Agent for the detection of esterolytic and/or proteolytic enzymes and phenoxyamino acid and phenoxypeptide esters suitable as substrates therefor, processes for their preparation, as well as their use for the production of agents according to the invention

In the diagnosis of diseases of the kidneys and of the urogenital tract, the detection of leukocytes in the urine is of great importance. Hitherto, this detection was carried out microscopically in that the number of leukocytes which were present in a definite volume of urine were counted under the microscope. However, this method is very time-consuming, laborious and tiring and, in addition, requires the use of trained personnel.

Therefore, for some time, it has been attempted to use enzymatic reactions as detection principle for leukocytes in various body fluids since the leukocytes possess a broad enzyme spectrum.

Agents for the detection of leukocytes in body fluids, in which the esterolytic and/or proteolytic activity present in the leukocytes are utilised for analytical purposes are known from published German Patent Specifications Nos. 2 826 965 and 2 836 644. Sulphonphthalein esters or azo dyestuff esters are thereby used as substrate for the leukocyte esterases and/or proteases. The coloured materials liberated in the case of the enzymatic reaction are evaluated

according to generally known methods. The agents described in these documents are still too insensitive. They display too long reaction times for the lower limit of detection so that the practical use is still bound up with certain disadvantages, in particular too long waiting times in the carrying out of the test.

Various methods for the detection of proteases and esterases are also known from the histo- and cytochemical enzymology (cf., for example, A.G.E. Pearse, Histochemistry, Theoretical and Applied, 3rd ed., Churchill Livingstone, Edinburgh-London-New York 1968). In principle, there are thereby also used colourless or weakly coloured esters which, by enzymatic splitting, mostly break down into a colourless acid and an also colourless alcohol (phenol) component. The latter is then reacted, in a reaction following the enzymatic saponification, to give coloured products, e.g. by coupling with diazonium salts or oxidative reaction.

F. Schmalzl and H. Braunsteiner describe, for example, in Klin. Wschr. 46, 642 (1968) a specific cytochemical leukocyte esterase detection with naphthol-AS-D-chloroacetate as substrate and a diazonium salt for the formation of the coloured azo compound.

For an agent for the rapid and simple detection of leukocytes in body fluids, such as e.g. in urine, two component systems of this kind proved to be unsuitable. They react too insensitively. For example,

samples with 5000 Leukocytes/ µl. show no reaction. Furthermore, as is known, many compounds occurring in the urine, such as urobilinogen, stercobilinogen, bilirubin and others, react with diazonium salts. This is best shown by the commerically available patented tests for the detection of urobilinogen or bilirubin in the urine which use diazonium salts for the detection reaction. The diazonium salts used in the literature for the esterase detection show the described side reactions with other urine components and are, in some cases, not usable for a leukocyte test because of their own colour.

F. Sweetman and L. Ornstein describe in J. Histochem./Cytochem. 22, 327-339 (1974), as well as Ornstein et al. in Abstracts Histochem. Soc. 1973, p. 411, the coloration of various esterolytic and proteolytic enzymes on electrophoresis plates which serve for the purification and separation of the enzymes. There is hereby used the coupling of various naphthyl esters, inter alia 1-naphthyl-N-acetyl-D,L-alanine with diazotised para-rosaniline. In spite of the high starting concentration of the enzymes, which are obtained from 40 - 100 million cells/ml., and of the separation of disturbing secondary components, there are hereby required reaction times of over two hours for a coloration.

P.M. Starkey and A.J. Barrett are concerned in Biochem. J., 155, 265-271 (1976) with the character-

isation of an elastase isolated from human spleen cells and prepared pure. The cleavage activity of this enzyme towards various naphthyl esters and nitroaniline derivatives is described. From immunological investigations, the conclusion is drawn that this elastase agrees with that from human neutrophilic leukocytes. The authors carry out their investigations solely with regard to the chemical properties of leukocyte enzymes but not for the detection of unknown amounts of such enzymes.

Furthermore, there are known various esterase detection principles on the basis of a buffer, of a particular diazonium salt, as well as of a suitable ester. For example, in W.J. WILLIAMS et al., Haematology, 2nd edition 1977, Chapter A25: Leukocyte esterases, pages 1633-1636, there is described naphthol chloroacetate in combination with multiple diazotised dyestuffs and a phosphate buffer for the detection of an esterase activity. In U.S. Patent No. 3 905 872 there is claimed a combination of naphthyl phosphate or phenyl phosphate with a diazonium salt for the determination of the alkaline phosphatase. However, all these previously known esterase detection systems have proved not to be usable for the detection of the esterolytic and/or proteolytic enzyme activity of the

leukocytes in body fluids.

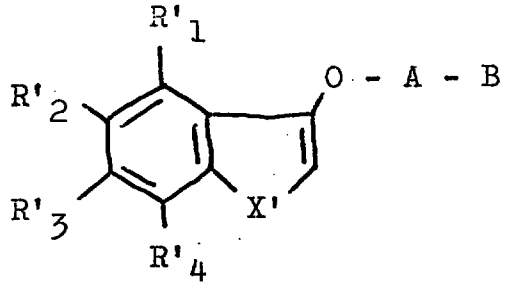
Now, it was the task of the present invention to make available an agent for the detection of esterolytic and/or proteolytic enzymes with a combination of esters and diazonium salts as reaction partners for these enzymes with which these can be detected in a shorter time in a simple and easily handled way and which show no disturbing side reactions with other components of the sample to be investigated.

This task was solved in that a particular combination of suitable esters and specially substituted diazonium salts is employed, whereby, surprisingly, the diazonium salts used do not enter into side reactions with other urine components, such as for example bilirubin and urobilinogen, but rather couple specifically and rapidly the phenolic components resulting in the case of the splitting of the ester used, with formation of a coloured compound.

The suitable esters must be sufficiently reactive with regard to the esterolytic and/or proteolytic enzymes in order that they are split as quickly as possible into the acid and alcohol components (under which phenols are also to be understood). The esters are also to be so selected that the liberated alcohol components couple well and completely with the diazonium salts used. The reactivity of the diazonium salts must also be so adjusted by suitable choice of the substituents

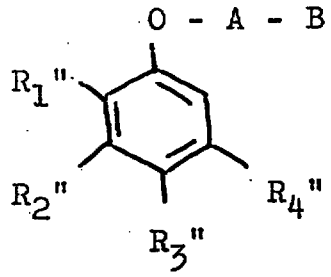
that there admittedly takes place a sufficiently rapid coupling of the alcohol components liberated from the esters used according to the invention but no reaction with other components of the test solution.

The subject of the present invention is the use of agents for the detection of esterolytic and/or proteolytic enzymes, consisting of an absorbent carrier, a film layer, a powder mixture, a lyophilisate, a solution or a reagent tablet, containing a suitable esterase and/or protease detection principle, a buffer, as well as possibly further usually employed additive materials, for the detection of leukocytes in urine, whereby the agent is added to the urine to be investigated and the resultant colour formation is assessed visually or photometrically, characterised in that, as esterase and/or protease detection principle, there is used a combination of a specially substituted diazonium salt and a suitable ester, the reactivity of which is atuned to one another by suitable choice of the substituents in such a manner that there admittedly takes place a sufficiently rapid coupling with the alcohol components liberated from the ester but no reaction with other components of the test solution, whereby the ester is selected from the group of compounds of the general formula II



(II)

in which R'_1, R'_2, R'_3, R'_4, which each can be the same or different, each signifies a hydrogen or a halogen atom, a lower alkyl, a lower alkoxy, an aryl, an aralkyl, an aralkoxy, a hydroxyl, a carboxyl, a carboxy-lower-alkoxy, an aralkoxycarbonyl, an aralkoxy-carbonyl-lower-alkoxy, a nitro or a lower acylamino group or in each case two neighbouring substituents a benzo-annellated radical possibly substituted by halogen, X' a sulphur atom or an imino group possibly substituted by a lower alkyl, an aryl, an aralkyl or an acyl radical, A an amino acid or a peptide residue, B a nitrogen protective group usual in peptide chemistry or derived therefrom, or of compounds of the general formula III

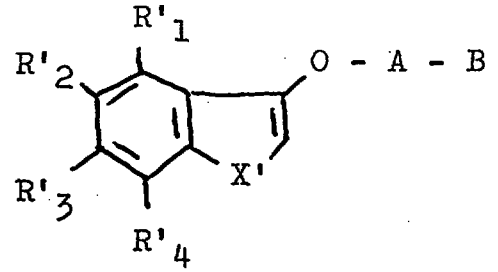


(III)

in which $R_1"$, $R_2"$, $R_3"$, $R_4"$, which can be the same or different, each signify hydrogen, a lower alkyl, a lower alkoxy, alkylamino or dialkylamino group, whereby

two neighbouring substituents can also represent an annellated benzene ring, A and B have the above-given meaning.

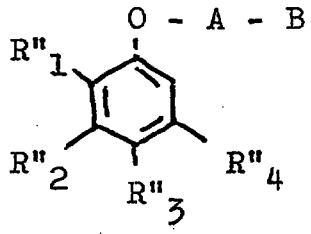
A further subject of the present invention are agents for the detection of esterolytic and/or proteolytic enzymes in leukocytes in urine, consisting of an absorbent carrier, a film layer, a powder mixture, a lyophilisate, a solution or a reagent tablet, containing a suitable esterase and/or protoease detection principle, a buffer, as well as possibly further additive materials usually employed, characterised in that, as esterase or protease principle, there is used a combination of a specially substituted diazonium salt and a suitable ester, the reactivity of which is adjusted to one another by suitable choice of the substituents in such a manner that there admittedly takes place a sufficiently rapid coupling with the alcohol components liberated from the ester but no reaction with other components of the test solution, whereby the ester is selected from the group of the compounds of the general formula II



(II)

in which R'_1, R'_2, R'_3, R'_4, which can be the same or different, each signify a hydrogen or halogen atom, a lower alkyl, a lower alkoxy, an aryl, ar aralkyl, an

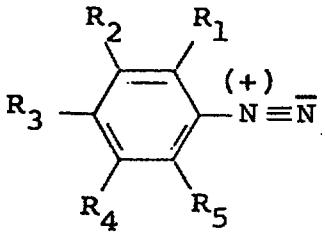
aralkoxy, a hydroxyl, a carboxyl, a carboxy-lower-alkoxy, an aralkoxycarbonyl, an aralkoxycarbonyl-lower-alkoxy, a nitro or a lower acylamino group or in each case two neighbouring substituents a benzo-annelated radical possibly substituted by halogen, X' a sulphur atom or an imino group possibly substituted by a lower alkyl, an aryl, an aralkyl or an acyl radical, A an amino acid or a peptide residue, B a nitrogen protective group usual in peptide chemistry or derived therefrom, or of compounds of the general formula III



(III)

in which $R"_1$, $R"_2$, $R"_3$, $R"_4$, which can be the same or different, can each represent hydrogen, a lower alkyl, a lower alkoxy, alkylamino or dialkylamino group, whereby two neighbouring substituents can also represent an annelated benzene ring, A and B have the above-given meaning, whereby when $R_1"$ and $R_2"$ or $R_2"$ and $R_3"$ is an annelated benzene ring, B does not represent an acetyl or benzyloxycarbonyl group.

Suitable substituted diazonium salts in the meaning of the present invention are, for example, compounds of the general formula I



(I)

in which R_1 signifies a lower alkyl, a lower alkoxy, a lower alkylmercapto, an N-morpholino, an N-thiomorpholino, an N-pyrrolidino, a possibly N'-alkylated N-piperazino, an N-piperidino group, halogen or hydrogen, R_3 a lower alkyl, a lower alkoxy, an aryloxy, a lower alkylmercapto, alkylamino, dialkylamino, a hydroxyl, an N-morpholino, N-thiomorpholino, N-pyrrolidino, a possibly N'-alkylated N-piperazino, N-piperidino, phenylamino, a phenyl group possibly substituted with a lower alkyl or lower alkoxy radical, halogen or hydrogen, R_2, R_3, R_4, which can be the same or different, each signify a lower alkyl, a lower alkoxy, a lower alkylmercapto group, halogen or hydrogen, whereby R_1 and R_2 together can represent an annelated benzene ring, and X a stabilising anion.

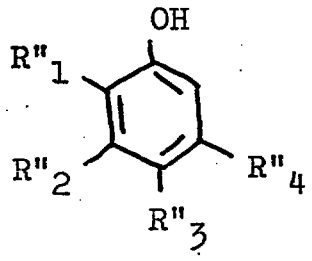
The esters of the general formulae II and III are saponified rapidly and completely by esterases and/or proteases, for example by the esterases and proteases occurring in the neutrophilic granulocytes. The liberated phenol components react well, quickly and selectively with the low electrophilic diazonium salts of the general formula I. Therefore, according

to the principle according to the invention, there can be produced stable and rapidly-indicating agents for the detection of leukocytes in body fluids. Furthermore, it has been shown that the agents according to the invention are also outstandingly suitable for the general detection of proteolytic enzymes, such as for example of elastase, chymotrypsin or trypsin, in aqueous solutions or body fluids, such as e.g. whole blood, serum or liquor, pancreas secretion or aqueous faecal extracts.

The diazonium salts of general formula I are, in part, known compounds. Derivatives in which the substituents R_1 and/or R_2 represent N-thiomorpholine, N-pyrrolidino, a possibly N'-alkylated N-piperazino or N-piperidino group are new compounds. However, they can be prepared in analogy to the known substances according to per se known methods.

The compounds of the general formula II are described in published German Specification No. 2 854 987.

The esters of the general formula II are then new compounds when R_1" and R_2" or R_2" and R_3" is an annelated benzene ring, B is not an acetyl or benzyloxycarbonyl group. They can be prepared in that one reacts compounds of the general formula IV



(IV)

in which $R"_1$, $R"_2$, $R"_3$ and $R"_4$ have the above-given

meaning, with amino acids and peptides of the general formula V

$$HO\text{-}A\text{-}B \qquad (V),$$

in which A and B have the above-given meaning, or appropriate reactive derivatives hereof according to methods usual in peptide chemistry.

As reactive derivatives, there are used e.g. the acid chlorides or the mixed anhydrides usually employed in peptide synthesis, for example with chloroformic acid ethyl ester, or active esters.

Therefore, a further subject of the present invention are the new esters of the general formula III, as well as their use for the production of agents for the detection of esterolytic and/or proteolytic enzymes.

By halogen in the definition of R_1, R_2, R_3, R_4 and R_5 or of R'_1, R'_2, R'_3 and R'_4 is to be understood fluorine, chlorine, bromine or iodine, preferably chlorine and bromine.

The lower alkyl, alkoxy, alkylmercapto, alkyl- and dialkylamino groups in the definitions of R_1-R_5, R'_1-R'_4, as well as R''_1-R''_4 contain 1 to 5, preferably 1 to 3 carbon atoms, whereby the corresponding methyl radicals are quite especially preferred.

As stabilising anion X, tetrafluoroborate, tetrachlorozincate or perchlorate is preferred.

By an aralkoxy group in the definition of R'_1, R'_2, R'_3 and R'_4, as well as of an aralkyl group in

the definition of X', are to be understood e.g. phenyl and naphthyl groups substituted by oxy-lower alkyl or lower alkyl radicals, whereby the alkyl radicals contain 1 to 5, preferably 1 to 3 carbon atoms. The benzyloxy or the benzyl radical are especially preferred.

As lower acylamino group of R'_1, R'_2, R'_3 and R'_4, there come into question the amide groupings of the lower aliphatic carboxylic acids with 1 to 5, preferably 1 to 3 carbon atoms. The acetylamino radical is especially preferred.

As acyl radical in the definition of X', there come into question the residues of aliphatic carboxylic acids with 1 to 5, preferably 1 to 3 carbon atoms or also of aromatic carboxylic acids, such as for example of benzoic or naphthoic acids. Acetyl and benzoyl radicals are especially preferred.

By an aryl or aralkyl radical in the definition of R'_1, R'_2, R'_3, R'_4 and X' are preferably to be understood the phenyl or naphthyl group or the benzyl radical.

As amino acid residue in the definition of A, there preferably come into question the residues of the natural α-amino acids in their L- or D-form or also in their racemic form. Especially preferred are the residues of glycine, alanine, valine, leucine, isoleucine, phenylalanine and tyrosine, whereby in each case the L-form is quite especially preferred.

Free hydroxy groups possibly present can be acylated, preferably acetylated.

By a peptide residue in the definition of A are to be understood e.g. di-, tri-, tetra- and penta-peptides, preferably di- and tripeptides, whereby, as amino acid components, the above-mentioned amino acids preferably find use.

As nitrogen protective groups usual in peptide chemistry in the definition of B are to be understood e.g. acyl, oxycarbonyl, thiocarbonyl, sulphonyl, sulphenyl, vinyl, cyclohexyl, phosphoryl or carbamoyl groups.

The diazonium salts of general formula I and the esters of general formulae II and III used according to the invention are employed in concentrations of 10^{-4} mol/l. to 10^{-1} mol/l., preferably 10^{-3} mol/l. to 10^{-2} mol/l. of impregnation solution-coating mass or fluid to be investigated.

A further component of the agent according to the invention for the detection of proteolytic enzymes and especially of the leukocyte proteases is a suitable buffer system. For these, there come into question e.g. phosphate, borate, barbiturate, tris-(hydroxymethyl)-aminomethane (= tris), 2-amino-2-methylorpandiol-1,3 (= amediol) or amino acid buffers, whereby pH value and capacity must be so selected that, in the measurement solution or on the test strip, a pH value of 6 - 10,

preferably 7 - 9, is adjusted.

A further component of the agent according to the invention for the detection of proteolytic enzymes can be a wetting agent since a more homogeneous colour distribution and, in part, more brilliant colours can hereby be achieved. There can be used cationic- but also anionic-active, as well as amphoteric and non-ionic wetting agents in concentrations of 0.05 - 2% (w/v), preferably 0.1 - 1% (w/v).

As further component of the agent according to the invention, as stabiliser there can serve a phosphoric or phosphonic acid amide of the general formula VI

$$R_7 - \overset{\overset{R_6}{|}}{\underset{|}{P}} = O \qquad \text{(VI)}$$
$$\underset{R_8}{|}$$

in which R_6 signifies dialkylamino, an alkoxy, an aryloxy, an alkyl or an aryl group or an N-morpholino radical and R_7 and R_8 a dialkylamino group or an N-morpholino radical.

As alkoxy or alkyl group in the definition of R_6, there come into question hydrocarbon radicals with up to 10 carbon atoms.

By aryl or aryloxy group in the definition of R_6 are to be understood phenyl or naphthyl radicals possibly substituted by halogen, lower alkyl or alkoxy groups.

With the help of compounds of the general formula VI, there is obtained a surprising stabilisation of the formulations.

The phosphoric and phosphonic acid amides of general formula VI are known compounds, they find use e.g. in accepted German Patent Application No.2 235 127 as stabilisers of test strip formulations which work on the basis of a peroxidase detection.

The stabilisers of the phosphoric and phosphonic acid amide type of general formula VI are added to the aqueous or preferably the organic impregnation solution in concentrations of 1 - 20% (w/v), preferably 5 - 15% (w/v).

Surprisingly, it was found that the reaction times of the diagnostic agent according to the invention for the detection of proteolytic enzymes, especially proteolytic leukocyte enzymes, can be considerably shortened when, additionally to the diazonium salts, esters and the previously mentioned adjuvant materials, one or more activators are used. As activators suitable for the agent according to the invention, there have proved to be the compounds described and claimed in EP-A 14 929.

The activators are used in concentrations of 0.5 - 10%, preferably 1 - 5% (w/v), in the impregnation solution.

For the production of the agent according to the invention, there are impregnated e.g. absorbent carriers, preferably filter paper, cellulose or synthetic resin fleece, with solutions of the necessary reagents usually employed for the production of test strips (substrate, buffer, possibly wetting agent) in easily volatile solvents, such as e.g. water, methanol, ethanol or acetone. This expediently takes place in two separate steps: first, it is impregnated with an aqueous solution which contains the buffer and other water-soluble additive materials. Thereafter, it is impregnated with a solution of the protease substrate of the general formula II or III, diazonium salts of the general formula I and activators. However, the impregnation can also be carried out in another sequence or with another composition of the two impregnation solutions.

The finished test papers can be used as such or stuck in per se known manner on to handles or preferably sealed in between synthetic resins and fine meshworks according to DE-C- 2 118 455.

For the production of film-coated test strips, all reagents are introduced into the solution of dispersion of a film-forming substance, such as e.g. polyvinyl ester or polyamide, and homogeneously mixed. The mixture is coated in a thin layer on to a synthetic resin carrier and dried. The film-coated test strips

according to the invention are, after drying, cut up and can be used as such or are stuck on to handles in per se known manner or e.g. sealed in between synthetic resins and fine meshworks according to DE-C 2 118 455.

The diagnostic agent according to the invention for the detection of proteolytic enzymes, especially of leukocyte proteases, in the form of powder mixtures or reagent tablets can be produced in that the above-described components of the test are mixed with usual galenical additive materials and granulated. Additive materials of this kind are e.g. carbohydrates, such as e.g. mono-, oligo- or polysaccharides, or sugar alcohols, such as e.g. mannitol, sorbitol or xylitol, or other soluble inert compounds, such as polyethylene glycols or polyvinylpyrrolidone. The powder mixtures or reagent tablets have, in general, an end weight of about 50 - 200 mg., preferably 50 - 80 mg.

For the production of lyophilisates in the total weight of, in each case, about 5 - 20 mg., preferably about 10 mg., a solution is freeze dried which, besides all reagents needed for the test, contain usual structure formers, such as e.g. polyvinylpyrrolidone, and possibly further filling materials, such as e.g. mannitol, sorbitol or xylitol.

The diagnostic agent according to the invention in the form of a solution preferably contains all

reagents needed for the test. As solvent, there come into question water or mixtures of water with a water-soluble organic solvent, such as e.g. methanol, ethanol, acetone or dimethylformamide. For storage stability reasons, it can be advantageous to divide the reagents needed for the test into two or more solutions which are first mixed together in the case of the actual investigation.

The so produced diagnostic agents make it possible, after dipping into the fluid to be investigated or after the addition of the body fluid in question, to detect the presence of proteolytic enzymes, especially of the leukocyte proteases, rapidly and simply via a colour formation which can be assessed visually or photometrically, e.g. remission photometrically or in a cuvette. Since the activity of the leukocyte proteases per cell can be regarded as an essentially constant value, from the intensity of the colour formation, there can be determined the leukocyte concentration of the investigated body fluid. With the diagnostic agent according to the invention, there are thereby encompassed not only intact but also lysed leukocytes since the activity of the leukocyte proteases remains fully maintained even after the lysis of the leukocytes. Consequently, a lysis error does not occur.

Example 1

Filter paper (e.g. Schleicher & Schüll 23 SL) is

successively impregnated with the following solutions and then dried at 60°C.:

Solution 1

0.2 molar borax-hydrochloric acid buffer pH 8.

10% phosphoric acid trimorpholide

Solution 2

2×10^{-3} mol/l. substrate

10^{-2} mol/l. diazonium salt

dissolved in acetone/1-decanol 98:2

Substrates

S 1: 3-[N-(toluene-4'-sulphonyl)-L-alanyloxy]-indole

S 2: 3-[N-(toluene-4'-sulphonyl)-L-alanyloxy]-1-methoxy-naphthalene

S 3: 1-[N-(toluene-4'-sulphonyl)-L-alanyloxy]-4-methoxy-naphthalene

S 4: 1-[N-(toluene-4'-sulphonyl)-L-alanyloxy]-4-isopropoxy-naphthalene

S 5: 1-[N-(toluene-4'-sulphonyl)-L-alanyloxy]-4-pentoxy-naphthalene

S 6: 1-[N-(toluene-4'-sulphonyl)-L-alanyloxy]-3-dimethyl-aminobenzene

S 7: 1-[N-(toluene-4'-sulphonyl)-L-alanyloxy]-3-diethyl-aminobenzene

S 8: 1-[N-(toluene-4'-sulphonyl)-L-alanyloxy]-3,5-dimethoxybenzene

S 9: 1-[N-(toluene-4'-sulphonyl)-L-alanyloxy]-3-methyl-5-methoxybenzene

Diazonium salts

D 1: 2,4-dimethoxybenzenediazonium tetrafluoroborate

D 2: 4-methoxynaphthalene-1-diazonium tetrafluoroborate

D 3: 2,5-dimethoxy-4-dimethylaminobenzenediazonium tetrafluoroborate

D 4: 4-dimethylaminobenzenediazonium tetrafluoroborate

One obtains papers which, in the case of dipping into leukocyte-containing urines, indicate 100 leukocytes/μl. in about 3 minutes with the colours set out in the Table. The assessment can also take place remission photometrically.

substrate	diazonium salt	colour
S1	D1	red-brown
S1	D2	red-brown
S1	D3	blue-grey
S1	D4	green
S2	D1	red
S2	D2	bright red
S2	D3	violet
S2	D4	lilac
S3	D1	red-violet
S3	D2	violet
S3	D3	blue-grey
S4	D1	lilac
S5	D1	lilac
S6	D1	bright red
S7	D1	bright red
S8	D1	red-violet
S9	D1	red-violet

Example 2

Filter paper is successively impregnated with the following solutions and dried at 60°C.

Solution 1

0.1 molar borate buffer pH 8

Solution 2

2×10^{-3} mol/l. 2-methoxy-4-(N-morpholino)-benzene-diazonium tetrachlorozincate

2×10^{-3} mol/l. substrate +

10% phosphoric acid trimorpholide in ethanol/1-decanol 98:2

+ substrate

A: 3-[N-benzyloxycarbonyl-L-alanyloxy]-indole

B: 3-[N-benzyloxycarbonyl-L-alanyloxy]-5-methylindole

C: 3-[N-benzyloxycarbonyl-L-alanyloxy]-7-methylindole

D: 3-[N-benzyloxycarbonyl-L-alanyloxy]-4-chloroindole

E: 3-[N-benzyloxycarbonyl-L-alanyloxy]-5-bromoindole

F: 3-[N-(toluene-4'-sulphonyl)-L-alanyloxy]-indole

G: 3-[N-(toluene-4'-sulphonyl)-L-alanyloxy]-5-methoxy-indole

H: 3-[N-(4'-acetamidobenzenesulphonyl)-L-alanyloxy]-indole

I: 3-[N-(4-methoxytosyl)-L-alanyloxy]-indole

K: [N-(toluene-4'-sulphonyl)-L-alanyloxy]-benzene

L: 1-[N-(toluene-4'-sulphonyl)-L-alanyloxy]-naphthalene

M: 1-[N-(toluene-4'-sulphonyl)-L-alanyloxy]-3-methoxy-benzene

With the so obtained reagent papers, 100 leukocytes/μl. can be detected in leukocyte-containing urines.

Reagent papers with comparable reactivity can also be obtained when, instead of the above-mentioned diazonium salt, one (uses)

2-methoxy-4-(N-pyrrolidino)-benzenediazonium tetrafluoroborate

2-methoxy-4-(N-piperidino)-benzenediazonium tetrafluoroborate

2,5-dimethoxy-4-(N-morpholino)-benzendiazonium tetrafluoroborate

4-methoxy-2-(N-morpholino)-benzenediazonium tetrafluoroborate

2-methoxy-4-[N-(N-methyl)-piperazino]-benzenediazonium tetrafluoroborate

2-methoxy-4-(N-thiomorpholino)-benzenediazonium tetrafluoroborate.

Example 3

The following parent solutions are prepared:

Solution 1

2×10^{-3} mol/l. 1-[N-(toluene-4'-sulphonyl)-L-alanyloxy]-4-isopropoxynaphthalene

10^{-2} mol/l. 2,4-dimethoxybenzenediazonium tetrafluoroborate

in acetone/1-decanol 98:2

Solution 2

0.2 molar borax-hydrochloric acid buffer

10% phosphoric acid trimorpholide

After, in each case, the activators given below are added in the there-given concentrations either to Solution 1 or to Solution 2, filter papers are impregnated with the Solutions 1 and 2 and, in each case, dried at 60°C.

In the case of the testing in isotonic common salt solution with 300 leukocytes/μl., the tests react in the given times

activator	mg. activator addition to 20 ml. of solution 1	solution 2	reaction time [sec.]
without	-	-	54
4-azafluorene	33	-	17
benzo-(h)-quinoline	36	-	8
quinine	33	-	32
1,2-bis-4-pyridyl-ethylene	37	-	34
1-decanol	400	-	5
1-tetradecanol	400	-	4
sodium nitroprusside	-	31	38
potassium hexacyano-ferrate (II)	-	40	30

Example 4

Filter paper is impregnated with the following solutions and, after the impregnation, in each case dried at 60°C.

Solution 1

2×10^{-3} mol/l. 3-(N-toluene-4'-sulphonyl-L-alanyloxy)-indole

2×10^{-3} mol/l. 2-methoxy-4-morpholinobenzenediazonium tetrachlorozincate

in acetone/1-deconal 98:2

Solution 2

0.2 molar borax-hydrochloric acid buffer pH 8

10% phosphoric acid trimorpholide.

In a further experiment, in Solution 2, phosphoric acid trimorpholide was omitted.

The samples with phosphoric acid trimorpholide remain, after a stressing with 60°C. for 2 days, uncoloured and provide good reactions with leukocyte-containing solutions. The samples without phosphoric acid trimorpholide show grey discolorations.

Example 5

In known manner, there was produced a reagent tablet which contained the following components:

2 mg. 3-(N-toluene-4'-sulphonyl-L-alanyloxy)-indole

2 mg. 2-methoxy-4-(N-morpholino)-benzenediazonium tetrachlorozincate

1.5 mg. potassium dihydrogen phosphate

30 mg. disodium hydrogen phosphate dihydrate

20 mg. mannitol.

This tablet was dissolved in 2 ml. leukocyte-containing urine and well mixed up. In the presence of leukocytes, a red-violet coloration occurred. 100 Leukocytes could thus be detected in about 2 minutes.

If, before the tablet addition and during the reaction, the urine was warmed to 37°C., then, in the same time, 20 leukocytes could be detected.

Example 6

1-[N-(Toluene-4'-sulphonyl)-L-alanyloxy]-3,5-dimethoxy-benzene

For the reaction according to the active ester process, 14.6 g. (0.06 mole) N-(toluene-4-sulphonyl)-L-alanine and 12.2 g. (0.09 mole) N-hydroxybenzotriazole are dissolved in 300 ml. abs. ethyl acetate, cooled to 0°C. and mixed with 12.4 g. (0.06 mole) dicyclohexyl-carbodiimide. For the formation of the active ester, one stirs for 2 hours at 0°C., then for a further 2 hours at room temperature. After the addition of 6.2 g. (0.04 mole) 3,5-dimethoxyphenol and 5.5 ml. (0.04 mole) triethylamine, the mixture is stirred for 15 hours at room temperature. The N,N'-dicyclohexylurea formed is filtered off with suction. The filtrate is evaporated in a vacuum at a maximum bath temperature of 50°C. The residue is taken up in 200 ml. ethyl acetate and

successively washed, in each case three times, with, in each case, 100 ml. 5% citric acid solution and then correspondingly with, in each case, 100 ml. 5% sodium hydrogen carbonate solution. The organic phase is, after drying with sodium sulphate, evaporated in a vacuum. The oily crude product is purified column chromatographically on a silica gel column with a toluene-ethyl acetate mixture (4:1). After evaporation of the corresponding collected fractions, the residue is dissolved in a little methylene chloride and precipitated out by the addition of ether. There are obtained 5.8 g. (38%) 1-[N-(toluene-4'-sulphonyl)-L-alanyloxy]-3,5-dimethoxybenzene; colourless crystals, m.p. 110°C.

α_D^{20} : -52.5° (c = 1%, acetone)

In analogous manner, by reaction of N-(toluene-4-sulphonyl)-L-alanine with the correspondingly substituted phenols or naphthols, one obtains the following substances:

6.1 [N-(toluene-4'-sulphonyl)-L-alanyloxy]-benzene, colourless crystals, m.p. 113°C.

α_D^{20}: -63.8° (c = 1%, acetone)

6.2 1-[N-(toluene-4'-sulphonyl)-L-alanyloxy]-3-dimethylaminobenzene

colourless, amorphous powder

α_D^{20}: -36.6° (c = 1%, methanol)

TLC: finished plate silica gel (elution agent: toluene-dioxan 2:1, detection: UV, R_F value: 0.68)

6.3 1-[N-(toluene-4'-sulphonyl)-L-alanyloxy]-3-methoxy-benzene

colourless crystals, m.p. 60 - 61°C.

α_D^{20}: -62.9° (c = 1%, methanol)

6.4 1-[N-(toluene-4'-sulphonyl)-L-alanyloxy]-naphthalene

colourless viscous oil

α_D^{20}: -2.73° (c = 1%, methanol)

TLC: finished plate silica gel (elution agent: toluene-dioxan 4:1, detection: UV, R_F value: 0.53)

6.5 1-[N-(toluene-4'-sulphonyl)-L-alanyloxy]-4-methoxy-naphthalene

colourless crystals, m.p. 136°C.

α_D^{20}: -42.6° (c = 1%, acetone)

6.6 1-[N-(toluene-4'-sulphonyl)-L-alanyloxy]-4-isopropoxynaphthalene

colourless crystals, m.p. 93 - 96°C.

α_D^{20} : -38.0° (c = 1%, acetone)

6.7 1-[N-(toluene-4'-sulphonyl)-L-alanyloxy]-4-pentoxy-naphthalene

colourless crystals, m.p. 87°C.

α_D^{20}: -36.9° (c = 1%, acetone)

6.8 1-[N-(toluene-4'-sulphonyl)-L-alanyloxy]-3-diethyl-aminobenzene

colourless crystals, m.p. 83 - 84°C.

α_D^{20}: -59.4° (c = 1%, methanol)

TLC: finished plate silica gel (elution agent: xylene-methyl ethyl ketone 1:1, R_F value: 0.68)

6.9 1-[N-(toluene-4'-sulphonyl)-L-alanyloxy]-3-methyl-5-methoxybenzene

colourless crystals, m.p. 79 - 81°C.

α_D^{20}: 62.2° (c = 1%, methanol)

TLC: finished plate (elution agent: chloroform-methanol 20:1, R_F value: 0.79)

Example 7

2-[N-(Toluene-4'-sulphonyl)-L-alanyloxy]-4-methoxy-naphthalene

Solution 1

For the preparation of the acid chloride according to the one-step method, 19.44 g. (0.08 mole) N-(toluene-4-sulphonyl)-L-alanine are dissolved in 100 ml. abs. dimethylformamide and cooled to -20°C. 5.81 ml. (0.08 mole) Thionyl chloride are then pipetted thereto, with stirring and cooling, and the reaction mixture left for 30 minutes in a cold bath at -20°C.

Solution 2

11.0 ml. (0.08 mole) triethylamine are added to a solution of 6.96 g. (0.04 mole) 4-methoxynaphthol-2 in 50 ml. abs. dimethylformamide. The mixture is cooled to -20°C.

Reaction

One pours solution 1 into solution 2 and stirs, with the exclusion of water, for about 4 hours at -20°C., thereafter leaves the mixture overnight in a refrigerator.

For working up, the reaction solution is evaporated in a vacuum at a maximum bath temperature of 50°C. The residue is taken up in about 150 ml. ethyl acetate and successively washed, in each case twice, with, in each case, 100 ml. 5% citric acid and then with, in each case, 100 ml. 5% sodium hydrogen carbonate solution. After drying with sodium sulphate, the organic phase is evaporated in a vacuum. The crude product is purified column chromatographically on a silica gel column with a toluene-ethyl acetate mixture (4:1). After evaporation of the corresponding collected fractions in a vacuum, the residue is dissolved in a little methylene chloride and then precipitated with an ether-ligroin mixture (1:2). There are obtained 2.8 g. (18%) 2-[N-(toluene-4'-sulphonyl)-L-alanyloxy]-4-methoxynaphthalene; colourless crystals, m.p. 119°C.

α_D^{20}: -57.9° (c = 1%, acetone).

Example 8

2-Methoxy-4-(N-morpholino)-benzenediazonium tetrachlorozincate

5-Chloro-2-nitroanisole is reacted with a 1.5 times molar excess of morpholine in toluene as solvent by several hours heating under reflux (10 - 14 hours). The reaction can also be expediently carried out in morpholine as solvent. For this purpose, the 5-chloro-2-nitroanisole is mixed with the 5 to 10 fold volume of morpholine. Possibly demethylated product is

separated off by shaking with aqueous sodium hydroxide solution or post-methylated by reaction with diazomethane.

The nitro compound obtained is reduced to the amide in the usual way with palladium/charcoal in methanol or tin (II) chloride in hydrochloric acid and this is diazotised. The diazonium compound is converted into the tetrachlorozincate in known manner in hydrochloric acid solution by the addition of concentrated zinc chloride solution or, by the addition of tetrafluoroboric acid, is converted into the tetrafluoroborate and isolated as such.

m.p. 170 - 172°C. (tetrachlorozincate)

166 - 168°C. (tetrafluoroborate)

Example 9

4-Methoxy-2-(N-morpholino)-benzenediazonium tetrafluoroborate

18.76 g. (0.1 mole) 3-Chloro-4-nitroanisole, m.p. 52°C., are heated under reflux for 4 hours with 87.1 g. (1 mole) morpholine. Thereafter, one cools to room temperature, adds 100 ml. ice water to the reaction solution, filters off with suction the separated yellow reaction product, washes with ice-cooled 10% acetic acid and dries the material obtained at 60°C. in a vacuum. There result 19.5 g. of a mixture of N-(3-hydroxy-6-nitrophenyl)-morpholine and N-(3-methoxy-6-nitrophenyl)-morpholine. One dissolves

this product in methylene chloride and adds thereto an ethereal solution of diazomethane. After standing for two days at room temperature, the excess diazomethane is destroyed by the dropwise addition of 2N acetic acid, the organic phase is shaken up several times with 2N aqueous sodium hydroxide solution and the solvent evaporated off in a vacuum. One obtains 19.1 g. (= 80.2%) N-(3-methoxy-6-nitrophenyl)-morpholine, m.p. 84 - 86°C. Subsequently, this substance is suspended in 250 ml. methanol and, after the addition of 1.9 g. palladium-charcoal (10%), hydrogenated at 20 - 30°C.

After filtering off the catalyst with suction, one evaporates substantially and converts the liberated base into the hydrochloride by the addition of ethereal hydrochloric acid. There crystallise 20.6 g. (= 73.1%) N-(3-methoxy-6-aminophenyl)-morpholine dihydrochloride, m.p. 237 - 240°C.

One suspends this material at -5°C. in 96 ml. 6N hydrochloric acid and adds dropwise thereto, within 30 minutes, a solution of 6 g. sodium nitrite in 12 ml. water; one adds the resultant brown-red diazonium salt solution at 0°C., with stirring, to 120 ml. 35% tetrafluoroboric acid. After standing for several hours, one obtains 19.5 g. (= 63.5% of theory) 4-methoxy-2-(N-morpholino)-benzenediazonium tetrafluoroborate; yellow crystals, m.p. 115 - 116°C. (decomp.).

In analogous manner, one obtains

a) from 5-chloro-2-nitroanisole and thiomorpholine, 2-methoxy-4-(N-thiomorpholino)-benzenediazonium tetrafluoroborate

m.p. 106 - 108°C. (decomp.)

The intermediate product 2-amino-5-(N-thiomorpholino)-anisole dihydrochloride is prepared as follows:

Into a 1 l. three-necked flask, with stirrer, one introduces 100 g. tin II chloride dihydrate in 400 ml. 6N hydrochloric acid and adds thereto portionwise at room temperature, with stirring, 25.4 g. (1 mole) 2-nitro-5-(N-thiomorpholino)-anisole. Thereafter, one heats for 30 minutes to 75°C., cools to room temperature and drops the reaction solution, with ice cooling, into 750 ml. 30% aqueous sodium hydroxide solution. One extracts the liberated base several times with ether and converts this, after drying and partial evaporation of the solvent, by addition of ethereal hydrochloric acid, into the hydrochloride. One obtains 24.9 g. (= 83.8% of theory) 2-amino-5-(N-thiomorpholino)-anisole dihydrochloride, m.p. 218 - 220°C.

b) from 5-chloro-2-nitroanisole and piperidine, 2-methoxy-4-(N-piperidino)-benzenediazonium tetrafluoroborate

m.p. 123 - 125°C. (decomp.).

c) from 5- chloro-2-nitroanisole and pyrrolidine, 2-methoxy-4-(N-pyrrolidino)-benzenediazonium tetrafluoroborate

m.p. 137 - 139°C. (decomp.)

d) from 5-chloro-2-nitroanisole and N-methylpiperazine, 2-methoxy-4-[N-(N'-methyl)-piperazino]-benzenediazonium tetrafluoroborate dihydro-tetrafluoroborate

m.p. 163°C. (decomp.)

In the case of the preparation of this compound, the diazotisation is carried out with amyl nitrite in methanol. For this purpose, 39.6 g. (0.1 mole) 2-amino-5-(N-methylpiperazino)-anisole dihydro-tetrafluoroborate are dissolved in 175 ml. methanol, 11.6 g. (0.1 mole) amyl nitrite in 25 ml. methanol added thereto and 60 ml. 35% tetrafluoroboric acid slowly added dropwise at 0°C., with stirring. Upon standing, there crystallise 24.8 g. (= 51.7% of theory) of brownish crystals of 2-methoxy-4-[N-(N'-methyl)-piperazino]-benzenediazonium tetrafluoroborate dihydro-tetrafluoroborate

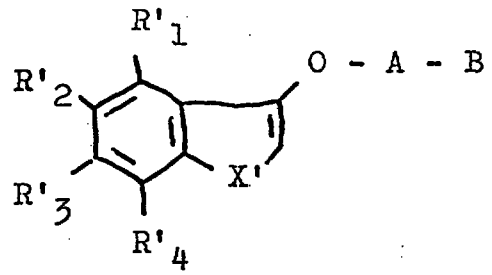
m.p. 163°C. (decomp.)

e) from 5-chloro-2-nitroanisole and morpholine, 2-methoxy-4-(N-morpholino)-benzenediazonium tetrafluoroborate

m.p. 166 - 168°C. (decomp.)

Patent Claims

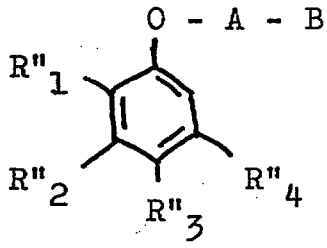
1. Use of agents for the detection of esterolytic and/or proteolytic enzymes, consisting of an absorbent carrier, a film layer, a powder mixture, a lyophilisate, a solution or a reagent tablet, containing a suitable esterase and/or protease detection principle, a buffer, as well as possibly further usually employed additive materials for the detection of leukocytes in urine, whereby the agent is added to the urine to be investigated and the resulting colour formation is assessed visually or photometrically, characterised in that as esterase and/or protease detection principle, there is used a combination of a specially substituted diazonium salt and a suitable ester, the reactivity of which is atuned to one another by suitable choice of the substituents in such a manner that admittedly a sufficiently rapid coupling takes place with the alcohol components liberated from the ester but no reaction with other components of the test solution, whereby the ester is selected from the group of compounds of the general formula II



(II),

in which R'_1, R'_2, R'_3, R'_4, which can be the same or different, each signify a hydrogen or halogen atom, a

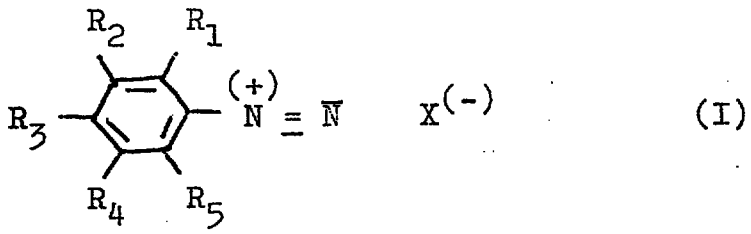
lower alkyl, a lower alkoxy, an aryl, an aralkyl, an aralkoxy, a hydroxyl, a carboxyl, a carboxy-lower-alkoxy, an aralkoxycarbonyl, an aralkoxycarbonyl-lower-alkoxy, a nitro or a lower acylamino group or in each case two neighbouring substituents a benzo-annellated radical possibly substituted by halogen, X' a sulphur atom or an imino group possibly substituted by a lower alkyl, an aryl, an aralkyl or an acyl radical, A an amino acid or a peptide residue, B a nitrogen protective group usual in peptide chemistry or derived therefrom, or of the compounds of the general formula III



(III),

in which $R"_1$, $R"_2$, $R"_3$, $R"_4$, which can be the same or different, each signify hydrogen, a lower alkyl, a lower alkoxy, alkylamino or dialkylamino group, whereby two neighbouring substituents can also represent an annellated benzene ring, A and B have the above-given meaning.

2. Use of agents according to claim 1, characterised in that the esterase and/or protease detection principle contains as diazonium salt a compound of the general formula I



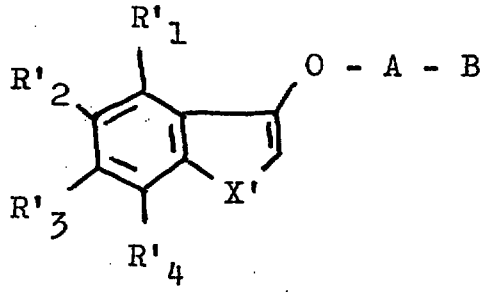
(I)

in which R_1 signifies a lower alkyl, a lower alkoxy, a lower alkylmercapto, an N-morpholino, an N-thiomorpholino, an N-pyrrolidino, a possibly N'-alkylated N-piperazino, an N-piperidino group, halogen or hydrogen, R_3 a lower alkyl, a lower alkoxy, an aryloxy, a lower alkylmercapto, alkylamino, dialkylamino, a hydroxyl, an N-morpholino, N-thiomorpholino, N-pyrrolidino, N-piperidino, phenylamino, a phenyl group possibly substituted with a lower alkyl or lower alkoxy radical, halogen or hydrogen, R_2, R_4, R_5, which can be the same or different, each signify a lower alkyl, a lower alkoxy, a lower alkylmercapto group, halogen or hydrogen, whereby R_1 and R_2 together can represent an annellated benzene ring, and X a stabilising anion.

3. Use according to claim 1, characterised in that, as additional adjuvant agents, there are used wetting agents, stabilisers, activators, film formers, galenical additive materials and/or structure formers.

4. Agent for the detection of esterolytic and/or proteolytic enzymes in leukocytes in urine, consisting of an absorbent carrier, a film layer, a powder mixture, a lyophilisate, a solution or a reagent tablet, containing a suitable esterase and/or

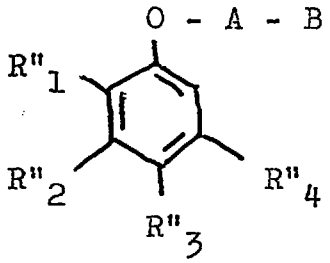
protease detection principle, a buffer, as well as possibly further additive materials usually employed, characterised in that, as esterase and/or protease detection principle, there is used a combination of a specially substituted diazonium salt and a suitable ester, the reactivity of which is atuned to one another by suitable choice of the substituents in such a manner that admittedly a sufficiently rapid coupling takes place with the alcohol components liberated from the ester but no reaction with other components of the test solution, whereby the ester is selected from the group of compounds of the general formula II



(II)

in which R'_1, R'_2, R'_3, R'_4, which can be the same or different, each signify a hydrogen or halogen atom, a lower alkyl, a lower alkoxy, an aryl, an aralkyl, an aralkoxy, a hydroxyl, a carboxyl, a carboxy-lower-alkoxy, an aralkoxycarbonyl, an aralkoxycarbonyl-lower-alkoxy, a nitro or a lower acylamino group, or in each case two neighbouring substituents a benzo-annellated radical possibly substituted by halogen, X' a sulphur atom or an imino group possibly substituted by a lower alkyl, an aryl, an aralkyl or an acyl radical, A an

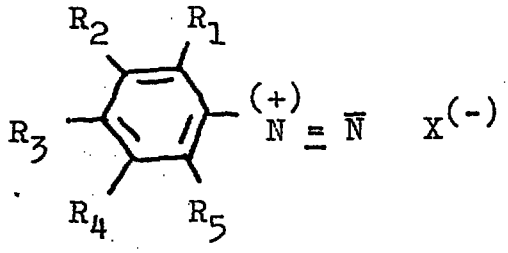
amino acid or peptide residue, B a nitrogen protective group usual in peptide chemistry or derived therefrom, or of the compounds of the general formula III



(III),

in which R''_1, R''_2, R''_3, R''_4, which can be the same or different, each signify hydrogen, a lower alkyl, a lower alkoxy, alkylamino or dialkylamino group, whereby two neighbouring substituents can also represent an annellated benzene ring, A and B have the above-given meaning, whereby, when R''_1 and R''_2 or R''_2 and R''_3 is an annellated benzene ring, B does not represent an acetyl or benzyloxycarbonyl group.

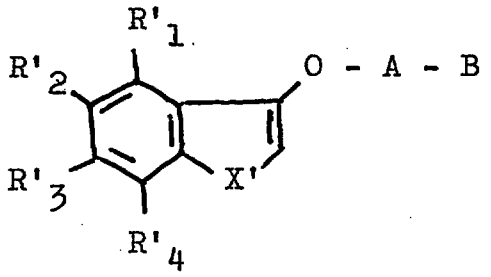
5. Agent according to claim 4, characterised in that as esterase and/or protease detection principle, there is used a combination of a diazonium salt of the general formula I



(I),

in which R_1 signifies a lower alkyl, a lower alkoxy, a lower alkylmercapto, an N-morpholino, an N-thiomorpholino, an N-pyrrolidino, a possibly N'-alkylated

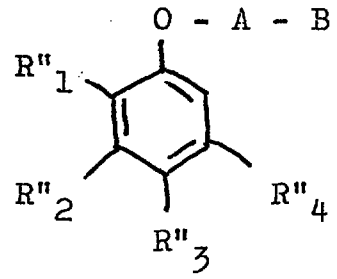
N-piperazino, an N-piperidino group, halogen or hydrogen, R_3 a lower alkyl, a lower alkoxy, an aryloxy, a lower alkylmercapto, alkylamino, dialkylamino, a hydroxyl, an N-morpholino, N-thiomorpholino, N-pyrrolidino, a possibly N-alkylated N-piperazino, N-piperidino, phenylamino, a phenyl group possibly substituted with a lower alkyl or lower alkoxy radical, halogen or hydrogen, R_2, R_4, R_5, which can be the same or different, each signify a lower alkyl, a lower alkoxy, a lower alkylmercapto group, halogen or hydrogen, whereby R_1 and R_2 can together represent an annellated benzene ring, and X a stabilising anion, and an ester selected from the group of compounds of the general formula II



(II),

in which R'_1, R'_2, R'_3, R'_4, which each can be the same or different, each signify a hydrogen or halogen atom, a lower alkyl, a lower alkoxy, an aryl, an aralkyl, an aralkoxy, a hydroxyl, a carboxyl, a carboxy-lower-alkoxy, an aralkoxycarbonyl, an aralkoxycarbonyl-lower-alkoxy, a nitro or a lower acylamino group or in each case two neighbouring substituents a benzo-annellated radical possibly substituted by halogen, X' a sulphur atom or an imino group possibly substituted

by a lower alkyl, an aryl, an aralkyl or an acyl radical, A an amino acid or a peptide residue, B a nitrogen protective group usual in peptide chemistry or derived therefrom, or of the compounds of the general formula III

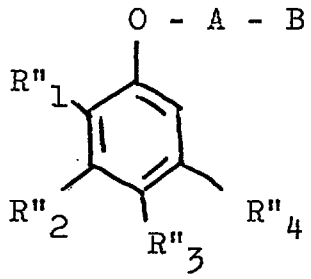


(III)

in which $R"_1$, $R"_2$, $R"_3$, $R"_4$, which can be the same or different, each signify hydrogen, a lower alkyl, a lower alkoxy, alkylamino or dialkylamino group, whereby two neighbouring substituents can also represent an annellated benzene ring, A and B have the above-given meaning, whereby, when $R"_1$ and $R"_2$ or $R"_2$ and $R"_3$ is an annellated benzene ring, B does not represent an acetyl or benzyloxycarbonyl group.

6. Agent according to claim 4, characterised in that, as addition adjuvent agents, there are used wetting agents, stabilisers, activators, film formers, galenical additive materials and/or structure formers.

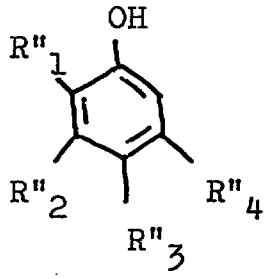
7. Phenoxyamino acid esters and peptide esters of the general formula III



(III)

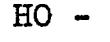
in which R''_1, R''_2, R''_3, R''_4, which can be the same or different, each signify hydrogen, a lower alkyl, a lower alkoxy, alkylamino or dialkylamino group, whereby two neighbouring substituents can also represent an annellated benzene ring, A and B have the above-given meaning, whereby, when R''_1 and R''_2 or R''_2 and R''_3 is an annellated benzene ring, B does not represent an acetyl or benzyloxycarbonyl group.

8. Process for the preparation of phenoxyamino acid esters and peptide esters according to claim 7, characterised in that one reacts compounds of the general formula IV



(IV)

in which R''_1, R''_2, R''_3 and R''_4 have the above-given meaning, with amino acids and peptides of the general formula V



(V)

in which A and B have the above-given meaning, or suitable reactive derivatives thereof according to

methods usual in peptide chemistry.

9. Use of compounds according to claim 7 for the preparation of agents according to claim 4.