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Synthesis of Oligonucleotide-Peptide Conjugates Carrying the c-*myc* Peptide Epitope as Recognition System.

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Oligonucleotide-peptide conjugates **1-3** were prepared by sequential addition of the appropriate Boc-protected amino acids followed by nucleoside phosphoramidites in the same support. These molecules are designed to be used for triplex formation and for the directed assembly of nanomaterials. The structure of the desired oligonucleotide-peptide conjugates was confirmed by mass spectrometry on small oligonucleotide-peptide conjugates, by gel electrophoresis and by hybridization with complementary oligonucleotide. Oligonucleotides carrying the c-*myc* peptide were specifically recognized by the anti c-*myc* monoclonal antibody.

Introduction.- Oligonucleotide-peptide conjugates are chimeric molecules made by oligonucleotides covalently linked to peptide sequences. They are produced to transfer some of the biological or/and biophysical properties of peptides to synthetic oligonucleotides [1][2]. For example, the introduction of peptides into oligonucleotide sequences resulted in the introduction of a higher number of multiple nonradioactive labels [3], in improving cellular uptake of antisense oligonucleotides [4], in improving binding to DNA [5][6], RNA [7] and proteins [8], and in the preparation of sequence-specific artificial nucleases [9]. Recently, we described the introduction of epitope peptide sequences into oligonucleotides as nonradioactive labeling system [10]. Specifically, oligonucleotides carrying c-*myc* tag-sequence were prepared using a post-synthetic conjugation protocol based on the reactivity of the thiol groups. In this method, a thiolated oligonucleotide reacted with the c-*myc* peptide carrying a maleimido group. The resulting oligonucleotide-peptide conjugates were

recognized by an anti-c-*myc* monoclonal antibody and detection was achieved through interaction with a peroxidase-conjugate antibody and a chemiluminiscent substrate [10].

Among the biological molecules, oligonucleotides have been used as templates to assemble inorganic nanocrystals. The hybridization properties of the oligonucleotides permit the assembly of gold nanoparticles at distances determined by the length of the oligonucleotides and also the formation of three-dimensional networks [11][12][13][14]. In addition, DNA can be metallized to form conducting wires between electrodes [15]. These results have rise interest on oligonucleotides carrying a second recognition system for the assembly of complex nanoparticle networks [16]. Most of the experiments were performed using the recognition system of biotin and streptavidin because of the high affinity of the biotin-streptavidin interaction [17]. Recently, we described the use of branched oligonucleotides carrying biotin to direct one single nanoparticle to the middle of a lineal DNA duplex using the biotin-streptavidin system [18-20].

Here we described the stepwise synthesis of oligonucleotides carrying the c-*myc* peptide sequence using one single support (*schemes 1* and *2*). Our aim is the preparation of oligonucleotide-peptide conjugates (*scheme 1*) consisting of two sequences connected by a non-DNA material such as hexaethyleneglycol (=3,6,9,12,15-pentaoxaheptadecane-1,17-diol) ($(EG)_6$). This type of oligonucleotide may be used to assemble nanomaterials as described previously [18] [20]. Moreover, the presence of the peptide in these oligonucleotides may direct nanoparticles to the middle of the structure using antibodies against the peptide. The possibility of using the large diversity of available antibodies and

their many epitope sequences makes oligonucleotide-peptide conjugates good candidates for the directed assembly of complex nanoparticle networks.

Results. - *1. Synthesis of Oligonucleotide-Peptide Conjugates***.** Oligonucleotide-peptide sequences **1**-**3** (*Table 1*) were prepared essentially as described for the synthesis of oligonucleotides carrying nuclear localization peptide (NLS) sequences [21, 22]. First, the peptide sequence was assembled using Boc-amino acids on a sarcosyl-polyethyleneglycol-polystyrene (PEG-PS) support carrying the 5-aminopentyl linker [23]. The amino group of lysine was protected with the trifluoroacetyl (Tfa) group [24]. The carboxylic group of Glu and Asp were protected with the 9*H*-fluoren-9-ylmethyl (Fm) group, and the hydroxy group of serine was protected with the acetyl (Ac) group. Once the peptide sequence was completed, a linker molecule was added to convert the last amino group of the peptide into a hydroxyl group protected by a dimethoxytrityl ($(MeO)_2Tr$) group. For this purpose, the active ester of the $(MeO)_2Tr$ -protected derivative of 6-hydroxy hexanoic acid was used. After the addition of the linker, aliquots of the support were placed inside the synthesis columns and the assembly of the desired oligonucleotide sequences was performed. Phosphoramidites were dissolved either in dry CH_2Cl_2 or dry MeCN.

First we assembled five thymidines to the peptide support (sequence **1**, *Table 1*). Using PEG-PS supports the coupling of the nucleoside phosphoramidites may be influence by the solvent used in the reactions. Previously, we observed phosphoramidite coupling reactions to be more efficient when phosphoramidites were dissolved in dry CH_2Cl_2 [21, 22] but in this case coupling efficiencies were equally high (>98%) using both solvents. A similar

effect was observed during the synthesis of oligonucleotides carrying trifunctional amino acids at the 3'-end [23] and we believe that differences on coupling yields are due to the influence of the amino acids on the swelling of the PEG-PS support. In general, we observed low coupling yields in MeCN if lysine residues are protected with the (9*H*-fluoren-9-ylmethoxy)carbonyl (Fmoc) group. After the removal of the protecting groups with aq. ammonia, the $(MeO)_2$Tr-protected oligonucleotide-peptide **1** was purified by reversed phase HPLC using standard trityl-on and trityl-off protocols. One major peak was observed that had the expected molecular weight.

The parallel hairpin **2** (*Table 1*) was prepared using standard phosphoramidites for the purine strand and reversed phosphoramidites for the assembly of the loop and the pyrimidine strand [25]. The overall yield was 70 % and the desired conjugate was isolated from truncated sequences by HPLC using trityl-on and trityl-off protocols. The effect on the triplex stability of the presence of the c-*myc* peptide sequence on the hairpin is shown in *Table 2*. The same melting temperatures are observed for triplex formed by peptide-containing hairpin (**2**) as for the triplex formed by the control hairpin without the peptide (B22) at pH 4.5 and pH 6.0. This indicates that the peptide sequence at the terminal position has no influence on the triplex stability of the hairpin. This was expected, because the peptide is attached on a terminal position of the hairpin. The same peptide sequence, linked through an internal C residue of an oligonucleotide, induces destabilization of the duplex of approximately 10 ºC [10].

The branched oligonucleotide **3** (*Table 1*) having two equal sequences in each arm and the c-*myc* peptide sequence in the middle isolated from the oligonucleotide sequences by hexaethyleneglycol molecules was prepared. In

order to obtain this molecule a symmetric branching molecule [26] was added to the peptide-support followed by successive additions of reversed phosphoramidites at 0.2 M concentration in dry CH_2Cl_2 until the desired sequence was assembled [19]. Coupling yields, measured by the absorbance of the $(MeO)_2Tr$ cation released in each detritylation, were around 95%. The desired conjugate was isolated from truncated sequences by HPLC obtaining a broad peak in the area of the $(MeO)_2Tr$-containing products which was collected. The $(MeO)_2Tr$ group was removed with acetic acid and the resulting product was repurified. Analysis of the purified product by polyacrylamide gel electrophoresis show a broad band with less mobility than the corresponding 40 mer as described for other oligonucleotide-peptide conjugates [1, 27]. Melting experiments performed with duplexes of oligonucleotide **3** with their complementary oligonucleotide gave the same melting temperatures when compared with their linear analogues (*Table 3*).

2. Immunodetection of Oligonucleotide-Peptide Conjugates. Nonradioactive detection of oligonucleotides carrying the c-*myc* peptide was assayed by on a dot-blot format. Serial dilutions of the oligonucleotide-peptide conjugates (from 3 μg) were applied to a nitrocellulose membrane using a manifold attached to a suction device. After immobilization of the oligonucleotide by UV radiation, the samples were incubated with an anti-c-*myc* monoclonal antibody [28], followed by incubation with a secondary antibody conjugated to horseradish peroxidase, and developed with a chemiluminescent substrate. *Fig. 2* shows the autoradiography of the detection of oligonucleotides carrying the c-*myc* peptide prepared in this work (Lanes 2-4) as well as several controls such as

peptide c-*myc* alone (Lane 1), oligonucleotide without peptide (Lane 5) and oligonucleotide carrying a peptide non-related with c-*myc* (Lane 6). Positive dots were obtained for the oligonucleotides carrying the c-*myc* peptide. Peptide c-*myc* alone and pentathymidine sequence **1** gave a positive dot only at the highest concentration. This is due to low efficiency of the attachment of these compounds to the nitrocellulose membrane. Oligonucleotide sequences **2** and **3** gave a positive dot up to 90-180 ng. Control oligonucleotides without peptide and with a non-related peptide gave no signal.

It is important to remark that the detection limit on the oligonucleotide-peptide conjugates prepared in this work using the stepwise method is similar to the conjugates prepared by postsynthetic conjugation [10]. This confirm the integrity of the peptide during oligonucleotide synthesis and ammonia deprotection. Although mass spectrometry gave the expected mass, this method will not detect racemization of the amino acids. On the contrary the recognition of the antibody to the oligonucleotide-peptide conjugates is a good proof of the correct quirality of the amino acids.

Discussion. The ability to control the assembly of nanomaterials from well-defined units is a key step that is expected to allow the exploitation of the technological potential of these materials. The use of the hybridisation properties of oligonucleotides is a promising approach [11][12][13][14][16][17][18]. Our aim in this paper was the synthesis of oligonucleotides carrying peptide epitopes. This type of chimeric molecule carry two well-defined recognition systems: the natural affinity to their complementary DNA sequence and the antibody-epitope interaction. Although this may be achieved easily using small ligands such as

introducing biotin in oligonucleotides, the number of available epitope-antibody recognition systems is practically unlimited. So, a larger molecular diversity can be obtained with oligonucleotide-peptide conjugates.

We show that oligonucleotide carrying the c-*myc* peptide as model sequence for a peptide epitope could be prepared by sequential addition of Boc-amino acids protected with base labile groups in their side chains followed by the addition of phosphoramidites. Moreover, complex oligonucleotides such as branched oligonucleotides with two identical arms carrying the c-*myc* peptide at the central position are amenable to chemical synthesis. This new class of oligonucleotides have potential interest as templates for the assembly of nanomaterials

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Experimental Part

General. Phosphoramidites and ancillary reagents used during oligonucleotide synthesis were from *Applied Biosystems* (*PE Biosystems Hispania S.A.,* Spain), *Cruachem Ltd.* (Scotland), and *Glen Research Inc*. (USA). Amino acid derivatives were from *Bachem* (Switzerland) and *Novabiochem* (Switzerland). The rest of the chemicals were purchased from *Aldrich, Sigma* or *Fluka* (Spain). Long-chain-alkyl-amine controlled-pore glass (LCAA-CPG) was

purchased from *CPG, Inc*. (*New Jersey,* USA). Amino-polyethyleneglycol-polystyrene (PEG-PS) was purchased from *PerSeptive* (now *Applied Biosystems*, USA). Solvents were from *S.D.S*. (France). NAP-10 columns (Sephadex G-25) were purchased from *Pharmacia Biotech*. Oligonucleotide sequences were synthesized on a *Applied Biosystems-392* DNA synthesizer (*Applied Biosystems,* USA). UV-Visible spectra and melting temperatures: *Shimadzu UV-2101PC* spectrophotometer equipped with a temp. controller *SPR-8*. Mass spectra (matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF), and electrospray were provided by the mass spectrometry service at the University of Barcelona.

*Synthesis of Oligonucleotide-Peptide Conjugates***.** The c-*myc* tag sequence (N-terminal Glu-Gln-Lys-Leu-Ile-Ser-Glu-Glu-Asp-Leu-Asn C-terminal) was assembled on a 0.19 mmol scale using a homemade manual synthesizer and amino-PEG-PS (0.19 mmol/g) as the solid support. First, Fmoc-sarcosine was coupled using Ph_3P and 2,2'-dithiobis[5-nitropyridine] as coupling agents [29]. Then, the handle butanedioic acid mono [5-$(MeO)_2$Tr-aminopent-1-yl] ester was coupled to the resin as described elsewhere [21-23]. The loading of the support, as measured by the absorbance of the $(MeO)_2$Tr cation, was 0.1 mmol/g. The peptide sequence was assembled on the 5-aminopentyl-succinyl-sarcosyl-PEG-PS support using Boc-chemistry [21-23]. Protection of the side chains was as follows: The ε-amino group of lysine was protected with the Tfa group, the carboxyl groups of glutamic and aspartic acid were protected with the Fm group, and the hydroxyl group of serine was protected with the Ac group. The peptide was elongated in *N,N*-dimethylformamide (DMF) using a 5-fold excess of Boc-amino acid, 10-fold

excess of $EtN(i\text{-}Pr)_2$, and 5-fold excess of [(1*H*-benzotriazol-1-yl)oxy]tris(pyrrolidino)phosphonium hexafluorophosphate (PyBOP) for 1 h at room temperature. In order to remove the Boc group, the support was treated with a 30% CF_3COOH soln. in CH_2Cl_2 for 30 min and neutralized with $EtN(i\text{-}Pr)_2$-CH_2Cl_2 (1:19). After the assembly of the peptide sequence an extra alanine and a derivative of 6-hydroxyhexanoic acid were added as spacers to separate the peptide sequence from the oligonucleotide and to convert the last amino group to a hydroxyl group [21-23]. The assembly of the oligonucleotide chain requires the presence of the hydroxyl group. The 6-hydroxyhexanoic linker carried a $(MeO)_2Tr$ group for protection of the hydroxyl group and it was a 4-nitrophenyl ester (5-fold excess in DMF for 1 h). Characterization of the peptide support was performed by treatment of an aliquot with conc. ammonia and analysis of the resulting product by mass spectrometry. MALDI-TOF of the resulting peptide: 1588.4 $[M+H]^+$; calc. for $C_{69}H_{118}N_{16}O_{26}$:1587.6.

Synthesis of Oligonucleotide-Peptide Sequences **1** and **2**. Oligonucleotide sequences were assembled on a 1 µmol scale. The phosphoramidites were dissolved either in dry CH_2Cl_2 or in dry MeCN, giving 0.1 M solutions (using either reverse phosphoramidites for the pyrimidine chain growth or standard phosphoramidites for the purine chain elongation). The coupling time was increased to 5 min, capping and oxidation times to 1 min and the detritylation step to 2 min. Coupling yields were >95%. The last $(MeO)_2Tr$ protecting group was not removed. After the assembly, some of the protecting groups (Fmoc, Fm, CH_2CH_2CN) were removed with a 0.5M soln. of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) in MeCN [21-23]. The conjugates were liberated from the solid

support by conc. ammonia (with drops of dioxane) in an overnight treatment at 55ºC, with concomitant removal of the nucleobase protecting groups. After filtration of the solid supports, the solutions were evaporated to dryness, the residues were dissolved in water and the conjugates were purified using standard two-step HPLC. Yields after purification. **1**: 8 OD units, **2**: 9 OD units. **3**: 34 OD units. Electrospray of **1**: 621.29 $[M+Na-6H]^{5-}$, 775.74 $[M+Na-5H]^{4-}$, 1034.63 $[M+Na-4H]^{3-}$, and 1585.41 $[M+Na-3H]^{2-}$ giving a molecular weight of 3108.2; calc. for $C_{119}H_{183}N_{26}O_{61}P_5$ 3108.4. MALDI-TOF of **2**: 9859.29 $[M+17Na-16H]^{+}$; calc. for $C_{313}H_{410}N_{105}Na_{17}O_{190}P_{25}$: 9847.41.

Synthesis of Oligonucleotide-Peptide Sequence **3**. After the addition of 6-hydroxyhexanoic acid, the symmetric doubler phosphoramidite (*Cruachem* or *Glen Research*) was added. The oligonucleotide sequence was then assembled using reversed phosphoramidites. A hexaethyleneglycol molecule (*Cruachem* or *Glen Research*) was added between the symmetric doubler and the first nucleotide. For the synthesis of the oligonucleotide part we used a special cycle with the following changes: increased coupling time (5 min), and double coupling using double concentration (0.2 M) of phosphoramidites. In addition phosphoramidites were dissolved in dry CH_2Cl_2. Scale of synthesis : 1 μmol. The last $(MeO)_2Tr$ group was not removed to facilitate HPLC purification. After the assembly, the support was treated with DBU [21-23] and the conjugate was liberated from the solid support by concentrated ammonia (with drops of dioxane) in an overnight treatment at 55ºC. The conjugate was purified by HPLC obtaining a broad peak in the area of the $(MeO)_2Tr$-containing products, which was collected. The $(MeO)_2Tr$ was removed with acetic acid and the resulting product

was repurified obtaining 44 O.D. units at 260 nm (1.3 mg). The homogeneity of the purified product was checked by PAGE (data not shown).

*Melting Experiments***.** Melting experiments were performed as follows. Solutions of equimolar amounts of the appropriate oligonucleotides were mixed either in a soln. containing 0.15M NaCl, 0.05M Tris·HCl buffer of pH 7.5 (in experiments related with duplex formation) or in a soln. containing 1M NaCl and 0.1M sodium phosphate / citric acid buffer of pH 4.5 or pH 6.0 (in experiments related with triplex formation). The DNA concentration was determined by UV absorbance measurements (260 nm) at 90 ºC, using for the DNA coil state the following extinction coefficients: 7500, 8500, 12500, and, 15000 M^{-1} cm^{-1} for C, T, G, and, A, respectively. The solutions were heated to 90 ºC, allowed to cool slowly to room temperature, and stored at 4ºC until UV was measured. UV absorption spectra and melting experiments (absorbance vs temperature) were recorded in 1 cm path-length cells using a spectrophotometer, with a temperature controller and a programmed temperature increase rate of 0.5 ºC/min. Melting curves were recorded at 260 nm and melting temperatures were measured at the maximum of the first derivatives of the melting curves. Results: see *Table 2* and *Table 3*.

Immunodetection of the Oligonucleotide-Peptide Conjugates. Serial dilutions (from 3µg) of the oligonucleotide-peptide conjugates dissolved in water were applied to a nitrocellulose membrane (0.2µm, *BioRad*) using a manifold attached to a suction device. After drying at 70 ºC for 30minutes, the membrane was UV cross-linked for 2.5 minutes. The membrane was blocked with PBS

containing 1% Tween 20 for 1h at room temperature and incubated with culture supernatant from anti-c-*myc* murine hybridoma clone 9E10 (24.6mg/ml, [28]) for 1h at room temperature. After washing twice with PBS containing 0.1%Tween-20 for 15 minutes, the membrane was incubated with a horseradich peroxidase-conjugated goat anti-mouse antibody (*Jackson*, 0.9 mg/mL) at a 1:20000 dilution for 1h. Finally, after several washes with PBS containing Tween-20, the membrane was developed in ECL (*Amersham*) and exposed to X-ray film.

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Table 1: *Sequences of the Synthesized Oligonucleotide-Peptide Conjugates*

	Sequence[a)]
1	5'TTTTT-3'-hexyl-CONH-AEQKLISEEDLN-CONH-$(CH_2)_5$-OH
2	3'-TCTCCTCCTTC-TTTT-5'-5'GAAGGAGGAGA-3'-hexyl-CONH-AEQKLISEEDLN-CONH-$(CH_2)_5$-OH
3	3'-CGTAACTCGCTACGTCCGTC-$(EG)_6$-(bpp-hexyl-CONH-AEQKLISEEDLN-CONH-$(CH_2)_5$-OH)-$(EG)_6$-CTGCCTGCATCGCTCAATGC-3'

[a)] $(EG)_6$: hexaethyleneglycol, bpp: [-PO_3-$O(CH_2)_4$-CONH-CH_2]$_2$-$CHOPO_3$-

Table 2. Melting Temperatures (T_m) of Triplexes Formed by Oligonucleotide **2** and a Control Sequence.

<table>
<tr><th>Triplex</th><th></th><th>T_m [°][a])
pH 4.5</th><th>T_m [°][a])
pH 6.0</th></tr>
<tr><td>B22 + S11WC</td><td>3'-CTTCCTCCTCT-5'
|||||||||||
GAAGGAGGAGA-3'
$(EG)_6$ :::::::::::
CTTCCTCCTCT-3'</td><td>63</td><td>45</td></tr>
<tr><td>2 + S11WC</td><td>3'-CTTCCTCCTCT-5'
|||||||||||
T GAAGGAGGAGA-PEPTIDE
T :::::::::::
T CTTCCTCCTCT-3'</td><td>64</td><td>45</td></tr>
</table>

[a]) S11WC: 5'-CTTCCTCCTCT-3'; B22: 3'-AGAGGAGGAAG-5'-$(EG)_6$-5'-CTTCCTCCTCT 3', 0.1M sodium phosphate and citric acid, 1M NaCl, error in T_m is ±1 °.

Table 3: Melting Temperatures (T_m) of Duplexes Formed by Oligonucleotide **3** and the Linear Control Sequence.

Oligonucleotide	T_m [°][a])
3	71
5'-CTGCCTGCATCGCTCAATGC-3'	72

[a]) Complementary sequence: 3'-GAC GGA CGT AGC GAG TTA CG-5', 0.050M Tris·HCl, 0.15M NaCl, pH 7.5, error in T_m is ±1 °.

LEGENDS

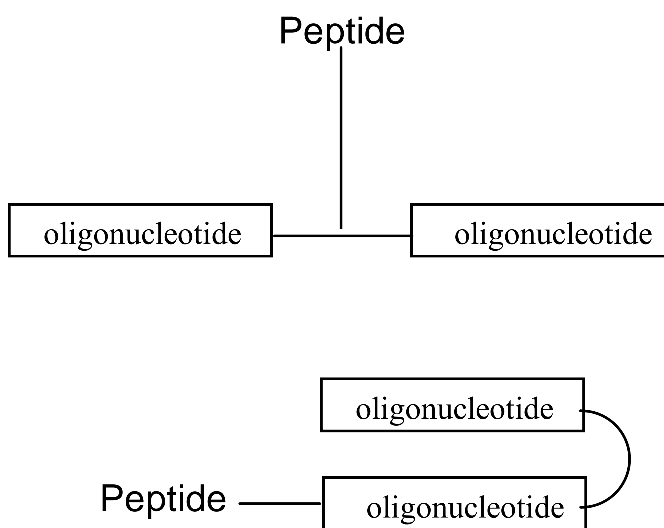
Scheme 1: *Schematic Representation of Oligonucleotide-Peptide Conjugates Described in this Paper*.

Scheme 2. *Outline of the Synthesis of the Oligonucleotide-Peptide Conjugate* **3**.

Fig. 1: *HPLC profiles of* $(MeO)_2$*Tr-containing oligonucleotides* **1** (a) *and* **2** (b). Truncated sequences without $(MeO)_2$Tr groups had a retention time of less than 5 minutes. Benzamide eluted at 7-8 minutes. The desired oligonucleotide-peptide conjugate with the $(MeO)_2$Tr group eluted at 10-14 minutes.

Fig. 2: *Immunodetection of Oligonucleotide-Peptide Conjugates*. Serial ½ dilutions from 3µg of oligonucleotide-peptide conjugates were applied to the blot and immunodetection performed with anti c-*myc* antibody. Lane 1, peptide c-*myc*; Lane 2, oligonucleotide **2**; Lane 3, oligonucleotide **1**; Lane 4, oligonucleotide **3**; Lane 5, oligonucleotide control containing 24 bases without peptide; Lane 6, dodecamer carrying the nuclear localization sequence (QAKKKKLDK); Lane 7, blank.

Scheme 1



Scheme 2

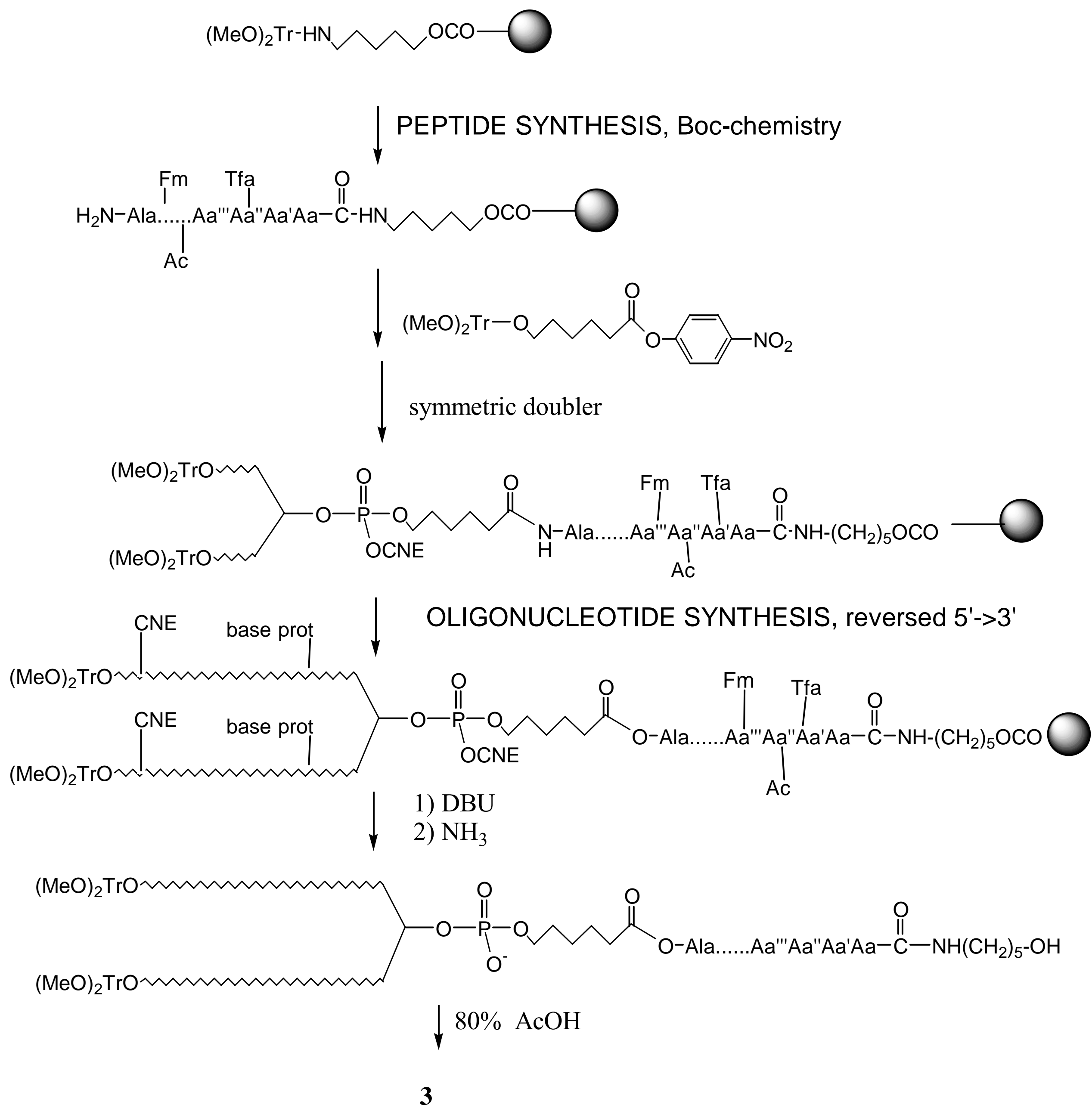


Figure 1

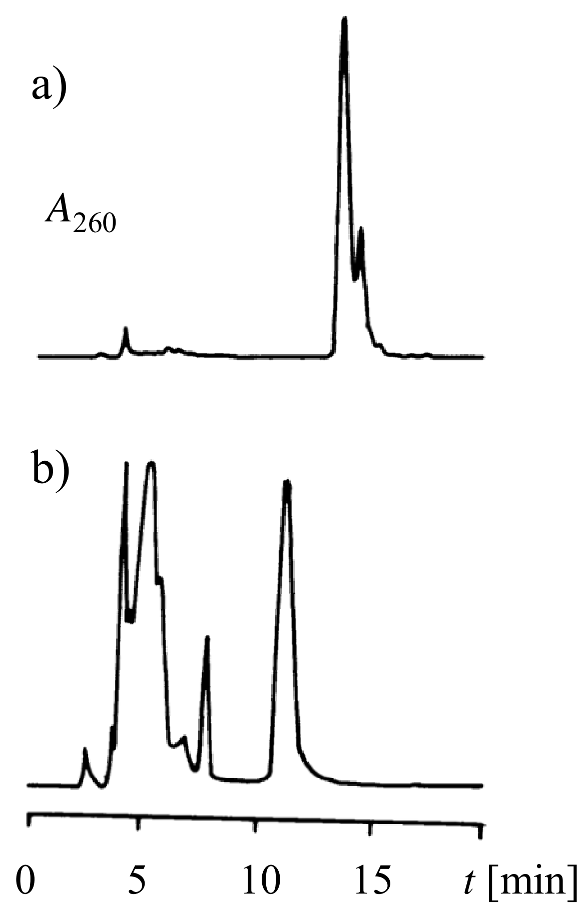


Figure 2

a b c d e f g

1
2
3
4
5
6
7