



(86) Date de dépôt PCT/PCT Filing Date: 2009/03/13  
(87) Date publication PCT/PCT Publication Date: 2009/09/24  
(45) Date de délivrance/Issue Date: 2017/08/15  
(85) Entrée phase nationale/National Entry: 2010/07/20  
(86) N° demande PCT/PCT Application No.: EP 2009/053002  
(87) N° publication PCT/PCT Publication No.: 2009/115465  
(30) Priorités/Priorities: 2008/03/19 (EP08425175.0);  
2008/05/07 (US61/051,088)

(51) Cl.Int./Int.Cl. *C12N 15/11* (2006.01),  
*A61K 31/7088* (2006.01), *A61K 48/00* (2006.01),  
*A61P 9/00* (2006.01), *A61P 35/00* (2006.01),  
*C12N 5/02* (2006.01)  
(72) Inventeurs/Inventors:  
STEIN, AARON CY, US;  
IACOBELLI, MASSIMO, IT  
(73) Propriétaire/Owner:  
GENTIUM S.R.L., IT  
(74) Agent: RICHES, MCKENZIE & HERBERT LLP

(54) Titre : OLIGONUCLEOTIDES A FONCTION PHOSPHODIESTER ET LEURS UTILISATIONS THERAPEUTIQUES  
(54) Title: SYNTHETIC PHOSPHODIESTER OLIGONUCLEOTIDES AND THERAPEUTICAL USES THEREOF

(57) **Abrégé/Abstract:**

A composition of phosphodiester oligonucleotides of various defined sizes has been created that mimics the effects of defibrotide. The composition essentially consists of mixtures of synthetic phosphodiester oligonucleotides comprising Nmers ranging from 40 mers to 65 mers. The phosphodiester oligonucleotides are preferably heteropolymers composed of either A, G, C, and T at each position but may also be homopolymers, i.e. the same base may be present at each position in the oligonucleotide. These mixtures are effective in the treatment of cancer and other diseases.

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization  
International Bureau



(43) International Publication Date  
24 September 2009 (24.09.2009)

PCT

(10) International Publication Number  
**WO 2009/115465 A1**

(51) International Patent Classification:

*C12N 15/11* (2006.01)      *C12N 5/02* (2006.01)  
*A61K 31/7088* (2006.01)    *A61P 9/00* (2006.01)  
*A61K 48/00* (2006.01)      *A61P 35/00* (2006.01)

(21) International Application Number:

PCT/EP2009/053002

(22) International Filing Date:

13 March 2009 (13.03.2009)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

08425175.0      19 March 2008 (19.03.2008)      EP  
61/051,088      7 May 2008 (07.05.2008)      US

(71) Applicant (*for all designated States except US*): **GEN-TIUM SPA** [IT/IT]; Piazza XX Settembre, 2, I-22079 Villa Guardia (CO) (IT).

(72) Inventors; and

(75) Inventors/Applicants (*for US only*): **STEIN, Aaron Cy** [US/US]; 11, Dolphin Road, New City, New York NY-10956 (US). **IACOBELLI, Massimo** [IT/IT]; Via Forze Armate, 260/3, I-20152 Milano (IT).

(74) Agents: **PISTOLESI, Roberto** et al.; Via Marina, 6, I-20121 Milano (IT).

(81) Designated States (*unless otherwise indicated, for every kind of national protection available*): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (*unless otherwise indicated, for every kind of regional protection available*): ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:

— *of inventorship (Rule 4.17(iv))*

Published:

— *with international search report (Art. 21(3))*

(54) Title: SYNTHETIC PHOSPHODIESTER OLIGONUCLEOTIDES AND THERAPEUTICAL USES THEREOF

(57) Abstract: A composition of phosphodiester oligonucleotides of various defined sizes has been created that mimics the effects of defibrotide. The composition essentially consists of mixtures of synthetic phosphodiester oligonucleotides comprising Nmers ranging from 40 mers to 65 mers. The phosphodiester oligonucleotides are preferably heteropolymers composed of either A, G, C, and T at each position but may also be homopolymers, i.e. the same base may be present at each position in the oligonucleotide. These mixtures are effective in the treatment of cancer and other diseases.



WO 2009/115465 A1

**SYNTHETIC PHOSPHODIESTER OLIGONUCLEOTIDES AND  
THERAPEUTICAL USES THEREOF**

**FIELD OF THE INVENTION**

5

The invention relates to mixtures of synthetic phosphodiester oligonucleotides called Nmers ranging from 40 mers to 65 mers and, in particular, to using these oligonucleotides to treat diseases, including cancer. The phosphodiester oligonucleotides are preferably heteropolymers composed of  
10 either A, G, C, and T at each position but may also be homopolymers, *i.e.* the same base may be present at each position in the oligonucleotide.

**BACKGROUND OF THE INVENTION**

The term defibrotide identifies a complex mixture of single stranded  
15 oligonucleotides (15-80mer, average 45mer) obtained by extraction from animal and/or vegetable tissue and, in particular, from the intestines of a pig or cow (US 3,770,720 and US 3,899,481). Defibrotide, which has an average molecular weight of  $16.5 \pm 2.5$  kDa, is normally used in the form of a salt of an alkali metal, generally sodium. It is principally used for its antithrombotic activity (US  
20 3,829,567) although it may be used in different applications, such as, for example, the treatment of acute renal insufficiency (US 4,694,134) and the treatment of acute myocardial ischemia (US 4,693,995). Additional literature on defibrotide is cited below.

US 5,081,109 discloses the use of defibrotide to treat peripheral  
25 arteriopathies in advanced phase (phase III and IV).

US 5,116,617 discloses methods of strengthening capillaries in humans comprising topically applying compositions containing defibrotide.

US 5,977,083 discloses that various disease states can be treated by modifying the dose of defibrotide in response to observed fluctuations (e.g.,  
30 increase, decrease, appearance, disappearance) in normal, disease and repair markers.



US 6,046,172 discloses oligodeoxyribonucleotides of animal origin, having a molecular weight comprised between 4000 and 10000 Daltons, which can be obtained by fractionation of polydeoxyribonucleotides or otherwise by chemical or enzymatic depolymerization of high molecular weight  
5 deoxyribonucleic acids.

US 6,699,985 and US 5,624,912 disclose a method of using defibrotide to treat various disease conditions, including HIV infection.

US 7,338,777 discloses a method of determining the biological activity of defibrotide.

10 EP1276497 discloses a method of increasing the amount of stem cells and progenitor cells in the peripheral blood of a mammal by the administering defibrotide in combination or in temporal proximity with at least one haematopoietic factor (such as G-CSF) having the capacity to mobilise haematopoietic progenitors.

15 WO2005023273 discloses the anti-tumor action of defibrotide.

WO2006094916 describes the use of defibrotide for treating angiogenesis-dependent tumors.

Additionally, a review article, "Defibrotide, a Polydisperse Mixture of Single Stranded Phosphodiester Oligonucleotides with Lifesaving Activity in  
20 Severe Hepatic Veno-occlusive Disease: Clinical Outcomes and Potential Mechanisms of Action," by Kornblum *et al.* (*Oligonucleotides*, 16:105-114 (2006)), discusses defibrotide and its use in treating veno-occlusive disease (VOD).

In 1998, Dr. Paul Richardson of the Dana-Farber Cancer Institute in  
25 Boston, Massachusetts began using defibrotide as a treatment for severe hepatic VOD after bone marrow transplantation. The drug was dosed intravenously with almost no toxicity, and it cured about 40%-50% of patients in a disease that was hitherto fatal in 95% of cases. Subsequent multi-institutional trials in the United States and Europe have confirmed the efficacy of defibrotide, although its  
30 mechanism of action is unknown.

US 4,985,552 and US 5,223,609 describe a process for the production of defibrotide which enables a product to be obtained which has constant and well

defined physico-chemical characteristics and is also free from any undesired side-effects.

EP1325162 discloses a method for determining the biological activity of defibrotide.

5       For decades the dogma has been that phosphodiester oligonucleotides cannot be used as drugs because of nuclease digestion, but this attitude neglects the large quantities in which they can be administered to patients due to their low toxicity. Clinical experience with defibrotide clearly indicates that these types of molecules can be given with therapeutic efficacy. However, there is some  
10 question as to whether or not defibrotide can be reproduced identically, as it is a natural product. Therefore, there is a need in the art to develop a composition that has the same effect as defibrotide but that will be able to be identically reproduced.

## SUMMARY OF THE INVENTION

The present invention is directed to a method of treating a disease or condition comprising administering a mixture of synthetic phosphodiester oligonucleotides having a length of from about 40 bases to about 65 bases, preferably from about 40 bases to about 60 bases, even more preferably from about 45 bases to about 60 bases, from about 45 bases to about 55 bases or from about 50 bases to about 55 bases.

The invention also includes pharmaceutical compositions which consist essentially of the synthetic phosphodiester oligonucleotides having an average length as set forth above and a pharmaceutical carrier (and optionally pharmaceutically acceptable excipients and/or adjuvants) and no other ingredient which materially affects the activity of the synthetic phosphodiester oligonucleotides.

In the method of the invention said oligonucleotides may be single stranded; the sequences of said oligonucleotides may DNA and/or RNA sequences; the sequences of said oligonucleotides may also be random sequences.

In the method of the invention the purine bases of said oligonucleotides may be selected from guanine, adenine, xanthine and hypoxanthine and the pyrimidine bases may be selected from cytosine, thymine, methylcytosine and uracil; the sugar of said oligonucleotides may be selected from ribose and deoxyribose.

In the method of the invention the disease or condition may be veno-occlusive disease, thrombotic thrombocytopenic purpura, tumors, angiogenesis-dependent tumors, or a disease or condition that benefits from use of a blood anticoagulant; the method may also be used for increasing the amount of stem cells and progenitor cells in the peripheral blood of a mammal when said phosphodiester oligonucleotides are administered in combination or in temporal proximity with at least one haematopoietic factor having the capacity to mobilise haematopoietic progenitors.

In yet another aspect, the present invention provides a pharmaceutical formulation which is an aqueous solution and which consists essentially of a mixture of synthetic phosphodiester oligonucleotides having a length of from about 40 bases to about 65 bases, in which said synthetic phosphodiester oligonucleotides have different sequences and same or different length.



In yet another aspect, the present invention provides the use of a pharmaceutical agent for treating veno-occlusive disease, the pharmaceutical agent containing a single active ingredient consisting of single stranded nucleic acids having random sequences and lengths from 40 bases to 65 bases, wherein the pharmaceutical agent is for administration to a patient afflicted with veno-occlusive disease.

In yet another aspect, the present invention provides the use of a pharmaceutical composition for treating veno-occlusive disease, the pharmaceutical composition consisting essentially of: single stranded nucleic acids having random sequences and lengths from 40 bases to 65 bases and excluding nucleic acids having lengths less than 40 bases and greater than 65 bases; and one or more pharmaceutically acceptable excipients and/or adjuvants, wherein the pharmaceutical composition is for administration to a patient afflicted with veno-occlusive disease.

These and other aspects of the present invention will become apparent upon reference to the following detailed description and attached drawings.

## DETAILED DESCRIPTION

### DEFINITIONS

5 The following definitions are given for a better understanding of the present invention:

A nucleotide is a chemical compound that consists of 3 portions: a heterocyclic base, a sugar, and one or more phosphate groups. In the most common nucleotides, the base is a derivative of purine or pyrimidine and the sugar is the pentose (five-carbon sugar) deoxyribose or ribose. Nucleotides are the  
10 monomers of nucleic acids such as DNA or RNA.

Oligonucleotides are short sequences of nucleotides, typically with twenty or fewer bases. Automated synthesizers allow the synthesis of oligonucleotides up to 160 to 200 bases. The length of a synthesized base is usually denoted by 'mer' (from 'Greek' meros "part"). For example, a fragment of 25 bases would be called  
15 a 25-mer.

A phosphodiester bond is a group of strong covalent bonds between the phosphorus atom in a phosphate group and two other molecules over two ester bonds. Phosphodiester bonds make up the backbone of the strands of DNA and RNA.

20 DNA and RNA are long polymers of simple units called nucleotides, with a backbone made of sugars and phosphate groups joined by phosphodiester bonds. Attached to each sugar is one of four types of molecules called bases. In DNA and RNA, the phosphodiester bond is the linkage between the 3' carbon atom and the 5' carbon of the ribose sugar.

25 DNA is often double stranded and normally contains two types of purine bases, guanine and adenine, and two types of pyrimidine bases, cytosine and thymine. In certain cases, purine and pyrimidine bases can be replaced by their mutated forms: guanine and adenine may be replaced by xanthine and hypoxanthine, respectively, whereas cytosine may be replaced by methylcytosine.  
30 RNA is very similar to DNA, but differs in a few important structural details: RNA is typically single stranded, while DNA is typically double stranded. Also,



RNA nucleotides contain ribose sugars while DNA contains deoxyribose; furthermore, RNA contains uracil instead of thymine which is present in DNA.

A random nucleotide sequence is a nucleotide sequence essentially containing an equal mixture of two different purine bases and two different pyrimidine bases wherein, at each position of the sequence, each purine or pyrimidine base has a  $25\% \pm 5$  probability of being present, preferably  $25\% \pm 2$ , more preferably  $25\% \pm 1$ .

As used herein, the term “isolated” means that the material being referred to has been removed from the environment in which it is naturally found, and is characterized to a sufficient degree to establish that it is present in a particular sample. Such characterization can be achieved by any standard technique, such as, *e.g.*, sequencing, hybridization, immunoassay, functional assay, expression, size determination, or the like. Thus, a biological material can be “isolated” if it is free of cellular components, *i.e.*, components of the cells in which the material is found or produced in nature.

An isolated organelle, cell, or tissue is one that has been removed from the anatomical site (cell, tissue or organism) in which it is found in the source organism. An isolated material may or may not be “purified”. The term “purified” as used herein refers to a material (*e.g.*, a nucleic acid molecule or a protein) that has been isolated under conditions that detectably reduce or eliminate the presence of other contaminating materials. Contaminants may or may not include native materials from which the purified material has been obtained. A purified material preferably contains less than about 90%, less than about 75%, less than about 50%, less than about 25%, less than about 10%, less than about 5%, or less than about 2% by weight of other components with which it was originally associated.

The practice of the present invention will employ, unless indicated specifically to the contrary, conventional methods of molecular biology, cell biology and protein chemistry within the skill of the art, many of which are described below for the purpose of illustration. Such techniques are explained fully in the literature. See, *e.g.*, Sambrook, *et al.*, “Molecular Cloning: A Laboratory Manual” (2nd Edition, 1989); “DNA Cloning: A Practical Approach,

vol. I & II" (D. Glover, ed.); "Oligonucleotide Synthesis" (N. Gait, ed., 1984);  
"Nucleic Acid Hybridization" (B. Hames & S. Higgins, eds., 1985); Perbal, "A  
Practical Guide to Molecular Cloning" (1984); Ausubel *et al.*, "Current protocols  
in Molecular Biology" (New York, John Wiley and Sons, 1987); and Bonifacino  
5 *et al.*, "Current Protocols in Cell Biology" (New York, John Wiley & Sons, 1999).

The term "about" means within an acceptable error range for the particular  
value as determined by one of ordinary skill in the art, which will depend in part  
on how the value is measured or determined, *i.e.*, the limitations of the  
measurement system. For example, "about" can mean within an acceptable  
10 standard deviation, per the practice in the art. Alternatively, "about" can mean a  
range of up to  $\pm 20\%$ , preferably up to  $\pm 10\%$ , more preferably up to  $\pm 5\%$ , and  
more preferably still up to  $\pm 1\%$  of a given value. Alternatively, particularly with  
respect to biological systems or processes, the term can mean within an order of  
magnitude, preferably within 2-fold, of a value. Where particular values are  
15 described in the application and claims, unless otherwise stated, the term "about"  
is implicit and in this context means within an acceptable error range for the  
particular value.

In the context of the present invention insofar as it relates to any of the  
disease conditions recited herein, the terms "treat", "treatment", and the like mean  
20 to prevent or relieve or alleviate at least one symptom associated with such  
condition, or to slow or reverse the progression of such condition. For example,  
within the meaning of the present invention, the term "treat" also denotes to arrest,  
delay the onset (*i.e.*, the period prior to clinical manifestation of a disease) and/or  
reduce the risk of developing or worsening a disease. The term "protect" is used  
25 herein to mean prevent, delay or treat, or all, as appropriate, development or  
continuance or aggravation of a disease in a subject.

The phrase "pharmaceutically acceptable", as used in connection with  
compositions of the invention, refers to molecular entities and other ingredients of  
such compositions that are physiologically tolerable and do not typically produce  
30 untoward reactions when administered to an animal such as a mammal (*e.g.*, a  
human). Preferably, as used herein, the term "pharmaceutically acceptable"  
means approved by a regulatory agency of the Federal or a state government or



listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in mammals, and more particularly in humans.

As used herein, the expression “mixture of synthetic phosphodiester oligonucleotides” means a mixture of synthetic phosphodiester oligonucleotides which may have the same and/or different sequences. According to a first embodiment, the mixture may include both oligonucleotides having the same sequence and oligonucleotides having different sequences; on their turn, such oligonucleotides having different sequences may have the same or different lengths. According to a second embodiment, the mixture may consist of oligonucleotides having different sequences but the same length.

The terms “administering” or “administration” are intended to encompass all means for directly and indirectly delivering a compound to its intended site of action.

The term “animal” means any animal, including mammals and, in particular, humans.

### BRIEF DESCRIPTION OF THE DRAWINGS

The attached figures are included solely to illustrate the preferred embodiment of the present invention without limiting the invention in any manner whatsoever, wherein:

FIG. 1A is a band intensity showing competition by defibrotide and defibrotide molecular weight fractions for binding of C1RNH<sup>32</sup>P-OdT<sub>18</sub> to bFGF.

FIG. 1B is a plot of the normalized band intensity versus the log of the defibrotide or defibrotide molecular weight fractions concentrations.

FIG. 2 is a chart and table showing a comparison of K<sub>c</sub> values for defibrotide and defibrotide molecular weight fraction and Nmer competitors of C1RNH<sup>32</sup>P-OdT<sub>18</sub> binding to bFGF.

FIG. 3A is a band intensity showing modification of PDGF BB by alkylating oligodeoxynucleotide, C1RNH<sup>32</sup>P-OdT<sub>18</sub>.

FIG. 3B is a plot of relative band intensity versus reactive oligodeoxynucleotide concentration.



FIG. 3C is a double reciprocal plot of the data in FIG. 3B.

FIG. 4 is a chart and table showing a comparison of the  $K_c$  values for defibrotide, defibrotide molecular weight fraction and Nmer competitors of C1RNH<sup>32</sup>P-OdT<sub>18</sub> binding to bFGF.

5 FIG. 5 is a chart and table showing a comparison of the  $K_c$  values for Nmer and Tmer competitors of C1RNH<sup>32</sup>P-OdT<sub>18</sub> binding to VEGF.

FIG. 6 is a chart and table showing a comparison of the  $K_c$  values for Nmer and Tmer competitors of C1RNH<sup>32</sup>P-OdT<sub>18</sub> binding to laminin.

10 FIG. 7 is a chart and table showing a comparison of the  $K_c$  values of Nmer and Tmer competitors of C1RNH<sup>32</sup>P-OdT<sub>18</sub> binding to laminin.

FIG. 8A is a chart of inhibition of bFGF-mediated HMEC-1 proliferation by defibrotide.

FIG. 8B is a chart of the inhibitory effects of defibrotide on cell growth in the absence of bFGF.

15 FIG. 9A is a chart of inhibition of bFGF-mediated HMEC-1 proliferation by Nmers.

FIG. 9B is a chart of the inhibitory effects of Nmers on cell growth in the absence of bFGF.

20 FIG. 10 is a chart showing the effect of defibrotide and Nmers on the partial thromboplastin time (PTT).

FIG. 11A is a chart showing the dose-dependent release of TFPI to conditioned medium by exposure of HMEC-1 cells to increasing concentrations of defibrotide for 24 hours.

25 FIG. 11B is a chart showing the time-course of the TFPI release to conditioned medium induced by 5  $\mu$ M defibrotide.

FIG. 11C is a chart showing the dose-dependent release of TFPI to conditioned medium by exposure of HMEC-1 cells to increasing concentrations of defibrotide for 30 minutes.

30 FIG. 12A is a chart showing the dose-dependent release of TFPI into conditioned medium by exposure of HMEC-1 cells to increasing concentrations of defibrotide molecular weight fractions.

FIG. 12B is a chart showing the time-course of the TFPI release into conditioned medium induced by 5  $\mu$ M defibrotide.

FIG. 13 is a chart showing the ability of defibrotide and Nmers to substitute for heparin in the bFGF + heparin-stimulated proliferation of FGFR2-transfected C11 cells.

### DETAILED DESCRIPTION OF THE INVENTION

Oligonucleotides, such as defibrotide, can bind to proteins that bind to heparin. As used herein, the term heparin means low-affinity heparin. Synthetic analogs of defibrotide can be made that have comparable or higher activity than the natural product, and these analogs have anti-cancer activity because of their ability to bind to heparin-binding growth factors. Three heparin-binding proteins of great importance to cancer cells include basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF) and laminin; the composition of the present invention can bind to these proteins with nanomolar affinity, yet this binding is not sequence-specific.

The present composition is based on the surprising finding that mixtures of synthetic phosphodiester oligonucleotides having a length of from about 40 mers to about 65 mers recapitulate the properties of defibrotide and may thus be used as a synthetic alternative to such an active principle. The oligonucleotides of the present invention may preferably have a length of about 40-60 mers, preferably of about 45-60 mers; according to the better embodiment of the invention, they may have a length of about 45-55 mers, preferably of about 50-55 mers.

The purine bases of the oligonucleotides of the present invention are preferably selected from guanine, adenine, xanthine and hypoxanthine and the pyrimidine bases are selected from cytosine, thymine, methylcytosine and uracil. According to one embodiment, the sequences would be composed of a mixture of each genetic base (A, G, C, and T) at each position in the oligonucleotides; preferably, they would be random sequences. According to another embodiment the sequences would consist of the same base (such as thymidine, i.e. Tx) at each position in the oligonucleotides (known as the Tm series, or Tmers). According to



a further embodiment, the sugar of the present oligonucleotides is selected from ribose and deoxyribose.

According to another embodiment, the oligonucleotides of the present invention consists of DNA and/or RNA sequences.

5 According to a preferred embodiment, the oligonucleotides of the present invention are single stranded.

As it will be apparent from the experimental section, the present inventors have surprisingly found that the fractions of defibrotide having low molecular weight and, in particular, those having a molecular weight lower than 40 kDa, are  
10 those having the lower ability to bind to heparin-binding growth factors. Such a finding has thus allowed for the selection of well-defined mixtures of oligonucleotides that can be easily and identically reproduced and that can mimic the effects of defibrotide.

The mixtures of the present invention can thus be used to treat mammalian  
15 patients, preferably human, afflicted with those diseases which would be treated by administering defibrotide, such as VOD, thrombotic thrombocytopenic purpura (TTP), tumors, angiogenesis dependent tumors (such as multiple myeloma or breast carcinoma); those mixtures might also be used as blood anticoagulant or for increasing the amount of stem cells and progenitor cells in the peripheral blood of  
20 a mammal when administered in combination or in temporal proximity with at least one hematopoietic factor having the capacity to mobilize hematopoietic progenitors.

The mixtures of oligonucleotides of the present invention may be administered in the same way as defibrotide; preferably, they would be  
25 administered by injection, preferably intravenously, by means of an aqueous solution. Such aqueous solution may have oligonucleotide concentrations from 5 to 60 micromoles/liter, preferably from 10 to 50 micromoles/liter.

## EXAMPLES

30 The present invention will be better understood by reference to the following non-limiting examples.



## MATERIALS AND METHODS

Generation of synthetic phosphodiester oligonucleotides In order to create the series of Nmers, a DNA sequencing machine (commonly available on the market, by, for example ABI or Millipore) was used. Equal amounts (as measured by molarity) of each base (adenine, cytosine, guanine, and thymine) were used in the sequencing reaction. The machine was programmed to make random lengths of single-stranded DNA ranging in size from 25 bases to 200 bases, and each base was chosen at random from the four genetic bases.

### *Cell culture*

SV40-transformed HMEC-1 cells were obtained from the CDC in Atlanta, GA. They were grown in MCDB 131 media supplemented with 10% heat inactivated fetal bovine serum (FBS), 10 ng/ml EGF, 1 µg/mL hydrocortisone, 100 U/mL penicillin G sodium and 100 µg/ml streptomycin sulfate. The mycoplasma-free human melanoma cell line 518A2 was obtained from Dr. Volker Wacheck of the University of Vienna in Austria. Cells were grown in DMEM supplemented with 10% heat inactivated FBS and 100 U/ml penicillin G sodium and 100 µg/ml streptomycin sulfate. The human hepatic stellate LX2 cell line was generated by SV40 T antigen spontaneous immortalization in low serum conditions, and was provided by Dr. Scott L. Friedman of the Mount Sinai School of Medicine in New York. LX2 cells were grown in DMEM supplemented with 1% heat inactivated FBS and 100 U/ml penicillin G sodium and 100 µg/ml streptomycin sulfate. The stock cultures were maintained at 37°C in a humidified 5% CO<sub>2</sub> incubator.

### *Generation of defibrotide, defibrotide molecular weight fractions, and synthetic phosphodiester oligonucleotides*

Defibrotide, a highly complex polydisperse material composed of single-stranded phosphodiester polydeoxyribonucleotides (molecular weight is  $16.5 \pm 2.25$  kDa), was prepared via controlled depolymerization of DNA extracted from porcine intestinal tissue, and was provided by Gentium (Como, Italy). Defibrotide

molecular weight fractions, which is defibrotide isolated from porcine intestinal tissue and then fractionated, (A2, E2, G2, I2, L2 with molecular weights 9,353; 12,258; 16,761; 21,840; and 26,190 Daltons, respectively) were also supplied by Gentium. Nmers (a series of synthetic phosphodiester oligonucleotides of various, defined lengths,) and Tmers (a series of phosphodiester homopolymers of thymidine of defined length) were synthesized, purified via the procedure detailed above and supplied by Trilink Biotechnologies (San Diego, CA).

*Recombinant proteins and cell culture materials*

Recombinant human bFGF and VEGF165, platelet-derived growth factor-BB (PDGF BB) and heparin-binding epidermal growth factor-like growth factor (HB-EGF) were purchased from R&D Systems (Minneapolis, MN). Laminin was obtained from Sigma-Aldrich (St. Louis, MO). DMEM, MCDB 131, M199 medium, and FBS were obtained from Invitrogen (Carlsbad, CA). Fibronectin-coated plates and Matrigel were purchased from BD Bioscience (Bedford, MA). The IMUBIND Total TFPI ELISA kit was obtained from American Diagnostica (Stanford, CT).

*Synthesis of the alkylating oligodeoxynucleotide probe CIRNH<sup>32</sup>p-OdT<sub>18</sub>*

Ten OD U of OdT<sub>18</sub> were 5'-labeled with [<sup>32</sup>P]phosphate by reaction with 5'-polynucleotide kinase. Excess ATP was separated from the reaction product by Sephadex G25 chromatography in 0.1 M lithium perchlorate. The oligonucleotide was then precipitated by addition of 2% LiClO<sub>4</sub>/acetone and dissolved in water at a concentration of 200 OD U/μl. The oligonucleotide was then precipitated by the addition of 8% aqueous solution of cetyltrimethylammonium bromide solution and dried. 6.5 mg of p-(benzylamino)-N-chloroethyl-N-methylamine (CIRNH<sub>2</sub>) in 20μl of dimethylformamide, followed by 8 mg of dipyridyl disulfide and 9.5 mg of triphenylphosphine was then added to the dried oligonucleotide. After 2 hours, the oligonucleotide was precipitated by addition of 2% LiClO<sub>4</sub>/acetone, dissolved in 25 μl of 1M NaCl, precipitated with ethanol and dried. The final product was redissolved in water, and stored at -80°C.



*Modification of heparin-binding proteins by CIRNH<sup>32</sup>P-OdT<sub>18</sub>*

Modification of heparin-binding proteins by CIRNH<sup>32</sup>P-OdT<sub>18</sub> was accomplished by the method of Yakubov *et al.* (Oligonucleotides interact with recombinant CD4 at multiple sites. J. Biol. Chem. 1993 268:19918-18823). Initially, bFGF (50 nM concentration), PDGF BB (500 nM), VEGF (150 nM), laminin (50 nM) or HB-EGF (400 nM) was incubated in 0.1 M Tris-HCl, pH 7.4, containing 10-20  $\mu$ M CIRNH<sup>32</sup>P-OdT<sub>18</sub>. Defibrotide, defibrotide molecular weight fractions, Tmers or Nmers were used at increasing concentrations as competitors of the binding of the probe phosphodiester oligonucleotide to the proteins. After 1 hour at 37°C, one volume of a buffer containing 10% glycerol, 4% 2-mercaptoethanol, 4% SDS and 0.2% bromophenol blue was added, and SDS-PAGE was performed. The gels were dried and exposed to Kodak X-ray film until bands were visualized. The film was developed, and band densities were quantitated by laser densitometry.

15 *Proliferation assay*

Confluent HMEC-1 cells were treated in their place for 24 hours in M199 medium containing 2.5% FBS, and then seeded ( $2 \times 10^4$  cells/well) in Fibronectin-coated 96-well plates (in M199 medium supplemented with 2.5% FBS). Subsequently, the medium was then replaced with fresh medium containing either 20 ng/mL bFGF alone, defibrotide or Nmers with or without bFGF. After 3 days treatment at 37°C, the cell growth was evaluated by sulforhodamine B staining. All experiments were carried out in quadruplicate.

25 *Determination of Tissue Factor Pathway Inhibitor (TFPI) release*

HMEC-1 cells were seeded in 24-well plates in M199 medium containing 2.5% FBS at a density of  $10 \times 10^4$  cells/well. The cells were treated with either defibrotide or defibrotide molecular weight fractions, or Nmers for different time intervals. Then, the conditioned cell media was collected, centrifuged at 10,000g for 10 minutes to remove cell debris, and the concentration of Tissue Factor Pathway Inhibitor (TFPI) in the medium was measured using an ELISA assay as described by the manufacturer.



## RESULTS

*Defibrotide molecular weight fractions and Nmers interact similarly with heparin binding proteins*

5 Defibrotide, defibrotide molecular weight fractions and Nmers interact with heparin-binding proteins that are important in tumor growth, viability, angiogenesis, and migration. The assessment of the ability of defibrotide, defibrotide molecular weight fractions and Nmers to bind to heparin binding proteins was accomplished via a competition assay. In the first step, an  
10 alkylating,  $^{32}\text{P}$ -labeled phosphodiester 18mer homopolymer of thymidine (ClRNH $^{32}\text{P}$ -OdT<sub>18</sub>) was synthesized. This molecule was mixed with bFGF, PDGF BB, BB, VEGF, laminin or HB-EGF, incubated in 0.1 M Tris-HCl, pH 7.4, containing 10-20  $\mu\text{M}$  of labeled probe and with increasing concentrations of defibrotide, defibrotide molecular weight fractions or Nmers. The mixture was  
15 then separated by gel electrophoresis and autoradiographed. Defibrotide, defibrotide molecular weight fractions and Nmers were competitors of the binding of ClRNH $^{32}\text{p}$ -OdT<sub>18</sub>, and thus of the alkylation of the protein by the radioactively labeled oligonucleotide. The value of  $K_d$  for ClRNH $^{32}\text{P}$ -OdT<sub>18</sub> for each of these proteins has previously been determined: the average  $K_d$  for bFGF is 0.5  $\mu\text{M}$   
20 (Guvakova, *et al.*, "Phosphorothioate oligodeoxynucleotides bind to basic fibroblast growth factor, inhibit its binding to cell surface receptors, and remove it from low affinity binding sites on extracellular matrix", J. Biol. Chem., 1995, (270) 2620-2627) and the average  $K_d$  for laminin is 14  $\mu\text{M}$  (Khaled, *et al.* "Multiple mechanisms may contribute to the cellular antiadhesive effects of  
25 phosphorothioate oligodeoxynucleotides", Nucl. Acids Res., 1996, (24) 737-745). In order to determine the  $K_d$  for VEGF165, the concentration dependence of the modification of VEGF by ClRNH $^{32}\text{P}$ -OdT<sub>18</sub> was examined (Fig. 3A). These results are depicted in Fig. 3B, where the concentration of modifying oligodeoxynucleotide is plotted as a function of gel band intensity. The  
30 association of VEGF with the modifying oligodeoxynucleotide exhibits approximate saturation binding and can be described by a single-site binding equation of the Michaelis-Menton type. Fig. 3C depicts the double-reciprocal

replot of the data in Fig. 3B. These data are linear ( $R^2 = 0.98$ ), and the line intersects the abscissa corresponding to an apparent  $K_d$  value of 33.9  $\mu\text{M}$ . Similar experiments were performed for PDGF BB and HB-EGF. The  $K_d$ s are 4.5 and 8.7  $\mu\text{M}$ , respectively.

5  $K_c$  was calculated from equation I as described by Cheng and Prusoff:

Equation 1 
$$K_c = IC_{50} / (1 + [CIRNH^{32}P\text{-OdT}_{18}] / K_d)$$

10 In Fig. 1A, competition for binding to bFGF is shown. As per Equation 1, a plot of the normalized intensity of the gel band versus competitor concentration was linear (Fig. 1B). The  $IC_{50}$  was determined by inspection. Similar competition for binding of different competitors to all proteins of interest was also determined. The values of  $K_c$ , determined in an identical manner, are summarized in the Tables to Figures 2, 4, 5, 6, and 7.

15

*Nmers, in a length dependent fashion, and defibrotide inhibit the ability of heparin-binding growth factors to maximally stimulate the growth of SV40-transformed HMEC-1 cells in tissue culture*

Cytokine-stimulated cell growth was determined by using sulforhodamine B (SRB). These experiments were performed in SV40-transformed HMEC-1 cells, whose growth is stimulated by bFGF. The cells were in 0% serum for 24 hours before being treated with bFGF in M199 medium containing 2.5% FBS in order to up-regulate bFGF cell surface receptors, and then incubated in medium containing 20 ng/mL bFGF with or without increasing concentrations of defibrotide or Nmers for 3 days. As shown in Fig. 8 and 9, both Nmers, in a length-dependent fashion (length of about 45 nucleotides and greater having an effect) and defibrotide cause a small (and in the case of Nmers, length-dependent), decrease in maximal bFGF-induced cell proliferation. The rate of proliferation of the HMEC-1 cells increased by 60-70% after bFGF-treatment, compared to the non-stimulated group, compared to the bFGF control. The inhibitory effect of defibrotide, when added 1 hour before bFGF, was not significantly different from that observed when added at the same time (data not shown).

20

25

30



*Evaluation of Defibrotide and Nmers toxicity*

A major toxicity of defibrotide and Nmers, coagulopathy and bleeding, results from the binding of the oligonucleotides to heparin-binding members of the coagulation cascade and inhibition of their function. This anticoagulant effect was evaluated by partial thromboplastin time (PTT). Plasma from healthy volunteers was mixed with various concentrations of defibrotide or Nmers, and a standard PTT assay was performed. As shown in Fig. 10, defibrotide and Nmers do not cause significant elevation of the PTT. Only at high concentration of defibrotide, N50 or N60 (~100  $\mu$ M) was there prolongation of the PTT (1.5-1.7 times compared to control) observed (Fig. 10). For a longer Nmer, N80, this effect was seen even at a 25  $\mu$ M concentration.

*Defibrotide, defibrotide molecular weight fractions and Nmers increase Tissue Factor Pathway Inhibitor (TFPI) synthesis and release from HMEC-1 cells*

To investigate how defibrotide affects the acute and long-term release of TFPI, which is a protein that diminishes coagulopathy, from HMEC-1 cells, both concentration and time-course studies were performed. Conditioned medium from HMEC-1 cells was collected at selected time intervals, and TFPI levels determined using an ELISA assay as described by the manufacturer. As shown in Fig. 11A, 12.5  $\mu$ M defibrotide caused a time-dependent increase of TFPI into the medium, with a substantial amount released after 20-30 minutes (5-6-fold increase compared to control cells). During the acute phase (30 minutes), stimulation of HMEC-1s with increasing concentrations of defibrotide caused a concentration-dependent increase of TFPI release, which plateaued at a 12.5  $\mu$ M defibrotide concentration (Fig. 11C). A 24 hour-incubation of the cells with 12.5  $\mu$ M defibrotide molecular weight fractions or Nmers caused a 7-8-fold increase in the TFPI in the medium compared to unstimulated cells.

*Determination of mitogenesis in C11 cells*

C11 clones are BAF3 mouse lymphoid cells that have been engineered to overexpress fibroblast growth factor receptor 1 (FGFR-1), to which bFGF binds



with high (pM) affinity. These cells were obtained from D. Ornitz (Washington University, St. Louis). These cells have an absolute requirement for bFGF for proliferation; furthermore, it has long been known that heparin is also required for the activity of the bFGF. It had been previously demonstrated that DF and the  
5 Nmers could remove bFGF from its low affinity (nM) binding sites on extracellular matrix (Guvakova, *et al.*, "Phosphorothioate oligodeoxynucleotides bind to basic fibroblast growth factor, inhibit its binding to cell surface receptors, and remove it from low affinity binding sites on extracellular matrix", J. Biol. Chem., 1995, (270) 2620-2627). The inventors now wanted to determine if DF  
10 and Nmers could interfere with the binding of bFGF to its high affinity binding sites. Accordingly, C11 cells were washed twice with RPMI media lacking IL-3.  $2.2 \times 10^4$  cells were plated per well in 48-well plates. bFGF (final 1 nM) and DF or Nmers (final 10  $\mu$ M) or Heparin (1  $\mu$ g/mL) were added in a total volume 200  $\mu$ L. The cells (n = 3 for each experiment) were then incubated for 2-3 days, and  
15 stained with sulforhodamine blue (SRB). Cell numbers were normalized to control (proliferation in the absence of either bFGF, heparin or oligonucleotide). As can be seen in Figure 13, bFGF or heparin by themselves have little or no effect on cell proliferation after 3 days. The activity of bFGF is potentiated by both heparin and DF, demonstrating that DF can take the place of heparin.  
20 However, DF does not affect the binding of bFGF to its high-affinity binding sites. The Nmers, in a length-dependent manner, can also take the place of DF or heparin, but their activity is not quite as great as DF until a length of approximately 80mer is reached.

25

## CONCLUSIONS

The synthetic phosphodiester oligonucleotides (Nmers) of the present invention can virtually recapitulate the properties of defibrotide.

Nmers and defibrotide has been evaluated and compared with respect to  
30 their abilities to bind to heparin-binding proteins (including bFGF, PDGF BB, VEGF165, laminin, and HB-EGF), and to cause TFPI release from HMEC-1 cells. The Nmers may be administered via i.v. infusion in normal saline or 5% dextrose

in water to a patient afflicted with cancer or VOD (or other diseases which would be treated by administering defibrotide) at a dose of 10 mg/kg to 60 mg/kg of body weight daily in a simple dose or in divided doses for approximately 14 days. The dose may be adjusted depending on the individual patient's response to the particular course of therapy.

The values of  $K_c$  for bFGF and PDGF and Nmers of various lengths (Fig. 2, 4, 5, 6, 7) demonstrate that an Nmer length approximately of at least 40 mers is sufficient for maximum Nmer activity. Such  $K_c$  values also demonstrate that longer Nmers add little to the overall heparin-binding protein affinity; consequently, based both on their higher weight/dose ratio and on the difficulty to synthesize them, Nmers having a length approximately of more than 65 mers appear to be useless as an alternative to defibrotide.

The synthetic phosphodiester oligonucleotides having a length of from about 40 mers to about 65 mers may thus be used as an alternative to defibrotide and, in particular, they may be used in all the therapeutic applications disclosed above in the chapter entitled "background of the invention".

One of the advantages of the present invention is that the dosage of the related pharmaceutical formulations can be determined in function of the concentration of the synthetic phosphodiester oligonucleotides rather than in function of the biological activity, as it currently happens for oligonucleotide mixtures of extractive origin.

A further advantage is represented by the fact that the present invention provides for the administration of active sequences only; thus, if compared for instance to oligonucleotide mixtures of extractive origin, it provides for the administration of less oligonucleotides per dosage, with evident advantages in terms of efficacy, safety and side-effects.

Having described the present invention, it will now be apparent that many changes and modifications may be made to the above-described embodiments without departing from the scope of the present invention.



## Claims

1. Use of a pharmaceutical composition for treating veno-occlusive disease, the pharmaceutical composition containing a single active ingredient consisting of single stranded nucleic acids having random sequences and lengths from 40 bases to 65 bases, wherein the pharmaceutical composition is for administration to a patient afflicted with veno-occlusive disease.
2. The use of claim 1 wherein the composition further comprises one or more pharmaceutically acceptable excipients and/or adjuvants.
3. The use of claim 1 wherein the composition is for intravenous administration as an aqueous solution.
4. The use of claim 3 wherein the composition further comprises normal saline or dextrose in water.
5. The use of claim 3 wherein the composition is for administration in a dosage form selected to deliver between 10 mg and 60 mg of the nucleic acids per kilogram of the patient's body weight.
6. The use of claim 5 wherein the composition is for administration for 14 days.
7. The use of claim 3 wherein the nucleic acids are present in the aqueous solution at a concentration of between 5 and 60 micromoles per liter.
8. Use of a pharmaceutical composition for treating veno-occlusive disease, the pharmaceutical composition consisting essentially of:
  - single stranded nucleic acids having random sequences and lengths from 40 bases to 65 bases and excluding nucleic acids having lengths less than 40 bases and greater than 65 bases; and
  - one or more pharmaceutically acceptable excipients and/or adjuvants,
  - wherein the pharmaceutical composition is for administration to a patient afflicted with veno-occlusive disease.

9. The use of claim 8 wherein the composition is for intravenous administration as an aqueous solution.
10. The use of claim 9 wherein the composition is for administration in a dosage form selected to deliver between 10 mg and 60 mg of the nucleic acids per kilogram of the patient's body weight.
11. The use of claim 10 wherein the composition for administration for 14 days.
12. The use of claim 9 wherein the nucleic acids are present in the aqueous solution at a concentration of between 10 and 50 micromoles per liter.
13. The use of claim 1 wherein the single stranded nucleic acids are synthetic.
14. The use of claim 8 wherein the single stranded nucleic acids are synthetic.



1/14

bFGF

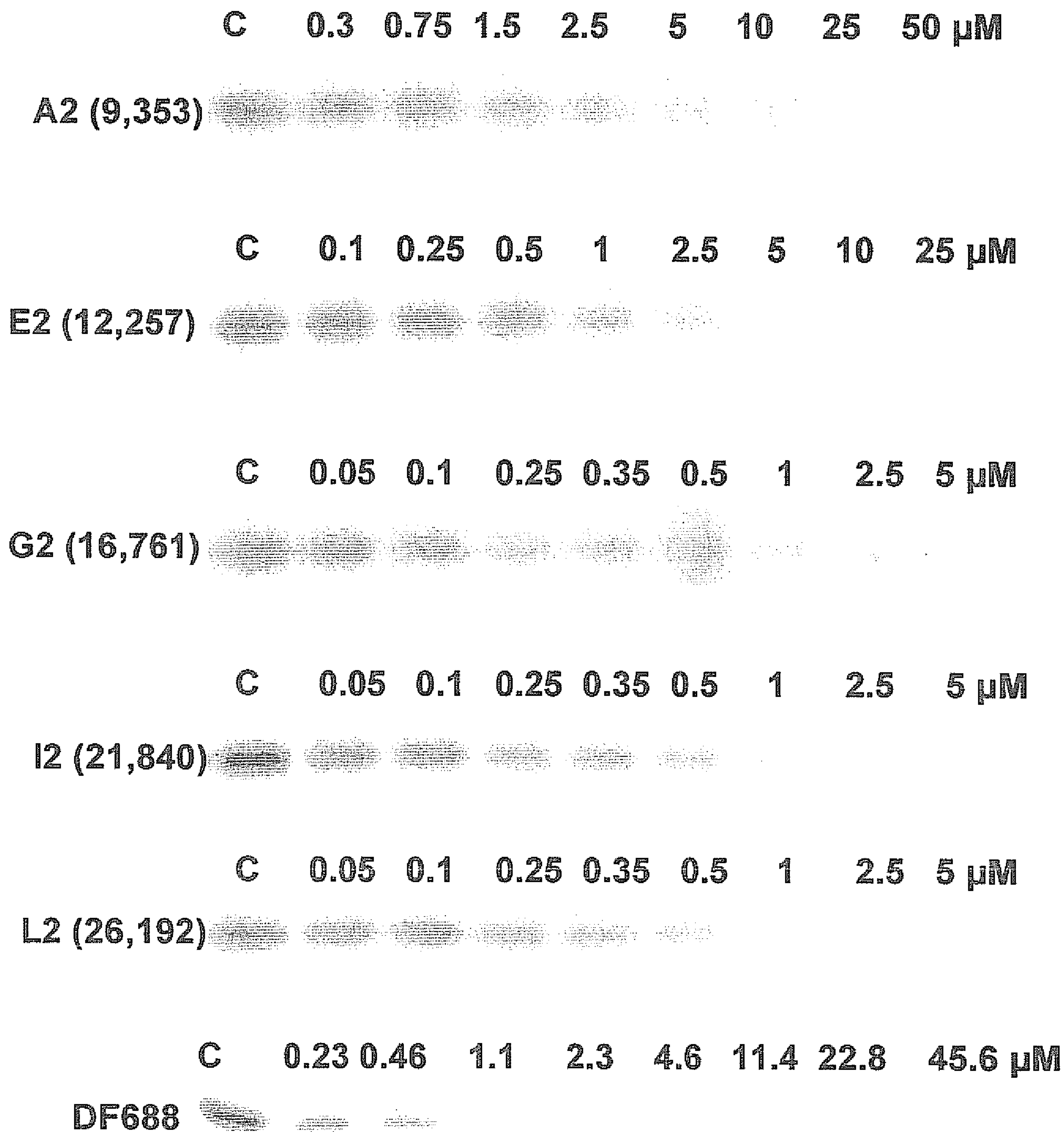


Fig. 1A

2/14

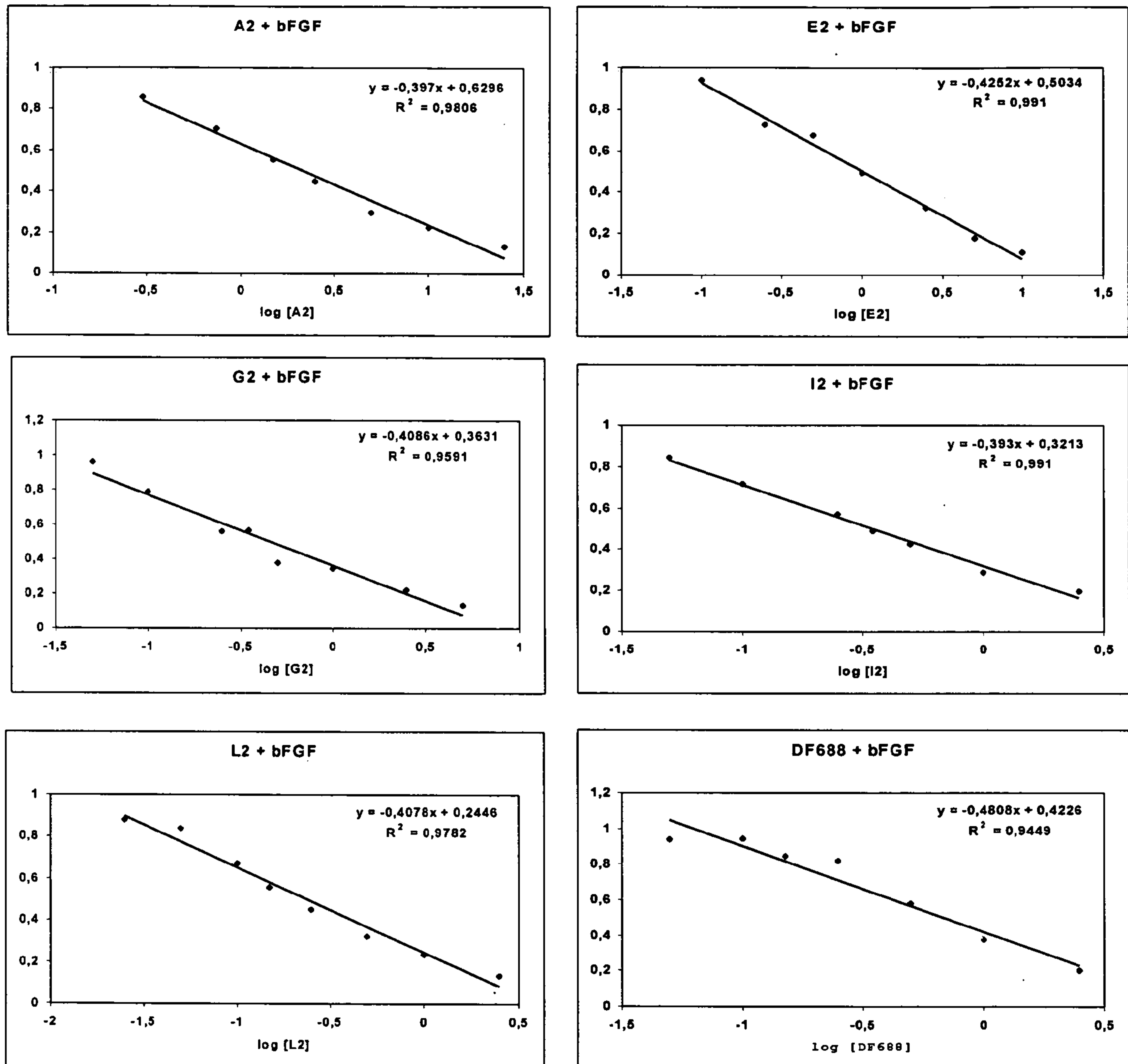


Fig. 1B



DF	Kc, nM
A2 (9,353)	100
E2 (12,257)	48.6
G2 (16,761)	22
I2 (21,840)	16.7
L2 (26,192)	11.2
DF688	32.4
N35	134
N40	33.5
N50	33
N60	40
N80	7.1

Oligo	Kc, nM
T25	1120
T40	840
T50	590
T80	300
T100	10
T120	16
T150	21
T200	2

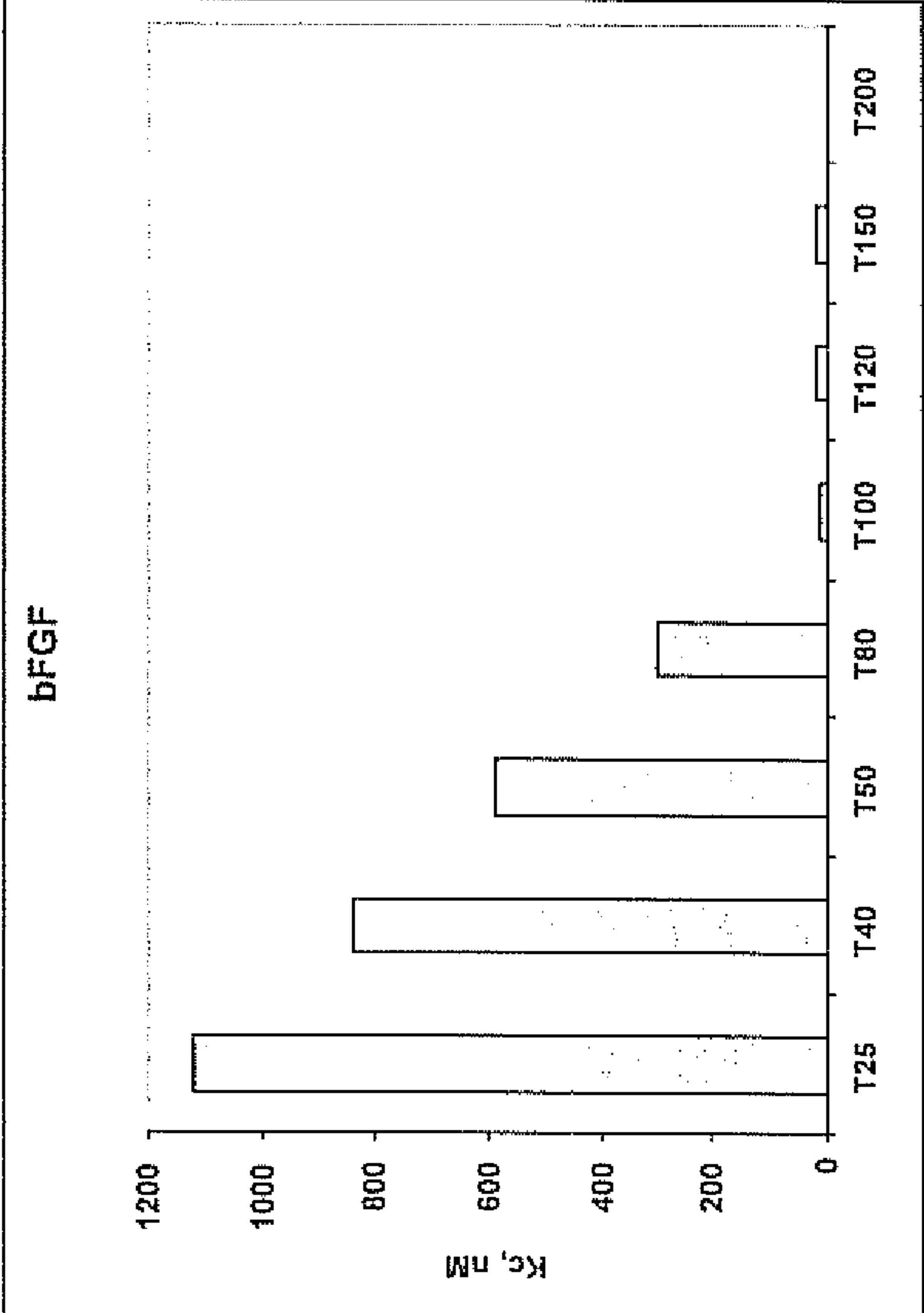
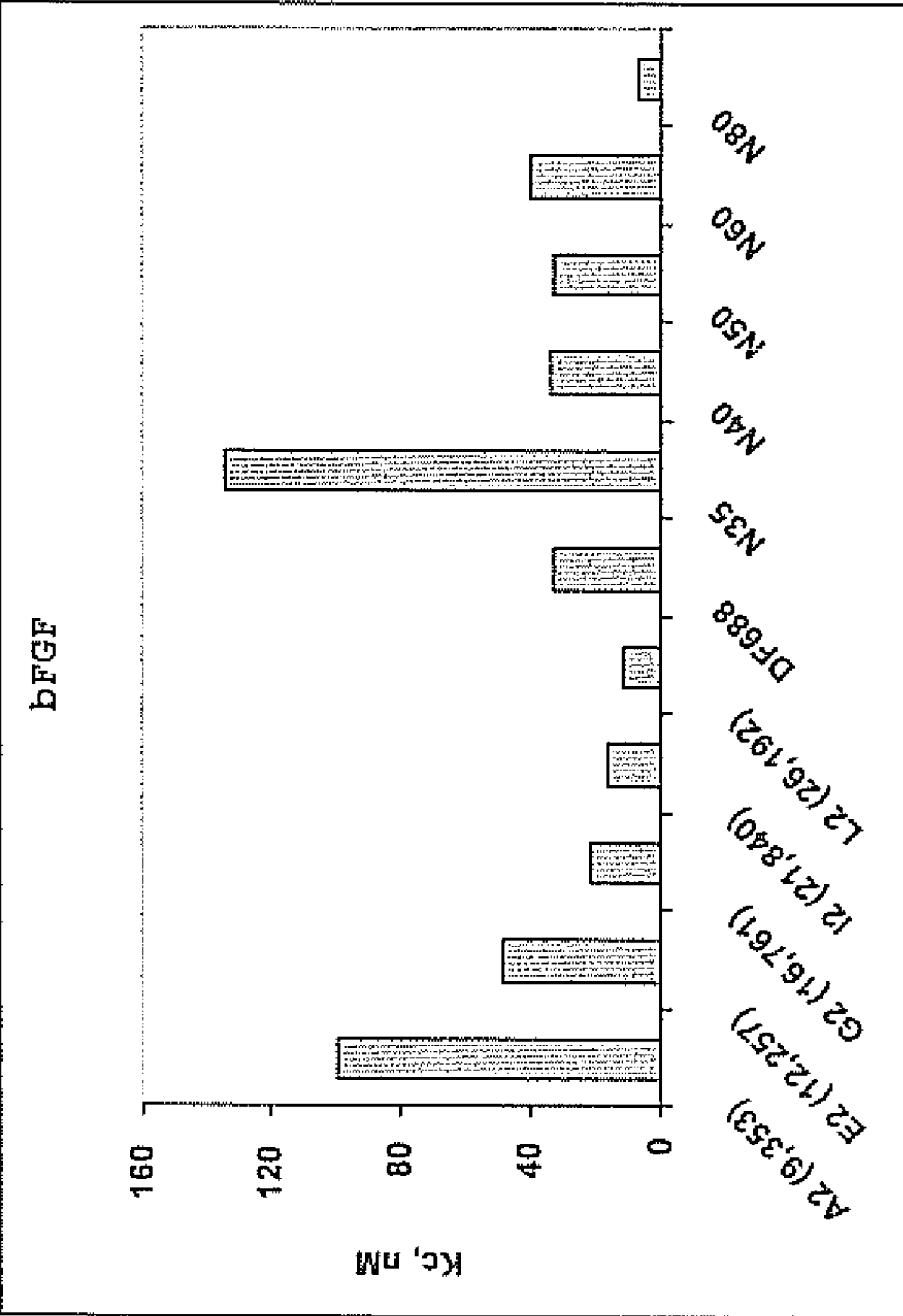
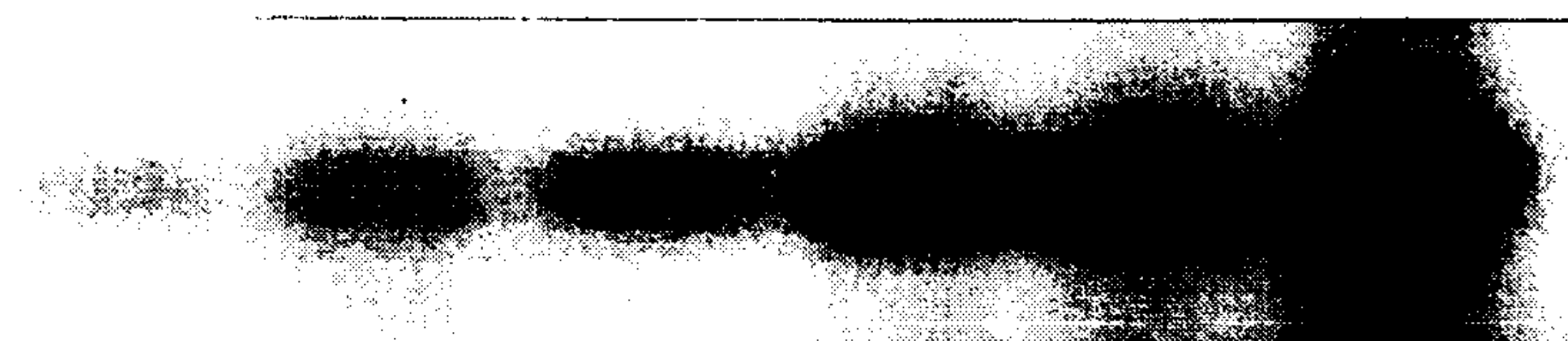
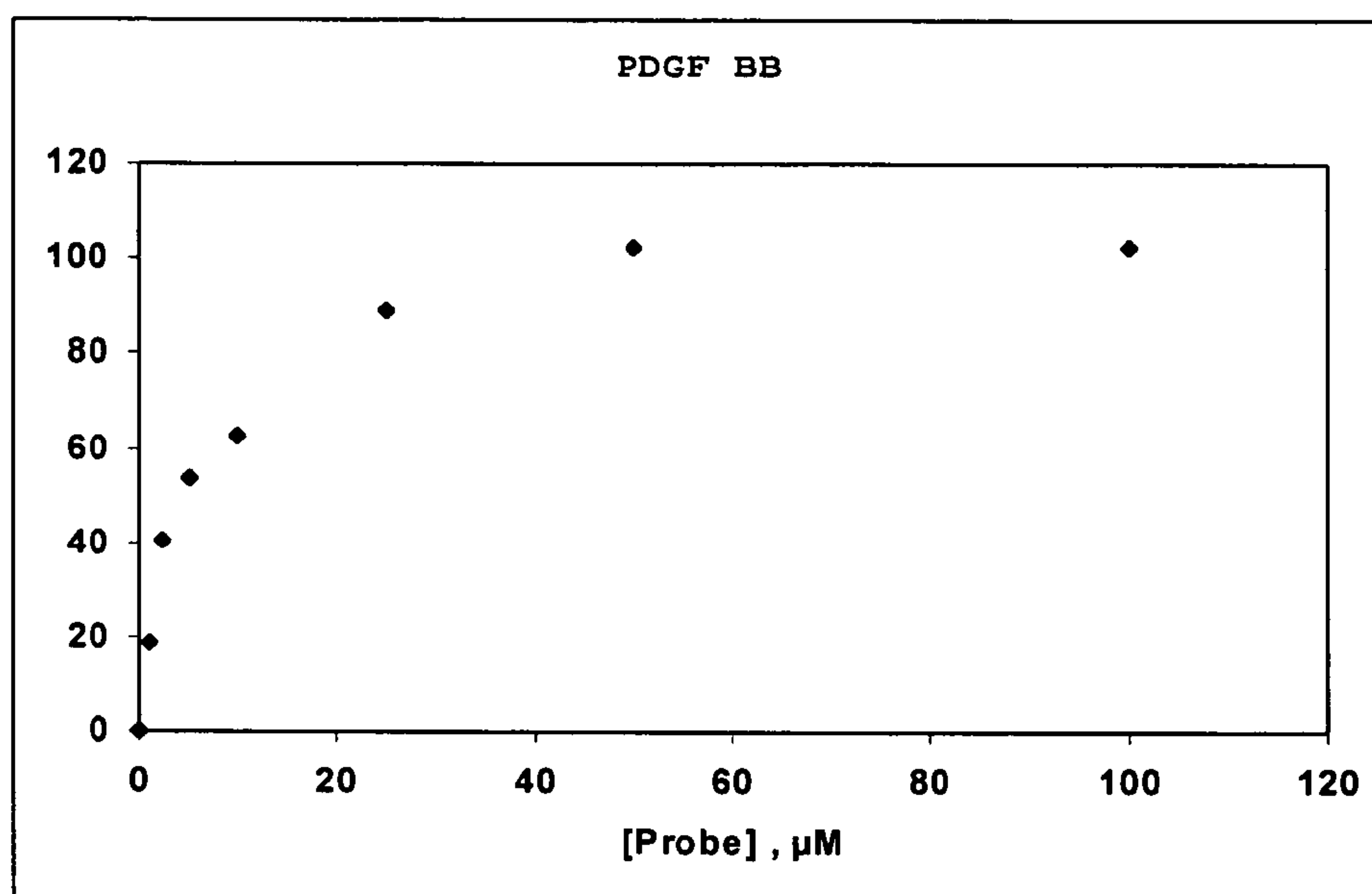
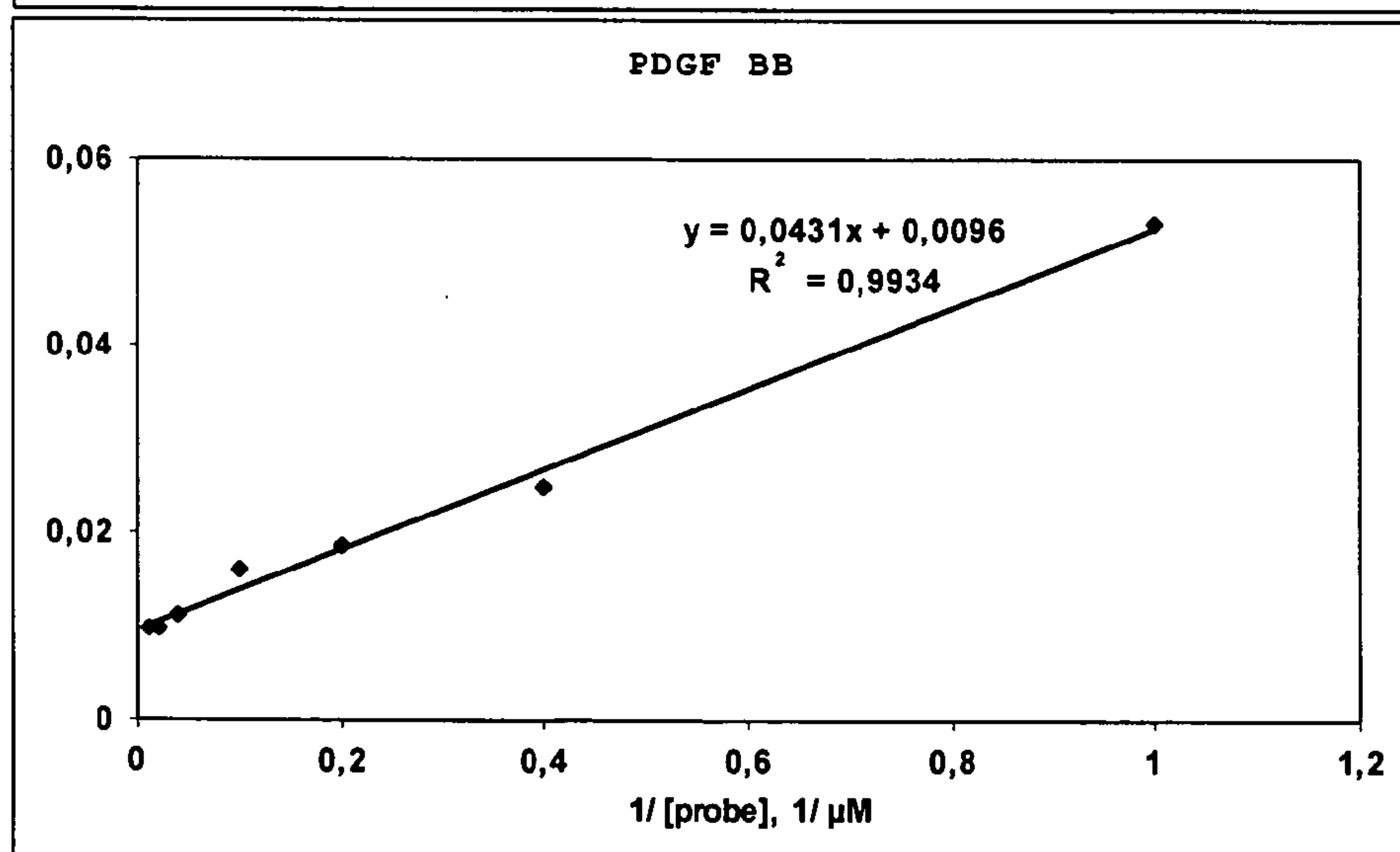
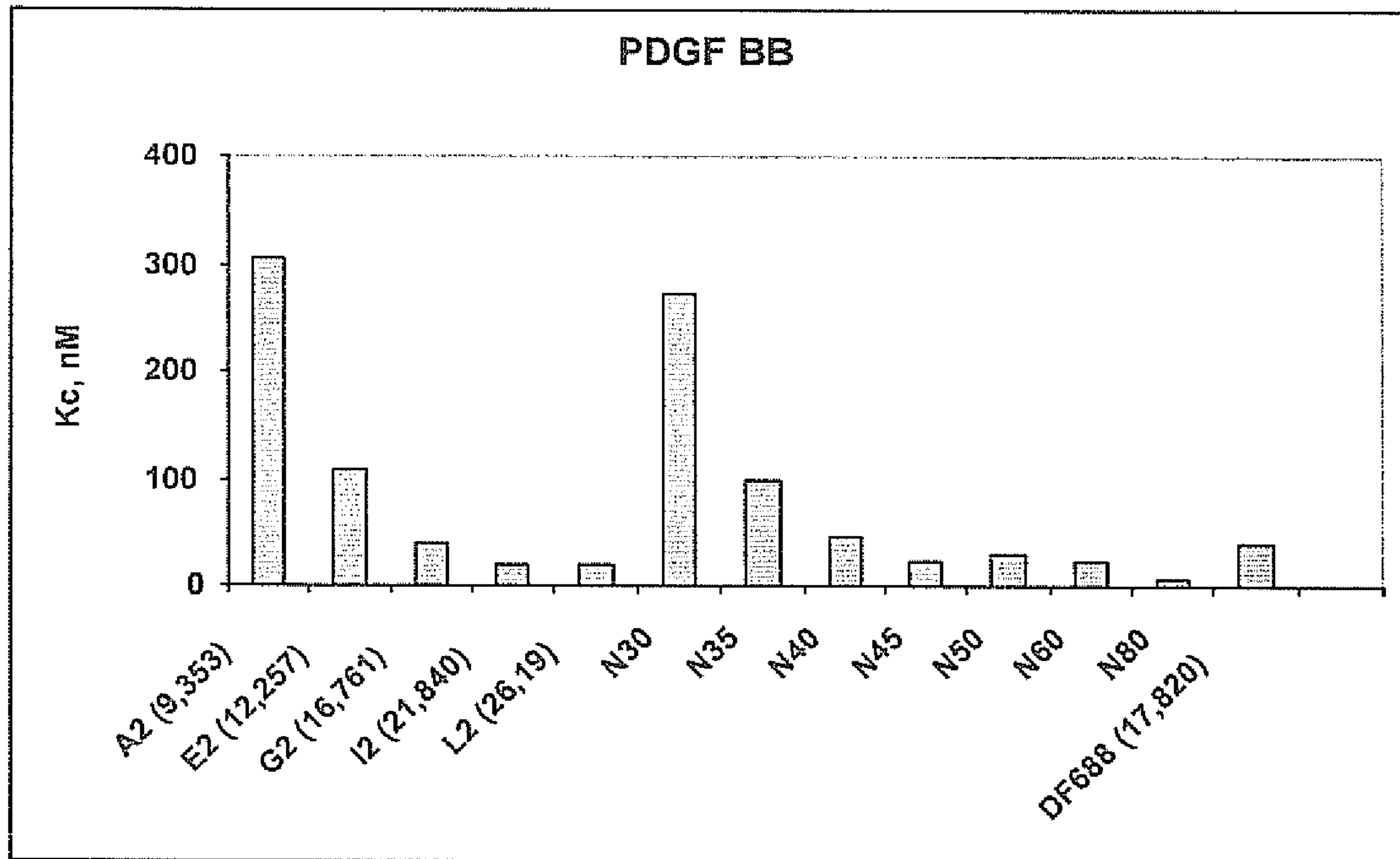


Fig. 2

**4/14****A) 0.1 0.25 0.5 1 2.5 5 10 25 50 100  $\mu\text{M}$** **B)****C)****Fig. 3**



5/14



DF	Kc, nM
A2 (9,353)	304.3
E2 (12,257)	108.7
G2 (16,761)	38.5
I2 (21,840)	20.2
L2 (26,192)	20.5
N30	272
N35	99
N40	47
N45	22
N50	29
N60	23
N80	6
DF688 (17,820)	39.8

Fig. 4

6/14

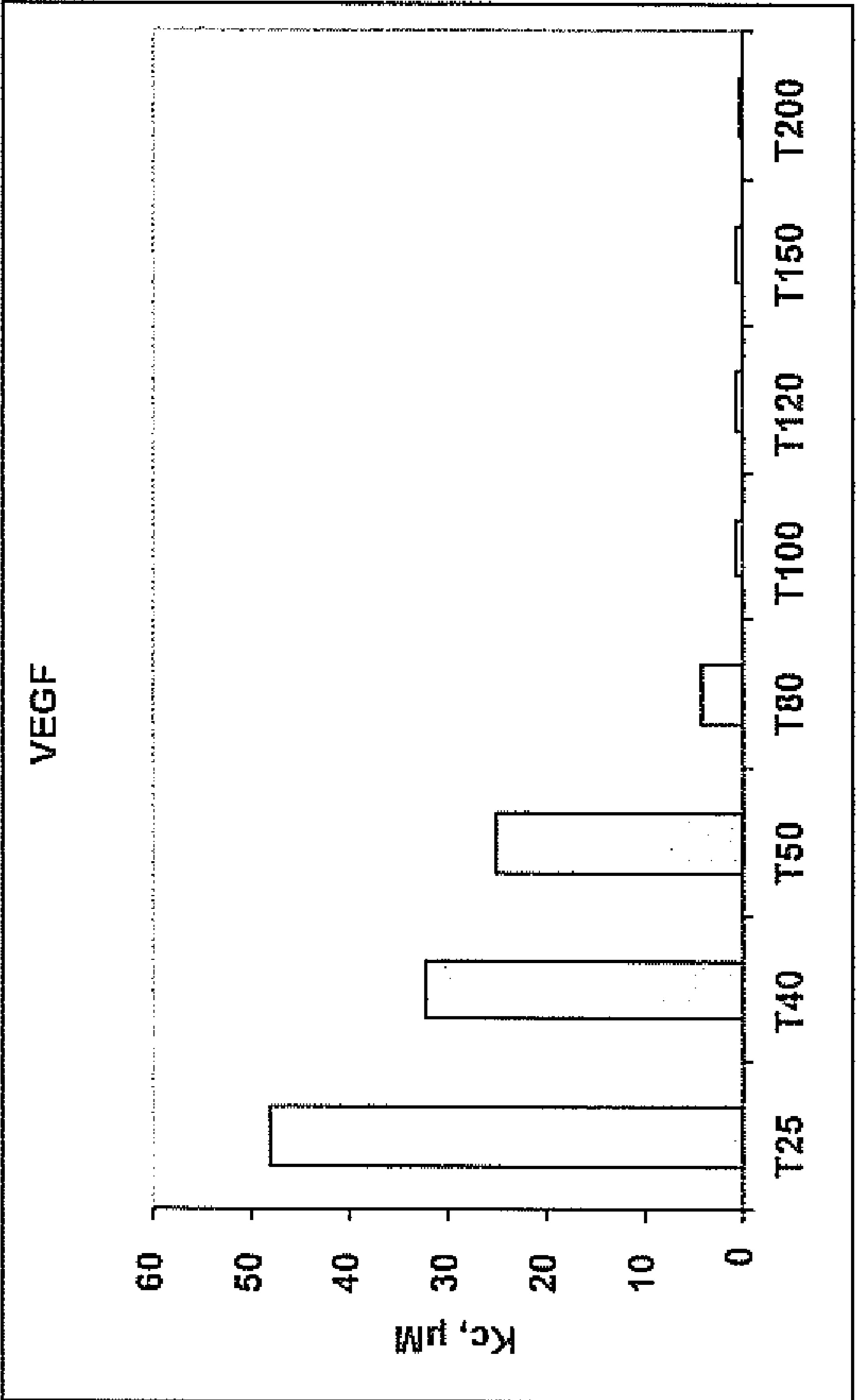
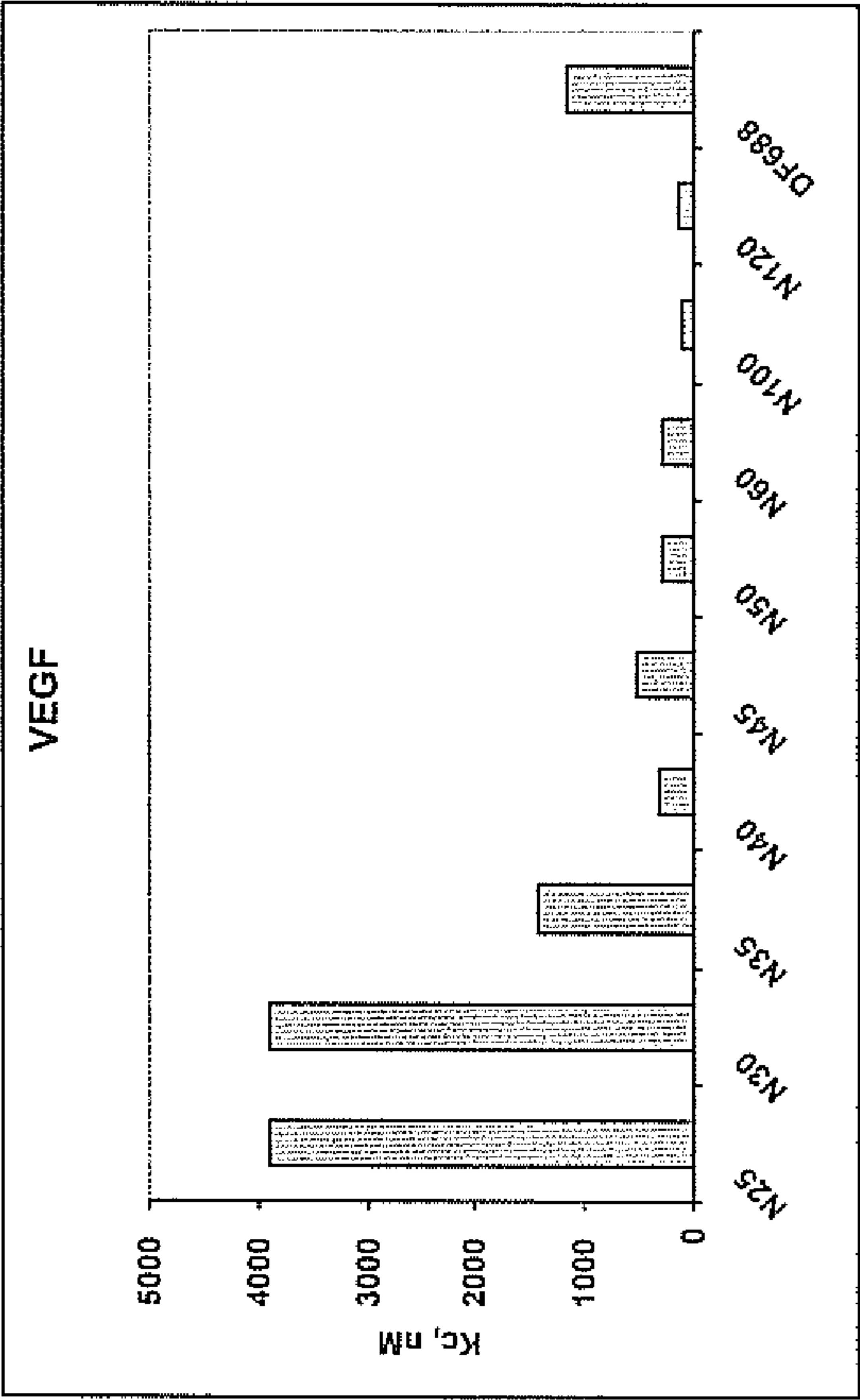
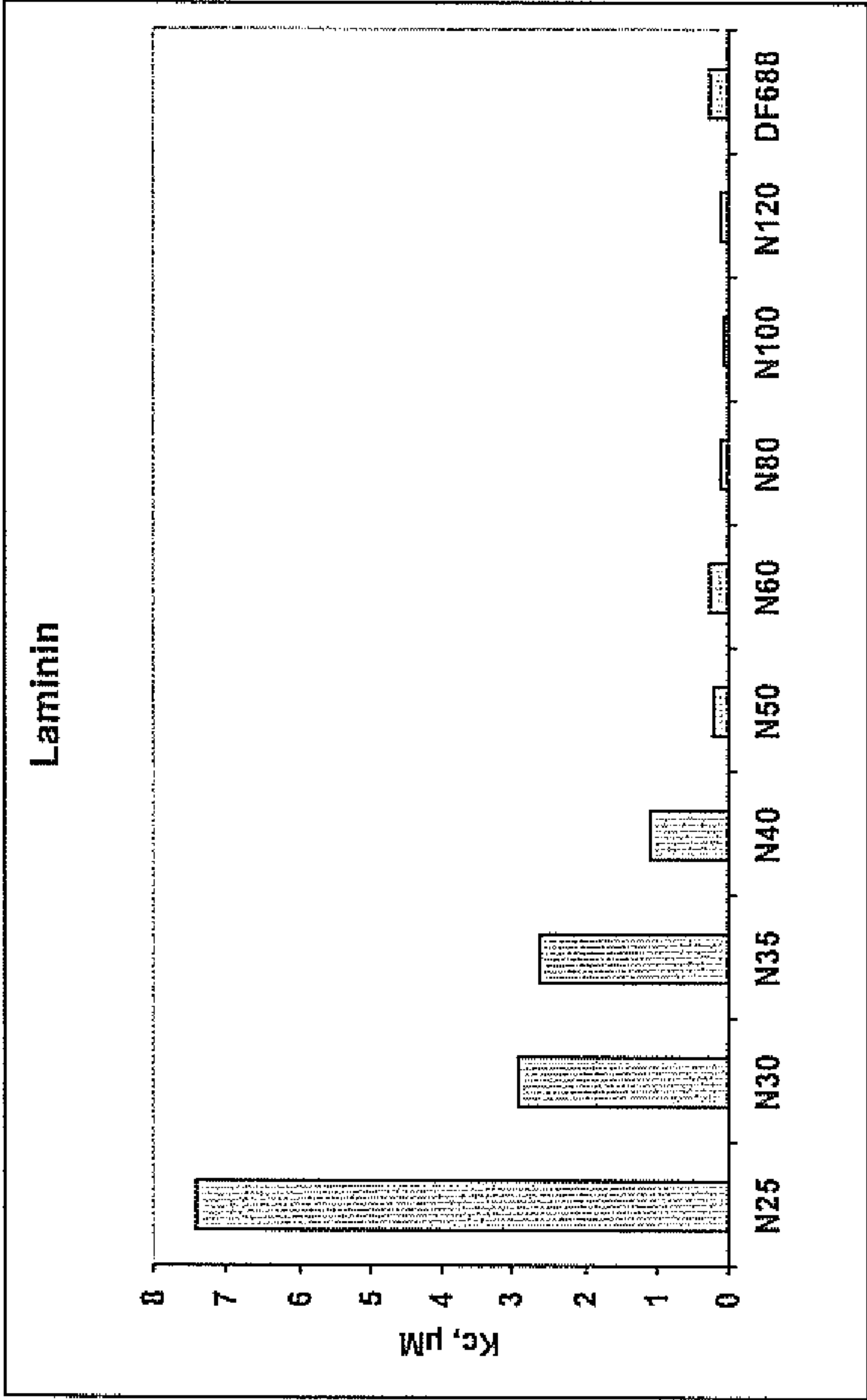


Fig. 5

Oligo	K <sub>C</sub> , μM
N25	7.4
N30	2.92
N35	2.6
N40	1.11
N50	0.185
N60	0.237
N80	0.097
N100	0.059
N120	0.092
DF688	0.27



Oligo	K <sub>C</sub> , μM
T25	53
T40	15.6
T50	13.3
T80	6.7
T100	0.6
T120	0.38
T150	0.11
T200	0.1

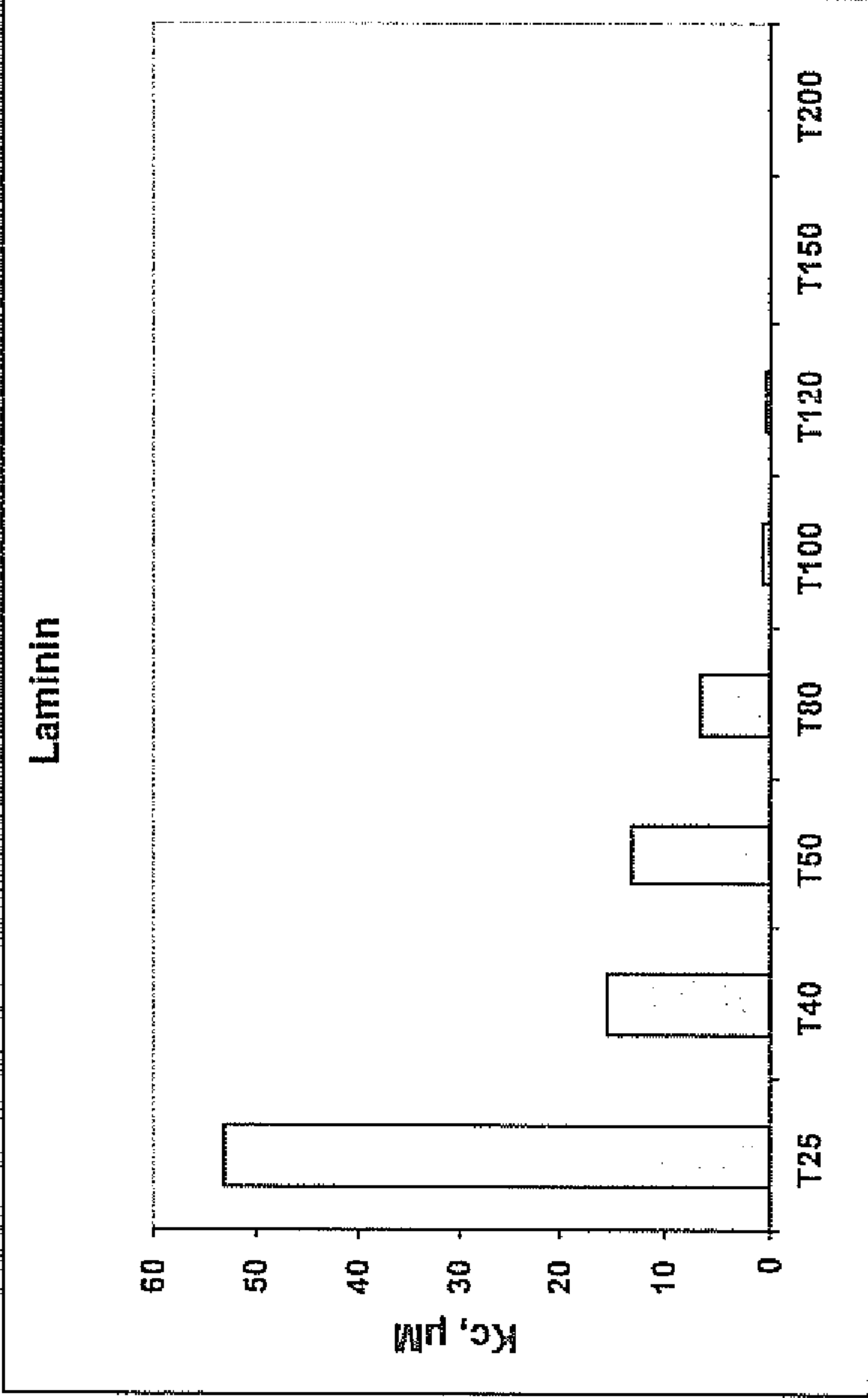
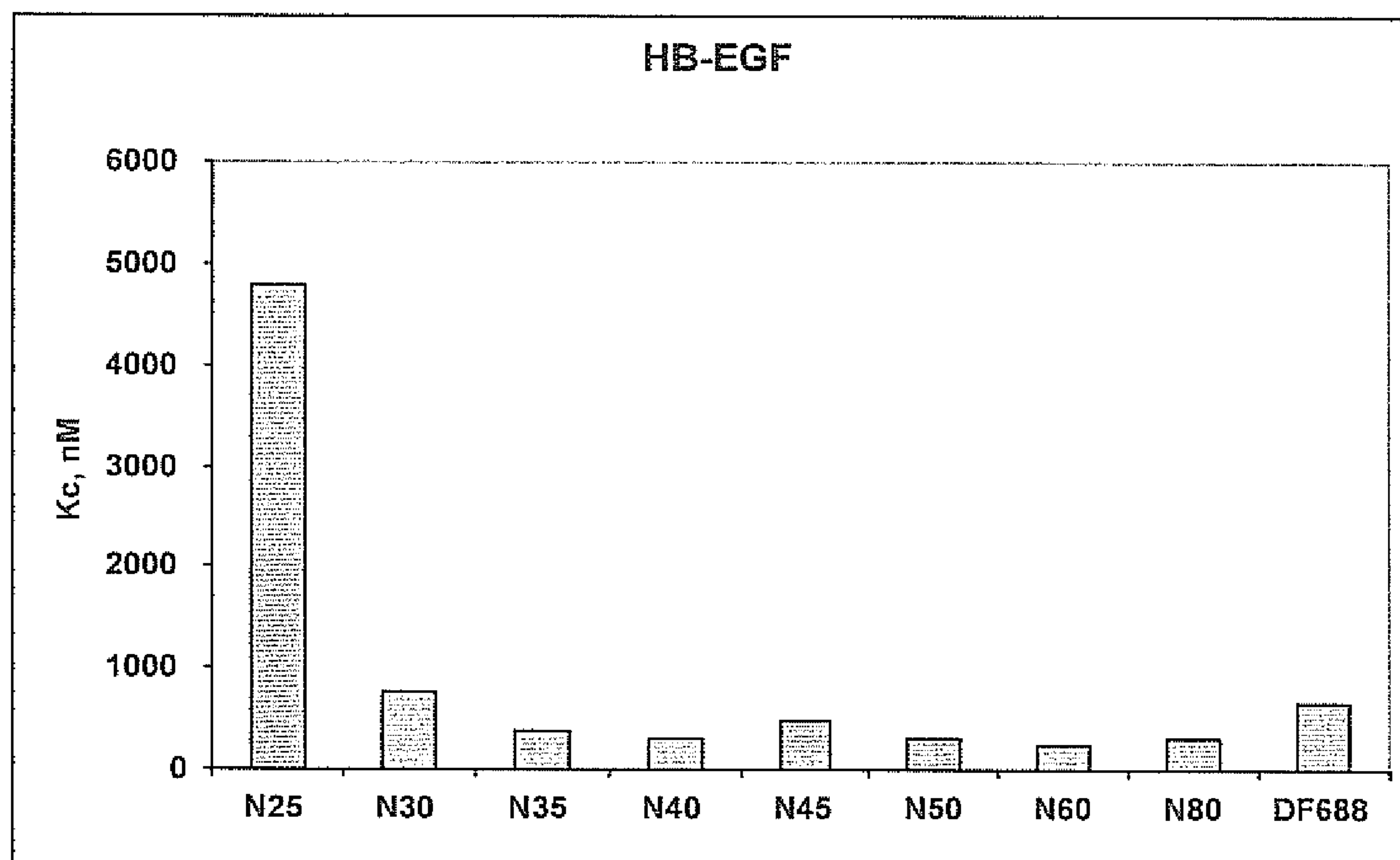


Fig. 6



8/14



Oligo	Kc, nM
N25	4800
N30	757
N35	396
N40	318
N45	490
N50	313
N60	252
N80	321
DF688	648

Fig. 7

9/14

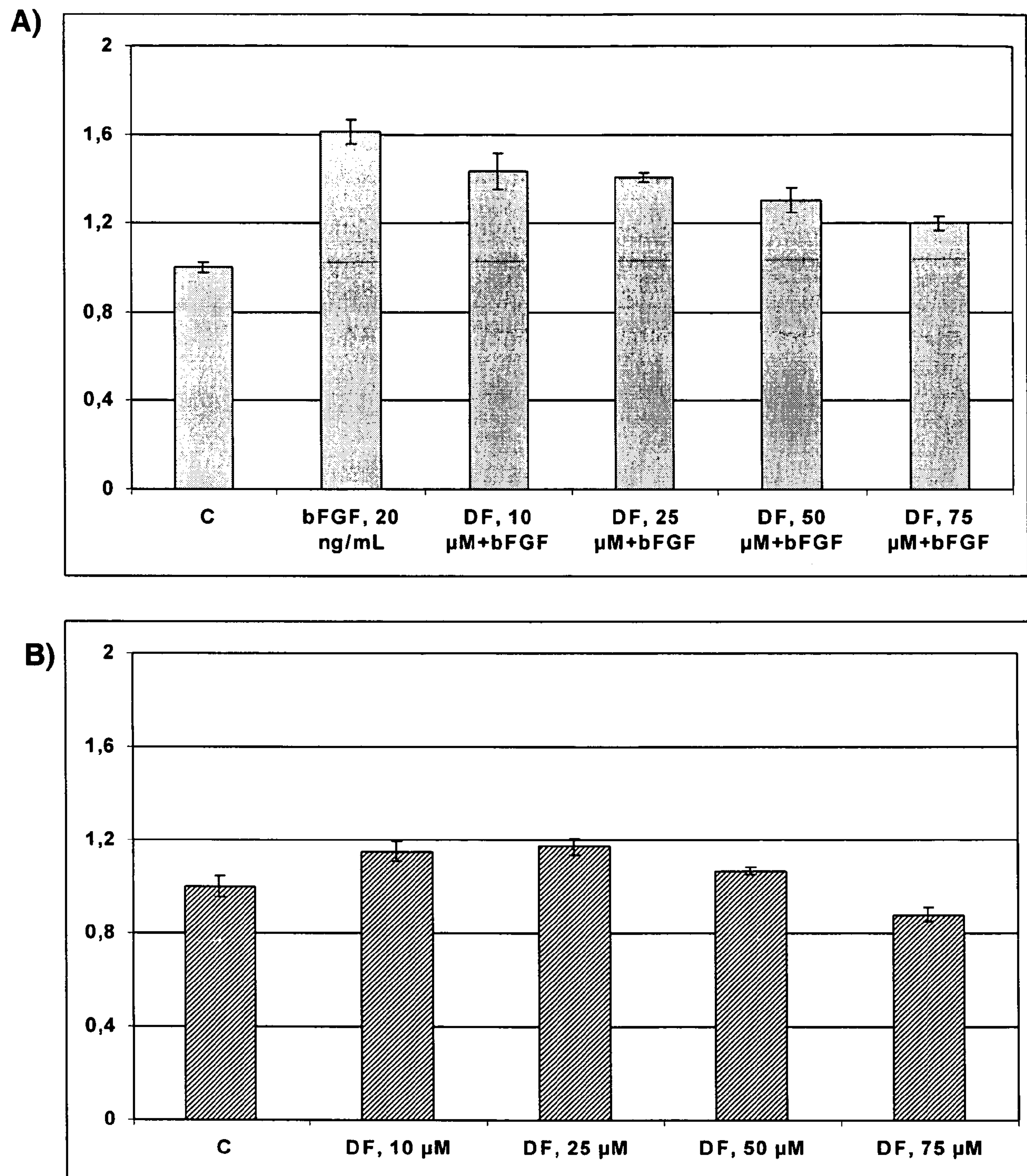
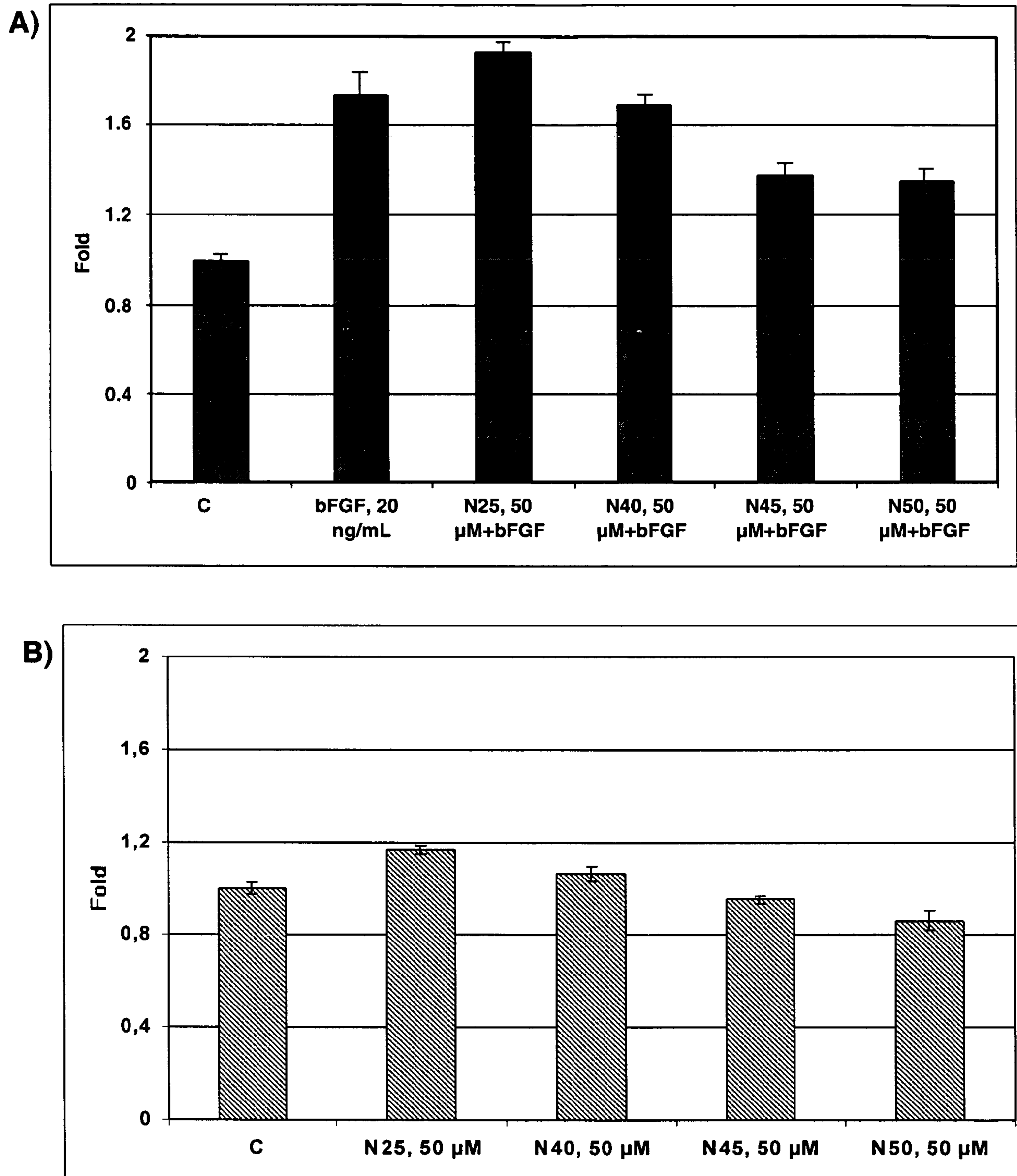


Fig. 8



**10/14****Fig. 9**



11/14

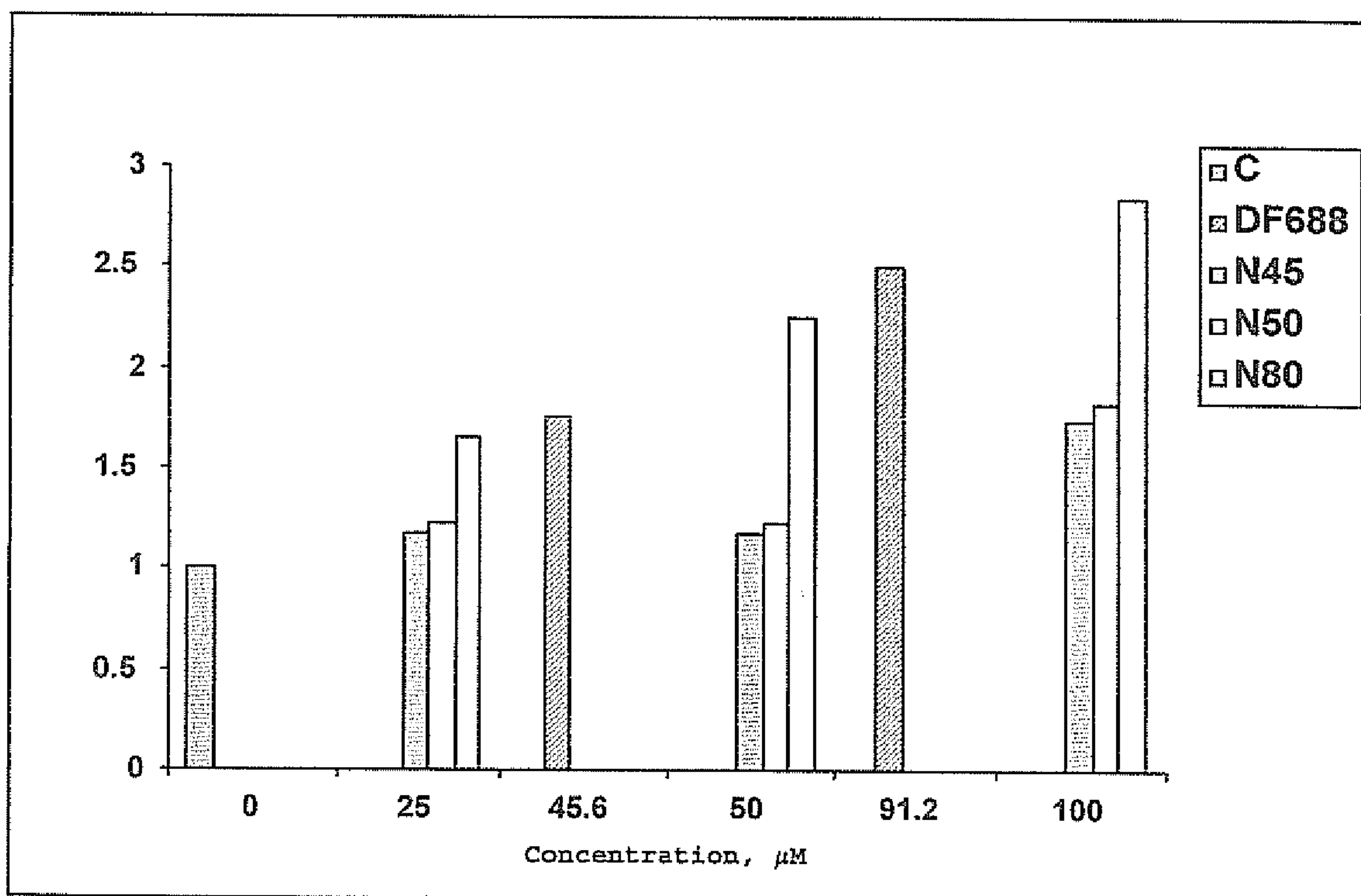
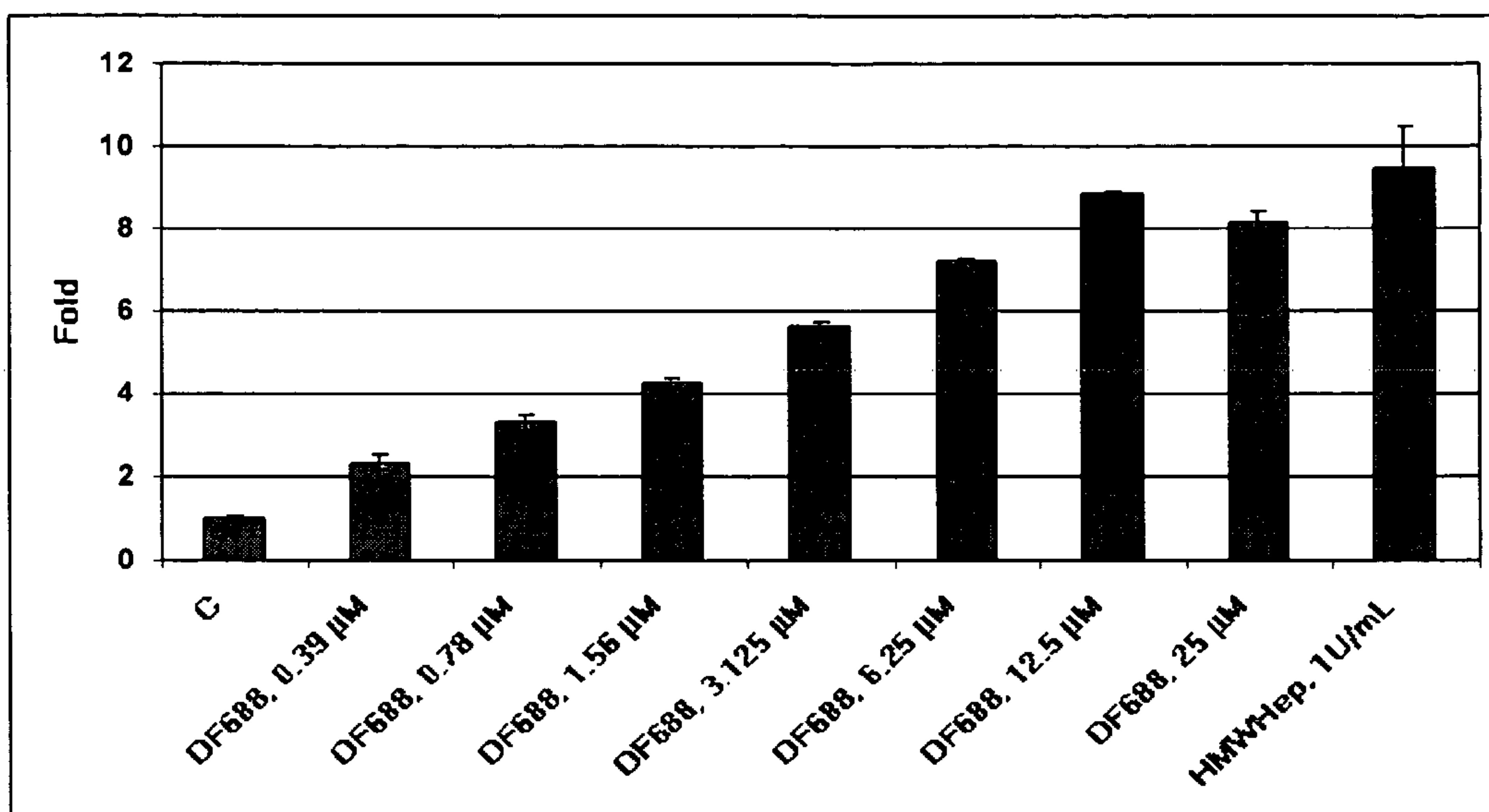


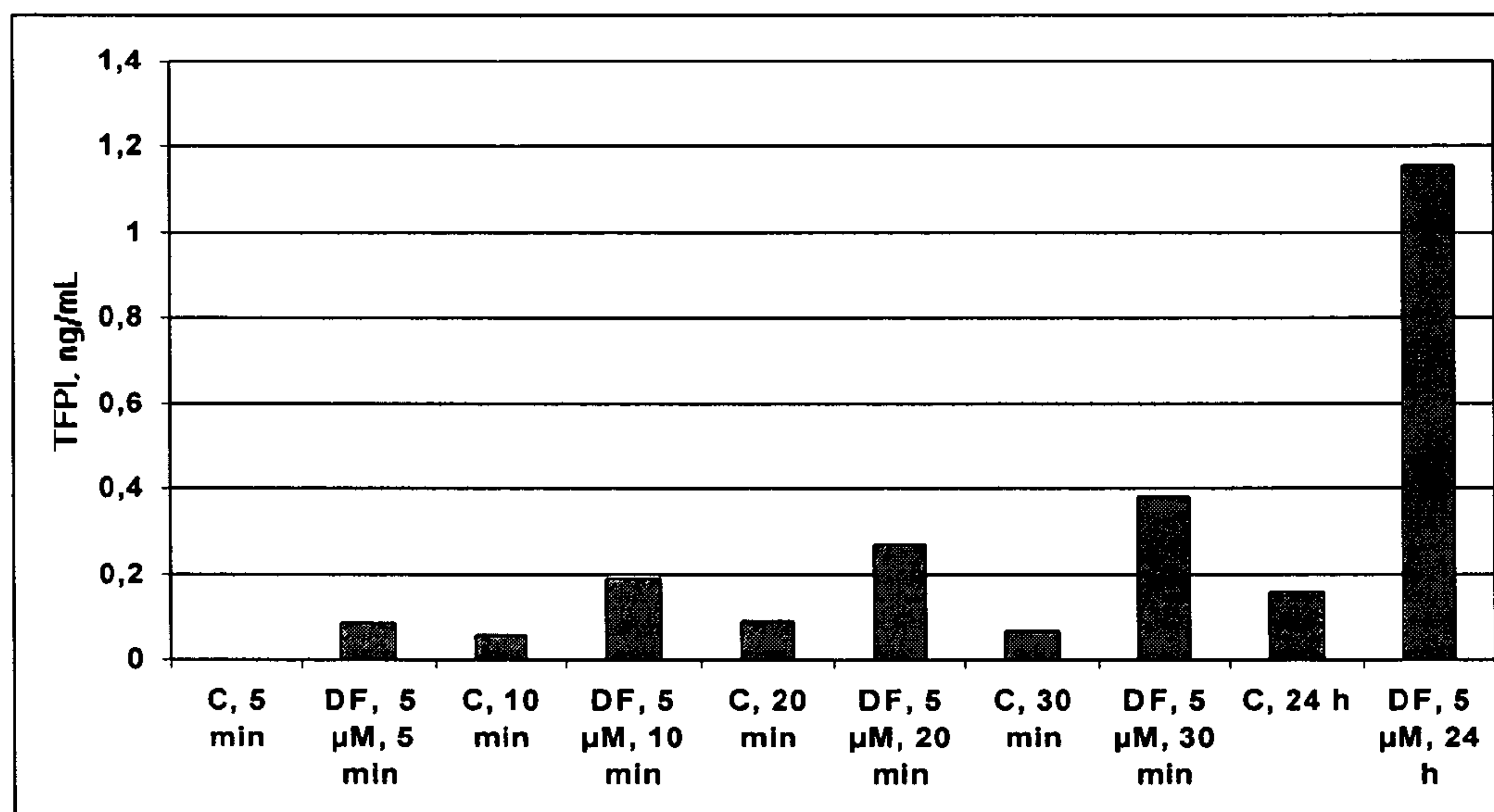
Fig. 10

12/14

A)



B)



C)

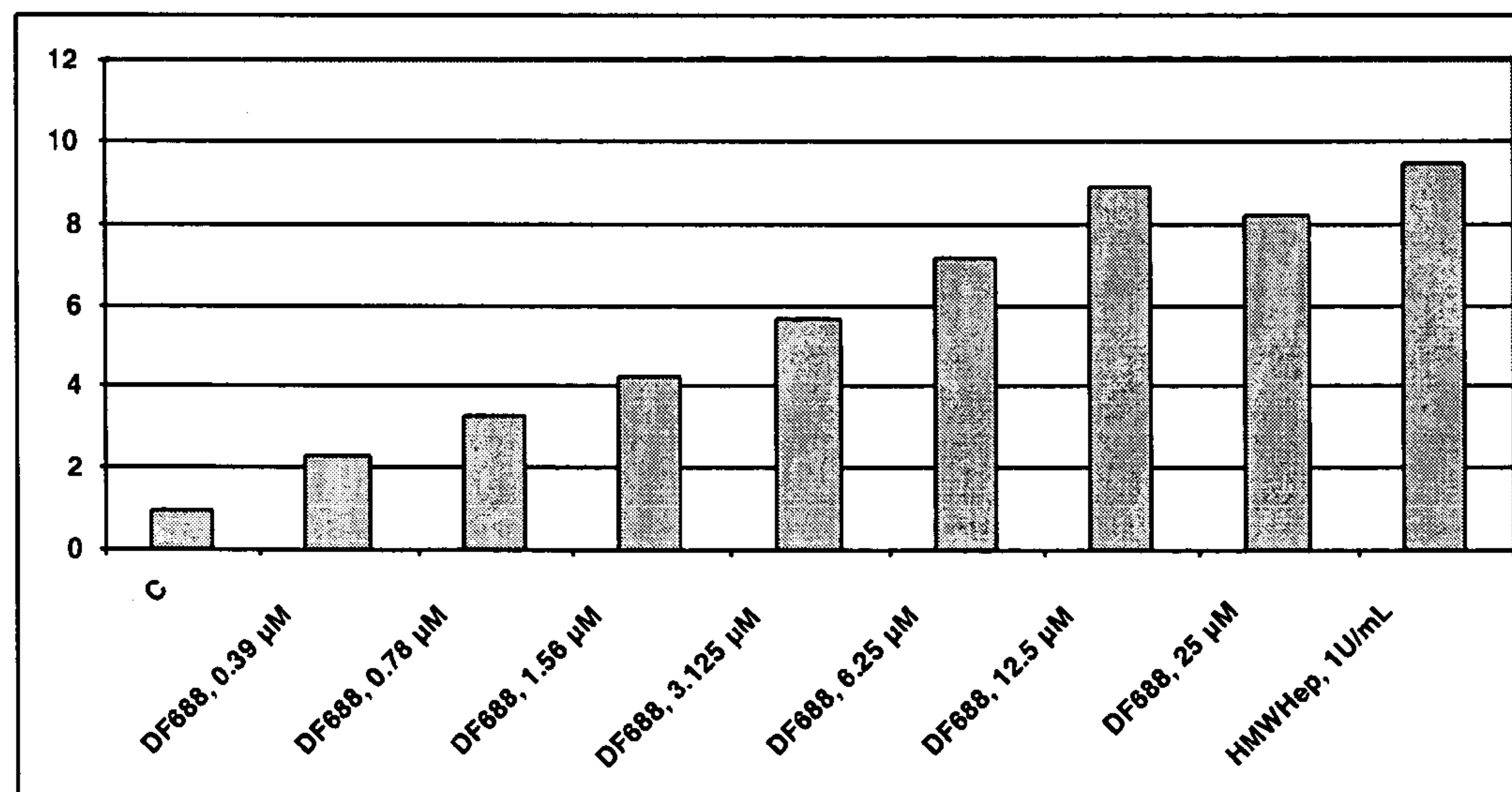


Fig. 11

13/14

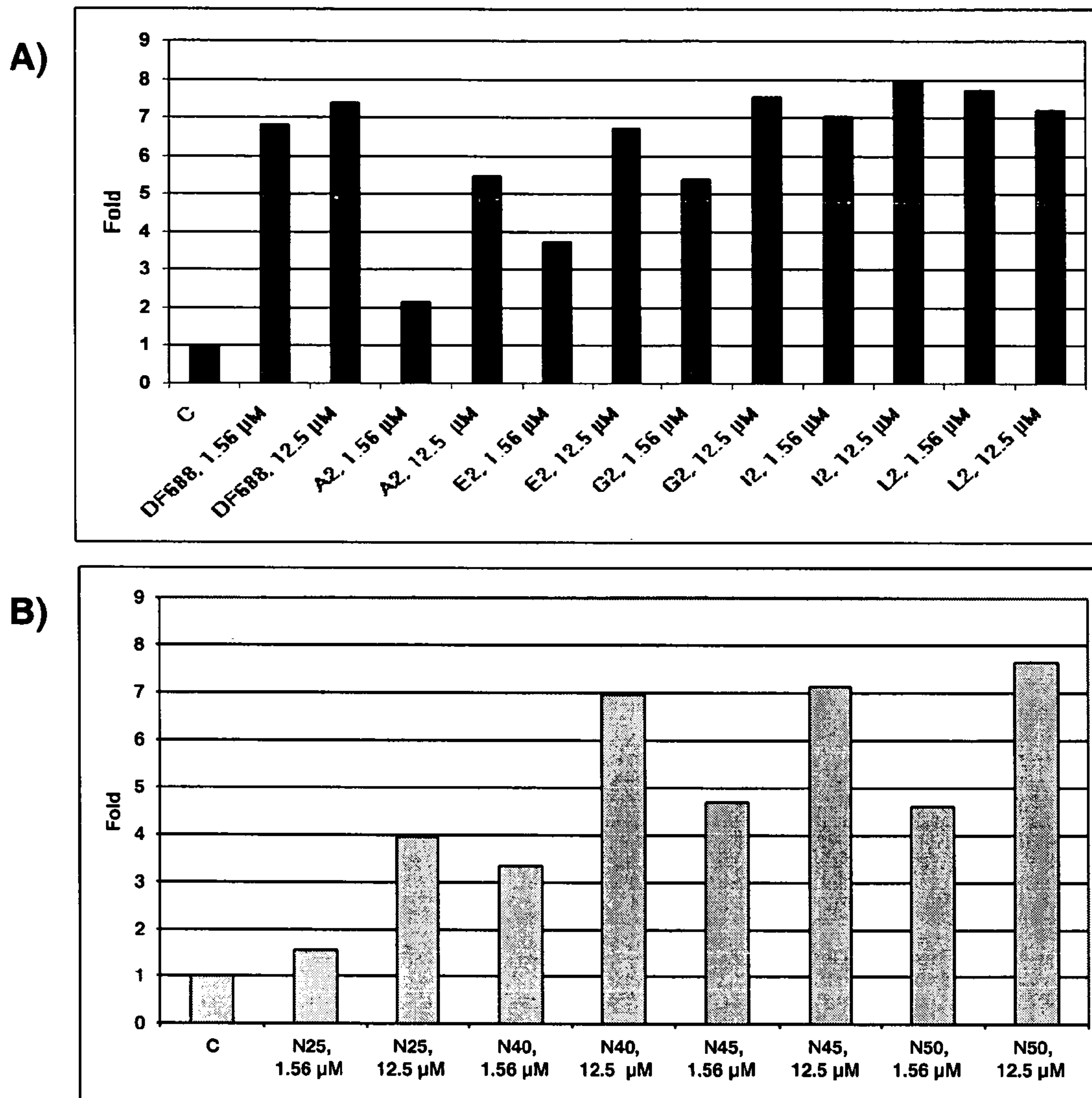


Fig. 12



14/14

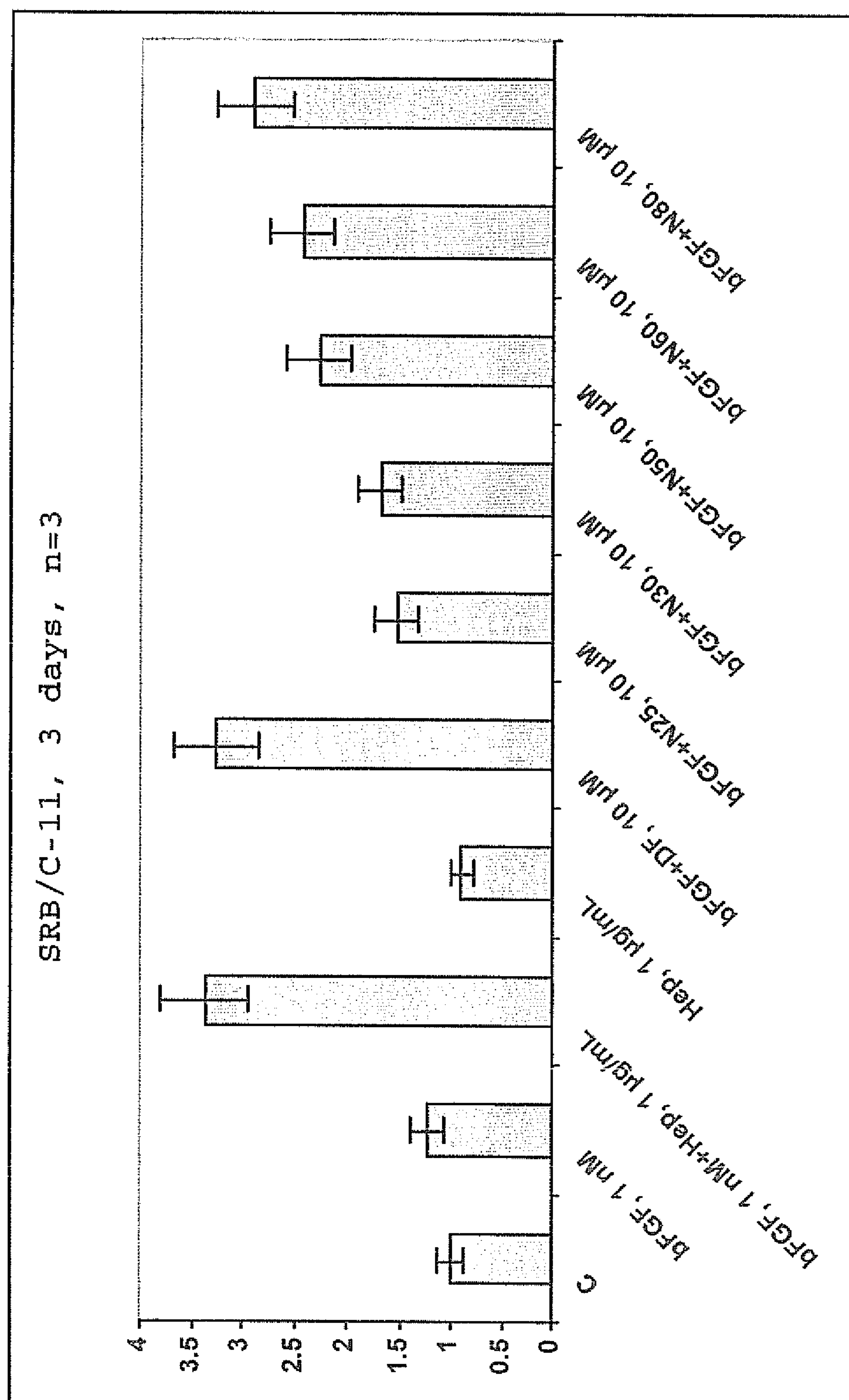


Fig. 13