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Application of Metal-Free Triazole Formation in the Synthesis of Cyclic RGD–DTPA Conjugates

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The tandem 1,3-dipolar cycloaddition-retro-Diels–Alder (tandem crDA) reaction is presented as a versatile method for metal-free chemoselective conjugation of a DTPA radiolabel to N-δ-azido-cyclo(-Arg-Gly-Asp-D-Phe-Orn-) *via oxanorbornadiene derivatives. To this end, the behavior of several trifluoromethyl-substituted oxanorbornadiene derivatives in the 1,3-dipolar cycloaddition was studied and optimized to give a clean and efficient method for bio-orthogonal ligation in an aqueous environment. After radioisotope treatment, the resulting ^{111}In-labeled c(RGD)-CF_3-triazole-DTPA conjugate was subjected to preliminary biological evaluation and showed high affinity for $\alpha_v\beta_3$ (IC_{50} = 192 nM) and favorable pharmacokinetics.*

Introduction

Functionalized cyclic RGD-containing peptides are of significant diagnostic and therapeutic interest because of their versatile application in both tumor targeting and tumor imaging. The major underlying mechanism responsible for the interest in such peptides is based on the binding affinity of the Arg-Gly-Asp (RGD) motif to integrin receptors, that is, alpha-beta heterodimeric cell-surface receptors which are overexpressed on developing capillary cells.[1,2] The interaction of integrins with specific matrix ligands is fundamental to invasion and formation of tumor-induced angiogenesis[3,4] and metastasis.[5] Based on the fact that the RGD sequence serves as a recurring motif for cell attachment in a large number of adhesive extracellular matrix, blood, and cell-surface proteins, a variety of RGD mimetics have been prepared for binding to $\alpha_v\beta_3$ integrin of the endothelium and tumor cells, leading to inhibition of cell-matrix interaction,[6] interruption of signal transmission,[7] disturbed cell migration,[8,9] and regression[10,11] or apoptosis[12,13] of tumor cells. Whereas linear and flexible RGD-containing peptides bind to a number of integrin receptor subtypes, including αIIbβ_3, $\alpha_v\beta_3$, and $\alpha_v\beta_5$,[14] the constrained cyclic RGD-containing peptides selectively bind to the $\alpha_v\beta_3$ subtype.[15] As a result, cyclic RGD derivatives have become an important therapeutic target for the diagnosis of various solid tumors. Various modifications have been introduced to cyclic RGD peptides to improve receptor binding affinity (for example, polyvalent constructs)[16,17,18] and pharmacokinetic modifiers (for example; polymer conjugates[19,20] and glycosylated derivatives[21,22]).

Cyclic pentapeptides are frequently used in tumor targeting[23] and imaging[24] as well as for stimulation of cell adhesion.[25] Similarly, the ε-amino group of the lysine residue in *cyclo*(-RGDfK-) is frequently used for modification with ligands for radiolabeling (^{18}F, ^{125}I, ^{64}Cu, ^{99m}Tc, ^{111}In)[26] or with other biologically relevant moieties.[27] However, conjugation strategies for chemoselective functionalization of cyclic RGD peptides with radiolabels or bioactive compounds are limited. Conjugation can be achieved with typical peptide coupling reagents,[28] or with other methods such as thioester or thioether coupling,[29] bromoacetyl derivatization,[30] or oxime ligation,[31] but most of the typical conjugation procedures suffer from poor selectivity or low yield. The synthesis of triazole-linked cyclic RGD-conjugates by copper-catalyzed, azide-alkyne cycloaddition has also recently been described.[18,22] Nevertheless, there still exists a strong demand for a bioconjugation reaction with increased efficiency and chemoselectivity involving mutually reactive conjugation partners that are synthetically readily accessible.[18] We recently reported on a copper-free tandem 1,3-dipolar cycloaddition–retro-Diels–Alder (tandem crDA) ligation, resulting in a stable [1,2,3]-triazole linkage.[32] The methodology was applied to ligate several bio(macro)molecules, under physiological conditions, including a linear RGD peptide. It appeared to us that the absence of copper during conjugation is particularly favorable for the introduction of ligands for radiolabeling such as DOTA or DTPA when metal-free conditions are crucial. For example, the tandem crDA paves the way for coupling of DTPA constructs to azido-functionalized cyclic RGD peptides resulting in conjugates for application in tumor targeting and imaging. In this paper, we wish to report on the efficient crDA-ligation of radiolabeled oxanorbornadiene-DTPA conjugates for functionalization of an *N*-δ-azido-derivative of

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cyclo(-Arg-Gly-Asp-D-Phe-Orn-). Additionally, the efficiency of the crDA approach was further enhanced and a preliminary biological evaluation of the radiolabeled DTPA-*c*RGD conjugates is described.

Results and Discussion

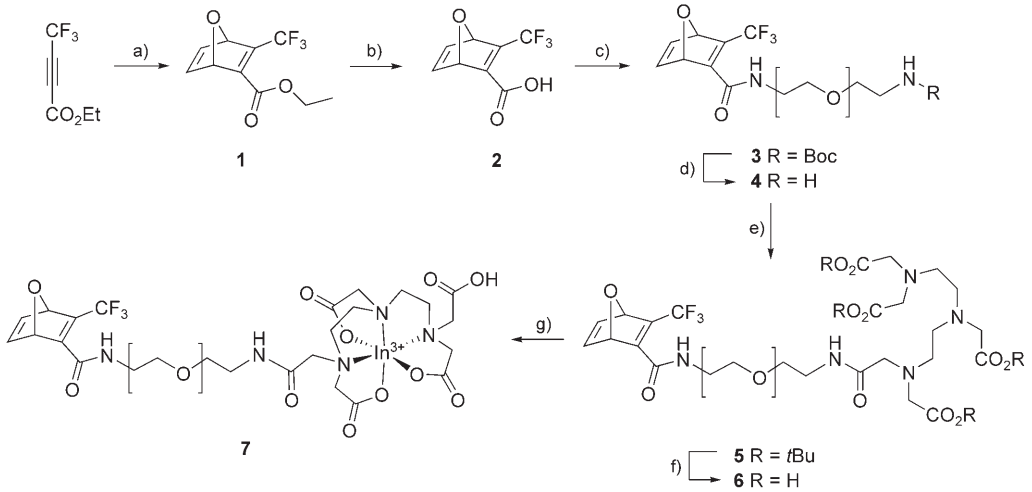
Synthesis

Our approach for the synthesis of the oxanorbornadiene-spacer-DTPA **6** is depicted in Scheme 1. Oxanorbornadiene **2** was prepared according to literature procedures[32,33] and was coupled to a short amino-modified ethylene glycol spacer. The particular ethylene glycol spacer was chosen, both for distancing the bulky diethylenetriaminepentaacetic acid (DTPA) ligand from the reactive oxanorbornadiene, and to potentially improve the pharmacokinetic characteristics of the conjugate.[19b,34] Secondly, as a result of the amino-modification of the spacer, an amide-linked conjugate **3** was obtained and was projected to display greater stability over the earlier reported esters under physiological conditions. After Boc-deprotection of compound **3**, the resulting free amine **4** was coupled to tetra-*O*-*tert*-butyl protected DTPA to give compound **5** in a reasonable yield (64%). Finally, the desired oxanorbornadiene-DTPA conjugate (oxanor-DTPA, **6**) was obtained in a sluggish reaction (four days) in quantitative yield after removal of the *tert*-butyl groups with concentrated TFA in CH_2Cl_2. Next, our attention was focused on the synthesis of *N*-δ-azido-*cyclo*(-Arg-Gly-Asp-D-Phe-Orn-) **12** (Scheme 2). Haubner et al.[35] demonstrated that substitution of the valine residue in the parent peptide *c*(-RGDfV-) for a lysine residue did not affect the activity or selectivity, enabling this position for any anchoring without interference with biological activity.[36] Consequently, the valine residue was substituted for an azide-functionalized ornithine derivative. *N*-δ-azido-Fmoc-L-ornithine (**8**) was prepared by diazotransfer reaction on Fmoc-L-Orn-OH and *N*-α-Fmoc-D-phenylalanine (**9**) was synthesized by Fmoc-protection of H-D-Phe-OH in an excellent yield (91%). The linear pentapeptide was synthesized using Fmoc-based solid-phase peptide synthesis (SPSS) on a trityl resin according to a modified literature procedure.[31] Cleavage of the protected peptide from the resin gave *N*-δ-azido-(Arg(PMC)-Gly-Asp(O*t*Bu)-D-Phe-Orn-OH) (**10**) in an overall yield of 29%. Subsequent cyclization under the influence of diphenylphosphorylazide (DPPA) followed by crystallization resulted in the desired *N*-δ-azido-*cyclo*(-Arg(PMC)-Gly-Asp(O*t*Bu)-D-Phe-Orn-) (**11**) in an excellent yield (87%). In the final step, *N*-δ-azido-*cyclo*(-Arg-Gly-Asp-D-Phe-Orn-) (**12**) was obtained by deprotection of PMC and *tert*-butyl protecting groups in concentrated trifluoroacetic acid (TFA). Characterization by ^{1}H NMR and LCMS analysis showed that the desired product was obtained with a purity of more than 97%.

Conjugation studies

The conjugation behavior of the cyclic RGD peptide with DTPA-functionalized oxanorbornadiene in the tandem crDA was studied by means of ^{1}H NMR spectroscopy, LCMS analysis, and HPLC with radiolabel detection. Monitoring the reaction by NMR spectroscopy was done by dissolving oxanor-DTPA (**6**) in a mixture of D_2O and CD_3OD which was subsequently added to a slight excess of cyclic RGD peptide **12** (1.5 equiv), leading to a final concentration of 6.6 μM. Under these conditions, 1,3-dipolar cycloaddition retro-Diels–Alder-reaction of **6** and **12** was expected to lead, via furan elimination, to triazole adduct **13** (Scheme 3). Compound **13**, although depicted as a single compound in Scheme 3, was expected to be formed as a mixture of regioisomeric products $\mathbf{A_1}$ and $\mathbf{A_2}$ as a result of the lack of regioselectivity in the first step of the crDA process, that is, the 1,3-dipolar cycloaddition (Scheme 4).

The reaction mixture was placed in a 400 MHz NMR-apparatus and conversion was monitored by integration of the furan protons with respect to the disappearing bridgehead protons of the oxanorbornadiene. First of all, the reaction was found to proceed rather slowly, requiring four days for complete disap-



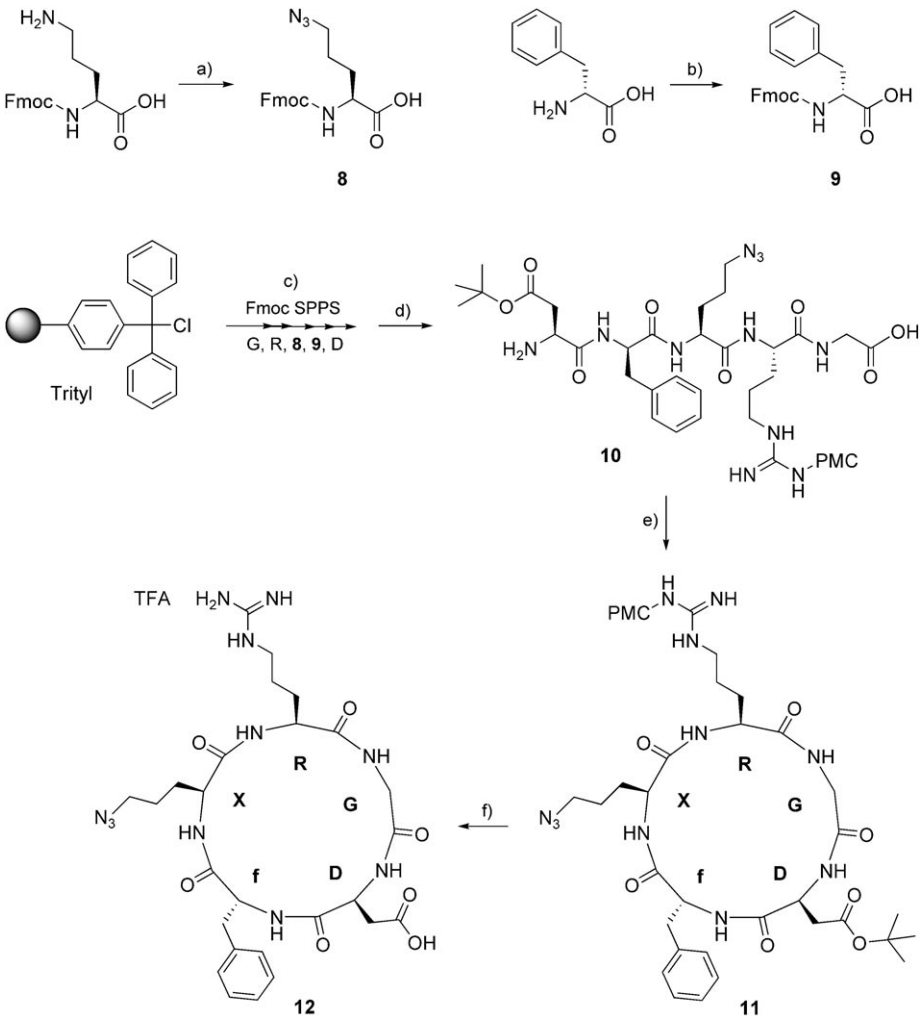
Scheme 1. a) and b) Literature procedures[32] (60% over 2 steps); c) 1-*N*-Boc-3,6-dioxa-8-octane-1,8-diamine, EDC, DMAP, DMF (64%); d) TFA, CH_2Cl_2 (99%); e) tetra-*O*-*tert*-Bu-DTPA, EDC, DMAP, CH_2Cl_2 (81%); f) TFA, CH_2Cl_2 (99%); g) NH_4OAc buffer (pH 5.5), $InCl_3$.

pearance of **6**. After this time, LCMS analysis (UV detection of the RGD motif) of the reaction mixture (Figure 1 A) revealed two signals for the desired triazole-containing conjugate **13** as a mixture of two regioisomers (55.5%, $\mathbf{A_1}$ and $\mathbf{A_2}$ ratio 1:1.5), along with residual cyclic RGD peptide (initially 1.5 equiv, found; 34.2%, Figure 1 B). Surprisingly, a third product was also observed (10.1%) and based on mass spectral analysis the structure was assigned to a 1*H*-1,2,3-triazole product **B** (R^2 = H, Scheme 4) resulting from undesired cycloaddition on the unsubstituted double bond of the oxanorbonadiene moiety, followed by CF_3-substituted furan elimination by retro-Diels–Alder reaction.

In addition to analysis by NMR spectroscopy and LCMS, HPLC analysis with radiolabel detection was performed to confirm complete substrate conversion. Monitoring the reaction by radiolabel detection required a radiolabeled DTPA-complex. ^{111}In was selected as a suitable radiolabel because of its long half-life (^{111}In, $t_{1/2}$ = 67.2 h) and its stability in the DTPA chelate. Thus, the oxanor-DTPA conjugate (**6**) was labeled with ^{111}In by addition of approximately 100 μCi $^{111}InCl_3$ to a solution of **6** in a metal-free NH_4OAc buffer (pH 5.5). The mixture was incubated for one hour at room temperature after which the ^{111}In-oxanor-DTPA complex (**7**, Scheme 1) was formed, concluded by a radiochemical purity check using an HPLC fitted with an in-line NaI detector (Figure 1 D). A total volume of 50 μL of the ^{111}In-labeled oxanor-DTPA complex (ca. 10 μCi) was used to perform the tandem crDA reaction with *N*-δ-azido-*cyclo*(-Arg-Gly-Asp-D-Phe-Orn-) (**12**).

Figure 1 E clearly shows the conversion of ^{111}In-oxanor-DTPA (**7**) into the two isomers of *c*(RGD)-CF_3-triazole-DTPA conjugate **13** (marked with *). In correspondence with LCMS analysis, approximately 14% of the undesired ^{111}In-DTPA-labeled CF_3-furan formed concomitantly with product **B** was also detected.

In order to suppress cycloaddition on the unsubstituted side of the amide-oxanorbornadiene system, optimization of the tandem crDA process was undertaken. To this end, a small series of methyl-substitut-



Scheme 2. a) TfN_3, $CuSO_4$, H_2O (64%); b) FmocCl, DiPEA, 1,4-dioxane (91%); c) Fmoc-based SPPS; d) TFA, TIPS, H_2O (90:4.75:4.75) (29% over two steps); e) DPPA, $NaHCO_3$, DMF (87%); f) TFA, CH_2Cl_2 (93%).

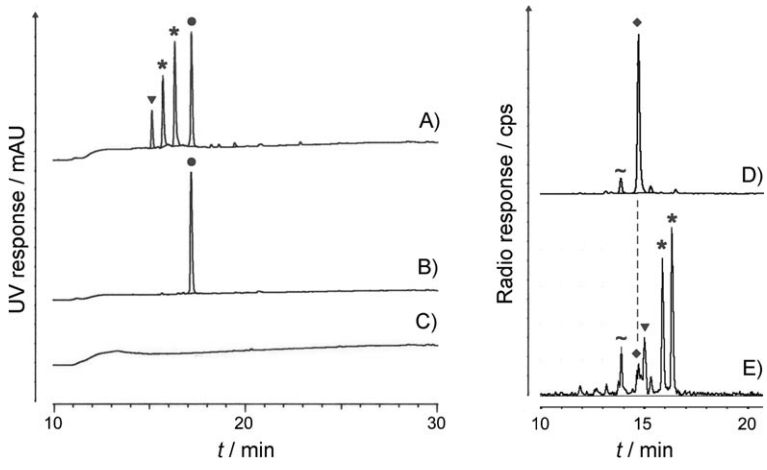
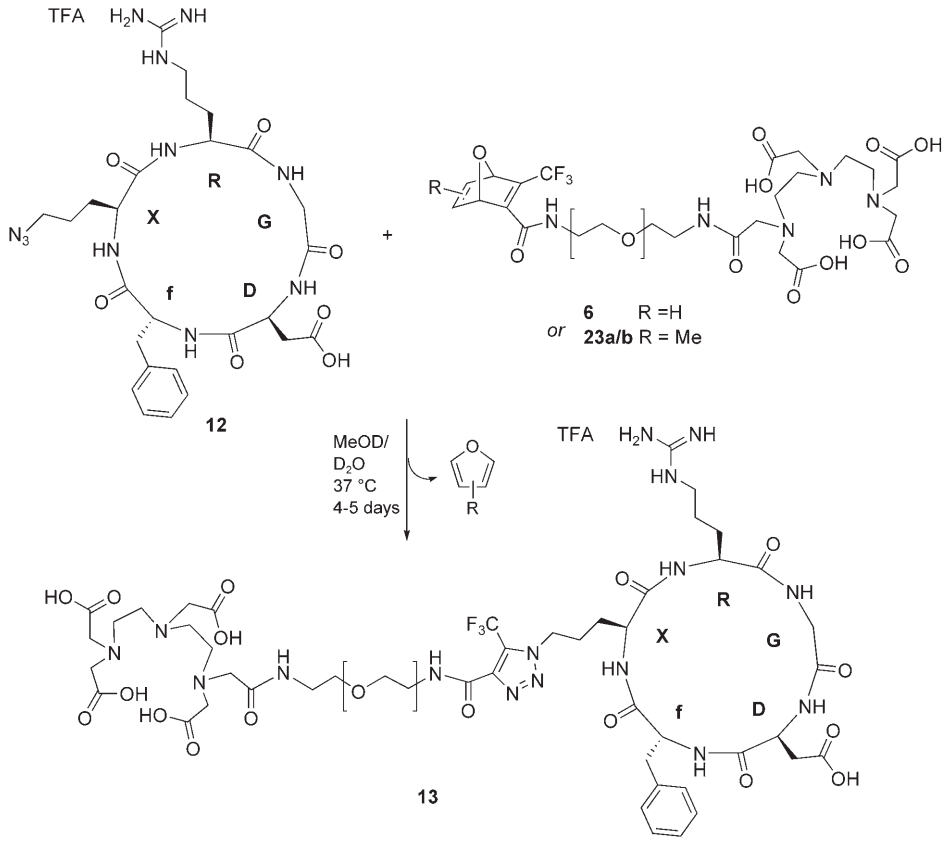


Figure 1. LCMS chromatograms; A = crude crDA reaction of **12** and **6** after four days: (▼) = isomer B (10.1%), (*) = isomer $\mathbf{A_1}$ (21.3%), (*) = isomer $\mathbf{A_2}$ (33.2%), and (●) = residual *c*(RGD) **12** (34.2%). B = *c*RGD **12**. C = oxanor-DTPA derivative **6**. Radiolabel traces; D = (◆) ^{111}In-oxanor-DTPA complex (**7**). E = reaction mixture comprised of (~) = byproduct in starting material (◆) = **7**, (▼) = ^{111}In-DTPA-labeled CF_3-furan formed concomitantly with product **B**, (*) = isomer $\mathbf{A_1}$, (*) = isomer $\mathbf{A_2}$.

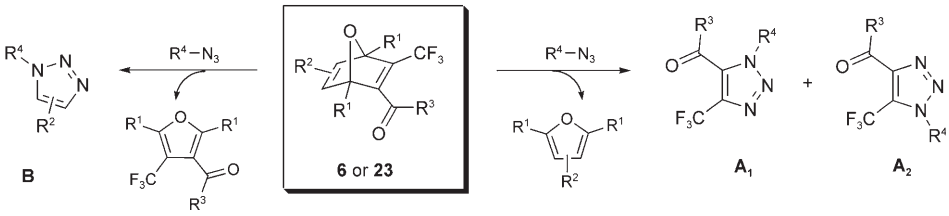
ed oxanorbornadienes was synthesized (**14**–**17**, Scheme 5) and the reaction characteristics in the tandem crDA reaction evaluated by ^{1}H NMR spectroscopy using benzyl azide in CD_3OD as a model system (Table 1).

Reaction of ethyl 4,4,4-trifluorobutynoate with 3-methylfuran resulted in the formation of two regioisomers (**14a** and **14b**) in good yield (72%). Saponification of **14a** and **14b** was followed by coupling of the resulting acids **15a** and **15b** to glycine methyl ester, leading to compounds **16a** and **16b** as a model system for the amide-linked glycol spacer. For comparison, the unsubstituted oxanorbornadiene glycine methyl ester derivative (**18**) was also synthesized. Apart from that, 1,4-dimethyloxanorbornadiene derivative **17** was prepared starting from 2,5-dimethylfuran.

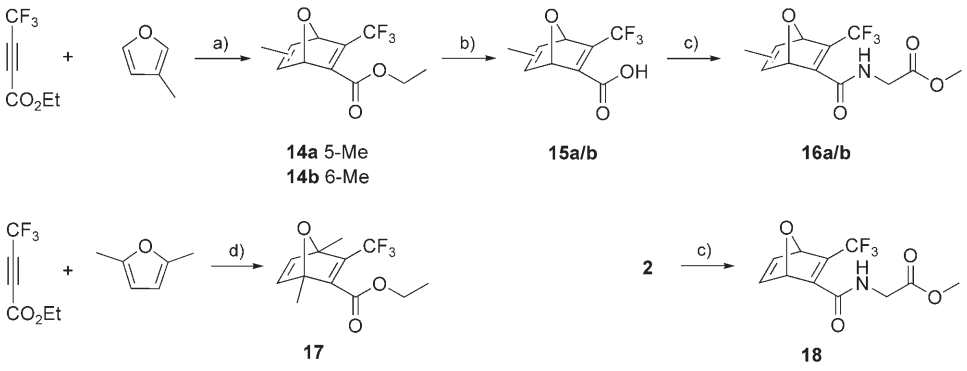
The oxanorbornadiene derivatives (**1** and **14**–**18**) were reacted with benzyl azide in the tandem crDA reaction. Product formation in the tandem crDA reaction with benzyl azide (R^4-N_3) and the specification of R^1–R^3 is illustrated in Scheme 4. The kinetic data obtained from ^{1}H NMR spectroscopy are depicted in Table 1. The cycloaddition reaction of the ethyl ester oxanorbornadiene derivative **1** with benzyl azide gave clean conversion to the desired isomers $\mathbf{A_1}$ and $\mathbf{A_2}$ (Table 1, entry 1).[37] Subjecting the methyl-substituted oxanorbornadiene **14a/b** to the tandem crDA reaction conditions (entry 2) resulted, apart from the expected cycloaddition product, in the partial formation oxaquadricyclanes (**19a/b**, Scheme 6).[38] The unexpected oxaquadricyclane formation can be attributed to an intramolecular migration of the electron-rich methyl-substituted double bond to the accepting electron-deficient ester-substituted double bond.



Scheme 3. Conjugation experiment of *N*-δ-azido-*cyclo*-(Arg-Gly-Asp-D-Phe-Orn) **12** with oxanor-DTPA (**6**) or Me-oxanor-DTPA (**23**) resulting in *c*(RGD)-CF_3-triazole-DTPA conjugate **13** (major 1,4-isomer shown).



Scheme 4. Reaction pathways for the formation of triazole compounds $\mathbf{A_1}$, $\mathbf{A_2}$, and **B** in the tandem crDA reaction.

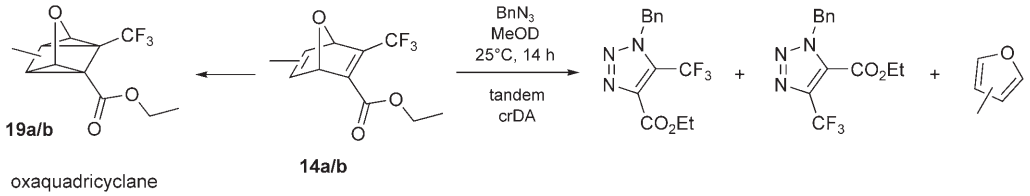


Scheme 5. a) Neat, 40 °C (**14a/b** 72%); b) THF, 1 M NaOH, (**15a/b** 80%); c) H-Gly-OMe, DMAP, EDC, CH_2Cl_2 (74% for **16a/b**; 56% for **18**); d) 1,4-dioxane, reflux (91%).

Table 1. Products and kinetic data of reactions between oxanorbornadiene derivatives **1** and **14**–**18** and benzyl azide.[a]

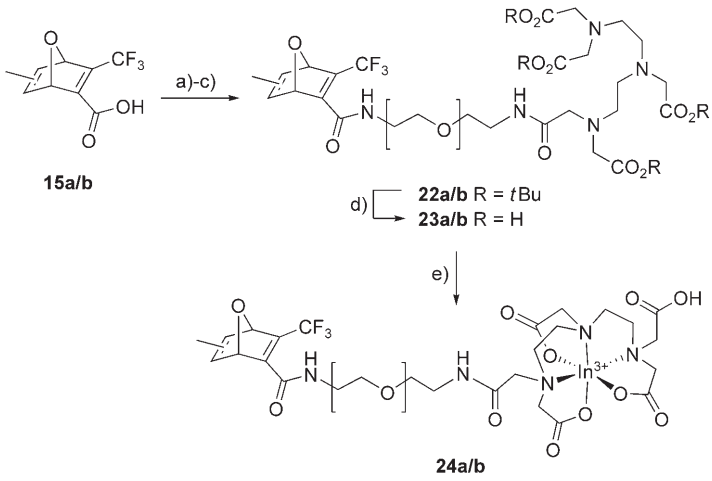
	Compound	R^1	R^2	R^3	Equiv N_3	**A** [%]	$\mathbf{A_1}$:$\mathbf{A_2}$	**B** [%]	$t_{1/2}$ [min]	Rate [$\times 10^4$ M^{-1} s^{-1}]
1	**1**	H	H	OEt	0.99	97	1:1.5	3	205	8.7 ± 0.09
2[b]	**14**	H	Me	OEt	1.20	n.d.	n.d.	n.d.	n.d.	n.d.
3	**15**	H	Me	OH	1.04	> 99	1:1.3	trace	360	4.2 ± 0.18
4	**17**	Me	H	OEt	1.39	94	1:1.2	6	490	2.6 ± 0.10
5	**18**	H	H	Gly-OMe	1.32	84	1:2.4	16	590	1.9 ± 0.03
6	**16**	H	Me	Gly-OMe	1.08	97	1:2.1	3	> 900	1.5 ± 0.01

[a] At 25 °C and 100 mM in CD_3OD. Obtained by monitoring the reactions with ^{1}H NMR spectroscopy (400 MHz). R^1, R^2, and R^3 as depicted in Scheme 4 (R^4 = Bn). [b] Unstable, partial oxaquadricyclane formation. n.d. = not determined.



Scheme 6. Formation of oxa-quadricyclanes **19a/b** from compound **14a/b** upon subjection to tandem crDA reaction conditions.

Much to our satisfaction, upon performing the crDA reaction with the less electron-withdrawing carboxylic acid derivative **15a/b** (entry 3), neither quadricyclane nor undesired isomer **B** formation was observed. The reaction rate, however, slightly decreased compared to the ethyl ester oxanorbornadiene derivative **1**. The oxanorbornadiene with methyl substituents on both bridgehead positions (i.e., compound **17**) gave comparable amounts of isomer **B** with respect to compound **1** (entries 4 and 1, respectively). However, the rate of the reaction dropped by a factor of four, which suggests that substitution at the bridgehead position merely affects the rate of the tandem crDA reaction. Repeating the experiments with the desired, more stable, amide bond at the 3-position of the oxanorbornadiene (that is, compound **18**) considerably increased the amount of undesired isomer **B** compared to compound **1** (16% and 3%, respectively). Apart from that, the rate of the cycloaddition reactions diminished considerably as a fivefold decrease in reaction rate was observed. A similar decrease in reaction rate was found for the amide containing Me-oxanorbornadiene (**16**, entry 6), though with the same order of magnitude as the unsubstituted oxanorbornadiene glycine conjugate **18** (1.5 and 1.9, respectively). Gratifyingly, almost full suppression of isomer **B** formation was achieved with the glycine-substituted Me-oxanorbornadiene (**16a/b**). Taking everything into consideration, the monomethyl-substituted oxanorbornadiene was considered the most suitable reaction partner for conjugation to *N*-δ-azido-*cyclo*(-Arg-Gly-Asp-D-Phe-Orn-) **12** as the reaction rate in the tandem crDA reaction is not negatively influenced, whereas formation of the undesired isomer **B** is effectively suppressed. To this end, a mixture of methyl-substituted oxanorbornadiene-DTPA complex (Me-oxanor-DTPA, **23a** and **23b**) was synthesized from compound **15a/b** in a four-step reaction sequence (Scheme 7). Radiolabeling of compound **23a/b** gave the desired ^{111}In-Me-oxanor-DTPA complex (**24a** and **24b**) in the final step.



Scheme 7. a) 1-*N*-Boc-3,6-dioxa-octane-1,8-diamine, EDC, DMAP, CH_2Cl_2 (47% for **20a/b**); b) TFA, CH_2Cl_2 (99% for **21a/b**); c) tetra-*O*-*tert*-Bu-DTPA, EDC, DMAP, CH_2Cl_2 (44% for **22a/b**); d) TFA, CH_2Cl_2 (99%); e) NH_4OAc buffer (pH 5.5), $InCl_3$.

The unlabeled Me-oxanor-DTPA complex **23a/b** and *N*-δ-azido-*cyclo*(-Arg-Gly-Asp-D-Phe-Orn-) (**12**) were used in the tandem crDA reaction and monitored by ^{1}H NMR spectroscopy. Similar reaction conditions as mentioned before were applied to give nearly full conversion to conjugate **13** after five days at 37 °C. After lyophilization of the reaction mixture, the sample was subjected to LCMS analysis (Figure 2). Much to our satisfaction, only three peaks were observed, comprising the two isomers of *c*(RGD)-CF_3-triazole-DTPA conjugate **13** (51.4%, $\mathbf{A_1}$ and $\mathbf{A_2}$ 1:1.5, Scheme 4) along with residual *c*RGD peptide

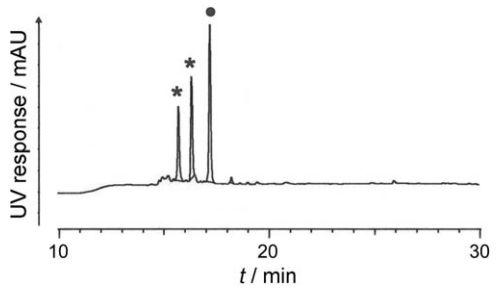


Figure 2. LCMS chromatogram of the crude crDA reaction of **23** and **12** after five days (*) = isomer $\mathbf{A_1}$ (22.4%), (*) = isomer $\mathbf{A_2}$ (29.0%), and (●) = *c*(RGD) (**12**, 48.2%).

(48.2%, initially 1.5 equiv used) and no undesired 1-*c*(RGD)-1*H*-1,2,3-triazole (isomer **B**) could be detected.

Solid-phase $\alpha_v\beta_3$ binding assay

The affinities of the *c*(RGD)-CF_3-triazole-DTPA conjugate **13** and DOTA-E-[*c*(RGDfK)]$_2$ (reference compound) for the $\alpha_v\beta_3$ integrin receptor were determined in a solid-phase competitive binding assay by using ^{111}In-DOTA-E-[*c*(RGDfK)]$_2$ as a tracer.[39,40]

Both ligands showed concentration-dependent inhibition of ^{111}In-DOTA-E-[*c*(RGDfK)]$_2$ binding to $\alpha_v\beta_3$, resulting in sigmoid curves. The IC_{50} values found for DOTA-E-[*c*(RGDfK)]$_2$ and *c*-(RGD)-triazole-DTPA conjugate **13** were 125 nM and 192 nM, respectively. Comparing the IC_{50} value of conjugate **13** with other triazole-containing monomeric or dimeric *c*(RGD)-conjugates,[18,22] similar results were obtained, indicating a limited effect on $\alpha_v\beta_3$ receptor binding for CF_3-containing triazole-conjugates (Figure 3).

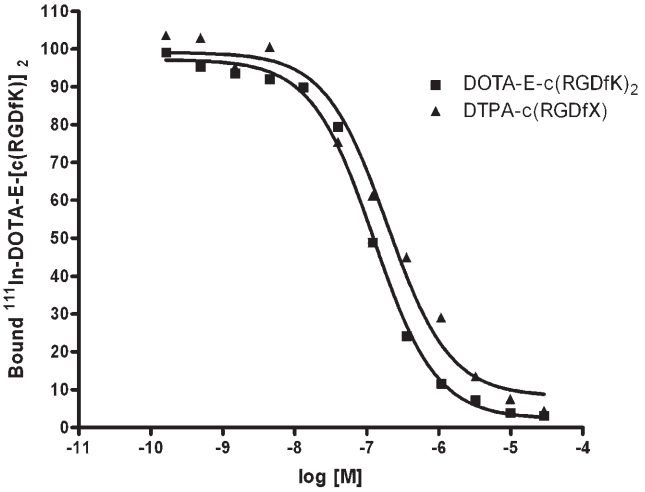


Figure 3. Competition of specific binding of ^{111}In-DOTA-E-[*c*(RGDfK)]$_2$ to $\alpha_v\beta_3$ with DOTA-E-[*c*(RGDfK)]$_2$ (■) and DTPA-triazole-*c*(RGD) (**13**) (▲).

Lipophilicity studies

The DTPA-triazole-*c*(RGD)-conjugate showed a log*P* value of -2.89 ± 0.38 obtained from *n*-octanol/water partition coefficient measurements, a number comparable to previously reported log*P* values for poly(ethylene glycol)[19] or glucose[21]-functionalised cyclic RGD derivatives.

Conclusions

Tandem cycloaddition–retro-Diels–Alder reaction of substituted oxanorbornadienes and functionalized azides is a powerful tool for constructing complex bioconjugates by forming stable triazole adducts. The 1,3-dipolar cycloaddition of DTPA-functionalized oxanorbornadiene and *N*-δ-azido-*cyclo*(-Arg-Gly-Asp-D-Phe-Orn-) resulted in the formation of the desired *c*(RGD)-CF_3-triazole-DTPA conjugate and a small amount of an unexpected 1-*c*(RGD)-1*H*-1,2,3-triazole. Introduction of methyl substituents on the oxanorbornadiene suppressed cycloaddition on the unfunctionalized side of the oxanorbornadiene systems, thereby effectively eliminating the formation of undesired 1-*c*-(RGD)-1*H*-1,2,3-triazole. Preliminary biological evaluation of the *c*(RGD)-CF_3-triazole-DTPA conjugate showed a good IC_{50} value and favorable hydrophilicity that could result in positive pharmacokinetic behavior in vivo. A detailed biological evaluation of the *c*(RGD)-CF_3-triazole-DTPA conjugate and further optimization of the new ligation method is currently under investigation in our laboratory.

Experimental Section

Instruments and methods: Unless otherwise stated, all chemicals were obtained from commercial sources and used without further purification. $^{111}InCl_3$ was obtained from Tyco Mallinckrodt, Petten, The Netherlands. Analytical thin layer chromatography (TLC) was performed on Merck precoated silica gel 60 F-254 plates (layer thickness 0.25 mm) with visualization by ultraviolet (UV) irradiation at $\lambda = 254$ nm and/or $\lambda = 366$ nm and/or staining with $KMnO_4$. Preparative thin layer chromatography (Prep-TLC) was performed on Merck precoated silica gel 60 F-254 plates (layer thickness 1.00 mm) with concentration zone and visualization by UV irradiation at $\lambda = 254$ nm and/or $\lambda = 366$ nm. Purifications by silica gel chromatography were performed using Acros (0.035–0.070 mm, pore diameter ca. 6 nm) silica gel. Unless otherwise stated, all experiments were performed under ambient atmosphere and temperature. The water used in the biological procedures was deionised using a Labconco Water Pro PS purification system. THF was distilled under nitrogen from sodium/benzophenone. CH_2Cl_2 was distilled under nitrogen from CaH_2. FTIR spectra were recorded on an ATI Matson Genesis Series FTIR spectrometer fitted with an ATR cell. The vibrations (ν) are given in cm^{-1}. NMR spectra were recorded on Bruker DPX200 (200 MHz and 50 MHz for ^1H and ^{13}C, respectively), Bruker DMX300 (300 MHz and 75 MHz for ^1H and ^{13}C, respectively) and Varian Inova 400 spectrometers. ^1H NMR chemical shifts are reported in parts per million (ppm) relative to a residual proton peak of the solvent, $\delta = 3.31$ for CD_3OD, $\delta = 7.26$ for $CDCl_3$, and $\delta = 4.79$ for D_2O. Broad peaks are indicated by the addition of br. Coupling constants are reported as a *J* value in Hertz (Hz). The number of protons (*n*) for a given resonance is indicated as *n*H, and is based on spectral integration values. ^{13}C NMR chemical shifts are reported in ppm relative to CD_3OD ($\delta = 49.0$) or $CDCl_3$ ($\delta = 77.0$). Electrospray LC/MS analysis was performed on a Shimadzu LC/MS 2010A system, equipped with a Zorbax Extend C18 column, 3.5 um, 4.6×150 mm, Agilent Technologies, Palo Alto, CA,

USA, eluting with a mobile phase gradient-profile: 0–5 min 10% acetonitrile/90% water (0.1% TFA), 5–30 min gradient to 95% acetonitrile/5% water (0.1% TFA), 30–40 min 95% acetonitrile/5% water (0.1% TFA). Matrix-assisted laser desorption/ionisation time-of-flight (MALDI-ToF) spectra were measured on a Bruker Biflex III spectrometer and samples were prepared from MeOH solutions using indoleacrylic acid (IAA) (20 mg mL^{-1}) as a matrix. LCQ/MS analysis was performed using Thermo scientific Advantage LCQ linear ion-trap electrospray (ESI-MS). Electrospray ionisation time-of-flight (ESI-ToF) spectra were measured with a JEOL AccuToF.

Radiolabeling experiments: The DTPA-linked oxanorbornadiene systems (**6** and **23a/b**) were labeled with ^{111}In by dissolving oxanor-DTPA **6** or Me-oxanor-DTPA **23a/b** (5 μg, 7.0 nmol) in 5 μL H_2O. Subsequently, metal-free NH_4OAc buffer (90 μL, 0.25 M, pH 5.5) and 5 μL (~100 μCi) $^{111}InCl_3$ were added to each of the reaction mixtures. The mixtures were allowed to incubate for 1 h at RT after which the radiochemical purity was checked by HPLC (HP1100 series, LC system, Agilent Technologies, Palo Alto, CA, USA) using a RP-C18 column (5 μm, 4.6 mm × 250 mm, Alltech, Deerfield, IL, USA) eluated with a gradient mobile phase (0–100% B over 20 min, solvent A = 0.1% TFA in water, solvent B = 0.1% TFA in acetonitrile) at 1 mL min^{-1}. The radioactivity of the eluates was monitored with an in-line NaI radiodetector (Raytest GmbH, Straubenhardt, Germany.)

Conjugation studies

General procedure for reactions between oxanorbornadiene derivatives and benzyl azide compounds monitored by ^{1}H NMR spectroscopy: A solution of an oxanorbornadiene derivative (0.05 mmol) in a deuterated solvent (0.5 mL) was added to a test tube containing benzyl azide (various equivalents). The mixture was briefly mixed with a vortex and then added to an NMR tube. Directly after the addition, the tube was placed in a Varion Inova 400 NMR apparatus at 25 °C and reaction was monitored following a preset measurement schedule.

*General procedure for the synthesis of c(RGD)-CF$_3$-triazole-DTPA conjugate **13** via cycloaddition reaction between oxanorbornadiene derivatives and* N-δ-azido-cyclo(-Arg-Gly-Asp-D-Phe-Orn-) (**12**) *monitored by ^{1}H NMR spectroscopy*: A solution of oxanor-DTPA (**6**) or Me-oxanor-DTPA (**23a/b**; 2.82 and 2.88 mg, respectively, 3.97 μmol) in CD_3OD/D_2O (0.5 mL 0.1 mL^{-1}) was added to a test tube containing cyclic RGD peptide **12** (4.3 mg, 5.96 μmol). The mixture was briefly mixed using a vortex and added to an NMR tube. The tube was then directly placed in a Varion Inova 400 NMR apparatus at 37 °C and the reaction was monitored following a preset measurement schedule. After completion of the reaction the resulted mixtures were lyophilized and analyzed by HRMS and electrospray LC/MS analysis performed on a Shimadzu LC/MS 2010A system; HRMS(ESI+): *m/z* calcd for $C_{50}H_{74}F_3N_{16}O_{19}$: 1257.5112 $[M+H]^+$, found 1257.5235.

General procedure for cycloaddition reactions between ^{111}In-labeled oxanorbornadiene derivatives and N-δ-azido-cyclo(-Arg-Gly-Asp-D-Phe-Orn-) (**12**) *monitored by HPLC with an in-line NaI radiodetector*: All tandem crDA reactions were performed at 37 °C in a total volume of 50 μL with 0.5 μg (0.70×10^{-3} μmol) ^{111}In-labeled DTPA (10 μCi) unless described otherwise. An incubator was used to warm the reactions to 37 °C for four days. The conversion was checked by HPLC using a RP-C18 column eluted with a gradient mobile phase (0–100% B over 20 min, solvent A = 0.1% TFA in water, solvent B = 0.1% TFA in acetonitrile) at 1 mL min^{-1}. The radioactivity of the eluates was monitored by using an in-line NaI radiodetector.

Octanol/water partition coefficient: For the lipophilicity determination, approximately 70000 cpm ^{111}In-DTPA-*c*RGD was diluted to a volume of 3 mL with phosphate-buffered saline (PBS) and an equal volume of *n*-octanol was added to obtain a binary phase system. After mixing the two layers vigorously for ten seconds and gently for another 2 min, the two layers were separated by centrifugation (500 G, 5 min). Three 250 μL samples were taken from each layer and their activity was measured in a 3^{II} well type NaI gamma counter (Wallac 1480-Wizard 3). The log*P* value was determined in two independent experiments.

Solid-phase $\alpha_v\beta_3$ binding assay: Affinity of the DTPA-cRGD conjugate (**13**) and the conventional DOTA-E-[*c*RGDfK)]$_2$ for the $\alpha_v\beta_3$ integrin was determined using a solid-phase competitive binding assay using ^{111}In-DOTA-E-[cRGDfK)]$_2$ as a tracer. Labeling was performed following the procedure described by Boerman et al.[41] $^{111}InCl_3$ (90 μCi μg^{-1}) was added to 5 μg DOTA-E-[cRGDfK)]$_2$ dissolved in 100 μL metal free HEPES buffer (1 M, pH 6.0). The mixture was heated to 100 °C during 15 min after which the chemical purity was determined with HPLC. Microtiter 96-well vinyl assay plates (Corning B.V., Schiphol-Rijk, The Netherlands) were coated with a solution of purified human integrin $\alpha_v\beta_3$ (150 ng mL^{-1}) in Triton X-100 Formulation (Chemical International, Temecula, CA, USA) in coating buffer (25 mM Tris·HCl (pH 7.4), 150 mM NaCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$ and 1 mM $MnCl_2$) for 18 h at 4 °C (100 μL per well). After washing the plate twice with binding buffer (coating buffer supplemented with 0.1% BSA (bovine serum albumin)), all wells were blocked with 200 μL blocking buffer (coating buffer supplemented with 1% BSA) during 3.5 h at RT. The wells were washed twice with binding buffer and subsequently incubated with 100 μL binding buffer containing 200000 cpm of ^{111}In-DOTA-E-[cRGDfK)]$_2$ and appropriate dilutions of nonlabeled DOTA-E-[*c*RGDfK)]$_2$ or DTPA-*c*RGD conjugate dissolved in 20 μL binding buffer for 1 h at 37 °C. The competitive displacement study of the dilutions was performed in triplicate. Finally, the plate was washed three times, the wells were cut, and the radioactivity in each well was counted using a γ-counter (Wallac 1480-Wizard® 3, Perkin–Elmer, Boston, MA, USA). The IC_{50} values of both the DOTA-E-[*c*RGDfK)]$_2$ and DTPA-cRGD conjugate were determined by nonlinear regression (GraphPad Pris 4.0 Software Package, San Diego, CA, USA).

Statistical analysis: The mean values for the solid phase binding assay and the lipophilicity studies are given ± standard deviation (S.D.). Statistical analysis was performed by using a Welch's corrected unpaired t-test or one-way analysis of variance with GraphPad InStat software (version 4.00, GraphPad Software). The level of significance was set at $p < 0.05$.

Synthesis

*3-Trifluoromethyl-7-oxa-bicyclo[2.2.1]hepta-2,5-diene-2-carboxylic acid (**2**)*: According to the literature procedure:[32] Ethyl 2-fluorobut-2-ynoate (1.00 g, 0.86 mL, 6.02 mmol) yielded 726 mg of 2 (60% over two steps) as a white solid.

*Fmoc-Orn(N$_3$)-OH (**8**)*: Modified literature procedure:[42] CH_2Cl_2 (3 mL) and triflic anhydride (1.3 mL, 7.73 mmol) at 0 °C were added to a solution of sodium azide (1.00 g, 15.4 mmol) in water (3 mL), while the mixture was vigorously stirred. After being stirred for 2 h at 0 °C the phases were separated, and the aqueous phase was extracted with CH_2Cl_2 (5 mL). The combined organic phases were washed with saturated aqueous $NaHCO_3$-solution (5 mL). The resulting triflic azide solution was added dropwise to a solution of Fmoc-Orn-OH·HCl (1.00 g, 2.57 mmol), $CuSO_4$ (16 mg, 64 μmol) and $NaHCO_3$ (436 mg, 5.19 mmol) in water (9 mL) followed by the addi-

tion of methanol until the mixture became a suspension. The mixture was stirred for 4 h at RT. The organic solvents were removed in vacuo and the resulting suspension was acidified with 1 M aqueous HCl to pH 3 after which the precipitate was isolated by filtration and washed with water. The residue was dissolved in ether, dried over Na_2SO_4, and concentrated in vacuo. Purification was performed by flash chromatography (CH_2Cl_2/methanol/AcOH 98:2:0.3, 98:5:0.5) and the product was coevaporated with toluene (4×) yielding a white solid (621 mg, 64%). ^{1}H NMR (CD_3OD, 200 MHz) δ = 7.83 (m, 2H), 7.72 (m, 2H), 7.24 (m, 4H), 4.41 (d, J = 6.3 Hz, 2H), 4.29 (m, 1H), 4.19 (m, 1H), 4.29 (m, 1H), 4.19 (m, 1H), 3.39 (m, 1H), 1.90 (m, 2H), 1.75 (m, 2H); LRMS (ESI−) m/z calcd for $C_{20}H_{19}O_4N_4$: 379.1 $[M-H]^-$ found 378.9. FTIR ν_{max} film: 2109, 1718, 1630, 1238 cm^{-1}.

*Fmoc-D-Phe-OH (**9**)*: According to the literature procedure:[43] H-D-Phe-OH (530 mg, 1.37 mmol) yielded 1.13 g (91%) Fmoc-D-Phe-OH as a white solid after purification by crystallization from EtOAc.

*H-Asp(OtBu)-D-Phe-Orn(N_3)-Arg(PMC)-Gly-OH (**10**)*: Compound **9** was synthesized by standard solid-phase peptide synthesis (SPPS) using a modified literature procedure:[31] A solution of Fmoc-Gly-OH (0.174 g, 0.58 mmol) and DIPEA (235 μL, 1.35 mmol) in dry CH_2Cl_2 (2.5 mL) was added to a suspension of trityl chloride resin (300 mg of 1.5 mmol g^{-1} loaded resin) in dry CH_2Cl_2 (2.5 mL) and the mixture was shaken at RT for 2.5 h. MeOH (470 μL) and DIPEA (94 μL) were added and the mixture was shaken for another 15 min. The resin was washed with NMP (3×2.5 mL), CH_2Cl_2 (5×2.5 mL), and MeOH (3×2.5 mL) and dried in vacuo. The Fmoc-protected resin was suspended in a solution of 20% piperidine in NMP (2×20 mL) and shaken for 5 and 15 min. The resin was washed with NMP (6×20 mL). A positive Kaiser test[44] indicated completion of the peptide coupling reaction. After filtering and washing with DMF (3×20 mL), the next amino acid was coupled by adding a mixture of Fmoc-Arg(PMC)-OH (960 mg, 1.45 mmol, 2.5 equiv), TBTU (465 mg, 1.45 mmol, 2.5 equiv), HOBt (196 mg, 1.45 mmol, 2.5 equiv), and DIPEA (0.67 mL, 4.1 mmol, 7 equiv) dissolved in NMP (100 mL) to the resin. The reaction mixture was shaken at RT for 90 min and washed with NMP (6×20 mL). A negative Kaiser test indicated completion of the peptide coupling reaction. The deprotection-coupling sequence was repeated with the following amino acids: Fmoc-Orn(N_3)-OH (551 mg, 1.45 mmol, 2.5 equiv), Fmoc-D-Phe-OH (561 mg, 1.45 mmol, 2.5 equiv), and Fmoc-Asp(O*t*Bu)-OH (596 mg, 1.45 mmol, 2.5 equiv). For the coupling of Fmoc-D-Phe-OH 2,4,6-collidine (1.9 mL, 14.5 mmol, 25 equiv) was used instead of DIPEA. The resin was washed with DCM (4×20 mL) and treated with a solution of TFA, H_2O, and triisopropylsilane (TIPS; 90:4.75:4.75; 20 mL) for 3×10 min. After removal of the resin by filtration, the filtrates were combined and stirred for another 2.5 h. The collected filtrates were evaporated, coevaporated with toluene, and lyophilized from *tert*-BuOH/H_2O/dioxane yielding a white foam (126 mg, 29%). ^{1}H NMR (400 MHz, CD_3OD) δ = 7.33–7.23 (m, 5H), 4.59 (t, J = 8.0 Hz, 1H), 4.39 (dd, J = 9.5, 4.4 Hz, 1H), 4.19 (t, J = 6.5 Hz, 1H), 4.00 (dd, J = 10.5, 3.9 Hz, 1H), 3.85 + 3.62 (AB-system J = 17.0 Hz, 2H), 3.20–3.14 (m, 4H), 3.02 (d, J = 8.0 Hz, 2H), 2.83–2.77 (m, 2H), 2.67 (t, J = 6.7 Hz, 2H), 2.56 (s, 3H), 2.55 (s, 3H), 2.10 (s, 3H), 1.95–1.80 (m, 4H), 1.80–1.73 (m, 1H), 1.69–1.60 (m, 1H), 1.58–1.49 (m, 2H), 1.47 (s, 9H), 1.31 (s, 6H), 1.22–1.08 (m, 2H); LCMS (EI+) purity +97%, m/z calcd for $C_{44}H_{66}N_{11}O_{11}S$: 955.5 $[M+H]^+$, found 956.6.

*Cyclo-[Asp(O*t*Bu)-D-Phe-Orn(N_3)-Arg(PMC)-Gly] (**11**)*: Diphenylphosphorylazide (68 μL, 0.31 mmol) and $NaHCO_3$ (43.9 mg, 0.52 mmol) were added to a solution of the linear peptide **10** (100 mg, 0.10 mmol) in dry DMF (21 mL) and the mixture was stirred for 44 h at RT. The mixture was filtered, diluted with EtOAc (100 mL), and washed with saturated aqueous NH_4Cl-solution (2×100 mL) and brine (2×100 mL). The organic phase was dried over Na_2SO_4 and concentrated. The material was stirred with Et_2O for 10 min and the white precipitate was collected by filtration, washed with Et_2O (3×), and dried, yielding a white solid (85 mg, 87%). ^{1}H NMR (400 MHz, $CDCl_3$/CD_3OD/D_2O) δ = 7.28–7.18 (m, 5H), 4.70 (dd, J = 8.2 Hz, 1H), 4.55 (d, J = 8.0 Hz, 1H), 4.21 (brs, 1H), 4.21 + 3.34 (AB-system, J = 14.9 Hz, 2H), 3.94 (dd, J = 10.4 Hz, 1H), 3.14–3.11 (m, 4H), 2.96 (d, J = 8.0 Hz, 2H), 2.74 (dd, J = 16.1, 8.2 Hz, 1H), 2.63 (t, J = 6.9 Hz, 2H), 2.52 (s, 3H), 2.51 (dd, J = 16.1, 8.2 Hz, 1H) 2.50 (s, 3H), 2.06 (s, 3H), 1.85–1.77 (m, 3H), 1.76–1.67 (m, 1H), 1.64–1.55 (m, 1H), 1.52–1.41 (m, 3H), 1.39 (s, 9H), 1.28 (s, 6H) 1.21–1.12 (m, 2H); LCMS (EI+) purity +97%, m/z calcd for $C_{44}H_{64}N_{11}O_{10}S$: 937.5 $[M+H]^+$, found 938.7.

*Cyclo-[Asp-D-Phe-Orn(N_3)-Arg-Gly] (**12**)*: The cyclic peptide **11** (84 mg, 90 μmol) was dissolved in a mixture of TFA/H_2O 95:5 (5 mL) and the mixture was stirred for 3 h. The solvent was removed and the residue was co-evaporated with toluene. The material was dissolved in H_2O (15 mL), extracted with EtOAc (2×10 mL), and the aqueous phase was evaporated to dryness. The product was lyophilized out of H_2O/dioxane, yielding a white solid (61 mg, 93%). ^{1}H NMR (400 MHz, D_2O): δ = 7.42–7.21 (m, 5H), 4.64 (dd, J = 10.0, 6.0 Hz, 1H), 4.37 (dd, J = 9.1, 5.6 Hz, 1H), 4.21 + 3.52 (AB-system, J = 15.0 Hz, 2H), 3.92 (dd, J = 10.4, 4.8 Hz, 1H), 3.25–3.15 (m, 4H), 3.09 (dd, J = 13.1, 6.2 Hz, 1H), 3.00–2.90 (m, 2H), 2.75 (dd, J = 16.8, 6.5 Hz, 1H), 1.92–1.62 (m, 3H), 1.59–1.47 (m, 3H), 1.26–1.16 (m, 2H); ^{13}C NMR (50 MHz, D_2O (dioxane residue used as reference) δ = 174.9, 174.8, 173.7, 173.3, 172.0, 171.9, 157.3, 136.6, 129.8 (2C), 129.5 (2C), 127.9, 67.19 (dioxane), 55.8, 55.6, 53.1, 50.7, 50.2, 44.1, 41.1, 37.5, 34.9, 28.1, 27.9, 25.2, 25.1; HRMS (ESI+): m/z calcd for $C_{26}H_{38}N_{11}O_7$ $[M+H]^+$ 616.2956, found 616.2907. FTIR ν_{max} film: 3274, 2098, 1640, 1126 cm^{-1}.

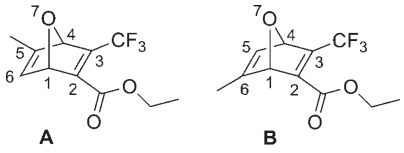
*N-{Boc}-N′-{3-trifluoromethyl-7-oxa-bicyclo[2.2.1]hepta-2,5-diene-2-carboxyl}-3,6-dioxaoctane-1,8-diamine (**3**)*: 4-(dimethylamino)-pyridine (DMAP, 41.1 mg, 0.34 mmol) was added to a solution of oxanorbornadiene **2** (34.7 mg, 0.17 mmol) and 1-*N*-Boc-3,6-dioxa-8-octane-1,8-diamine (41.8 mg, 0.17 mmol) in CH_2Cl_2 (1.5 mL). The mixture was cooled to 0 °C and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC·HCl, 21 mg, 0.11 mmol) was added slowly. The mixture was stirred for 30 min at 0 °C and 16 h at RT. The reaction mixture was acidified with HCl (2 M) to a pH of 1–2 and extracted with CH_2Cl_2 (2×5 mL). The combined organic layers were dried over $MgSO_4$, concentrated in vacuo, and purified by preparative TLC (CH_2Cl_2/MeOH 9:1) resulting in compound **3** as a slightly yellow oil (47.8 mg, 64%). R_F = 0.50 (CH_2Cl_2/MeOH 9:1). ^{1}H NMR (400 MHz, CD_3OD) δ = 7.30 (dd, J = 5.3, 1.9 Hz, 1H), 7.21 (dd, J = 5.3, 1.9 Hz, 1H), 5.65 (t, J = 1.6, 1.6 Hz, 1H), 5.55 (m, 1H), 3.59 (s, 4H) 3.56 (dd, J = 10.5, 5.0 Hz, 2H), 3.49 (t, J = 5.7, 5.7 Hz, 2H), 3.45 (dd, J = 11.3, 5.5 Hz, 2H), 3.20 (t, J = 5.7 Hz, 2H), 1.41 (s, 9H); ^{13}C NMR (50 MHz, CD_3OD): δ = 165.2, 158.5, 144.7, 143.6, [126.6, 121.2] CF_3, 87.1, 84.4, 80.1, 71.3, 70.3, 41.2, 40.4, 28.8; LRMS (ESI+) m/z calcd for $C_{19}H_{28}F_3N_2O_6$: 437.1 $[M+H]^+$, found 437.1.

*N,N′-{3-trifluoromethyl-7-oxa-bicyclo[2.2.1]hepta-2,5-diene-2-carboxyl}-3,6-dioxaoctane-1,8-diamine·TFA (**4**)*: Trifluoroacetic acid (TFA, 0.5 mL, 2.85 mmol) was added dropwise to a cooled solution (0 °C) of **3** (47.8 mg, 0.11 mmol) in dry CH_2Cl_2 (2 mL). The reaction was stirred at 0 °C for 1 h after which the reaction was complete. The solvent was evaporated and the crude mixture was dissolved in H_2O (5 mL) and dioxane (5 mL) and freeze-dried to afford compound **4** as a light yellow solid (49.0 mg, +99%). R_F = 0.09 (CH_2Cl_2/MeOH 9:1). ^{1}H NMR (400 MHz, CD_3OD): δ = 7.30 (ddd, J = 5.3, 1.9 Hz, 0.7 Hz, 1H), 7.22 (ddd, J = 5.3, 1.9, 0.7 Hz, 1H), 5.66 (s, 1H),

5.55 (s, 1H), 3.76 (t, $J=4.9$ Hz, 2H) 3.64, (s, 4H), 3.57 (t, $J=5.9$ Hz, 2H), 3.38–3.55 (m, 4H), 3.09 (t, $J=5.7$ Hz, 2H); ^{13}C NMR (50 MHz, CD_3OD): $\delta=165.3$, 156.2, 144.6, 143.7, 127.8, [126.5, 121.2] CF_3, 87.1, 84.5, 71.3, 70.3, 67.9, 40.7, 40.3; HRMS (ESI+) *m/z* calcd for $C_{14}H_{20}F_3N_2O_4$: 337.1375 $[M+H]^+$, found 337.1376.

*N-{2-O-tert-butyl-DTPA-acetamide},N′-{3-trifluoromethyl-7-oxa-bicyclo-[2.2.1]hepta-2,5-diene-2-carboxyl}-3,6-dioxaoctane-1,8-diamine (**5**)*: Compound **4** (49 mg, 0.11 mmol) was dissolved in dry CH_2Cl_2 (2 mL), and 4-(dimethylamino)pyridine (DMAP, 26.6 mg, 0.22 mmol) and diethylenetriamine-*N,N,N′′,N′′*-tetra-*tert*-butyl acetate-*N′*-acetic acid ((DTPA-*tert*-butyl ester) 67.8 mg, 0.11 mmol) were added. The mixture was cooled to 0 °C followed by addition of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC·HCl, 23.1 mg, 0.12 mmol). After being stirred for 1 h at 0 °C, the mixture was allowed to warm to RT and was stirred for an additional 4 h. The reaction mixture was quenched with 1 M HCl (2 mL) and the water layer was extracted with CH_2Cl_2 (2×2 mL). The combined organic layers was dried over Na_2SO_4 and subsequently evaporated. The crude product was purified by preparative TLC ($MeOH/CH_2Cl_2$ 1:9) to obtain pure product as a colorless oil (38 mg, 81%). ^{1}H NMR (400 MHz, $CDCl_3$): $\delta=8.23$ (br s, 1H, NH), 7.32 (dd, $J=5.3$, 2.1 Hz, 1H), 7.13 (dd, $J=5.3$, 2.1 Hz, 1H) 6.81 (br s, 1H, NH), 5.62 (dd, $J=$ 1.4, 0.5 Hz, 2H), 3.62–3.50 (m, 8H), 3.49–3.42 (m, 4H), 3.39 (s, 8H), 3.12 (s, 2H), 2.77 (t, $J=6.6$ Hz, 4H), 2.61 (t, $J=6.5$ Hz, 4H), 1.43 (s, 36H); ^{13}C NMR (50 MHz, $CDCl_3$): $\delta=172.2$, 170.5 (4C), 162.2, 143.7, 141.9, 86.0, 83.5, 81.0 (4C), 70.5, 70.0, 69.7, 69.4, 58.5, 55.8 (4C), 53.7, 53.4, 52.1 (2C), 39.4, 38.6, 28.1; LRMS (ESI+) *m/z* calcd for $C_{44}H_{74}F_3N_5O1_3$: 936.52 $[M+H]^+$, found 936.40.

*N-{2-DTPA-acetamide},N′-{3-trifluoromethyl-7-oxa-bicyclo[2.2.1]hepta-2,5-diene-2-carboxyl}-3,6-dioxaoctane-1,8-diamine (**6**)*: TFA (200 μL, 1.14 mmol) was added to a solution of **5** (49 mg, 0.11 mmol) in DCM (2 mL). This mixture was stirred for five days (until MS analysis did not show starting material). The product was obtained after lyophilized from H_2O/dioxane as a white solid (28 mg, +99%). ^{1}H NMR (400 MHz, CD_3OD): $\delta=7.32$ (dd, $J=5.3$, 1.9 Hz, 1H), 7.23 (dd, $J=5.3$, 1.9 Hz, 1H), 5.67 (s, 1H), 5.58 (s, 1H), 4.24 (br s, 1H, NH), 3.66 (s, 2H) 3.62–3.57 (m, 16H) ppm 3.50–3.37 (m, 8H), 3.20 (br s, 4H); ^{13}C NMR (50 MHz, CD_3OD): $\delta=174.31$, 174.28, 174.15,174.14, 167.2, 164.9, 155.9, 144.3, 143.4, 127.6 (q, CF_3), 86.9, 84.2, 71.1, 71.0, 70.04, 70.00, 67.9, 55.8 (4C), 53.8 (2C), 50.9, 50.8, 40.2, 40.1; LRMS (ESI−) *m/z* calcd for $C_{28}H_{39}F_3N_5O_{13}$: 710.25 $[M-H]^-$, found 710.30.



*5-Methyl-3-trifluoromethyl-7-oxa-bicyclo[2.2.1]hepta-2,5-diene-2-carboxylic acid ethyl ester (**14a**) and 6-methyl-3-trifluoromethyl-7-oxa-bicyclo[2.2.1]hepta-2,5-diene-2-carboxylic acid ethyl ester (**14b**)*: A mixture of ethyl 4,4,4-trifluorobutynoate (0.61 g, 3.65 mmol) and 3-methylfuran (0.30 g, 3.65 mmol) was stirred under an Ar atmosphere for four days. The crude mixture was washed out with ether, concentrated, and purified by column chromatography (EtOAc/*n*-heptane 1:5), resulting in a mixture of two regioisomers (ratio 1:1.4 for **14b** and **14a** respectively) as a slightly yellow oil (0.65 g, 72%). ^{1}H NMR (300 MHz, $CDCl_3$) peaks assigned to compound **14a**: $\delta=$ 6.65 (m, 1H), 5.38 (s, 1H), 5.30 (d, $J=1.5$ Hz, 1H), 4.28 (m, 2H), 1.99 (d, $J=2.0$ Hz, 3H), 1.31 (t, $J=6.9$ Hz, 3H); Peaks assigned to compound **14b**: $\delta=6.58$ (m, 1H), 5.61 (s, 1H), 5.56 (d, $J=1.5$ Hz, 1H), 4.27 (m, 2H), 2.05 (d, $J=2.0$ Hz, 3H), 1.32 (t, $J=6.9$ Hz, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) $\delta=162.1$, 162.3, 156.2, 154.8, 152.0 (q), 151.2 (q), 150.8, 150.3, 133.7,134.5, 122.2 (q, CF_3), 121.5 (q, CF_3), 88.6, 87.4 (d), 85.9, 84.8, 61.7 (2C), 14.3, 14.2, 14.0 (2C). LRMS (ESI+) *m/z* calcd for $C_{11}H_{12}F_3O_3$ $[M+H]^+$ 249.1, found 249.0.

*5-Methyl-3-trifluoromethyl-7-oxa-bicyclo[2.2.1]hepta-2,5-diene-2-carboxylic acid (**15a**) and 6-methyl-3-trifluoromethyl-7-oxa-bicyclo-[2.2.1]hepta-2,5-diene-2-carboxylic acid (**15b**)*: A mixture of **14a** and **14b** (0.30 g, 1.22 mmol) was dissolved in THF (16 mL) and cooled to 0 °C. 1 M NaOH (aq) (1.22 mL) was added dropwise and the mixture was stirred overnight at RT. TLC analysis showed some starting material remaining after reacting overnight so another 1.22 mL NaOH (aq) (1 M) was added. After 30 min the reaction was completed and the volume of THF was reduced to 50% of the original volume by evaporation using a nitrogen flow. The mixture was diluted with HCl (aq) (5 mL, 1 M) and extracted with EtOAc (2× 75 mL). The organic layer was dried over Na_2SO_4 and evaporated under reduced pressure to obtain a yellow solid (0.26 g, 95%). A mixture of two regioisomers was obtained in a ratio of 1:1.4 for **15b** and **15a** respectively. ^{1}H NMR (300 MHz, $CDCl_3$) peaks assigned to compound **15a**: $\delta=6.67$ (t, $J=2.0$ Hz, 1H), 5.56 (s, 1H), 5.34 (d, $J=1.5$ Hz, 1H), 2.01 (d, $J=2.0$, 3H); Peaks assigned to compound **15b**: $\delta=6.59$ (t, $J=2.0$ Hz, 1H), 5.60 (s, 1H), 5.42 (d, $J=$ 1.5 Hz, 1H), 2.05 (d, $J=2.0$ Hz, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) $\delta=$ 166.7, 166.4, 150.9 (q), 149.9 (q), 134.4, 133.2, 121.2 (q, CF_3), 121.0 (q, CF_3), 88.3, 87.5, 84.9, 84.8, 14.1, 14.0; LRMS (ESI−) *m/z* calcd for $C_9H_6F_3O_3$: 219.1 $[M-H]^-$, found 219.0.

*5-Methyl-3-trifluoromethyl-7-oxa-bicyclo[2.2.1]hepta-2,5-diene-2-carboxyl glycine methyl ester (**16a**) and 6-methyl-3-trifluoromethyl-7-oxa-bicyclo[2.2.1]hepta-2,5-diene-2-carboxyl glycine methyl ester (**16b**)*: Oxanorbornadiene carboxylic acids **15a** and **15b** (87.5 mg, 0.39 mmol), glycine methyl ester·HCl (158 mg, 1.26 mmol), and 4-(dimethylamino)pyridine (DMAP, 183 mg, 1.50 mmol) were dissolved in CH_2Cl_2 (16 mL) and cooled to 0 °C. 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC·HCl, 80 mg, 0.42 mmol) was added and the reaction mixture was stirred at 0 °C for 30 min. The mixture was allowed to warm to RT and was stirred for an additional 16 h. The reaction was quenched with a 10% aqueous solution of citric acid (20 mL) and extracted with EtOAc (2×20 mL). The combined organic layers were washed with brine (20 mL), dried over Na_2SO_4 and concentrated in vacuo. The crude mixture was purified by preparative TLC (CH_2Cl_2/MeOH 9:1) resulting in a mixture of compounds **16a** and **16b** (ratio of 1.4:1) as a colorless oil (85 mg, 74%). ^{1}H NMR (300 MHz, $CDCl_3$) peaks assigned to compound **16a**: $\delta=6.69$ (m, 1H), 5.56 (br s, 1H), 5.31 (d, $J=1.5$ Hz, 1H), 4.13 (m, 2H), 3.78 (s, 3H), 1.98 (d, $J=1.5$ Hz, 3H); Peaks assigned to compound **16b**: $\delta=6.56$ (m, 1H), 5.56 (m, 1H), 5.37 (br s, 1H), 4.13 (m, 2H), 3.78 (s, 3H), 2.09 (d, $J=1.5$ Hz, 3H); ^{13}C NMR (75 Mhz, $CDCl_3$): $\delta=169.4$, 162.2, 153.9, 134.3, 133.2, 89.4, 87.1, 86.7, 84.3, 52.6, 41.4, 14.0; HRMS (ESI+) *m/z* calcd for $C_{12}H_{12}O_4NF_3Na$ $[M+Na]^+$ 314.0616, found 314.0597.

*1,4-Dimethyl-3-trifluoromethyl-7-oxa-bicyclo[2.2.1]hepta-2,5-diene-2-carboxylic acid ethyl ester (**17**)*: 2,5-dimethylfuran (279 μL, 2.62 mmol) was added to a solution of ethyl 4,4,4-trifluoro-2-butynoate (436 mg, 2.62 mmol) in dioxane (0.5 mL). The mixture was heated to 103 °C under a nitrogen atmosphere and stirred overnight. The reaction mixture was cooled and diluted with CH_2Cl_2 after which all the solvents were evaporated. The crude product was purified by column chromatography ($MeOH/CH_2Cl_2$ 1:9) to obtain the pure product as a white solid (238 mg, 91%). ^{1}H NMR (300 MHz, $CDCl_3$): $\delta=7.01$ (d, $J=5.1$ Hz, 1H), 6.91 (d, $J=5.1$ Hz,

1H), 4.30 (m, 2H), 1.81 (s, 3H), 1.74 (s, 3H), 1.31 (t, $J=7.1$ Hz, 2H); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta=163.7$, 154.6, 149.0 (q, $J=35$ Hz), 147.5, 146.7, 121.9 (q, CF_3, $J=270$ Hz), 92.6, 91.4, 61.6, 15.1, 14.8, 13.9. Both HRMS and LRMS techniques were employed to acquire the mass of the described compound. Unfortunately, none of the techniques used gave a comprehensible mass spectrum.

*3-Trifluoromethyl-7-oxa-bicyclo[2.2.1]hepta-2,5-diene-2-carboxyl-glycine methyl ester (**18**)*: Oxanorbornadiene carboxylic acid 2 (20.6 mg, 0.1 mmol), H-Gly-OMe·HCl (13.8 mg, 0.11 mmol), and 4-(dimethylamino)-pyridine (DMAP, 24.2 mg, 0.2 mmol) were dissolved in 2 mL CH_2Cl_2 and cooled to 0 °C. 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC·HCl, 21 mg, 0.11 mmol) was added and the reaction mixture was stirred at 0 °C for 30 min. The mixture was allowed to warm to RT and was stirred for an additional 16 h. The reaction was quenched with 2 mL HCl (aq) (2 M) and extracted with EtOAc (2×5 mL). The combined organic layers were washed with brine (5 mL), dried over Na_2SO_4, and concentrated in vacuo. The crude mixture was purified by preparative TLC (CH_2Cl_2/MeOH 9:1) resulting in compound **18** as a slightly yellow solid (15.5 mg, 56%). $R_f=0.55$ (CH_2Cl_2/MeOH 9:1); ^{1}H NMR (400 MHz, $CDCl_3$): $\delta=7.33$ (dd, $J=5.3$, 2.0 Hz, 1H), 7.16 (dd, $J=5.3$, 2.0 Hz, 1H), 6.41 (br s, 1H, NH), 5.68 (m, 2H), 4.15 (dq, $J=18.5$, 18.5, 18.5, 5.2 Hz, 2H) 3.80 (s, 3H); ^{13}C NMR (50 MHz, $CDCl_3$) $\delta=166.6$, 154.9, 154.2, 150.7, 144.0, 142.7, [124.8, 118.6] CF_3, 85.1, 84.3; HRMS (ESI+) *m/z* calcd for $C_{11}H_{10}F_3NaNO_4$: 300.0460 $[M+Na]^+$, found 300.0459; FTIR ν_{max} (film): 2924, 1744, 1636, 1169, 1117, 886 cm^{-1}.

N-*{Boc}*-N′-*{5-methyl-3-trifluoromethyl-7-oxa-bicyclo[2.2.1]hepta-2,5-diene-2-carboxyl}-3,6-dioxaoctane-1,8-diamine (**20a**) and* N-*{Boc}*-N′-*{6-methyl-3-trifluoromethyl-7-oxa-bicyclo[2.2.1]hepta-2,5-diene-2-carboxyl}-3,6-dioxaoctane-1,8-diamine (**20b**)*: 4-(dimethylamino)-pyridine (DMAP, (50.6 mg, 0.41 mmol) was added to a solution of a mixture of compounds **15a** and **15b** (49 mg, 0.21 mmol) and 1-*N*-Boc-3,6-dioxa-8-octane-1,8-diamine (50.9 mg, 0.21 mmol) in dry CH_2Cl_2 (1.5 mL). The mixture was cooled to 0 °C, and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC·HCl, 44.1 mg, 0.23 mmol) was added slowly. The mixture was stirred for 30 min at 0 °C and 16 h at RT. The reaction mixture was acidified with HCl (2 M) to a pH of 1–2 and extracted with CH_2Cl_2 (2×5 mL). The combined organic layers were dried over $MgSO_4$, concentrated in vacuo, and purified by preparative TLC (CH_2Cl_2/MeOH 9:1) resulting in compounds **20a** and **20b** as a slightly yellow oil (44.4 mg, 47%) A mixture of two regioisomers was obtained in a ratio of 1:1.4 for **20b** and **20a** respectively. ^{1}H NMR (300 MHz, $CDCl_3$) peaks assigned to compound **20a**: $\delta=6.70$ (d, $J=6.7$ Hz, 1H), 6.44 (br s, 1H, NH), 5.54 (s, 1H), 5.28 (s, 1H), 4.96 (br s, 1H, NH), 3.60–3.52 (m, 10H), 3.30 (q, $J=5.2$ Hz, 2H), 1.97 (d, $J=1.6$ Hz, 3H), 1.44 (s, 9H); Peaks assigned to compound **20b**: $\delta=6.55$ (d, $J=6.6$ Hz, 1H), 6.44 (br s, 1H, NH), 5.54 (s, 1H), 5.34 (s, 1H), 4.96 (br s, 1H, NH), 3.30 (q, $J=5.2$ Hz, 2H), 3.60–3.52 (m, 10H), 3.30 (q, $J=5.2$ Hz, 2H), 2.10 (d, $J=1.9$ Hz, 3H), 1.44 (s, 9H); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta=162.4$, 155.9, 154.5, 153.8, 134.8, 133.1, 122.8 (q, CF_3), 89.4, 86.7, 84.1, 70.4, 70.2, 70.1, 69.4, 40.3, 39.4, 28.4, 14.0; HRMS (ESI+): *m/z* calcd for $C_{20}H_{30}F_3N_2O_6$ 451.2056 $[M+H]^+$, found 451.2078.

N,N′-*{5-methyl-3-trifluoromethyl-7-oxa-bicyclo[2.2.1]hepta-2,5-diene-2-carboxyl}-3,6-dioxaoctane-1,8-diamine (**21a**) and* N,N′-*{6-methyl-3-trifluoromethyl-7-oxa-bicyclo[2.2.1]hepta-2,5-diene-2-carboxyl}-3,6-dioxaoctane-1,8-diamine (**21b**)*: Trifluoroacetic acid (TFA, 0.5 mL, 2.85 mmol) was added dropwise to a cooled solution (0 °C) of a mixture of **20a** and **20b** (42 mg, 0.093 mmol) in dry CH_2Cl_2 (2 mL). The reaction was stirred at 0 °C for 1 h after which the reaction was complete. The solvent was evaporated and the crude mixture was dissolved in H_2O (5 mL) and dioxane (5 mL) and freeze-dried to afford compound **21a** and **21b** as a light yellow solid (43.1 mg, +99%). A mixture of two regioisomers was obtained in a ratio of 1:1.4 for **21b** and **21a** respectively. ^{1}H NMR (400 MHz, $CDCl_3$) peaks assigned to compound **21a** $\delta=7.84$ (br s, 3H, NH_3), 7.92 (br s, 1H, NH), 6.66 (s, 1H), 5.52 (s, 1H), 5.27 (s, 1H), 3.70–3.46 (m, 10H), 3.15 (m, 2H), 1.96 (s. 3H); Peaks assigned to compound **21b** $\delta=7.84$ (br s, 3H, NH_3), 7.92 (br s, 1H, NH), 6.55 (s, 1H), 5.52 (s, 1H), 5.31 (s, 1H), 3.70–3.46 (m, 10H), 3.15 (s, 2H), 2.06 (s, 3H); ^{13}C NMR (50 MHz, $CDCl_3$) $\delta=159.1$, 154.0, 151.1, 134.8, 133.1, 105.0, 103.0, 101.0, 100.0, 86.8, 86.5, 40.3, 14.0; HRMS (ESI+): *m/z* calcd for $C_{15}H_{22}F_3N_2O_4$ $[M+H]^+$ 351.1532, found 351.1541

N-*{2-*O-tert-*butyl-DTPA-acetamide}*,N′-*{5-methyl-3-trifluoromethyl-7-oxa-bicyclo[2.2.1]hepta-2,5-diene-2-carboxyl}-3,6-dioxaoctane-1,8-diamine·TFA (**22a**) and* N-*{2-*O-tert-*butyl-DTPA-acetamide}*,N′-*{6-methyl-3-trifluoromethyl-7-oxa-bicyclo[2.2.1]hepta-2,5-diene-2-carboxyl}-3,6-dioxaoctane-1,8-diamine·TFA (**22b**)*: Compounds **21a** and **21b** (39 mg, 0.084 mmol) were dissolved in dry CH_2Cl_2 (2 mL), and 4-(dimethylamino)-pyridine (DMAP, 20.4 mg, 0.17 mmol) and diethylenetriamine-*N*,*N*,*N*′′,*N*′′-tetra-*tert*-butyl acetate-*N*′-acetic acid ((DTPA-*tert*-butyl ester) 53 mg, 0.084 mmol) were added. The mixture was cooled to 0 °C followed by addition of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC·HCl, 17.5 mg, 0.09 mmol). After 1 h stirring at 0 °C the mixture was allowed to warm to RT and was stirred for an additional 4 h. The reaction mixture was quenched with 1 M HCl (2 mL) and the water layer was extracted with CH_2Cl_2 (2×2 mL). The combined organic layers was dried over Na_2SO_4 and subsequently evaporated. The crude product was purified by preparative TLC (CH_2Cl_2/MeOH 9:1) to obtain pure product as a light brown oil (35 mg, 44%). A mixture of two regioisomers was obtained in a ratio of 1:1.4 for **22b** and **22a**, respectively. ^{1}H NMR (400 MHz, $CDCl_3$) peaks assigned to compound **22a** $\delta=$ 8.22 (br s, 1H, NH), 6.75 (br s, 1H, NH), 6.69 (t, $J=1.9$ Hz, 1H), 5.53 (s, 1H), 5.27 (s, 1H), 3.60–3.39 (m, 18H), 3.11 (s, 2H), 2.77 (t, $J=$ 6.5 Hz, 2H), 2.61 (t, $J=6.5$ Hz, 2H), 1.96 (d, $J=1.3$ Hz, 3H), 1.43 (s, 36H); Peaks assigned to compound **22b** $\delta=8.22$ (br s, 1H, NH), 6.75 (br s, 1H, NH), 6.54 (t, $J=1.9$ Hz, 1H), 5.51 (s, 1H), 5.33 (s, 1H), 3.60–3.39 (m, 18H), 3.11 (s, 2H), 2.77 (t, $J=6.5$ Hz, 2H), 2.61 (t, $J=$ 6.5 Hz, 2H), 2.09 (d, $J=1.7$ Hz, 3H), 1.43 (s, 36H); ^{13}C NMR (75 MHz, $CDCl_3$) $\delta=172.2$, 170.5, 162.5, 155.2 (q), 153.7, 142.5, 134.4, 122.8 (q, CF_3), 89.3, 86.7, 84.0, 81.0, 70.5, 70.0, 69.8, 69.4, 58.6, 55.8, 53.8, 52.1, 39.4, 38.6, 28.1, 14.0; HRMS (ESI+): *m/z* calcd for $C_{45}H_{75}F_3N_5O_{13}$ $[M+H]^+$ 950.5313, found 950.5361.

N-*{2-DTPA-acetamide}*,N′-*{5-methyl-3-trifluoromethyl-7-oxa-bicyclo-[2.2.1]hepta-2,5-diene-2-carboxyl}-3,6-dioxaoctane-1,8-diamine (**23a**) and* N-*{2-DTPA-acetamide}*,N′-*{6-methyl-3-trifluoromethyl-7-oxa-bicyclo[2.2.1]hepta-2,5-diene-2-carboxyl}-3,6-dioxaoctane-1,8-diamine (**23b**)*: TFAA (200 μL) was added to a solution of compounds **22a** and **22b** (25 mg, 0.026 mmol) in CH_2Cl_2 (2 mL). This mixture was stirred for six days (until MS analysis did not show starting material). The product was obtained as a white solid in quantitative yield (+99%) after lyophilization from H_2O/dioxane. A mixture of two regioisomers was obtained in a ratio of 1:1.4 for **23b** and **23a** respectively. ^{1}H NMR (400 MHz, CD_3OD) peaks assigned to compound **23a** $\delta=6.96$ (t, $J=1.7$ Hz, 1H), 5.50 (s, 1H), 5.36 (s, 1H), 4.31 (br s, 2H), 3.66–3.58 (m, 18H), 3.49–3.42 (m, 2H), 3.30–3.15 (m, 8H), 1.99 (d, $J=1.5$ Hz, 3H); Peaks assigned to compound **23b** $\delta=6.63$ (m, 1H), 5.56 (s, 1H), 5.30 (s, 1H), 4.31 (br s, 2H), 3.66–3.58 (m, 18H), 3.48–3.42 (m, 2H,), 3.28–3.14 (m, 8H), 2.07 (d, $J=1.7$ Hz, 3H); ^{13}C NMR (75 MHz, CD_3OD) $\delta=174.6$ (4C), 167.2, 165.6, 155.8, 135.4, 90.5, 87.7, 84.1, 71.4, 71.3, 70.3, 68.2, 55.9, 55.6, 54.3, 50.9, 40.5, 40.4, 14.0; HRMS (ESI+): *m/z* calcd for $C_{29}H_{43}F_3N_5O_{13}$ $[M+H]^+$ 726.2809, found 726.2837.

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Keywords: bioconjugation · cyclic-RDG · cycloadditions · oxanorbornadiene · tandem crDA

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