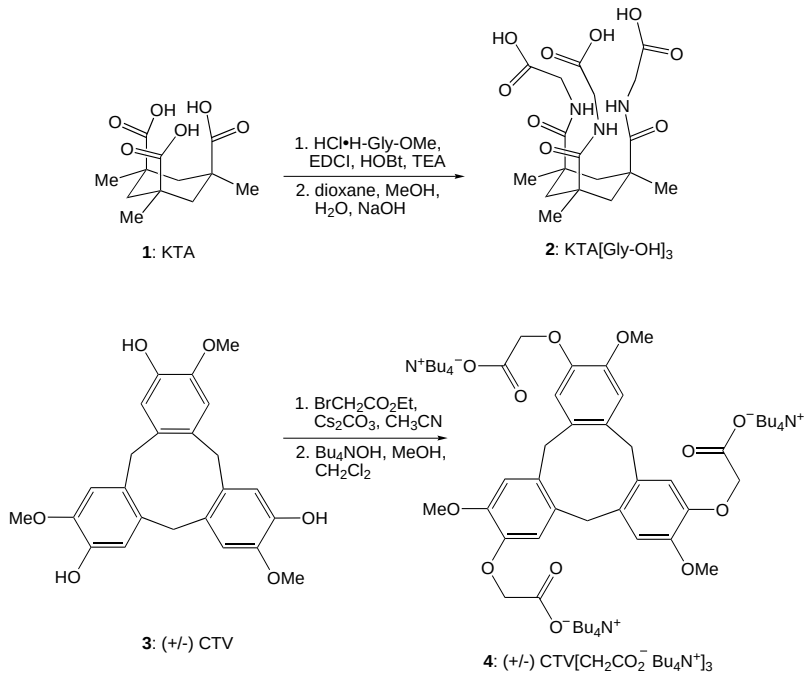
In this study we describe a new chiral triacid scaffold, based upon a cone-shaped cyclotriveratrylene (CTV, **3**) structure. Both the model and native collagen sequences were attached to this scaffold. The latter were synthesized using an orthogonal protecting group strategy. This new scaffold was compared to the Kemp's triacid scaffold. Analysis of the collagen mimics was performed by CD spectroscopy. The influence of the spacer length between the scaffold molecule and the collagen peptide as well as the effect of the two enantiomers of the CTV unit upon the triple helix stability was also assessed.

Results

Collagen peptides containing short naturally occurring or native sequences do not fold into triple helices at low temperatures. Triple helix formation can be induced by a high content of the imino acids hydroxyproline (O) and proline, and by increasing the length of the composing peptides. Stabilization of the triple helix can be achieved by assembly of the three composing peptides to a scaffold molecule. To study the assembly of a native collagen sequence with two Pro-Hyp-Gly (POG) repeats at both termini, a new triacid scaffold based upon a CTV unit that is compound **4** was synthesized (Scheme 1). The Kemp's triacid (KTA[Gly-OH]$_3$) scaffold **2** was chosen for comparison. The CD spectra and the stabilizing characteristics of these two triacid scaffold structures were compared.

Previous work by the group of Goodman[7] already showed the stabilizing properties of the KTA[Gly-OH]$_3$ template **2** for collagen structures composed solely of Gly-Pro-Hyp trimers or alkyl- and aryl peptoid-containing peptides. So far, assembly on KTA **1** of collagen peptides containing *native* sequences has not been reported. In our approach, the collagen peptides were assembled on KTA or CTV scaffolds **2** and **4** (Scheme 1) in solution, and therefore protection of the functional groups in the amino acids side chains was required. The collagen peptide, containing the native collagen sequence, was synthesized using Fmoc chemistry on the solid phase using a Rink amide linker. The stabilizing POG sequences were conveniently introduced by segment condensation using Fmoc-POG-OH. This tripeptide was synthesized starting from Boc-Pro-OH (**5**) and H-Hyp-OMe. The imino acids were coupled with BOP resulting in the dipeptide Boc-Pro-Hyp-OMe[10] **6** (Scheme 2). After saponification of the methyl ester and coupling of H-Gly-OMe, Boc-Pro-Hyp-Gly-OMe (**8**) was obtained. Removal of the Boc group and ester hydrolysis was achieved by acidic treatment, followed by introduction of the Fmoc protection group to give Fmoc-POG-OH (**9**) in 31% overall yield over five steps. As described by Ottl et al.,[11] there is no need for protection of the hydroxy function in hydroxyproline, and the use of this tripeptide building block significantly improved the purity and yield of collagen peptides containing POG repeats.

The new CTV triacid scaffold **4**[12] was prepared starting from the CTV triol **3** (Scheme 1) by coupling with α-bromo ethylacetate, followed by saponification of the CTV triester with Bu_4NOH.[16] The reference tem-



Scheme 1. Synthesis of (+/-)-CTV triacid scaffold and KTA(Gly-OH)$_3$ scaffold.

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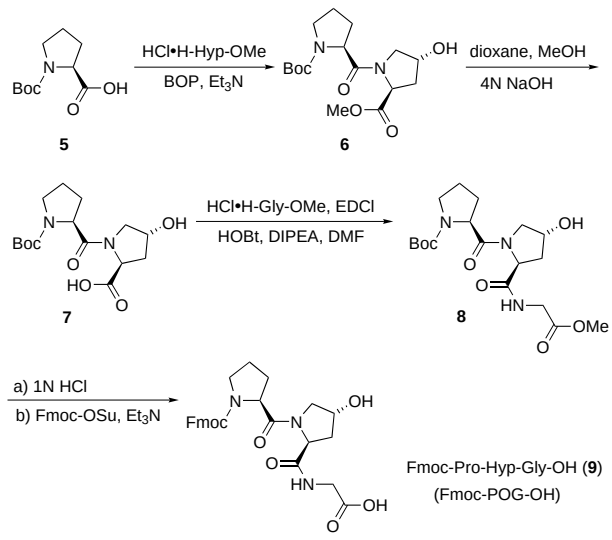


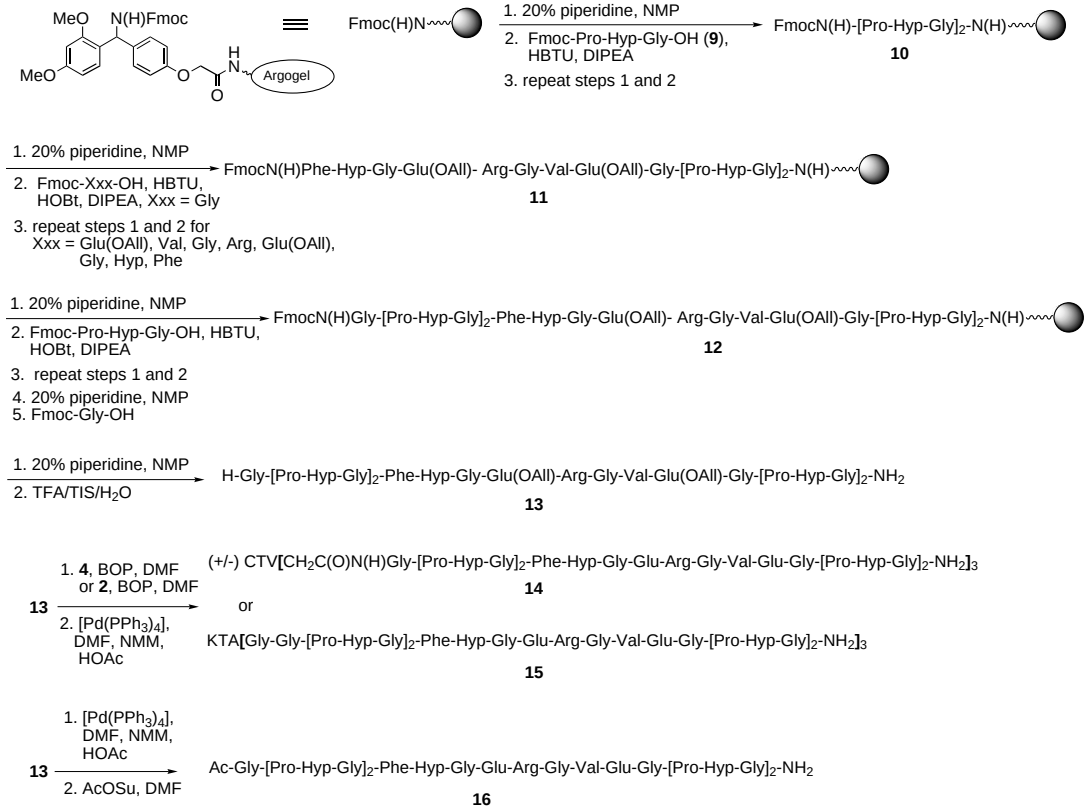
Scheme 2. Synthesis of Fmoc-Pro-Hyp-Gly-OH (Fmoc-POG-OH).

plate structure, KTA(Gly-OH)$_3$ (**2**), was synthesized from KTA **1** by a procedure described by Feng et al.[7]

The synthesis of H-Gly-[Pro-Hyp-Gly]$_2$-Phe-Hyp-Gly-Glu(OAll)-Arg-Gly-Val-Glu(OAll)-Gly-[Pro-Hyp-Gly]$_2$-NH$_2$ (**13**) is depicted in Scheme 3. Subsequently, the peptide was coupled at its N-terminus to both scaffolds **2** and **4** using BOP as a coupling reagent. The CTV triacid scaffold **4** was coupled as its Bu_4N salt, which is soluble in DMF.[16] After coupling, the allyl protection groups were removed by treatment with [Pd(PPh$_3$)$_4$]. The close proximity of the three peptide chains seemed to have no effect on this deprotection step. Final purification of the product by gel filtration and RP-HPLC yielded the two desired assembled collagen peptides **14** and **15** (Scheme 3, Table 1). The correct composition of the collagen structures was verified by MS. As a reference peptide for triple helical folding, the acetylated peptide **16** (Scheme 3, Table 1) was prepared. This was synthesized starting from the allyl-protected peptide **13**. The allyl protection groups were removed, and subsequently the N-terminus was acetylated using AcOSu.[13]

CD analysis of the collagen peptide assembled to either the CTV-triacid scaffold **4**, or KTA(Gly-OH)$_3$ (**2**), showed that both scaffolds are capable of assisting in the folding of the peptides into a triple helix. Moreover, assembly using the scaffolds increased the stability of the helix significantly compared with the non-assembled peptide. As is depicted in Figure 1, the CD spectrum exhibited a positive band at 223 nm and a large negative band at 200 nm for the KTA-assembled compound **15**, and 224 nm and 200 nm for the CTV-assembled compound **14**, respectively. A high ratio of these two bands (ratio positive over negative "R_{pn}" values) and a cooperative melting transition curve is indicative for the proper triple helical conformation.[6] R_{pn} values are 0.15 for the KTA-assembled compound **15** and 0.14 for the CTV assembled compound **14**, measured in 10 mM HOAc. In H_2O, the values are 0.15 and 0.15, respectively.

Melting curves were determined by monitoring the ellipticity at the maximal wavelength (positive peak) as a function of the temperature. Calculation of the first derivative of the melting curve leads to the melting temperature (T_m). The T_m



Scheme 3. Synthesis of the collagen peptides containing the $\alpha_2\beta_1$ integrin recognition sequence GFOGERGVE.

Table 1. Electrospray (ES) and MALDI-TOF mass spectrometry, and CD data of peptides.[a]

	Compounds	Calcd mass (m/z)	MS results	T_m [°C]	R_{pn}
13	H-G-$(POG)_2$FOGE(All)RGVE(All)G$(POG)_2$-NH_2	1084.5 $[M+2H]^{2+}$	1084.5	n.d.	n.d.
14a	(+ or −)CTV-[G-$(POG)_2$FOGERGVEG$(POG)_2$-$NH_2]_3$	1698.3 $[M+4H]^{4+}$	1698.4	< 5	n.d.
14b	other diastereomer	1358.8 $[M+5H]^{5+}$	1358.7	19; 23[b]	0.14
15	KTA-[GG-$(POG)_2$FOGERGVEG$(POG)_2$-$NH_2]_3$	1328.2 $[M+5H]^{5+}$	1328.1	20; 21[b]	0.15
16	Ac-G-$(POG)_2$FOGERGVEG$(POG)_2$-NH_2	1065.5 $[M+2H]^{2+}$	1065.9	< 5	n.d.
19	H-G-$(POG)_5$-NH_2	1410.7 $[M+H]^+$	1411.0	n.d.	n.d.
22	Ac-G-$(POG)_5$-NH_2	1453.7 $[M+H]^+$	1453.7	9.6	0.12
24	H-GG-$(POG)_5$-NH_2	1467.7 $[M+H]^+$	1467.1	n.d.	n.d.
26	Ac-GG-$(POG)_5$-NH_2	1510.7 $[M+H]^+$	1510.8	9.2	0.11
20a	(+ or −)CTV-[G-$(POG)_5$-$NH_2]_3$	1586.7 $[M+3H]^{3+}$	1587.0	37	0.10
20b	other diastereomer	1607.7 $[M+3Na]^{3+}$	1607.7	50	0.10
25a	(+ or −)CTV-[GG-$(POG)_5$-$NH_2]_3$	1233.0 $[M+4H]^{4+}$	1233.0	55	0.12
25b	other diastereomer	1248.8 $[M+3Na+H]^{4+}$	1248.8	58	0.12
30a	(−) CTV-[GG-$(POG)_5$-$NH_2]_3$	1248.8 $[M+3Na+H]^{4+}$	1249.3	55	0.12
30b	(+) CTV-[GG-$(POG)_5$-$NH_2]_3$	1243.5 $[M+2Na+2H]^{4+}$	1243.5	58	0.11
21	KTA-[GG-$(POG)_5$-$NH_2]_3$	1151.8 $[M+4H]^{4+}$	1152.5	62	0.14

[a] Melting temperatures (T_m) were determined in 10 mM AcOH at a concentration of 0.5 mg mL^{-1}. R_{pn}: Ratio of the positive value (at 223 or 224 nm, see text) over the negative value (at ≈200 nm). [b] Same measurements in H_2O instead of AcOH. n.d. : not determined.

for the CTV-assembled peptide is 19.3 °C and 20.3 °C for the KTA-assembled compound, both measured in 10 mM HOAc (Figure 1). The melting temperatures in H_2O are 23.0 and 21.4 °C, respectively. In contrast, the acetylated single chain peptide did not adopt a triple helical conformation at 5 °C, since no thermal transition could be measured; this indicates that the presence of other amino acids—as are present in this native sequence—than those of the POG repeats (see below) hamper the formation of triple helices.

During the HPLC purification of CTV[$CH_2C(O)N(H)$Gly-[Pro-Hyp-Gly]$_2$-Phe-Hyp-Gly-Glu-Arg-Gly-Val-Glu-Gly-[Pro-Hyp-Gly]$_2$-$NH_2]_3$ (**14**) two peaks with equal mass eluting very close to each other could be separated. CD analysis at 5 °C of both compounds revealed that only one of the products had the expected triple helical conformation, whereas the other product did not. The CD spectrum of this latter product resembled the CD spectrum of the acetylated single chain peptide, and no temperature-dependent transition could be measured (Figure 2). Since the CTV triacid scaffold that we used was a racemic mixture of (+) and (−)-CTV, we most likely separated the diastereomeric collagen mimics originating from the two CTV enantiomers. Apparently, there is a difference in assisting the correct folding of the collagen peptide into a triple helix between the two CTV enantiomers. The orientation of the side chains of the CTV core is for one enantiomer presumably more favorable for adopting the right-handed triple helix conformation.

To further explore the different stabilizing properties of the two CTV enantiomers, as well as the difference in stabilizing properties between the CTV triacid and KTA(Gly-OH)$_3$ scaffolds, we synthesized collagen model peptides H-Gly-(Pro-Hyp-Gly)$_5$-NH_2 (**19**) and H-Gly-Gly-(Pro-Hyp-Gly)$_5$-

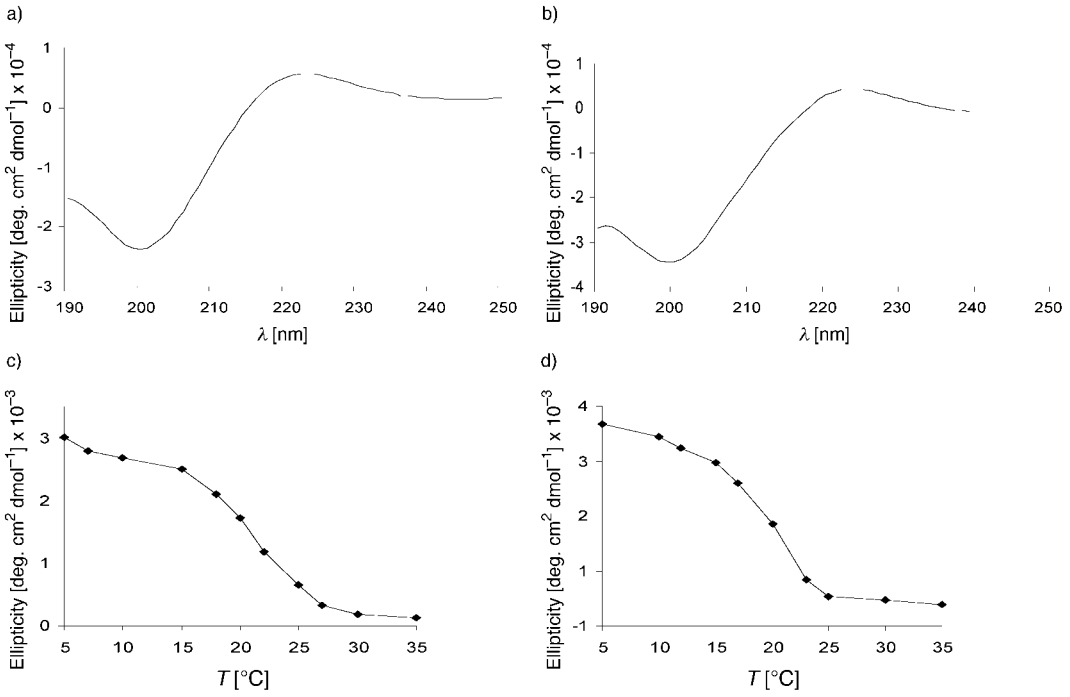


Figure 1. A) CD spectrum of KTA[GG$(POG)_2$FOGERGVEG$(POG)_2$-$NH_2]_3$ (**15**), at a concentration of 0.5 mg mL^{-1} in 10 mM HOAc at 5 °C. B) CD spectrum of CTV[G$(POG)_2$FOGERGVEG$(POG)_2$-$NH_2]_3$ (**14**), at a concentration of 0.5 mg mL^{-1} in 10 mM HOAc at 5 °C. C) Melting temperature measurement of KTA[GG$(POG)_2$FOGERGVEG$(POG)_2$-$NH_2]_3$ (**15**). D) Melting temperature measurement of CTV[G$(POG)_2$FOGERGVEG$(POG)_2$-$NH_2]_3$ (**14**).

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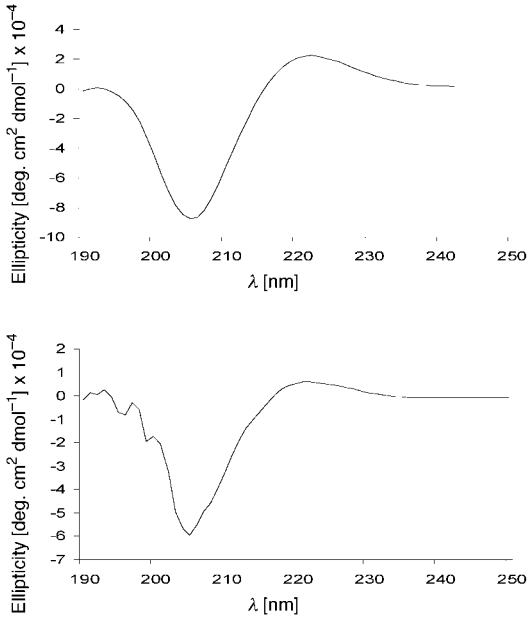
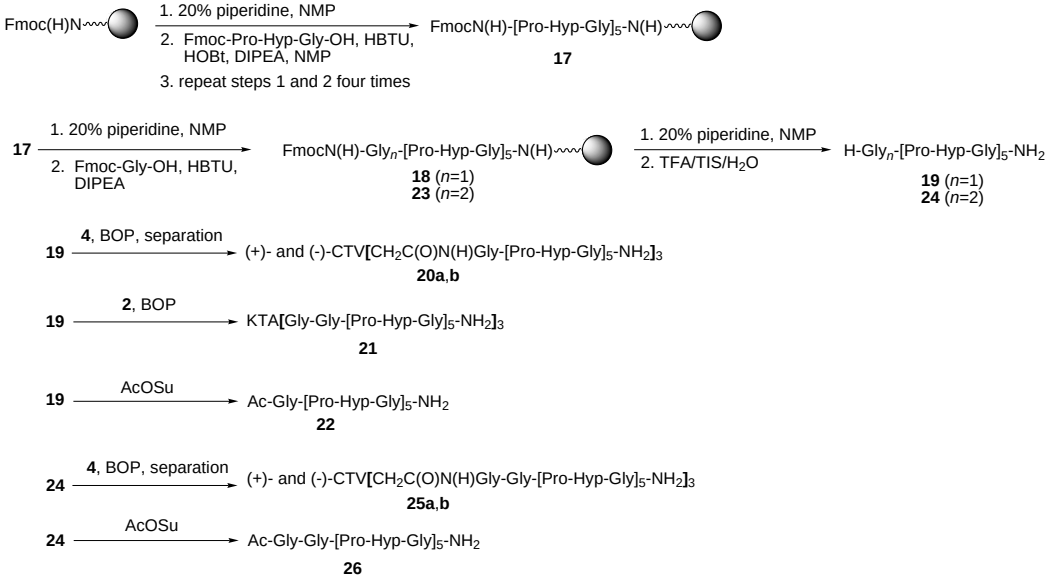


Figure 2. CD spectrum of (−)CTV[G(POG)$_2$FOGERGVEG(POG)$_2$-NH$_2$]$_3$, at a concentration of 0.5 mg mL^{-1} in 10 mM HOAc at 5 °C (top). CD spectrum of Ac-(POG)$_2$FOGERGVEG(POG)$_2$-NH$_2$, at a concentration of 0.5 mg mL^{-1} in 10 mM HOAc at 5 °C (bottom).

NH$_2$ (**24**) (Scheme 4, Table 1). The model peptides were successfully coupled in solution to racemic CTV triacid using BOP to give **20ab** and **25ab**, respectively. KTA(Gly-OH)$_3$ was coupled to the model peptide H-Gly-(Pro-Hyp-Gly)$_5$-NH$_2$ to give **21**. The influence of the spacer length (Gly and Gly-Gly) between the CTV triacid scaffold and the peptide was studied. A certain degree of flexibility of the spacer is needed to allow for one residue staggering of the three peptide chains, which is necessary for a triple helical formation. After synthesis of CTV[Gly-(Pro-Hyp-Gly)$_5$-NH$_2$]$_3$, starting from racemic CTV triacid, both diastereomers could be separated by RP-HPLC. A comparison of the CD data of the most stable CTV[Gly-Gly(Pro-Hyp-Gly)$_5$-NH$_2$]$_3$ molecule and the KTA-assembled equivalent (KTA[Gly-Gly-(Pro-Hyp-Gly)$_5$-NH$_2$]$_3$), revealed that, alike the native collagen peptide attached to these scaffolds, the thermal stability of both compounds measured in water is almost equal (i.e., $T_m = 58$ and 62 °C, respectively). The other, slightly less stable CTV[Gly-Gly(Pro-Hyp-Gly)$_5$-NH$_2$]$_3$ structure **25a** or **25b**, did also show a temperature-dependent transition, which was somewhat lower ($T_m =$ 55 °C). A shortening of the spacer to only one glycine residue, caused a marked decrease in the thermal stability of the CTV-assembled peptides. The T_m of the most stable CTV[Gly(Pro-Hyp-Gly)$_5$-NH$_2$]$_3$ decreased to 50 °C, (Table 1) whereas the T_m of the other diastereomer dropped to 37 °C (Table 1). All these thermal transitions were fully reversible upon cooling to 4 °C.

To determine the absolute configuration of the CTV enantiomer, present in the two CTV diastereomers, it was decided to couple the diastereomers separately to the model peptide. The required CTV enantiomers were obtained by resolution of the racemic CTV triol **3**, which was achieved by chromatographic separation of the camphanic acid triesters of CTV **27** as described by Canceill et al.[14, 15] The optically active CTV triols **28a** and **28b** were converted into the corresponding triacid scaffolds **29a** and **29b**, respectively, as depicted in Scheme 5.

After coupling of the chiral CTV templates to H-Gly-Gly-(Pro-Hyp-Gly)$_5$-NH$_2$ leading to **30a** and **30b** (Figures 3 and Figure 4), respectively, and concomitant CD analysis, the melting temperatures were determined. As expected, the T_m values did match perfectly the T_m values of the compounds that were purified from the mixture of diastereomers. Based upon these data we could assign (+)-CTV as the best triple helix stabilizing CTV triacid scaffold leading to collagen mimic **30b**, with a T_m of 58 °C (Table 1). The (−)-CTV assembled peptide in collagen mimic **30a** showed a T_m of 55 °C (Table 1). The orientation of the side chains of (+)-CTV seem to accomodate the right-handed folding of the superhelix the best. The orientation of the side chains of (−)-CTV might be less favorable, and at least two glycine residues were



Scheme 4. Synthesis of the collagen peptides containing the model peptides G$_n$(POG)$_5$.

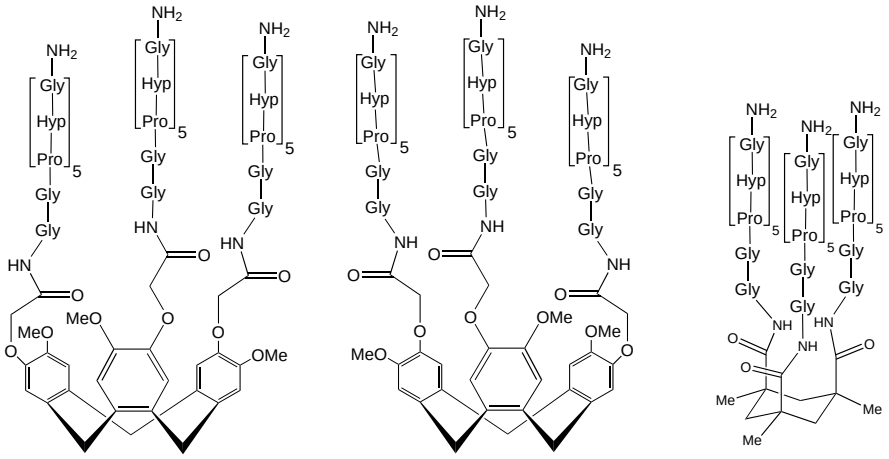


Figure 3. (−)-CTV[Gly-Gly-(Pro-Hyp-Gly)$_5$-NH$_2$]$_3$ (**30a**), (+)-CTV[Gly-Gly-(Pro-Hyp-Gly)$_5$-NH$_2$]$_3$ (**30b**) and KTA[Gly-Gly-(Pro-Hyp-Gly)$_5$-NH$_2$]$_3$.

needed to allow the three assembled peptides to fold into a right-handed triple helix.

Discussion

A synthetic collagen mimic can serve as a valuable tool to study the interaction of collagens with other proteins in more detail at the molecular level. Therefore, a synthetic protocol to synthesize small and stable native collagen structures was developed. Using one new and one known scaffold molecule, small native collagen sequences were induced to fold into a triple helical conformation. This was accomplished by assembly of three collagen peptides on triacid scaffold structures and extension of the native sequence with POG repeats. The peptides were prepared on the solid phase by an orthogonal synthesis strategy. Cleavage from the resin left the carboxylic

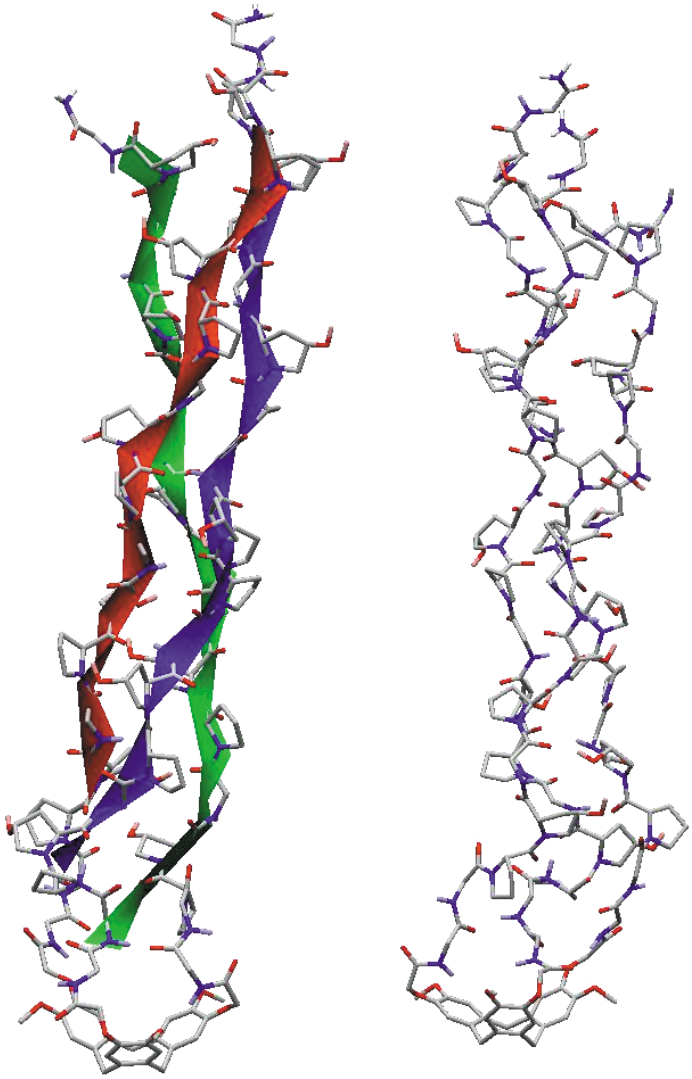
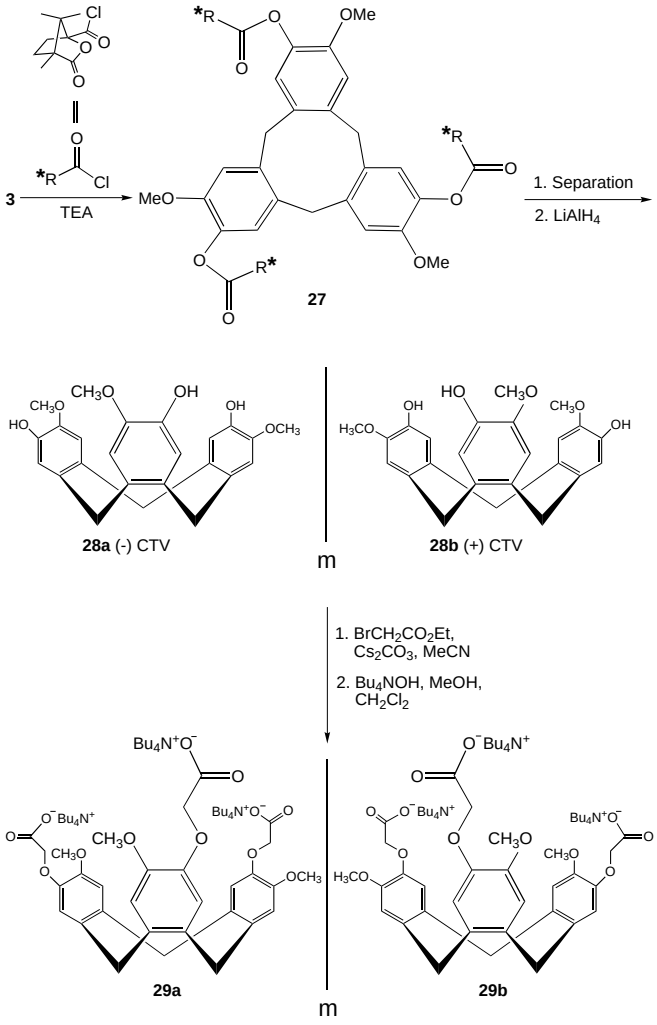


Figure 4. Molecular models constructed using MacroModel[29] of (−)-CTV[Gly-Gly-(Pro-Hyp-Gly)$_5$-NH$_2$]$_3$ (**30a**, left), (+)-CTV[Gly-Gly-(Pro-Hyp-Gly)$_5$-NH$_2$]$_3$ (**30b**, right) clearly showing the triple helix structure.



Scheme 5. Optical Resolution of the CTV triacid scaffold.

0947-6539/02/0820-4618 $ 20.00+.50/0 *Chem. Eur. J.* **2002**, *8*, No. 20

acid moieties of the amino acid side chains protected. This enabled coupling of the amino-terminus to the triacid scaffolds.

The necessity for stabilization of collagen peptides containing native sequences is mostly due to the destabilizing effect of non-imino acids on the collagen triple helix. As was illustrated by studies of the group of Brodsky,[17] replacement of two imino acids in a POG multimer by two non-imino acids residues, commonly found in the native collagen molecule, resulted in a dramatic destabilization of the triple helix.

The idea of an organized assembly of three peptide chains to increase the stability of the collagen triple helix has been described by several groups. A strong reduction of entropy loss involved in triple helix formation is thought to be the driving force for the increased triple helical stability. Assembly has been achieved using a template structure such as the Kemp's triacid, the tripodal amine TREN or di-amino acids.[7, 18–21] Tanaka et al.[20] constructed a collagen molecule by crosslinking both the C- and N-terminus by a Lys-Lys dimer template. Alternatively, the three peptides can be assembled by disulfide bridges, which also allowed assembly of heterotrimeric constructs.[22] Directed self-assembly, without covalent linkages between the peptides, has been achieved by peptide amphiphiles.[23]

A comparison of the various stabilizing strategies is difficult, since no consensus sequences were used. In this study we showed the stabilizing properties of a new triacid scaffold, compared with the known Kemp's triacid, using two different kinds of collagen peptides. One peptide we used contains the $\alpha_2\beta_1$ integrin recognition sequence GFOGERGVE (residues 502–510 of human type I collagen α1(I) chain[24]). The other peptides were composed of POG repeating units. The high imino acid content in these peptides resulted in a different handedness (seven-fold symmetry) of the helix compared with the natural collagen molecule (ten-fold symmetry).[25] Both scaffolds significantly increased the T_m of the triple helical structures, compared with non-assembled peptides (Table 1). The KTA(G-OH)$_3$ was already used for studying the structure and stability of collagen and collagen-like triple helices.[7] The CTV triacid scaffold, which has C_3 symmetry, has not been used before to induce collagen structures. Our data demonstrated that the CTV triacid can indeed stabilize the triple helical folding of a collagen peptide. This was shown by CD melting curves of the triple helical structure of either an assembled peptide containing a native collagen motif, or an assembled collagen model peptide. Due to the different eluting properties of the two CTV diastereomers after coupling to the peptides, we were able to study the effect of each of both diastereomers.

Our data showed that (−)-CTV was less potent than (+)-CTV in assisting the correct folding of the collagen peptides. In case of the native collagen motif, no folding into a triple helical structure could be measured for (−)-CTV. However, due to the high thermal stability of the triple helix of the model peptide H-G$_n$(POG)$_5$-NH$_2$ ($n = 1,2$), a triple helical structure of (−)-CTV could be measured (Table 1). The remarkable difference in stability of the CTV triacid diastereomers coupled to either H-G(POG)$_5$-NH$_2$ or H-GG(POG)$_5$-NH$_2$ illustrated the necessity for a certain degree of flexibility to allow the correct folding. The different stabilizing properties of the two CTV diastereomers may be explained by the orientation of the side chains of the CTV cone, which probably point in the opposite direction. A certain flexibility is therefore likely necessary for both one amino acid residue staggering and right-handed folding of the triple helix. This was also shown by comparing the stability of the two CTV triacid scaffolds coupled to H-G(POG)$_5$-NH$_2$. In case of (−)-CTV coupled to H-G(POG)$_5$-NH$_2$, the melting temperature was 37 °C compared with 50 °C for the other CTV diastereomer (Table 1). If the flexibility was limited to one glycine, the first residues of the peptide will probably have to function as the spacer to compensate for the less ideal orientation of the side chains. Since the difference in T_m for the two CTV[G(POG)$_5$-NH$_2$]$_3$ diastereomers is significantly larger than for both CTV[GG(POG)$_5$-NH$_2$]$_3$ diastereomers, the two glycines can probably also compensate partly for the less ideal orientation of the side chains of (−)-CTV in order to induce the right-handed superhelix formation. The large difference in T_m for the two CTV-collagen derived (CTV[G(POG)$_5$-NH$_2$]$_3$) peptides also correlates with the large difference in thermal stability found for CTV[G(POG)$_2$FOGERGVEG(POG)$_2$-NH$_2$]$_3$.

The two peptides used in this study differ also in their triple helical twist. High resolution crystal structures of collagen peptides show that peptides composed of repetitive POG triplets generate a seven-fold (7_5) symmetry, whereas the native collagen peptides, possessing imino-acid-poor regions, generate a ten-fold (10_7) symmetry.[25, 28] This sequence dependent helical twisting may thus also effect our conclusions, since we compare the triple helical folding of both a native sequence and an imino-acid-rich peptide G$_n$(POG)$_5$. However, since we only observe a small difference in thermal stability between the KTA(G-OH)$_3$ template and the (+)-CTV triacid scaffold, it is not expected that the helical twisting effects the stability data.

Comparison of the structures of the two triacid scaffolds showed that the CTV template is much larger. The distance between the three CTV-cone side chains is approximately 8–10 Å. For KTA the distance between these three functionalities is much shorter and more close to the interchain distances of the three peptides in the triple helix (≈3 Å). This could explain that for the CTV triacid as a scaffold, the full length of the G$_2$ spacer was necessary for a proper folding of the whole peptide into a triple helix.

The moderate to low yields of the collagen mimics were mostly due to difficult RP-HPLC purifications. HPLC purification of collagen structures can be difficult, as was also shown by others. RP-HPLC of such structures can result in peak broadening resulting from aggregation or conformational effects.[26] Especially collagen structures composed of POG repeats are prone to partial denaturation by the HPLC system.[27] It was shown that conformations may differ at different HPLC conditions. In our case, the collagen structures containing the native sequences could be purified somewhat easier by RP-HPLC than the collagen structures composed of only POG repeats. The latter structures gave broad peaks. In addition, purification of collagen mimics which were synthesized from the racemic CTV triacid scaffold

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was even more difficult, due to the different retention times of the two CTV diastereomers.

Conclusion

We have introduced a new chiral scaffold that induces the folding of collagen peptides into a triple helical structure. Although both enantiomers are capable of folding collagen peptides into a triple helix, CD data shows that the (+)-CTV enantiomer is a better triple helix-stabilizing scaffold than (−)-CTV. In addition to POG repeats, the CTV scaffold was also effective in stabilizing the triple helix of *native* collagen sequences, comparable to the ability of KTA. The latter was demonstrated for the first time in this paper. Thus, the CTV scaffold seems a valuable addition to the presently available limited number of rigid scaffolds, which can be used for the organization of large peptide sequences as is described here as well as smaller peptides.[30]

Experimental Section

Synthesis of Fmoc-POG-OH (9)

Boc-Pro-Hyp-OMe (6): HCl·H-Hyp-OMe (3.9 g; 26.9 mmol, 1.05 equiv) was dissolved in dry DMF (90 mL), and the solution was cooled to 0 °C. Subsequently Et_3N (11.6 mL; 83.3 mmol), Boc-Pro-OH (5.5 g; 25.6 mmol) and BOP (11.3 g; 25.6 mmol) were added. After 4 h, the cooling bath was removed, and the mixture was stirred for 2 d at RT. Next, the mixture was concentrated in vacuo, and the residue was dissolved in EtOAc, and was washed once (successive washing with aqueous solutions resulted in severe loss of product, due to the aqueous solubility of this compound) with 5% $KHSO_4$, 5% $NaHCO_3$ and brine. The product was purified by column chromatography (EtOAc) to give Boc-Pro-Hyp-OMe as a colorless oil (6.0 g, 68%). $[\alpha]_D = -109.5$ ($c = 1$, MeOH) [lit.:[10] −112.6 ($c = 0.8$, MeOH); $R_f = 0.15$ (EtOAc); ^{1}H NMR ($CDCl_3$, 300 MHz): $\delta = 1.40$, 1.43 (2 × s, 9H, Boc), 1.79–2.58 (m, 6H, Pro-γ, Pro-β, Hyp-β), 3.35–3.82, 4.00, 4.04, 4.20–4.71 (m, 7H, Pro-δ, Pro-α, Hyp-δ, Hyp-α, Hyp-γ), 3.72 (s, 3H, OMe); ^{13}C NMR ($CDCl_3$, 75 MHz): $\delta = 24.00$, 28.29, 29.03, 37.30, 46.81, 52.01, 54.53, 57.33, 57.39, 70.11, 79.84, 154.62, 171.28, 172.79.

Boc-Pro-Hyp-OH (7): Boc-Pro-Hyp-OMe (6.0 g; 18.3 mmol) was dissolved in dioxane/MeOH/4N NaOH (14:5:1 *v*/*v*/*v*) and the mixture was stirred for 16 hours at RT. The mixture was concentrated in vacuo and the residue was dissolved in H_2O. The aqueous solution was acidified to pH 2 with 2N $KHSO_4$, followed by successive extractions with EtOAc. After removal of the solvents in vacuo, Boc-Pro-Hyp-OH was obtained as a white foam (4.6 g, 80%). $[\alpha]_D = -66.2$ ($c = 1$, $CHCl_3$); $R_f = 0.10$ (CH_2Cl_2/MeOH 9:1); ^{1}H NMR ($CDCl_3$, 300 MHz): $\delta = 1.39$, 1.43 (2 × s, 9H, Boc), 1.76–2.40 (m, 6H, Pro-γ, Pro-β, Hyp-β), 3.34–3.91 (m, 4H, Pro-δ, Hyp-δ), 4.36–4.72 (m, 3H, Pro-α, Hyp-α, Hyp-γ); ^{13}C NMR ($CDCl_3$, 75 MHz): $\delta = 24.08$, 28.36, 29.11, 36.82, 46.99, 54.75, 57.61, 58.16, 70.1, 80.51, 154.98, 172.47, 173.81; ES-MS: m/z: calcd for $C_{15}H_{24}N_2O_6 + Na^+$: 350.2; found: 351.1.

Boc-Pro-Hyp-Gly-OMe (8): Boc-Pro-Hyp-OH (4.6 g; 14.0 mmol), HCl·H-Gly-OMe (2.0 g; 15.9 mmol) and HOBt·H_2O (2.3 g; 15.0 mmol) were dissolved in DMF (50 mL). Subsequently, DIPEA (2.7 mL) was added, and the mixture was cooled on ice. EDCI (3.0 g; 15.6 mmol) was then added, and after cooling for another 4 h the solution was stirred at room temperature overnight. The solvents were then removed in vacuo, and the residue was suspended in CH_2Cl_2 (350 mL) and filtered. The CH_2Cl_2 solution was washed twice with 5% $NaHCO_3$ (10 mL). The organic layer was dried over anhydrous Na_2SO_4. After removal of the solvent in vacuo, the residue was purified by column chromatography (CH_2Cl_2/MeOH, gradient from 2% to 5% MeOH) to give Boc-Pro-Hyp-Gly-OMe as a white foam (5.1 g, 90%). $R_f = 0.22$ (CH_2Cl_2/MeOH 9:1 *v*/*v*); ^{1}H NMR ($CDCl_3$, 300 MHz): $\delta = 1.40$, 1.44 (2 × s, 9H, Boc), 1.76–2.54 (m, 6H, Pro-γ, Pro-β, Hyp-β), 3.36–4.80 (m, 9H, Gly-α, Pro-δ, Hyp-δ, Pro-α, Hyp-α, Hyp-γ), 3.69, 3.70 (2 × s, 3H, OMe), 7.76, 8.50 (2 × m, 1H, Gly-NH); ^{13}C NMR ($CDCl_3$, 75 MHz): $\delta = 24.01$, 28.20, 29.15, 36.22, 40.91, 46.79, 54.69, 51.86, 57.94, 58.35, 70.19, 79.84, 154.80, 170.15, 172.01, 172.39; ES-MS: m/z: calcd for $C_{18}H_{29}N_3O_7 + Na^+$: 421.2; found: 422.4.

Fmoc-Pro-Hyp-Gly-OH (9): Boc-Pro-Hyp-Gly-OMe (5.1 g; 10.1 mmol) was dissolved in 1N HCl (100 mL), and the mixture was stirred for 3 d. Subsequently, the mixture was concentrated in vacuo, and the residue was dissolved in CH_3CN/H_2O (80 mL, 1:1 *v*/*v*). The pH was adjusted to pH 9–9.5 using TEA. A solution of of Fmoc-OSu (4.3 g; 12.7 mmol) in CH_3CN (75 mL) was then added in one portion. Stirring was continued for 1 h, and the pH of the solution was maintained at pH 8.5–9. Subsequently 2N HCl was added until pH 7.5, and the solvents were removed in vacuo. The residue was dissolved in 2% $NaHCO_3$ and was washed three times with EtOAc. The aqueous phase was acidified to pH 3 with 2N HCl, and the mixture was stored overnight at 4 °C, after which a white precipitate was formed. The precipitate was dried, and crystallized from hot CH_3CN to give Fmoc-Pro-Hyp-Gly-OH (4.03 g, 63%). M.p. 179–182 °C (lit.:[11] 177–181 °C); $R_f = 0.19$ ($CHCl_3$/MeOH/AcOH 40:10:1 *v*/*v*/*v*); RP-HPLC (C18, 300 Å, gradient of 10–60% B in 50 min): $t_R = 29$ min; ES-MS: m/z: calcd for $C_{27}H_{29}N_3O_7 + H^+$: 508.54; found: 508.55, 530.35 $[M+Na]^+$.

Synthesis and analysis of linear peptides: All peptides were synthesized as C-terminal amides by solid phase peptide synthesis on an ABI 433A peptide synthesizer using Argogel Rink-amide resin and the Fastmoc 0.25 mmol protocol. All amino acids were obtained from Alexis or Novabiochem and are of the L-configuration. Removal of the Fmoc group was monitored at 301 nm. Sequences of Pro-Hyp-Gly were introduced by segment condensation using 2 equiv of Fmoc-Pro-Hyp-Gly-OH. Cleavage of the peptide from the resin was carried out with TFA/TIS/H_2O (92.5:2.5:5 *v*/*v*/*v*). To obtain the acetylated compounds, the cleaved peptide was treated with 1.5 equiv of AcOSu in DMF/DIPEA. Removal of the allyl protection group was performed in solution by treatment for 72 h with $[Pd(PPh_3)_4]$ and DMF/AcOH/NMM (50:10:1 *v*/*v*/*v*) under Ar atmosphere,[12] followed by applying the reaction mixture to a PD10 (sephadex G25) gel filtration column. All peptides were purified by preparative RP-HPLC (C18, 300 Å), using a gradient from A (0.1% TFA in H_2O) to 80% B (0.085% TFA in 95% CH_3CN/H_2O). Analysis was performed by ES or MALDI-TOF MS.

Purification and analysis of assembled peptides: After coupling of the peptides to the triacid scaffolds, the reaction mixture was dissolved in H_2O/CH_3CN (4:1 *v*/*v*) and was dialyzed (membrane cutoff 3500) against H_2O (3 × 2 L). Allyl protection groups of the assembled peptides were removed by treatment for 72 h with $[Pd(PPh_3P)_4]$ as described above. Final purification was performed by preparative RP-HPLC (C18, 300 Å), using a gradient from A (0.1% TFA in H_2O) to 60% B (0.085% TFA in 95% CH_3CN/H_2O). Analysis was performed by ES or MALDI-TOF MS.

CD spectroscopy: Spectra were recorded on a OLIS spectropolarimeter using a 0.01 mm pathlength quartz cuvette. The cuvette was placed in a thermally controlable holder. Samples were dissolved in H_2O or in 10 mM AcOH at a concentration of 0.5 mg mL^{-1} and were stored at 4 °C at least 24 h prior to recording the measurements. Spectra were obtained by averaging 10 scans. Melting temperatures were obtained by data collection of the positive maximum at 220–225 nm at a heating rate of 0.25 °C min^{-1} and 15 min equilibration at the temperature of data collection.

(−)-2,7,12-Trimethoxy-3,8,13-[tris(acetoxyethyl)oxy]-10,15-dihydro-5*H*-tribenzo[a,d,g]cyclononene: The synthesis of the cyclotriveratrylene triol (racemic mixture) and the optical resolution of this triol has been described by Canceill et al.[13, 14] The thus obtained (+)-triol (125 mg; 0.31 mmol) was dissolved in dry CH_3CN (30 mL), and the mixture was cooled to 4 °C. Subsequently Cs_2CO_3 (600 mg; 1.84 mmol) was added, and after stirring for 5 min bromoacetic ethyl ester (120 µL) was added. The mixture was allowed to warm to room temperature, and was stirred for 2 d. The solvent was removed without heating (to avoid racemisation of CTV) in vacuo, and the residue was dissolved in EtOAc and 1N HCl. The EtOAc layer was washed with brine and dried over anhydrous Na_2SO_4. After removal of the solvent in vacuo, again without heating, the residue was purified by column chromatography (CH_2Cl_2/Et_2O, gradient from 5 to 10% Et_2O) to give the desired compound (93 mg, 46%). $[\alpha]_D = -11°$ ($c = 1$, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 200 MHz): $\delta = 1.23$ (t, 9H), 3.52 (d, 3H), 3.88 (s, 9H, OCH_3), 4.19 (m,6H), 4.59 (d, 6H), 4.71 (d, 3H), 6.87 (d, 6H, arom H); ^{13}C NMR ($CDCl_3$, 75 MHz): $\delta = 14.08$, 36.33, 55.98, 61.08, 67.56, 113.74, 118.00, 131.42, 134.03, 145.94, 148.70, 169.40.

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(+)-2,7,12-Trimethoxy-3,8,13-[tris(acetoxyethyl)oxy]-10,15-dihydro-5*H*-tribenzo[a,d,g]cyclononene: The procedure was as described for the (−)-enantiomer. Yield: 70 mg (50%). $[\alpha]_D = +8^\circ$ ($c = 1$, $CHCl_3$); ^1H NMR ($CDCl_3$, 200 MHz): $\delta = 1.23$ (t, 9H), 3.52 (d, 3H), 3.88 (s, 9H, OCH_3), 4.19 (m, 6H), 4.59 (d, 6H), 4.70 (d, 3H), 6.87 (d, 6H, ar H); ^{13}C NMR ($CDCl_3$, 75 MHz): $\delta =$ 14.08, 36.33, 55.98, 61.08, 67.56, 113.74, 118.00, 131.42, 134.03, 145.94, 148.70, 169.40.

CTV[G(POG)$_2$FOGERGVEG(POG)$_2$-NH$_2$]$_3$: The lineair peptide H-G(POG)$_2$FOGE(OAll)RGVE(OAll)G(POG)$_2$-NH$_2$]$_3$ (25 mg, 12 μmol), which was prepared as desribed above, and the NBu_4 salt of CTV triacid[16] (2.33 mg; 4 μmol) were dissolved in DMF (0.75 mL). Subsequently Et_3N (7.5 μL) was added, and the mixture was cooled to 4 °C. After 5 min, BOP (9 mg; 20.4 μmol) in DMF (100 μL) was added, and the mixture was stirred at RT. After 48 h the mixture was concentrated in vacuo and the residue was dissolved in H_2O/CH_3CN (5 mL). After HPLC purification, the separate diastereomers (2 mg each) were obtained. ES-MS: *m*/*z*: calcd for $C_{303}H_{426}N_{78}O_{102} + 5H^+$: 1358.8; found: 1358.7 (diastereomer 1) and *m*/*z*: calcd for $C_{303}H_{426}N_{78}O_{102} + 4H^+$: 1698.3; found: 1698.4 (diastereomer 2). All other assembled peptides were synthesized as described above and are listed in Table 1

Acknowledgements

This project was supported by grant PL963517 of the European Commission.

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Received: April 22, 2002 [F4032]