JOC Article

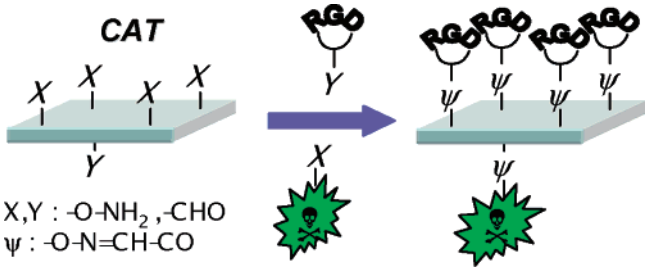
Chemoselectively Addressable Template: A Valuable Tool for the Engineering of Molecular Conjugates

Elisabeth Garanger, Didier Boturyn,* Olivier Renaudet, Eric Defrancq, and Pascal Dumy*

LEDSS, UMR CNRS 5616, ICMG FR-2607, Université Joseph Fourier, BP 53, 38041 Grenoble Cedex 9, France

didier.boturyn@ujf-grenoble.fr; pascal.dumy@ujf-grenoble.fr

Received December 12, 2005



We herein report the modular design and the synthesis of new molecular conjugates, which can combine a cell targeting function (ligand domain) with potential cytotoxic molecules (effector domain). The present approach utilizes a cyclic peptide template, Chemoselectively Addressable Template (CAT) as a key intermediate. These CAT molecules exhibit two independent and chemically addressable domains which permits the sequential and regioselective assembly of different ligand and/or effector domains. The attachment of various units to the template was achieved by the formation of iterative oxime bonds. The chemoselective oxime bonds were produced by the reaction of glyoxylyl aldehyde groups obtained from serine precursors. The process was further developed to prevent transoximation reactions. RAFT-(*c*[-RGDfK-])$_4$, a synthetic vector targeting the tumor-associated a $\alpha_V\beta_3$ integrin was prepared and coupled to either a cytotoxic peptide or oligonucleotide as an illustration of present approach. The potential application of this approach has been further demonstrated by the synthesis of high molecular weight compounds such as RAFT(*c*[-RGDfK-])$_{16}$, a $\alpha_V\beta_3$-targeting ligand of high valency index.

Introduction

Chemotherapy has been the main alternative for the systemic treatment of advanced or metastatic cancers for more than 50 years. Nevertheless, common chemotherapeutic drugs cause serious side effects due to the toxicity to normal cells and noncancerous tissues. Benefits of the drugs are also often limited because of intrinsic or acquired resistance of tumor cells. The efficiency of the treatment can be increased by escalating the doses, but this option worsens to the toxicity problem and thus rarely considered. Vectorization of therapeutic molecules to primary tumors and to their metastases therefore represents a major challenge for improving current cancer therapies.[1] Development of tools endowed with tumor-targeting functions and carrying cytotoxic components will enable the specific delivery of chemotherapeutics to malignant tissues, thereby increasing their local efficacy while limiting their peripheral toxicity. Drug carriers can also pave the way for the use of new classes of therapeutic agents based on biomolecules (e.g., antisense DNA, siRNA, antimicrobial peptide, toxin protein). Biomolecules that fight specifically against protagonists of neoplastic development frequently have physicochemical properties that are unfavorable to the crossing of biological and enzymatic barriers, thereby limiting their use as drugs. Typically, efforts in developing antisense oligonucleotides as drugs have failed as a result of their poor cellular uptake. From these considerations, molecular conjugate vectors that are capable of reaching the desired target cells in vivo and breaching the membrane barrier represent a challenge and a valuable paradigm for future diagnosis and therapeutic applications. Such conjugates are composed of a cell targeting function (the "ligand domain") providing the homing function of the vector and are linked to a functional unit such as a toxic molecule or a probe.

* To whom correspondence should be addressed. Tel: +33-476-635-545 Fax: +33-476-514-946.

(1) Magrath, I. T. *Int. J. Cancer* **1994**, *56*, 163−166.

10.1021/jo0525480 CCC: $33.50
Published on Web 02/10/2006

In this context, we have recently developed a nonviral system named RAFT(*c*[−RGDfK−])$_4$ targeting the tumor-associated $\alpha_V\beta_3$ integrin.[2] The core of the vector is composed of a cyclic decapeptide template (RAFT) presenting two distinct domains (upper and lower face of the template).[3] These domains allow the spatial control of the attached functions to the vector thereby preventing steric hindrance. The multimeric presentation of *cyclo*[−RGDfK−] moieties[4] provides the targeting duty and promotes multivalent interactions of the vector with the target receptors, hence promoting receptor-mediated internalization.[2] The lower face of the RAFT(*c*[−RGDfK−])$_4$ vector is devoted to the transport of the molecules of biological interest (cytotoxic or contrast agents, i.e., "effector" molecules).[5] These encouraging results lead us to explore the properties of this vector prototype to carry biomolecules such as nucleic acids, peptides or proteins inside the cells.

Incompatibilities between chemistries of peptides, nucleic acids, carbohydrates, and other organic compounds represent major hurdles in synthesizing such conjugates. To overcome these limitations, the fragment-coupling approach using chemoselective ligations has emerged as a general and suitable strategy.[6] Formation of chemoselective bonds, such as disulfides, thioesters, thiazolidines, hydrazones, or oximes, proved highly efficient for the preparation of various relevant macromolecules such as synthetic vaccines,[7] synthetic proteins,[8] antiviral drugs,[9] or anticancer agents.[10] Previous works have focused on the use of the oxime linkage allowing the conjugation of peptides, carbohydrates, and oligonucleotides.[11] The oxime bond results from the reaction between an aldehyde and an oxyamine function under mild conditions (pH 3−5)[12] and has been shown to be stable in vivo.[13] The oxime strategy has proven successful for the synthesis of artificial proteins,[8] for the engineering of biomimetic surfaces,[14] as well as for the sensitive structure analysis of oligosaccharides.[15] More recently, it has been used for the design of our prototype of $\alpha_V\beta_3$ integrin-targeting vector where it proved flexible and adapted to the synthesis of a broad series of compounds.[2]

Pursuing our work, we envisioned to conjugate effector elements to the RAFT(*c*[−RGDfK−])$_4$ vector. This implies two successive oxime ligations for the sequential functionalization of the RAFT core with the targeting *c*[−RGDfK−] peptides and with the active molecule. Some examples of successive oxime formations have been already reported. The consecutive formation of two oxime bonds requires orthogonal protecting groups, either at the aminooxy function or at the aldehyde group, allowing mostly three strategies. The first one consists of using orthogonal protection schemes for distinguishing the functions involved in the successive steps. For instance, addressable aminooxy functions were incorporated into a peptide to successively attach a variety of aldehyde-containing compounds[16] or dialdehyde compounds were used to form successive oximes with appropriate aminooxy derivatives for the preparation of synthetic vaccines[17] and 3′,5′-bifunctionalized oligonucleotide conjugates.[18] This strategy naturally required regioselective protecting groups whose removal conditions are suitable for the oxime bond. From these and further studies, it appeared that the oxime bond obtained from aliphatic aldehyde is sensitive to acidic conditions (pH < 4) that cause its hydrolysis.[17,18] Conversely, the second strategy involves masked oxyamine functions. This permitted Reymond and co-workers to form linear oxime oligomers by iterative couplings from aldehyde building blocks bearing precursors of oxyamines.[19] The method adopted by Rose and co-workers, allows two consecutive oxime reactions thanks to the use of a serine as a masked glyoxylyl aldehyde.[20] The latter is then obtained after a periodate oxidation under mild conditions, preserving the oxime from hydrolysis.[21] Additionally, the oxime bond formed from a glyoxylyl aldehyde is much more stable than an oxime originating from an aliphatic aldehyde. It is noteworthy that undesirable transoximation reactions, favored by temperature and acidic catalysis, can occur between the oxime bond and the aminooxy-containing compound.[17,22] These side reactions, also named "oxime exchanges",

(2) Boturyn, D.; Coll, J.-L.; Garanger, E.; Favrot, M.-C.; Dumy, P. *J. Am. Chem. Soc.* **2004**, *126*, 5730−5739.

(3) (a) Dumy, P.; Eggleston, I. M.; Cervigni, S.; Sila, U.; Sun, X.; Mutter, M. *Tetrahedron Lett.* **1995**, *38*, 1255−1258. (b) Peluso, S.; Ruckle, T.; Lehmann, C.; Mutter, M.; Peggion, C.; Crisma, M. *ChemBioChem* **2001**, *2*, 432−437 (c) Dumy, P.; Eggleston, I. M.; Esposito, G.; Nicula, S.; Mutter, M. *Biopolymers* **1996**, *39*, 297−308.

(4) (a) Haubner, R.; Gratias, R.; Diefenbach, B.; Goodman, S. L.; Jonczyk, A.; Kessler, H. *J. Am. Chem. Soc.* **1996**, *118*, 7461−7472. (b) Haubner, R.; Finsinger, D.; Kessler, H. *Angew. Chem., Int. Ed. Engl.* **1997**, *36*, 1374−1389.

(5) Garanger, E.; Boturyn, D.; Jin, Z.; Dumy, P.; Favrot, M.-C.; Coll, J.-L. *Mol. Therapy* **2005**, *12*, 1168−1175.

(6) For reviews on chemoselective ligations, see: (a) Dawson, P. E.; Kent, S. B. H. *Annu. Rev. Biochem.* **2000**, *69*, 923−960. (b) Lemieux, G. A.; Bertozzi, C. R. *Trends Biotechnol.* **1998**, *16*, 506−513.

(7) (a) Verez-Bencomo, V.; Fernandez-Santana, V.; Hardy, E.; Toledo, M. E.; Rodríguez, M. C.; Heynngnezz, L.; Rodriguez, A.; Baly, A.; Herrera, L.; Izquierdo, M.; Villar, A.; Valdés, Y.; Cosme, K.; Deler, M. L.; Montane, M.; Garcia, E.; Ramos, A.; Aguilar, A.; Medina, E.; Toraño, G.; Sosa, I.; Hernandez, I.; Martinez, R.; Muzachio, A.; Carmenates, A.; Costa, L.; Cardoso, F.; Campa, C.; Diaz, M.; Roy, R. *Science* **2004**, *305*, 522−525. (b) Zeng, W.; Jackson, D. C.; Murray, J.; Rose, K.; Brown, L. E. *Vaccine* **2000**, *18*, 1031−1039. (c) Rose, K.; Zeng, W.; Brown, L. E.; Jackson, D. C. *Mol. Immunol.* **1995**, *32*, 1031−1037. (d) Zeng, W.; Ghosh, S.; Macris, M.; Pagnon, J.; Jackson, D. C. *Vaccine* **2001**, *19*, 3843−3852.

(8) (a) Rose, K. *J. Am. Chem. Soc.* **1994**, *116*, 30−33. (b) Canne, L. E.; Ferré-D' Amaré, A. R.; Burley, S. K.; Kent S. B. H. *J. Am. Chem. Soc.* **1995**, *117*, 2998−3007. (c) Hartley, O.; Gaertner, H.; Wilken, J.; Thompson, D.; Fish, R.; Ramos, A.; Pastore, C.; Dufour, B.; Cerini, F.; Melotti, A.; Heveker, N.; Picard, L.; Alizon, M.; Mosier, D.; Kent, S. B.; Offord, R. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 16460−16465.

(9) Naicker, K. P.; Li, H.; Heredia, A.; Song H.; Wang, L.-X. *Org. Biomol. Chem.* **2004**, *2*, 660−664.

(10) Henry, M. D.; Wen, S.; Silva, M. D.; Chandra, S.; Milton, M.; Worland, P. J. *Cancer Res.* **2004**, *64*, 7995−8001.

(11) (a) Forget, D.; Boturyn, D.; Defrancq, E.; Lhomme, J.; Dumy, P. *Chem. Eur. J.* **2001**, *7*, 3976−3984. (b) Zatsepin, T. S.; Stetsenko, D. A.; Arzumanov, A. A.; Romanova, E. A.; Gait, M. J.; Oretskaya, T. S. *Bioconjugate Chem.* **2002**, *13*, 822−830. (c) Renaudet, O.; Dumy, P. *Org. Lett.* **2003**, *5*, 243−246. (d) Grigalevicius, S.; Chierici, S.; Renaudet, O.; Lo-Man, R.; Deriaud, E.; Leclerc, C.; Dumy, P. *Bioconjugate Chem.* **2005**, *16*, 1149−1159. (e) Singh, Y.; Renaudet, O.; Defrancq, E.; Dumy, P. *Org. Lett.* **2005**, *7*, 1359−1362.

(12) Jenks, W. P. *J. Am. Chem. Soc.* **1959**, *81*, 475−481.

(13) (a) Nardin, E. H.; Calvo-Calle, J. M.; Oliveira, G. A.; Clavijo, P.; Nussenzweig, R.; Simon, R.; Zeng, W.; Rose, K. *Vaccine* **1998**, *16*, 590−600. (b) Nardin, E. H.; Calvo-Calle, J. M.; Oliveira, G. A.; Nussenzweig, R. S.; Schneider, M.; Tiercy, J.-M.; Loutan, L.; Hochstrasser, D.; Rose, K. *J. Immunol.* **2001**, *166*, 481−489.

(14) Chen, X.; Soo Lee, G.; Zettl, A.; Bertozzi, C. R. *Angew. Chem., Int. Ed.* **2004**, *43*, 6112−6116.

(15) Zhao, Y.; Kent S. B. H.; Chait, B. T. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 1629−1633.

(16) Cervigni, S. E.; Dumy, P.; Mutter, M. *Angew. Chem., Int. Ed. Engl.* **1996**, *35*, 1230−1232.

(17) (a) Cremer, G.-A.; Bureaud, N.; Lelièvre, D.; Piller, V.; Piller, F.; Delmas, A. *Chem. Eur. J.* **2004**, *10*, 6353−6360. (b) Lelievre, D.; Buré, C.; Laot, F.; Delmas, A. *Tetrahedron Lett.* **2001**, *42*, 235−238.

(18) Edupuganti, O. P.; Singh, Y.; Defrancq, E.; Dumy, P. *Chem. Eur. J.* **2004**, *10*, 5988−5995.

(19) Renaudet, O.; Reymond, J.-L. *Org. Lett.* **2003**, *5*, 4693−4696.

(20) Chen, J.; Zeng, W.; Offord, R.; Rose, K. *Bioconjugate Chem.* **2003**, *14*, 614−618.

(21) Geoghegan, K. F.; Stroh, J. G. *Bioconjugate Chem.* **1992**, *3*, 138−146.

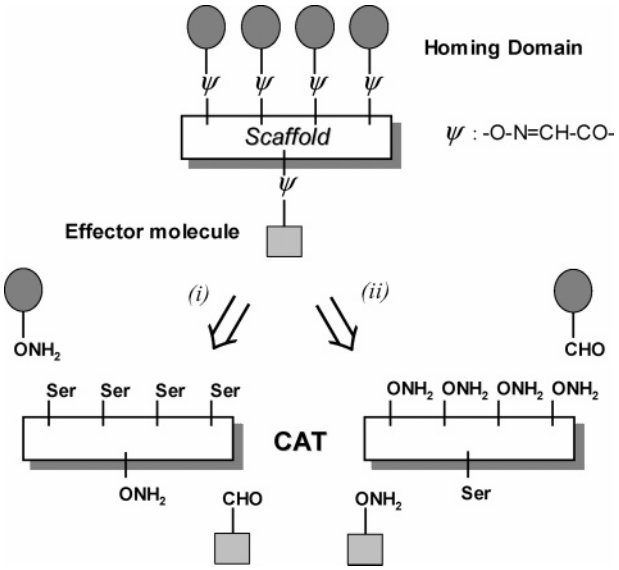


FIGURE 1. Chemoselectively addressable templates (CATs) resulting from two alternative retrosynthetic schemes: (i) the downside-up strategy and (ii) the upside-down strategy.

have been rarely mentioned in the literature, and their occurrence during the successive ligations has never been studied previously.

In this work, we describe the synthesis of molecular conjugate vectors through a process implying two successive oxime bond formations from glyoxylyl aldehyde and excluding side reactions. The synthetic scheme involves a key intermediate, namely chemoselectively addressable template (CAT), presenting free aminooxy residues on one side of the cyclopeptide scaffold and masked aldehyde groups (serine residues) on the other side (Figure 1). The CAT was first employed in a model reaction in order to detect oxime exchange byproducts during the coupling. The procedure was then applied to the conjugation of the RAFT-(c[−RGDfK−])$_4$ vector to either a cytotoxic peptide or an oligonucleotide. The strategy was finally validated and extended to functionalization with up to sixteen simultaneous oxime bonds formation to access the macromolecule RAFT(c[−RGDfK−])$_{16}$, a high valency $\alpha_V\beta_3$-integrin targeting compound.

Results and Discussion

Two strategies were considered to build the different conjugates, depending on the nature of the effector to be carried by the vector (Figure 1): (i) the first oxime bond was performed on the lower face of the CAT containing aminooxy function for grafting the effector molecule prior to the targeting entities (*cyclo*[−RGDfK−] peptides); (ii) the oxime ligation was first carried out on the upper face to introduce the targeting peptides prior to the effector molecule.

Bis-conjugation via the "Downside-Up" Strategy (Figure 1, Strategy i). CATs **1** and **2** represent the key intermediates that were used to perform the first oxime ligation on the lower face of the template (Scheme 1). These were prepared by either a combination of solid- and solution-phase synthesis (compound **1**) or by a solid-phase synthesis alone (compound **2**). Linear decapeptide sequences were assembled using the standard Fmoc/*t*-Bu approach, and their cyclization was performed as described earlier.[2] Convenient choice of lysine side-chain protections and their relative positioning within the peptide primary sequence provided cyclodecapeptides **3** and **4** containing orthogonally protected attachment sites pointing to opposite faces of the template backbone (PG1 and PG2 groups in Scheme 1).

To prepare compound **5**, Boc protecting groups of **3** were removed by treatment with trifluoroacetic acid (TFA), and Boc-Ser(*t*-Bu)-OH moieties were incorporated next using standard PyBOP coupling chemistry. On the other face of the template, Alloc moieties were removed using the well-established Pd^0/$PhSiH_3$ procedure,[23] and Boc-protected aminooxy functions were introduced using the succinimide ester of *N*-Boc-*O*-(carboxymethyl)hydroxylamine. The key CAT compound **1**, bearing free aminooxy functions and fully deprotected serine residues, was obtained after acidic deprotection of **5** with TFA followed by RP-HPLC purification in 20% overall yield. The CAT **2** was synthesized using an entirely solid-phase supported process. Such efforts in developing "all-on-support syntheses" are valuable for limiting workup steps and reducing the duration of the synthesis. D-Glutamine (q) was used as the first amino acid at the C-terminus of the linear decapeptide and linked through its side-chain to an acid labile resin (Rink Amide MBHA). The D-amino acid permits cyclization on solid support with a type II β-turn, required for a conformationally defined scaffold. *p*-Nitrobenzyloxycarbonyl (pNZ) and 1-(4,4-dimethyl-2,6-dioxocyclohexylidene)ethyl (Dde) protecting groups were chosen for their orthogonality and their compatibility with the solid support. To introduce protected serines, pNZ groups were first removed using the reducing agent $SnCl_2$[24] and Boc-Ser(*t*-Bu)-OH was further coupled. Subsequent hydrazinolysis to cleave Dde groups followed by coupling of the Boc-protected aminooxy residues afforded compound **6** on the solid support. Standard acid conditions using triisopropylsilane (TIS) and water as scavengers gave CAT **2** in 20% overall yield after RP-HPLC purification.

To investigate possible occurrence of transoximation reactions as has been previously observed,[22] we first set up a model reaction to study the stepwise chemoselective ligations via oxime bonds (Scheme 2). For this purpose, RGD-peptides containing suitable functions (i.e., aldehyde or aminooxy compounds **7** and **8**, respectively, in Scheme 2) were prepared by solid-phase synthesis as described earlier [25] and successively assembled onto the CATs **1** and **2**. The first oxime ligation between the aldehyde-containing peptide **7** and CAT **1** was carried out in aqueous solution at pH 4.6 to maximize the rate of oxime formation.[12] Mild periodate oxidation was then used to generate aldehydic functions from serine moieties,[21] yielding compound **9** in 70% yield after RP-HPLC purification. It must be noted that under the oxidation conditions the oxime bonds were found to be stable.

To assess the ability of aminooxy peptide **8** to react selectively with template **9** without transoximation reaction, the second chemoselective ligation was carried out and carefully monitored by RP-HPLC (Figure 2). In parallel, control peptide **10**, the

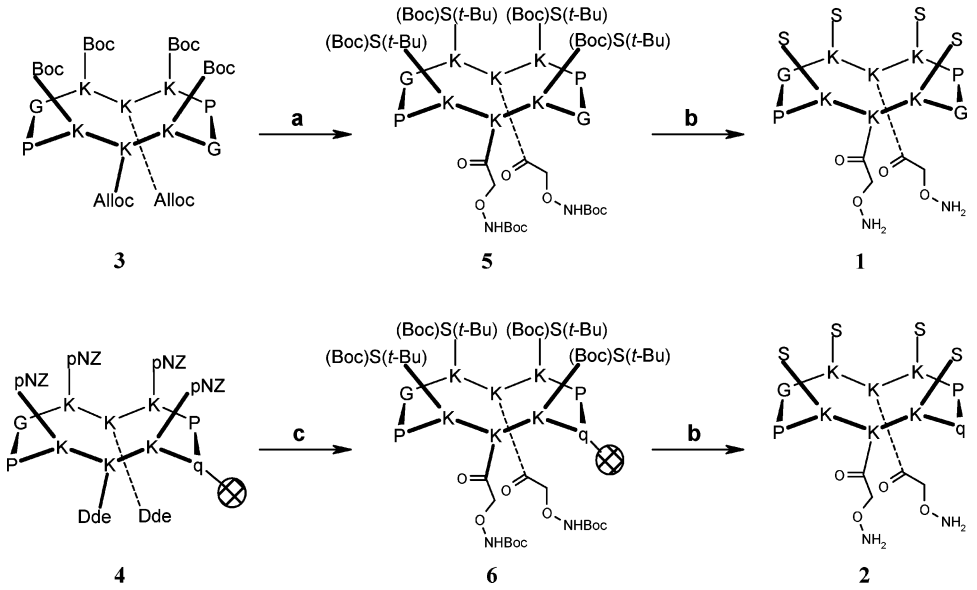
(22) (a) Polyakov, V. A.; Nelen, M. I.; Nazarpack-Kandlousy, N.; Ryabov, A. D.; Eliseev A. V. *J. Phys. Org. Chem.* **1999**, *12*, 357−363. (b) Axelson, M. *Anal. Biochem.* **1978**, *86*, 133−141. (c) Shao, H.; Crnogorac, M. M.; Kong, T.; Chen, S.-Y.; Williams, J. M.; Tack, J. M.; Gueriguian, V.; Cagle, E. N.; Carnevali, M.; Tumelty, D.; Paliard, X. Miranda, L. P.; Bradburne, J. A.; Kochendoerfer G. G. *J. Am. Chem. Soc.* **2005**, *127*, 1350−1351.

(23) Thieret, N.; Alsina, J.; Giralt, E.; Guibé, F.; Albericio, F. *Tetrahedron Lett.* **1997**, *38*, 7275−7278.

(24) Hocker, M. D.; Caldwell, C. G.; Macsata, R. W.; Lyttle, M. H. *Peptide Res.* **1995**, *8*, 310−315.

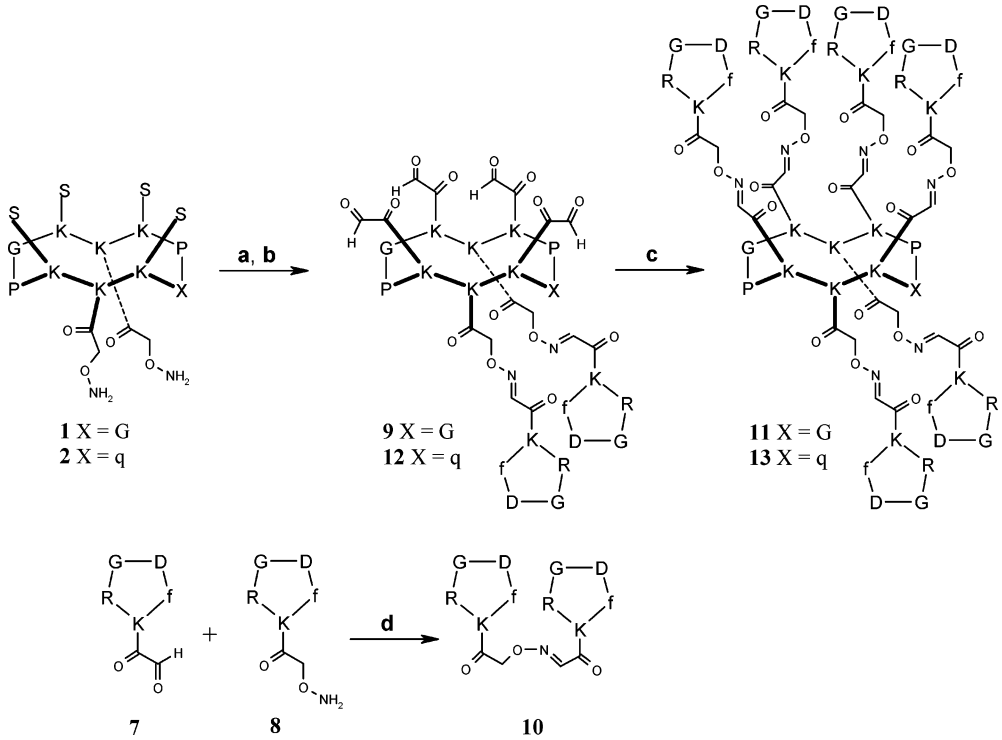
(25) Boturyn, D.; Dumy, P. *Tetrahedron Lett.* **2001**, *42*, 2787−2790.

SCHEME 1. CATs 1 and 2[a]



[a] Reagents and conditions: (a) 50% TFA, CH_2Cl_2, 15 min; BocSer(*t*-Bu)OH, PyBOP, DIPEA, DMF, 30 min; $Pd(PPh_3)_4$, $PhSiH_3$, CH_2Cl_2, 1 h; $BocNHOCH_2CO$-Succ, DIPEA, DMF, 30 min; (b) 95% TFA, TIS, H_2O; (c) $SnCl_2$, PhOH, AcOH, DMF, 1 h; BocSer(*t*-Bu)OH, PyBOP, DIPEA, DMF, 30 min; 2% hydrazine, DMF, 30 min; $BocNHOCH_2CO$-Succ, DMF, 30 min.

SCHEME 2. Model Reaction to Study Oxime Exchange: Successive Oxime Ligations of RGD Peptides[a]



[a] Reagents and conditions: (a) 0.1 M AcO^-Na^+ pH 4.6/CH_3CN (1:1), **7**; (b) $NaIO_4$, H_2O; (c) 0.1 M AcO^-Na^+ pH 4.6/CH_3CN (1:1), **8**; (d) 0.1 M AcO^-Na^+ pH 4.6/CH_3CN (1:1).

byproduct that could result from an oxime exchange, was prepared from RGD-peptides **7** and **8** (Scheme 2). An excess of aminooxy peptide **8** (6 equiv) was used to form the multitopic ligand architecture. The reaction proceeded to completion to yield the desired conjugate **11** as the major product. No byproduct **10** could be detected (Figure 2A). Subsequent purification gave compound **11** in 70% isolated yield. The peptide **13** was obtained from CAT **2** using an identical strategy in 50% overall yield. These conjugates **11** and **13** were characterized by ES-MS and the observed molecular weights were found in excellent agreement with the calculated values.

This preliminary study demonstrated that transoximation reactions did not occur during the second coupling reaction even when an excess of aminooxy compound was used. Therefore, with an easy and efficient technique for stepwise oxime ligations in hand, the CAT intermediate **1** with two functional domains

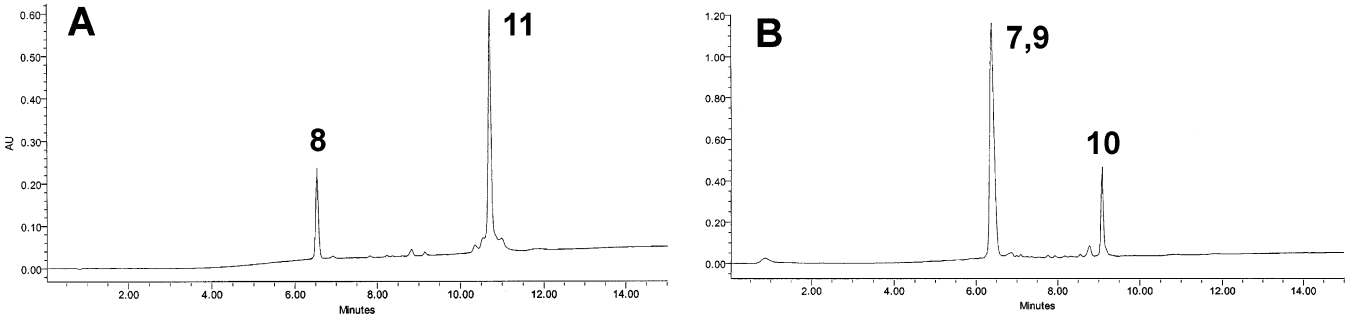
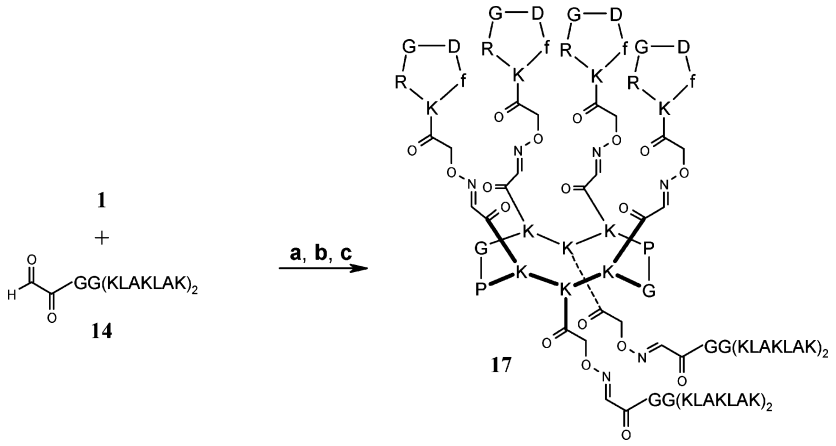


FIGURE 2. RP-HPLC profiles of reaction mixtures of (A) conjugate **11** and (B) byproduct **10**.

SCHEME 3. Preparation of KLA-Conjugated RAFT(*c*[−RGDfK−])$_4$ Vector 17 from CAT 1[a]



[a] Reagents and conditions: (a) 0.1 M AcO$^-$Na$^+$ pH 4.6; (b) $NaIO_4$, H_2O; (c) 0.1 M AcO$^-$Na$^+$ pH 4.6/CH_3CN (1:1), **8**.

was used to prepare a conjugate combining the multivalent cluster of $\alpha_V\beta_3$-specific RGD-peptides and the antimicrobial peptide $(KLAKLAK)_2$, **26**. This peptide is commonly named "KLA" and has been reported to induce apoptosis of tumor cells in vitro and in vivo.

The preparation of the "KLA" peptide **14** bearing an aldehyde moiety at the N-terminal was accomplished by solid-phase synthesis followed by subsequent periodate oxidation at the N-terminal serine residue.

Chemoselective introduction of "KLA" peptide **14** on CAT **1** was carried out in sodium acetate buffer (pH 4.6) to obtain the lower face-functionalized compound **15** in 50% yield after purification (Scheme 3).[27] It is worth noting that from **15** it could be still possible to vary the nature of the targeting ligands, thus allowing the specific delivery of the toxic KLA peptide to different kind of tumors/tissues/organs. Subsequent oxidative cleavage at serine residues using an excess of $NaIO_4$-generated template **16** presenting four aldehyde functions. Finally, coupling reaction with **8** (6 equiv) using the same conditions as above gave the desired conjugate **17** in 80% yield. In this last step, we did not detect any oxime exchange. This compound along with other derivatives is currently undergoing thorough biological evaluations. Preliminary results suggest a strong antiproliferative activity for **17** on HEK293(β_3) cells. At 100 μM, **17** inhibits cellular growth by 80% while neither KLA alone or *c*[−RGDfK(KLA)−] have any effect even at 400-fold higher doses.[28]

Bis-conjugation via the "Upside-Down" Strategy (Figure 1, Strategy ii). To incorporate high molecular weight elements (nucleic acids, proteins) into our vector, it is essential to perform their ligation in the final step, thus allowing easier manipulation and purification. Contrary to the first approach, the $\alpha_V\beta_3$-targeting components were attached to the scaffold first. This strategy has advantage that it permits the ligation of a pool of different effector elements from a common intermediate at the last step. CAT **18** represents the key intermediate used to perform the oxime ligation on the upper face of the template first (Scheme 4).

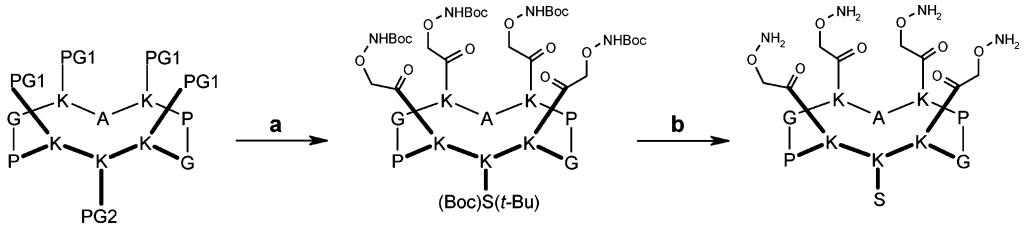
The preparation of CAT **18** was performed in a five-step procedure from cyclodecapeptide **19** containing orthogonal Boc and Alloc protecting groups on the upper and lower face, respectively. This compound **19** was synthesized using a protocol similar to that for compound **3**. To append the aminooxy moieties on the upper face of the template, Boc protecting groups from **19** were first removed and the crude compound was acylated using the succinimide ester of *N*-Boc-*O*-(carboxymethyl)hydroxylamine (Scheme 4). The Alloc group was then eliminated under standard conditions. The subsequent coupling of protected serine and the removal of all protecting groups gave the key CAT **18** in 42% overall yield after RP-HPLC purification.

(26) Mai, J. C.; Mi, Z.; Kim, S.-H.; Ng, B.; Robbins, P. D. *Cancer Res.* **2001**, *61*, 7709−7712. (b) Javadpour, M. M.; Juban, M. M.; Lo, W. C.; Bishop, S. M.; Alberty, J. B.; Cowell, S. M.; Becker, C. L.; McLaughlin, M. L., *J. Med. Chem.* **1996**, *39*, 3107−3113.

(27) After reaction, 2 equiv of benzyloxyamine was added to trap the slight excess of remaining aldehyde **16** and to facilitate the HPLC separation.

(28) Jin, Z.; Garanger, E.; Boturyn, D.; Favrot, M.-C.; Dumy, P.; Coll, J.-C. Unpublished results.

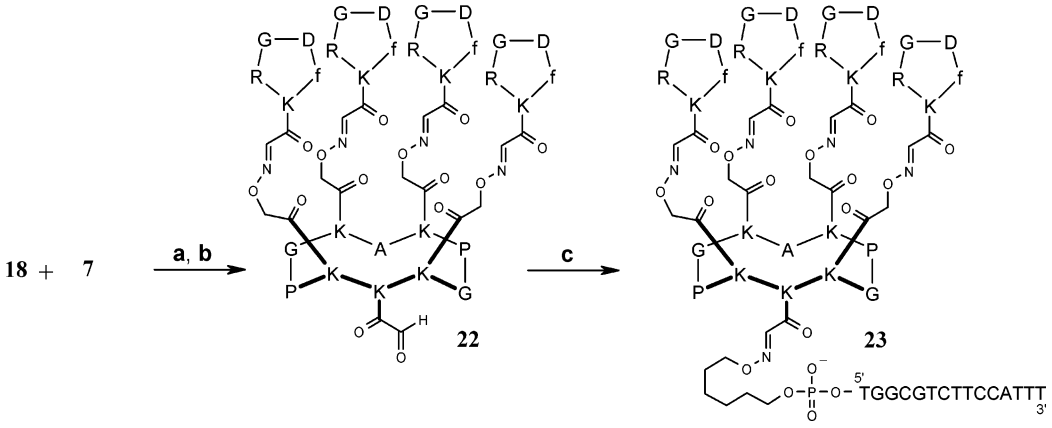
SCHEME 4. CAT 18[a]



19 PG1 = Boc, PG2 = Alloc **20** **18**

[a] Reagents and conditions: (a) 50% TFA, CH_2Cl_2, 15 min; BocNHOCH$_2$CO-Succ, DMF, 30 min; Pd(PPh$_3$)$_4$, PhSiH$_3$, CH_2Cl_2/DMF (2:1), 1 h; BocSer(*t*-Bu)OH, PyBOP, DIPEA, DMF, 30 min; (b) 95% TFA, 2.5% TIS, 2.5% H_2O, 2 h.

SCHEME 5. Conjugation of Oligonucleotide 21 to the RAFT(*c*[−RGDfK−])$_4$ Vector from CAT 18[a]



[a] Reagents and conditions: (a) 0.1 M AcO$^-$Na$^+$ pH 4.6/CH_3CN (1:1); (b) $NaIO_4$, H_2O; (c) 80% aqueous AcOH, d(5′XTGGCGTCTTCCATTT3′) **21** [X designs the 5′-trityl-protected aminooxy linker].

Conjugation with the oligonucleotide component was performed from the scaffold **22** bearing an aldehyde group on the lower face and four *c*[−RGDfK−] peptides on the upper face (Scheme 5). Compound **22** was obtained from CAT **18** in 36% overall yield after first oxime ligation with aldehyde peptide **7** and subsequent periodate oxidation. The oligonucleotide [29] bearing an aminooxy function at the 5′-end was prepared by automated DNA synthesis according to the previously reported method.[11a] The 5′-conjugation was achieved using a one-pot cleavage of the trityl group and coupling reaction. This procedure has the advantage of eliminating the handling of the highly reactive aminooxy moiety. The reaction was carried out in 80% aqueous acetic acid in the presence of the peptide derivative **22** containing the aldehyde function. The liberation of the aminooxy moiety at the 5′-end resulted in the immediate conjugation with the aldehyde **22** through the formation of the oxime bond. The unprotected oligonucleotide intermediate has never been observed. The course of the reaction was monitored by analytical RP-HPLC and revealed the formation of a major compound (Figure 3A). Purification by RP-HPLC gave conjugate **23** that was characterized by ES-MS analysis (Figure 3B). The effect of attachment to the template on the hybridization properties of the oligonucleotide was investigated by melting temperature (Tm) measurements. The conjugate **23** was hybridized with the complementary strand 5′AAATGGAAGACGCCA3′ and the melting temperature of the resulting duplex was determined at two different NaCl concentrations (50 mM and 100 mM). The "natural" oligonucleotide 5′TGGCGTCTTCCATTT3′ was studied as control. The "natural" oligonucleotide showed T_m values of 52.0 and 58.0 °C at NaCl concentrations of 50 and 100 mM, respectively. The conjugate **23**, under similar conditions gave almost identical values (T_m values, 52.5 and 58.5 °C, respectively). It is thus evident from the T_m studies that the attachment of the oligonucleotide to the vector does not perturb the duplex stability. The results reported herein are consistent with the earlier reported observations for the oligonucleotide 5′-conjugates and further emphasize the fact that 5′-conjugation does not induce any instability in the duplex even with a bulky group such as our scaffold.

Synthesis of the Macromolecule RAFT(c[−RGDfK−])$_{16}$ 24. We finally extended this general strategy to systems displaying oxime bonds in ever-increasing numbers. This involves the simultaneous formation of a large number of oxime bonds when oximes are already present in the starting molecules. It is obviously crucial in such syntheses to completely abolish occurrence of transoximation reactions (if one coupling was even accompanied by the presence of 2% of byproduct, a complex mixture would be obtained when performing 10−20 simultaneous couplings). For this purpose, we designed the hexadecavalent ligand RAFT(*c*[−RGDfK−])$_{16}$ **24** containing 20 oxime bonds. The latter was devised from the template **25** containing four aldehyde groups and from the polylysine peptide **26**. The first oxime ligation step involved the grafting of four copies of the polylysine core to the template which enabled to introduce 16 masked aldehyde groups on the same face of the

(29) The sequence was known as an inhibitor of luciferase gene expression and was chosen as an example of antisense oligonucleotide. Antopolsky, M.; Azhayeva, E.; Tengvall, U.; Auroila, S.; Jääskeläinen, I.; Rönkkö, S.; Honkakoski, P.; Urtti, A.; Lönnberg, H.; Azhayev, A. *Bioconjugate Chem.* **1999**, *10*, 598−606.

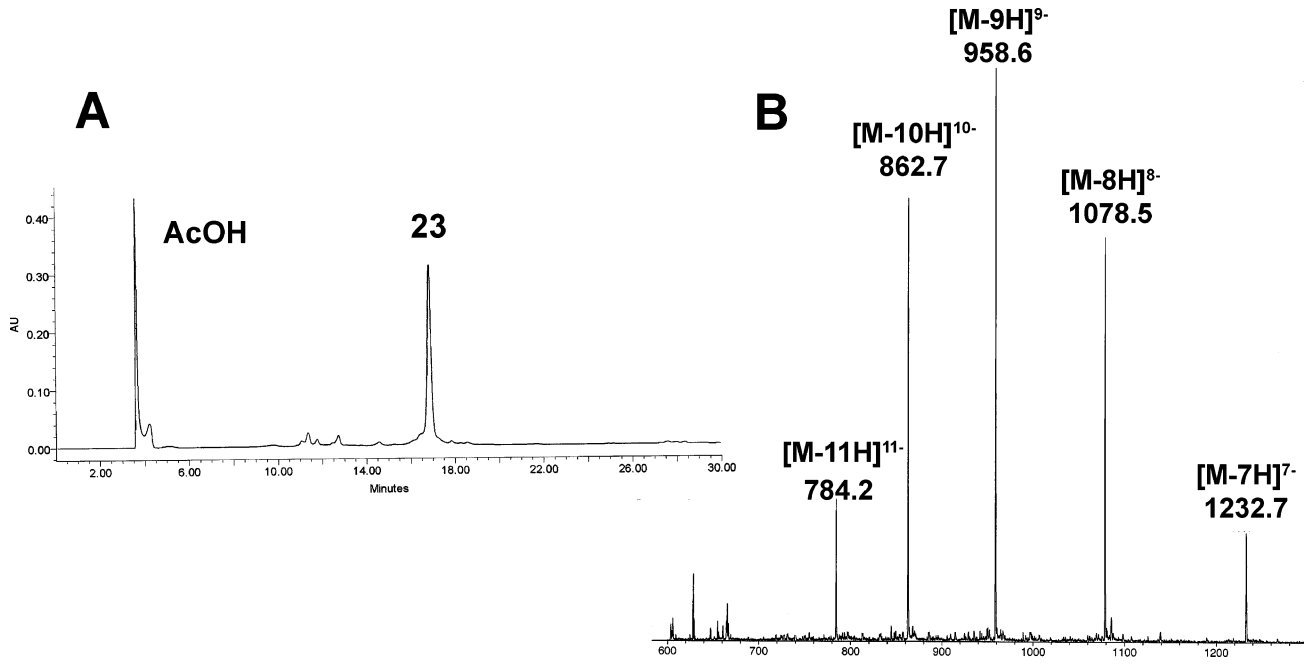
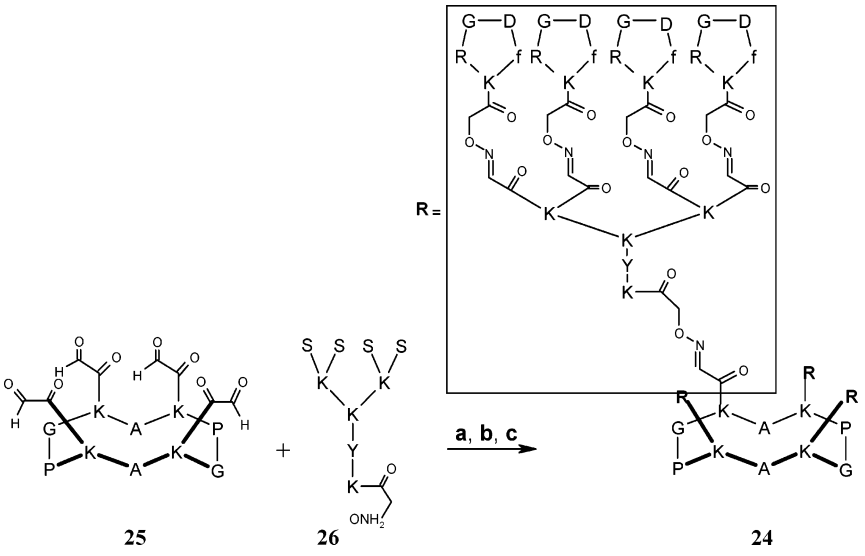


FIGURE 3. (A) RP-HPLC profile of the reaction mixture containing compound **23**. (B) ES-MS analysis of purified compound **23**.

SCHEME 6. Synthesis of the Macromolecule 24[a]



[a] Reagents and conditions: (a) 0.1 M AcO^-Na^+ pH 4.0/CH_3CN (1:1); (b) $NaIO_4$, H_2O; (c) 0.1 M AcO^-Na^+ pH 4.0/CH_3CN (5:1), **8**.

CAT. The second ligation step further led to the coupling of the sixteen *c*[−RGDfK−] monomers. From a biological point of view, the resulting hexadecavalent structure **24** is of great interest to study the effect of a high valency RGD-containing scaffold on $\alpha_V\beta_3$ receptors targeting and clustering (Scheme 6).

We first synthesized CAT **25** using a similar procedure as described for CAT **1** and prepared the polylysine peptide **26** containing an oxyamino group and four masked aldehyde functions (serine residues) at the α and ϵ amines of the *N*-terminal lysines. This kind of peptide has been extensively exploited earlier for the preparation of synthetic vaccines.[13a,30] The first chemoselective ligation of the CAT molecule **25** with an excess of peptide **26** followed by oxidation of the sixteen serine residues provided the desired template in an excellent 93% overall yield. The next chemoselective ligation using large excess (24 equiv) of *c*(−RGDfK[$COCH_2ONH_2$]−) **8** gave the RAFT(*c*[−RGDfK−])$_{16}$ **24** in 86% yield. We could not detect the formation of oxime exchange byproducts. The macromolecule was characterized by mass spectroscopy without ambiguity since we found the deconvoluted mass in total agreement with the calculated mass (15614.9). The formation of this fully synthetic high molecular weight structure demonstrates the high efficiency of sequential oxime ligations from glyoxylyl compounds. This method could be great value for facile access to dendrimers also.

Conclusion

In summary, we describe an efficient method for access to fully synthetic vectors combining $\alpha_V\beta_3$ integrin-targeting func-

(30) Shao, J.; Tam, J.-P. *J. Am. Chem. Soc.* **1995**, *117*, 3893−3899.

tions with diverse functional elements. The approach is based on the preparation of a key intermediate, the chemoselectively addressable template (CAT), containing two independently addressable domains allowing successive oxime ligations: the sequential functionalization of the vector with the targeting ligands and with the functional biomolecules. Complex structures are efficiently obtained without any oxime exchange. The modular synthetic approach used in the present work can be applied to a broad range of purposes, such as the targeted delivery of nucleic acids or the diagnostic of tumors and metastases, by suitably adapting both domains on the template. Chemical synthesis of such well-defined constructs is worthwhile as it may provide the plasticity required for their design. Additionally, such entirely synthetic vectors are devoid of any viral component and hence it is expected that synthesis of such molecular conjugate vectors will likely become the rule in future biomolecule delivery, not only on account of their better uptake ability but also because they provide a way to better control the biomolecule distribution.

Experimental Section

General Procedure for Solid-Phase Peptide Synthesis. Assembly of fully protected peptides was carried out using the Fmoc/*t*-Bu strategy either manually in a glass reaction vessel fitted with a sintered glass frit or automatically on a synthesizer. Coupling reactions were performed by using 1.5−2 equiv of *N*-α-Fmoc-protected amino acid (relative to the resin loading) activated in situ with 1.5−2 equiv of PyBOP and 3−4 equiv of diisopropylethylamine (DIPEA) in DMF (10 mL/g resin) for 30 min. The coupling efficiency in manual synthesis was assessed by Kaiser and/or TNBS tests. *N*-α-Fmoc protecting groups were removed by treatment with a piperidine/DMF solution (1:4) for 10 min (10 mL/g resin). The process was repeated three times and the completeness of deprotection verified by UV absorption of the piperidine washings at 299 nm. Synthetic linear peptides were recovered directly upon acid cleavage (1% TFA in CH_2Cl_2) from Sasrin and ChloroTrityl resins. The resins were treated for 3 min repeatedly until the resin beads became dark purple. The combined washings were concentrated under reduced pressure and white solid peptides were obtained by precipitation from ether. They were analyzed by RP-HPLC and, if necessary, purified by preparative RP-HPLC.

General Procedure for Cyclization Reactions. Linear peptides (0.5 mM) were dissolved in DMF, and the pH value was adjusted to 8−9 by addition of DIPEA. PyBOP (1.2 equiv) was added and the solution stirred at room temperature for 1 h. Solvent was removed under reduced pressure and the residue dissolved in the minimum of CH_2Cl_2. Ether was added to precipitate the peptide. The latter was then triturated and washed with ether (×3). The crude material obtained was used in the following steps without further purification.

CAT 1. Starting with cyclic decapeptide **3** (247 mg, 0.15 mmol), Boc moieties were removed using a solution containing 50% TFA in CH_2Cl_2 (100 mL) for 15 min at room temperature. The crude product was concentrated, triturated and washed with ether. Boc-Ser(*t*-Bu) was introduced using the general procedure for solid-phase peptide synthesis. Alloc groups were removed in dry CH_2Cl_2 (13 mL) under argon by adding successively phenylsilane (703 mg, 6.5 mmol) followed after 3 min by $Pd(PPh_3)_4$ (75 mg, 65 μmol). The reaction mixture was stirred for 1 h at room temperature. The solvent was removed under reduced pressure. The crude product was dissolved in minimum amount of a solution containing a CH_2Cl_2/CH_3OH (1:1). Ether was added to precipitate the crude product. The latter was triturated and washed with ether (3×). This material was then dissolved in a solution containing 50% aqueous CH_3CN and lyophilized. This crude peptide was then treated with $BocNHOCH_2CO$-Succ (82.9 mg, 0.29 mmol) and DIPEA (pH 8.0) in DMF solution (13 mL). The reaction mixture was stirred for 30 min at room temperature and then concentrated under reduced pressure. The crude product was triturated and washed with ether. Finally, removal of all protecting groups was carried out in a solution containing TFA/TIS/H_2O (95:2.5:2.5) for 2 h at room temperature. The crude product was concentrated, triturated and washed with ether. The product was then purified by RP-HPLC to obtain compound **1** as white powder (69 mg, 30.6 μmol, 20% overall yield). Mass spectrum (ES-MS, positive mode): calcd 1571.8, found 1571.9.

CAT 2. Removal of PnZ group from cyclic decapeptide **4 (**0.34 mmol/g) on solid support (500 mg) was carried out in a solution containing $SnCl_2$ (2 M), acetic acid (1.6 mM), and phenol (10 mM) in DMF (10 mL/g) for 1 h at room temperature. The procedure was repeated three times, and the resin was washed with solutions of DMF, CH_2Cl_2/triethylamine (9/1), DMF, and CH_2Cl_2. Boc-Ser(*t*-Bu) was introduced using the general procedure for solid-phase peptide synthesis. Removal of the Dde group was carried out in a solution containing 2% hydrazine in DMF (20 mL/g) for 30 min at room temperature. The procedure was repeated four times, and the resin was washed with DMF. Finally, $BocNHOCH_2CO$-Succ (2 equiv) was added in DMF (10 mL/g). The reaction was carried out for 30 min at room temperature. The resin was washed with DMF and CH_2Cl_2, and the free peptide was recovered directly upon acid cleavage using a solution containing TFA/TIS/H_2O (95:2.5:2.5) (10 mL/g) for 2 h at room temperature. The acid solution was removed under reduced pressure, and the residue obtained was dissolved in a minimum amount of CH_2Cl_2. Ether was added to precipitate the peptide. CAT peptide **2** was isolated as white powder after purification by RP-HPLC (56 mg, 34 μmol, 20% overall yield estimated from the Rink Amide MBHA loading). Mass spectrum (ES-MS, positive mode): calcd 1642.9, found 1643.3.

Peptide Conjugate 9. RGD-containing peptide **7** (1 mg, 1.3 μmol) was added to a solution of peptide **1** (1 mg, 0.44 μmol) in 200 μL of 0.1 M AcO^-Na^+, pH 4.6/CH_3CN (1:1). After 6 h, sodium periodate (1.9 mg, 8.8 μmol) was added to the solution. The reaction mixture was stirred for 1 h and then purified by RP-HPLC to obtain compound **9** as white powder (0.9 mg, 0.3 μmol, 70% overall yield). Mass spectrum (ES-MS, positive mode): calcd 2731.0, found 2730.3

Control Peptide 10. RGD-containing peptides **7** and **8** were obtained as described earlier.[25] Peptide **8** (2 mg, 2.2 μmol) was added to a solution containing peptide **7** (1.7 mg, 2.2 μmol) in aqueous buffer (400 μL, 50% 0.1 M AcO^-Na^+, pH 4.6/50% CH_3CN). The reaction mixture was stirred for 6 h and purified by RP-HPLC to obtain peptide **10** as a white powder (3 mg, 1.9 μmol, 88%). Mass spectrum (ES-MS, positive mode): calcd 1318.4, found 1318.5.

Peptide Conjugate 11. RGD-containing peptide **8** (1.3 mg, 1.4 μmol) was added to a solution of peptide **9** (0.9 mg, 0.3 μmol) in 200 μL of 0.1 M AcO^-Na^+, pH 4.6/CH_3CN (1:1). After 4 days, the product was purified by RP-HPLC to obtain compound **11** as a white powder (1.3 mg, 0.21 μmol, 70% overall yield). Mass spectrum (ES-MS, positive mode): calcd 5365.8, found 5366.3.

"KLA" Peptide 14. The linear sequence H-SGGKLAKLAK-KLAKLAK-OH was synthesized as described in the literature[26b] and following the general procedure for solid-phase peptide synthesis. Oxidation at the *N*-terminal residue (159 mg, 0.063 mmol) was performed with $NaIO_4$ (34 mg, 0.160 mmol) in water (8 mL). The reaction mixture was stirred for 6 h at room temperature and then purified by RP-HPLC to obtain compound **14** as white solid powder (113 mg, 0,048 mmol, 76%). Mass spectrum (ES-MS, positive mode): calcd 1694.2, found 1693.8.

Peptide Conjugate 17. KLA-containing peptide **14** (92 mg, 38.7 μmol) was added to a solution of peptide **1** (34.5 mg, 15.3 μmol) in 3 mL of aqueous buffer (0.1 M AcO^-Na^+, pH 4.6). After 24 h, benzyloxyamine (4.7 mg, 29.2 μmol) was added. The reaction was stirred for 1 h, and the product was purified by RP-HPLC. Oxidation of the product was carried out by using sodium periodate (7.7 mg,

36 μmol) for 1 h, and the product was purified again by RP-HPLC to obtain aldehyde-containing compound **16** as white powder (51.9 mg, 8.4 μmol) in 55% overall yield. RGD-containing peptide **8** 18 mg (20 μmol) was added to a solution containing **16** (26 mg, 4.2 μmol) in 1.5 mL of aqueous buffer (50% 0.1 M AcO^-Na^+, pH 4.6/50% CH_3CN). After 1 day, the product was purified by RP-HPLC to obtain compound **17** as a white powder (31.3 mg, 3.38 μmol, 80% overall yield). Mass spectrum (ES-MS, positive mode): calcd 7434.7, found 7433.9.

CAT 18. Starting with cyclic decapeptide **19** (126 mg, 83.8 μmol), Boc moieties were removed using a solution containing 50% TFA in CH_2Cl_2 (10 mL) for 15 min at room temperature. The crude product was concentrated, triturated, and washed with ether. $BocNHOCH_2CO$-Succ (102 mg, 0.35 mmol) and DIPEA (62 μL) were added to a DMF solution (8 mL) containing the peptide to adjust the pH value at 8.0. The reaction was stirred for 30 min at room temperature and then concentrated under reduced pressure. The crude product was triturated and washed with ether. The Alloc group was removed in a dry CH_2Cl_2/DMF mixture (2:1; 6 mL) under argon by adding successively phenylsilane (200 mg, 1.8 mmol) followed after 3 min by $Pd(PPh_3)_4$ (75 mg, 3.5 μmol). The reaction mixture was stirred for 1 h. at room temperature, and the solvent was evaporated under reduced pressure. The crude product was dissolved in the minimum amount of solution containing a mixture of CH_2Cl_2/CH_3OH (1:1). Ether was added to precipitate the product. This was triturated and washed with ether (3×). Boc-Ser(*t*-Bu) was then introduced on half of the material using the general procedure for solid-phase peptide synthesis. Removal of all protecting groups was finally carried out in a solution containing TFA/TIS/H_2O (95:2.5:2.5) for 2 h at room temperature. The crude product was concentrated, triturated, and washed with ether. The product was then purified by RP-HPLC to obtain compound **18** as white powder (35 mg, 17.8 μmol, 42% overall yield). Mass spectrum (ES-MS, positive mode): calcd 1399.6, found 1399.8.

Peptide Conjugate 22. RGD-containing peptide **7** (19 mg, 37 μmol) was added to a solution of peptide **18** (10 mg, 5.1 μmol) in 2 mL of aqueous buffer (50% 0.1 M AcO^-Na^+, pH 4.6/50% CH_3CN). After 6 h, sodium periodate (5 mg, 22 μmol) was added. The reaction mixture was stirred for 1 h, and the product was purified by RP-HPLC to obtain compound **22** as a white powder (8 mg, 1.8 μmol, 36% overall yield). Mass spectrum (ES-MS, positive mode): calcd 3953.3, found 3953.5.

Peptide−Oligonucleotide Conjugate 23. The oligonucleotide **21** (18 OD, 0.55 mg, 0.11 μmol) was dissolved in an 80% aqueous AcOH solution (0.8 mL), and a solution of the peptide **22** (3 equiv, 1.46 mg, 0.33 μmol) in water (0.2 mL) was added to it. The reaction mixture was stirred overnight at room temperature, and acetic acid was lyophilized. The crude mixture was then purified by HPLC to obtain conjugate **23** in 67% yield (12 OD, 0.64 mg, 0.07 μmol). Mass spectrum (ES-MS, negative mode): calcd 8637.3, found 8636.9.

CAT 25.[11c] Starting with cyclic decapeptide *cyclo*[−PGK(Boc)-AK(Boc)PGK(Boc)AK(Boc)−] (163.6 mg, 0.12 mmol), Boc moieties were removed using a solution containing 50% TFA in CH_2Cl_2 (100 mL) for 30 min at room temperature. The crude product was concentrated, triturated and washed with ether. Introduction of Boc-Ser(*t*-Bu) was performed using the general procedure for solid-phase peptide synthesis and immediately followed by removal of Boc and *t*-Bu protecting groups in TFA. The deprotection reaction was carried out for 1 h at room temperature. The crude product was concentrated, triturated, washed with ether, and obtained as white powder (160 mg, 0,09 mmol, 75% overall yield). Oxidation at serine moieties was performed by using $NaIO_4$ (64 mg, 0.3 mmol) for 30 min at room temperature. The product was then purified by RP-HPLC to obtain compound **25** as a white powder (80 mg, 0.07 mol, 58% overall yield). Mass spectrum (ES-MS, positive mode): calcd 1187.3, found 1187.2.

Hexadecavalent RGD Peptide 24. Peptide **26** (33 mg, 19.5 μmol) was added to a solution containing compound **25** (5 mg, 4.3 μmol) dissolved in 1 mL of sodium acetate buffer (0.1 M, pH 4.0) and acetonitrile (1:1). The reaction was stirred for 2 h at room temperature. The product was isolated after purification by RP-HPLC as a white powder. This product (31.9 mg, 4.0 μmol) was dissolved in 8 mL of water, and sodium periodate (18.5 mg, 86 μmol) was added to it. The reaction mixture was stirred for 1 h at room temperature. The intermediate compound was purified by RP-HPLC and isolated as white powder in quantitative yield (20.6 mg, 4.0 μmol, 93% overall yield). After, the product obtained above (14.5 mg, 2.86 μmol) was dissolved in 6 mL of a solution containing sodium acetate buffer (0.1M, pH 4.0) and acetonitrile (5:1). The RGD derivative **8** (62.1 mg, 68.6 μmol) was added to it and the reaction was stirred for 24 h. Multidentate RGD-containing peptide **24** was isolated as white powder after purification by RP-HPLC (42.8 mg, 2.45 μmol, 86%). Mass spectrum (ES-MS, positive mode): calcd 15614.9, found 15614.9.

Melting Studies. The melting curves (absorbance versus temperature) were measured at 260 nm on a Lambda 5 UV−vis spectrophotometer equipped with a Perkin-Elmer C570−070 temperature controller. A sweep rate of 1 °C·min^{-1} (from 10 to 80 °C) was employed. The experiments were carried out by mixing equimolar amounts of the two strands in 10 mM sodium phosphate buffer (pH 7) containing 1 mM EDTA and 50 mM or 100 mM NaCl. The samples were heated to 80 °C for 5 min and then cooled slowly before the experiment. The ODN concentration was 1.3 μM, and experiments were done in duplicate.

Acknowledgment. The work was supported by the Région Rhône-Alpes, the Centre National de la Recherche Scientifique (CNRS), and the Institut Universitaire de France (IUF). Financial assistance from La Ligue Nationale Contre le Cancer (to E.G.) is gratefully acknowledged. We thank Yashveer Singh for careful reading of this manuscript.

Supporting Information Available: Synthetic procedures for **3**, **7**, **8**, **12**, **13**, **19**, **21,** and **26**, procedure for oligonucleotides synthesis, HPLC chromatograms and MS spectra of compounds **1**, **2**, **9**−**13**, **17**, **18**, and **22**−**24**. This material is available free of charge via the Internet at http://pubs.acs.org.

JO0525480