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(54) **Titre : OLIGONUCLEOTIDES CATIONIQUES, PROCEDES AUTOMATISES POUR LES PREPARER ET LEURS UTILISATIONS**
 (54) **Title: CATIONIC OLIGONUCLEOTIDES, AUTOMATED METHODS FOR PREPARING SAME AND THEIR USES**

(57) **Abrégé/Abstract:**

The invention relates to oligonucleotide-oligocation molecules $A_i B_j H$ that can be synthesized via automated phosphoramidite chemistry having oligonucleotide moieties A_i and oligocation moieties B_j , wherein A_i is an i -mer oligonucleotide residue, with $i = 5$ to 50 , where nucleotide A is an oligomer with naturally or non naturally occurring nucleobases and/or pentafuranosyl groups and/or native phosphodiester bonds, for example selected from the group comprising deoxyribo, ribo, locked (LNA) nucleotides as well as their chemical modifications or substitutions such as phosphorothioate, 2'-fluoro, 2'-O-alkyl, or a marker group such as a fluorescent agent, B_j is a j -mer organic oligocation moiety, with $j = 1$ to 50 , where B is selected from the group comprising $\bullet - \text{HPO}_3 - \text{R}^1 - (\text{X} - \text{R}^2)_n - \text{X} - \text{R}^3 - \text{O} -$, where R^1 , R^2 and R^3 , identical or different, are lower alkylene, X is NH or $\text{NC}(\text{NH}_2)_2$, n varies from 1 to 5 and $n_1 = 2$ to 20 , $\bullet - \text{HPO}_3 - \text{R}^4 - \text{CH}(\text{R}^5 \text{X}^1) - \text{R}^6 - \text{O} -$, where R^4 is lower alkylene, R^5 and R^6 , identical or different, are lower alkylene and X^1 is putrescine, spermidine or spermine residue, $\bullet - \text{HPO}_3 - \text{R}^7 - (\text{aa})_{n_2} - \text{R}^8 - \text{O} -$, where R^7 is lower alkylene and R^8 is lower alkylene, serine, a natural aminoalcohol, $(\text{aa})_{n_2}$ is a peptide containing natural aminoacids with cationic side chains, such as Arginine, Lysine, Ornithine, - Histidine, Diaminopropionic acid and $n_2 = 2$ to 20 .

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(54) Title: CATIONIC OLIGONUCLEOTIDES, AUTOMATED METHODS FOR PREPARING SAME AND THEIR USES

(57) Abstract: The invention relates to oligonucleotide-oligocation molecules $A_i B_j H$ that can be synthesized via automated phosphoramidite chemistry having oligonucleotides moieties A_i and oligocations moieties B_j , wherein A_i is an i -mer oligonucleotide residue, with $i = 5$ to 50 , where nucleotide A is an oligomer with naturally or non naturally occurring nucleobases and/or pentafuranosyl groups and/or native phosphodiester bonds, for example selected from the group comprising deoxyribo, ribo, locked (LNA) nucleotides as well as their chemical modifications or substitutions such as phosphorothioate, 2'-fluoro, 2'-O-alkyl, or a marker group such as a fluorescent agent, B_j is a j -mer organic oligocation moiety, with $j = 1$ to 50 , where B is selected from the group comprising $\bullet - \text{HPO}_3 - \text{R}^1 - (\text{X} - \text{R}^2)_{n1} - \text{X} - \text{R}^3 - \text{O} -$, where R^1 , R^2 and R^3 , identical or different, are lower alkylene, X is NH or $\text{NC}(\text{NH}_2)_2$, n varies from 1 to 5 and $n1 = 2$ to 20, $\bullet - \text{HPO}_3 - \text{R}^4 - \text{CH}(\text{R}^5 \text{X}^1) - \text{R}^6 - \text{O} -$, where R^4 is lower alkylene, R^5 and R^6 , identical or different, are lower alkylene and X^1 is putrescine, spermidine or spermine residue, $\bullet - \text{HPO}_3 - \text{R}^7 - (\text{aa})_{n2} - \text{R}^8 - \text{O} -$, where R^7 is lower alkylene and R^8 is lower alkylene, serine, a natural aminoalcohol, $(\text{aa})_{n2}$ is a peptide containing natural aminoacids with cationic side chains, such as Arginine, Lysine, Ornithine, - Histidine, Diaminopropionic acid and $n2 = 2$ to 20.

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CECI EST LE TOME 1 DE 2
CONTENANT LES PAGES 1 À 19

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NOM DU FICHER / FILE NAME :

NOTE POUR LE TOME / VOLUME NOTE:

“Cationic oligonucleotides, automated methods for preparing same and their uses”

5

The invention relates to cationic oligonucleotides, i.e, oligonucleotide-oligocation molecules, also called cationic oligonucleotides in the description (irrespective of their global charge) that can be synthesized stepwise on an oligonucleotide synthesizer. It also pertains to their use, in molecular biology,
10 diagnostics and therapeutic applications.

Oligonucleotides find an extremely large number of applications in molecular biology and diagnostics, and may become a very selective class of drugs for the treatment of a vast palette of diseases.

Oligonucleotides are polyanions that exert their specific activity following
15 hybridization to a complementary sequence borne by another polyanionic nucleic acid.

As drug candidates, they must also be capable of crossing the anionic cell membrane.

Simple electrostatic considerations imply that hybridization energy and cell
20 binding could benefit from the addition of cationic groups to the oligonucleotide structure.

Towards this goal, many synthetic approaches for introducing ammonium or guanidinium residues into oligonucleotides have been explored: phosphate backbone replacement, ribose or nucleic base modification, and end conjugation of a
25 polycation. However, hybridization specificity, nucleic acid-processing enzyme activity as well as metabolite toxicity concerns all point to the block approach, where the polycation is appended to an otherwise natural oligonucleotide, as the best solution. Unfortunately, stepwise automated synthesis of oligonucleotide-cationic peptide conjugates is not yet routine. On the other hand, conjugation chemistry
30 between preformed large blocks is not straightforward, especially in water, where « super » zwitterions raise intractable solubility, purification and characterization problems. Moreover, molecular biology and diagnostics applications require fast and straightforward synthesis of any given base sequence linked to any organic cation length.

The inventors have found that an online, computer driven, synthesis of oligonucleotide-oligocation molecules was possible by plugging vials containing properly activated and protected oligocationic derivatives to an oligonucleotide synthesizer in addition to those of the four natural bases.

5 An object of the invention is thus to provide new cationic oligonucleotides.

Another object of the invention is to provide a high yield, automated synthesis of said cationic oligonucleotides.

In a further object, the invention relates to the applications of said cationic oligonucleotides, particularly in molecular biology, diagnostics and therapeutics.

10 The invention thus relates to mixed oligonucleotide oligocation molecules that can be synthesized *via* automated phosphoramidite chemistry, i.e., polyphosphodiester.

More particularly, the cationic oligonucleotides A_iB_jH of the invention have oligonucleotides moieties A_i and oligocations moieties B_j , wherein

15 . A_i is an i -mer oligonucleotide residue, with $i = 5$ to 50 , where nucleotide A is an oligomer with naturally or non naturally occurring nucleobases and/or pentafuranosyl groups and/or native phosphodiester bonds,

. B_j is a j -mer organic oligocation moiety, with $j = 1$ to 50 , where B is selected from the group comprising

- 20
- - $HPO_3-R^1-(X-R^2_n)_{n1}-X-R^3-O-$, where R^1 , R^2_n and R^3 , identical or different, are lower alkylene, X is NH or $NC(NH_2)_2$, n varies from 1 to 5 and $n1 = 2$ to 20 ,
 - - $HPO_3-R^4-CH(R^5X^1)-R^6-O-$, where R^4 is lower alkylene, R^5 and R^6 , identical or different, are lower alkylene and X^1 is putrescine, spermidine or spermine residue,
- 25
- - $HPO_3-R^7-(aa)_{n2}-R^8-O-$, where R^7 is lower alkylene and R^8 is lower alkylene, serine, a natural aminoalcohol, $(aa)_{n2}$ is a peptide containing natural aminoacids with cationic side chains, such as Arginine, Lysine, Ornithine, Histidine, Diaminopropionic acid and $n2 = 2$ to 20 .

30 "Lower alkyl" and "lower alkylene", as used in the description and the claims, preferably designate an optionally substituted C1-C5 linear or branched alkyl or alkylene radical, respectively.

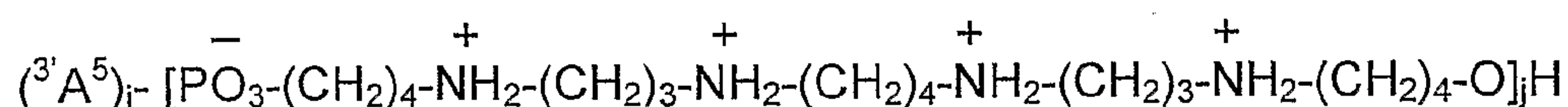
A is for example selected from the group comprising deoxyribo, ribo, locked (LNA) nucleotides as well as their chemical modifications or substitutions such as phosphorothioate (also designated thiophosphate), 2'-fluoro, 2'-O-alkyl or a marker group such as a fluorescent agent.

5 Mixed oligonucleotide-oligocation molecules of the invention have $3'A^{5'}$ - B sequence.

Other molecules of the invention have B - $3'A^{5'}$ sequence.

Still other molecules of the invention have B - $3'A^{5'}$ - B or $3'A^{5'}$ - B - $3'A^{5'}$ sequence.

10 Such a sequence is illustrated in the examples by an oligonucleotide – spermine molecule having the following structure:



15 wherein A, i and j are as above defined.

Molecules with A being a phosphorothioate nucleotide are particularly advantageous in view of their biological applications, since phosphorothioate oligonucleotides are not hydrolyzed in biological fluids.

20 The above defined cationic oligonucleotides form fast and stable complexes with their complementary sequence in a strand replacement context and even in a plasmid strand invasion context, as illustrated by the examples.

Due to end conjugation, sequence selectivity remains as high as for natural nucleotides.

25 Accordingly, the cationic oligonucleotides of the invention are of great interest for molecular biology, research reagents and diagnostics applications, such as PCR, real-time PCR, genotyping, *in situ* hybridization and DNA chips.

Such applications are then also covered by the invention and comprise the use of oligonucleotide-oligocation molecules such as above defined.

30 In contrast to anionic oligonucleotides, cationic oligonucleotides of the invention are shown in the examples to spontaneously enter the cytoplasm and nucleus of living cells.

In view of their enhanced hybridization and cell permeation properties, they are also useful for therapeutic approaches, such as those mediated by antisense and siRNA degradation of messenger RNA, by exon skipping during messenger RNA

maturation, by triple helix formation with chromatin, by chromatin strand invasion (gene correction)...

The invention thus also relates to pharmaceutical compositions comprising an effective amount of oligonucleotide-oligocation molecules such as above defined, in association with a pharmaceutically acceptable carrier.

The invention also relates to a method of treatment comprising using an effective amount of oligonucleotide-oligocation molecules such as above defined, in association with a pharmaceutically acceptable carrier.

The above defined mixed oligonucleotide-oligocation molecules are advantageously stepwise synthesized on an oligonucleotide synthesizer, *via* the phosphoramidite route, according to a method comprising

- plugging vials containing activated and protected oligocations B to an oligonucleotide synthesizer, in addition to vials of oligonucleotides A such as above defined, or the reverse,
- stopping the synthesis, when the desired length is obtained,
- cleaving the oligomers from the solid support, and
- removing the protecting groups.

The invention is closely related to the phosphoramidite reagents used in the automated synthesis for the construction of oligocation repeated block B. The following phosphoramidite reagents can be used for this purpose

- $P(OR^9)(N(R^{10})_2)-O-R^1-(X-R^2_n)_{n1}-X-R^3-O-Prot$, where R^1 , R^2 , R^3 , n and $n1$ are as above defined, X is suitably protected NH or $NC(NH_2)_2$, R^9 is $-CH_2CH_2CN$, or lower alkyl, R^{10} is lower alkyl, or $-N(R^{10})_2$ is pyrrolidino, piperidino or morpholino group, and $Prot$ is a protecting group used in oligonucleotide synthesis, such as DMT, MMT;
- $P(OR^9)(N(R^{10})_2)-O-R^4-CH(R^5X^1)-R^6-O-Prot$, where R^4 , R^5 , R^6 are lower alkylene, X^1 is suitably protected putrescine, spermidine or spermine, R^9 and R^{10} are as above defined;
- $P(OR^9)(N(R^{10})_2)-O-R^7-(aa)_{n2}-R^8-O-Prot$, where R^7 , R^8 , R^9 , R^{10} , $n2$, and $Prot$ are as above defined, $(aa)_{n2}$ is a peptide containing natural aminoacids with suitably protected cationic side chains, such as Arginine, Lysine, Ornithine, Histidine, Diaminopropionic acid and $n2= 2$ to 20 .

Suitably protected NH or NC (NH₂)₂ means that protecting groups are present on the amino or guanidine residue, respectively, to render their functionality inert to chemical reaction conditions to which the reagent is exposed.

Such protecting group are for example phthalimide (PHTH), trifluoroacetate, allyloxycarbonyl (Alloc), benzyloxycarbonyl (CBZ), chlorobenzyloxycarbonyl, t-butyloxycarbonyl (Boc), fluorenylmethoxycarbonyl (Fmoc) and isonicotinyloxy (i-Noc) groups.

According to an embodiment of the invention, stepwise synthesis of the oligonucleotide sequence is followed by stepwise synthesis of the oligocation moiety to obtain compounds having sequence (³A⁵- B).

According to another embodiment, reverse steps are performed, the stepwise synthesis of oligocation moiety being followed by stepwise synthesis of the oligonucleotide sequence to obtain compounds of (B - ³A⁵) sequence.

According to still another embodiment, mixed sequences are synthesized.

In particular, oligonucleotide sequences capped at both ends (B - ³A⁵- B) can resist exonucleases in biological fluids, and cation-interrupted sequences (³A⁵- B - ³A⁵) allow targeting of vicinal nucleic acid sequences.

By using naturally occurring amines like spermine, or peptides such as oligoarginines, potential toxicity of metabolites is avoided. Spermine is indeed present at millimolar concentration in cells and its end-alkylation is harmless. Moreover, basic peptide sequences are present in many nuclear proteins.

The activated and protected oligocations B are advantageously obtained by protecting the amino groups of a polyamine, followed by α , ω -bis hydroxylalkylation, leading to diols compatible with oligonucleotide synthesis.

Classical DMT and phosphoramidite elongation chemistry is advantageously implemented together with base-labile TFA protecting groups.

The chemically protected diols are new products and enter into the scope of the invention.

The invention particularly relates to the intermediates selected from the group comprising

$P(OR^9)(N(R^{10})_2)-O-R^1-(X-R^2_n)_{n1}-XR^3-O-Prot$, where R¹, R², R³, n and n1 are as above defined, X is suitably protected NH or NC(NH₂)₂, R⁹ is -CH₂CH₂CN, or lower alkyl, R¹⁰ is lower alkyl, or -N(R¹⁰)₂ is pyrrolidino, piperidino or morpholino

group, and Prot is a protecting group used in oligonucleotide synthesis such as DMT, MMT;

- $P(OR^9)(N(R^{10})_2)-O-R^4-CH(R^5X^1)-R^6-O-Prot$, where R^4 , R^5 , R^6 are lower alkylene, X^1 is suitably protected putrescine, spermidine or spermine, R^9 and R^{10} are as above defined;
- $P(OR^9)(N(R^{10})_2)-O-R^7-(aa)_{n2}-R^8-O-Prot$, where R^7 , R^8 , R^9 , R^{10} , $n2$, and Prot are as above defined, $(aa)_{n2}$ is a peptide containing natural aminoacids with suitably protected cationic side chains, such as Arginine, Lysine, Ornithine, Histidine, Diaminopropionic acid and $n2= 2$ to 20 .

10 Other characteristics and advantages of the invention are given hereinafter. In particular, the synthesis of decamer oligonucleotidic sequences (A_{10}) with spermine (S), designated by $A_{10}S_n$ in the following will be given by way of illustration, without limiting the invention. In the examples, it will be referred to Figures 1 to 14, which represent, respectively:

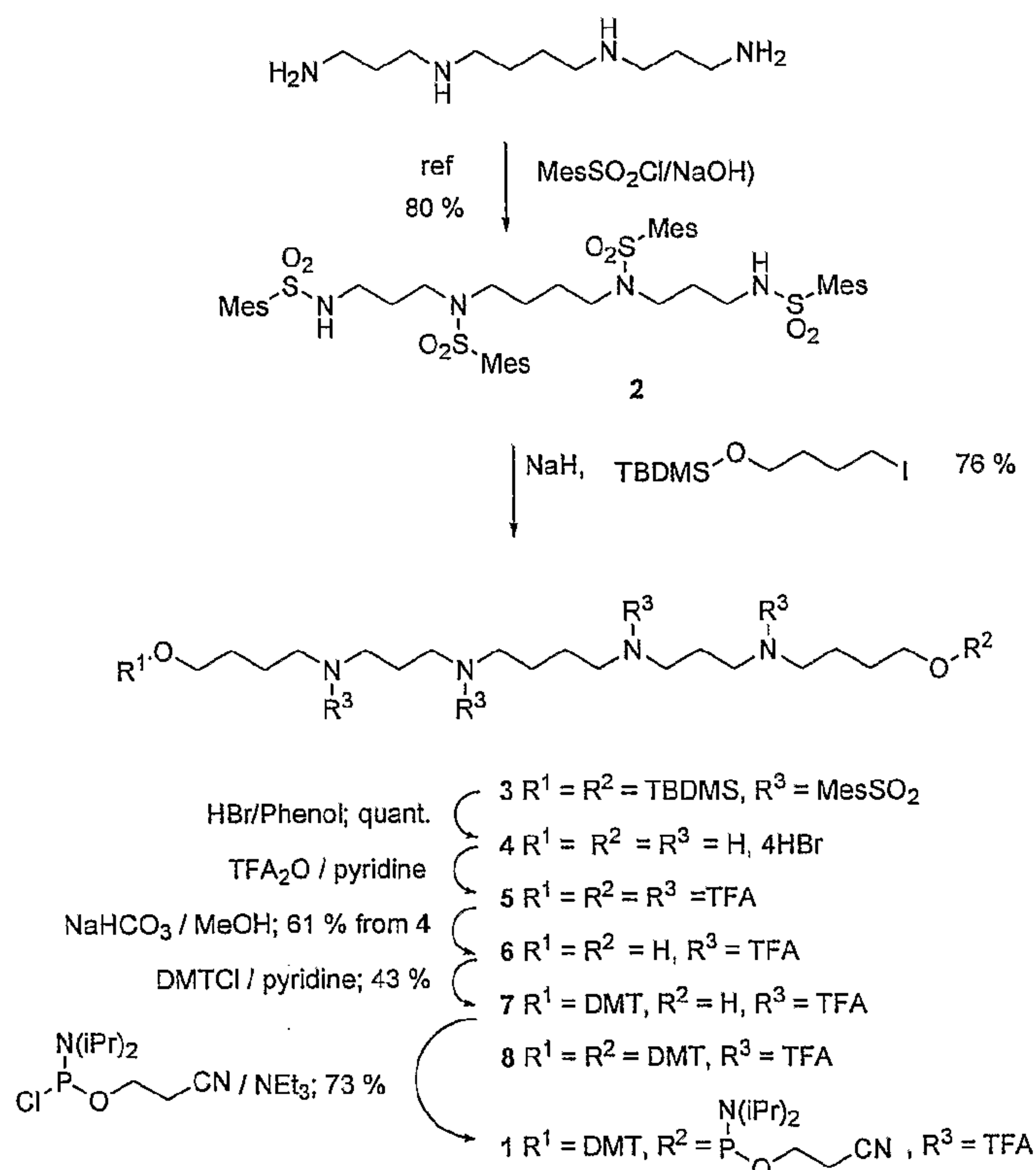
- 15 - Figure 1, HPLC analysis of cationic oligonucleotides $N_{10}S_n$ ($n=1-2$) on a reverse – phase column,
- Figure 2, HPLC analysis of purified oligonucleotides $N_{10}S_n$ ($n=1-6$) on an anion exchange column,
- Figure 3, analysis of $N_{10}S_n$ ($n=1-6$) electrophoretic mobility by polyacrylamide gel electrophoresis,
- 20 - Figure 4, spontaneous exchange of N_{10} with $N_{10}\cdot C_{10}$ at various temperatures,
- Figure 5, strand exchange between N_{10} and $N_{10}S_n$ as revealed by polyamide gel electrophoresis
- Figure 6, melting temperatures of $N_{10} S_n. C_{10}$ duplexes (where C is the nucleotide complementary to N),
- 25 - Figure 7 : comparative results of melting temperatures of duplexes formed by $N_{10}S_n$ ($n=0-6$) with $5'GTGGC\text{A}TCGC3'$ and with $5'GTGGC\text{G}TCGC3'$
- Figure 8, ES-MS analysis of purified $N_{10}S_n$ ($n=1-6$) oligonucleotides,
- Figure 9, HPLC traces of phosphorothioate oligonucleotides $N_{12}S_{11}F$ (9A) and $N_{12}S_2F$ (9B),
- 30 - Figure 10, MALDI-TOF MS spectra of $N_{12}S_2F$ (10 A) and $N_{12}S_{11}F$ (10 B),
- Figure 11, HPLC traces of $N_{14}S_4F$ (11A) and $N_{20}S_5F$ (11B), respectively
- Figure 12, MALDI-TOF MS spectra of $N_{14}S_4F$ (12A) and $N_{20}S_5F$ (12B),

- Figure 13, strand invasion of pGL2 and pGL3 plasmids by N₁₄S_nF (13A) and N₂₀S_nF (13B).
- Figures 14A and 14B, penetration of the cationic oligonucleotide F-S₁₈N₁₉ into HeLa cells.

5

Example 1: Synthesis of phosphoramidite spermine synthon

The spermine tethered phosphoramidite 1 was synthesized from spermine as shown in following Scheme 1:



10

(Mes = 2,4,6-trimethylphenyl; TBDMS = *t*-butyldimethylsilyl; TFA = CF₃CO-;

DMT = 4, 4'-dimethoxytrityl)

Tetrakis(mesitylsulfonyl)spermine 2, prepared from spermine, was bis-alkylated to 3. After complete deprotection of 3 in acidic conditions, the crude bis(C4-OH)spermine tetrahydrobromide 4 was fully protected by trifluoroacetic anhydride in pyridine, then the two terminal ester group of 5 were hydrolyzed in neutral conditions to diol 6. Mono tritylation of 5 was performed in statistical way using one molar equivalent of DMTCl reagent to afford 7 in 43 % yield. Unreacted diol 6 and bis-trityl compound 8 were recovered and re-equilibrated in mild acidic conditions

15

(trifluoroacetic acid in dichloromethane) to afford 7. Phosphitylation of 7 gave the desired phosphoramidite 1.

N^1, N^4, N^9, N^{12} -Tetrakis (mesitylsulfonyl)spermine (2): This compound was prepared according to the reference: Bergeron *et al. J. Med. Chem.* 2001, 44, 232-244.

N^1, N^{12} -Bis[4-(*t*-butyldimethylsilyloxy)butyl]- N^1, N^4, N^9, N^{12} -tetrakis(mesitylsulfonyl)-spermine (3): Sodium hydride (60%, 1.0 g, 25 mmol) was added in portions with stirring under N_2 at 0 °C to a solution of 2 (9.31 g, 10.0 mmol) in DMF (20 mL). After stirring at room temperature for 30 min, *t*-butyl(4-iodobutoxy)dimethylsilane (7.86 g, 25 mmol) was added in one portion. The mixture was stirred overnight at room temperature and then partitioned between $H_2O-CH_2Cl_2$ (100 mL/100 mL). Organic phase was separated and the aqueous phase was extracted three times with CH_2Cl_2 (50 mL). Combined organic phases were washed with $NaHCO_3$ (1 M) solution and then dried on $MgSO_4$. After evaporation, pasty residue was purified by flash chromatography with 1:4 AcOEt:cyclohexane as eluant. The fractions containing 3 were evaporated to a pasty oil which was further washed with cold pentane to eliminate fast moving impurity and then pumped in vacuo to afford 9.97 g (76%) of 3 as an oil: TLC (AcOEt/cyclohexane 1:4): $R_f = 0.28$. – IR (KRS-5): 2937, 1604, 1471, 1320, 1151, 1101, 838, 777, 657, 578 cm^{-1} . – 1H NMR (300 MHz, $CDCl_3$): $\delta = -0.01$ (s, 12 H), 0.85 (s, 18 H), 1.20–1.45 (m, 12 H), 1.62 (m, 4 H), 2.28 (s, 6 H), 2.29 (s, 6 H), 2.53 (s, 12 H), 2.54 (s, 12 H), 2.90–3.10 (m, 16 H), 3.42 (t, $J = 6.1$ Hz, 4 H), 6.91 (s, 4 H), 6.92 (s, 4 H). – ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 4.7, 18.9, 21.6, 23.4, 23.5, 24.1, 24.9, 25.7, 26.6, 30.4, 43.5, 43.6, 45.6, 45.7, 62.9, 132.59, 132.64, 133.8, 140.7, 143.0, 143.1$ – MS-ESI (MeOH): $m/z = 1325.85 [M + Na]^+, 1303.83 [M + H]^+$. – $C_{66}H_{110}N_4O_{10}S_4Si_2$ (Mw = 1304.03) calcd. C 60.79, H 8.50, N 4.30, S 9.84; found C 60.74, H 8.55, N 4.21, S 9.63.

N^1, N^{12} -Bis(4-hydroxybutyl)spermine tetrahydrobromide (4): Hydrogen bromide in acetic acid (33% wt solution, 80 mL, 1.4 mol) was added dropwise to a solution of 3 (9.87 g, 7.57 mmol) and phenol (29.0 g, 0.31 mol, 40 equiv.) in CH_2Cl_2 (80 mL). The reaction mixture was stirred overnight at room temperature. On cooling with an ice bath, cold water (100 mL) was added with stirring. Organic layer was separated and extracted three times with water (20 mL). Combined aqueous layers were washed five times with CH_2Cl_2 (30 mL) and evaporated to driness. Resulting humid solid residue was suspended in ether, triturated with spatula and the supernatant

ether layer was discarded. These operations were repeated (five times) until a solid suspension was obtained. After evaporation and drying in vacuo, compound 4 was obtained as a solid (5.32 g). This crude material was used without further purification: ^1H NMR (300 MHz, D_2O): $\delta = 1.75\text{--}2.10$ (m, 12 H), 2.27 (m, 4 H), 3.15-3.35 (m, 16 H), 3.76 (t, $J = 12.2$ Hz, 4 H). – ^{13}C NMR (75 MHz, D_2O): $\delta = 22.9, 23.2, 23.4, 29.0, 45.0, 45.2, 47.7, 48.3, 61.5$. – MS-ESI (MeOH): $m/z = 347.39$ $[\text{M} + \text{H}]^+$.

N^1, N^{12} -Bis(4-(trifluoroacetoxy)butyl)- N^1, N^4, N^9, N^{12} -tetrakis(trifluoroacetyl)spermine (5) (from 4 with $\text{TFA}_2\text{O}/\text{NEt}_3$): To a suspension of 4 (5.3 g, 7.6 mmol) in CH_2Cl_2 (50 mL), triethylamine (11.5 g, 114 mmol, 15 equiv.) was added in one portion. The mixture was cooled on an ice-bath and trifluoroacetic anhydride (19.1 g, 90.9 mmol, 12 equiv.) was added dropwise with stirring under N_2 . The mixture was stirred at room temperature for 3.5 h. After cooling on an ice-bath, the resulting solution was washed three times with cold water (20 mL), dried on MgSO_4 and then evaporated to afford an oily residue (11.7 g) which contains as secondary product of this reaction, $(\text{TFA})_2\text{C}=\text{CH}-\text{NEt}_2$ (ref Schreber, S. L., *Tetrahedron Lett.* 1980, 21, 1027). This was eliminated by two successive flash chromatography (eluant 1:1 – 60:40 AcOEt: cyclohexane and then 5-10% $\text{Et}_2\text{O}/\text{CH}_2\text{Cl}_2$) to afford 5 (5.59 g, 81%) as an oil: TLC (AcOEt/cyclohexane 1:1): $R_f = 0.25$. – IR (KRS-5): 2955, 1789, 1690, 1467, 1352, 1197, 1147, 759, 731, 692 cm^{-1} . – ^1H NMR (300 MHz, CDCl_3): $\delta = 1.52\text{--}2.06$ (m, 16 H), 3.33-3.49 (m, 16 H), 3.38 (m, 4 H). – ^{13}C NMR (75 MHz, CDCl_3): This spectrum is complicated by rotational isomerism of four amide groups. Only high intensity resonance signals are described as following: $\delta = 23.3, 23.9, 24.1, 24.8, 25.3, 25.6, 26.0, 26.55, 26.61, 44.4, 44.8, 45.7, 46.1, 46.4, 47.3, 48.0, 56.6, 67.3, 67.5, 116.6$ (q, $J = 288$ Hz), 156.9, 157.4, 157.8, 158.6.

N^1, N^{12} -Bis(4-hydroxybutyl)- N^1, N^4, N^9, N^{12} -tetrakis(trifluoroacetyl)spermine (6): To a solution of 5 (5.39 g, 5.84 mmol) in MeOH (50 mL), NaHCO_3 (0.1 g, solid) was added in one portion and the resulting suspension was stirred for 2 h at room temperature. After evaporation, oil residue was dissolved in CH_2Cl_2 (affording a suspension of some fibrous NaHCO_3) and purified by flash chromatography eluting with 5-10% MeOH/ CH_2Cl_2 to afford 3.61 g (85%) of 6 as an oil: TLC (MeOH 5%/CH₂Cl₂): $R_f = 0.14$. (MeOH 10%/CH₂Cl₂): $R_f = 0.45$. – ^1H NMR (300 MHz, CDCl_3): $\delta = 1.51\text{--}2.02$ (m, 18 H), 3.33-3.51 (m, 16 H), 3.68 (m, 4 H). – MS-ESI (MeOH): m/z

= 753.33 [M + Na]⁺. – C₂₆H₃₈F₁₂N₄O₆•H₂O (Mw = 748.60) calcd. C 41.72, H 5.39, N 7.48, F 30.45; found C 41.97, H 5.26, N 7.37, F 30.14.

Preparation of 6 from 4 (with TFA₂O/pyridine, then NaHCO₃): To a suspension of 4 (15.3 g, 22.8 mmol) in CH₂Cl₂ (100 mL) and pyridine (44 mL, 0.54 mol),
5 trifluoroacetic anhydride (46 mL, 0.33 mol) was added dropwise with cooling on an ice bath and with stirring under N₂. The mixture was stirred at room temperature for 3 h. The excess of trifluoroacetic anhydride was decomposed by addition of cold water (100 mL) with cooling on an ice bath, then the resulting solution was extracted with CH₂Cl₂ (four times 100 mL + 50 mL + 25 mL × 2). The combined extracts were
10 washed with cold water (50 mL × 3), dried on MgSO₄ and then evaporated to afford crude 5 (19.4 g, 92 %) as oil. This oil was dissolved in MeOH (100 mL). NaHCO₃ (solid, 0.1 g) was added and the suspension was stirred overnight. After evaporation of solvent, the residue was purified by flash chromatography with 5-7 % MeOH:CH₂Cl₂ as eluant to afford 10.1 g (61%) of 6 as an oil.

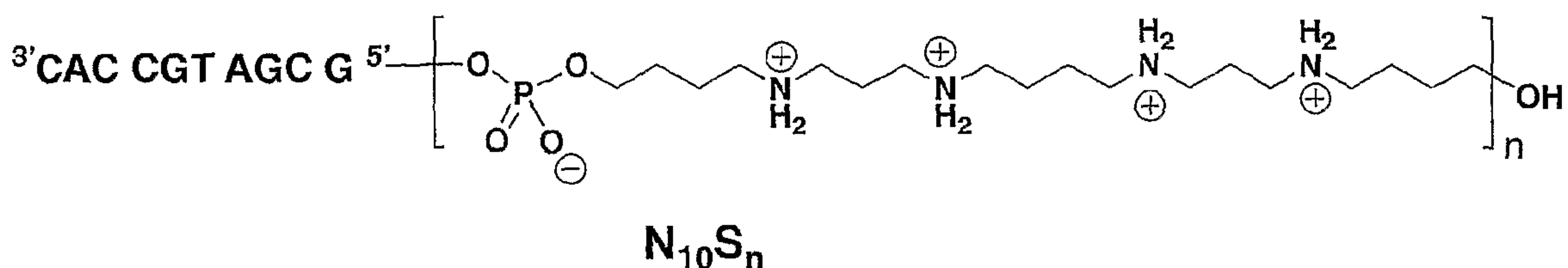
15 *N*¹-[4-(Dimethoxytrityloxy)butyl]-*N*¹²-(4-hydroxybutyl)-*N*¹, *N*⁴, *N*⁹, *N*¹²-tetrakis(trifluoro-acetyl)spermine (7): To a solution of 6 (1.46 g, 2.00 mmol) in pyridine (3 mL), DMTCl (757 mg, 2.23 mmol) was added using 1 mL of pyridine to rinse. The reaction mixture was stirred for 4 h at room temperature under N₂ and then pyridine was repeatedly removed by coevaporation with toluene. Residue was
20 purified by two successive flash chromatography (eluant 2-5% MeOH/CH₂Cl₂ and then 10-15% acetone/CH₂Cl₂) to afford 7 (879 mg, 43%) as foam and bis-DMT derivative 8 (648 mg, 24%). Starting diol 6 was also recovered (350 mg, 24%). Data of 7: TLC (acetone/CH₂Cl₂ 1:9): *R*_f = 0.20. – ¹H NMR (300 MHz, CDCl₃): δ = 1.51–2.03 (m, 17 H), 3.11 (m, 2 H), 3.32-3.51 (m, 16 H), 3.71 (m, 2 H), 3.81 (s, 6H), 6.84
25 (m, 4 H), 7.19-7.46 (m, 9 H). – MS-ESI (MeOH): *m/z* = 1055.52 [M + Na]⁺. – C₄₇H₅₆F₁₂N₄O₈ (Mw = 1032.95) calcd. C 54.65, H 5.46, N 5.42, F 22.07; found C 54.46, H 5.58, N 5.37, F 21.63.

Compound (7) from diol (6) and bis-DMT derivative (8): To a solution of 6 (1.4 g, 1.9 mmol) and 8 (2.5 g, 1.9 mmol) in CH₂Cl₂, trifluoroacetic acid (50 μL, 0.6 mmol)
30 was added and stirred at room temperature for 30 min. The solution was washed three times with Na₂CO₃ 1 M solution, dried on MgSO₄ and evaporated. Residue was separated by flash chromatography (column diameter: 50 mm, SiO₂ height: 15 cm) using successively 5% AcOEt/CH₂Cl₂ (750 mL), 33% AcOEt/CH₂Cl₂ (500 mL), 7%

MeOH/CH₂Cl₂ (500 mL) and 10% MeOH/CH₂Cl₂ (500 mL) to afford 8 (1.1 g), 7 (1.2 g) and 6 (1.3 g).

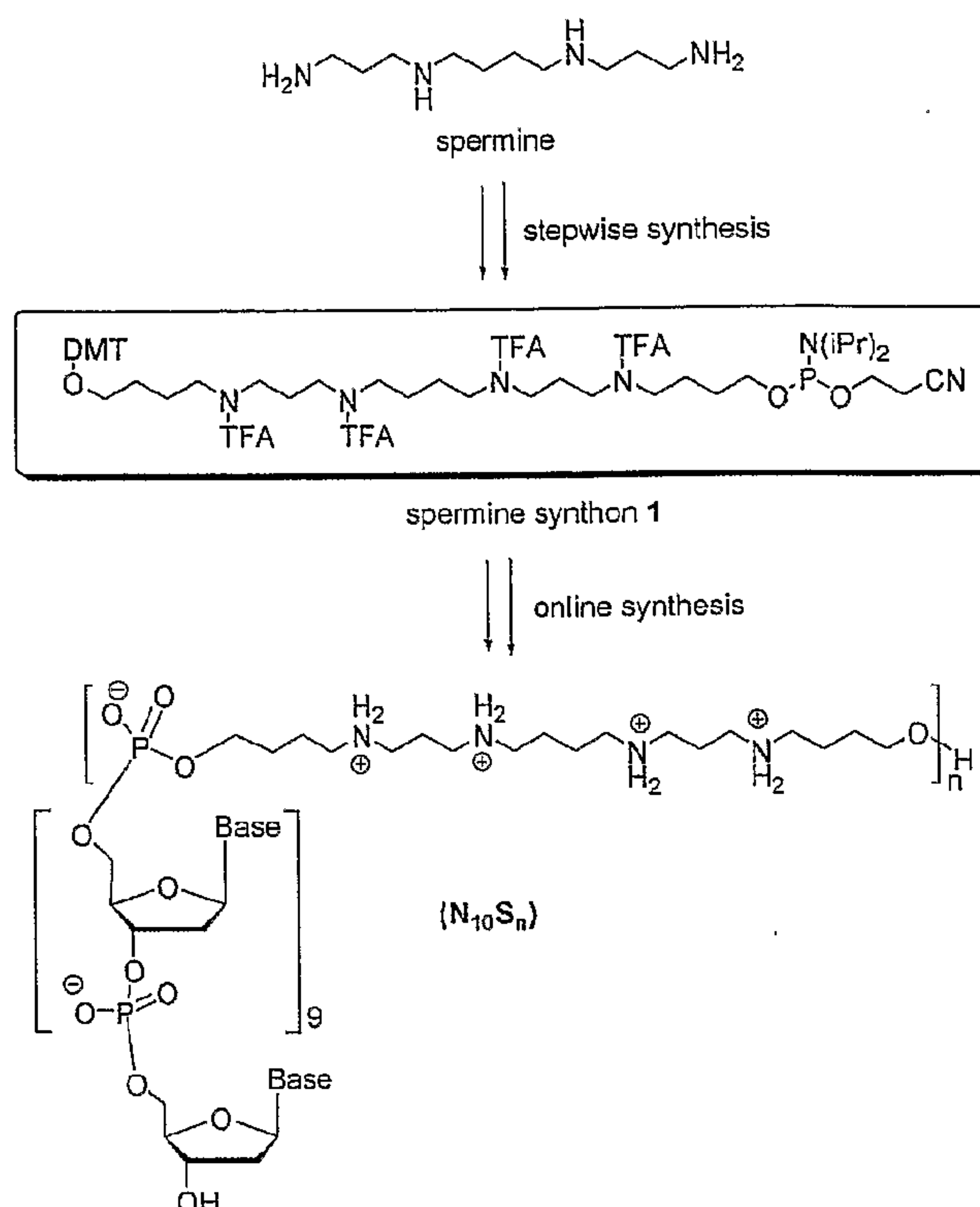
Spermine tethered phosphoramidite (1): To a solution of 7 (844 mg, 817 μmol) and triethylamine (230 μL, 1.65 mmol, 2 equiv.) in CH₂Cl₂ (4 mL), 2-cyanoethyl-(*N,N*-diisopropylamino)chlorophosphite (205 μL, 0.92 mmol, 1.1 equiv.) was added and the mixture was stirred under N₂ at room temperature for 40 min. The reaction mixture was passed through SiO₂ column (diameter: 20 mm, height: 15 cm) saturated with NEt₃ (NEt₃ 1% in CH₂Cl₂:cyclohexane 1:2; 400 mL) using NEt₃ 1% in CH₂Cl₂:cyclohexane 1:2 (125 mL) and then NEt₃ 1% in CH₂Cl₂:cyclohexane 1:1 100 mL to give 1 (735 mg, 73%) as an oil: ¹H NMR (200 MHz, CDCl₃): δ = 1.13-1.35 (m, 12 H), 1.51-2.06 (m, 16 H), 2.66 (t, *J* = 6.4 Hz, 2 H), 3.11 (m, 2 H), 3.32-3.98 (m, 20 H), 3.81 (s, 6H), 6.84 (m, 4 H), 7.15-7.51 (m, 9 H). – ³¹P NMR (81 MHz, CDCl₃): 148.06, 148.13, 148.19, 148.3 (splitting due to amide rotational isomerism).

Example 2: Synthesis, purification and characterization of decamer oligonucleotides having formula



Said oligonucleotides will be hereinafter designated by N₁₀S_n (N₁₀ = an oligonucleotide moiety; S = a spermine residue and n = 1-6).

Automated Synthesis: A series of decamer oligonucleotides of identical sequences N₁₀ = 3' CACCGTAGCG 5' appended with increasing numbers of spermine residues S was synthesized using standard solid-phase cyanoethyl phosphoramidite chemistry on a Expedite DNA synthesizer, according to the following scheme:



the last N moiety being a nucleoside according to the classical oligonucleotide synthesis.

Reagents used for automated DNA synthesis were purchased from Glen Research (Eurogentec).

During the automated synthesis, the standard 1 μ mol coupling cycle was used, except for coupling of the spermine phosphoramidite 1 which was done with prolonged coupling time (15 min) and using a slightly more concentrated phosphoramidite solution (90 mg amidite in 1 mL acetonitrile).

Trityl fractions were collected, diluted and analyzed in a spectrophotometer to determine the stepwise coupling yields.

The coupling yields of the four natural nucleotides exceeded 97 %, while the yields of the spermine phosphoramidite coupling were between 90 and 96 % in the above coupling conditions.

In all cases, the DMT-ON (ON=oligonucleotide) mode was used, keeping the 5'-end DMT group uncleaved on oligomers for purification-identification purposes.

Post-synthetic treatment: After automated synthesis, cleavage from the solid support and complete deprotection of oligomers were done using standard conditions (treatment with concentrated aqueous ammonia for 90 min at room temperature for cleavage and then overnight at 55 °C for deprotection).

Purification: The first two anionic oligonucleotides $N_{10}S_1$ and $N_{10}S_2$ were initially purified in DMT-on state by standard HPLC procedure on a reverse-phase nucleosil C-18 column (Macherey-Nagel 10 × 250 mm) with a linear gradient of acetonitrile (5-35% in 20 min) in 20 mM ammonium acetate solution (pH 7). Purified oligonucleotides were then detritylated by treatment with AcOH/H₂O = 4/1 (500 mL) at r.t. for 20 min. After dilution with water (5 mL), DMT-OH was eliminated by ether extraction (3 × 2 mL) and the aqueous phase was concentrated to afford the oligomers.

The HPLC analysis of oligonucleotides $N_{10}S_1$ and $N_{10}S_2$ is given in Figure 1 a reverse-phase nucleosil C-18 column (Macherey-Nagel 4.6 × 250 mm) with a linear gradient of acetonitrile (5-35% in 20 min) in 20 mM ammonium acetate solution (pH 7): a) $N_{10}S_1$, crude, DMT-ON; b) $N_{10}S_1$, purified c) $N_{10}S_2$, crude, DMT-ON; d) $N_{10}S_2$, purified. *Benzamide; **Truncated sequences.

The neutral oligomer $N_{10}S_3$ and the cationic oligomers $N_{10}S_4$, $N_{10}S_5$ and $N_{10}S_6$ (with or without DMT group) were purified using Poly-Pak IITM (Glen Research/Eurogentec) columns according to the instruction given by manufacturer except for the final oligonucleotide elution which was done with acetonitrile/concentrated aqueous ammonia/water (20:4:80). The fractions containing the oligonucleotide could be revealed using a TLC plate. After gathering the fractions, solvents were removed by lyophilization. The oligomers thus obtained were generally contaminated by benzamide. It was eliminated by extraction with ether (three times) after dissolution in diluted aqueous ammonia solution (50 mM). The purified oligonucleotides were dissolved in diluted aqueous ammonia solution (50 mM), and their concentration was determined using the following extinction coefficient (260 nm, mol⁻¹dm³cm⁻¹):

$$\varepsilon = (15.4 N_A + 11.5 N_G + 7.4 N_C + 8.7 N_T) \times 0.9 \times 10^3.$$

The HPLC analysis of purified oligonucleotides is given in Figure 2: anion exchange column (Dionex PA-100 9 × 250 mm) with a linear gradient of NaCl (100-350 mM over 10 min) /NaOH 25mM (pH 12.4): a) $N_{10}S_1$, b) $N_{10}S_2$, c) $N_{10}S_3$, d) $N_{10}S_4$, e) $N_{10}S_5$, f) $N_{10}S_6$.

Due to the conjugation chemistry employed, each polyamine comes with a phosphate group, hence contributing for a net additional cationic charges. Seven

oligonucleotides, $(N_{10}S_n)^{3n-9}$ $n=0...6$, with overall charges -9,-6,-3,0,+3,+6,+9 when fully ionized, where thus available in amounts ranging from 80 to 250 nanomoles.

Electrophoretic mobility:

Their migration in an electric field at pH7 was studied by polyacrylamide gel electrophoresis and revealed by silver mirror staining. Compounds (0.5 nmol) in 10 μ L loading buffer (10mM HEPES pH 7.4, 150mM NaCl, glycerol) were loaded onto a nondenaturing polyacrylamide gel (15% in TAE pH 7). Electrophoresis was run at 5 V/cm for 17 h at 4°C. Silver staining was performed according to Rabilloud et al, Electrophoresis, 1987,9, 288-291. The results are given in Figure 3. Oligonucleotide N₁₀ (lane 1) without spermine was moving fast towards the anode and showed only faint silver staining in conditions where polyamine-containing oligonucleotides were revealed.

Spontaneous exchange of N₁₀ with N₁₀•C₁₀

Oligonucleotide C₁₀ (where C is the nucleotide complementary to N) (50pmol or 500pmol) was added to the fluorescent N₁₀•C₁₀* duplex solution (50pmol in HEPES 10mM pH 7.4, NaCl 150mM). The mixtures were incubated 4 h at 37°C, 20°C or 10°C and loaded onto a nondenaturing polyacrylamide gel (15% in TAE pH 7). Electrophoresis was performed at 4°C for 17 h at 5 V/cm. C₁₀* fluorescence was detected by scanning the gel using a Typhoon 8600 Imager. As shown by the results given in Figure 4, the spontaneous exchange of N₁₀ with N₁₀•C₁₀ is not significant at 10°C.

Strand exchange between N₁₀ and N₁₀ S_n

The strand replacement capacity of N₁₀S_n towards the natural duplex N₁₀•C₁₀ was tested in physiological salt conditions.

Spermine conjugates N₁₀S_n (50 or 500 pmol) were added to a fluorescent N₁₀•C₁₀* duplex solution (50pmol in 10mM HEPES pH 7.4, 150mM NaCl). The mixtures were incubated 4 h at 10°C and loaded onto a nondenaturing polyacrylamide gel (15% in TAE pH 7). Electrophoresis was performed at 4°C for 17 h at 5 V/cm. Fluorescence was detected by scanning the gel using a Typhoon 8600 Imager.

Spermine conjugation had a profound effect on the strand exchange reaction as shown in Figure 5. The band corresponding to N₁₀•C₁₀* became weaker as the number of spermine residues of the competing N₁₀S_n increased, in favour of a slower-moving, less anionic N₁₀S_n•C₁₀* complex. This effect was especially

pronounced for $N_{10}S_3$, i.e., for conjugates which no longer bear a formal negative charge. Indeed, spermine is clipping duplex DNA structures by forming an interstrand network of NH_2^+ bidentate hydrogen bonds in the minor groove, hence will favour $N_{10}S_n$ binding over N_{10} . Yet an additional favourable kinetic factor may operate when strand exchange occurs in a preformed $(N_{10}S_n)^{3n-9}/(N_{10}\cdot C_{10})^{18-}$ electrostatic complex, which can be the case for $n>3$.

Melting temperatures of $N_{10}S_n\cdot C_{10}$ duplexes

Stabilities of double stranded nucleic acids were compared by measuring their melting temperature, i.e. the temperature where complementary strands cooperatively fall apart. Optical density (O.D.) was thereof recorded at 260 nm of solutions of $N_{10}S_n\cdot C_{10}$ vs. temperature T.

Melting temperatures T_m were measured in HEPES 10 mM pH 7.4 (black line, rhombi) and in HEPES 10 mM pH 7.4 + 150 mM NaCl (grey line, cercles). Melting profiles of all duplexes (3.75 nmol in 1 ml buffer) were obtained using a CARY 4000 Spectrophotometer equipped with a temperature control unit by gradually heating the samples ($1^\circ C/min$) while recording their absorbance at 260 nm. Duplex melting results in a hyperchromic shift and T_m is the temperature where the first derivative curve $dO.D./dT = f(T)$ reaches its maximum. The results are given in Figure 5.

The natural duplex melted at $T_m = 30^\circ C$ in 10 mM HEPES pH 7.4 (Figure 5). Conjugation of increasing numbers of spermines led to remarkable T_m increases. $N_{10}S_6\cdot C_{10}$ melted at $T_m = 75.2^\circ C$, some $45^\circ C$ higher than the natural duplex. The $T_m = f(n)$ curve showed a sigmoidal shape with an inflection for the neutral $N_{10}S_3$ oligonucleotide.

Melting temperatures were also recorded in physiological salt conditions. The $T_m = f(n)$ curve appeared much damped and, remarkably, crossed the previous curve for $N_{10}S_3$. Thus for $n<3$, both $N_{10}S_n$ and C_{10} oligonucleotides are anionic and repel each other in the duplex; increasing the solution salt concentration shields repulsive forces hence increases T_m . For $n>3$ $N_{10}S_n$ becomes cationic and attracts C_{10} ; here salt-induced electrostatic shielding decreases stability.

For the neutral $N_{10}S_3$, duplex stability is independent of salt concentration.

Comparison of melting temperatures of duplexes formed by $N_{10}S_n$ ($n=0-6$) with $5'GTGGC\underline{A}TCGC^3'$ and with $5'GTGGC\underline{G}TCGC^3'$

A single base pair mismatch discrimination of the oligonucleotide-spermine conjugates was tested. Within the sequence context of $C_{10} = 5'GTGGC\underline{A}TCGC3'$, literature data recommended a centrally-located A-to-G conversion as being the most stringent test.

5 Melting temperatures T_m were measured in HEPES 10 mM pH 7.4 + NaCl 150 mM. Melting profiles of all duplexes (3.75 nmol in 1 mL buffer) were obtained using a CARY 4000 Spectrophotometer equipped with a temperature control unit by gradually heating the samples ($1^\circ\text{C}/\text{min}$) while recording their absorbance at 260 nm. T_m is the temperature where the first derivative curve $dO.D./dT = f(T)$ reaches its
10 maximum. The results are given on Figure 7 (rhombi correspond to $5'GTGGC\underline{A}TCGC3'$ and triangles to $5'GTGGC\underline{G}TCGC3'$).

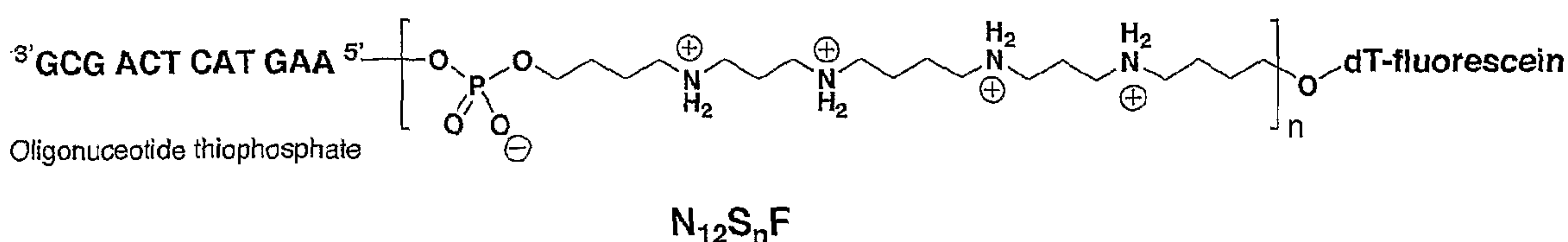
The transition temperature of the natural $N_{10}\cdot C_{10}$ duplex in 150 mM NaCl fell from 50.6°C to 42.9°C , i.e. $\Delta T_m = 7.7^\circ\text{C}$ when the mismatch was present. In principle, stability increase due to nonspecific, end-conjugated electrostatic forces should not
15 impair base pair specificity, which is expressed as $\Delta\Delta G$. This is indeed what was observed, as the complementary and mismatch target oligonucleotide showed quasi-parallel $T_m = f(n)$ curves with average $\Delta T_m = 7.9^\circ\text{C}$.

ES-MS analysis of purified $N_{10}S_n$ oligonucleotides.

Oligonucleotides were dissolved in 50 % aqueous acetonitrile (v/v) containing
20 1 % triethylamine at a final concentration of 5×10^{-5} M. 100 mL aliquots were introduced into the ion source of an Applied Biosystems Mariner 5155 mass spectrometer at a flow rate of 5 mL/min. The results are given in Figure 8 (insets: deconvoluted spectra): a) $N_{10}S_1$, b) $N_{10}S_2$, c) $N_{10}S_3$, d) $N_{10}S_4$, e) $N_{10}S_5$, f) $N_{10}S_6$. Ionization of the neutral and cationic oligomers $N_{10}S_{3-6}$ became more difficult and it
25 was necessary to accumulate several spectra to obtain acceptable signal-to noise ratio.

Example 3 : Synthesis, purification and characterization of 12-mer thiophosphate oligonucleotides having formula

30



Said oligonucleotides will be thereafter designated by $N_{12}S_nF$ (N = a 12-mer oligonucleotide thiophosphate moiety; S = a spermine residue and $n = 2$ or 11 ; F = fluorescein conjugated to thymine).

5 *Automated Synthesis:* Twelve-mer thiophosphate oligonucleotides of sequence $N_{12} = 3'GCGACTCATGAA5'$ appended with two or 11 spermine residues S were synthesized using solid-phase cyanoethyl phosphoramidite chemistry on an Expedite DNA synthesizer. Ultramild CE phosphoramidites and ultramild supports (Glen Research / Eurogentec) were used in order to avoid oligomer cleavage during
10 work-up. A standard sulfurizing reagent (Glen Research/Eurogentec) was used to generate the phosphorothioate linkages in the 12-mer oligonucleotide moiety. Fluorescein-dT phosphoramidite (Glen Research/Eurogentec) was used for 5'-end labelling. Spermine phosphoramidite coupling was performed using the coupling protocol described in example 2.

15 Trityl fractions were collected, diluted and analyzed in a spectrophotometer to determine the stepwise coupling yields.

In all cases, the DMT-ON mode was used, keeping the 5'-end DMT group uncleaved on oligomers for purification-identification purposes.

20 *Post-synthetic treatment:* After automated synthesis, cleavage from the solid support and complete deprotection of oligomers were performed by treatment with concentrated aqueous ammonia overnight at room temperature.

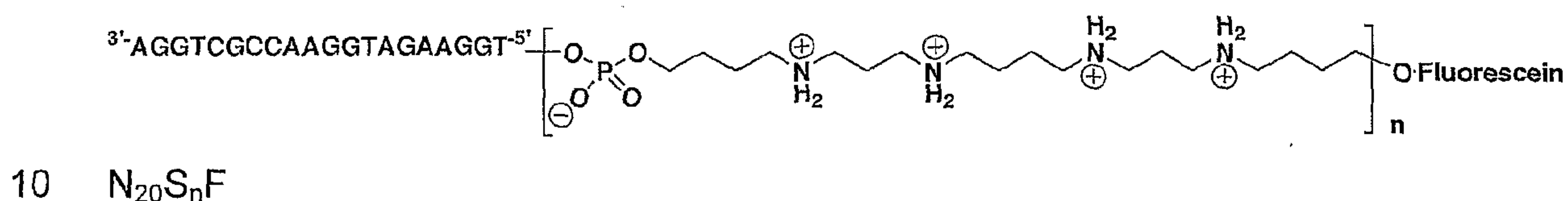
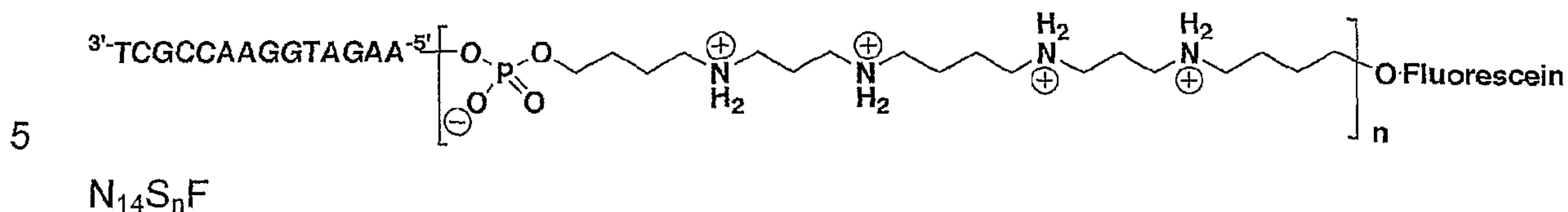
Purification: DMT-ON compounds $N_{12}S_2F$ and $N_{12}S_{11}F$ were purified using Poly-Pak IITM columns (Glen Research/Eurogentec) according to instructions given by the manufacturer.

25 Purified oligonucleotides $N_{12}S_nF$ ($n = 2, 11$) were analyzed on an anion exchange column (SAX1000-8) in aqueous basic conditions (100 mM ammonia, pH 11) using a NaCl gradient (0.75-2.5 M in 20 min). HPLC traces are shown in Figure 9 (A: $N_{12}S_{11}F$, B: $N_{12}S_2F$).

MALDI-TOF MS analysis of purified oligonucleotides.

30 Oligonucleotides were dissolved in 500 μ L of deionized water. The sample and HPA matrix were mixed together on the plate. Once crystallized, the sample was analyzed with a BRUKER Ultraflex MS apparatus. Results are given in Figure 10 A : $N_{12}S_2F$ calc 5460 , found 5459 (upper) and Figure 10 B : $N_{12}S_{11}F$ calc : 9135 found : 9125 (lower).

Example 4 : Plasmid DNA strand invasion with 14-mer and 20-mer fluorescent oligonucleotides



Compounds shown above will be thereafter designated by $N_{14}S_nF$ (N = an oligonucleotide moiety; S = a spermine residue with $n = 2-4$; F = a fluorescein residue) and by $N_{20}S_nF$ (N = an oligonucleotide moiety; S = a spermine residue with $n = 3-5$; F = a fluorescein residue).

15 These fluorescent oligonucleotides were synthesized following the procedure described in example 2. 5'-Fluorescein phosphoramidite (Glen Research/Eurogentec) was used for 5'-end labelling. Analytical HPLC traces and MALDI-TOF MS spectra for the most substituted $N_{14}S_4F$ and $N_{20}S_5F$ compounds are shown in figures 11 and 12 as proofs of purity and structure ($N_{14}S_4F$ calc 6470, found
20 6478; $N_{20}S_5F$ calc 8813, found 8815), respectively.

Oligonucleotide sequences of $N_{14}S_nF$ and $N_{20}S_nF$ were chosen within the Luciferase gene sequence of the pGL3 control plasmid (Promega). To assess the sequence specificity of strand invasion, pGL2 control plasmid (Promega) was used. The GL2 Luciferase sequence is 95% identical to GL3, and the sequences targeted
25 by $N_{14}S_nF$ and $N_{20}S_nF$ contain respectively one and two mismatches.

The ability of $N_{14}S_nF$ and $N_{20}S_nF$ to strand-invade pGL3 and not pGL2 plasmids was tested in physiological salt and temperature conditions.

Fluorescent conjugates $N_{14}S_nF$ and $N_{20}S_nF$ (8.65 pmol) were added to a solution of plasmid (1.5 μg , 0.43 pmol in 10 mM HEPES pH 7.4, 150 mM NaCl). The
30 mixtures were incubated 24 h at 37°C and loaded onto an agarose gel (1.3% in TAE pH 7.4). Electrophoresis was performed at room temperature for 45 min after what

fluorescein green emission was detected by scanning the gel using a Typhoon 8600 Imager. A red fluorescence picture of the gel was taken on an UV transilluminator following a 15 min incubation in ethidium bromide solution. The results are given in Figure 13.

5 Red and green fluorescences are evidence of double stranded plasmid DNA and fluorescent oligonucleotide, respectively. Their colocalization with pGL3 and not with pGL2 is thus evidence for strand invasion. Compounds $N_{14}S_3F$ and $N_{20}S_nF$ showed a faint green fluorescent band associated with the plasmid when incubated with pGL3 and not with pGL2.

10

Example 5 : Penetration of cationic oligonucleotides into cells.

Hela cells, grown in 10% (v/v) fetal calf serum containing MEM medium, were plated at $50-60 \times 10^3$ cells/well into 4-well chambered borosilicate Lab-Tek dishes one day prior to the experiment. Complete medium was replaced by 0.5ml serum-free
15 MEM medium. A 5'-cationic fluorescein-conjugated oligonucleotide F- $S_{18}N_{19}$ (where N_{19} is TCGAAGTACTCAGCGTAAG) formulation was prepared in sterile PBS. It was added to the cells to a final concentration of $2 \mu M$. Four hours later, the medium was replaced by 1ml of fresh serum-containing medium. A first picture was taken with a
20 Zeiss axiovert 25 fluorescence microscope, equipped with a FITC filter (Figure 14 A, left). All cells became fluorescent, with some fluorescence located in intracellular vacuoles and, most importantly, also spread throughout the cytoplasm and nucleus. After 24h, the medium was replaced with 1ml of phenol red-free MEM medium. Propidium iodide (1mM in water) was added to a final concentration of $10 \mu M$. Ten
25 minutes later, a second picture was taken showing a majority of propidiumless healthy cells that were still fluorescent (Figure 14 B, right). The control cells that were incubated in similar conditions with F-N19 oligonucleotide showed no fluorescence.

The invention thus provides a versatile automatic synthesis of cationic oligonucleotides that form fast and stable complexes with their complementary sequence even in a strand invasion context. Due to end conjugation, sequence
30 selectivity remains as high as for natural oligonucleotides. Moreover, thanks to their cationic nature, intracellular delivery does not require complex formation with cationic carrier molecules. Taken together, these properties make oligonucleotide-oligocation conjugates attractive alternatives to oligonucleotides for molecular biology, diagnostics as well as therapeutic applications.

DEMANDE OU BREVET VOLUMINEUX

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NOTE POUR LE TOME / VOLUME NOTE:

WHAT IS CLAIMED IS:

1. Oligonucleotide-oligocation molecules comprising a $A_i B_j H$ sequence or a sequence selected from the group of: a ${}^3A_i{}^5-B_j$ sequence, a $B_j-{}^3A_i{}^5$ sequence, a $B_j-{}^3A_i{}^5-B_j$ sequence, a ${}^3A_i{}^5-B_j-{}^3A_i{}^5$ sequence and combinations thereof that can be synthesized via automated phosphoramite chemistry having oligonucleotides moieties A_i and oligocations moieties B_j wherein

A_i is an i -mer oligonucleotide residue, with $i = 5$ to 50 , where nucleotide A is an oligonucleotide with nucleobases and/or pentafuranosyl groups and/or native phosphodiester bonds and/or phosphorothioate, or 2'-fluoro modifications or substitutions of the nucleobases or pentafuranosyl groups or phosphodiester bonds or 2'-O-alkyl modifications or substitutions of the nucleobases or pentafuranosyl groups or phosphodiester bonds,

B_j is a j -mer organic oligocation repeated block, with $j=2$ to 50 , where B is selected from the group consisting of :

$HPO_3-R^1-(X-R^2_n)_{n1}-X-R^3-O-$, where R^1 , R^2_n and R^3 , identical or different are a linear or branched C1 to C5 alkylene groups, X is NH or $NC(NH_2)_2$ n varies from 1 to 5 and $n1=2$ to 20,

$HPO_3-R^4-CH(R^5X^1)-R^6-O-$, where R^4 is a linear or branched C1 to C5 alkylene, R^5 and R^6 , identical or different, are a linear or branched C1 to C5 alkylene groups and X^1 is putrescine, spermidine or spermine residue, and

$-HPO_3-R^7-(aa)_{n2}-R^8-O-$, where R^7 is a linear or branched C1 to C5 alkylene group and R^8 is a linear or branched C1 to C5 alkylene group, serine or an amino alcohol, $(aa)_{n2}$ is a peptide containing amino acids with cationic side chains and $n2=2$ to 20 wherein B groups are oligomerized by a stepwise synthesis through a phosphodiester linkage to form B_j and wherein A_i is linked to B_j through a phosphodiester linkage.

2. The molecules of claim 1, wherein the A_i oligonucleotide is selected from the group consisting of deoxyribonucleotides, ribonucleotides, and locked (LNA) nucleotides.

3. The molecules of claim 2, wherein, A_i or B_j further contains a marker.
4. The molecules of claim 3, wherein said marker is a fluorescent agent.
5. The molecules of claim 1, having a ${}^3A_i{}^5-B_j$ sequence.
6. The molecules of claim 1, having a $B_j-{}^3A_i{}^5$ sequence.
7. The molecules of claim 1, having $B_j-{}^3A_i{}^5-B_j$ or ${}^3A_i{}^5-B_j-{}^3A_i{}^5$ sequences and combinations thereof.
8. A method for obtaining oligonucleotide-oligocation molecules according to claim 1, by using a stepwise synthesis on an oligonucleotide synthesizer, via the phosphoramidite route, comprising
 - (i) plugging vials containing activated and protected oligocations B into an oligonucleotide synthesizer, in addition to vials of oligonucleotide A, or the reverse,
 - (ii) stopping the synthesis when the desired length is obtained,
 - (iii) cleaving the oligomers from the solid support, and
 - (iv) removing the protecting groups.
9. The method of claim 8, wherein phosphoramidite reagents are selected from the group comprising
 - $P(OR^9)(N(R^{10})_2)-O-R^1-(X-R^2_n)_{n1}-X-R^3-O-Prot$, where R^1, R^2, R^3 , identical or different are a linear or branched C1 to C5 alkylene groups, X is a protected NH or $NC(NH_2)_2$, R^9 is CH_2CH_2CN or a linear or branched C1 to C5 alkyl group, R^{10} is a linear or branched C1 to C5 alkyl group, or $-N(R^{10})_2$ is pyrrolidino, piperidino or morpholino group, and Prot is a protecting group used in oligonucleotide synthesis selected from the group comprising 4,4-dimethoxytrityl and methylcyclopentadienyl manganese tricarbonyl, n varies from 1 to 5 and $n1=2$ to 20;
 - $P(OR^9)(N(R^{10})_2)-O-R^4-CH(R^5X^1)-R^6-O-Prot$ where R^4, R^5, R^6 are a linear or branched C1 to C5 alkylene groups, X^1 is a protected putrescine, spermidine or spermine, R^9 is CH_2CH_2CN or a linear or branched C1 to C5 alkyl group and R^{10} is a linear or branched C1 to C5 alkyl group;

•P(OR⁹)(NR¹⁰)₂-O-R⁷-(aa)_{n2}-R⁸-O-Prot, where R⁷ is a linear or branched C1 to C5 alkylene group, R⁸ is a linear or branched C1 to C5 alkyl group, a serine or an amino alcohol, R⁹ is CH₂CH₂CN or a linear or branched C1 to C5 alkyl group, R¹⁰ is a linear or branched C1 to C5 alkyl group, (aa)_{n2} is a peptide containing amino acids with cationic side chains selected from the group comprising Arginine, Lysine Ornithine, Histidine and Diaminopropionic acid and n₂=2 to 20, and Prot is a protecting group used in oligonucleotide synthesis selected from the group comprising 4,4- dimethoxytrityl and methylcyclopentadienyl manganese tricarbonyl.

10. The method of claim 9, wherein stepwise synthesis of the oligonucleotide sequence is followed by stepwise synthesis of the oligocation moiety to obtain compounds having sequence (³A⁵_i-B_j).
11. The method of claim 9, wherein stepwise synthesis of the oligonucleotide sequence is followed by stepwise synthesis of the oligocation moiety to obtain compounds having sequence (B_j-³A⁵_i)sequence.
12. The method of claim 9, comprising the synthesis of mixed sequences.
13. The method of claim 12, comprising the synthesis of oligonucleotide sequences capped at both ends (B_j-³A⁵_i-B_j) or oligonucleotide sequences cation-interrupted sequences (³A⁵_i-B_j-³A⁵_i).
14. The method of claim 9, wherein the activated and protected oligocations B are obtained by protecting the amino groups of a polyamine, followed by α,ω-bis hydroxyalkylation, leading to diols compatible with oligonucleotide synthesis.
15. Use of an oligonucleotide-oligocation molecule according to any one of claims 1 to 7 in PCR, real-time PCR, genotyping, *in situ* hybridization or the manufacture of DNA chips.

16. Pharmaceutical compositions comprising an oligonucleotide-oligocation molecule according to any one of claims 1 to 7, in association with a pharmaceutically acceptable carrier.

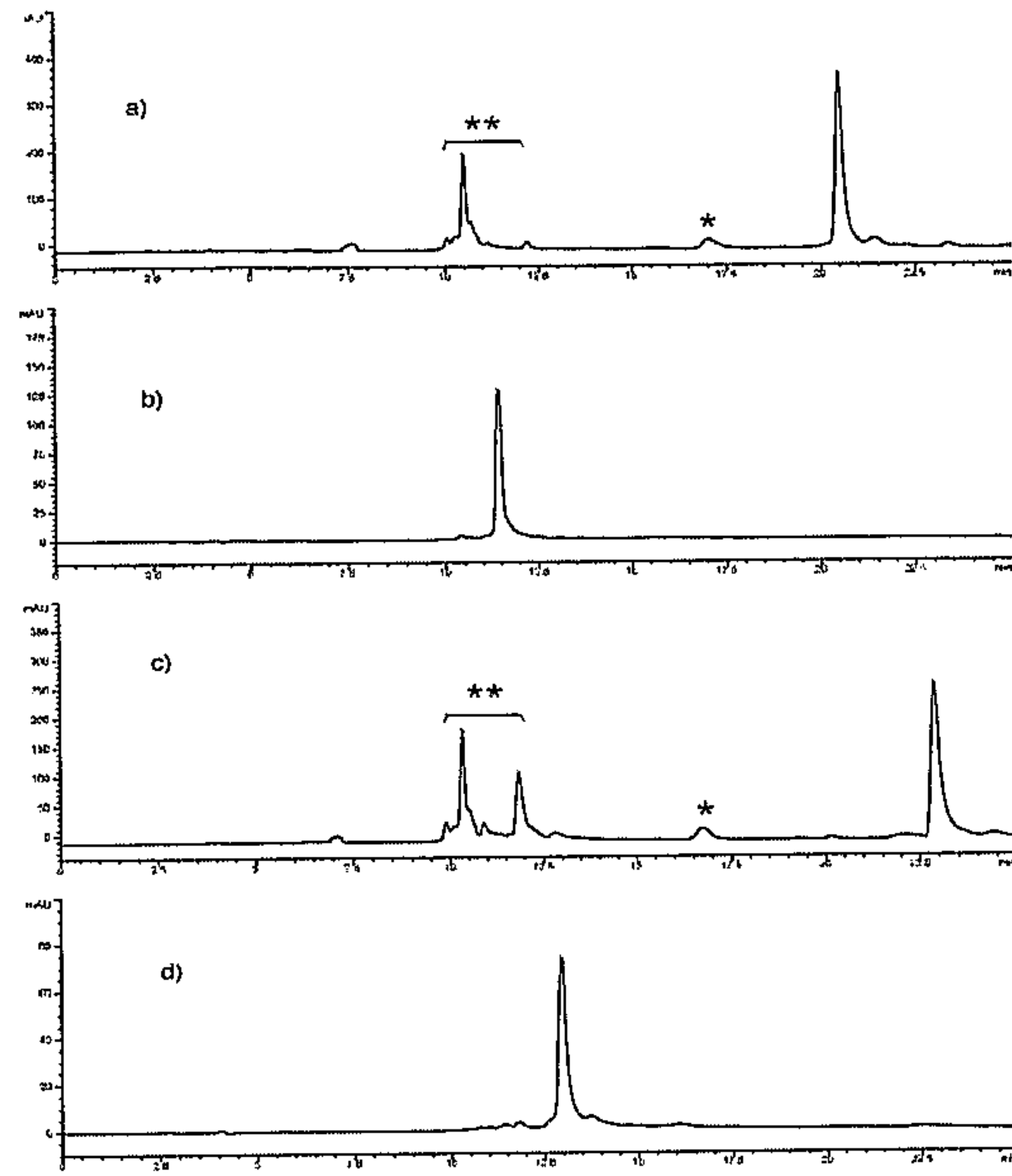


Figure 1

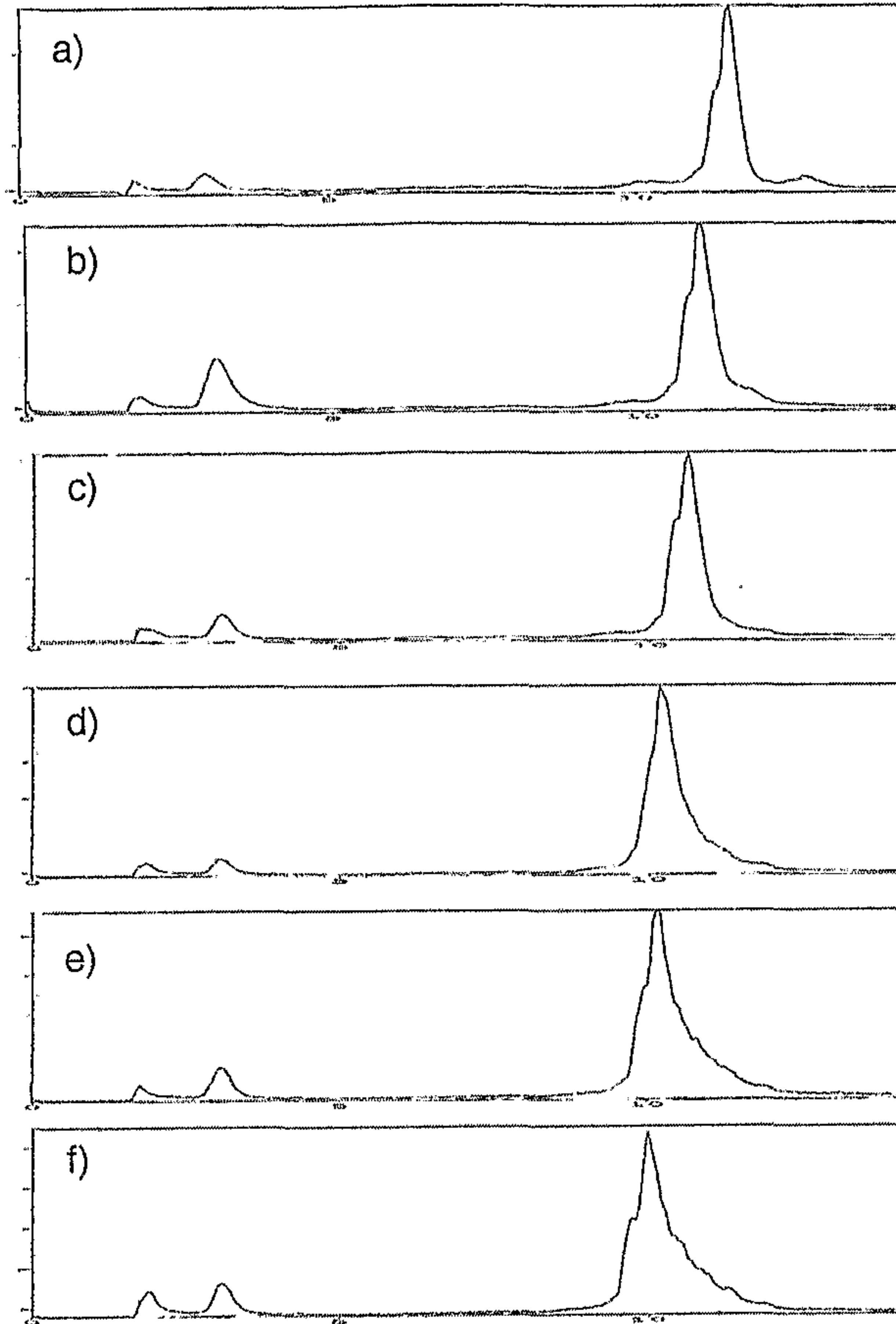


Figure 2

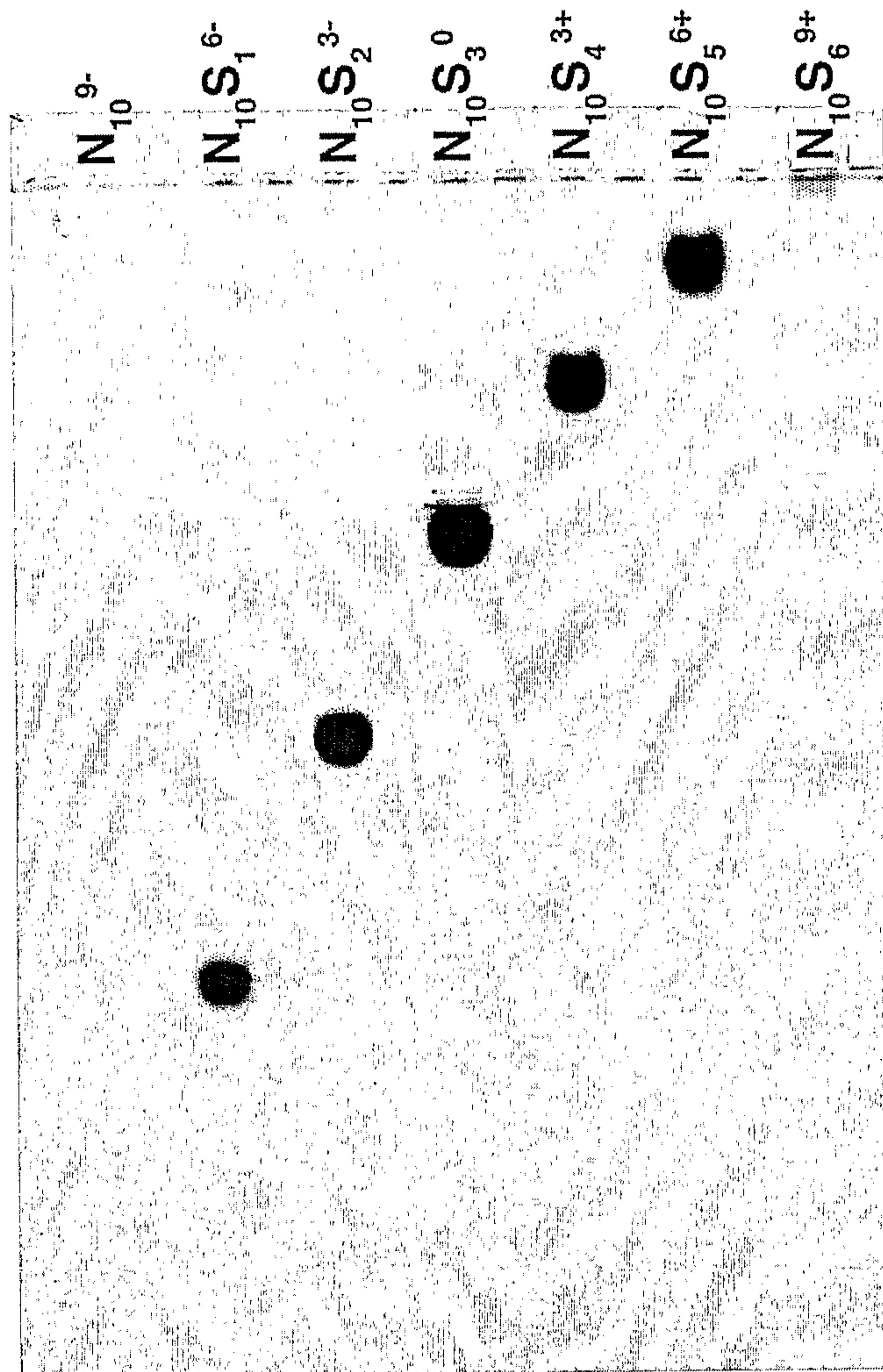


Figure 3

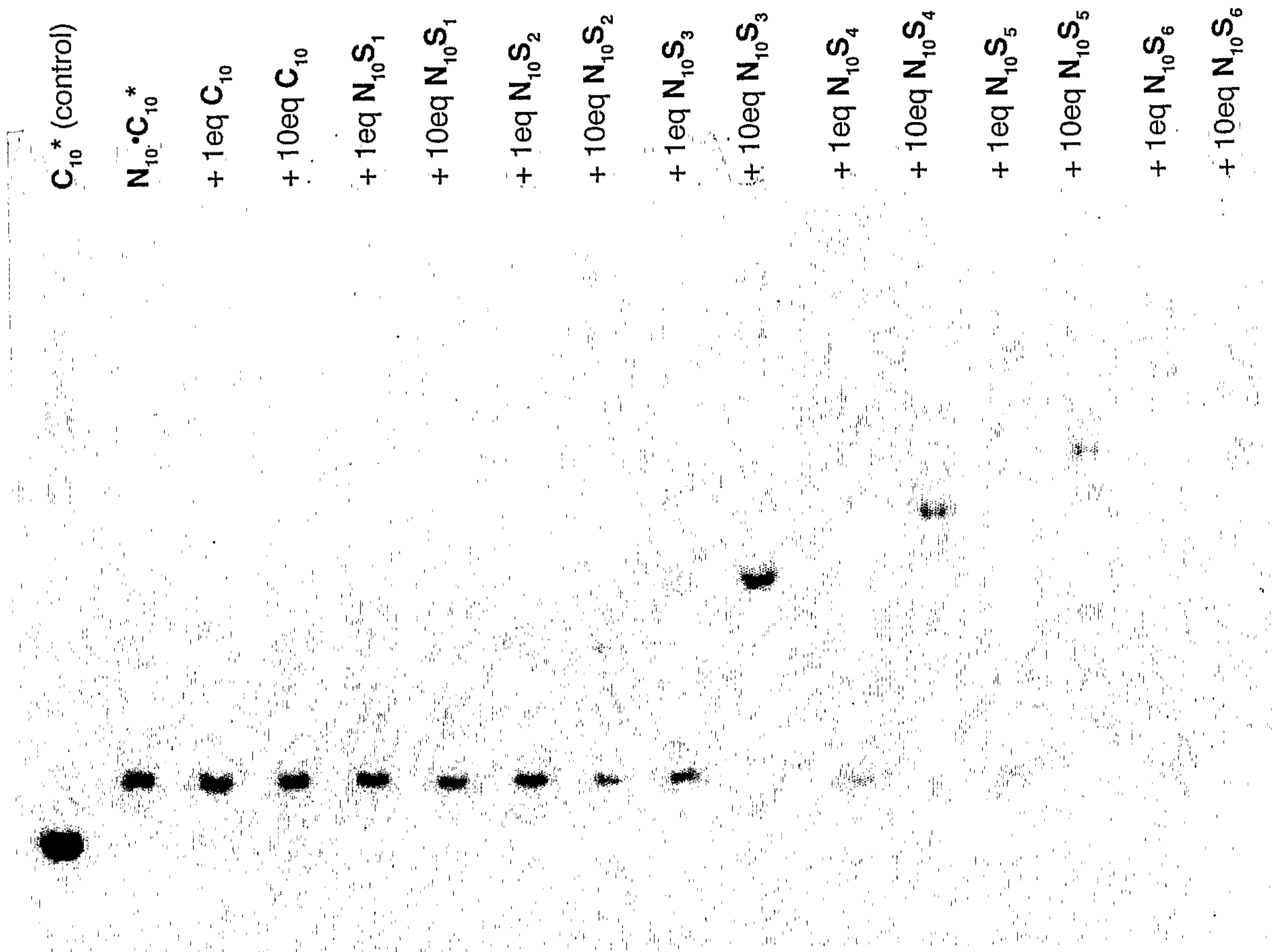


Figure 4

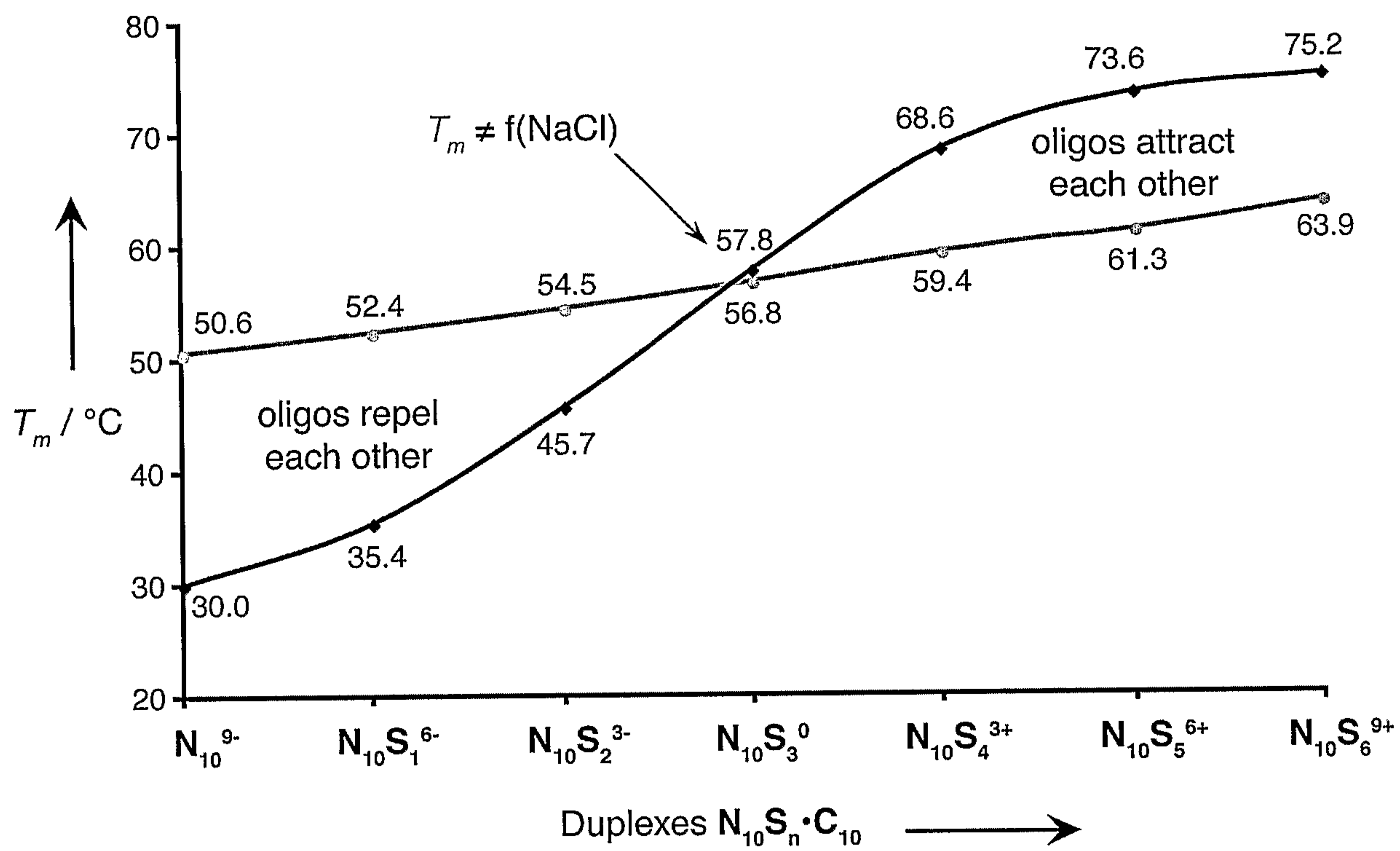


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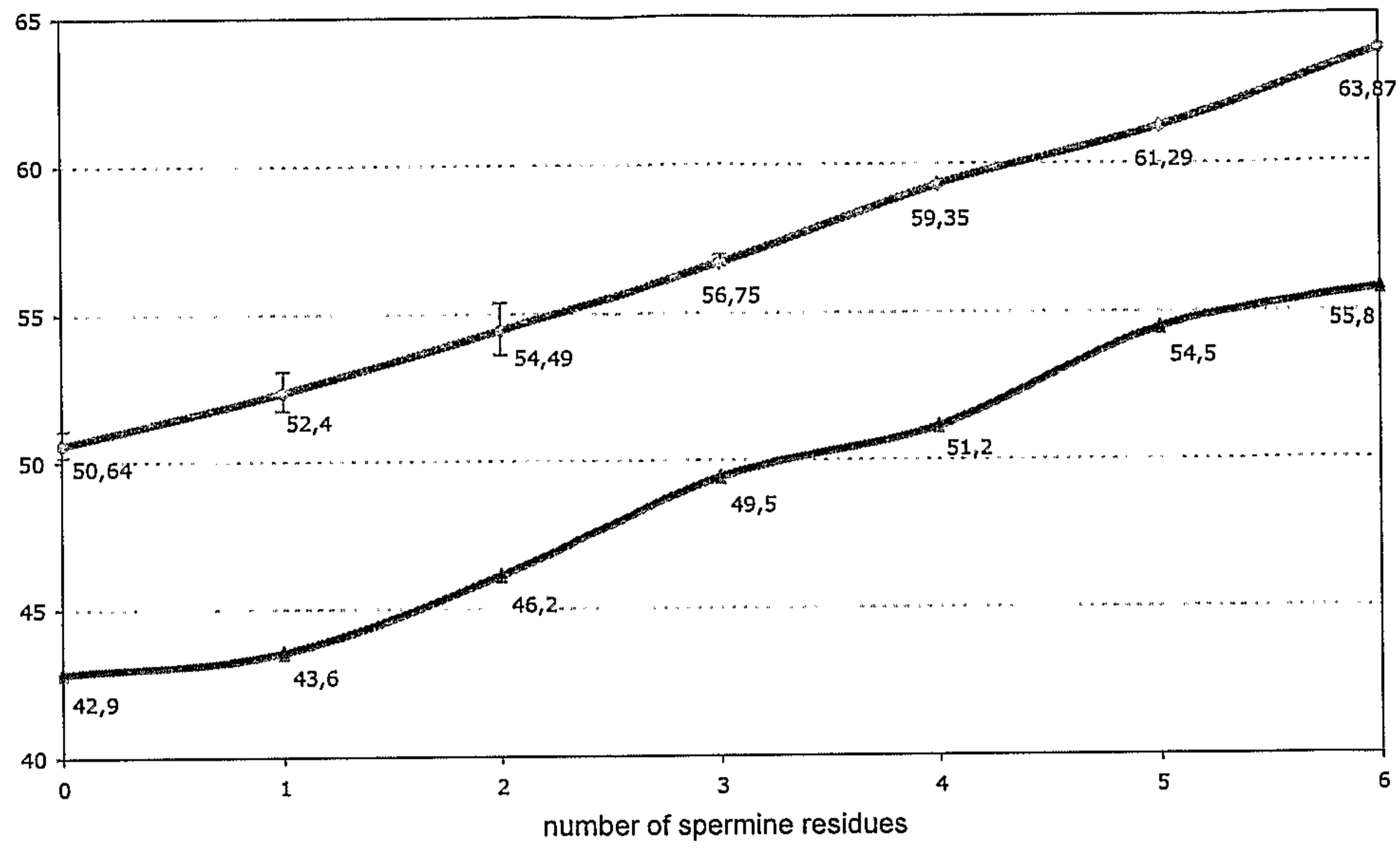


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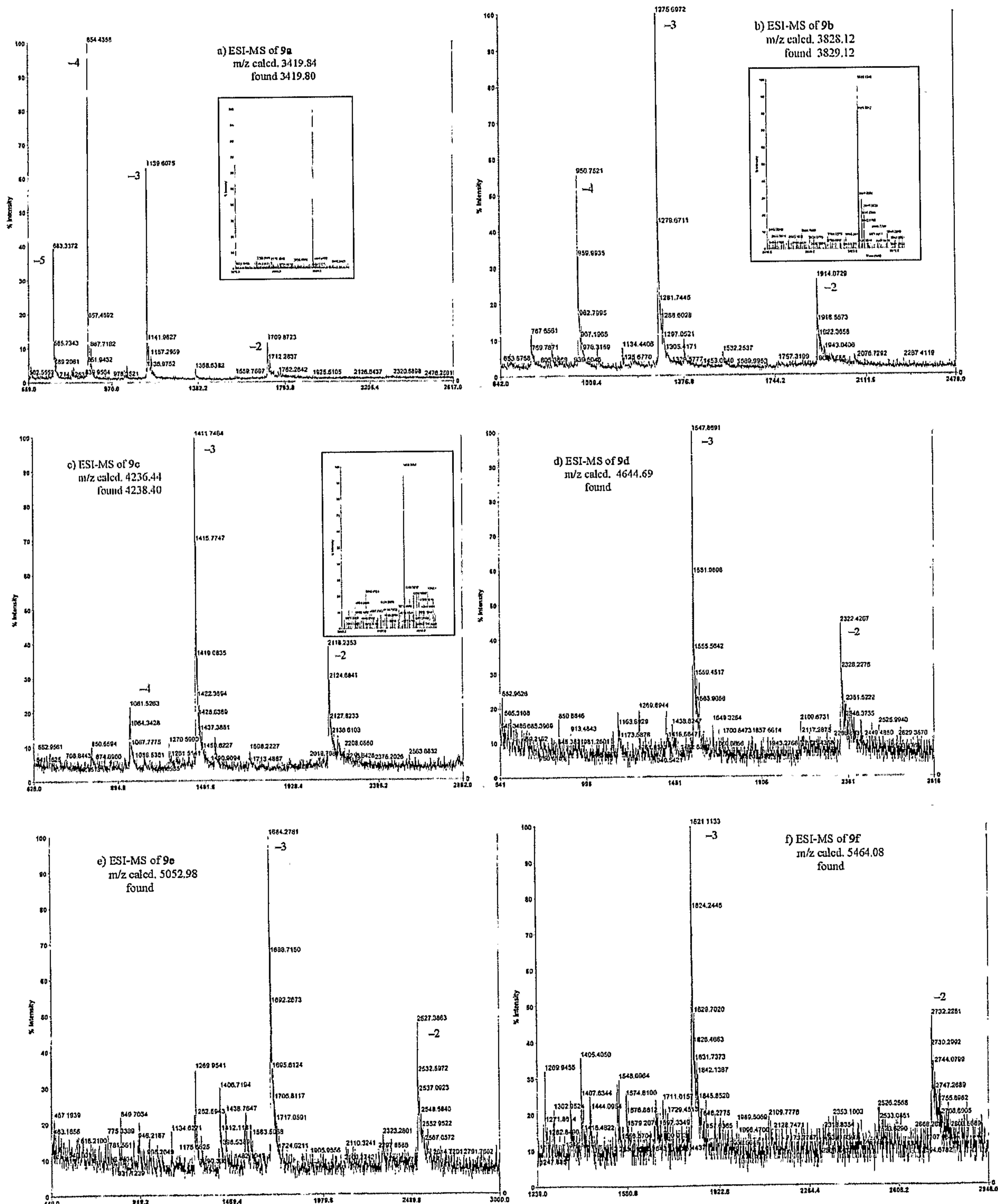


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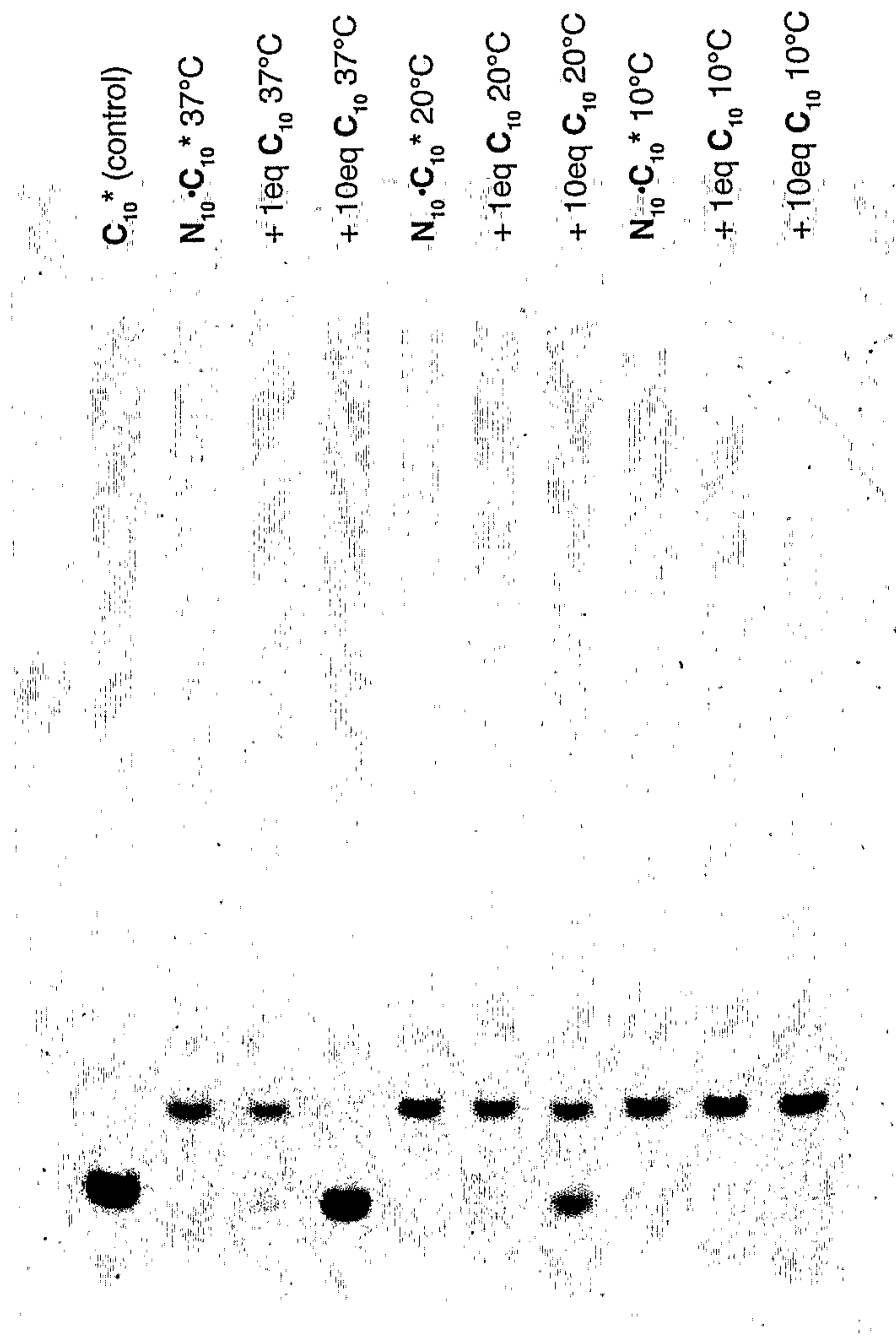


Figure 8

Figure 9

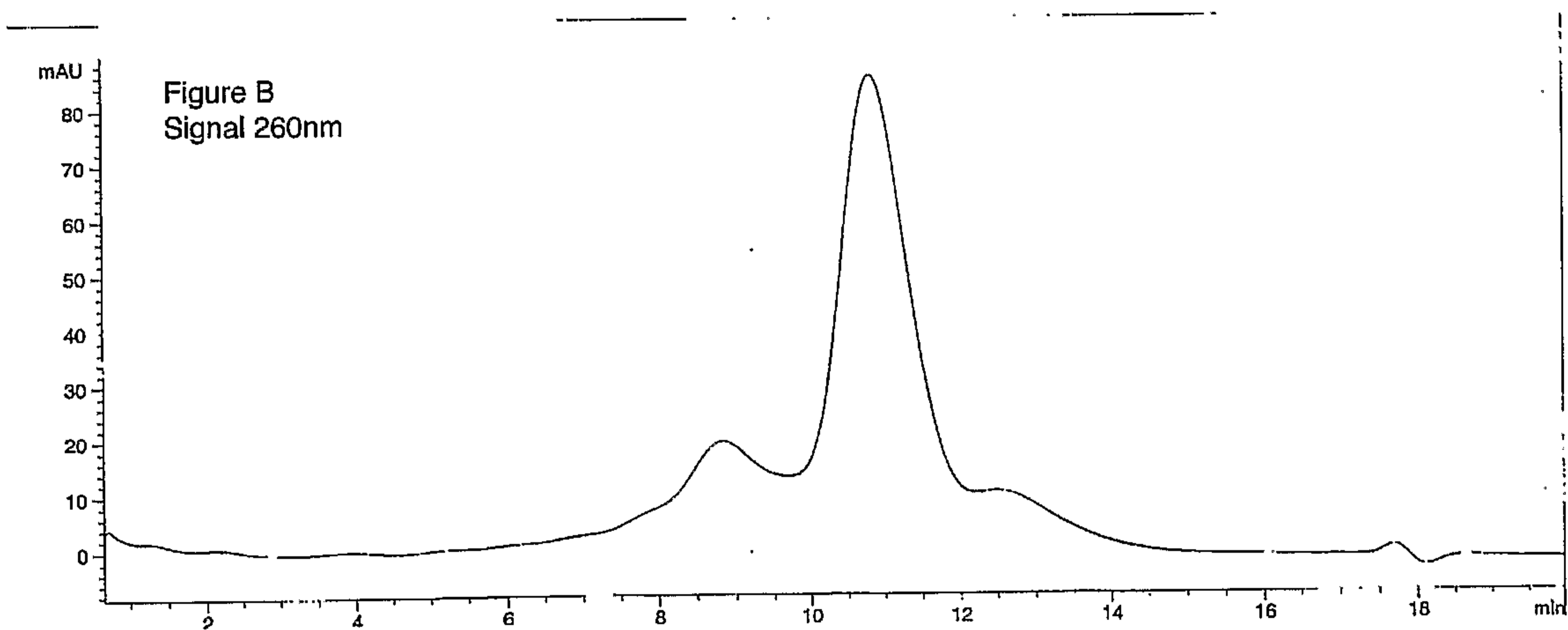
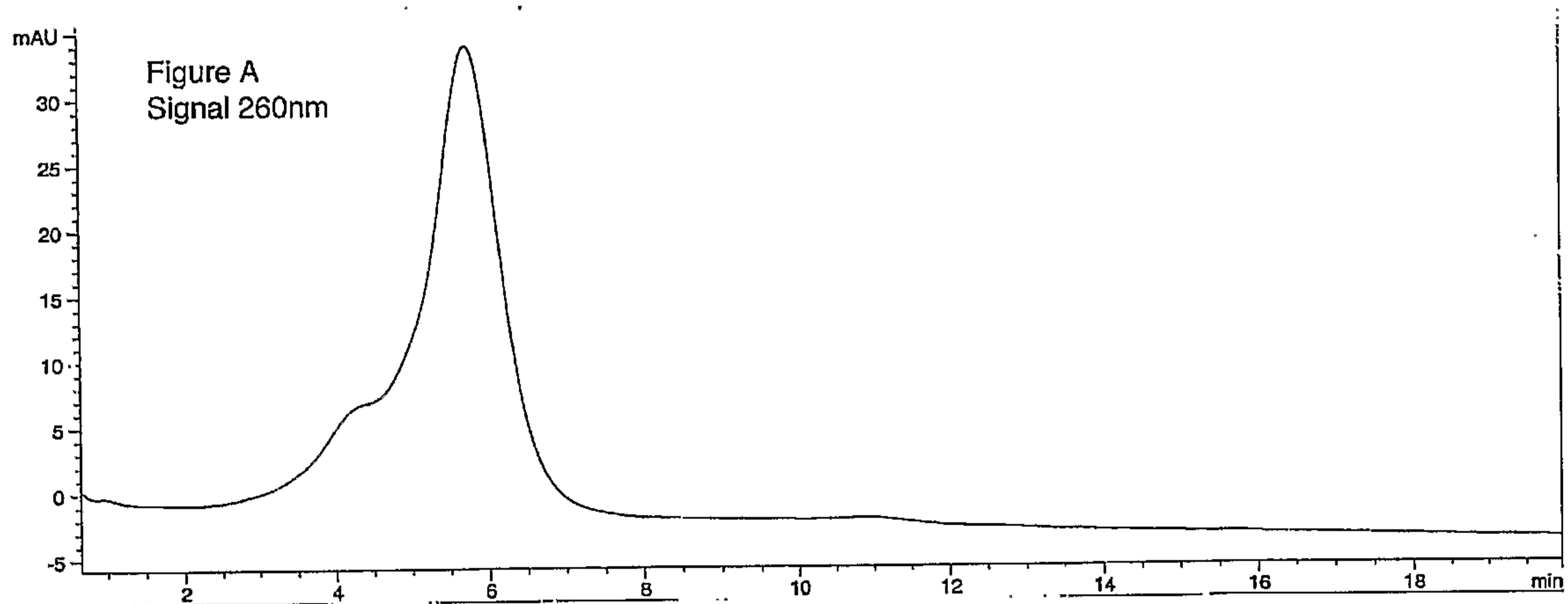


Figure 10 A

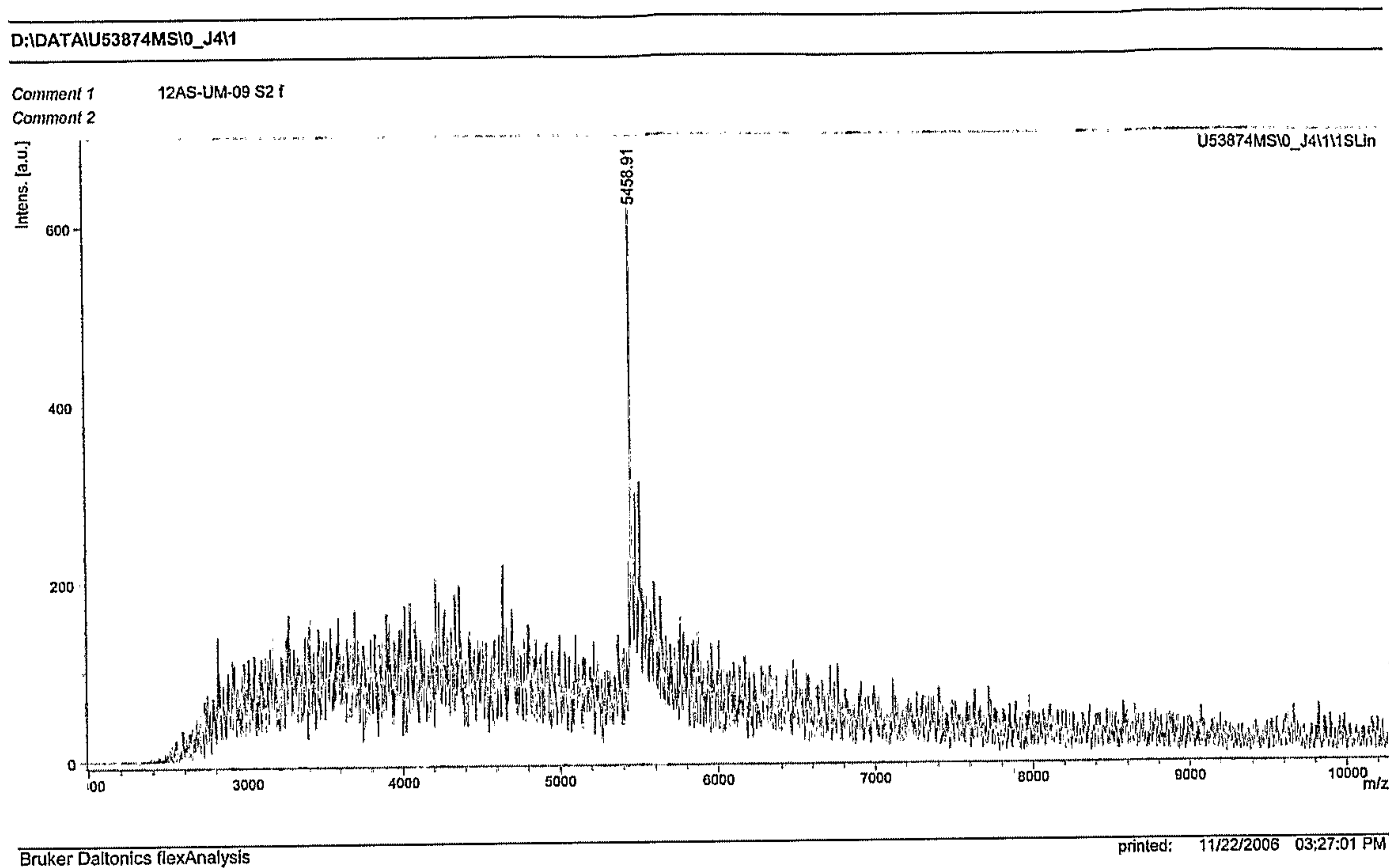


Figure 10 B

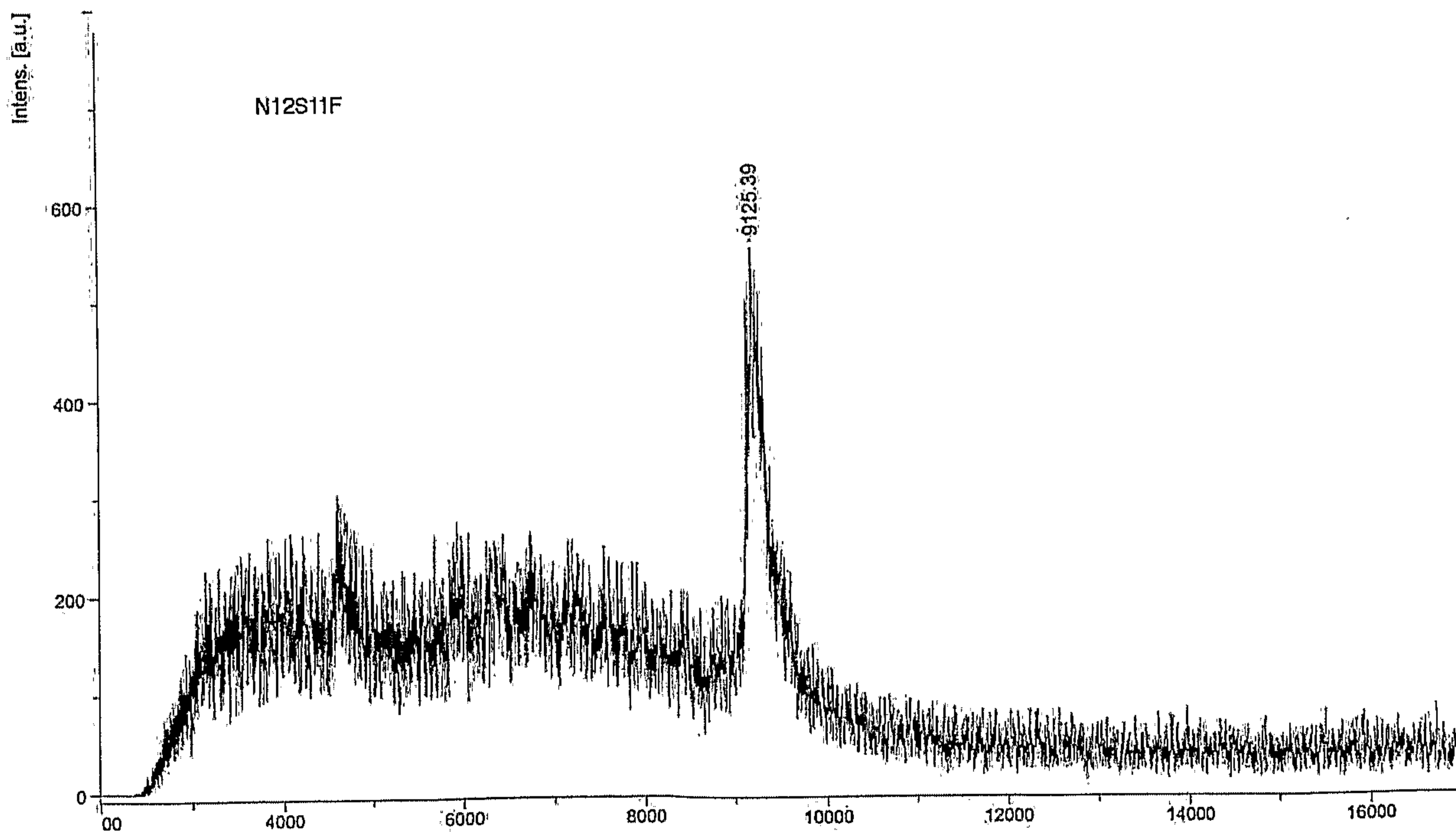


Figure 11

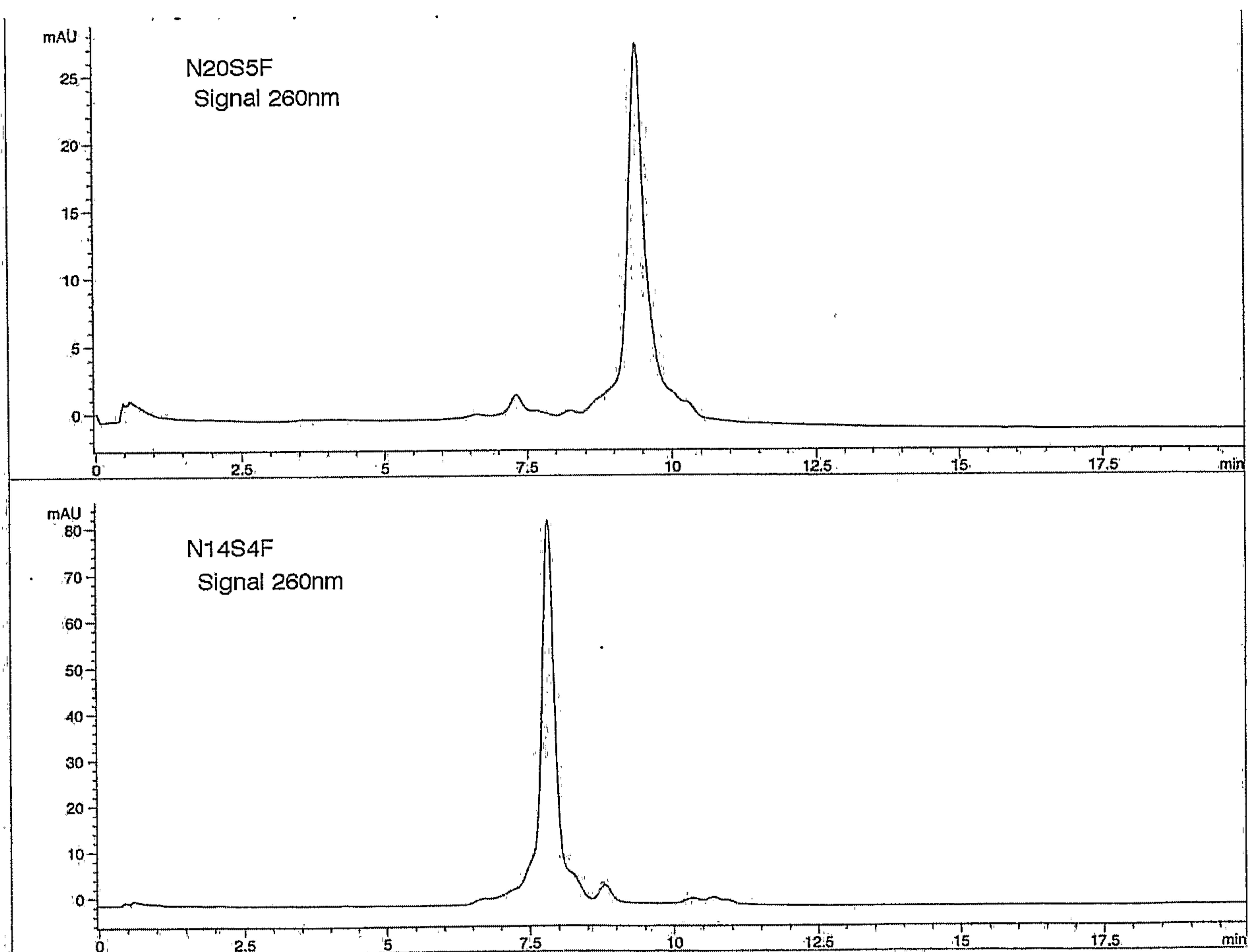


Figure 12 A

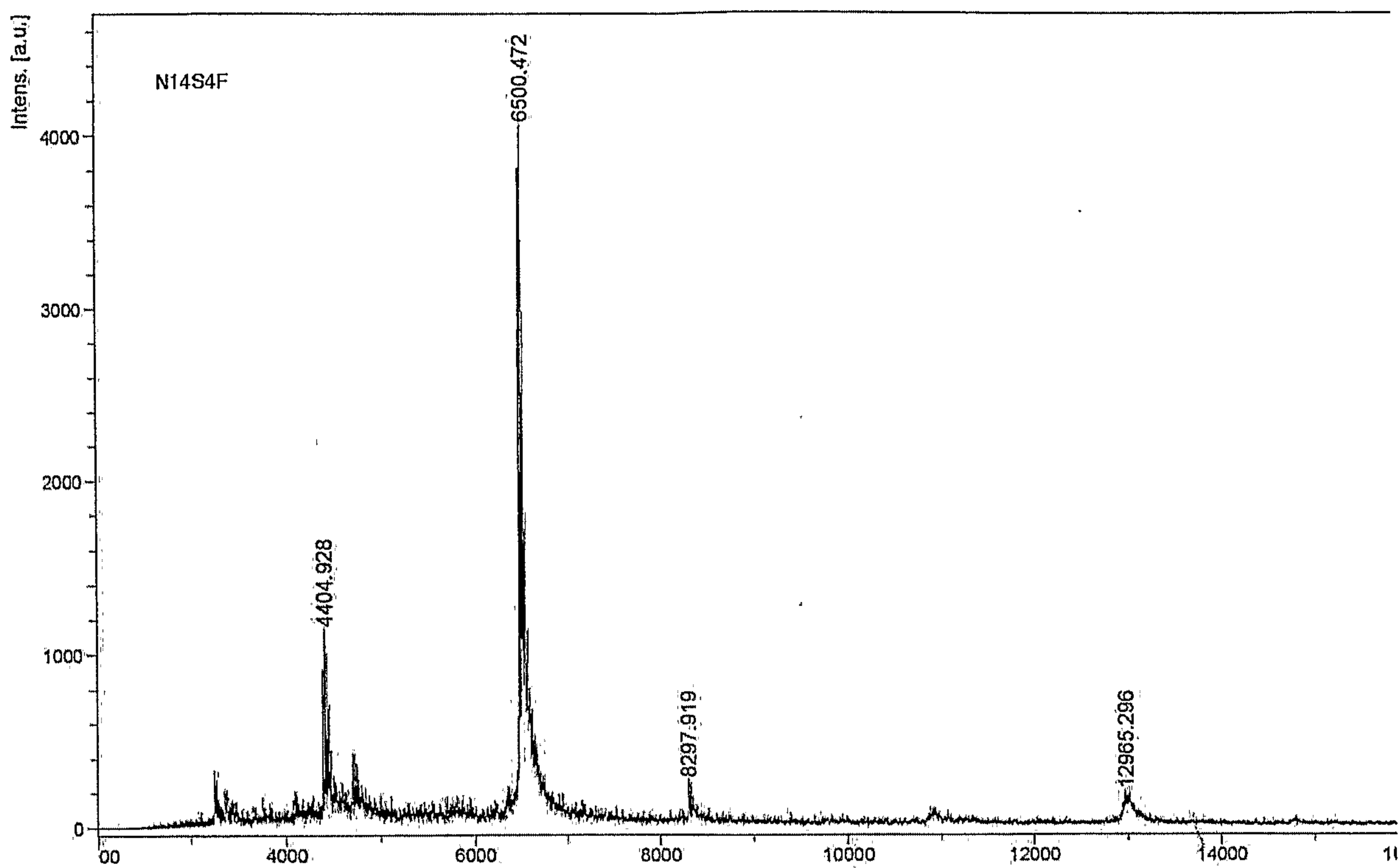


Figure 12 B

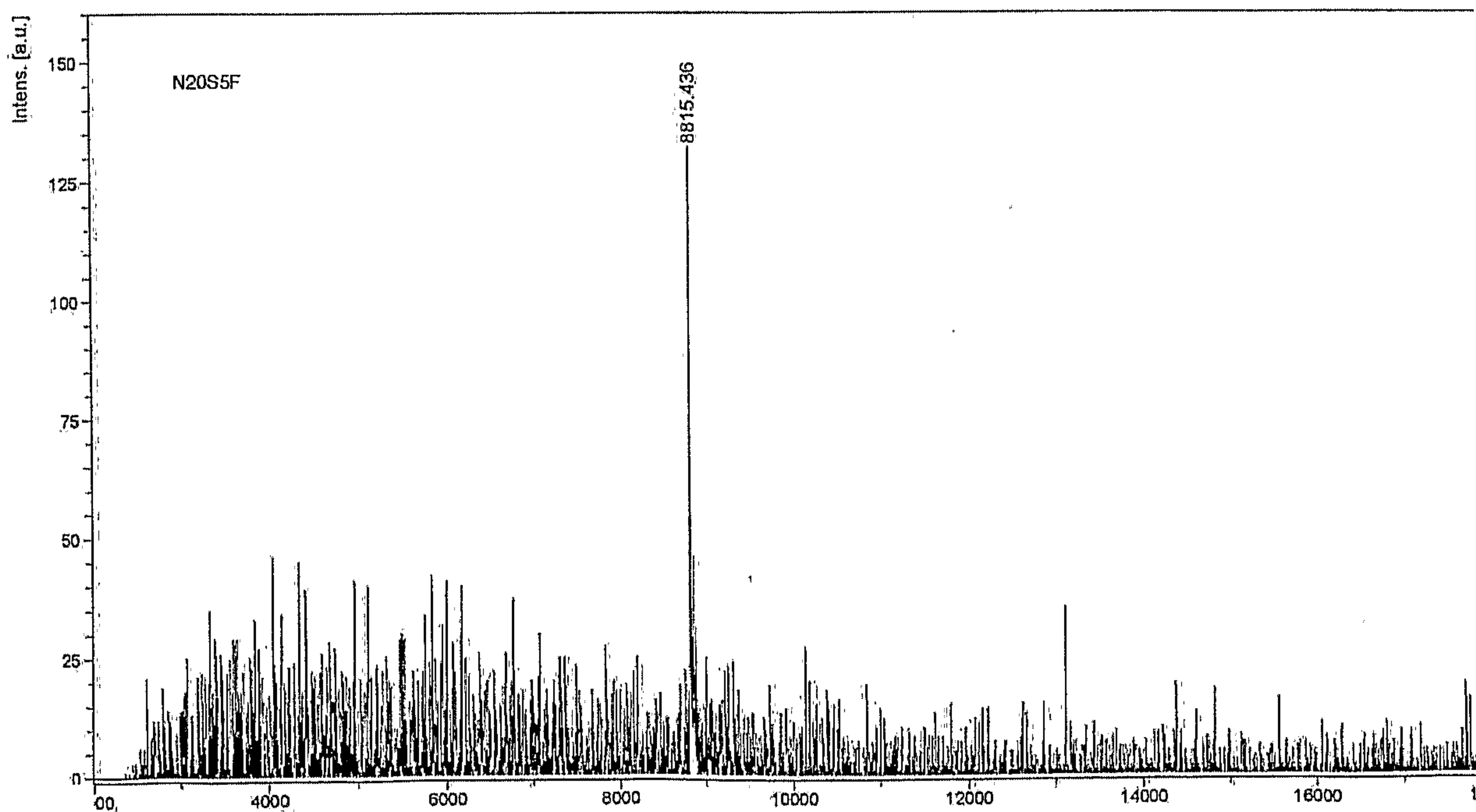
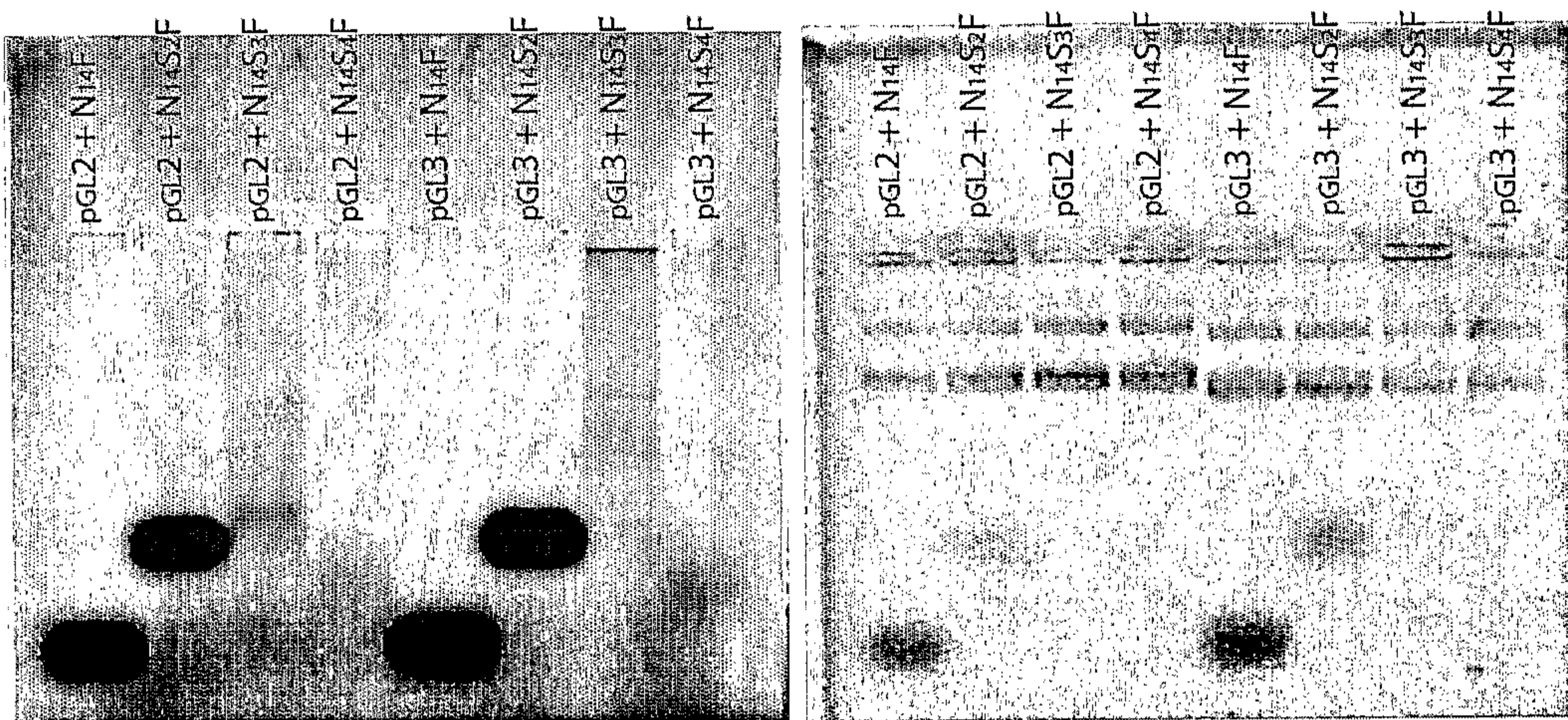


Figure 13

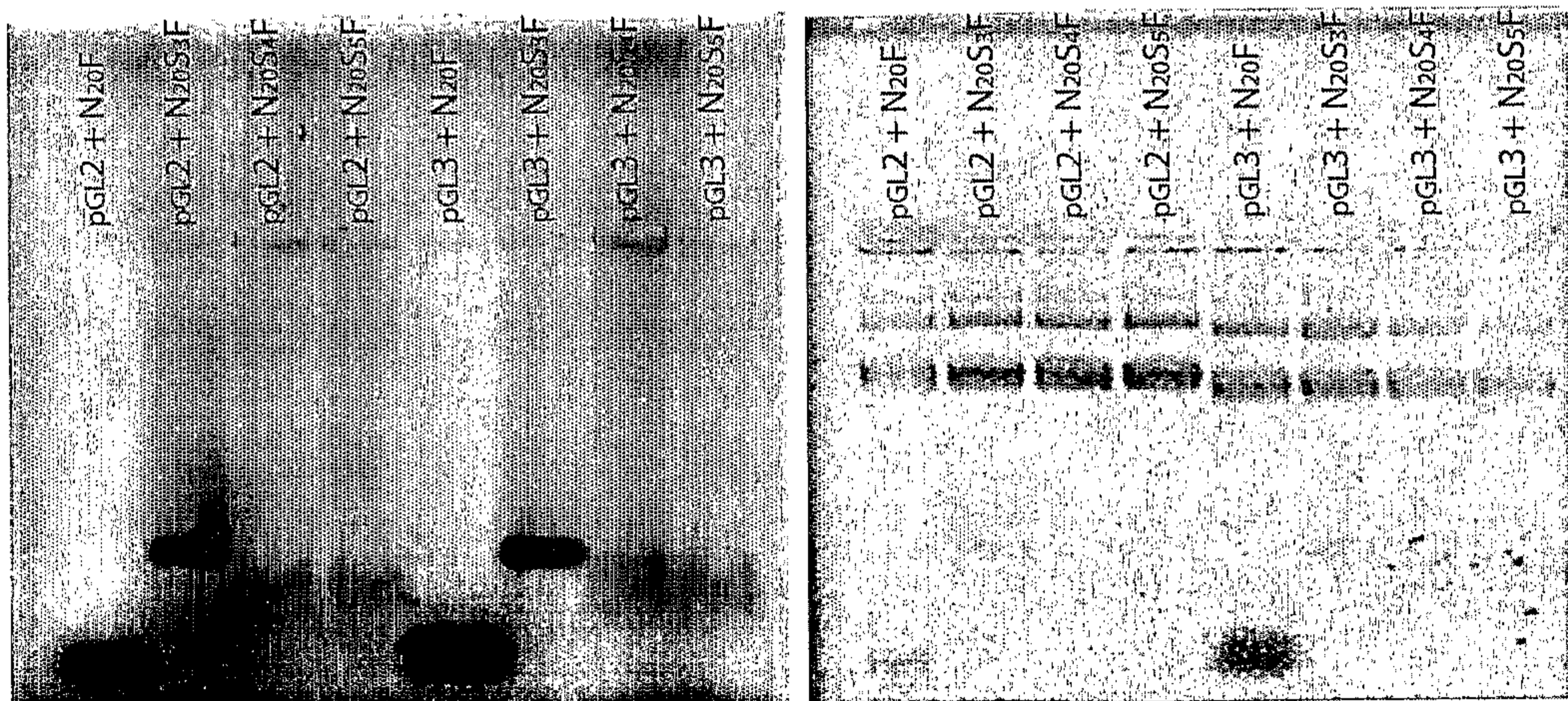
A



Green Fluorescence

Ethidium Bromide Staining

B



Green Fluorescence

Ethidium Bromide Staining

Figure 14

A

B

