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(54) **Title**: METHOD FOR MEASUREMENT OF CALCIUM IONS

(57) **Abstract**: The present invention relates to a reagent for determination of calcium and to a determination method using that reagent. More particularly, it relates to a reagent for determination of calcium comprising a mono-nitro substituted BAPTA-type chelator (BAPTA =1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid). A determination method which permits accurate determination of calcium in a sample, such as a blood sample (e.g. whole blood, plasma or serum) or any other aqueous liquid sample (e.g. cerebrospinal fluid, lymph, salivary juice or urine) and thus is especially useful for clinical diagnoses as described.

Method for measurement of calcium ions

Background of the Invention

The present invention relates to a reagent for determination of calcium and to a determination method using that reagent. More particularly, it relates to a reagent for determination of calcium comprising a mono-nitro substituted BAPTA-type chelator (BAPTA = 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid). A determination method which permits accurate determination of calcium in a sample, such as a blood sample (e.g. whole blood, plasma or serum) or any other aqueous liquid sample (e.g. cerebrospinal fluid, lymph, salivary juice or urine) and thus is especially useful for clinical diagnoses as described.

Blood or serum calcium levels, respectively, are of significant diagnostic value and may have important treatment implications.

The reference range for calcium ions is very narrow, 2.20 to 2.55 mmol/L, and slight deviations above or below these levels are diagnostic of several physiological disorders. The two most common diseases associated with hypercalcaemia (elevated serum calcium) are hyperparathyroidism and malignancy, especially when the malignancy has metastasized to the skeleton and caused bone resorption (i.e. local destruction of the bone accompanied by release of calcium from the site of the metastatic lesion). Decreased serum calcium levels (hypocalcaemia) are commonly associated with hypoparathyroidism. About 1% of newborns have significant hypocalcaemia (serum calcium <1.75 mmol/L) with symptoms like irritability, twitching and convulsions which require immediate medical intervention.

Magnesium, like calcium, is one of the major elements found in the body. Impairments in the level of magnesium also lead to clinical symptoms some of which are very alike to the ones found with impaired levels of calcium. Given the nearly identical clinical symptoms of low serum calcium and low serum magnesium, it is imperative to delineate which element is causing the clinical symptoms. Often both serum calcium and magnesium measurements are necessary to determine which element or as the case may be whether both elements are out of normal range and it is imperative that magnesium does not interfere with the quantification of calcium.

The reference method for measuring calcium and magnesium, respectively, is atomic absorption. For routine measurements, atomic absorption is somewhat inconvenient, requires expensive instrumentation and a rather skilled operator to perform the assays in order to achieve sufficient precision and reproducibility.

The catalytic activity of certain enzymes strongly depends on the presence of calcium ions, thereby making it possible to quantify calcium via a measurement of calcium-dependent enzymatic activity. Methods for measurement of calcium ions based on enzymatic procedures have been e.g. described in US 6,068,971.

Present methods frequently used in the routine of clinical laboratories for measuring calcium are based on procedures employing chelating, color-producing agents like ortho-cresolphthalein complexone (o-CPC), arsenazo III, phosphonazo or chlorophosphonazo. Although at least one of these methods is often used in the clinical routine of a laboratory, each one has drawbacks.

The sensitivity of o-CPC methods is very dependent on pH. For maximum sensitivity the reaction is carried out at a pH of about 10,7. At these alkaline pH values, however, the reagent readily absorbs ambient carbon dioxide. The absorption of carbon dioxide which combines with water to form carbonic acid gradually reduces the reagent pH and eventually renders the reagent non-functional for calcium measurements. The gradual change in pH also requires more frequent calibration runs in order to insure a correct measurement. Also, o-CPC is rather non-selective and binds magnesium and other metals, like gadolinium. To eliminate magnesium interference at the levels normally encountered in serum, 8-hydroxyquinoline is added to chelate magnesium.

Methods for calcium detection based on Arsenazo III do not suffer from the problems of high pH and magnesium interference (depending on measurement pH) inherent in the o-CPC methods. Arsenazo III binds calcium under weakly acidic conditions, e.g. pH 5 to 6, and if the calcium measurement is made at a pH less than 7, binding of magnesium is negligible. Although arsenazo III eliminates many of the disadvantages of o-CPC methods, it suffers from rather low sensitivity and environmental concerns. Each mole of arsenazo III contains 2 moles of arsenic, and disposal of the arsenazo III reagents is a critical issue in many countries due to concerns of contamination of the water supply with arsenic.

JP-A-04-120464 discloses that calcium and magnesium can be quantified at the same time by using Chlorophosphonazo-III as a chelating and color-producing agent. In the case of Chlorophosphonazo-III, the pH range most suitable for its color change is weakly acidic and Chlorophosphonazo-III is a reagent containing no arsenic. Therefore, Chlorophosphonazo-III is advantageous with respect to problems caused by a high pH and toxicity. However, the simultaneous quantification of magnesium and calcium using Chlorophosphonazo-III poses a problem. This chelant usually is added to a sample to bind both magnesium and calcium thereby causing coloration. Thereafter EGTA is added to solely release the bound calcium, this release causing a change in color. Calcium then is quantified on the basis of the change in color. Chlorophosphonazo-III tends to also give high blank values, this fact limits the concentration range wherein the determination of calcium ions is possible.

BAPTA-type calcium-chelating agents have been described and used as buffer systems to e.g. control the concentration of intracellular calcium ions. Pethig, R. et al., (Cell Calcium 10 (1989) 491-498) have determined the dissociation constants of seven different BAPTA-type calcium buffers. They propose to use dibromo-BAPTA in physiological work with calcium.

As discussed above, various assay methods for measurement of calcium ions are known in clinical routine. Several reagents for calcium determination based on the use of a chelating and color-producing agent are available, with each one leaving room for further improvement.

Thus, there are unmet needs for reagents and for methods to quantitatively measure calcium in analytical samples. The ideal method should be based on a chelator that a) is stable under storage conditions and on board of an analyzer, b) does not contain toxic elements, like e.g. arsenic, c) has a relatively low reagent blank absorbance, d) does not bind to magnesium ions or other metal ions, like gadolinium, e) allows for rapid determinations and high sample throughput, and f) leads to a precise measurement of calcium ions over a broad measuring range.

It surprisingly has been found that mono-nitro-derivatives of BAPTA-type chelators exhibit quite advantageous properties rendering them very appropriate for measurement of calcium ions.

Summary of the Invention

The present invention provides a reagent that is very useful in the measurement of calcium ions and amongst other positive aspects, has excellent storage stability, is free from the problem of environmental pollution by arsenic, does not show interference by magnesium or gadolinium and permits rapid and accurate calcium determination over a broad range of concentrations.

The present invention relates to a method for determining the concentration of calcium ions in a sample, the method comprising the steps of mixing the sample with a solution comprising a mono-nitro-BAPTA-type chelator thereby binding calcium ions to the mono-nitro-BAPTA-type chelator, releasing calcium ions from the mono-nitro-BAPTA-type chelator, wherein said release causes a change in absorbance of the mono-nitro-BAPTA-type chelator, measuring the change in absorbance and using the change in absorbance measured for determining the concentration of calcium ions.

Also disclosed is a stable reagent composition for measurement of calcium containing a mono-nitro-BAPTA-type chelator and having a pH ranging from pH 8.5 to pH 11.5.

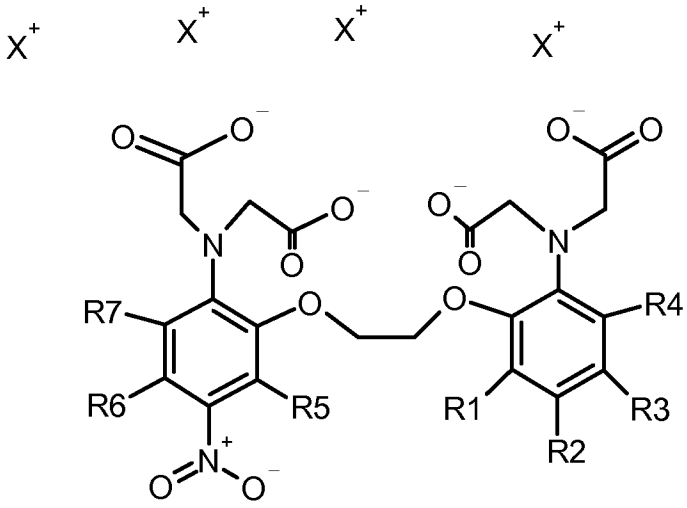
Further the present invention is directed to a kit comprising a reagent composition for measurement of calcium having a pH ranging from pH 8.5 to pH 11.5 and containing a mono-nitro-BAPTA-type chelator.

Detailed Description of the Invention

In a preferred embodiment the present invention relates to a method for determining the concentration of calcium ions in a sample, the method comprising the steps of mixing the sample with a solution comprising a mono-nitro-BAPTA-type chelator thereby binding calcium ions to the mono-nitro-BAPTA-type chelator, releasing calcium ions from the mono-nitro-BAPTA-type chelator, wherein said release causes a change in absorbance of the mono-nitro-BAPTA-type chelator, measuring the change in absorbance and using the change in absorbance measured for determining the concentration of calcium ions.

A method for determining the concentration of calcium ions in a sample is disclosed, the method comprising the steps of a) mixing the sample with a solution comprising a compound of Formula I

Formula I



wherein R1 is selected from hydrogen, halogen, carboxy, alkyl and formyl, R2 is independently selected from hydrogen, halogen, alkyl, alkoxy, morpholino, CN, carboxy and formyl, R3 is independently selected from hydrogen, halogen, N-alkyl sulfate, carboxy, alkoxy, phenyl, CN, CF3, and tertiary butyl, R4 is independently selected from hydrogen, halogen or alkyl, R5 and R7 independently are hydrogen or alkyl R6 is selected from hydrogen, alkyl, alkoxy and halogen, or wherein R3 and R4 form an aromatic bridge and X+, is a positively charged counter ion, thereby binding calcium ions to the compound, b) releasing calcium ions from the compound, wherein said release causes a change in absorbance of the compound, c) measuring the change in absorbance, and d) using the change in absorbance measured in (c) for determining the concentration of calcium ions.

The halogen mentioned as candidate substituent R1, R2, R3, R4 and/or R6 preferably is selected from Cl- Br- and F-.

In one preferred embodiment the substituent R1 and/or R2, and/or R3 is carboxy.

Alkyl as mentioned for R1, R2, R4, R5, R6, and/or R7 preferably is C1 to C3-alkyl.

Alkoxy as mentioned for R2, R3, and/or R6 preferably is methoxy or ethoxy.

The aromatic bridge between R3 and R4 preferably is part of a benzene ring system

The counter ion X+ preferably is selected from the group consisting of Na+, K+, Li+ and Cs+. Also preferred X+ is K+ or Na+.

As used herein, each of the following terms has the meaning associated with it in this section.

The articles "a" and "an" are used herein to refer to one or to more than one (i.e. to at least one) of the grammatical object of the article. By way of example, "a marker" means one marker or more than one marker. The term “at least” is used to indicate that optionally one or more further objects may be present.

The expression “one or more” denotes 1 to 50, preferably 1 to 20 also preferred 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, or 15.

A compound according to Formula I is capable of binding calcium ions. Upon binding or upon release of calcium a change in its spectral characteristics takes place. This change in spectral characteristics can be easily measured and is directly correlated to the concentration of calcium ions present in a sample.

It has been found that it is advantageous to first bind all calcium ions present in a sample to be analyzed to the compound of Formula I. Thereby a stable base line is obtained. Such stable baseline is especially valuable for measuring low concentrations of calcium ions. Upon release of the calcium ions from the compound according to Formula I a measureable change in its spectral characteristics is induced that can be used to exactly determine the concentration of calcium ions present in a sample.

The compound of Formula I can efficiently bind calcium ions over a broad pH range. Efficient calcium binding can be observed from about pH 5.0 to about pH 11.0. In one embodiment the method according to the present invention is practiced under conditions wherein the pH in the assay mixture comprising the compound according to Formula I and the sample is between pH 5.0 and pH 11.0. The reaction mixture contains at least an aliquot of a sample and a compound according to Formula I. In further preferred embodiments the pH in the reaction mixture will be from pH 5.0 or above, from pH 5.5 or above, from pH 6.0 or above from pH 6.5 or to pH 11 or below, also pH 10 or below pH 9.0 or below pH 8.0 or below.

The final concentration for a compound according to Formula I is adjusted to be high enough for reliable measurement of calcium ions in a sample. Due to the high sensitivity achieved by measuring calcium ions using a compound of Formula I, a clinical sample like serum or plasma may be diluted e.g. about 100-fold and still be reliably measured. As obvious to the skilled artisan the final concentration of a

compound according to Formula I in an assay mixture has to match the final concentration of calcium ions in such assay mixture. In a preferred embodiment the method disclosed will be practiced using a compound according to Formula I in a final concentration that is at least 1.5 fold the expected upper limit for the final concentration of calcium ions.

As mentioned, a sample having 5 mmol/L calcium ions should be reliably measured, this is the expected upper limit. In case such sample is diluted 1:100 the final concentration of calcium ions in the assay mixture will be 0.05 mmol/L. The final concentration of the compound according to Formula I should be at least 1.5-fold this concentration, i.e. 0.075 mmol/L. Also preferred the final concentration of a compound according to Formula I in an assay mixture will be at least 2-fold, 2.5-fold, 3-fold and at most 20-fold, 15-fold or 10-fold the calcium ion concentration as calculated for a sample having the expected upper limit of 5 mmol/L. Preferably the final concentration of a compound according to Formula I in an assay mixture will be at least 1.5-fold, 2-fold, 2.5-fold, 3-fold and at most 20-fold, 15-fold or 10-fold the molar concentration obtained by multiplying 5 mmol/L with the dilution factor for the sample.

It has been found that long-term stability in solution of a compound according to Formula I is best preserved at a pH of 8.5 or above. It is preferred to use a reagent comprising a compound according to Formula I that does not need to be freshly made or checked frequently for its functionality, therefore in one embodiment the method according to claim the present invention is practiced with a solution comprising the compound of Formula I having a pH in the range from pH 8.5 to pH 11.5.

In alternative preferred embodiments the method according to the present invention is performed with a solution comprising the compound of Formula I having a pH in the range from pH 8.5 to pH 11.0 or from pH 9.0 to pH 10.5.

In a preferred embodiment, the method according to the present invention is practiced with a compound according to Formula I, wherein R1 is either hydrogen or halogen.

In a preferred embodiment, the method according to the present invention is practiced with a compound according to Formula I, wherein R2 of is hydrogen, halogen, carboxy, morpholino or alkyl.

In a preferred embodiment, the method according to the present invention is practiced with a compound according to Formula I, wherein R3 is hydrogen, halogen, carboxy or alkoxy.

In one embodiment a compound according to Formula I for use in a method as disclosed in the present invention preferably should bind calcium ions with a binding constant of log k equal to 9.0 or lower. On the other hand the binding constant to calcium ions described as log k should be at least 4.0 or higher. Preferably the binding constant for calcium ions given in log k should be between 4.5 and 8.5, also preferred the log k will be between 5.0 and 8.0.

The log k is measured according to the procedure described in Harrison, S.M. and Bers, D.M., Biochimica et Biophysica Acta 925 (1987) 133 - 143. In brief, the calcium binding compound is buffered to pH 7.0 in 25mM Hepes-buffer. Temperature is kept constant at 20° C. Various concentrations of calcium ions are incubated with a constant amount of calcium binding compound. The fractions of free and bound calcium ions are determined and the affinity constant calculated by aid of a Scatchard plot.

As the skilled artisan will appreciate various combinations of substituents are possible. The substituents can be chosen and used to influence or modulate the binding constant of a compound according to Formula I. Electron-withdrawing groups will lead to a reduced binding constant whereas electron-donating groups in general will result in a stronger binding of calcium ions.

Preferably the substituents to the compound of Formula I are selected to result in a binding constant, given as log k, of 7.0 or less, also preferred of a log k between 4.0 and 7.0, of between 4.5 and 6.5 or of between 5.0 to 6.0.

It may well be possible to combine two ore more compounds according to Formula I in order to perform the method according to the present invention. In a preferred embodiment a single compound according to Formula I is used.

The method according to the present invention is based on back-titration, i.e. calcium ions are first bound to the compound of Formula I and released thereafter. The release of calcium ions from the compound according to Formula I is most easily achieved by use of a chelator that binds calcium ions stronger than the compound according to Formula I. In a preferred embodiment the chelator used for release of the calcium ions in step b) of the method disclosed herein above has a

binding constant that is 10-fold higher as compared to the compound of Formula I used in the method of calcium detection.

While it is e.g. possible to combine a calcium binding compound according to Formula I with a log k of 5.0 with a chelator having a log k of 6.0, it is preferred to use chelator having at least a log k of 7.0 or higher. As obvious to the skilled artisan the reagent used to release calcium ions from a compound according to Formula I is best chosen to have spectral characteristics that do not interfere with those of interest, e.g. with the absorption or emission spectrum of a compound according to Formula I.

Preferably the reagent/chelator used for release of calcium from the compounds according to Formula I in a method as disclosed herein is selected from di-, tri-, tetra-acetic acid derivatives, poly-phosphonic acids or phosphoric acid derivatives, 4,4'-Difluoro-BAPTA, 5,5'-Dibromo-BAPTA, 5,5'- Difluoro-BAPTA, 5-Methyl-5'-formyl-BAPTA, 5,5'-dimethyl-BAPTA, (1,2-Cyclohexylenedinitrilo)tetraacetic acid (CETA), citric acid, nitrilotriacetic acid (NTA), iminodiacetic acid (IDA), N-(2-hydroxyethyl)iminodiacetic acid (HIDA), N-(2-Hydroxyethyl)ethylenediamine-N,N',N'-triacetic acid (= HEDTA; CAS 150-39-0, log k = 8.14), CyDTA (= CAS 125572-95-4, log k = 12.50), TTHA (= CAS 869-52-3, log k = 10.06), Me-EDTA (= 1,2-Propanediamine-N,N,N',N'-tetraacetic acid, log k = 10.4), BAPTA (= CAS 73630-08-7, log K 6.97), DTPA (= Diethylentriaminopentaacetic acid; CAS-Nr.: 67-43-6, log k =10.8), EGTA (= Ethylene glycol-bis(2-aminoethylether)-N,N,N,N-tetraacetic acid; CAS-Nr.: 67-42-5, log k=10.9), DTPMP, (= Diethylentriaminpenta(methylenephosphonic acid); CAS-Nr.: 15827-60-8, log k =10.7) and EDPMP (= Ethylendiamintetra(methylenephosphonic acid); CAS-Nr.: 1429-50-1, log k =10.2).

Also preferred the method according to the present invention is practiced such that the release of calcium ions is triggered by EDTA, DTPA, EGTA, DTPMP and/or EDPMP.

The choice of the chelator used to release calcium ions from a compound according to Formula I is not critical, as long as the calcium ions bound to the compound of Formula I are released after addition of such chelator. The concentration of the chelator in the final reaction mixture preferably will be at least equimolar but not higher than 100-fold the concentration of the compound according to Formula I in this mixture. In order to be fully on the safe side, e.g. by compensating for minor

errors in pipetting, a surplus of chelator may be used. In one embodiment, while performing the method as disclosed herein, the concentration of the chelator in the final reaction mixture is higher than the final concentration for the compound of Formula I, e.g. between 1.5-fold and 50-fold or also preferred, between 2-fold and 10-fold.

A reagent used in routine clinical chemistry for measurement of calcium ions should be stable under transport and long term storage conditions. It has been found that a compound according to Formula I is not as stable at acidic or neutral pH as it is under alkaline buffer conditions. Reagent compositions comprising a compound according to Formula I should have at least a pH of 8.5 or the pH should be higher. In a preferred embodiment the present invention discloses a reagent for measurement of calcium ions having a pH ranging from pH 8.5 to pH 11.5, and containing a compound of Formula I as defined in claim 1.

In alternative preferred embodiments the reagent according to the present invention has a pH in the range from pH 8.5 to pH 11.0 or from pH 9.0 to pH 10.5.

Buffer systems that are appropriate to buffer a solution at a pH of 8.5 and/or higher are well-known to the skilled artisan. Preferably such buffer system will be selected from AMPD (= 2-Amino-2-Methyl-1.3-propanediol), CHES (= 2-(N-Cyclohexylamino)-ethanesulfonic acid), AMPSO (= 3-[Dimethyl(hydroxylmethyl)-methylamino]-2-hydroxypropanesulfonic acid), CAPSO (= 3-Cyclohexylamino)-2-hydroxy-1-propanesulfonic acid), CAPS (= 3-Cyclohexylamino)-2-propanesulfonic acid), a glycine buffer system, or a carbonate buffer system. Also preferred the reagent for measurement of calcium ions according to the present invention will comprise a buffer system, selected from CAPS or CAPSO.

As mentioned further above, the calcium concentration in the circulation is tightly regulated and physiological concentrations usually are between 2.20 to 2.55 mmol/L. Elevated levels of calcium ions in the circulation very rarely go above 4 mmol/L. For this reason reagents adapted to measure calcium ions in the circulation usually are manufactured to cover a measurement range of up to 5 mmol/L. The reagent for measurement of calcium should match the physiologically relevant concentrations. In urine, however, calcium concentrations may vary to a large extend. This requires that an assay for measurement of calcium also should cover a large measurement range.

In a preferred embodiment the reagent for measurement of calcium comprises a compound of Formula I in a concentration ranging from 0.10 mmol/L to 50 mmol/L.

As the skilled artisan will appreciate the final concentration of a compound of Formula I in the assay mixture, comprising the sample to be measured, must match the concentration of calcium ions in the sample. In one embodiment the concentration of the compound according to Formula I will be in the range of 0.10 mmol/L to 2 mmol/L. In alternative embodiments the concentration of the compound according to Formula I will be in the range from 0.1, 0.125, 0.15, or 0.2 to 2.0, 1.5 or 1 mmol/L. This reagent can be admixed with the sample to be measured used without further dilution.

In another embodiment a more concentrated reagent for measurement of calcium, based on the compound of Formula I, is provided. Such more concentrated reagent can be appropriately diluted for measurement of a sample. The concentrated form of a reagent according to the present invention will comprise a compound according to Formula I in a range from 0.5 to 50 mmol/L. In alternative embodiments the concentration of the compound according to Formula I in a more concentrated reagent for detection of calcium ions will be in the range from 0.5, 0.6, 0.7, 0.8, 0.9 or 1 mmol/L to 50, 40, 30, 20, 15, or 10 mmol/L.

In a further preferred embodiment, the reagent for measurement of calcium as disclosed in the present invention comprises a compound according to Formula I, wherein R1 is either hydrogen or halogen.

In a further preferred embodiment, the reagent for measurement of calcium as disclosed in the present invention comprises a compound according to Formula I, wherein R2 is hydrogen, halogen, carboxy, morpholino or alkyl.

In a further preferred embodiment, the reagent for measurement of calcium as disclosed in the present invention comprises a compound according to Formula I, wherein R3 of Formula I is hydrogen, halogen, carboxy or alkoxy.

It has been found to be advantageous to also add a detergent to a reagent for measurement of calcium ions, this may be due to reduction of interfering unspecific binding, to the reduction of foam and air bubbles or other positive influences. In a further preferred embodiment the present invention relates to a reagent for

measurement of calcium ions having a pH ranging from pH 8.5 to pH 11.5, containing a compound of Formula I as defined in claim 1 and a detergent.

As used herein, the term "detergent" means an ionic or a non-ionic detergent. Examples of detergents include, but are not limited to: sodium dodecyl sulphate (SDS), fatty acid salts, the Triton® family, octyl glycoside, 3-[(3-cholamidopropyl)dimethyl-ammonio]-1-propanesulfonate (CHAPS), sodium dodecyl maltoside (DM), lauryldiethylamine oxide (LDAO), NP-40 and the Tween® family, primary amines, amine acetates and hydrochlorides, quaternary ammonium salts, trimethylethyl ammonium bromide, amides of substituted diamines, diethanolaminopropylamine or diethylaminopropylamide, amides of cyclised diethylenetriamine, alkylaryl sulfonates, petroleum sulfonates, sulfonated glycerides, cholamides, sulfobetaines, alkyl glycosides, saponins, alkyl-polyethylene glycol ethers.

In one embodiment the detergent is a non-ionic detergent. Non-limiting examples of non-ionic detergents are Imbentin V413/91, Thesit, Triton® X-100, Triton® X-114, Brij® 35, Brij® 58, Tween® 20, Tween® 80, Nonidet® P-40, Octyl ß Glucoside and MEGA 8-Octanoyl-N-methylglucamide. In one embodiment the non-ionic detergent is selected from Brij® 35, Triton® X-100, Tween® 20, and Nonidet® P-40.

As mentioned further above, the concentration of calcium ions is an important parameter in clinical routine diagnostics. Reagents for measurement of calcium preferable are assembled in the form of a kit comprising at least one reagent that contains a calcium indicator, like the compounds currently used or a compound according to Formula I as described in this invention. In a further preferred embodiment, the present invention relates to a test kit for the measurement of calcium, the test kit containing a reagent comprising a compound according to Formula I and having a pH ranging from pH 8.5 to pH 11.5.

In many instances it will be advantageous to include at least two reagents into a kit tailored to measure calcium ions, a first reagent comprising a compound according to Formula I and having a pH ranging from pH 8.5 to pH 11.5.0 and a second reagent comprising a chelator. In a further preferred embodiment the present invention relates to a kit containing a first reagent comprising a compound according to Formula I and having a pH ranging from pH 8.5 to pH 11.5.0 and a

second reagent comprising a chelator for calcium ions. The kit may optionally also comprise a package insert and/or one or more additional reagents.

The following examples and Figures are provided to aid the understanding of the present invention, the true scope of which is set forth in the appended claims. It is understood that modifications can be made in the procedures set forth without departing from the spirit of the invention.

Description of the Figures

Figure 1 The synthesis of NM-BAPTA is schematically depicted in Figure 1.

Figure 2 Measurement of calcium ions according to the procedure given in Example 2, last column, on the Modular P analyzer of Roche Diagnostics, Germany. In the upper part (A) the theoretical value and the value actually measured are plotted against each other. In the lower part (B) the % recovery is given, i.e. the value actually measured is given as percentage of the expected (theoretical) value.

Figure 3 Measurement of calcium ions according to the procedure given in Example 2, middle column, on the cobas c501 analyzer of Roche Diagnostics, Germany. In the upper part (A) the theoretical value and the value actually measured are plotted against each other. In the lower part (B) the % recovery is given, i.e. the value actually measured is given as percentage of the expected (theoretical) value.

Figure 4 Measurement of calcium ions according to the procedure given in Example 2, last column, on the Modular P analyzer of Roche Diagnostics, Germany. The concentration of NM-BAPTA has been reduced to 90 % of the standard concentration. In the upper part (A) the theoretical value and the value actually measured are plotted against each other. In the lower part (B) the % recovery is given, i.e. the value actually measured is given as percentage of the expected (theoretical) value.

Figure 5 Measurement of calcium ions according to the procedure given in Example 2, last column, on the Modular P analyzer of Roche Diagnostics, Germany. The concentration of NM-BAPTA has been reduced to 80 % of the standard concentration. In the upper

part (A) the theoretical value and the value actually measured are plotted against each other. In the lower part (B) the % recovery is given, i.e. the value actually measured is given as percentage of the expected (theoretical) value.

Figure 6 The synthesis of NF-BAPTA is schematically depicted in Figure 6.

Figure 7 The absorbance spectra for NM-BAPTA and NF-BAPTA, respectively, both in the presence and absence of calcium ions, respectively, are given. The key to the four spectra is given underneath the Figure.

Example 1

Synthesis of NM-BAPTA

The synthesis of NM-BAPTA is schematically depicted in Figure 1.

a) 1-(2-Chloro-ethoxy)-2-nitro-benzene

2-Nitro-phenol (168,7 g) and toluene-4-sulfonic acid 2-chloroethyl ester (100 g) were dissolved in 500 ml DMF and were stirred for 1 h at 110-120°C after careful addition of 199 g potassium carbonate. The reaction mixture was poured in a mixture of crushed ice and water (8 l) which was vigorously stirred. The residue was filtered off, washed several times with water and dried.

Yield: 100-120 g

b) 4-Methyl-1-nitro-2-(2-(2-nitro-phenoxy)-ethoxy)-benzene

1-(2-Chloro-ethoxy)-2-nitro-benzene (116 g) and 5-methyl-2-nitro-phenol (88 g) were dissolved in 500 ml DMF and was stirred for 4 h at 90-110°C after careful addition of 160 g potassium carbonate. The reaction mixture was poured in a mixture of crushed ice and water (8 l) which was vigorously stirred. The residue was filtered off, washed several times with water and dried. The crude product was suspended in methanol and the pale yellow residue was again filtered off, washed with methanol and dried.

Yield: 150-165 g.

c) 2-(2-(2-Amino-phenoxy)-ethoxy)-4-methyl-phenylamine

100 g 4-methyl-1-nitro-2-(2-(2-nitro-phenoxy)-ethoxy)-benzene and 10 g palladium on charcoal were suspended in 3,5 l dioxane and hydrogenated at room temperature under a hydrogen pressure of 5,5 bar. After flashing three times with

nitrogen the catalyst was filtered off under a nitrogen atmosphere and the remaining solution was evaporated and the product was dried under vacuum.
Yield: 80 g

d) ((2-(2-(2-(Bis-methoxycarbonylmethyl-amino)-phenoxy)-ethoxy)-4-methyl-phenyl)-methoxycabonylmethyl-amino)-acetic acid methyl ester

2-(2-(2-Amino-phenoxy)-ethoxy)-4-methyl-phenylamine (80 g) were dissolved in 2,5 l DMF and 285 ml bromo-acetic acid methyl ester, 429 g potassium carbonate, and 36,8 g sodium iodide were added. The reaction mixture was heated up to 80°C for 2 h. After evaporation the remaining bromo-acetic acid methyl ester was removed from the product with hexane. The crude product was further purified by crystallization in methanol.
Yield: 93 g

e) ((2-(2-(2-(Bis-methoxycarbonylmethyl-amino)-5-nitro-phenoxy)-ethoxy)-4-methyl-phenyl)-methoxycabonylmethyl-amino)-acetic acid methyl ester

50 g ((2-(2-(2-(Bis-methoxycarbonylmethyl-amino)-phenoxy)-ethoxy)-4-methyl-phenyl)-methoxycabonylmethyl-amino)-acetic acid methyl ester were dissolved in 600 ml glacial acetic acid. Under vigorous stirring 91,5 ml 1 molar nitric acid in glacial acetic acid was added and in a second step 28 ml of concentrated sulfuric acid was added. The temperature increased up to 30°C. The reaction mixture was directly poured in a 10 l ice/water mixture. The residue was filtered off, washed several times with water and dried under vacuum. The crude product was further purified by column chromatography on silica gel first with hexane /acetic acid ethyl ester (1:1) as eluent and a second time with toluene/acetonitrile (1:1) as eluent. The product was finally crystallized from propan-2-ol.
Yield: 20 g

f) NM-BAPTA; Potassium salt of ((2-(2-(2-(bis-carboxymethyl-amino)-5-nitro-phenoxyl)-ethoxy)-4-methyl-phenyl)-carboxymethyl-amino)-acetic acid

9,5 g ((2-(2-(2-(Bis-methoxycarbonylmethyl-amino)-5-nitro-phenoxy)-ethoxy)-4-methyl-phenyl)-methoxycabonylmethyl-amino)-acetic acid methyl ester were dissolved in a mixture of water/methanol (230 ml each) and 160 ml 1 molar potassium hydroxide solution was added. The reaction mixture was refluxed for 1 h. After cooling down to room temperature and adding 250 ml water the solution's pH was adjusted to pH 3 and the methanol was evaporated. The product was isolated by solvent extraction with acidic acid ethyl ester. After evaporation the product was dried under vacuum.

The ((2-(2-(2-(bis-carboxymethyl-amino)-5-nitro-phenoxyl)-ethoxy)-4-methyl-phenyl)-carboxymethyl-amino)-acetic acid was dissolved in methanol and the equimolar potassium hydroxide in methanol was added. After evaporation and drying the appropriate potassium salt could be isolated.

Yield: 9 g

Example 2

General procedure for measurement of calcium ions with NM-BAPTA

The measurement of calcium ions with a mono-nitro-BAPTA-type compound according to Formula I is performed in a back-titration method.

An aliquot of the sample of interest is mixed with a solution comprising the mono-nitro-BAPTA-type compound and incubated. On the automatic analyzers of Roche Diagnostics, Germany, this reagent is called R1. The incubation is performed till a stable base-line signal is obtained. Usually a stable base-line signal is obtained in less than 10 min, mostly within 2 to 5 min.

The mixture of sample and R1 (optionally diluted, e.g. with distilled water) is then analyzed, i.e. the absorbance values at the most appropriate wave-length(s) is(are) or a spectrum is measured.

The calcium ions bound to a compound according to Formula I are then released by addition of a releasing agent, e.g. EDTA. This second reagent is called R2 on the automatic analyzers of Roche Diagnostics, Germany. Where required, the mixture can be further diluted with distilled water.

The final mixture of sample, R1 and R2 (optionally diluted, e.g. with distilled water) is then analyzed, i.e. the absorbance values at the most appropriate wave-length(s) is(are) or a spectrum is measured.

The change in absorbance is directly correlated to the concentration of calcium ions in the sample of interest and the concentration of calcium ions is calculated according to standard procedures.

In Table I an overview is given over the applications recommended for measurement of calcium ions on five different automated analyzers of Roche Diagnostics, Germany. The recommended applications for the Modular P and the Modular D analyzer are identical.

Table 1:

Recommended pipetting volumes (in µl) for measurement of calcium ions

	HiCo R1 Integra	HiCo R1 cobas	LoCo R1 Roche/Hitachi 902	LoCo R1 Modular D / Modular P
R1 (reagent 1)	20	20	250	180
D (distilled water)	100	130	---	---
S (sample)	3	3	4	3
D (distilled water)	30	---	---	---
R2 (reagent 2)	20	20	28	20
D (distilled water)	50	50	---	---
total volume of final mixture	223	223	282	203

HiCo R1 = high concentration R1 reagent;

LoCo R1 = low concentration R1 reagent;

Integra, cobas, Roche/Hitachi 902, Modular D, and Modular P are analyzer systems distributed by Roche Diagnostics, Germany.

Table 2:

Compositions of HiCo R1, LoCo R1 and R2, respectively

	buffer	pH	Brij-35	NaCl	NM-BAPTA	NaN_3
HiCo-R1	557 mmmol/L CAPSO	10.0	0.123 %	---	1.68 mmol/L	0.09 %
LoCo-R1	57 mmol/L CAPSO	10.0	0.012 %	0.9 %	0.20 mmol/L	0.09 %
R2	7.5 mmol/L K_3-EDTA	7.3	0.012 %	0.9 %	---	0.09 %

Example 3

Linearity of calcium measurement using NM-BAPTA

The method for measurement of calcium ions disclosed in the present invention is technically excellent, e.g. it shows a very high precision. This becomes e.g. evident if theoretically expected and actually measured values are compared.

Different concentrations of calcium ions (→ theoretical values) are compared to values actual measured in the new method.

Only two representative examples, with values measured on two different analyzers, Modular P and cobas c501 (both distributed by Roche Diagnostics), respectively, are given as Figures 2 and 3. These two figures demonstrate the outstanding technical quality/precision of the measurements. As obvious from Figures 2 and 3, all values actually measured are within 95 to 105% of the corresponding expected theoretical value, which translates to a rather exact measurement of calcium ions over the whole range of concentrations investigated.

Example 4

Determining the minimum concentration of NM-BAPTA

The concentration of calcium ions in the circulation only rarely exceeds 4 mmol/L. A reagent capable of measuring up to 5 mmol/L calcium in a reliable manner should be most appropriate to determine calcium ions in the circulation.

Various concentrations of calcium ions (expected values in Figures 4 and 5) have been measured on the Modular P instrument with the application described in the last column of Table 1. However, only 90 % or 80 %, respectively, of the regular concentration of NM-BAPTA have been used. As can be seen from Figures 4 and 5, respectively, even if only 80 % of NM-BAPTA are present in the detection mixture as compared to the standard procedure given in Example 2, calcium ions up to 5 mmol/L are still recovered at between 95 to 105 %. As obvious from Figure 5, values above 5 mM tend to be recovered with too low values if the concentration of NM-BAPTA is reduced to 80 %. This means that for measurement of the high concentrations of calcium as present in some pathological samples (in case such sample is diluted about 1:70 as in the above example) the final concentration of NM-BAPTA should be about 0.2 mmol/L in order to secure a correct measurement of calcium ions in those samples.

Example 5

pH-dependent stability of NM-BAPTA

A reagent for measurement of calcium should be as stable as possible both under transport conditions as well as on board of an analyzer.

In order to investigate its stability NM-BAPTA has been stored at different pH values. A short term stress model has been applied and the reagent containing NM-BAPTA stored at 35°C. The "stressed" reagent has been used in the measurement of calcium ions under otherwise identical conditions using aliquots of the same samples. The values measured after stressing the reagent have been compared to the values measured with a non-stressed reagent at day zero, i.e. the day when the temperature stress on the reagent was initiated.

The buffer systems used were 100mmol/L HEPES at pH 7.4, 50 mmol/L Tris at pH 8.0, 50 mmol/L $NaHCO_3$ at pH 10.0, 50 mmol/L Glycin at pH 9.8, and 40 mmol/L CAPSO at pH 10, respectively. The corresponding data are summarized in Tables 3 to 7.

Table 3:

Stability of NM-BAPTA in 100 mmol/L HEPES pH 7.4 at 35 °C

		day 0	day 0 Median	week1	week1 Median	Recovery from day 0
0.9 %NaCl	calibrator	0,11		0,07		
		0,10	0,10	0,17	0,15	
target	0,0	0,08		0,15		
Calibrator	calibrator	2,55		1,91		
		2,51	2,54	1,94	1,94	76,4%
target	2,5	2,54		1,95		
PNU	control	2,20		1,82		
lot	176136	2,07	2,08	1,64	1,69	81,3%
target	2,05	2,08		1,69		
PPU	control	3,08		2,29		
lot	174531	3,09	3,09	2,27	2,29	74,1%
target	3,32	3,12		2,29		
Human serum 1	sample	2,23		1,74		
		2,24	2,24	1,76	1,76	78,6%
		2,25		1,76		
Human serum 2	sample	2,14		1,66		
		2,14	2,14	1,60	1,64	76,6%
		2,12		1,64		
Human plasma 1	sample	1,82		1,47		
		1,82	1,82	1,42	1,42	78,0%
		1,82		1,40		
Human plasma 2	sample	1,79		1,42		
		1,80	1,80	1,40	1,40	77,8%
		1,84		1,40		

As obvious from Table 3, the reagent containing NM-BAPTA is not stable at pH 7.4. The recovery of calcium ions is only in range of 80% or below.

Table 4:

Stability of NM-BAPTA in 50 mmol/L TRIS pH 8.0 at 35 °C

As obvious from Table 4, the reagent containing NM-BAPTA has has a borderline, but still acceptable stability at pH 8.0. The recovery of calcium ions is mostly in the range of 80 % to 90 %.

		day 0	day 0 Median	week1	week1 Median	Recovery from day 0
0.9 %NaCl	calibrator	0,02		0,11		
		0,00	0,00	0,15	0,15	
target	0,0	0,00		0,16		
Calibrator	calibrator	2,49		2,10		
		2,48	2,49	2,13	2,12	85,1%
target	2,5	2,49		2,12		
PNU	control	2,04		1,80		
lot	176136	2,02	2,02	1,77	1,80	89,1%
target	2,05	2,01		1,80		
PPU	control	3,12		2,47		
lot	174531	3,09	3,09	2,46	2,47	79,9%
target	3,32	3,07		2,47		
Human serum 1	sample	2,21		1,97		
		2,20	2,21	1,93	1,97	89,1%
		2,22		1,97		
Human serum 2	sample	2,07		1,79		
		2,08	2,08	1,81	1,81	87,0%
		2,08		1,84		
Human plasma 1	sample	1,80		1,55		
		1,80	1,80	1,55	1,55	86,1%
		1,77		1,55		
Human plasma 2	sample	1,77		1,54		
		1,77	1,77	1,54	1,54	87,0%
		1,78		1,54		

Table 5:

Stability of NM-BAPTA in 50 mmol/L NaHCO3 pH 10.0 at 35 °C

		day 0	day 0 Median	week1	week1 Median	Recovery from day 0
0.9 % NaCl	calibrator	0,04		0,13		
		0,01	0,01	0,13	0,13	
target	0,0	0,01		0,11		
Calibrator	calibrator	2,48		2,53		
		2,48	2,48	2,57	2,57	103,6%
target	2,5	2,46		2,59		
PNU	control	2,04		2,04		
lot	176136	1,98	1,98	2,04	2,04	103,0%
target	2,05	1,98		2,03		
PPU	control	3,10		3,16		
lot	174531	3,08	3,08	3,20	3,17	102,9%
target	3,32	3,07		3,17		
Human serum 1	sample	2,18		2,21		
		2,21	2,18	2,26	2,24	102,8%
		2,18		2,24		
Human serum 2	sample	2,02		2,10		
		2,05	2,05	2,11	2,11	102,9%
		2,05		2,19		
Human plasma 1	sample	1,75		1,88		
		1,76	1,76	1,80	1,81	102,8%
		1,76		1,81		
Human plasma 2	sample	1,73		1,78		
		1,74	1,74	1,81	1,80	103,4%
		1,75		1,80		

Table 6:

Stability of NM-BAPTA in 50 mmol/L Glycin pH 9.8 at 35 °C

		day 0	day 0 Median	week1	week1 Median	Recovery from day 0
0.9 % NACL	calibrator	-0,03		-0,03		
		-0,05	-0,05	-0,05	-0,03	
target	0,0	-0,05		-0,03		
CACO3	calibrator	2,48		2,49		
		2,52	2,48	2,45	2,46	99,2%
target	2,5	2,47		2,46		
PNU	control	2,07		2,05		
lot	176136	2,07	2,07	2,08	2,05	99,0%
target	2,05	2,01		2,02		
PPU	control	3,11		3,07		
lot	174531	3,09	3,09	3,12	3,12	101,0%
target	3,32	3,04		3,12		
Human serum 1	sample	2,26		2,22		
		2,29	2,26	2,23	2,23	98,7%
		2,26		2,23		
Human serum 2	sample	2,34		2,24		
		2,32	2,34	2,25	2,25	96,2%
		2,34		2,28		
Human plasma 1	sample	1,91		1,75		
		1,88	1,89	1,73	1,73	91,5%
		1,89		1,73		
Human plasma 2	sample	1,92		1,69		
		1,76	1,76	1,72	1,71	97,2%
		1,75		1,71		

Table 7:

Stability of NM-BAPTA in 40 mmol/L CAPSO, pH 10.0 at 35 °C

		day 0	day 0 median	day 7	day 7 median	Recovery from day 0
0,9 % NaCl	calibrator	-0,02		-0,08		
		-0,01	-0,02	-0,11	-0,09	
target	0,0	-0,04		-0,09		
Calibrator	calibrator	2,50		2,47		
		2,58	2,57	2,47	2,47	95,9%
Sollwert	2,5	2,57		2,42		
PNU	control	2,21		2,14		
lot	179596	2,17	2,19	2,12	2,12	96,6%
target	2,12	2,19		2,08		
PPU	control	3,39		3,28		
lot	176287	3,35	3,35	3,30	3,29	98,2%
target	3,26	3,31		3,29		
Human serum 1	Probe	2,09		1,99		
		2,12	2,09	2,07	2,02	96,7%
		2,06		2,02		
Human serum 2	Probe	2,20		2,16		
		2,20	2,20	2,11	2,16	98,4%
		2,21		2,20		
Human plasma 1	Probe	2,04		2,07		
		2,06	2,06	2,03	2,03	98,4%
		2,08		1,97		
Human plasma 2	Probe	2,12		2,06		
		2,05	2,11	2,06	2,06	97,8%
		2,11		2,09		

As obvious from Tables 5 to 7 the reagent disclosed in the present application has an excellent stability at a pH around 10. The recovery of calcium ions after stressing such reagent at 35°C for one week is excellent and by large in the desired range of between 90 % to 110 %, mostly even between 95 % and 105 %. This high stability can be achieved irrespective of the buffer system used.

Example 6

No interference by magnesium in calcium measurements using NM-BAPTA

A sample comprising calcium ions in a physiological concentration of 2.53 mmol/L has been spiked with 0 to 15 mmol/L magnesium ions. The spiked samples were measured on a Roche/Hitachi 917 analyzer in an application as given in Example 2 for Modular P. Median results of triple determinations are given in Table 8 below.

Table 8:

No effect on calcium ion measurements by magnesium ions

Magnesium (mmol/L)	Calcium measured (mmol/L)	recovery (%)
0,0	2,53	100,0
1,5	2,52	99,6
3,0	2,52	99,6
4,5	2,55	100,8
6,0	2,55	100,8
7,5	2,55	100,8
9,0	2,55	100,8
10,5	2,55	100,8
12,0	2,54	100,4
13,5	2,54	100,4
15,0	2,54	100,4

As obvious from the recovery values given in Table 8, in the above experiment magnesium ions do not interfere with the measurement of calcium ions.

Example 7

No interference by gadolinium in calcium measurements using NM-BAPTA

One sample comprising calcium ions in a physiological concentration of about 2,35 mmol/L as well one sample with elevated level of calcium ions of about 3.40 mmmol/L have been spiked with different levels of Omniscan® (C1: 14 µL ad 986 µL, C2: 2,8 µL ad 997 µL). Omniscan® is a frequently used contrast agent comprising gadolinium. The spiked samples have been measured on a Hitachi 917 analyzer in an application as given in Example 2 for Modular P. Median results of triple determination are given in Table 9 below.

Table 9:

No effect on calcium ion measurements by gadolinium ions

	Calcium measured (mmol/L)	Median (mmol/L)	recovery of value without gadolinium (%)
C1_NORMAL	2,35	2,37	**101,3**
plus gadolinium	2,37		
	2,37		
C1_REF_NORMAL	2,32	2,34	
(no gadolinium)	2,34		
	2,34		
C2_NORMAL	2,33	2,37	**100,4**
plus gadolinium	2,37		
	2,37		
C2_REF_NORMAL	2,36	2,36	
(no gadolinium)	2,35		
	2,37		
C1_HIGH	3,42	3,40	**100,3**
plus gadolinium	3,40		
	3,40		
C1_REF_HIGH	3,39	3,39	
(no gadolinium)	3,39		
	3,33		
C2_HIGH	3,43	3,43	**100,6**
plus gadolinium	3,44		
	3,40		
C2_REF_HIGH	3,41	3,41	
(no gadolinium)	3,40		
	3,41		

As obvious from the recovery values given in Table 9, in the above experiment gadolinium ions do not interfere with the measurement of calcium ions, because all values measured are well with the 95 to 105 % recovery range.

Example 8

Synthesis of NF-BAPTA

The synthesis of NF-BAPTA is schematically depicted in Figure 6.

a) 1-(2-Chloro-ethoxy)-2-nitro-benzene

See Example 1

b) 4-Fluoro-1-nitro-2-(2-(2-nitro-phenoxy)-ethoxy)-benzene

1-(2-Chloro-ethoxy)-2-nitro-benzene (10 g) and 5-fluoro-2-nitro-phenol (7,86 g) were dissolved in 50 ml DMF and was stirred for 19 h at 90-110°C after careful addition of 13,82 g potassium carbonate. The reaction mixture was poured in a mixture of crushed ice and water (500 ml) which was vigorously stirred. The residue was filtered off, washed several times with water and dried. The crude product was suspended in methanol and the pale yellow residue was again filtered off, washed with methanol and dried.

Yield: 9,5 g.

c) 2-(2-(2-Amino-phenoxy)-ethoxy)-4-fluoro-phenylamine

9,48 g 4-fluoro-1-nitro-2-(2-(2-nitro-phenoxy)-ethoxy)-benzene and 1,5 g palladium on charcoal were suspended in 500 ml dioxane and 60 ml glacial acetic acid and hydrogenated at room temperature. After flashing three times with nitrogen the catalyst was filtered off under a nitrogen atmosphere and the remaining solution was evaporated and the product was dried under vacuum.

Yield: 7,52 g

d) ((2-(2-(2-(Bis-methoxycarbonylmethyl-amino)-phenoxy)-ethoxy)-4-fluoro-phenyl)-methoxycabonylmethyl-amino)-acetic acid methyl ester

2-(2-(2-Amino-phenoxy)-ethoxy)-4-fluoro-phenylamine (7,52 g) were dissolved in 250 ml DMF and 38,4 ml bromo-acetic acid methyl ester, 56 g potassium carbonate, and 2,13 g sodium iodide were added. The reaction mixture was heated up to 80°C for 21 h. After evaporation the remaining bromo-acetic acid methyl ester was removed from the product with hexane. The crude product was further purified by crystallization in methanol.

Yield: 4,14 g

e) ((2-(2-(2-(Bis-methoxycarbonylmethyl-amino)-5-nitro-phenoxy)-ethoxy)-4-fluoro-phenyl)-methoxycabonylmethyl-amino)-acetic acid methyl ester

2,5 g ((2-(2-(2-(Bis-methoxycarbonylmethyl-amino)-phenoxy)-ethoxy)-4-fluoro-phenyl)-methoxycabonylmethyl-amino)-acetic acid methyl ester were dissolved in 30 ml glacial acetic acid. Under vigorous stirring 4,54 ml 1 molar nitric acid in glacial acetic acid was added and in a second step 11,35 ml of concentrated sulfuric acid was added. The temperature increased up to 30°C. The reaction mixture was

directly poured in a 250 ml ice/water mixture. The residue was filtered off, washed several times with water and dried under vacuum. The crude product was further purified by column chromatography on silica gel first with hexane /acetic acid ethyl ester (1:1) as eluent and a second time with toluene/acetonitrile (1:1) as eluent.
Yield: 0,82 g

f) NM-BAPTA; Potassium salt of ((2-(2-(2-(bis-carboxymethyl-amino)-5-nitro-phenoxyl)-ethoxy)-4-fluoro-phenyl)-carboxymethyl-amino)-acetic acid

0,82 g ((2-(2-(2-(Bis-methoxycarbonylmethyl-amino)-5-nitro-phenoxy)-ethoxy)-4-mfluoro-phenyl)-methoxycabonylmethyl-amino)-acetic acid methyl ester were dissolved in a mixture of water/methanol (20 ml each) and 13,77 ml 1 molar potassium hydroxide solution was added. The reaction mixture was refluxed for 1 h. After cooling down to room temperature and adding 250 ml water the solution's pH was adjusted to pH 3 and the methanol was evaporated. The product was isolated by solvent extraction with acidic acid ethyl ester. After evaporation the product was dried under vacuum.

The ((2-(2-(2-(bis-carboxymethyl-amino)-5-nitro-phenoxyl)-ethoxy)-4-fluoro-phenyl)-carboxymethyl-amino)-acetic acid was dissolved in methanol and the equimolar potassium hydroxide in methanol was added. After evaporation and drying the appropriate potassium salt could be isolated.
Yield: 0,75 g

Example 9

Binding of calcium ions to NF-BAPTA.

The absorbance characteristics of NF-BAPTA in the presence and absence of calcium ions were analyzed using an Uvikon 930 photometer. Absorbance spectra were taken with 0.15 mmol/L NM-BAPTA or NF-BAPTA, respectively, in 50 mmol/L CAPSO (at pH 7), 0.9% NaCl and 0.01% Brij-35 either in the absence or in the presence of 0.09 mmol/L Ca2+, respectively.

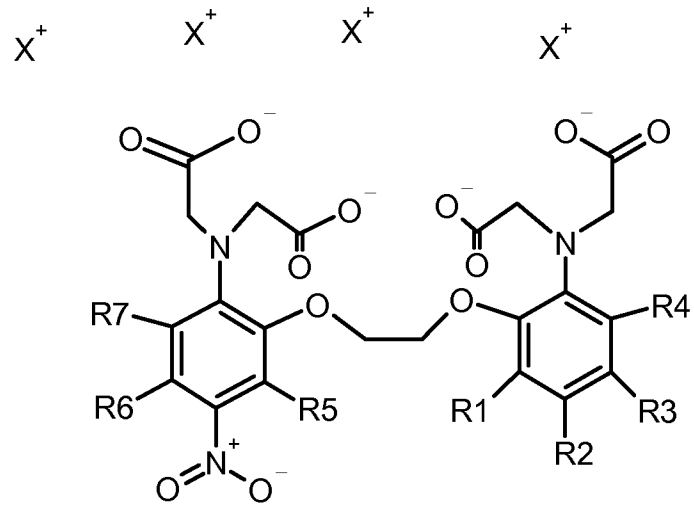
As shown in Figure 7, the absorbance spectra for NM-BAPTA or NF-BAPTA, respectively, are very much alike, indicating that both these compounds, i.e. compounds according to Formula I in general, can be used in the measurement of calcium ions.

Patent Claims

1. A method for determining the concentration of calcium ions in a sample, the method comprising the steps of

a) mixing the sample with a solution comprising a compound of Formula I

Formula I



wherein R1 is selected from hydrogen, halogen, carboxy, alkyl and formyl, R2 is independently selected from hydrogen, halogen, alkyl, alkoxy, morpholino, CN, carboxy and formyl, R3 is independently selected from hydrogen, halogen, N-alkyl sulfate, carboxy, alkoxy, phenyl, CN, CF3, and tertiary butyl, R4 is independently selected from hydrogen, halogen or alkyl, R5 and R7 independently are hydrogen or alkyl R6 is selected from hydrogen, alkyl, alkoxy and halogen, or wherein R3 and R4 form an aromatic bridge and X+ is a positively charged counter ion,

thereby binding calcium ions to the compound

b) releasing calcium ions from the compound, wherein said release causes a change in absorbance of the compound

c) measuring the change in absorbance

d) using the change in absorbance measured in (c) for determining the concentration of calcium ions.

2. The method according to claim 1, wherein the solution comprising the compound of Formula I has a pH in the range from pH 8.5 to pH 11.5

3. The method according to claim 1 or 2, wherein the solution comprising the compound of Formula I has a pH in the range from pH 9.0 to pH 10.5.

4. The method according to any of claims 1 to 3, wherein R1 of Formula I is either hydrogen or halogen.

5. The method according to any of claims 1 to 3, wherein R2 of Formula I is hydrogen, halogen or alkyl.

6. The method according to any of claims 1 to 3, wherein R3 of Formula I is hydrogen, halogen, carboxy or alkoxy.

7. The method according to any of claims 1 to 3, wherein the release of calcium ions in step (b) of claim 1 is triggered by a calcium chelating agent having at 20°C a binding constant for calcium ions of log k equal to 7.0 or above.

8. The method according to claim 7, wherein the release of calcium ions in step (b) of claim 1 is triggered by EDTA, DTPA, EGTA, DTPMP and/or EDPMP.

9. A reagent for measurement of calcium having a pH ranging from pH 8.5 to pH 11.5, containing a compound of Formula I as defined in claim 1.

10. The reagent of claim 9, comprising the compound of Formula I in a concentration ranging from 0.10 mM to 50mM.

11. The reagent of claim 9, wherein comprising a buffer system, wherein the buffer system is selected from AMPD (= 2-Amino-2-Methyl-1.3-propanediol), CHES (= 2-(N-Cyclohexylamino)-ethanesulfonic acid), AMPSO (= 3-[Dimethyl(hydroxylmethyl)-methylamino]-2-hydroxypropane-sulfonic acid), CAPSO (= 3-Cyclohexylamino)-2-hydroxy-1-propanesulfonic acid), CAPS (= 3-Cyclohexylamino)-2- propanesulfonic acid), a glycine buffer system or a carbonate buffer system.

12. The reagent according to any of claims 9 to 11, wherein R1 of Formula I is either hydrogen or halogen.

13. The reagent according to any of claims 9 to 11, wherein R2 of Formula I is hydrogen, halogen or alkyl.

14. The reagent according to any of claims 9 to 11, wherein R3 of Formula I is hydrogen, halogen, carboxy or alkoxy.

15. A test kit for the measurement of calcium, the test kit comprising the reagent according to any of claims 9 to 14 .

Fig. 1

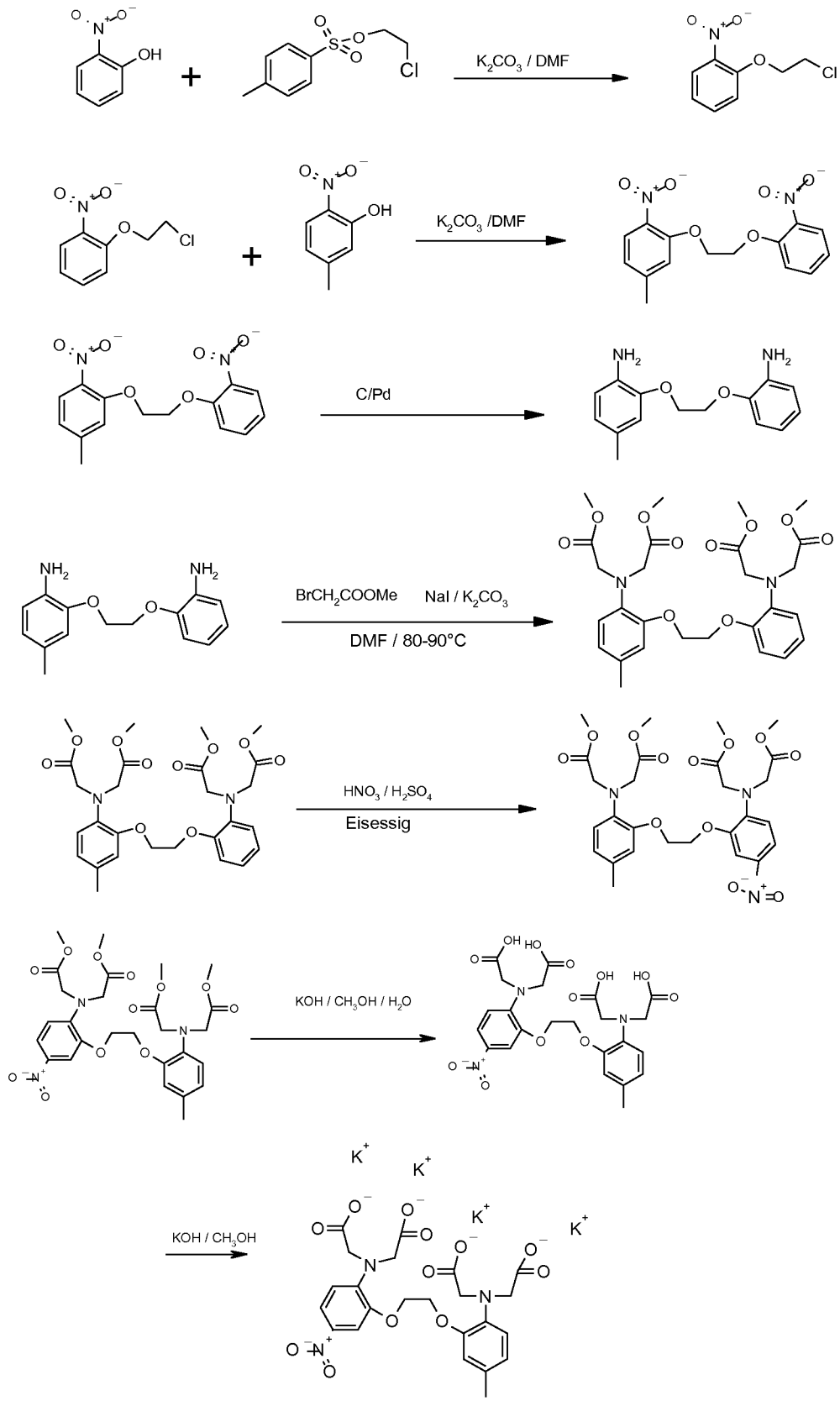
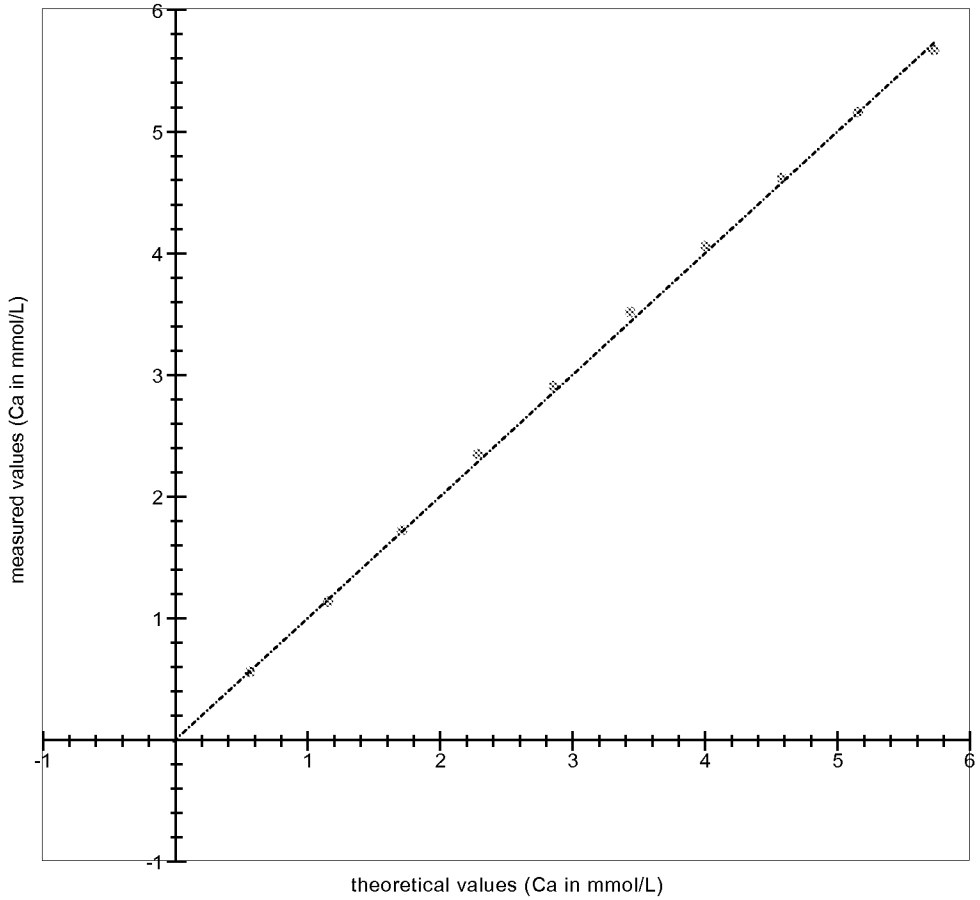

K2CO3 / DMF
K2CO3 /DMF
C/Pd
BrCH2COOMe
NaI / K2CO3
DMF / 80-90°C
HNO3 / H2SO4
Eisessig
KOH / CH3OH / H2O
KOH / CH3OH

Fig. 2

Modular P

A; B



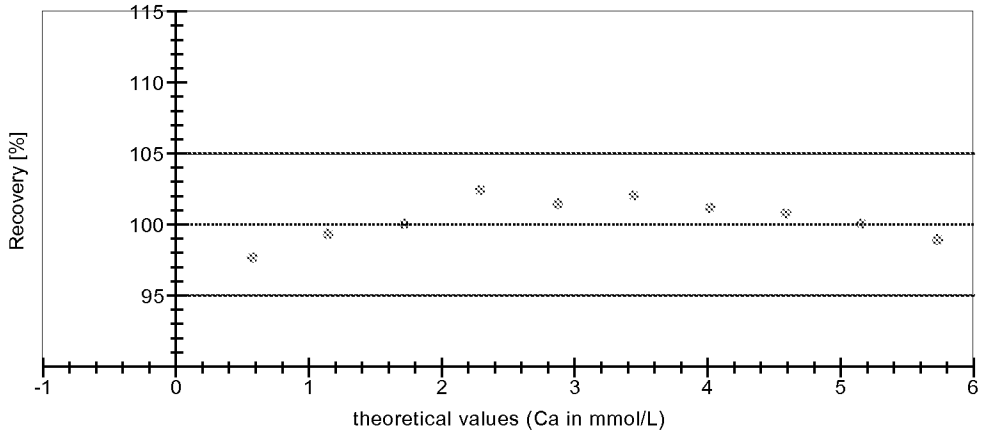
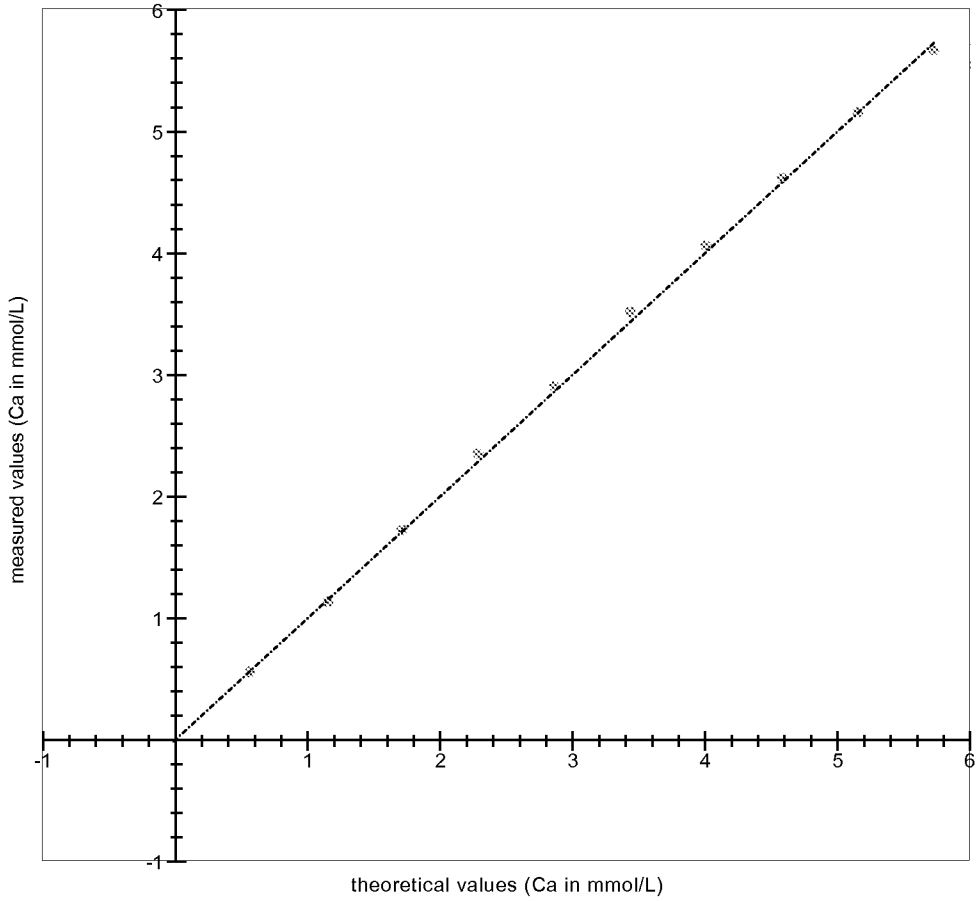


Fig. 3

cobas c501

A; B



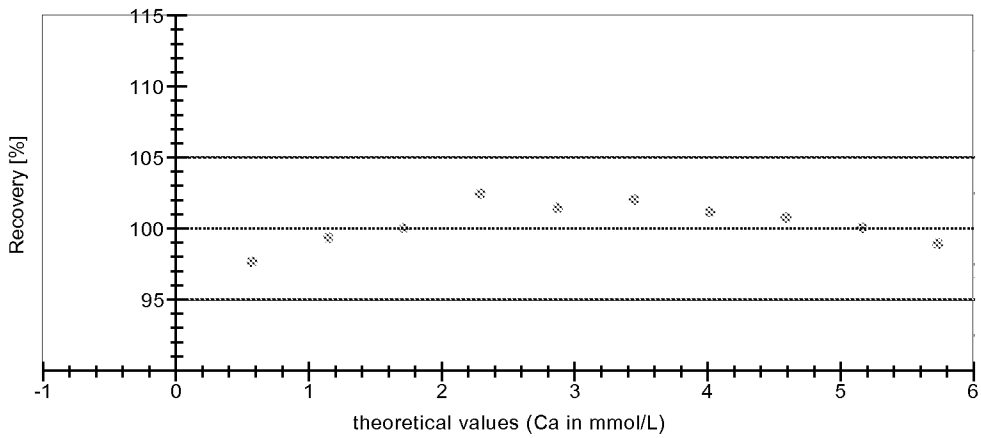
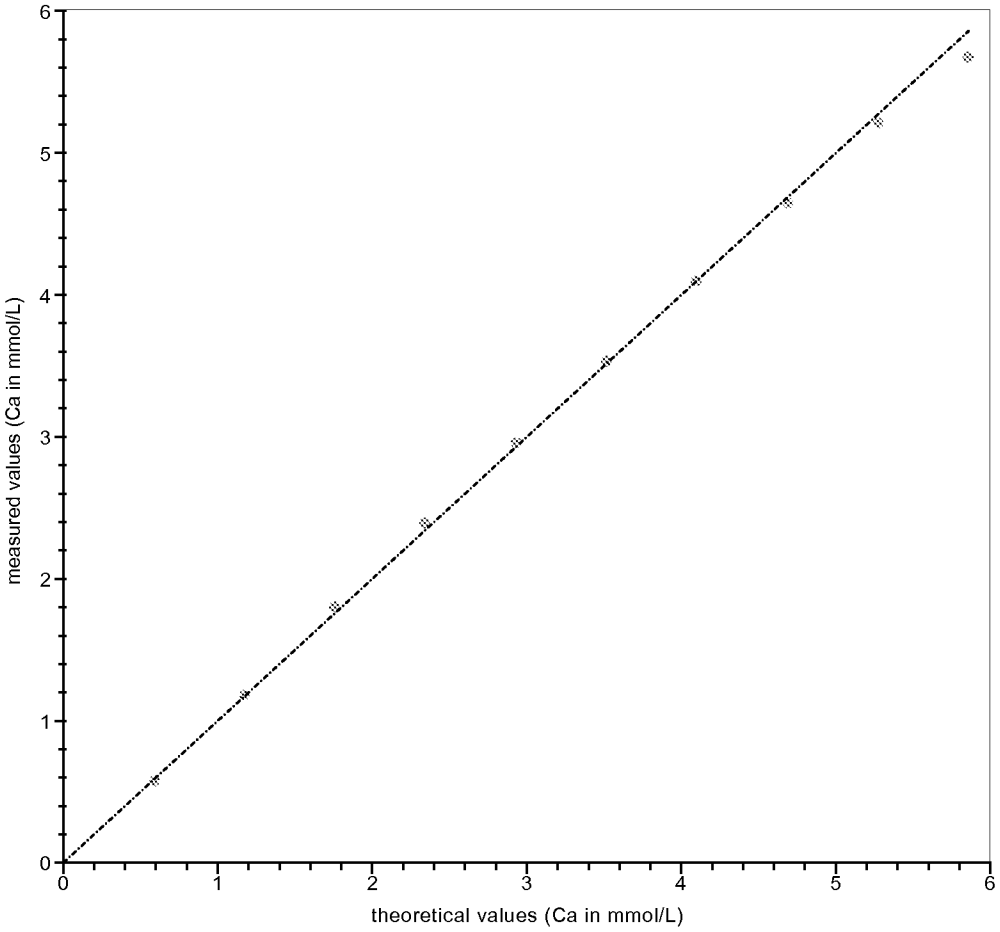


Fig. 4

Modular P 90% R1

A; B



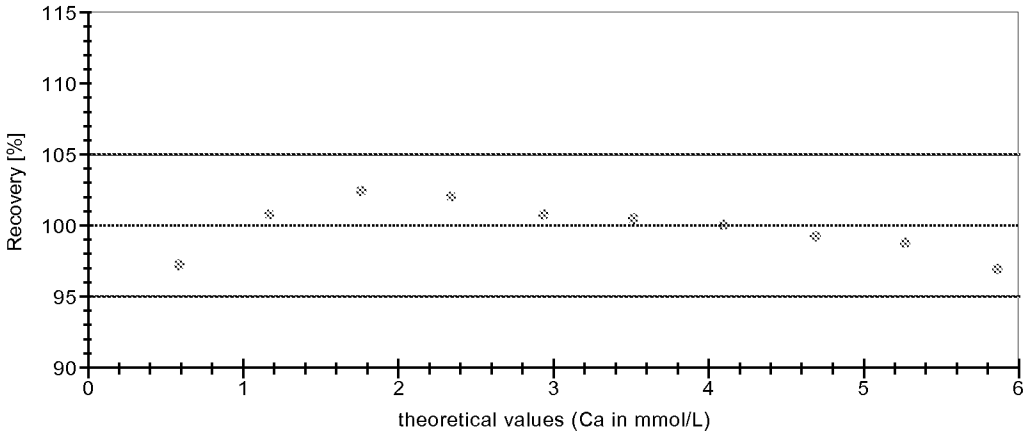
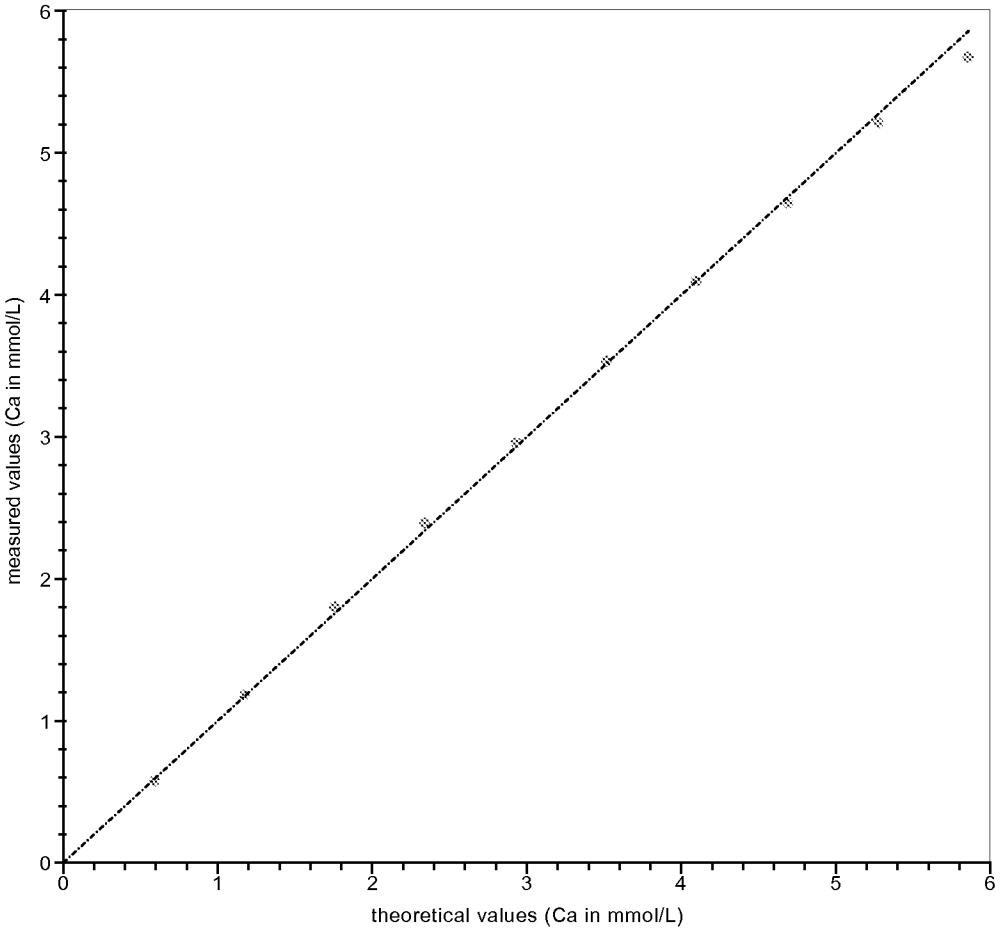


Fig. 5

Modular P 80% R1

A; B



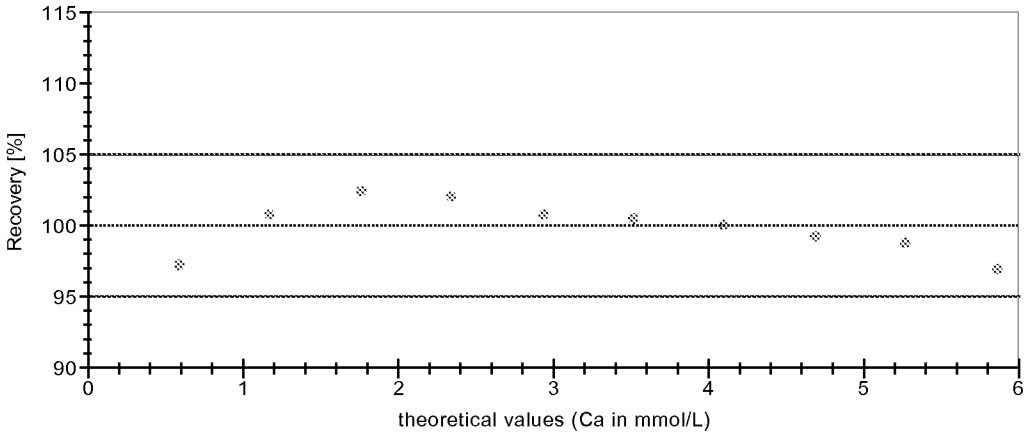


Fig. 6

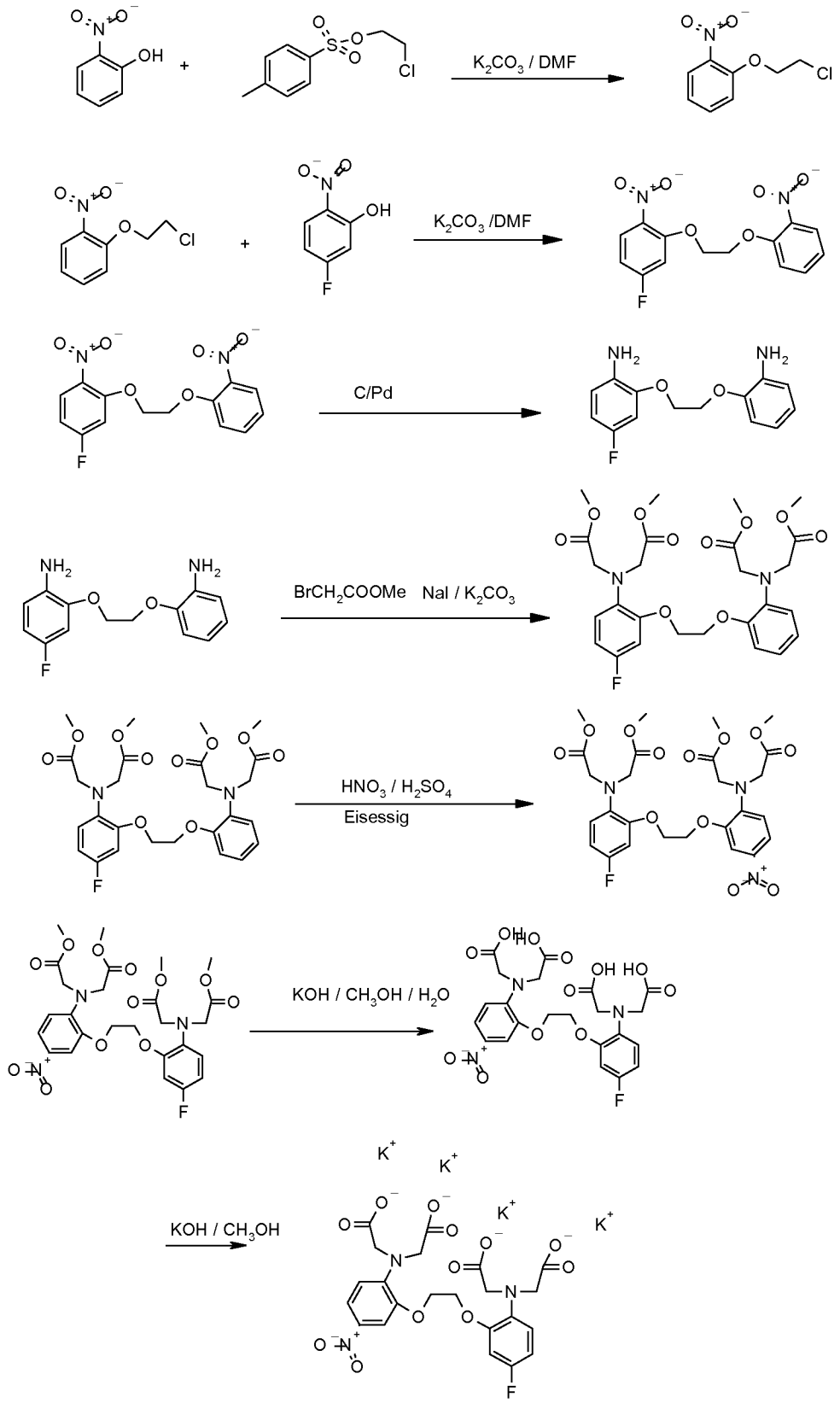
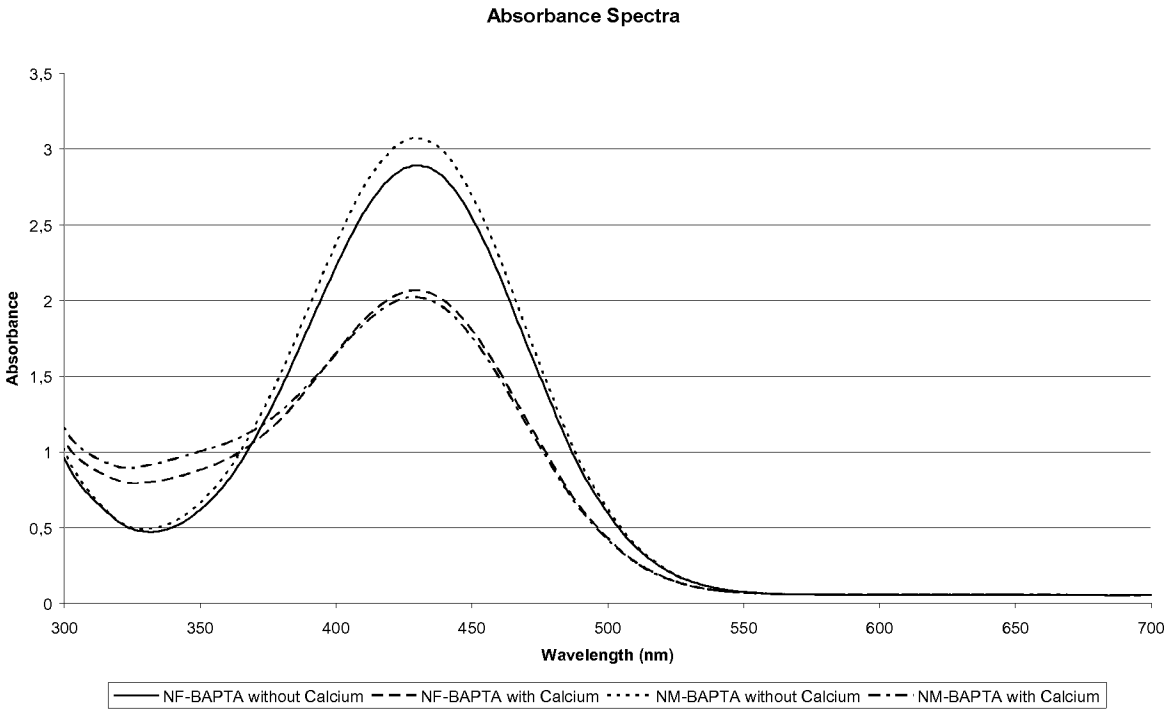

K2CO3 / DMF
K2CO3 /DMF
C/Pd
BrCH2COOMe NaI / K2CO3
HNO3 / H2SO4
Eisessig
KOH / CH3OH / H2O
KOH / CH3OH

Fig. 7


Absorbance Spectra
Absorbance
3,5
3
2,5
2
1,5
1
0,5
0
300
350
400
450
500
550
600
650
700
Wavelength (nm)
NF-BAPTA without Calcium
NF-BAPTA with Calcium
NM-BAPTA without Calcium
NM-BAPTA with Calcium

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2011/054713

A. CLASSIFICATION OF SUBJECT MATTER
INV. G01N33/84 C09B11/00
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
G01N C09B

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS, COMPENDEX, EMBASE, INSPEC, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MASINO LAURA ET AL: "Ligand binding and thermodynamic stability of a multidomain protein, calmodulin", PROTEIN SCIENCE, vol. 9, no. 8, August 2000 (2000-08), pages 1519-1529, XP002590318, ISSN: 0961-8368	9,12-15
Y	page 1527, column 2, paragraph 3	10,11

-/--

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
30 May 2011	14/06/2011

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Thumb, Werner

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	BARTH ANDREAS ET AL: "Decarboxylation is a significant reaction pathway for photolabile calcium chelators and related compounds.", PHOTOCHEMICAL & PHOTOBIOLOGICAL SCIENCES : OFFICIAL JOURNAL OF THE EUROPEAN PHOTOCHEMISTRY ASSOCIATION AND THE EUROPEAN SOCIETY FOR PHOTOBIOLOGY JAN 2006 LNKD- PUBMED:16395435, vol. 5, no. 1, January 2006 (2006-01), pages 107-115, XP002590319, ISSN: 1474-905X abstract page 110, column 1, paragraph 3 - column 2, paragraph 1 page 114, column 1, paragraph 7 - column 2, paragraph 4	1-15
Y	BETT I M ET AL: "A rapid micro-method for determining serum calcium", CLINICA CHIMICA ACTA, ELSEVIER BV, AMSTERDAM, NL LNKD- DOI:10.1016/0009-8981(59)90101-9, vol. 4, no. 3, 1 May 1959 (1959-05-01), pages 346-356, XP023390750, ISSN: 0009-8981 [retrieved on 1959-05-01] the whole document	1-15
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A	EP 0 594 047 A1 (MILES INC [US] BAYER AG [US]) 27 April 1994 (1994-04-27) compounds 54-58	1-15

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Information on patent family members

International application No
PCT/EP2011/054713

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