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(54) **COMPOSITIONS CONTENANT UN AGENT DE BLANCHIMENT
ET DES PROTEASES**

(54) **BLEACHING COMPOSITIONS COMPRISING PROTEASE
ENZYMES**

(57) La présente invention concerne des compositions de nettoyage contenant un agent de blanchiment et une enzyme proléolytique qui est une carbonyle hydrolase modifiée ayant une séquence d'acides aminés que l'on ne trouve pas dans la nature et où on a remplacé une pluralité de groupes acides aminés de la carbonyle hydrolase initiale, par d'autres acides aminés. Ce remplacement d'acides aminés de l'enzyme initiale concerne l'acide aminé en position +76, ainsi qu'un ou plusieurs des acides aminés dans les positions suivantes: +99, +101, +103, +104, +107, +123, +27, +105, +109, +126, +128, +135, +156, +166, +195, +197, +204, +206, +210, +216, +217, +218, +222, +260, +265 et/ou +274. Les positions numérotées des acides aminés correspondent à celles des acides aminés de la subtilisine naturelle de *Bacillus amyloliquefaciens* ou à celles d'autres carbonyle hydrolases ou subtilisines (par exemple subtilisine de *Bacillus lentus*).

(57) The invention herein provides bleaching compositions comprising a protease enzyme which is a carbonyl hydrolase variant having an amino acid sequence not found in nature, which is derived by replacement of a plurality of amino acid residues of a precursor carbonyl hydrolase with different amino acids, wherein said plurality of amino acid residues replaced in the precursor enzyme correspond to position +76 in combination with one or more of the following residues: +99, +101, +103, +104, +107, +123, +27, +105, +109, +126, +128, +135, +156, +166, +195, +197, +204, +206, +210, +216, +217, +218, +222, +260, +265, and/or +274, where the numbered positions correspond to naturally-occurring subtilisin from *Bacillus amyloliquefaciens* or to equivalent amino acid residues in other carbonyl hydrolases or subtilisins (such as *Bacillus lentus* subtilisin) and a bleaching agent.



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(54) Title: BLEACHING COMPOSITIONS COMPRISING PROTEASE ENZYMES			
(57) Abstract The invention herein provides bleaching compositions comprising a protease enzyme which is a carbonyl hydrolase variant having an amino acid sequence not found in nature, which is derived by replacement of a plurality of amino acid residues of a precursor carbonyl hydrolase with different amino acids, wherein said plurality of amino acid residues replaced in the precursor enzyme correspond to position +76 in combination with one or more of the following residues: +99, +101, +103, +104, +107, +123, +27, +105, +109, +126, +128, +135, +156, +166, +195, +197, +204, +206, +210, +216, +217, +218, +222, +260, +265, and/or +274, where the numbered positions correspond to naturally-occurring subtilisin from <u>Bacillus amyloliquefaciens</u> or to equivalent amino acid residues in other carbonyl hydrolases or subtilisins (such as <u>Bacillus lentus</u> subtilisin) and a bleaching agent.			

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BLEACHING COMPOSITIONS COMPRISING
PROTEASE ENZYMES

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FIELD OF THE INVENTION

The present invention relates to bleaching compositions, especially laundry detergents, and methods which employ one or more protease enzymes which are carbonyl hydrolase variants and a bleaching system with one or more bleaching agents, especially bleach
25 activators.

BACKGROUND OF THE INVENTION

Various types of enzymes have long been conventionally used in laundry detergents to assist in the removal of certain stains from fabrics. These stains are typically associated with lipid and protein
30 soils. The enzymes, however, have proven less effective against other types of soils and stains.

It has also long been known that peroxygen bleaches are effective for stain and/or soil removal from fabrics, but that such bleaches are temperature dependent. At a laundry liquor temperature
35 of 60°C, peroxygen bleaches are only partially effective. As the laundry liquor temperature is lowered below 60°C, peroxygen bleaches become relatively ineffective. As a consequence, there has been a

substantial amount of industrial research to develop effective bleaching systems.

5 By the present invention, it has been discovered that the combination of novel protease enzymes which are carbonyl hydrolase variants and bleaching agents, especially bleach activators, provide effective stain removal and/or dingy cleanup benefits. It is therefore an object of the present invention to provide bleaching compositions, especially laundry detergent compositions, having improved stain removal and/or dingy cleanup benefits and/or fabric cleaning benefits and/or bleaching properties.

10 These and other objects of the present invention will be apparent from the detailed description hereinafter.

BACKGROUND ART

15 U.S. Patent 4,634,551, Burns et al, issued January 6, 1987, discloses amido peroxyacid bleaching compounds and their precursors which are employed in the present invention. See also, U.S. Patent 4,852,989, Burns et al, issued August 1, 1989. U.S. Patent 5,069,809, Lagerwaard et al, issued December 3, 1991 disclose the combination of NOBS bleach activators with LIPOLASE, lipase enzymes. See EP Patent 341,947, Lagerwaard, et al, published November 15, 1989 for a discussion of the compatibility problems of lipase enzymes with certain bleaching systems. 20 U.S. Patent 4,545,784, Sanderson, issued October 8, 1985, discloses the absorption of activators onto sodium perborate monohydrate.

SUMMARY OF THE INVENTION

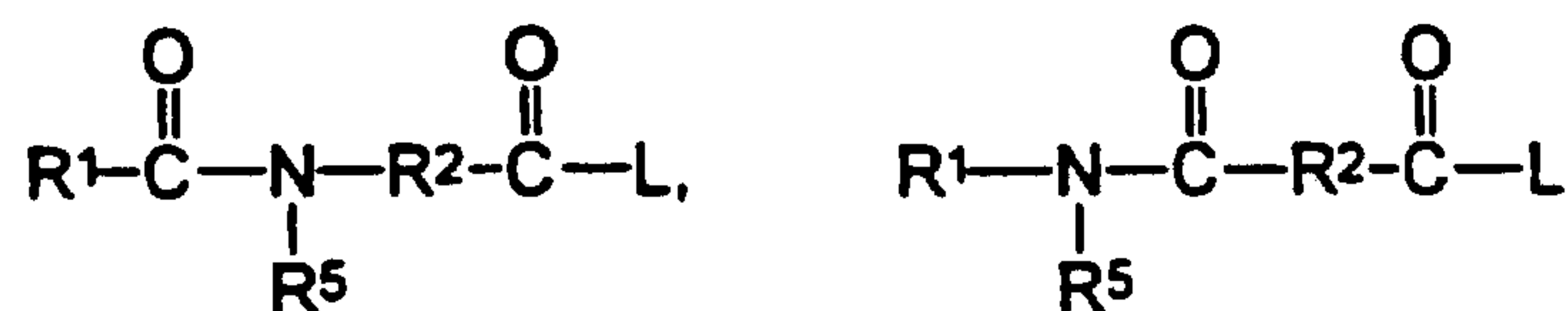
The invention herein provides a bleaching composition comprising:

25 (a) from about 0.0001% to about 10% by weight of the bleaching composition of a protease enzyme which is a carbonyl hydrolase variant having an amino acid sequence not found in nature, which is derived from a precursor carbonyl hydrolase comprising a substitution of a different amino acid for a plurality of amino acid residues at a position in said precursor carbonyl hydrolase equivalent to position +76 in *Bacillus amyloliquefaciens* subtilisin, in combination with one or more amino acid residue positions 30 equivalent to those selected from the group consisting of +99, +101, +103,

+104, +107, +123, +27, +105, +109, +126, +128, +135, +156, +166, +195, +197, +204, +206, +210, +216, +217, +218, +222, +260, +265, and/or +274 in *Bacillus amyloliquefaciens* subtilisin; provided that: i) when said carbonyl hydrolase variant includes a substitution of amino acid residues at positions equivalent to +76 and +195, there is also a substitution of an amino acid residue at one or more amino acid residue positions other than amino acid residue positions equivalent to positions +99; and ii) when said carbonyl hydrolase variant includes a substitution of amino acid residues at positions equivalent to +76 and +156, there is also a substitution of an amino acid residue at one or more amino acid residue positions other than amino acid residue positions equivalent to positions +99; and (b) a bleaching agent which either is an organic peroxyacid or is a combination of a bleach activator and a peroxygen compound capable of yielding hydrogen peroxide that can react with the activator to form an organic peroxyacid in situ in a bleaching solution formed from the composition; and (c) one or more cleaning composition materials compatible with the protease enzyme and bleaching agent.

In a further embodiment the invention provides for a fabric cleaning composition comprising: (a) from about 0.0001% to about 10% protease enzyme which is an N76D/S103A/V104I subtilisin variant derived from *Bacillus lentus* subtilisin; (b) from about 0.5% to about 20% of a bleaching system comprising at least about 0.1% by weight of a peroxygen bleaching compound capable of yielding hydrogen peroxide in an aqueous liquor and at least 0.1% by weight of one or more bleach activators, wherein said bleach activators are members selected from the group consisting of:

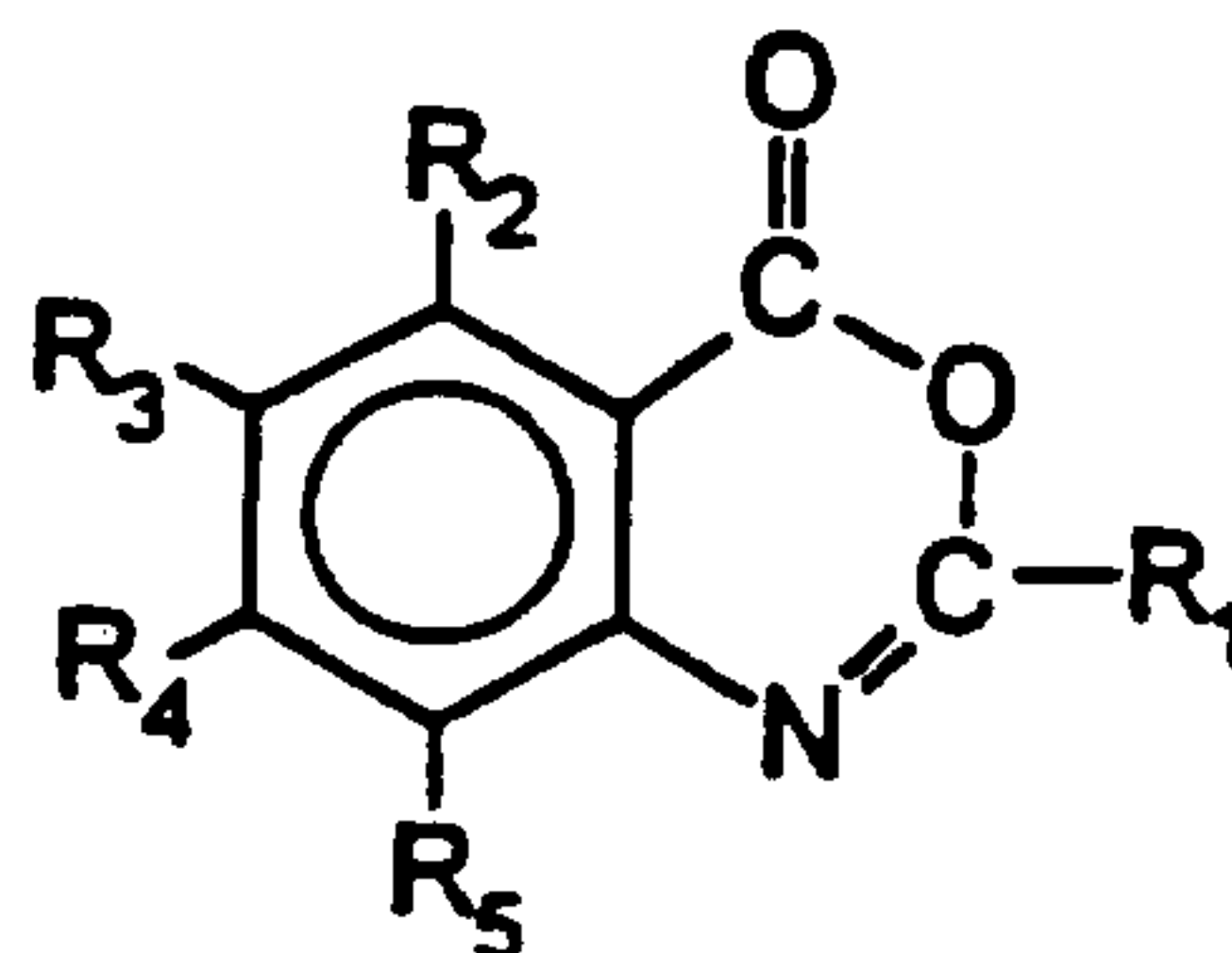
i) a bleach activator of the general formula:



2b

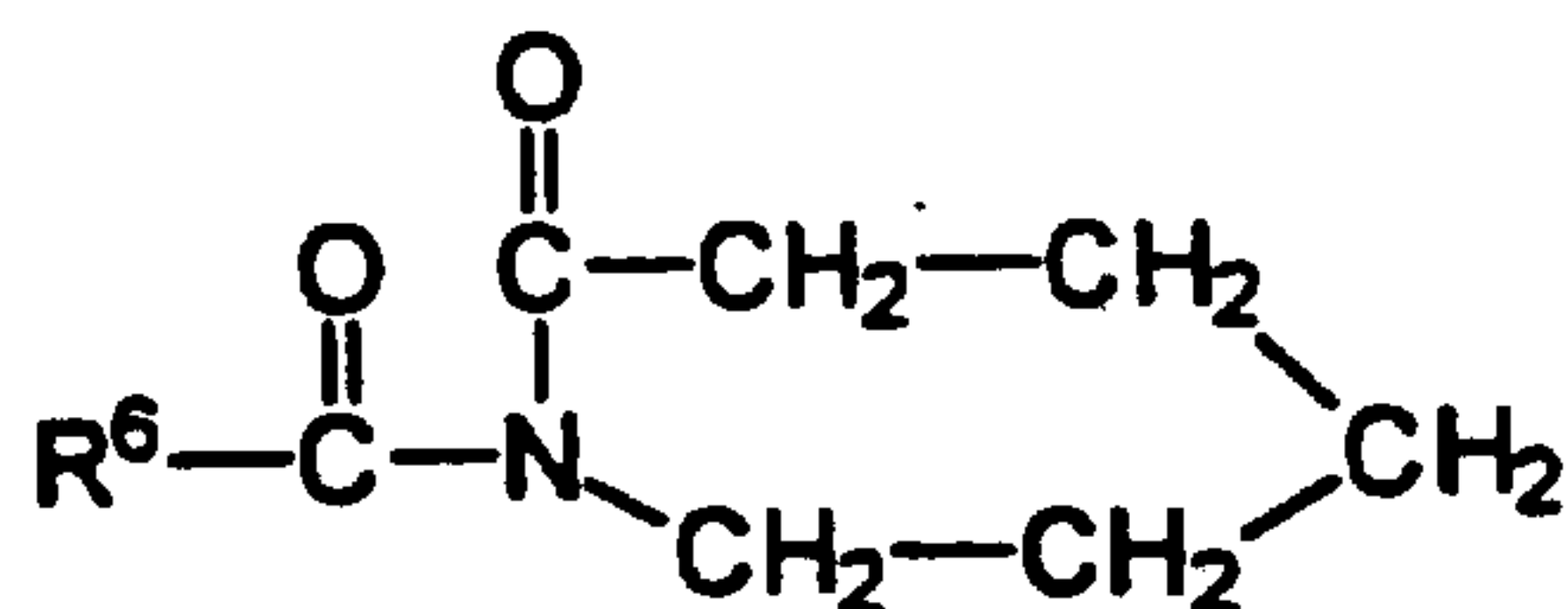
or mixtures thereof, wherein R^1 is an alkyl, aryl, or alkaryl group containing from about 1 to about 14 carbon atoms, R^2 is an alkylene, arylene or alkarylene group containing from about 1 to about 14 carbon atoms, R^5 is H or an alkyl, aryl, or alkaryl group containing from about 1 to about 10 carbon atoms, and L is a leaving group;

ii) a benzoxazin-type bleach activator of the formula:



wherein R_1 is H, alkyl, alkaryl, aryl, arylalkyl, and wherein R_2 , R_3 , R_4 , and R_5 may be the same or different substituents selected from H, halogen, alkyl, alkenyl, aryl, hydroxyl, alkoxyl, amino, alkylamino, $-COOR_6$, wherein R_6 is H or an alkyl group and carbonyl functions;

iii) a N-acyl caprolactam bleach activator of the formula:



wherein R^6 is H or an alkyl, aryl, alkoxyaryl, or alkaryl group containing from 1 to 12 carbons; and

iv) mixtures of i), ii) and iii);

- 15 (c) at least about 5% surfactant;
- (d) at least about 5% builder; and
- (e) optionally, one or more cleaning composition materials compatible with the protease enzyme and bleaching system selected from the group consisting of solvents, buffers, enzymes, soil release agents, clay soil

removal agents, dispersing agents, brighteners, suds suppressors, fabric softeners, suds boosters, enzyme stabilizers, dyes, perfumes, and mixtures thereof.

5 The invention also encompasses a method for cleaning fabrics comprising contacting, preferably with agitation, said fabrics with an aqueous liquor containing said bleaching compositions. The method can be carried out at temperatures below about 60°C but, of course, is quite effective at laundry temperatures up to the boil. The aqueous laundry liquor preferably comprises at least about 300 ppm of conventional detergent ingredients, as
10 well as at least about 25 ppm of bleach activator and at least about 25 ppm of bleaching compound. Preferably, said aqueous liquor comprises from about 900 ppm to about 20,000 ppm of the conventional detergent ingredients, from about 100 ppm to about 25,000 ppm of bleaching compound and from about 100 ppm to about 2,500 ppm of said bleach
15 activator.

The conventional detergent ingredients employed in said method comprise from about 1% to about 99.8%, preferably from about 5% to about 80%, of a deterative surfactant. Optionally, deterative compositions can also comprise from about 5% to about 80% of a detergent builder. Other optional
20 deterative ingredients are also encompassed by the fully-formulated detergent/bleach compositions provided by this invention.

All percentages, ratios and proportions are by weight, unless otherwise specified.

BRIEF DESCRIPTION OF THE DRAWINGS

25 Figs. 1 A-C depict the DNA and amino acid sequence for *Bacillus amyloliquefaciens* subtilisin and a partial restriction map of this gene (Seq. ID No. 6).

Fig. 2 depicts the conserved amino acid residues among subtilisins from *Bacillus amyloliquefaciens* (BPN)' and *Bacillus lentus* (wild-type).

Figs. 3A and 3B depict the amino acid sequence of four subtilisins. The top line represents the amino acid sequence of subtilisin from *Bacillus amyloliquefaciens* subtilisin (also sometimes referred to as subtilisin BPN') (Seq. ID No.7). The second line depicts the amino acid sequence of subtilisin from *Bacillus subtilis* (Seq. ID No.8). The third line depicts the amino acid sequence of subtilisin from *B. licheniformis* (Seq. ID No.9). The fourth line depicts the amino acid sequence of subtilisin from *Bacillus lentus* (also referred to as subtilisin 309 in PCT WO89/06276) (Seq. ID No.10). The symbol * denotes the absence of specific amino acid residues as compared to subtilisin BPN'.

Fig. 4 depicts the construction of plasmid GGA274.

Fig. 5 depicts the construction of GGT274 which is an intermediate to certain expression plasmids used in this application.

Figs. 6A and 6B depict the DNA and amino acid sequence of subtilisin from *Bacillus lentus* (Seq. ID No.11). The mature subtilisin protein is coded by the codons beginning at the codon GCG (334-336) corresponding to Ala.

Figs. 7A and 7B depict the DNA and amino acid sequence of a preferred embodiment of the invention (N76D/S103AV104I) (Seq. ID No.12). The DNA in this figure has been modified by the methods described to encode aspartate at position 76, alanine at position 103 and isoleucine at position 104. The mature subtilisin variant protein is coded by the codons beginning at the codon GCG (334-336) corresponding to Ala.

Fig. 8 depicts the construction of vector pBCDAICAT.

Fig. 9 depicts the construction of vector pUCCATFNA.

DETAILED DESCRIPTION OF THE INVENTION

The bleaching compositions employed in the present invention provide effective and efficient cleaning of fabrics which thereby removes stains and/or soils from the fabrics. The bleaching systems in combination with the protease enzyme are particularly efficient at removing most types of soils from the fabrics, including protein and lipid

soils, dingy soils, and heavy soil loads, especially from nucleophilic and body soils.

The protease enzymes, bleaching agents (including peroxyacids and bleaching systems) and cleaning composition materials useful herein, including preferred levels, are described in detail hereinafter.

(1) Bleaching Agent

The bleaching compositions herein contain a bleaching agent, which preferably comprises from about 0.5 to about 20 wt.% of the composition. The bleaching agent is either a substantially insoluble, preferably solid, organic peroxyacid, or a bleaching system comprising a bleach activator and a peroxygen bleaching compound capable of yielding hydrogen peroxide, or a combination of both. The peracid which is in the composition, or which is formed by the combination of activator and peroxygen compound, preferably has a corresponding carboxylic acid that has a Hydrophilic-Lipophilic Balance ("H.L.B.") value which ranges from about 3 to about 6.5. Therefore, a method that can be used to characterize the preferred peroxyacids (from activators or as preformed peroxyacids) which are useful in the present invention is the "H.L.B. Scale" such as that described in Davies, J.T., Proc 2nd Internat. Congr. Surface Activity 1, 426, Butterworths, London (1957). Such an H.L.B. Scale (Hydrophilic-Lipophilic Balance) has been used in the study of surface-active agents (surfactants) as a means to relate the distribution of a surface-active agent between a hydrophilic (water-like) and a lipophilic (oil-like) phase. In this manner, H.L.B. values can be used as an indication of the lipophilic (hydrophobic) character of the active bleaching species in the wash (i.e., the ability of the peroxyacid to partition out of the wash liquor and concentrate at the soil/fabric interface).

Set forth hereinafter in Table A are H.L.B. values which have been calculated for selected peroxyacids (as the corresponding carboxylic acids). The equation used to calculate the H.L.B. values can be set forth as:

$$\text{HLB} = \text{Sum (Hydrophilic Group Numbers)} - \text{Sum (Hydrophobic Group Numbers)} + 7.$$

The values for the Hydrophilic Group Numbers are $[-C(O)OH \text{ \& } -N(H)C(O)-=2.1]$ and the values for the Hydrophobic Group Numbers are [aliphatic/aromatic carbon = 0.475 & aliphatic carbon atoms between polar groups are 1/2 the value of an aliphatic carbon in a hydrocarbon chain = $(0.475)/2$]. For reference, an H.L.B. value >7 indicates that the material is preferentially water soluble and an H.L.B. value <7 indicates increasing surface-activity and hydrophobicity.

Table AH.L.B. Values Provided by Various Peroxyacids

<u>Activator/Preformed Peroxyacid</u>	<u>Abbreviation</u>	<u>Peroxyacid</u>	<u>H.L.B. Corresponding Carboxylic Acid</u>
Tetra Acetyl Ethylene Diamine	TAED	$CH_3C(O)OOH$	8.6
DiPeroxyDodecane Dioic Acid	DPDDA	$HOO(O)C(CH_2)-10-C(O)OOH$	6.5
Nonyl Amide of Peroxy Succinic Acid	NAPSA	$CH_3(CH_2)_8N(H)-C(O)(CH_2)_2C(O)-OOH$	6.4
BenzoylOxyBenzene Sulfonate	BOBS	$C_6H_5C(O)OOH$	6.3
Nonyl Amide of Peroxy Adipic Acid	NAPAA	$CH_3(CH_2)_8N(H)-C(O)(CH_2)_4C(O)-OOH$	6.0
NonanoylOxyBenzene Sulfonate	NOBS	$CH_3(CH_2)_7C(O)-OOH$	5.3

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DecanoylOxyBen- zene Sulfonate	DOBS	CH ₃ (CH ₂) ₈ C(O)- OOH	4.8
PerLauric Acid	PLA	CH ₃ (CH ₂) ₁₀ C- (O)OOH	3.9

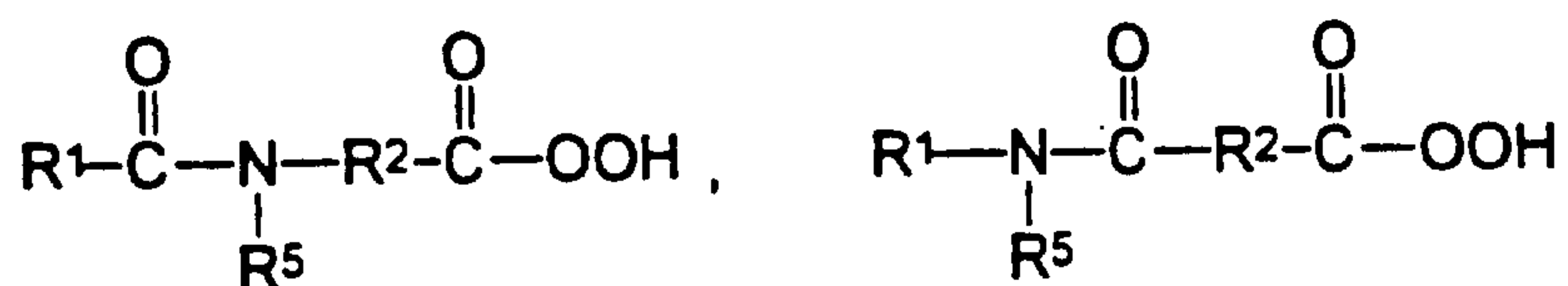
As noted hereinbefore, a preferred range of H.L.B. values (of the corresponding carboxylic acid) for the peroxyacids of the present invention (whether added directly or generated in situ) ranges from about 3.0 to about 6.5. A more preferred range of H.L.B. values (as the carboxylic acid) for the peroxyacids useful in the present invention (whether added directly or generated in situ) range from about 4.0 to 6.5. The most preferred range of H.L.B. values (as the carboxylic acid) for the peroxyacids of the present invention (whether added directly as generated in situ) ranges from about 4.0 to about 6.0.

10 (a) Peroxyacid

The present invention encompasses detergent compositions comprising an effective amount of the protease enzyme and a bleaching system comprising at least about 0.1%, preferably from about 0.1% to about 50%, by weight, of a substantially insoluble organic peroxyacid. The peroxyacid useful herein preferably comprises from about 0.5 to about 20, more preferably from about 1 to about 10, most preferably from about 2 to about 7, wt. % of the composition.

Preferred organic peroxyacids are selected from the group consisting of 4-nonylamino-4-oxoperoxybutyric acid; 6-(nonyl-amino)-6-oxoperoxyhexanoic acid; 1,12-diperoxydodecanedioic acid; heptyl sulfonylperpropionic acid; decylsulphonyl perpropionic acid; and heptyl-, octyl-, nonyl-, decyl-sulphonylperbutyric acid; and mixtures thereof.

Of the organic peroxyacids, amidoperoxyacids (amide substituted peroxycarboxylic acids) are preferred. Suitable amidoperoxyacids for use herein are described in U.S. Patents 4,634,551 and 4,686,063, both Burns et al., issued January 6, 1987 and August 11, 1987, respectively. Suitable amidoperoxyacids are of the formula:



wherein R^1 is an alkyl, aryl, or alkaryl group containing from about 1 to about 14 carbon atoms (preferably R^1 is an alkyl group containing from about 6 to about 12 carbon atoms), R^2 is an alkylene, arylene or alkarylene group containing from about 1 to about 14 carbon atoms (preferably R^2 is an alkylene group containing from about 1 to about 6 carbon atoms), and R^5 is H or an alkyl, aryl, or alkaryl group containing from about 1 to about 10 carbon atoms (preferably R^5 is H). More preferably, R^1 is an alkyl group containing from about 8 to about 10 carbon atoms, and R^2 is an alkylene group containing from about 2 to about 4 carbon atoms

Also suitable for use herein are peroxyfumarates, which are described in U.S. Patent 4,852,989, Burns et al., issued August 1, 1989, and sulfone peroxyacids (sulfone peroxydicarboxylic acids), which are described in U.S. Patents 4,758,369, 4,824,591, and 5,004,558, all Dryoff et al., issued July 19, 1988, April 25, 1989, and April 2, 1991, respectively.

Example I of U.S. Patent 4,686,063 contains one description of the synthesis of NAPSA, from column 8, line 40 to column 9, line 5, and NAPAA, from column 9, line 15 to column 9, line 65. At the end of the amidoperoxyacid synthesis, the reaction is quenched with water, filtered, washed with water to remove some excess sulfuric acid (or other strong acid with which the peroxyacid was made), and filtered again.

The amidoperoxyacid wet cake thus obtained can be contacted with a phosphate buffer solution at a pH between about 3.5 and 6, preferably between about 4 and 5, according to U.S. Patent 4,909,953, Sadlowski et al., issued March 20, 1990.

Other agents for storage stabilization or exotherm control can be added to the amidoperoxyacid before incorporation into the final product. For example, boric acid, an exotherm control agent disclosed in U.S. Patent 4,686,063, Burns, issued August 11, 1987 can be mixed with the amidoperoxyacid (which has been washed in phosphate buffer) in about a 2:1 peracid:boric acid ratio. The phosphate buffer washed amidoperoxyacid can also be

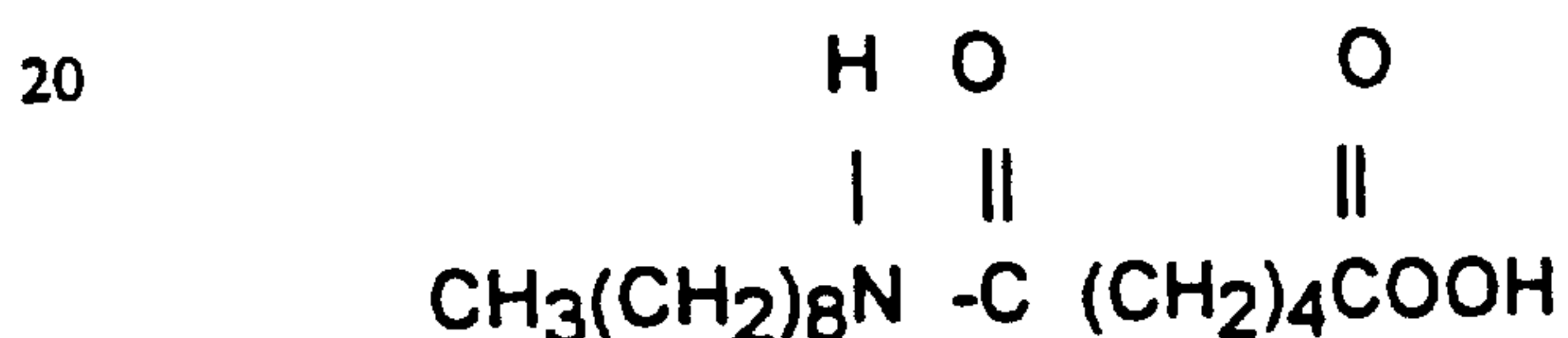
mixed with appropriate amounts of dipicolinic acid and tetrasodium pyrophosphate, a chelating stabilization system. Chelants can optionally be included in the phosphate buffer before contact with the wet cake.

5 The wet cake is preferably made up of particles with an average particle diameter of from about 0.1 to about 260 microns, preferably from about 10 to about 100 microns, and most preferably from about 30 to about 60 microns. Small particle size NAPAA crystals are desired herein. See U.S. Patent 5,055,218, Getty et al., issued October 8,
10 1991.

NAPAA filter cake herein is preferably washed twice in phosphate buffer. It has been found that two successive phosphate buffer washes lend optimal stability to NAPAA.

15 Particulate (solid), organic peroxyacids with a theoretical AvO (available oxygen) of between about 3 and about 12, most preferably between 5 and 7, are preferred.

Most preferred for use herein is NAPAA. Another name for the nonylamide of peroxyadipic acid ("NAPAA") is 6-(nonylamino)-6-oxoperoxycaproic acid. The chemical formula for NAPAA is:



The molecular weight of NAPAA is 287.4.

25 Detergent compositions and bleaching compositions containing NAPAA provide extremely effective and efficient surface bleaching of textiles. Stains and/or soils are removed from the textiles. These compositions are particularly effective at removing dingy soils from textiles.

30 NAPAA's polar amide or substituted amide moiety results in a peroxyacid which has a very low vapor pressure and thus possesses a low odor profile as well as excellent bleaching performance. It is believed that the polarity of the amide group results in a reduction of vapor pressure of the peroxyacid, and an increase in melting point.

35 NAPAA can be used directly as a bleaching agent. It has a reduced vapor pressure and a good odor profile in laundry applications.

NAPAA can be prepared by, for example, first reacting NAAA (monononyl amide of adipic acid), sulfuric acid, and hydrogen peroxide. The reaction product is quenched by addition to ice water followed by filtration, washing with distilled water, and final suction filtration to
5 recover the wet cake. Washing can be continued until the pH of the filtrate is neutral.

It is also preferred that the NAPAA pH (10% solids in water) be between about 4.2 and 4.8. Surprisingly, this pH results in more thermally stable particles.

10 (b) Bleaching Systems - Bleach Activator and Peroxygen Bleaching Compound

(i) Bleach Activators

The bleach activator for the bleaching systems useful herein preferably has the following structure:

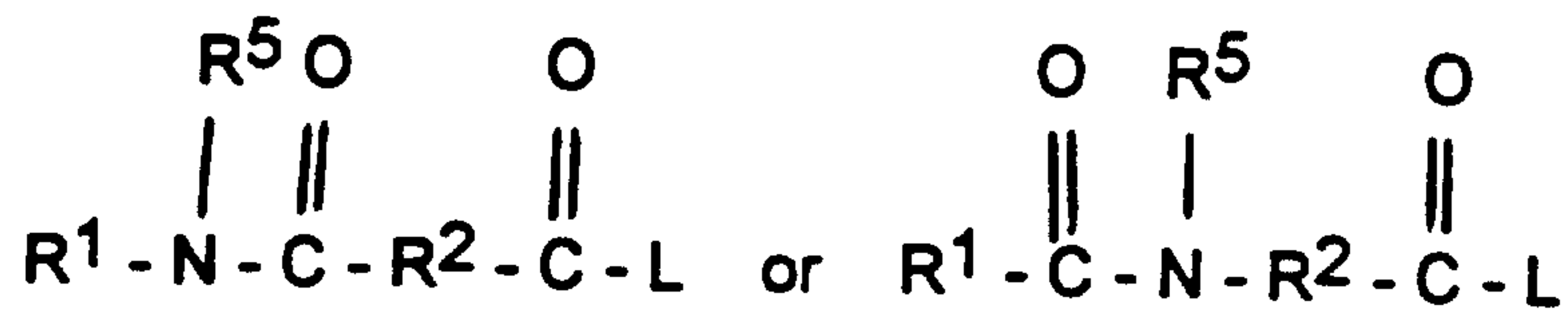


wherein R is an alkyl group containing from about 5 to about 18 carbon atoms wherein the longest linear alkyl chain extending from and
20 including the carbonyl carbon contains from about 6 to about 10 carbon atoms and L is a leaving group, the conjugate acid of which has a pKa in the range of from about 4 to about 13, preferably from about 6 to about 11, most preferably from about 8 to about 11.

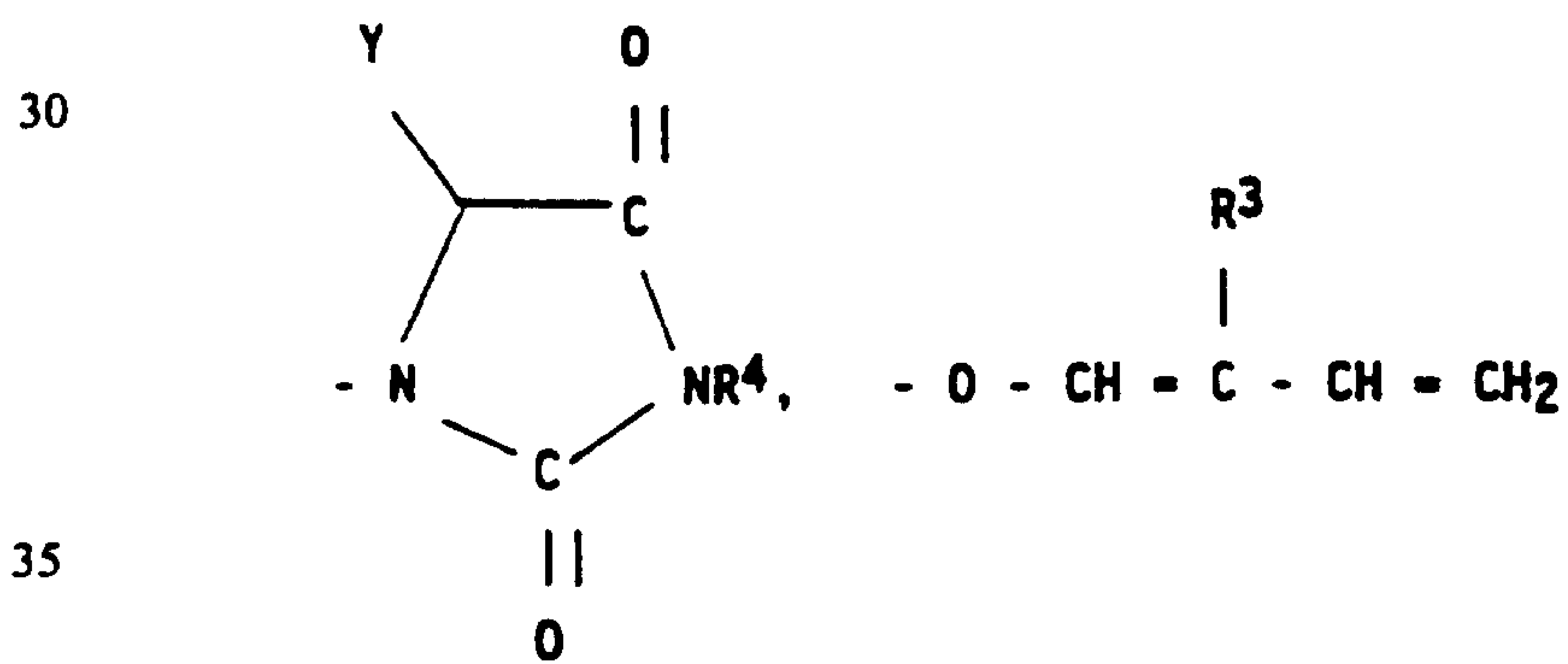
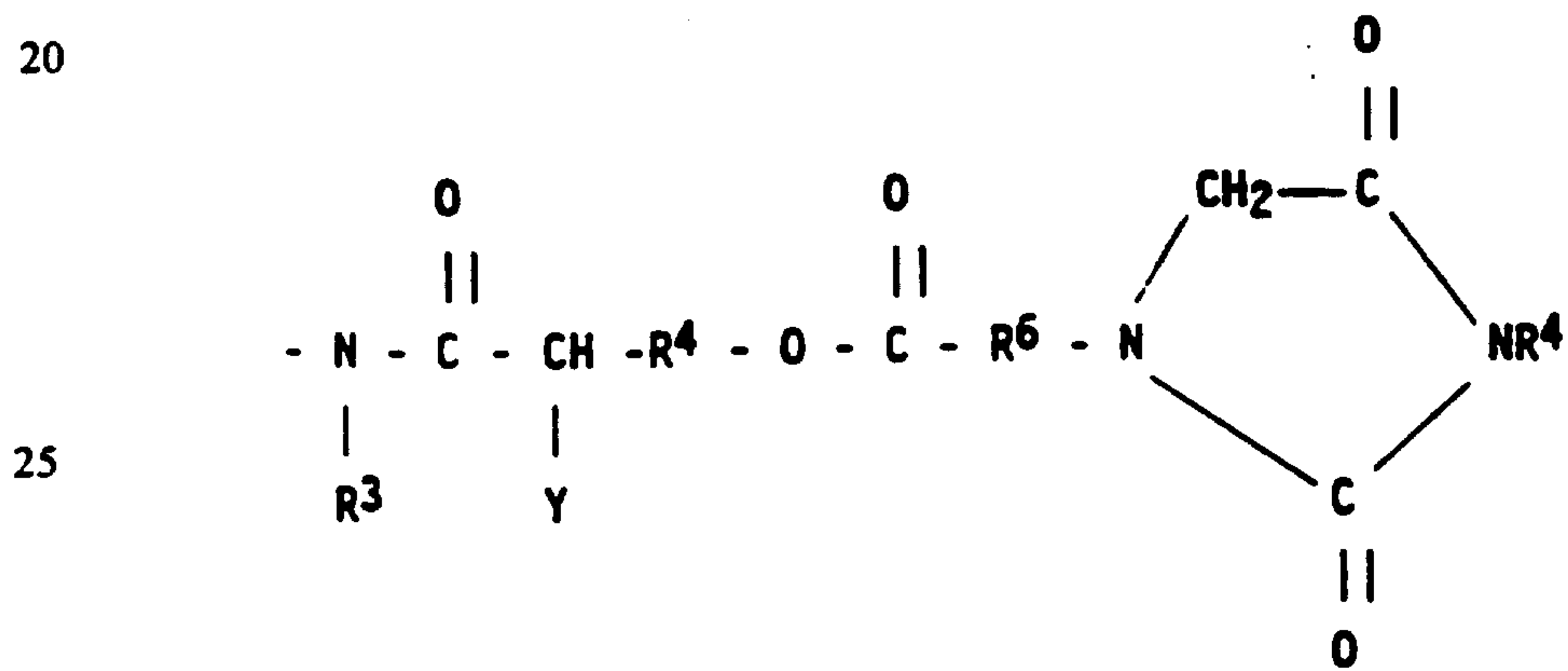
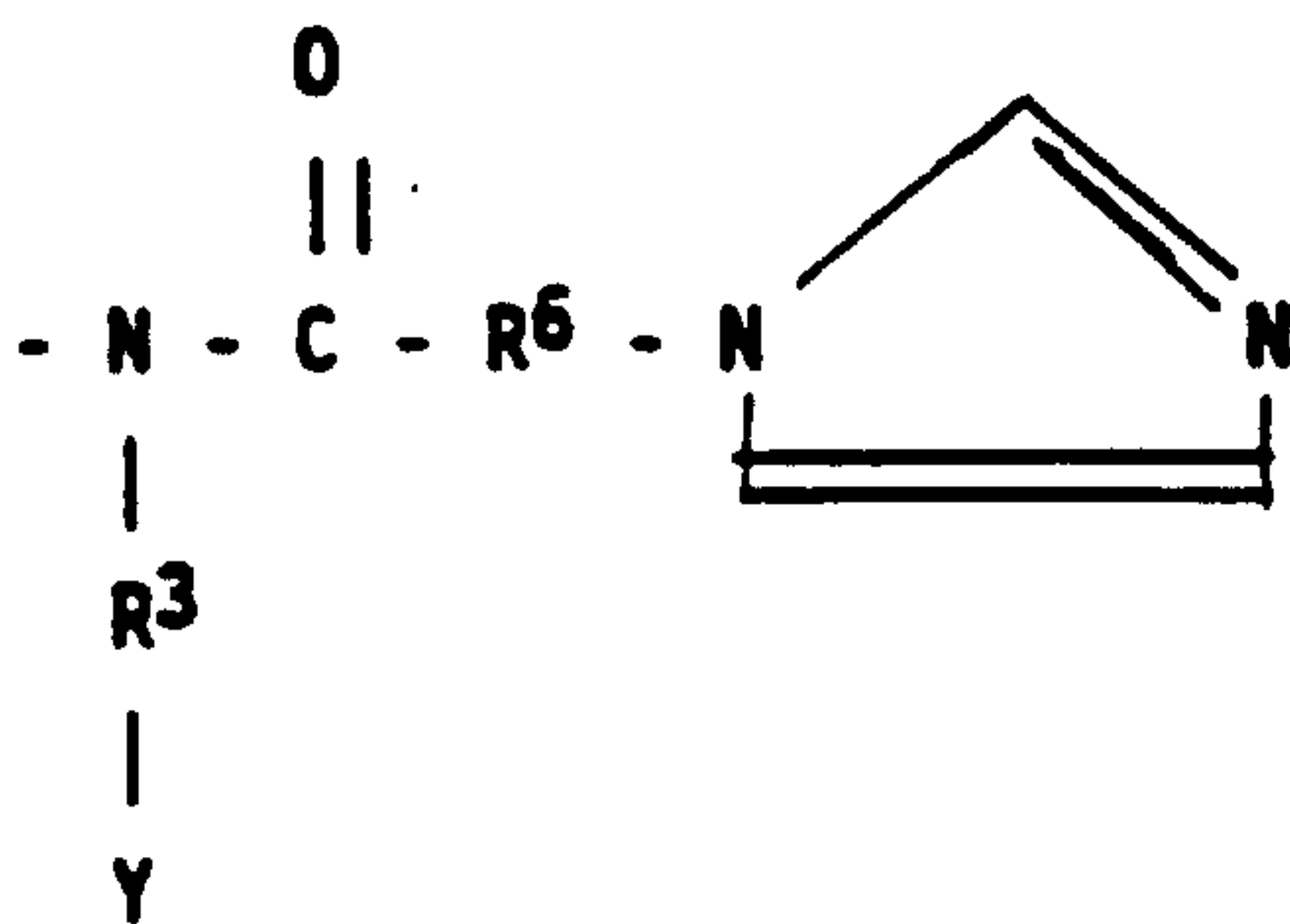
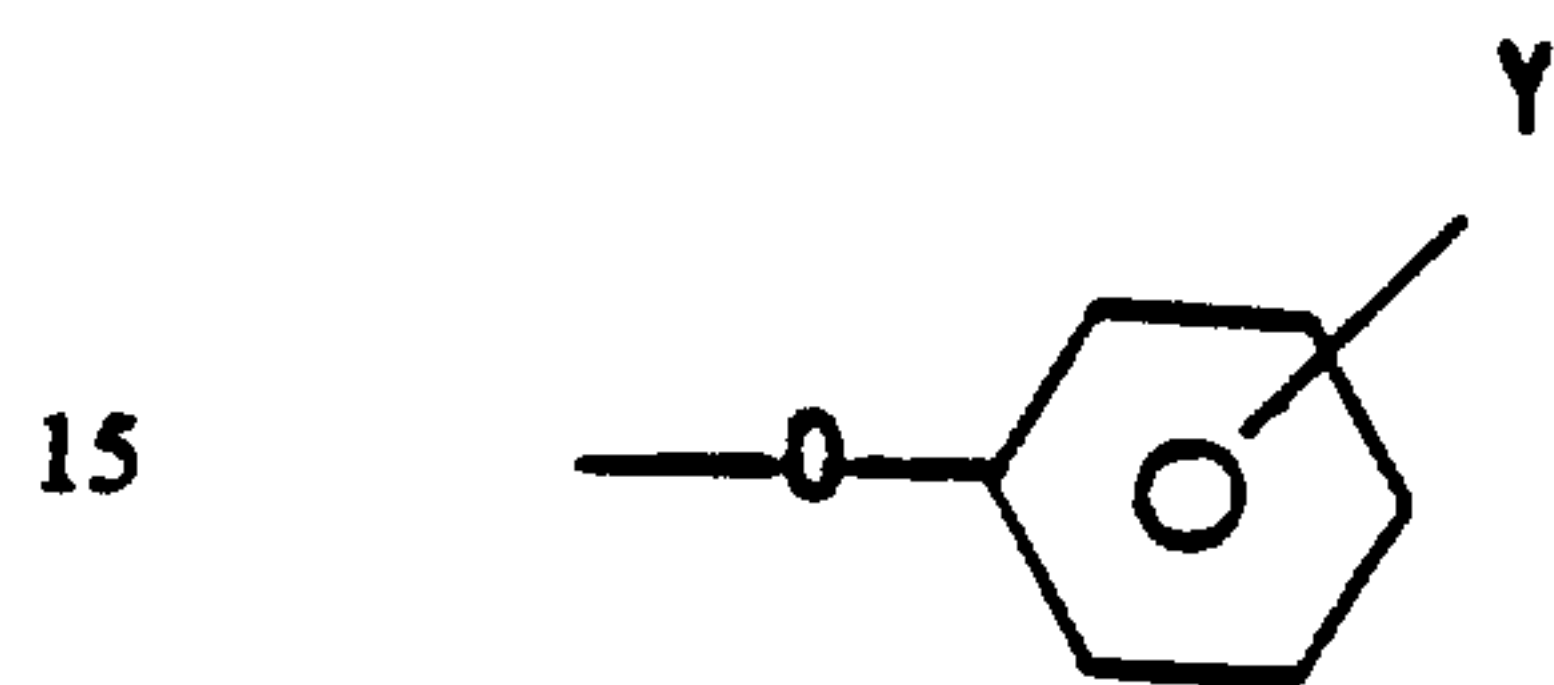
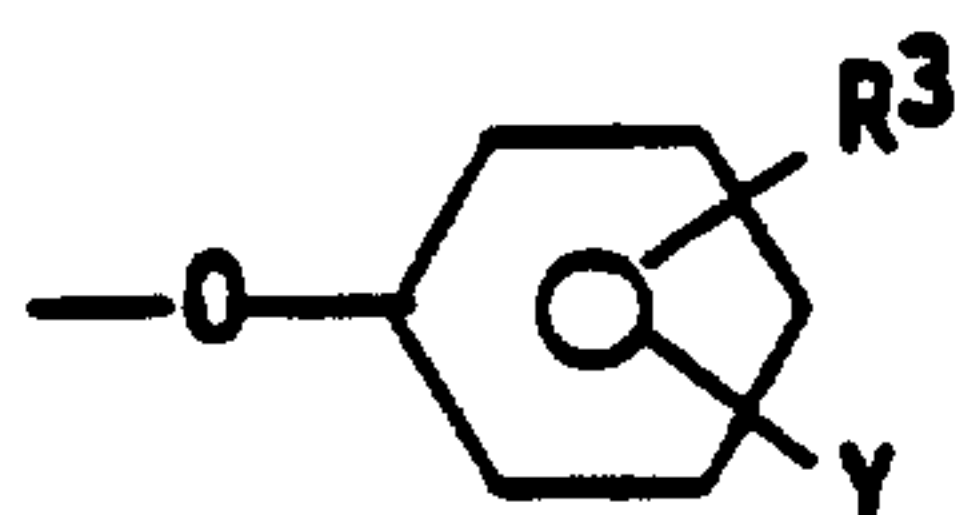
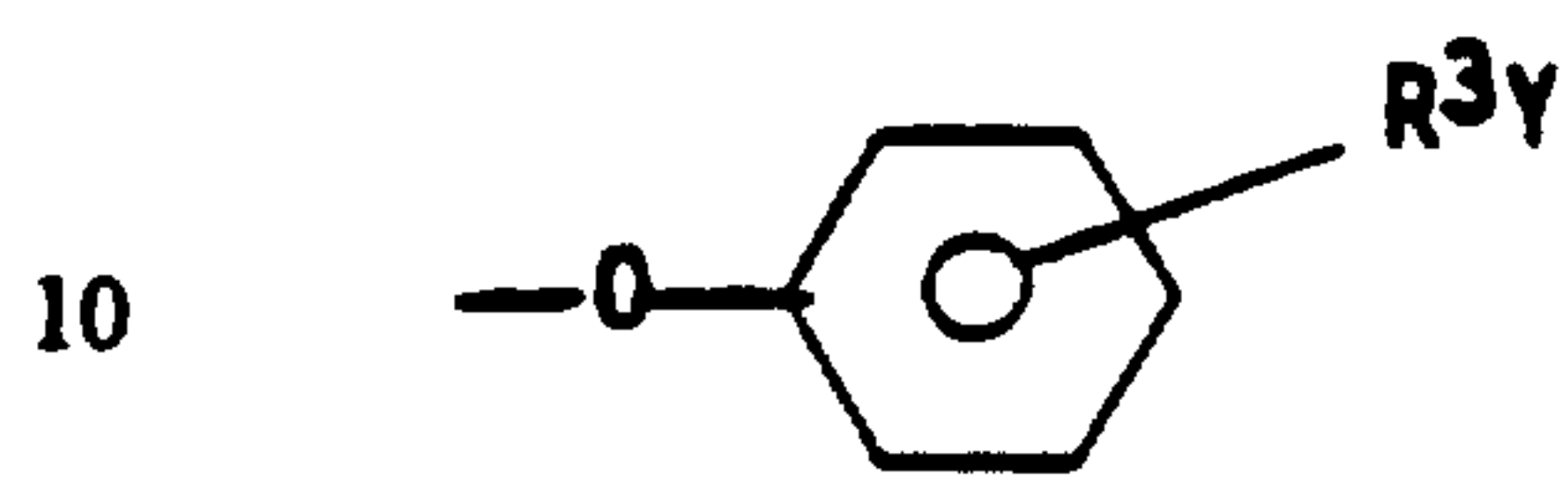
L can be essentially any suitable leaving group. A leaving group
25 is any group that is displaced from the bleach activator as a consequence of the nucleophilic attack on the bleach activator by the perhydroxide anion. This, the perhydrolysis reaction, results in the formation of the percarboxylic acid. Generally, for a group to be a suitable leaving group it must exert an electron attracting effect. This
30 facilitates the nucleophilic attack by the perhydroxide anion.

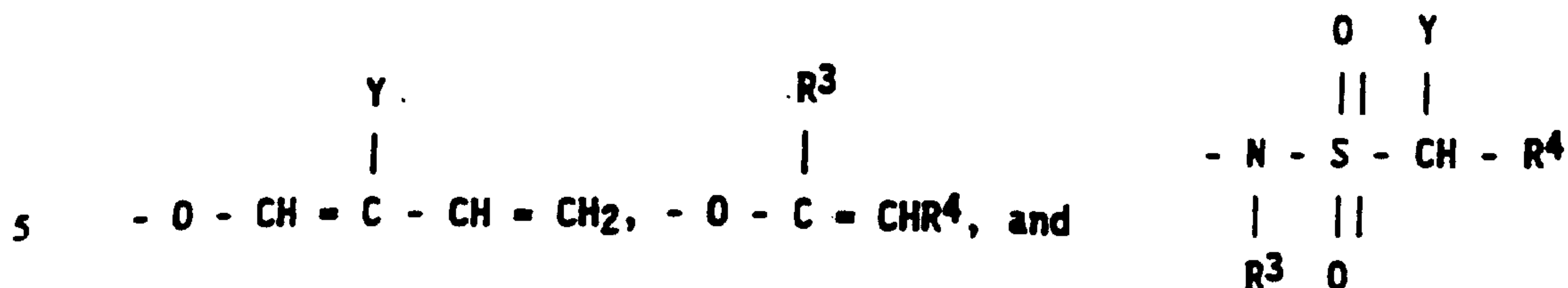
The L group must be sufficiently reactive for the reaction to occur within the optimum time frame (e.g., a wash cycle). However, if L is too reactive, this activator will be difficult to stabilize. These characteristics are generally paralleled by the pKa of the conjugate acid of the leaving
35 group, although exceptions to this convention are known.

Preferred bleach activators are those of the general formula:

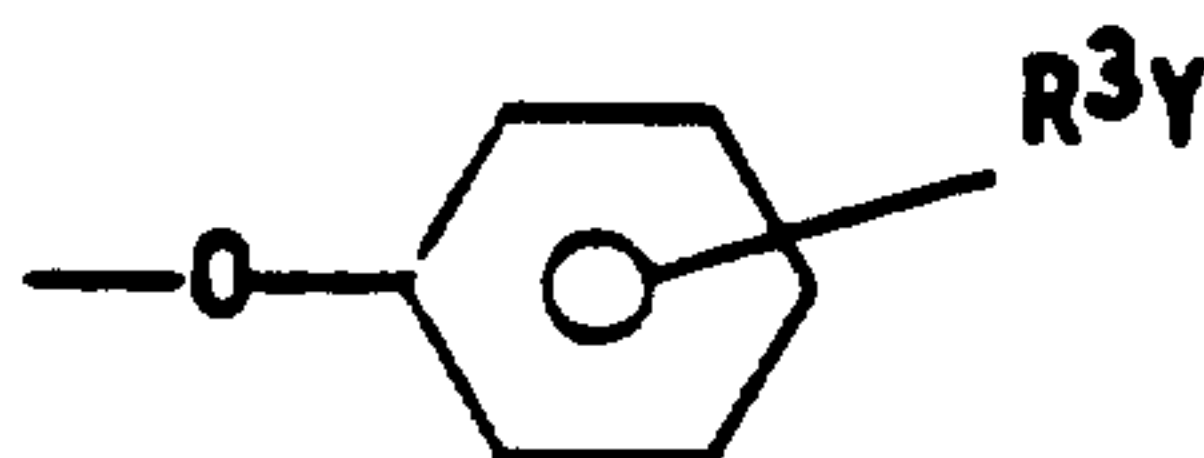


wherein R¹ is an alkyl group containing from about 6 to about 12 carbon atoms, R² is an alkylene containing from 1 to about 6 carbon atoms, R⁵ is H or alkyl, aryl, or alkaryl containing from about 1 to about 10 carbon atoms, and L is selected from the group consisting of:





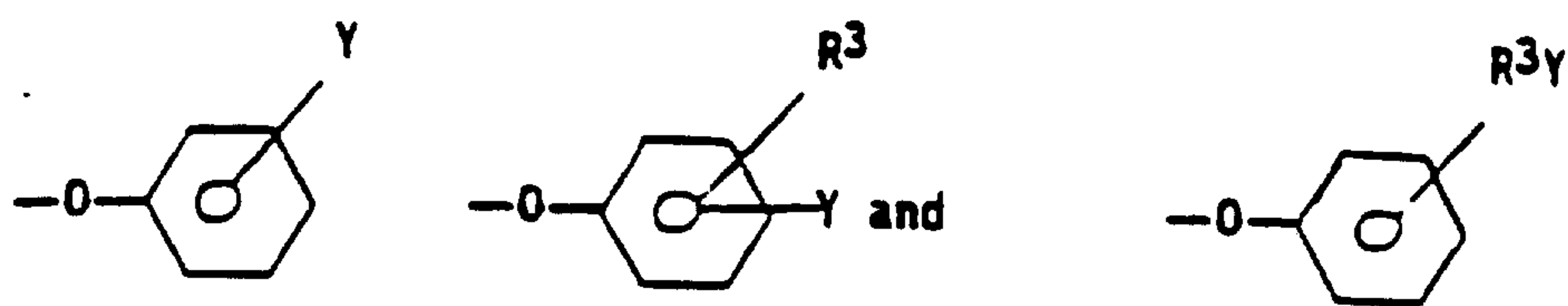
wherein R^6 is an alkylene, arylene, or alkarylene group containing from
 about 1 to about 14 carbon atoms, R^3 is an alkyl chain containing from
 about 1 to about 8 carbon atoms, R^4 is H or R^3 , and Y is H or a
 solubilizing group. Y is preferably selected from the group consisting of
 $-\text{SO}_3-\text{M}^+$, $-\text{COO}-\text{M}^+$, $-\text{SO}_4-\text{M}^+$, $(-\text{N}+\text{R}'_3)\text{X}^-$ and $\text{O} \leftarrow \text{N}(\text{R}'_3)$, wherein R'
 is an alkyl chain containing from about 1 to about 4 carbon atoms, M is
 a cation which provides solubility to the bleach activator and X is an
 anion which provides solubility to the bleach activator. Preferably, M is
 an alkali metal, ammonium or substituted ammonium cation, with
 sodium and potassium being most preferred, and X is an anion
 selected from the group consisting of halide, hydroxide, methylsulfate
 and acetate anions. More preferably, Y is $-\text{SO}_3-\text{M}^+$ and $-\text{COO}-\text{M}^+$. It
 should be noted that bleach activators with a leaving group that does
 not contain a solubilizing group should be well dispersed in the bleach
 solution in order to assist in their dissolution. Preferred is:



wherein R^3 is as defined above and Y is $-\text{SO}_3-\text{M}^+$ or $-\text{COO}-\text{M}^+$ wherein
 M is as defined above.

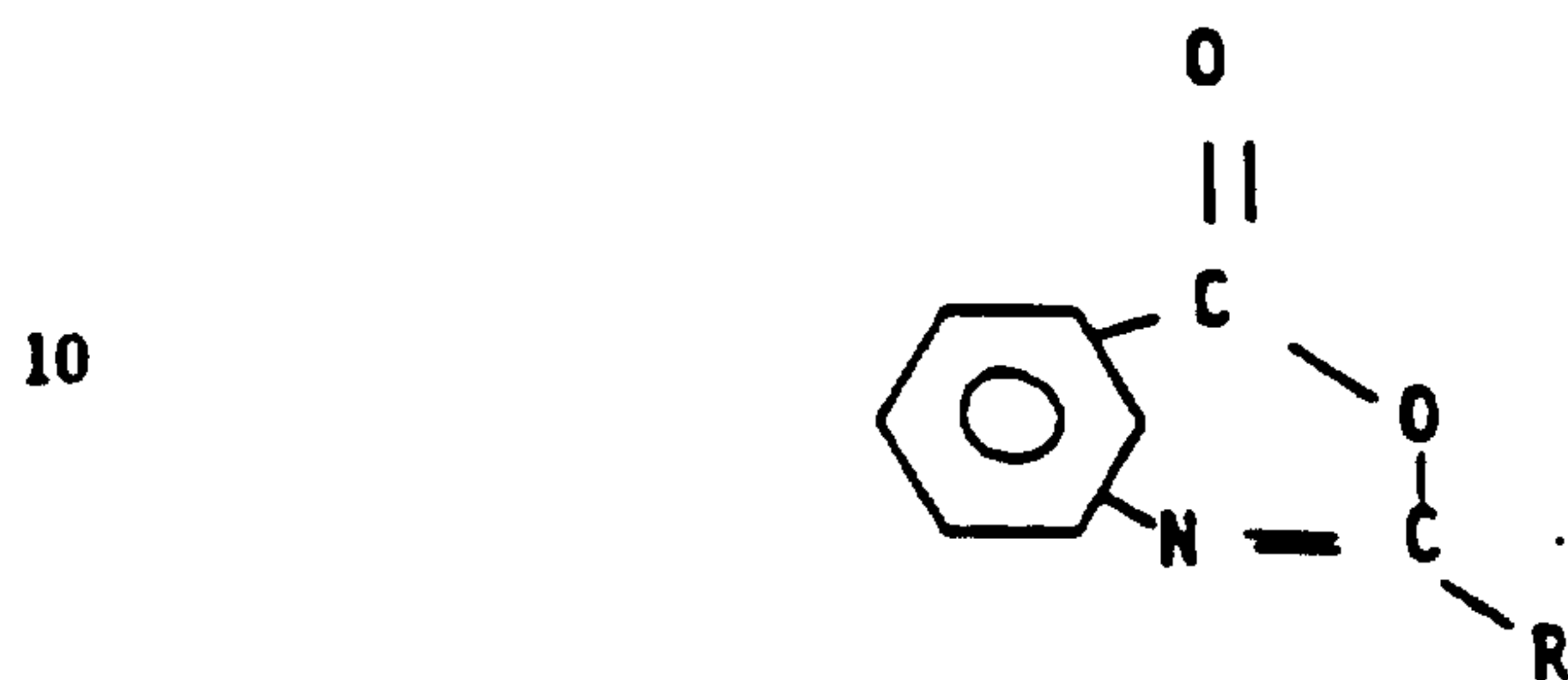
Especially preferred bleach activators are those wherein R^1 is a
 linear alkyl chain containing from about 6 to about 12 carbon atoms, R^2
 is a linear alkylene chain containing from about 2 to about 6 carbon
 atoms, R^5 is H, and L is selected from the group consisting of:

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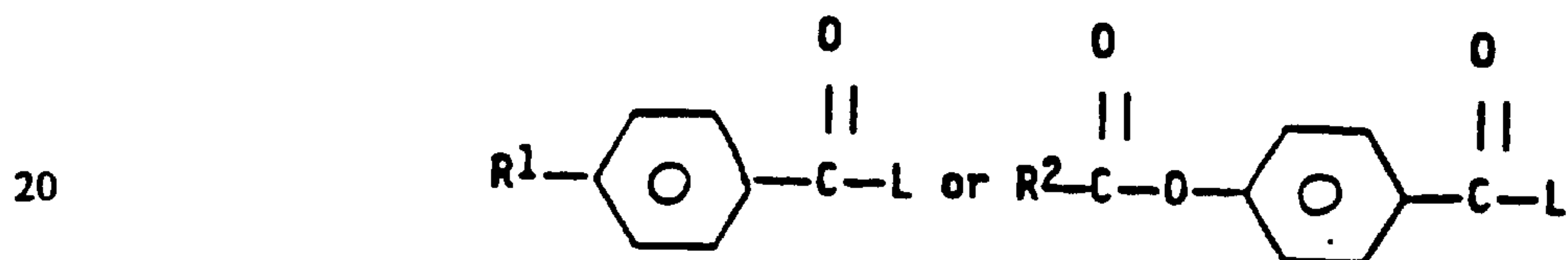
wherein R³ is as defined above, Y is -SO₃-M⁺ or -COO-M⁺ and M is as defined above.

A preferred bleach activator is:



wherein R is H, alkyl, aryl or alkaryl. This is described in U.S. Patent 4,966,723, Hodge et al.

Preferred bleach activators are:

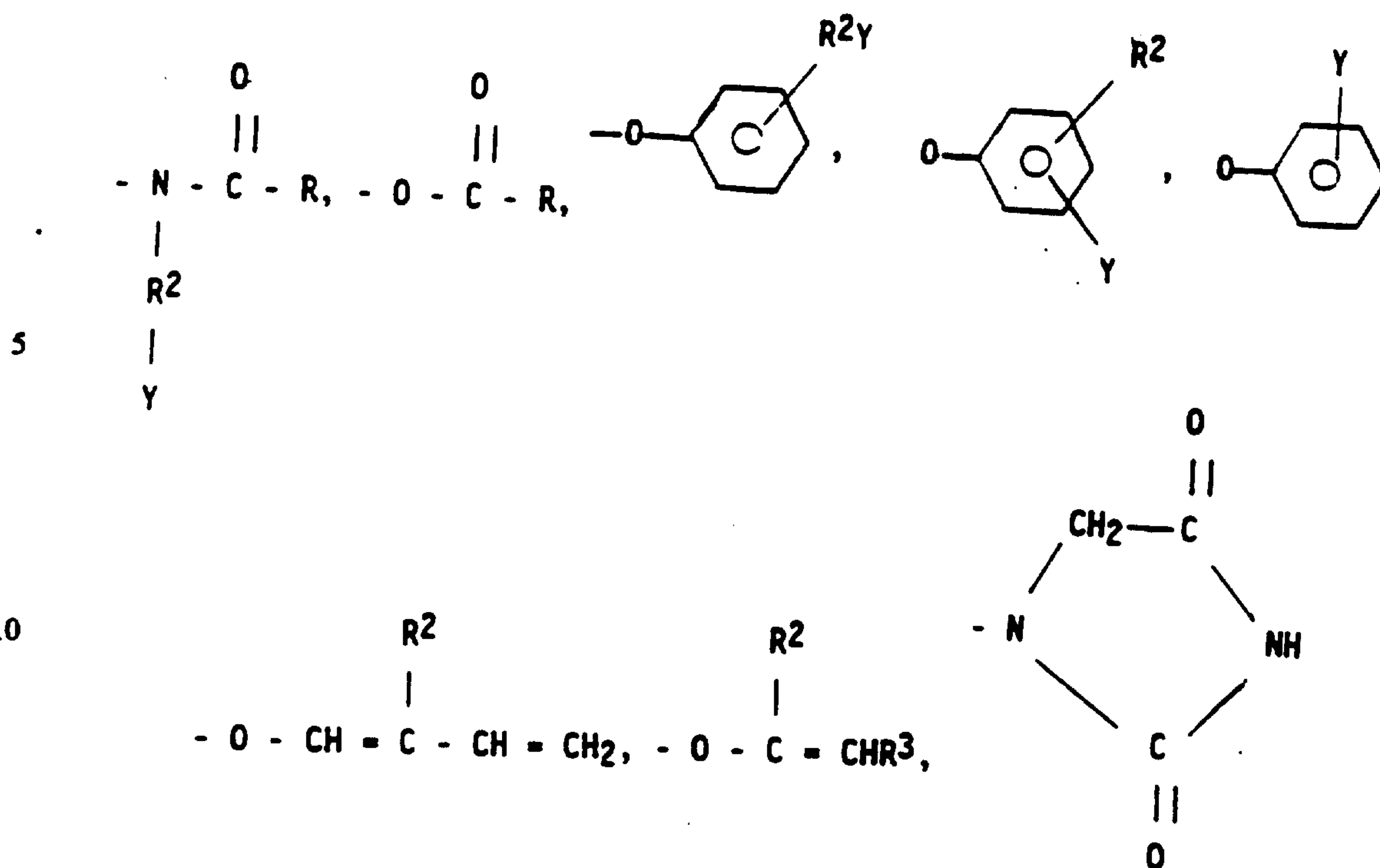


wherein R¹ is H or an alkyl group containing from about 1 to about 6 carbon atoms and R² is an alkyl group containing from about 1 to about 6 carbon atoms and L is as defined above.

Preferred bleach activators are also those of the above general formula wherein L is as defined in the general formula, and R¹ is H or an alkyl group containing from about 1 to about 4 carbon atoms.

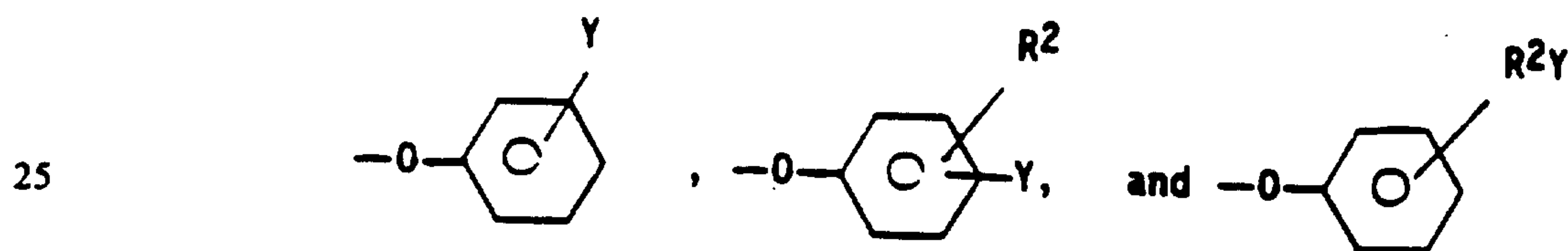
Even more preferred are bleach activators of the above general formula wherein L is as defined in the general formula and R¹ is a H.

More preferred bleach activators are those of the above general formula wherein R is a linear alkyl chain containing from about 5 to about 9 and preferably from about 6 to about 8 carbon atoms and L is selected from the group consisting of:



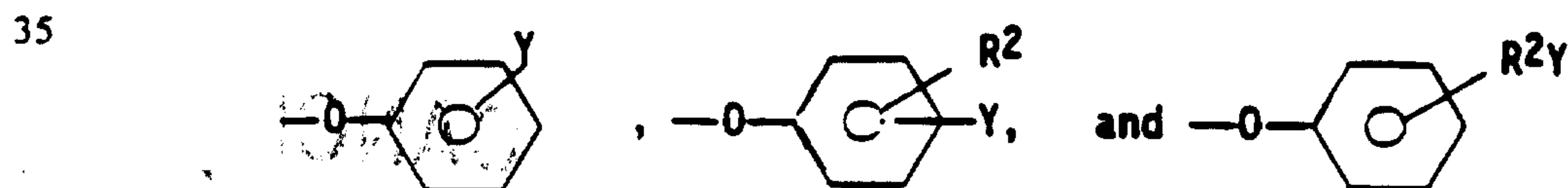
wherein R, R², R³ and Y are as defined above.

Particularly preferred bleach activators are those of the above general formula wherein R is an alkyl group containing from about 5 to about 12 carbon atoms wherein the longest linear portion of the alkyl chain extending from and including the carbonyl carbon is from about 6 to about 10 carbon atoms, and L is selected from the group consisting of:



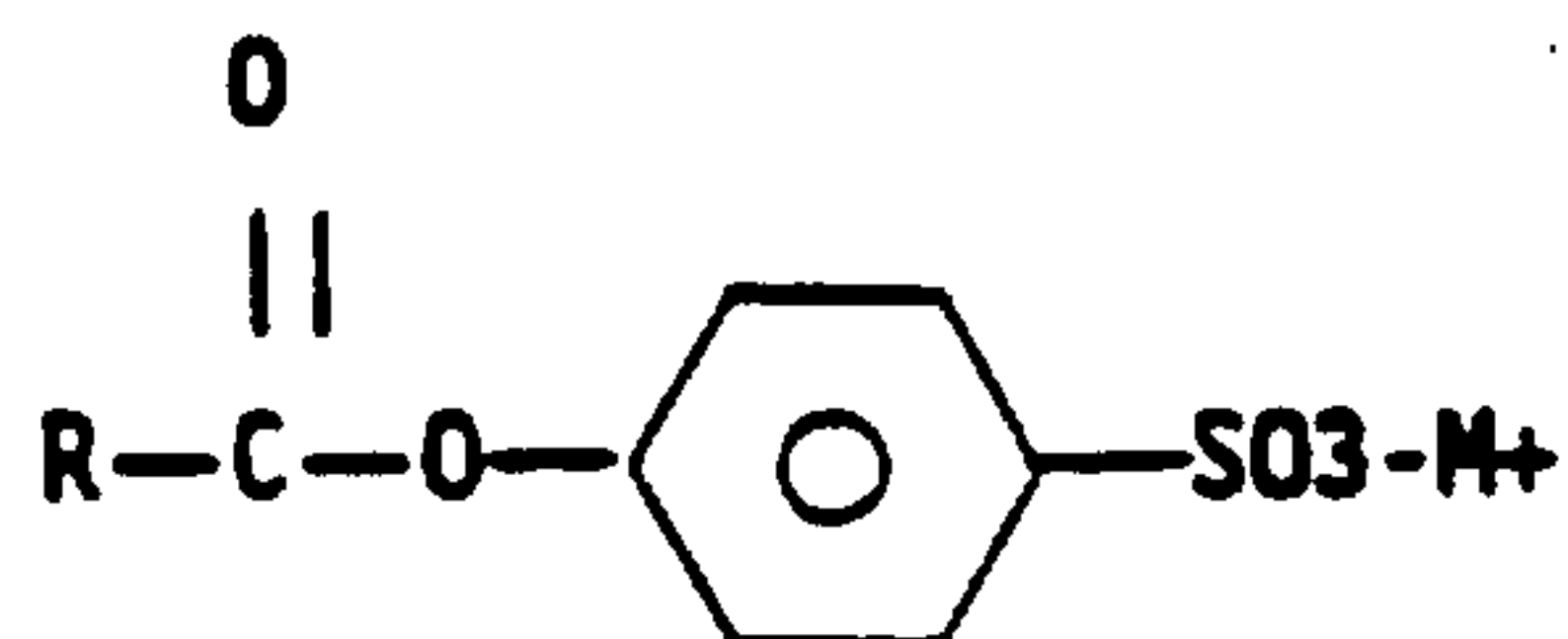
wherein R² is an alkyl chain containing from about 1 to about 8 carbon atoms, and Y is -SO₃M⁺ or -COO-M⁺ wherein M is an alkali metal, ammonium or substituted ammonium cation.

Especially preferred bleach activators are those of the above general formula wherein R is a linear alkyl chain containing from about 5 to about 9 and preferably from about 6 to about 8 carbon atoms and L is selected from the group consisting of:



wherein R^2 is as defined above and Y is $-\text{SO}_3\text{M}^+$ or $-\text{COO}-\text{M}^+$ wherein M is as defined above.

5 The most preferred bleach activators have the formula:



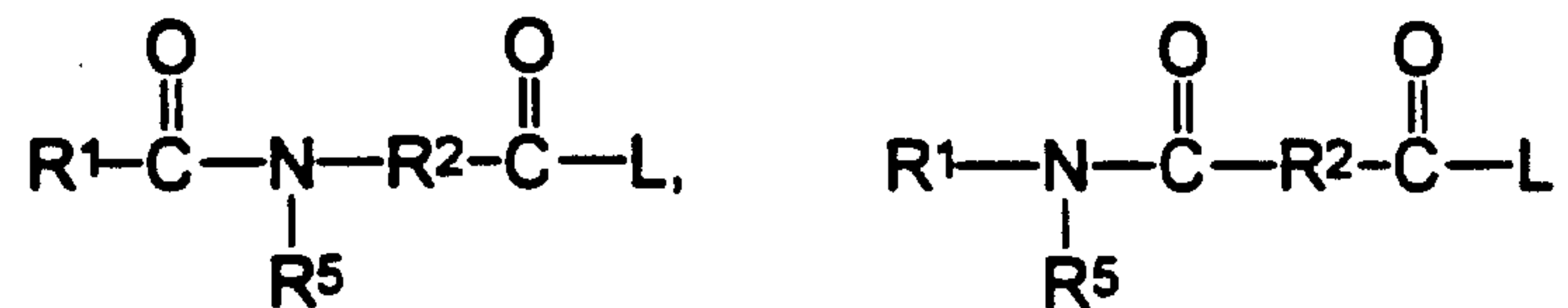
10

wherein R is a linear alkyl chain containing from about 5 to about 9 and preferably from about 6 to about 8 carbon atoms and M is sodium or potassium.

15 Preferably, the bleach activator herein is sodium nonanoyloxybenzenesulfonate (NOBS) or sodium benzoyloxybenzenesulfonate (BOBS).

Further particularly preferred for use in the present invention bleaching compositions are the following bleach activators which are particularly safe for use with machines having natural rubber parts.
 20 This is believed to be the result of not producing oily diacylperoxide (DAP) species by the perhydrolysis reaction of these amido acid-derived bleach activators, but rather forming insoluble crystalline solid DAP's. These solids are believed to not form a coating film and thus natural rubber parts are not exposed to DAP's for extended periods of
 25 time. These preferred bleach activators are members selected from the group consisting of:

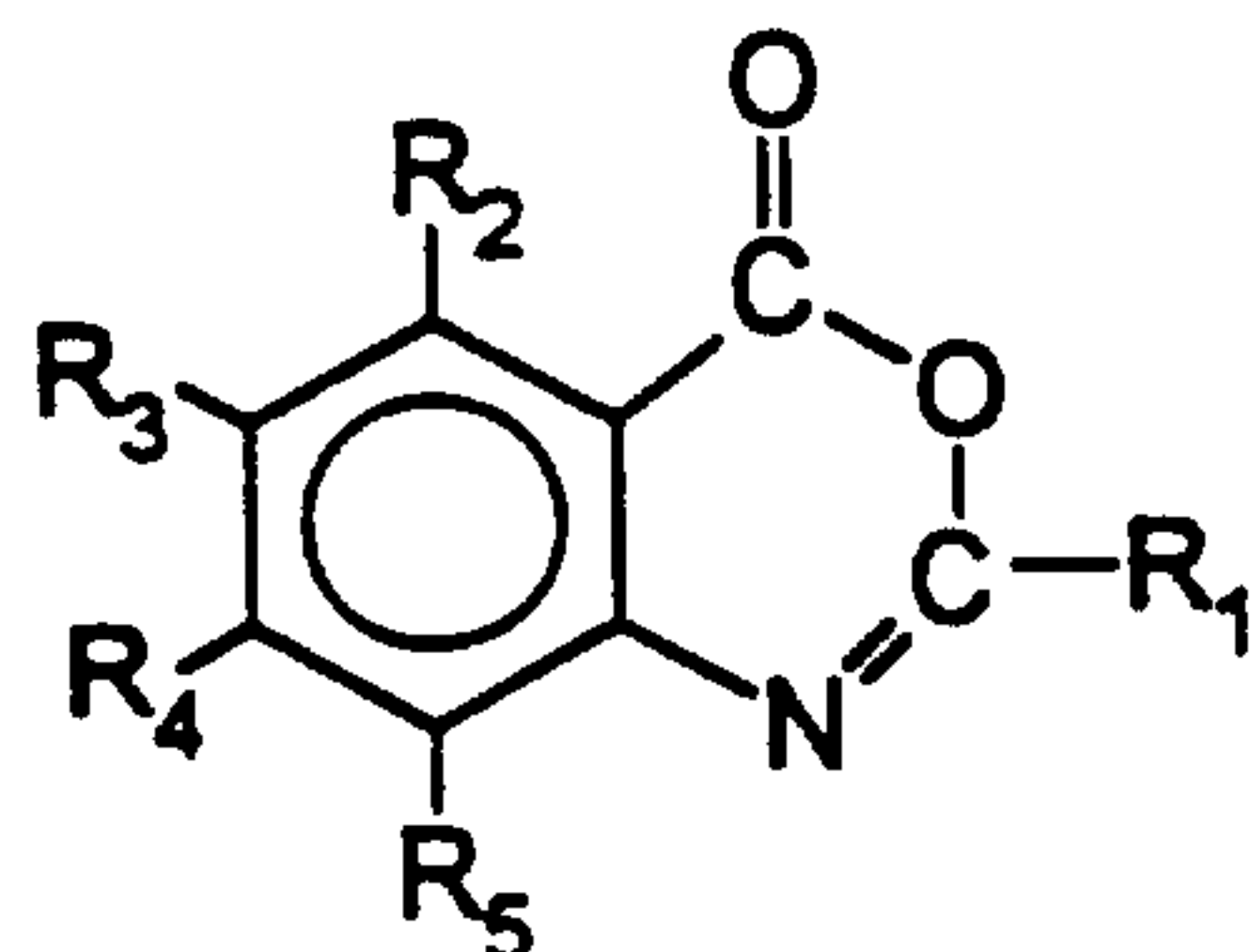
a) a bleach activator of the general formula:



30

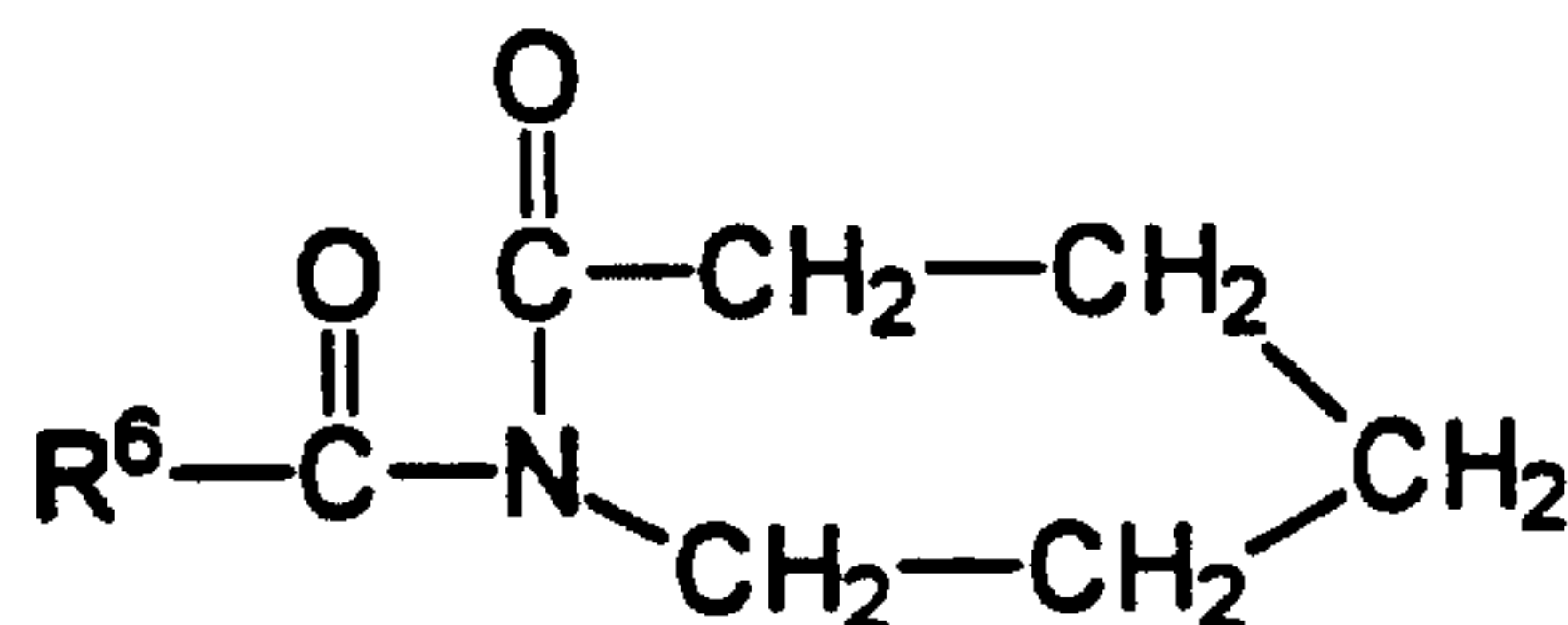
or mixtures thereof, wherein R^1 is an alkyl, aryl, or alkaryl group containing from about 1 to about 14 carbon atoms, R^2 is an alkylene, arylene or alkarylene group containing from about 1 to about 14 carbon atoms, R^5 is H or an alkyl, aryl, or alkaryl group containing from about 1 to about 10 carbon atoms, and L is a leaving group;

b) benzoxazin-type bleach activators of the general formula:



wherein R_1 is H, alkyl, alkaryl, aryl, arylalkyl, and wherein R_2 , R_3 , R_4 , and R_5 may be the same or different substituents selected from H, halogen, alkyl, alkenyl, aryl, hydroxyl, alkoxy, amino, alkylamino, COOR_6 (wherein R_6 is H or an alkyl group) and carbonyl functions;

c) N-acyl caprolactam bleach activators of the formula:



wherein R^6 is H or an alkyl, aryl, alkoxyaryl or alkaryl group containing from 1 to 12 carbons; and

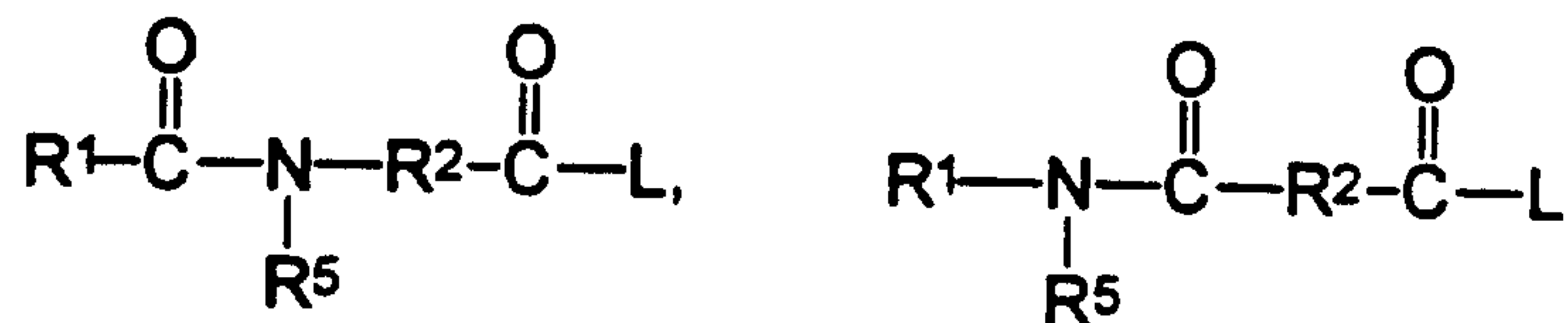
d) mixtures of a), b) and c).

Preferred bleach activators of type a) are those wherein R^1 is an alkyl group containing from about 6 to about 12 carbon atoms, R^2 contains from about 1 to about 8 carbon atoms, and R^5 is H or methyl. Particularly preferred bleach activators are those of the above general formulas wherein R^1 is an alkyl group containing from about 7 to about 10 carbon atoms and R^2 contains from about 4 to about 5 carbon atoms.

Preferred bleach activators of type b) are those wherein R_2 , R_3 , R_4 , and R_5 are H and R_1 is a phenyl group.

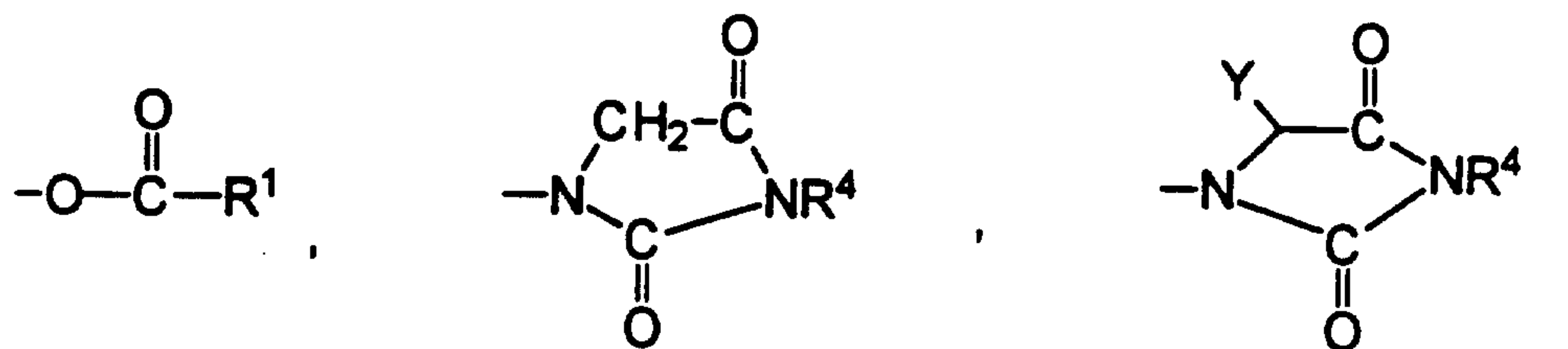
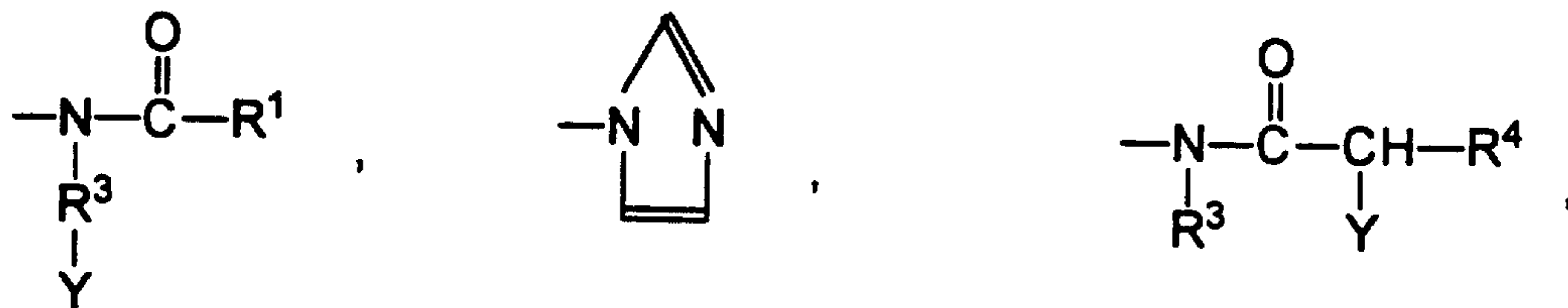
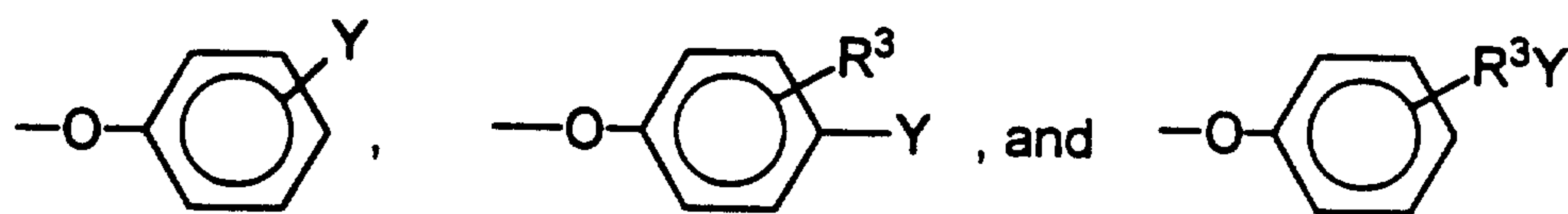
The preferred acyl moieties of said N-acyl caprolactam bleach activators of type c) have the formula $R^6\text{-CO-}$ wherein R^6 is H or an alkyl, aryl, alkoxyaryl, or alkaryl group containing from 1 to 12 carbons, preferably from 6 to 12 carbon atoms. In highly preferred embodiments, R^6 is a member selected from the group consisting of phenyl, heptyl, octyl, nonyl, 2,4,4-trimethylpentyl, decenyl and mixtures thereof.

- Amido Derived Bleach Activators - The bleach activators of type a) employed in the present invention are amide substituted compounds of the general formulas:



- 5 or mixtures thereof, wherein R^1 , R^2 and R^5 are as defined above and L can be essentially any suitable leaving group. Preferred bleach activators are those of the above general formula wherein R^1 , R^2 and R^5 are as defined for the peroxyacid and L is selected from the group consisting of:

10

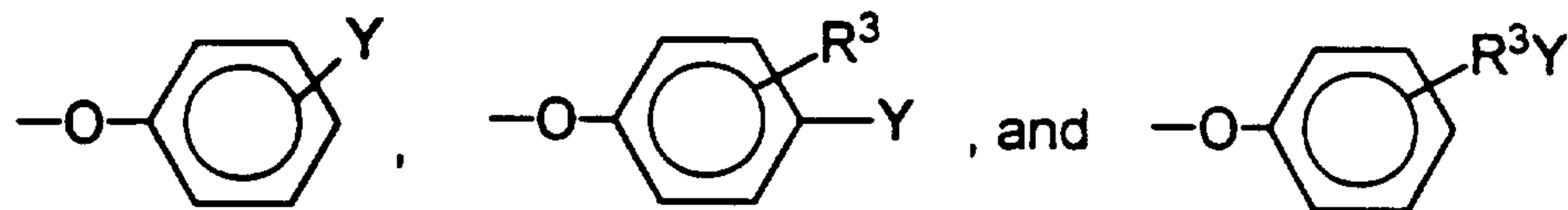


- 15 and mixtures thereof, wherein R^1 is an alkyl, aryl, or alkaryl group containing from about 1 to about 14 carbon atoms, R^3 is an alkyl chain

containing from 1 to about 8 carbon atoms, R^4 is H or R^3 , and Y is H or a solubilizing group.

The preferred solubilizing groups are $-\text{SO}_3^-\text{M}^+$, $-\text{CO}_2^-\text{M}^+$, $-\text{SO}_4^-\text{M}^+$, $-\text{N}^+(\text{R}^3)_4\text{X}^-$ and $\text{O} < \text{N}(\text{R}^3)_3$ and most preferably $-\text{SO}_3^-\text{M}^+$ and $-\text{CO}_2^-\text{M}^+$ wherein R^3 is an alkyl chain containing from about 1 to about 4 carbon atoms, M is a cation which provides solubility to the bleach activator and X is an anion which provides solubility to the bleach activator. Preferably, M is an alkali metal, ammonium or substituted ammonium cation, with sodium and potassium being most preferred, and X is a halide, hydroxide, methylsulfate or acetate anion. It should be noted that bleach activators with a leaving group that does not contain a solubilizing groups should be well dispersed in the bleaching solution in order to assist in their dissolution.

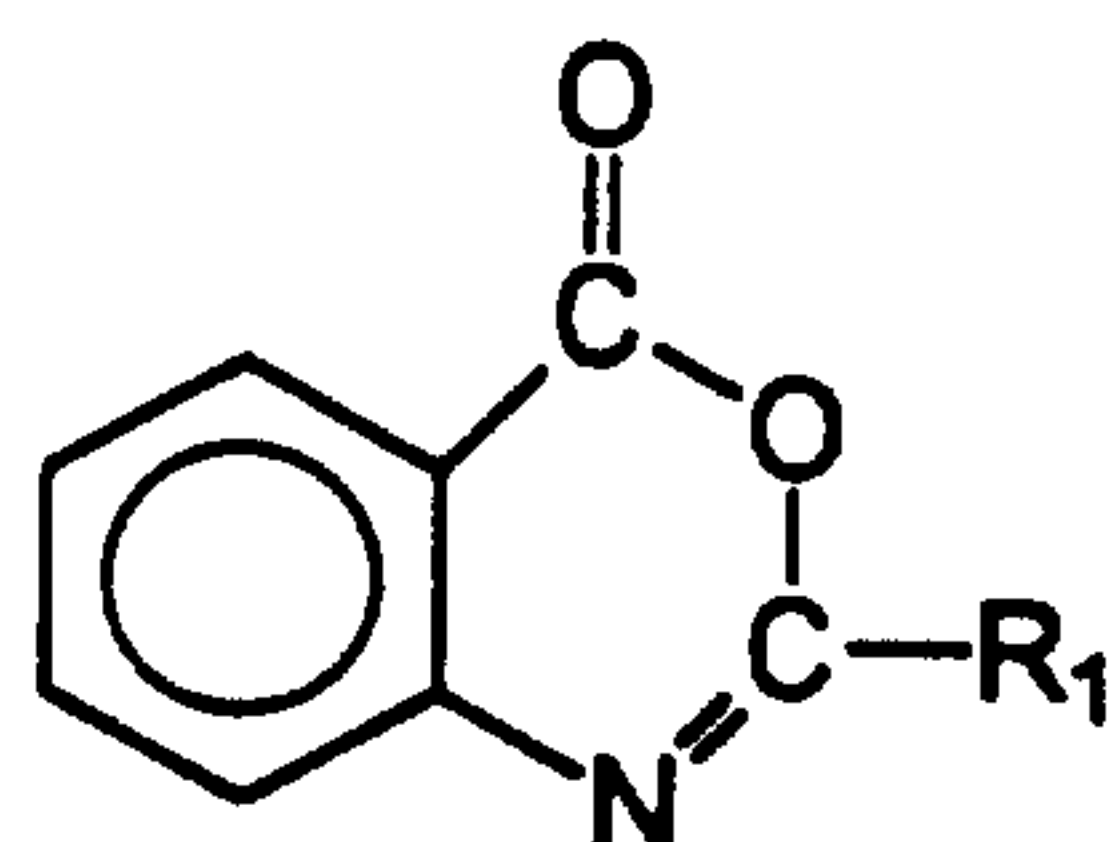
Preferred bleach activators are those of the above general formula wherein L is selected from the group consisting of:



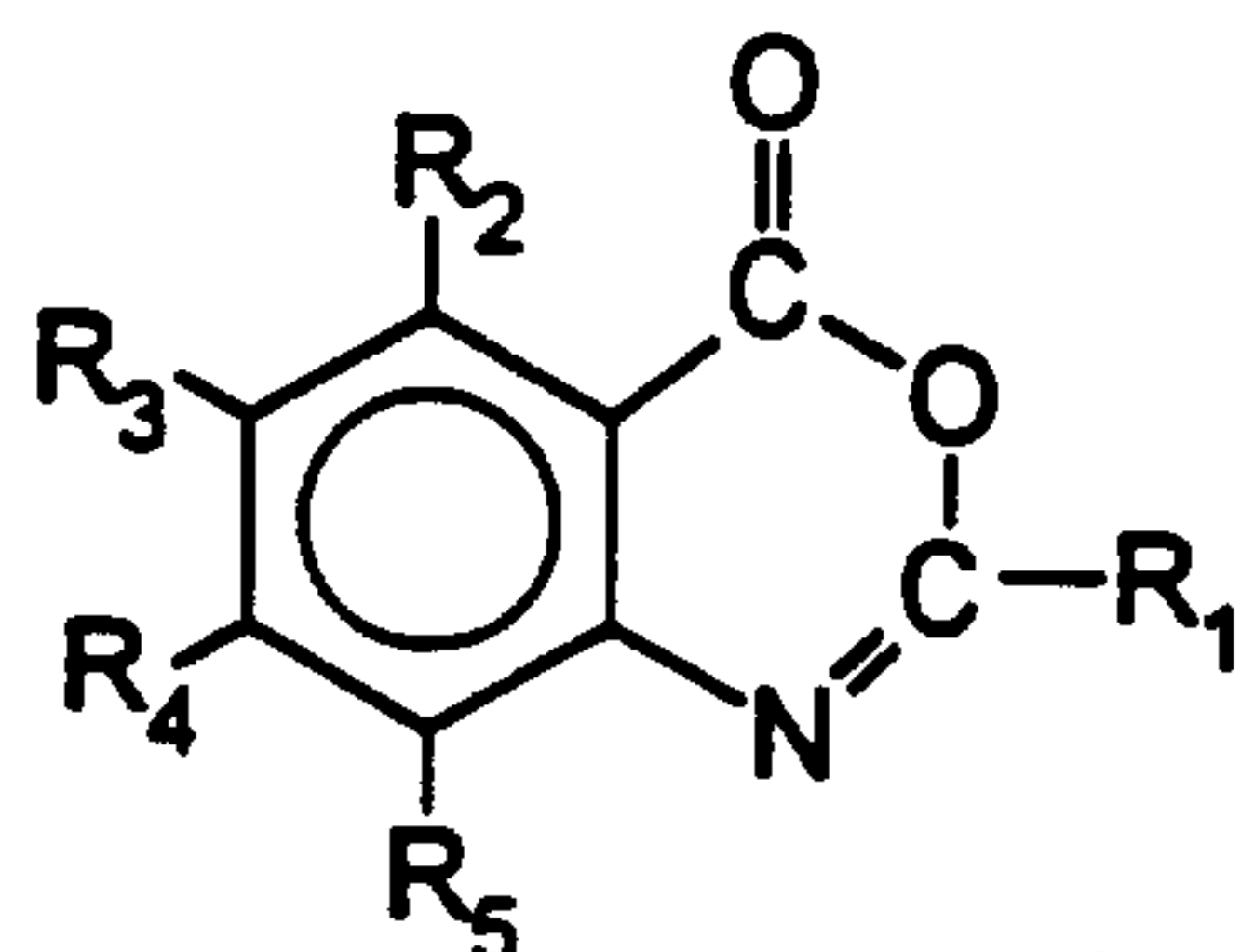
wherein R^3 is as defined above and Y is $-\text{SO}_3^-\text{M}^+$ or $-\text{CO}_2^-\text{M}^+$ wherein M is as defined above.

Another important class of bleach activators, including those of type b) and type c), provide organic peracids as described herein by ring-opening as a consequence of the nucleophilic attack on the carbonyl carbon of the cyclic ring by the perhydroxide anion. For instance, this ring-opening reaction in type c) activators involves attack at the caprolactam ring carbonyl by hydrogen peroxide or its anion. Since attack of an acyl caprolactam by hydrogen peroxide or its anion occurs preferably at the exocyclic carbonyl, obtaining a significant fraction of ring-opening may require a catalyst. Another example of ring-opening bleach activators can be found in type b) activators, such as those disclosed in U.S. Patent 4,966,723, Hodge et al, issued Oct. 30, 1990.

- Benzoxazin-type Bleach Activators - Such activator compounds disclosed by Hodge include the activators of the benzoxazin-type, having the formula:

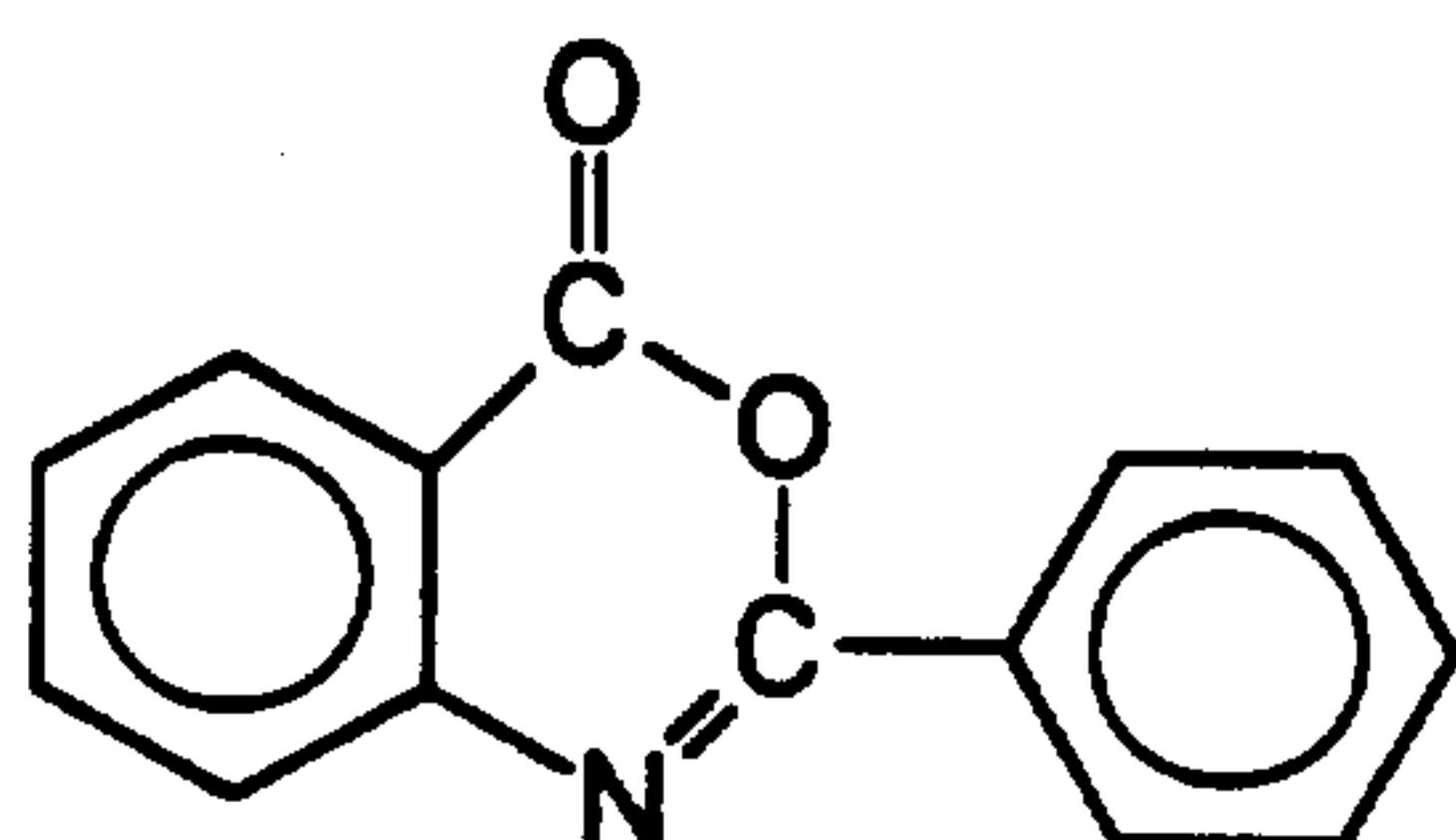


including the substituted benzoxazins of the type



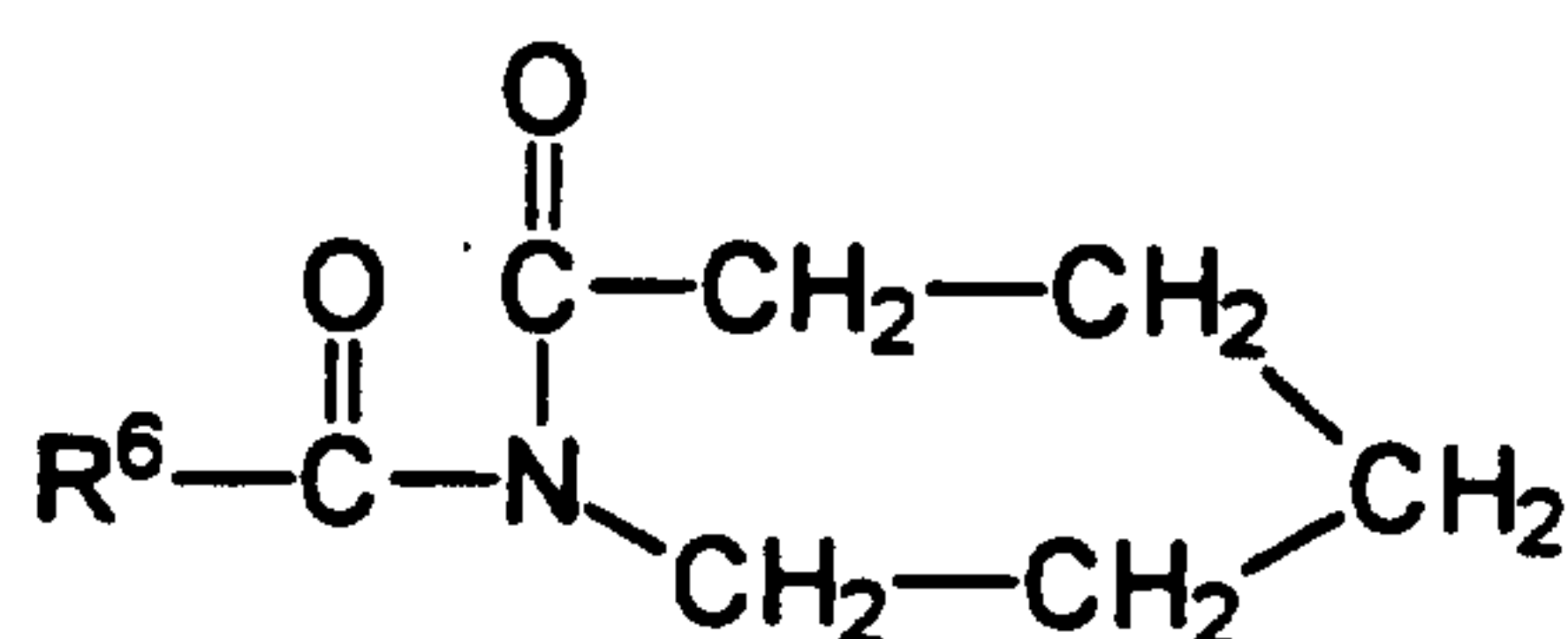
wherein R_1 is H, alkyl, alkaryl, aryl, arylalkyl, and wherein R_2 , R_3 , R_4 ,
 5 and R_5 may be the same or different substituents selected from H, halogen, alkyl, alkenyl, aryl, hydroxyl, alkoxy, amino, alkyl amino, COOR_6 (wherein R_6 is H or an alkyl group) and carbonyl functions.

A preferred activator of the benzoxazin-type is:



10 When the activators are used, optimum surface bleaching performance is obtained with washing solutions wherein the pH of such solution is between about 8.5 and 10.5 and preferably between 9.5 and 10.5 in order to facilitate the perhydrolysis reaction. Such pH can be obtained with substances commonly known as buffering agents, which
 15 are optional components of the bleaching systems herein.

- N-Acyl Caprolactam Bleach Activators - The N-acyl caprolactam bleach activators of type c) employed in the present invention have the formula:



20 wherein R^6 is H or an alkyl, aryl, alkoxyaryl, or alkaryl group containing from 1 to 12 carbons. Caprolactam activators wherein the R^6 moiety

contains at least about 6, preferably from 6 to about 12, carbon atoms provide hydrophobic bleaching which affords nucleophilic and body soil clean-up, as noted above. Caprolactam activators wherein R⁶ comprises from 1 to about 6 carbon atoms provide hydrophilic
5 bleaching species which are particularly efficient for bleaching beverage stains. Mixtures of hydrophobic and hydrophilic caprolactams, typically at weight ratios of 1:5 to 5:1, preferably 1:1, can be used herein for mixed stain removal benefits.

Highly preferred N-acyl caprolactams are selected from the group
10 consisting of benzoyl caprolactam, octanoyl caprolactam, nonanoyl caprolactam, 3,5,5-trimethylhexanoyl caprolactam, decanoyl caprolactam, undecenoyl caprolactam, and mixtures thereof. Methods for making N-acyl caprolactams are well known in the art.

Contrary to the teachings of U.S. Pat. 4,545,784, the bleach
15 activator is preferably not absorbed onto the peroxygen bleaching compound. To do so in the presence of other organic deterative ingredients could cause safety problems.

The bleach activators of type a), b) or c) will comprise at least about 0.1%, preferably from about 0.1% to about 50%, more preferably
20 from about 1% to about 30%, most preferably from about 3% to about 25%, by weight of bleaching system or detergent composition.

The preferred amido-derived and caprolactam bleach activators herein can also be used in combination with rubber-safe, enzyme-safe, hydrophilic activators such as TAED, typically at weight ratios of amido-
25 derived or caprolactam activators:TAED in the range of 1:5 to 5:1, preferably about 1:1.

The bleaching mechanism generally, and the surface bleaching mechanism in particular, are not completely understood. However, it is generally believed that the bleach activator undergoes nucleophilic
30 attack by a perhydroxide anion, which is generated from the hydrogen peroxide evolved by the peroxygen bleach, to form a peroxycarboxylic acid. This reaction is commonly referred to as perhydrolysis.

When the activators are used, optimum surface bleaching performance is obtained with washing solutions wherein the pH of such
35 solution is between about 8.5 and 10.5 and preferably between 9.5 and 10.5 in order to facilitate the perhydrolysis reaction. Such pH can be

obtained with substances commonly known as buffering agents, which are optional components of the bleaching systems herein.

(ii) The Peroxygen Bleaching Compound

The peroxygen bleaching systems useful herein are those
5 capable of yielding hydrogen peroxide in an aqueous liquor. These compounds are well known in the art and include hydrogen peroxide and the alkali metal peroxides, organic peroxide bleaching compounds such as urea peroxide, and inorganic persalt bleaching compounds, such as the alkali metal perborates, percarbonates, perphosphates,
10 and the like. Mixtures of two or more such bleaching compounds can also be used, if desired.

Preferred peroxygen bleaching compounds include sodium perborate, commercially available in the form of mono-, tri-, and tetra-
hydrate, sodium pyrophosphate peroxyhydrate, urea peroxyhydrate,
15 sodium percarbonate, and sodium peroxide. Particularly preferred are sodium perborate tetrahydrate, sodium perborate monohydrate and sodium percarbonate. Percarbonate is especially preferred because it is very stable during storage and yet still dissolves very quickly in the bleaching liquor. It is believed that such rapid dissolution results in the
20 formation of higher levels of percarboxylic acid and, thus, enhanced surface bleaching performance.

Highly preferred percarbonate can be in uncoated or coated form. The average particle size of uncoated percarbonate ranges from about 400 to about 1200 microns, most preferably from about 400 to about
25 600 microns. If coated percarbonate is used, the preferred coating materials include mixtures of carbonate and sulphate, silicate, borosilicate, or fatty carboxylic acids.

The peroxygen bleaching compound will comprise at least about 0.1%, preferably from about 1% to about 75%, more preferably from
30 about 3% to about 40%, most preferably from about 3% to about 25%, by weight of bleaching system or detergent composition.

The weight ratio of bleach activator to peroxygen bleaching compound in the bleaching system typically ranges from about 2:1 to 1:5. Preferred ratios range from about 1:1 to about 1:3.

35 The molar ratio of hydrogen peroxide yielded by the peroxygen bleaching compound to the bleach activator is greater than about 1.0,

more preferably greater than about 1.5, and most preferably from about 2.0 to about 10. Preferably, the bleaching compositions herein comprise from about 0.5 to about 20, most preferably from about 1 to about 10, wt.% of the peroxygen bleaching compound.

5 The bleach activator/bleaching compound systems herein are useful per se as bleaches. However, such bleaching systems are especially useful in compositions which can comprise various detergent adjuncts such as surfactants, builders and the like.

(2) Protease Enzymes:

10 The invention includes protease enzymes which are non-naturally-occurring carbonyl hydrolase variants having a different proteolytic activity, stability, substrate specificity, pH profile and/or performance characteristic as compared to the precursor carbonyl hydrolase from which the amino acid sequence of the variant is derived. The precursor
15 carbonyl hydrolase may be a naturally-occurring carbonyl hydrolase or recombinant hydrolase. Specifically, such carbonyl hydrolase variants have an amino acid sequence not found in nature, which is derived by replacement of a plurality of amino acid residues of a precursor carbonyl hydrolase with different amino acids. The plurality of amino acid residues
20 of the precursor enzyme correspond to position +76 in combination with one or more of the following residues +99, +101, +103, +104, +107, +123, +27, +105, +109, +126, +128, +135, +156, +166, +195, +197, +204, +206, +210, +216, +217, +218, +222, +260, +265, and/or +274, where the numbered position corresponds to naturally-occurring subtilisin
25 from *Bacillus amyloliquefaciens* or to equivalent amino acid residues in other carbonyl hydrolases or subtilisins, such as *Bacillus lentus* subtilisin.

 The carbonyl hydrolase variants which are protease enzyme useful in the present invention compositions comprise replacement of amino acid residue +76 in combination with one or more additional
30 modifications. Preferably the variant protease enzymes useful for the present invention comprise the substitution, deletion or insertion of amino acid residues in the following combinations: 76/99; 76/101; 76/103; 76/104; 76/107; 76/123; 76/99/101; 76/99/103; 76/99/104; 76/101/103; 76/101/104; 76/103/104; 76/104/107; 76/104/123; 76/107/123;
35 76/99/101/103; 76/99/101/104; 76/99/103/104; 76/101/103/104; 76/103/104/123; 76/104/107/123; 76/99/101/103/104; 76/99/103/104/123;

76/99/101/103/104/123; 76/103/104/128; 76/103/104/260;
76/103/104/265; 76/103/104/197; 76/103/104/105; 76/103/104/135;
76/103/104/126; 76/103/104/107; 76/103/104/210; 76/103/104/126/265;
and/or 76/103/104/222. Most preferably the variant enzymes useful for
5 the present invention comprise the substitution, deletion or insertion of an
amino acid residue in the following combination of residues: 76/99;
76/104; 76/99/104; 76/103/104; 76/104/107; 76/101/103/104;
76/99/101/103/104 and 76/101/104 of *B. amyloliquefaciens* subtilisin.

Variant DNA sequences encoding such carbonyl hydrolase or
10 subtilisin variants are derived from a precursor DNA sequence which
encodes a naturally-occurring or recombinant precursor enzyme. The
variant DNA sequences are derived by modifying the precursor DNA
sequence to encode the substitution of one or more specific amino acid
residues encoded by the precursor DNA sequence corresponding to
15 positions 76, 99, 101, 103, 104, 107, 123, 27, 105, 109, 126, 128, 135,
156, 166, 195, 197, 204, 206, 210, 216, 217, 218, 222, 260, 265 and/or
274, in *Bacillus amyloliquefaciens* or any combination thereof. Although
the amino acid residues identified for modification herein are identified
according to the numbering applicable to *B. amyloliquefaciens* (which has
20 become the conventional method for identifying residue positions in all
subtilisins), the preferred precursor DNA sequence useful for the present
invention is the DNA sequence of *Bacillus lentus* as shown in Fig. 6 (Seq.
ID No. 11).

These variant DNA sequences encode the insertion or substitution
25 of the amino acid residue 76 in combination with one or more additional
modification. Preferably the variant DNA sequences encode the
substitution or insertion of amino acid residues in the following
combinations: 76/99; 76/101; 76/103; 76/104; 76/107; 76/123;
76/99/101; 76/99/103; 76/99/104; 76/101/103; 76/101/104; 76/103/104;
30 76/104/107; 76/104/123; 76/107/123; 76/99/101/103; 76/99/101/104;
76/99/103/104; 76/101/103/104; 76/103/104/123; 76/104/107/123;
76/99/101/103/104; 76/99/103/104/123; 76/99/101/103/104/123;
76/103/104/128; 76/103/104/260; 76/103/104/265; 76/103/104/197;
76/103/104/105; 76/103/104/135; 76/103/104/126; 76/103/104/107;
35 76/103/104/210; 76/103/104/126/265; and/or 76/103/104/222. Most
preferably the variant DNA sequences encode for the modification of the

following combinations of residues: 76/99; 76/104; 76/99/104;
76/103/104; 76/104/107; 76/101/103/104; 76/99/101/103/104 and
76/101/104. These recombinant DNA sequences encode carbonyl
hydrolase variants having a novel amino acid sequence and, in general,
5 at least one property which is substantially different from the same
property of the enzyme encoded by the precursor carbonyl hydrolase
DNA sequence. Such properties include proteolytic activity, substrate
specificity, stability, altered pH profile and/or enhanced performance
characteristics.

10 The protease enzymes useful herein encompass the substitution
of any of the nineteen naturally occurring L-amino acids at the
designated amino acid residue positions. Such substitutions can be
made in any precursor subtilisin (procaryotic, eucaryotic, mammalian,
etc.). Throughout this application reference is made to various amino
15 acids by way of common one- and three-letter codes. Such codes are
identified in Dale, J.W. (1989), Molecular Genetics of Bacteria, John
Wiley & Sons, Ltd., Appendix B.

Preferably, the substitution to be made at each of the identified
amino acid residue positions include but are not limited to: substitutions
20 at position 76 including D, H, E, G, F, K, P and N; substitutions at
position 99 including D, T, N, Q, G and S; substitutions at position 101
including G, D, K, L, A, E, S and R; substitutions at position 103 including
Q, T, D, E, Y, K, G, R, S, and A; substitutions at position 104 including all
nineteen naturally-occurring amino acids; substitutions at position 107
25 including V, L, M, Y, G, E, F, T, S, A, N and I; substitutions at position
123 including N, T, I, G, A, C, and S; substitutions at position 27
including K, N, C, V and T; substitutions at position 105 including A, D,
G, R and N; substitutions at position 107 including A, L, V, Y, G, F, T, S
and A; substitutions at position 109 including S, K, R, A, N and D;
30 substitutions at position 126 including A, F, I, V and G; substitutions at
position 128 including G, L and A; substitutions at position 135 including
A, F, I, S and V; substitutions at position 156 including D, E, A, G, Q and
K; substitutions at position 166 including all nineteen naturally-occurring
amino acids; substitutions at position 195 including E; substitutions at
35 position 197 including E; substitutions at position 204 including A, G, C,
S and D; substitutions at position 206 including L, Y, N, D and E;

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substitutions at position 210 including L, I, S, C and F; substitutions at position 216 including V, E, T and K; substitutions at position 217 including all nineteen naturally-occurring amino acids; substitutions at position 218 including S, A, G, T and V; substitutions at position 222 including all nineteen naturally-occurring amino acids; substitutions at position 260 including P, N, G, A, S, C, K and D; substitutions at position 265 including N, G, A, S, C, K, Y and H; and substitutions at position 274 including A and S. The specifically preferred amino acid(s) to be substituted at each such position are designated below in Table I.

Although specific amino acids are shown in Table I, it should be understood that any amino acid may be substituted at the identified residues.

Table I

	<u>Amino Acid Residue</u>	<u>Preferred Amino Acid to be Substituted/Inserted</u>
15	+76	D,H
	+99	D,T,N,G
	+101	R,G,D,K,L,A,E
	+103	A,Q,T,D,E,Y,K,G,R
20	+104	I,Y,S,L,A,T,G,F,M,W,D,V,N
	+107	V,L,Y,G,F,T,S,A,N
	+123	S,T,I
	+27	K
	+105	A,D
25	+109	S,K,R
	+126	A,I,V,F
	+128	G,L
	+135	I,A,S
	+156	E,D,Q
30	+166	D,G,E,K,N,A,F,I,V,L
	+195	E
	+197	E
	+204	A,G,C
	+206	L
35	+210	I,S,C
	+216	V

	+217	H,I,Y,C,A,G,F,S,N,E,K
	+218	S
	+222	A,Q,S,C,I,K
	+260	P,A,S,N,G
5	+265	N,A,G,S
	+274	A,S

These protease enzymes containing *in vitro* mutations in *B. lentus* subtilisin at an amino acid residue equivalent to +76 in *Bacillus amyloliquefaciens* subtilisin produces subtilisin variants exhibiting altered stability (e.g., modified autoproteolytic stability) over precursor subtilisins. (See Tables IV and VI.)

Also, *in vitro* mutation at residues equivalent to +99, +101, +103, +104, +107, +123, +27, +105, +109, +126, +128, +135, +156, +166, +195, +197, +204, +206, +210, +216, +217, +218, +222, +260, +265, and/or +274 in *Bacillus amyloliquefaciens* subtilisin, alone or in combination with each other and in any combination with +76 mutations, produce subtilisin variants exhibiting altered proteolytic activity, altered thermal stability, altered pH profile, altered substrate specificity and/or altered performance characteristics.

Carbonyl hydrolases are protease enzymes which hydrolyze compounds containing



bonds in which X is oxygen or nitrogen. They include naturally-occurring carbonyl hydrolases and recombinant carbonyl hydrolases. Naturally-occurring carbonyl hydrolases principally include hydrolases, e.g., peptide hydrolases such as subtilisins or metalloproteases. Peptide hydrolases include α -aminoacylpeptide hydrolase, peptidylamino acid hydrolase, acylamino hydrolase, serine carboxypeptidase, metallocarboxypeptidase, thiol proteinase, carboxylproteinase and metalloproteinase. Serine, metallo, thiol and acid proteases are included, as well as endo and exo-proteases.

"Recombinant carbonyl hydrolase" refers to a carbonyl hydrolase in which the DNA sequence encoding the naturally-occurring carbonyl hydrolase is modified to produce a mutant DNA sequence which encodes

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the substitution, insertion or deletion of one or more amino acids in the carbonyl hydrolase amino acid sequence. Suitable modification methods are disclosed herein, and in U.S. Patent 4,760,025 (RE 34,606), U.S. Patent 5,204,015 and U.S. Patent 5,185,258.

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Subtilisins are bacterial or fungal carbonyl hydrolases which generally act to cleave peptide bonds of proteins or peptides. As used herein, "subtilisin" means a naturally-occurring subtilisin or a recombinant subtilisin. A series of naturally-occurring subtilisins is known to be
10 produced and often secreted by various microbial species. Amino acid sequences of the members of this series are not entirely homologous. However, the subtilisins in this series exhibit the same or similar type of proteolytic activity. This class of serine proteases shares a common amino acid sequence defining a catalytic triad which distinguishes them
15 from the chymotrypsin related class of serine proteases. The subtilisins and chymotrypsin related serine proteases both have a catalytic triad comprising aspartate, histidine and serine. In the subtilisin related proteases the relative order of these amino acids, reading from the amino to carboxy terminus, is aspartate-histidine-serine. In the chymotrypsin
20 related proteases the relative order, however, is histidine-aspartate-serine. Thus, subtilisin herein refers to a serine protease having the catalytic triad of subtilisin related proteases. Examples include but are not limited to the subtilisins identified in Fig. 3 herein.

"Recombinant subtilisin" refers to a subtilisin in which the DNA
25 sequence encoding the subtilisin is modified to produce a variant (or mutant) DNA sequence which encodes the substitution, deletion or insertion of one or more amino acids in the naturally-occurring subtilisin amino acid sequence. Suitable methods to produce such modification, and which may be combined with those disclosed herein, include those
30 disclosed in U.S. Patent 4,760,025 (RE 34,606), U.S. Patent 5,204,015 and U.S. Patent 5,185,258.

"Non-human carbonyl hydrolases" and the DNA encoding them may be obtained from many procaryotic and eucaryotic organisms. Suitable examples of procaryotic organisms include gram negative
35 organisms such as *E. coli* or *Pseudomonas* and gram positive bacteria such as *Micrococcus* or *Bacillus*. Examples of eucaryotic organisms from

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which carbonyl hydrolase and their genes may be obtained include yeast such as *Saccharomyces cerevisiae*, fungi such as *Aspergillus* sp. and non-human mammalian sources such as, for example, *bovine* sp. from which the gene encoding the carbonyl hydrolase chymosin can be
5 obtained. As with subtilisins, a series of carbonyl hydrolases can be obtained from various related species which have amino acid sequences which are not entirely homologous between the members of that series but which nevertheless exhibit the same or similar type of biological activity. Thus, non-human carbonyl hydrolase as used herein has a
10 functional definition which refers to carbonyl hydrolases which are associated, directly or indirectly, with procaryotic and eucaryotic sources.

A "carbonyl hydrolase variant" has an amino acid sequence which is derived from the amino acid sequence of a "precursor carbonyl hydrolase." The precursor carbonyl hydrolases (such as a subtilisin)
15 include naturally-occurring carbonyl hydrolases (subtilisin) and recombinant carbonyl hydrolases (subtilisin). The amino acid sequence of the carbonyl hydrolase variant is "derived" from the precursor hydrolase amino acid sequence by the substitution, deletion or insertion of one or more amino acids of the precursor amino acid sequence. Such
20 modification is of the "precursor DNA sequence" which encodes the amino acid sequence of the precursor carbonyl hydrolase (subtilisin) rather than manipulation of the precursor carbonyl hydrolase (subtilisin) enzyme *per se*. Suitable methods for such manipulation of the precursor DNA sequence include methods disclosed herein, as well as methods
25 known to those skilled in the art (see, for example, EP 0 328299, WO89/06279 and the US patents already mentioned).

Specific residues corresponding to position +76 in combination with one or more of the following positions +99, +101, +103, +104, +107,
30 +123, +27, +105, +109, +126, +128, +135, +156, +166, +195, +197, +204, +206, +210, +216, +217, +218, +222, +260, +265 and/or +274 of *Bacillus amyloliquefaciens* subtilisin are identified herein for mutation. Preferably the modified residues are selected from the following combinations: 76/99; 76/101; 76/103; 76/104; 76/107; 76/123;
35 76/99/101; 76/99/103; 76/99/104; 76/101/103; 76/101/104; 76/103/104; 76/104/107; 76/104/123; 76/107/123; 76/99/101/103; 76/99/101/104;

76/99/103/104; 76/101/103/104; 76/103/104/123; 76/104/107/123;
76/99/101/103/104; 76/99/103/104/123; 76/99/101/103/104/123;
76/103/104/128; 76/103/104/260; 76/103/104/265; 76/103/104/197;
76/103/104/105; 76/103/104/135; 76/103/104/126; 76/103/104/107;
5 76/103/104/210; 76/103/104/126/265; and/or 76/103/104/222; and most
preferably are 76/99; 76/104; 76/99/104; 76/103/104; 76/104/107;
76/101/103/104; 76/99/101/103/104 and 76/101/104. These amino acid
position numbers refer to those assigned to the mature *Bacillus*
amyloliquefaciens subtilisin sequence presented in Fig. 1. The protease
10 enzymes useful in the present invention, however, are not limited to the
mutation of this particular subtilisin but extends to precursor carbonyl
hydrolases containing amino acid residues at positions which are
"equivalent" to the particular identified residues in *Bacillus*
amyloliquefaciens subtilisin. Preferably, the precursor subtilisin is
15 *Bacillus lentus* subtilisin and the substitutions, deletions or insertions are
made at the equivalent amino acid residue in *B. lentus* corresponding to
those listed above.

A residue (amino acid) of a precursor carbonyl hydrolase is
equivalent to a residue of *Bacillus amyloliquefaciens* subtilisin if it is
20 either homologous (i.e., corresponding in position in either primary or
tertiary structure) or analogous to a specific residue or portion of that
residue in *Bacillus amyloliquefaciens* subtilisin (i.e., having the same or
similar functional capacity to combine, react, or interact chemically).

In order to establish homology to primary structure, the amino acid
25 sequence of a precursor carbonyl hydrolase is directly compared to the
Bacillus amyloliquefaciens subtilisin primary sequence and particularly to
a set of residues known to be invariant in subtilisins for which sequence
is known. Fig. 2 herein shows the conserved residues as between
amyloliquefaciens subtilisin and *B. lentus* subtilisin. After aligning the
30 conserved residues, allowing for necessary insertions and deletions in
order to maintain alignment (i.e., avoiding the elimination of conserved
residues through arbitrary deletion and insertion), the residues equivalent
to particular amino acids in the primary sequence of *Bacillus*
amyloliquefaciens subtilisin are defined. Alignment of conserved
35 residues preferably should conserve 100% of such residues. However,
alignment of greater than 75% or as little as 50% of conserved residues

is also adequate to define equivalent residues. Conservation of the catalytic triad, Asp32/His64/Ser221 should be maintained.

For example, in Fig. 3 the amino acid sequence of subtilisin from *Bacillus amyloliquefaciens*, *Bacillus subtilis*, *Bacillus licheniformis* (carlsbergensis) and *Bacillus lentus* are aligned to provide the maximum amount of homology between amino acid sequences. A comparison of these sequences shows that there are a number of conserved residues contained in each sequence. These conserved residues (as between BPN' and *B. lentus*) are identified in Fig. 2.

These conserved residues, thus, may be used to define the corresponding equivalent amino acid residues of *Bacillus amyloliquefaciens* subtilisin in other carbonyl hydrolases such as subtilisin from *Bacillus lentus* (PCT Publication No. W089/06279 published July 13, 1989), the preferred subtilisin precursor enzyme herein, or the subtilisin referred to as PB92 (EP O 328 299), which is highly homologous to the preferred *Bacillus lentus* subtilisin. The amino acid sequences of certain of these subtilisins are aligned in Figs. 3A and 3B with the sequence of *Bacillus amyloliquefaciens* subtilisin to produce the maximum homology of conserved residues. As can be seen, there are a number of deletions in the sequence of *Bacillus lentus* as compared to *Bacillus amyloliquefaciens* subtilisin. Thus, for example, the equivalent amino acid for Val165 in *Bacillus amyloliquefaciens* subtilisin in the other subtilisins is isoleucine for *B. lentus* and *B. licheniformis*.

Thus, for example, the amino acid at position +76 is asparagine (N) in both *B. amyloliquefaciens* and *B. lentus* subtilisins. In the preferred subtilisin variant useful in the invention, however, the amino acid equivalent to +76 in *Bacillus amyloliquefaciens* subtilisin is substituted with aspartate (D). A comparison of all the amino acid residues identified herein for substitution versus the preferred substitution for each such position is provided in Table II for illustrative purposes.

Table II

	<u>+76</u>	<u>+99</u>	<u>+101</u>	<u>+103</u>	<u>+104</u>	<u>+107</u>	<u>+123</u>
<i>B. amyloliquefaciens</i> (wild-type)	N	D	S	Q	Y	I	N

B. lentus (wild-type) N S S S V I N

Most Preferred Substitution D D R A I/Y V S

Equivalent residues may also be defined by determining homology
 5 at the level of tertiary structure for a precursor carbonyl hydrolase whose
 tertiary structure has been determined by x-ray crystallography.
 Equivalent residues are defined as those for which the atomic
 coordinates of two or more of the main chain atoms of a particular amino
 acid residue of the precursor carbonyl hydrolase and *Bacillus*
 10 *amyloliquefaciens* subtilisin (N on N, CA on CA, C on C and O on O) are
 within 0.13nm and preferably 0.1nm after alignment. Alignment is
 achieved after the best model has been oriented and positioned to give
 the maximum overlap of atomic coordinates of non-hydrogen protein
 atoms of the carbonyl hydrolase in question to the *Bacillus*
 15 *amyloliquefaciens* subtilisin. The best model is the crystallographic
 model giving the lowest R factor for experimental diffraction data at the
 highest resolution available.

$$R \text{ factor} = \frac{\sum_h |F_o(h)| - |F_c(h)|}{\sum_h |F_o(h)|}$$

Equivalent residues which are functionally analogous to a specific
 residue of *Bacillus amyloliquefaciens* subtilisin are defined as those
 20 amino acids of the precursor carbonyl hydrolases which may adopt a
 conformation such that they either alter, modify or contribute to protein
 structure, substrate binding or catalysis in a manner defined and
 attributed to a specific residue of the *Bacillus amyloliquefaciens* subtilisin.
 Further, they are those residues of the precursor carbonyl hydrolase (for
 25 which a tertiary structure has been obtained by x-ray crystallography)
 which occupy an analogous position to the extent that, although the main
 chain atoms of the given residue may not satisfy the criteria of
 equivalence on the basis of occupying a homologous position, the atomic
 coordinates of at least two of the side chain atoms of the residue lie with
 30 0.13nm of the corresponding side chain atoms of *Bacillus*
amyloliquefaciens subtilisin. The coordinates of the three dimensional
 structure of *Bacillus amyloliquefaciens* subtilisin are set forth in EPO
 Publication No. 0 251 446

and can be used as outlined above to determine equivalent residues on the level of tertiary structure.

Some of the residues identified for substitution, insertion or deletion are conserved residues whereas others are not. In the case of residues which are not conserved, the replacement of one or more amino acids is limited to substitutions which produce a variant which has an amino acid sequence that does not correspond to one found in nature. In the case of conserved residues, such replacements should not result in a naturally-occurring sequence. The carbonyl hydrolase variants useful in the present invention include the mature forms of carbonyl hydrolase variants, as well as the pro- and prepro-forms of such hydrolase variants. The prepro-forms are the preferred construction since this facilitates the expression, secretion and maturation of the carbonyl hydrolase variants.

"Prosequence" refers to a sequence of amino acids bound to the N-terminal portion of the mature form of a carbonyl hydrolase which when removed results in the appearance of the "mature" form of the carbonyl hydrolase. Many proteolytic enzymes are found in nature as translational proenzyme products and, in the absence of post-translational processing, are expressed in this fashion. A preferred prosequence for producing carbonyl hydrolase variants, specifically subtilisin variants, is the putative prosequence of *Bacillus amyloliquefaciens* subtilisin, although other subtilisin prosequences may be used. In the Examples, the putative prosequence from the subtilisin from *Bacillus lentus* (ATCC 21536) is used.

A "signal sequence" or "presequence" refers to any sequence of amino acids bound to the N-terminal portion of a carbonyl hydrolase or to the N-terminal portion of a prohydrolase which may participate in the secretion of the mature or pro forms of the hydrolase. This definition of signal sequence is a functional one, meant to include all those amino acid sequences encoded by the N-terminal portion of the subtilisin gene or other secretable carbonyl hydrolases which participate in the effectuation of the secretion of subtilisin or other carbonyl hydrolases under native conditions. The protease enzymes useful for the present invention utilize such sequences to effect the secretion of the carbonyl hydrolase variants as described herein. A preferred signal sequence used in the Examples comprises the first seven amino acid residues of

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the signal sequence from *Bacillus subtilis* subtilisin fused to the remainder of the signal sequence of the subtilisin from *Bacillus lentus* (ATCC 21536).

5 A "prepro" form of a carbonyl hydrolase variant consists of the mature form of the hydrolase having a prosequence operably linked to the amino terminus of the hydrolase and a "pre" or "signal" sequence operably linked to the amino terminus of the prosequence.

"Expression vector" refers to a DNA construct containing a DNA sequence which is operably linked to a suitable control sequence
10 capable of effecting the expression of said DNA in a suitable host. Such control sequences include a promoter to effect transcription, an optional operator sequence to control such transcription, a sequence encoding suitable mRNA ribosome binding sites and sequences which control termination of transcription and translation. The vector may be a
15 plasmid, a phage particle, or simply a potential genomic insert. Once transformed into a suitable host, the vector may replicate and function independently of the host genome, or may, in some instances, integrate into the genome itself. In the present specification, "plasmid" and "vector" are sometimes used interchangeably as the plasmid is the most
20 commonly used form of vector at present. However, included herein are such other forms of expression vectors which serve equivalent functions and which are, or become, known in the art.

The "host cells" used in the present invention generally are procaryotic or eucaryotic hosts which preferably have been manipulated
25 by the methods disclosed in U.S. Patent 4,760,025 (RE 34,606) to render them incapable of secreting enzymatically active endoprotease. A preferred host cell for expressing subtilisin is the *Bacillus* strain BG2036 which is deficient in enzymatically active neutral protease and alkaline protease (subtilisin). The construction of strain BG2036 is described in
30 detail in U.S. Patent 5,264,366. Other host cells for expressing subtilisin include *Bacillus subtilis* 1168 (also described in U.S. Patent 4,760,025 (RE 34,606) and U.S. Patent 5,264,366), as well as any suitable *Bacillus* strain such as *B. licheniformis*, *B. lentus*, etc.

35 Host cells are transformed or transfected with vectors constructed using recombinant DNA techniques. Such transformed host cells are

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capable of either replicating vectors encoding the carbonyl hydrolase variants or expressing the desired carbonyl hydrolase variant. In the case of vectors which encode the pre- or prepro-form of the carbonyl hydrolase variant, such variants, when expressed, are typically secreted
5 from the host cell into the host cell medium.

"Operably linked, " when describing the relationship between two DNA regions, simply means that they are functionally related to each other. For example, a presequence is operably linked to a peptide if it functions as a signal sequence, participating in the secretion of the
10 mature form of the protein most probably involving cleavage of the signal sequence. A promoter is operably linked to a coding sequence if it controls the transcription of the sequence; a ribosome binding site is operably linked to a coding sequence if it is positioned so as to permit translation.

15 The genes encoding the naturally-occurring precursor carbonyl hydrolase may be obtained in accord with the general methods known to those skilled in the art. The methods generally comprise synthesizing labeled probes having putative sequences encoding regions of the hydrolase of interest, preparing genomic libraries from organisms
20 expressing the hydrolase, and screening the libraries for the gene of interest by hybridization to the probes. Positively hybridizing clones are then mapped and sequenced. The *B. lentus* gene used in the Examples is cloned as described in Example 1 of U.S. Patent 5,185,258. The BPN' gene used in the Examples is cloned as described in Example
25 1 in RE 34,606.

The cloned carbonyl hydrolase is then used to transform a host cell in order to express the hydrolase. The hydrolase gene is then ligated into a high copy number plasmid. This plasmid replicates in hosts
30 in the sense that it contains the well-known elements necessary for plasmid replication: a promoter operably linked to the gene in question (which may be supplied as the gene's own homologous promotor if it is recognized, i.e., transcribed, by the host), a transcription termination and polyadenylation region (necessary for stability of the mRNA transcribed
35 by the host from the hydrolase gene in certain eucaryotic host cells) which is exogenous or is supplied by the endogenous terminator region

of the hydrolase gene and, desirably, a selection gene such as an antibiotic resistance gene that enables continuous cultural maintenance of plasmid-infected host cells by growth in antibiotic-containing media. High copy number plasmids also contain an origin of replication for the host, thereby enabling large numbers of plasmids to be generated in the cytoplasm without chromosomal limitations. However, it is within the scope herein to integrate multiple copies of the hydrolase gene into host genome. This is facilitated by procaryotic and eucaryotic organisms which are particularly susceptible to homologous recombination.

The genes used in the present examples are a natural *B. lentus* gene and a natural *B. amyloliquefaciens* gene. Alternatively, a synthetic gene encoding a naturally-occurring or mutant precursor carbonyl hydrolase (subtilisin) may be produced. In such an approach, the DNA and/or amino acid sequence of the precursor hydrolase (subtilisin) is determined. Multiple, overlapping synthetic single-stranded DNA fragments are thereafter synthesized, which upon hybridization and ligation produce a synthetic DNA encoding the precursor hydrolase. An example of synthetic gene construction is set forth in Example 3 of U.S. Patent 5,204,015.

Once the naturally-occurring or synthetic precursor carbonyl hydrolase gene has been cloned, a number of modifications are undertaken to enhance the use of the gene beyond synthesis of the naturally-occurring precursor carbonyl hydrolase. Such modifications include the production of recombinant carbonyl hydrolases as disclosed in U.S. Patent 4,760,025 (RE 34,606) and EPO Publication No. 0 251 446 and the production of carbonyl hydrolase variants described herein.

The following cassette mutagenesis method may be used to facilitate the construction and identification of the carbonyl hydrolase variants useful in the present invention, although other methods including site-directed mutagenesis may be used. First, the naturally-occurring gene encoding the hydrolase is obtained and sequenced in whole or in part. Then the sequence is scanned for a point at which it is desired to make a mutation (deletion, insertion or substitution) of one or more amino acids in the encoded enzyme. The sequences flanking this point are evaluated for the presence of restriction sites for replacing a short

segment of the gene with an oligonucleotide pool which when expressed will encode various mutants. Such restriction sites are preferably unique sites within the hydrolase gene so as to facilitate the replacement of the gene segment. However, any convenient restriction site which is not
5 overly redundant in the hydrolase gene may be used, provided the gene fragments generated by restriction digestion can be reassembled in proper sequence. If restriction sites are not present at locations within a convenient distance from the selected point (from 10 to 15 nucleotides), such sites are generated by substituting nucleotides in the gene in such
10 a fashion that neither the reading frame nor the amino acids encoded are changed in the final construction. Mutation of the gene in order to change its sequence to conform to the desired sequence is accomplished by M13 primer extension in accord with generally known methods. The task of locating suitable flanking regions and evaluating the needed
15 changes to arrive at two convenient restriction site sequences is made routine by the redundancy of the genetic code, a restriction enzyme map of the gene and the large number of different restriction enzymes. Note that if a convenient flanking restriction site is available, the above method need be used only in connection with the flanking region which does not
20 contain a site.

Once the naturally-occurring DNA or synthetic DNA is cloned, the restriction sites flanking the positions to be mutated are digested with the cognate restriction enzymes and a plurality of end termini-complementary oligonucleotide cassettes are ligated into the gene. The mutagenesis is
25 simplified by this method because all of the oligonucleotides can be synthesized so as to have the same restriction sites, and no synthetic linkers are necessary to create the restriction sites.

As used herein, proteolytic activity is defined as the rate of hydrolysis of peptide bonds per milligram of active enzyme. Many well
30 known procedures exist for measuring proteolytic activity (K. M. Kalisz, "Microbial Proteinases," Advances in Biochemical Engineering/Biotechnology, A. Fiechter ed., 1988). In addition to or as an alternative to modified proteolytic activity, the variant enzymes of the present invention may have other modified properties such as K_m , K_{cat} ,
35 K_{cat}/K_m ratio and/or modified substrate specificity and/or modified pH activity profile. These enzymes can be tailored for the particular

substrate which is anticipated to be present, for example, for hydrolytic processes such as laundry uses.

One objective can be to secure a variant carbonyl hydrolase having altered proteolytic activity as compared to the precursor carbonyl hydrolase, since increasing such activity (numerically larger) enables the use of the enzyme to more efficiently act on a target substrate. Also of interest are variant enzymes having altered thermal stability and/or altered substrate specificity as compared to the precursor. Preferably the carbonyl hydrolase to be mutated is a subtilisin. Specific amino acids useful to obtain such results in subtilisin-type carbonyl hydrolases at residues equivalent to +76, +99, +101, +103, +104, +107, +123, +27, +105, +109, +126, +128, +135, +156, +166, +195, +197, +204, +206, +210, +216, +217, +218, +222, +260, +265 and/or +274 or any combination thereof in *Bacillus amyloliquefaciens* subtilisin are presented in the Examples. In some instances, lower proteolytic activity may be desirable. Conversely, in some instances it may be desirable to increase the proteolytic activity of the variant enzyme versus its precursor. Additionally, increases or decreases (alteration) of the stability of the variant, whether alkaline or thermal stability, may be desirable. Increases or decreases in K_{cat} , K_m or K_{cat}/K_m are specific to the substrate used to determine these kinetic parameters.

Also, it has been determined that residues equivalent to +76 in combination with a number of other modifications in subtilisin are important in modulating overall stability and/or proteolytic activity of the enzyme. Thus, as set forth in the Examples, the Asparagine (N) in *Bacillus lentus* subtilisin at equivalent position +76 can be substituted with Aspartate (D) in the preferred protease enzymes in combination with modification of one or more of the following amino acid residues +99, +101, +103, +104, +107, +123, +27, +105, +109, +126, +128, +135, +156, +166, +195, +197, +204, +206, +210, +216, +217, +218, +222, +260, +265 and/or +274 to produce enhanced stability and/or enhanced activity of the resulting mutant enzyme.

The most preferred protease enzymes useful in this invention are set forth in the Examples. These include the following specific combinations of substituted residues: N76D/S99D; N76D/V104I; N76D/S99D/V104I; N76D/S103A/V104I; N76D/V104I/I107V;

N76D/V104Y/I107V and N76D/S101R/S103A/V104I. These substitutions are preferably made in *Bacillus lentus* (recombinant or native-type) subtilisin, although the substitutions may be made in any *Bacillus* subtilisin.

5 Based on the results obtained with this and other variant subtilisins, it is apparent that residues in carbonyl hydrolases (preferably subtilisin) equivalent to positions +76, +99, +101, +103, +104, +107, +123, +27, +105, +109, +126, +128, +135, +156, +166, +195, +197, +204, +206, +210, +216, +217, +218, +222, +260, +265 and/or +274 in
10 *Bacillus amyloliquefaciens* are important to the proteolytic activity, performance and/or stability of these enzymes and the cleaning or wash performance of such variant enzymes.

The following is presented by way of example for manufacturing protease enzymes useful in the present invention compositions.

15 Protease Manufacture Example

Construction for the Expression of GG36 Gene in *B. subtilis*

The cloning and the construction for expression of the subtilisin gene from *B. lentus* is performed essentially the same as that described in U.S. Patent 5,185,258. The plasmid GGA274 (described in Fig. 4 herein) is
20 further modified in the following manner, as shown in Fig. 5. The PstI site that is introduced during the construction of the GGA274 plasmid is removed by the oligonucleotide directed mutagenesis described below, with an oligonucleotide having the following sequence: 5'
GAAGCTGCAACTCGTTAAA 3' (Seq. ID No.1). The underlined "A"
25 residue eliminates the recognition sequence of restriction enzyme PstI and changes the corresponding amino acid residue from alanine to threonine at position 274. Threonine at position 274 is the wild-type residue originally found in the cloned *B. lentus* subtilisin gene sequences. The DNA segment encoding subtilisin is excised from the
30 plasmid GGA274 or its derivatives (GGT274 shown in Fig. 5) by EcoRI and BamHI digest. The DNA fragment is subcloned back into Bacteriophage M13-based vectors, such as MP19, for mutagenesis. After mutagenesis, the EcoRI and HindIII digest, followed by cloning, are performed to move the mutated subtilisin gene back into an expression
35 plasmid like GGA274 for the expression and the recovery of mutated subtilisin proteins.

Oligonucleotide-Directed Mutagenesis

Oligonucleotide-directed mutagenesis is performed as described in Zoller, M. et al. (1983), Methods Enzymol., 100:468-500. As an example, a synthetic oligonucleotide of the sequence 5'
5 GCTGCTCTAGACAATTCG 3' (Seq. ID No.2) is used to change the amino acid residue at position 76 from asparagine (N) to aspartic acid (D), or N76D. The underlined "G" and "C" residues denote changes from the wild-type gene sequence. The CA keeps the leucine at position +75 and changes the amino acid sequence to introduce an XbaI recognition site of the XbaI restriction enzyme (TCTAGA), while the change at GAC
10 changes asparagine at +76 to aspartate.

For mutagenesis at positions 99, 101, 103 and 104, different oligonucleotides can be used depending on the combination of mutations desired. For example, an oligonucleotide of the sequence 5'
15 GTATTAGGGGCGGACGGTCGAGGCGCCATCAGCTCGATT 3' (Seq. ID No.3) is used to simultaneously make the following changes: S99D; S101R; S103A and V104I in a single subtilisin molecule. Similarly, oligonucleotides of the sequence 5'
TCAGGTTCTGGTCTCGAGCGTTGCCCAAGGATTG 3' (Seq. ID No.4)
20 and 5' CACGTTGCTAGCTTGAGTTTAG 3' (Seq. ID No.5) are utilized to generate I107V and N123S, respectively. Again, the underlined residues denote changes from wild-type sequences which produce desired changes either in amino acid sequences or restriction enzyme recognition sequences.

25 Proteolytic Activity of Subtilisin Variants

Following the methods of Oligonucleotide-Directed Mutagenesis hereinbefore, the variants listed in Table III are made. Proteolytic activity of each of these subtilisin variants is shown in Table III. The kinetic parameters k_{cat} , K_M , and k_{cat}/K_M are measured for hydrolysis of the
30 synthetic peptide substrate succinyl-L-Ala-L-Ala-L-Pro-L-Phe-p-nitroanilide using the method described in P. Bonneau et al. (1991) J. Am. Chem. Soc., Vol. 113, No. 3, p. 1030. Briefly, a small aliquot of subtilisin variant stock solution is added to a 1 cm cuvette containing substrate dissolved in 0.1M Tris-HCL buffer, pH 8.6, and thermostated at
35 25°C. The reaction progress is followed spectrophotometrically by monitoring the absorbance of the reaction product p-nitroaniline at 410

nm. Kinetic parameters are obtained by using a non-linear regression algorithm to fit the reaction velocity and product concentration for each reaction to the Michaelis-Menten equation.

Table III

Kinetic Parameters k_{cat} , K_M and k_{cat}/K_M
Measured for Bacillus lentus Subtilisin and Variants

<u>Protease #</u>	<u>Enzyme</u> <u>Variants</u>	<u>k_{cat} (s^{-1})</u>	<u>K_M (M)</u>	<u>k_{cat}/K_M</u> <u>($s^{-1}M^{-1}$)</u>
-	<u>B. lentus</u> Subtilisin	170	0.00078	2.18×10^5
-	N76D	219	0.0008	2.74×10^5
1	N76D/S99D	88	0.00061	1.44×10^5
2	N76D/S101R	371	0.0013	2.85×10^5
3	N76D/S103A	400	0.0014	2.86×10^5
4	N76D/V104I	459	0.0011	4.17×10^5
5	N76D/I107V	219	0.0011	1.99×10^5
6	N76D/N123S	115	0.0018	6.40×10^4
7	N76D/S99D/S101R	146	0.00038	3.84×10^5
8	N76D/S99D/S103A	157	0.0012	1.31×10^5
9	N76D/S99D/V104I	247	0.00097	2.55×10^5
10	N76D/S101R/S103A	405	0.00069	5.90×10^5
11	N76D/S101R/V104I	540	0.00049	1.10×10^6
12	N76D/S103A/V104I	832	0.0016	5.20×10^5
13	N76D/V104I/I107V	497	0.00045	1.10×10^6
14	N76D/V104Y/I107V	330	0.00017	1.90×10^6
15	N76D/V104I/N123S	251	0.0026	9.65×10^4
16	N76D/I107V/N123S	147	0.0035	4.20×10^4
17	N76D/S99D/S101R/S103A	242	0.00074	3.27×10^5
18	N76D/S99D/S101R/V104I	403	0.00072	5.60×10^5
19	N76D/S99D/S103A/V104I	420	0.0016	2.62×10^5
20	N76D/S101R/S103A/V104I	731	0.00065	1.12×10^6
21	N76D/S103A/V104I/N123S	321	0.0026	1.23×10^5
22	N76D/V104I/I107V/N123S	231	0.003	7.70×10^4
23	N76D/S99D/S101R/S103A/V104I	624	0.00098	6.37×10^5
24	N76D/S99D/S103A/V104I/N123S	194	0.0043	4.51×10^4
25	N76D/S99D/S101R/S103A/V104I/N123S	311	0.0023	1.35×10^5

The results listed in Table III indicate that all of the subtilisin variants tested retain proteolytic activity. Further, detailed analysis of the data reveal that proteolytic activity is significantly altered for *Bacillus* *lentus* subtilisin by the various combinations of substitutions at amino

acid residues equivalent to positions 76, 99, 101, 103, 104, 107 and 123 in *Bacillus amyloliquefaciens*.

Thermal Stability of Subtilisin Variants

A comparison of thermal stability observed for *Bacillus lentus* subtilisin and the variants of the present invention made by the process of Oligonucleotide-Directed Mutagenesis hereinbefore is shown in Table IV. Purified enzyme, 15 ug/ml in 0.1 M glycine 0.01% Tween-80 pH 10.0, with or without 50 mM CaCl₂, is aliquotted into small tubes and incubated at 10°C for 5 minutes, 10°C to 60°C over 1 minute, and 60°C for 20 minutes. Tubes are then placed on ice for 10 minutes. Aliquots from the tubes are assayed for enzyme activity by addition to 1 cm cuvettes containing 1.2 mM of the synthetic peptide substrate succinyl-L-ala-L-Ala-L-Pro-L-Phe-p-nitroanilide dissolved in 0.1 M tris-HCL buffer, pH 8.6, thermostatted at 25°C. The initial linear reaction velocity is followed spectrophotometrically by monitoring the absorbance of the reaction product p-nitroaniline at 410 nm as a function of time. Data are presented as percent activity prior to heating. The results listed in Table IV indicate that a vast majority of variants exhibit thermal stability comparable to *Bacillus lentus* subtilisin (24 out of 26) in the test condition with 50mM CaCl₂ added. In the test condition without 50mM CaCl₂ added, a vast majority of variants (19 out of 26) are significantly more stable than *Bacillus lentus* subtilisin. Further, the variants N76D/S99D, N76D/V104I, N76D/S99D/V104I, N76D/S103A/V104I, N76D/V104I/I107V, N76D/V104Y/I107V and N76D/S101R/S103A/V104I are significantly more stable than the single substitution variant N76D in the test condition without 50mM CaCl₂ added.

Table IV

Thermal Stability Measured for *Bacillus lentus* Subtilisin and Variants
At pH 10, 60°C, +/- 50mM CaCl₂ Added

<u>Enzyme</u>	<u>% Initial Activity Remaining</u>	
	<u>-CaCl₂</u>	<u>+CaCl₂</u>
<u>B. lentus</u> Subtilisin	2	96
N76D	34	97
N76D/S99D	49	98
N76D/S101R	0	82
N76D/S103A	26	92

N76D/V104I	58	98
N76D/I107V	32	96
N76D/N123S	0	97
N76D/S99D/S101R	30	100
N76D/S99D/S103A	36	100
N76D/S99D/V104I	48	97
N76D/S101R/S103A	26	100
N76D/S101R/V104I	38	100
N76D/S103A/V104I	58	100
N76D/V104I/I107V	60	97
N76D/V104Y/I107V	48	74
N76D/V104I/N123S	0	98
N76D/I107V/N123S	16	100
N76D/S99D/S101R/S103A	38	100
N76D/S99D/S101R/V104I	33	100
N76D/S99D/S103A/V104I	38	98
N76D/S101R/S103A/V104I	40	99
N76D/S103A/V104I/N123S	1	98
N76D/V104I/I107V/N123S	3	99
N76D/S99D/S101R/S103A/V104I	36	99
N76D/S99D/S103A/V104I/N123S	2	95
N76D/S99D/S101R/S103A/V104I/N123S	0	100

Oligonucleotide-Directed Mutagenesis with
Single-Stranded DNA Template Generated from Phagemid

A. Construction of *B. lentus* Variants

The mutagenesis protocol is essentially the same as described
5 above in Oligonucleotide-Directed Mutagenesis. The single-stranded
DNA template is generated by phagemid method. To construct the
phagemid vector for generating the single-stranded DNA template we
first construct the vector pBCDAICAT. The flow chart of vector
construction is outlined in Figure 8. First, the *C1aI* to *C1aI* fragment
10 encoding the CAT gene from pC194 plasmid (Horinouchi, S. and
Weisblum, B., J. Bacteriol., 150:8-15, 1982) is cloned into the *AccI* site of
polylinker region of pUC19 (New England BioLabs, Beverly, MA) to make
plasmid pUCCHL and the *EcoRI*-*DraI* 0.6 KB fragment from the 5' end of
the GG36DAI encoding DNA is cloned into the *EcoRI* and *EcoRV* sites of

pBSKS plasmid (Stratagene, Inc., San Diego, CA) to make pBC2SK5. The single *EcoRI* site of the plasmid pBC2SK5 is eliminated by *EcoRI* digestion, followed by filling in catalyzed-by-T4 DNA polymerase, and religation to generate the plasmid pBC2SK-5R which does not have the *EcoRI* site. The *EcoRI*-*DraI* fragment which is cloned into pBCSK-5R is isolated as a *PstI*-*HindIII* fragment and cloned into the *PstI*-*HindIII* site of the pUCCHL (part of the polylinker of pUC19) to generate plasmid pUCCHL5R. The encoding sequence of GG36DAI gene is excised as an *EcoRI*-*BamHI* fragment and cloned into the *EcoRI*-*BamHI* sites of pUCCHL5R to make pUCCAT. The large *EcoRI*-*HindIII* fragment of pUCCAT is then cloned into the *EcoRI* and *HindIII* sites of BS2KS+ to generate the plasmid pBCDAICAT.

To generate single-stranded DNA, *E. coli*-containing pBCDAICAT are infected with phage R408 (obtained from Stratagene, San Diego, CA) following the protocol described in Russel, M., Kidd, S. and Kelley, M.R., GENE 45:333-338, 1986. Once the single-stranded DNA template is available, standard mutagenesis methods as described above in Oligonucleotide-Directed Mutagenesis are carried out. The construction of certain mutants is detailed below for illustrative purposes.

For the construction of *B. lentus* (GG36) N76D/S103A/V104I/L217H, an *EcoRI*-*BamHI* DNA fragment encoding GG36 N76D/S103A/V104I is used in the construction of pUCCAT (see Fig. 8) to generate the plasmid pBCDAICAT. After the single-stranded DNA template is made following the protocol described above, a mutagenesis primer with the following sequence

* *** ** x C1aI

5' TAT GCC AGC CAC AAC GGT ACT TCG ATG GCT 3' (Seq. ID No.13)

is used to make the L217H. As before, the underlined residues denote the nucleotide changes that are made and the x C1aI indicates that the existing C1aI site is eliminated after the mutagenesis. The mutagenesis protocol is as described in Oligonucleotide-Directed Mutagenesis hereinbefore. After the mutagenesis, plasmid DNA is first screened for the elimination of the C1aI site and those clones missing the C1aI site are subjected to DNA sequence analysis to verify the DNA sequence which made the L to H change at the 217th amino acid residue.

B. Construction of BPN' Variants and their Expression in *B. subtilis*

The construction of *B. amyloliquefaciens* (BPN') N76D/Q103A/Y104I/Y217L is done in a similar fashion except in two consecutive steps. N76D is first introduced into BPN' Y217L to make BPN' N76D/Y217L and a second mutagenesis is done to convert BPN' N76D/Y217L to BPN' N76D/Q103A/Y104I/Y217L. To generate the single-stranded DNA template for the first mutagenesis, an *EcoRI*-*Bam*HI fragment encoding BPN' Y217L subtilisin (derived from the Y217L plasmid described in Wells, J., et al., PNAS, 84, 5167, 1087) is used to construct a plasmid pUCCATFNA (see Fig. 9). The pUCCATFNA plasmid containing BPN' Y217L is used to construct the pBCFNACAT plasmid (Fig. 9). Single-stranded DNA is generated as described above. To generate BPN' N76D/Y217L, an oligonucleotide primer with the sequence

* *** ** *Xba*I

15 5' C ACA GTT GCG GCT CTA GAT AAC TCA ATC GGT G 3'
(Seq. ID No.14)

is used to generate the change N76D. Single-stranded DNA is then prepared from the pBCFNACAT plasmid containing the BPN' N76D/Y217L (the pBCFNACAT plasmid after N76D mutagenesis) and mutagenized with another oligonucleotide with the sequence

* *** ** *Pvu*II

5' GCT GAC GGT TCC GGC GCT ATT AGT TGG ATC ATT 3'
(Seq. ID No.15)

to obtain BPN' N76D/Q103A/Y104I/Y217L. All steps involved in the cloning, the single-stranded DNA preparation, the mutagenesis, and the screening for mutants are carried out as described above. Expression of the BPN' gene and its variants are achieved by integrating plasmid DNA (pBCFNACAT containing the different variants' BPN' gene) directly into a protease-deficient strain of *Bacillus subtilis* as described in RE 34,606.

30 Numerous variants are made as per the teachings of these Protease Manufacture Examples. Kinetics data and stability data are generated for such variants. The kinetics data are generated using the methods described hereinbefore and are provided in Table V. The stability data are generated as detailed herein. Results are shown in Table VI.

Thermal Stability Assay Procedure

Purified enzyme is buffer-exchanged into 0.1 M glycine pH 10.0, 0.01% Tween-80 by applying the enzyme to a column consisting of

Sephadex™ G-25 equilibrated with this buffer and eluting the enzyme from the column using the same buffer.

To a tube containing 0.1 M glycine, 0.01% Tween™-80 pH 10.0 thermostatted at 60°C, the buffer-exchanged enzyme is added to give a
5 final enzyme concentration of 15 ug/ml.

Aliquots are removed from the 60°C incubation at various times and immediately assayed for enzyme activity by addition to a 1 cm cuvette containing 1.2 mM of the synthetic peptide substrate succinyl-L-Ala-L-Ala-L-Pro-L-Phe-p-nitroanilide dissolved in 0.1 M tris-HCL buffer,
10 pH 8.6, thermostatted at 25°C. The initial linear reaction velocity is followed spectrophotometrically by monitoring the absorbance of the reaction product p-nitroaniline at 410 nm as a function of time.

Half-life, which is the length of time required for 50% enzyme inactivation, is determined from the first-order plot of reaction velocity as
15 a function of the time of incubation at 60°C.

The data are presented in Table VI as percent of the half-life determined for *Bacillus lentus* subtilisin (GG36) under identical conditions.

Table V

Enzyme	kcat (s ⁻¹)	KM (mM)	kcat/KM (s ⁻¹ M ⁻¹)
B. lentus subtilisin	170	0.78	2.20E+05
N76D/S103G/V104I*	380	1.4	2.70E+05
N76D/S103A/V104F	730	0.33	2.20E+06
N76D/S103A/V104N	790	2.8	2.80E+05
N76D/S103A/V104S	170	0.83	2.00E+05
N76D/S103A/V104T	370	1.9	2.00E+05
N76D/S103A/V104W	880	0.31	2.80E+06
N76D/S103A/V104Y	690	0.5	1.40E+06
K27R/N76D/V104Y/N123S	500	1.2	4.20E+05
N76D/S101G/S103A/V104I*	620	1.3	4.80E+05
N76D/S103A/V104I/S105A*	550	1.3	4.20E+05
N76D/S103A/V104I/S105D*	440	1.7	2.60E+05
N76D/S103A/V104T/I107A*	120	5.7	2.10E+04
N76D/S103A/V104T/I107L*	310	3.2	9.70E+04
N76D/S103A/V104I/L126A	90	2.2	4.10E+04
N76D/S103A/V104I/L126F	180	1.9	9.50E+04
N76D/S103A/V104I/L126I	100	2.4	4.20E+04
N76D/S103A/V104I/L126V	64	3.2	2.00E+04
N76D/S103A/V104I/S128G*	560	1.7	3.30E+05
N76D/S103A/V104I/S128L*	430	3.8	1.10E+05

N76D/S103A/V104I/L135A	140	0.76	1.80E+05
N76D/S103A/V104I/L135F	390	0.69	5.70E+05
N76D/S103A/V104I/L135I	110	0.73	1.50E+05
N76D/S103A/V104I/L135V	140	0.86	1.60E+05
N76D/S103A/V104I/S156E*	170	2.6	6.50E+04
N76D/S103A/V104I/S166D*	160	3.5	4.60E+04
N76D/S103A/V104I/D197E	510	1.4	3.60E+05
N76D/S103A/V104I/N204A*	530	1.1	4.80E+05
N76D/S103A/V104I/N204G*	580	1.4	4.10E+05
N76D/S103A/V104I/N204C*	370	1.3	2.90E+05
N76D/S103A/V104I/P210I*	500	1.2	4.20E+05
N76D/S103A/V104I/L217H*	80	0.63	1.30E+05
N76D/S103A/V104I/M222A	70	3.1	2.30E+04
N76D/S103A/V104I/M222S	80	3.1	2.60E+04
N76D/S103A/V104I/T260P	660	1.5	4.40E+05
N76D/S103A/V104I/S265N	590	1.3	4.50E+05
K27R/N76D/V104Y/I107V/N123S	220	1.4	1.60E+05
K27R/N76D/V104Y/N123S/D197E	430	1.1	3.90E+05
K27R/N76D/V104Y/N123S/N204C	400	1.1	3.60E+05
K27R/N76D/V104Y/N123S/Q206L	440	1.2	3.70E+05
K27R/N76D/V104Y/N123S/S216V	440	1.2	3.70E+05
K27R/N76D/V104Y/N123S/N218S	760	0.98	7.80E+05
K27R/N76D/V104Y/N123S/T260P	410	1.2	3.40E+05
K27R/N76D/V104Y/N123S/T274A	390	1	3.90E+05
N76D/S103A/V104I/L126F/S265N	170	2.1	8.10E+04
N76D/S103A/V104I/S156E/S166D*	40	6.3	6.40E+03
K27R/N76D/V104Y/N123S/G195E/G197E	410	0.98	4.20E+05
K27R/N76D/V104Y/N123S/G195E/N218S	540	0.66	8.20E+05
K27R/N76D/V104Y/N123S/D197E/N218S	770	0.79	9.80E+05
K27R/N76D/V104Y/N123S/N204C/N218S	610	0.99	6.20E+05
K27R/N76D/V104Y/N123S/Q206L/N218S	580	0.78	7.40E+05
K27R/N76D/V104Y/N123S/N218S/T260P	660	1	6.60E+05
K27R/N76D/V104Y/N123S/N218S/T274A	590	0.89	6.60E+05
K27R/N76D/V104Y/Q109S/N123S/N218S/T 274A	520	1	5.20E+05
K27R/N76D/V104Y/N123S/G195E/D197E/N 218S	460	0.65	7.10E+05
B. amyloliquefaciens subtilisin (BPN')	50	0.14	3.60E+05
BPN'-N76D/Y217L*	380	0.46	8.30E+05

* These mutants are made as per Oligonucleotide-Directed Mutagenesis with Single-Stranded DNA Template Generated from Phagemid, all others made as per Oligonucleotide-Directed Mutagenesis, hereinbefore.

Table VI

Enzyme	Thermal Stability (% half-life of native enzyme)
B. lentus subtilisin	100
N76D	590
N76D/S99D	840
N76D/S103A	390
N76D/V104I	660
N76D/I107V	710
N76D/N123S	70
N76D/S99D/S101R	610
N76D/S99D/S103A	590
N76D/S99D/V104I	910
N76D/S101R/S103A	930
N76D/S101R/V104I	500
N76D/S103A/V104I	460
N76D/S103G/V104I*	370
N76D/S103A/V104F	480
N76D/S103A/V104N	230
N76D/S103A/V104S	230
N76D/S103A/V104T	370
N76D/S103A/V104W	280
N76D/S103A/V104Y	400
N76D/V104I/I107V	940
N76D/V104Y/I107V	820
N76D/V104I/N123S	80
N76D/I107V/N123S	150
K27R/N76D/V104Y/N123S	100
N76D/S99D/S101R/S103A	570
N76D/S99D/S101R/V104I	1000
N76D/S99D/S103A/V104I	680
N76D/S101G/S103A/V104I*	390
N76D/S101R/S103A/V104I	470
N76D/S103A/V104I/S105A*	360
N76D/S103A/V104I/S105D*	370
N76D/S103A/V104T/I107A*	270
N76D/S103A/V104T/I107L*	230
N76D/S103A/V104I/N123S	110
N76D/V104I/I107V/N123S	220
N76D/S103A/V104I/L126A	270
N76D/S103A/V104I/L126F	950
N76D/S103A/V104I/L126I	410
N76D/S103A/V104I/L126V	320
N76D/S103A/V104I/S128G*	640
N76D/S103A/V104I/S128L*	760
N76D/S103A/V104I/L135A	230
N76D/S103A/V104I/L135F	200
N76D/S103A/V104I/L135I	510

N76D/S103A/V104I/L135V	500
N76D/S103A/V104I/S156E*	120
N76D/S103A/V104I/S166D*	590
N76D/S103A/V104I/D197E	460
N76D/S103A/V104I/N204A*	230
N76D/S103A/V104I/N204G*	240
N76D/S103A/V104I/N204C*	500
N76D/S103A/V104I/P210I*	1370
N76D/S103A/V104I/L217H*	60
N76D/S103A/V104I/M222A	520
N76D/S103A/V104I/M222S	490
N76D/S103A/V104I/T260P	490
N76D/S103A/V104I/S265N	360
K27R/N76D/V104Y/I107V/N123S	210
K27R/N76D/V104Y/N123S/D197E	120
K27R/N76D/V104Y/N123S/N204C	110
K27R/N76D/V104Y/N123S/Q206L	380
K27R/N76D/V104Y/N123S/S216V	140
K27R/N76D/V104Y/N123S/N218S	270
K27R/N76D/V104Y/N123S/T260P	40
K27R/N76D/V104Y/N123S/T274A	60
N76D/S99D/S101R/S103A/V104I	590
N76D/S99D/S103A/V104I/N123S	110
N76D/S103A/V104I/L126F/S265N	810
N76D/S103A/V104I/S156E/S166D*	220
K27R/N76D/V104Y/N123S/G195E/G197E	90
K27R/N76D/V104Y/N123S/G195E/N218S	250
K27R/N76D/V104Y/N123S/D197E/N218S	270
K27R/N76D/V104Y/N123S/N204C/N218S	460
K27R/N76D/V104Y/N123S/Q206L/N218S	1400
K27R/N76D/V104Y/N123S/N218S/T260P	310
K27R/N76D/V104Y/N123S/N218S/T274A	180
N76D/S99D/S101R/S103A/V104I/N123S	90
K27R/N76D/V104Y/Q109S/N123S/N218S/T274	230
K27R/N76D/V104Y/N123S/G195E/D197E/N21	240
B. amyloliquefaciens subtilisin (BPN')	100
BPN'-N76D/Y217L*	420

* These mutants are made as per Oligonucleotide-Directed Mutagenesis with Single-Stranded DNA Template Generated from Phagemid, all others made as per Oligonucleotide-Directed Mutagenesis, hereinbefore.

(3) Cleaning Composition Materials:

5 The cleaning compositions of the present invention also comprise, in addition to the bleaching agent and protease enzyme described hereinbefore, one or more cleaning composition materials compatible with the protease enzyme. The term "cleaning composition materials", as

used herein, means any liquid, solid or gaseous material selected for the particular type of cleaning composition desired and the form of the product (e.g., liquid; granule; spray composition), which materials are also compatible with the protease enzyme used in the composition. The
5 specific selection of cleaning composition materials are readily made by considering the surface, item or fabric to be cleaned, and the desired form of the composition for the cleaning conditions during use (e.g., through the wash detergent use). The term "compatible", as used herein, means the cleaning composition materials do not reduce the proteolytic
10 activity of the protease enzyme to such an extent that the protease is not effective as desired during normal use situations. Specific cleaning composition materials are exemplified in detail hereinafter.

An effective amount of one or more protease enzymes described above are included in compositions useful for cleaning a variety of
15 surfaces in need of proteinaceous stain removal. Such cleaning compositions include detergent compositions for cleaning hard surfaces, unlimited in form (e.g., liquid and granular); detergent compositions for cleaning fabrics, unlimited in form (e.g., granular, liquid and bar formulations); dishwashing compositions (unlimited in form); oral cleaning
20 compositions, unlimited in form (e.g., dentifrice, toothpaste and mouthwash formulations); and denture cleaning compositions, unlimited in form (e.g., liquid, tablet). As used herein, "effective amount of protease enzyme" refers to the quantity of protease enzyme described hereinbefore necessary to achieve the enzymatic activity necessary in
25 the specific cleaning composition. Such effective amounts are readily ascertained by one of ordinary skill in the art and is based on many factors, such as the particular enzyme variant used, the cleaning application, the specific composition of the cleaning composition, and whether a liquid or dry (e.g., granular, bar) composition is required, and
30 the like.

Preferably the cleaning compositions of the present invention comprise from about 0.0001% to about 10% of one or more protease enzymes, more preferably from about 0.001% to about 1%, more preferably still from about 0.001% to about 0.1%. Also preferably the
35 protease enzyme is present in the compositions in an amount sufficient to provide a ratio of mg of active protease per 100 grams of

composition to ppm theoretical Available O₂ ("AvO₂") from the peroxyacid in the wash liquor, referred to herein as the Enzyme to Bleach ratio (E/B ratio), ranging from about 1:1 to about 20:1. Several examples of various cleaning compositions wherein the protease enzymes may be employed are discussed in further detail below. All parts, percentages and ratios used herein are by weight unless otherwise specified.

(i) Optional Deterative Enzymes

The compositions and methods herein are effective with all manner of deterative enzymes in addition to the specified protease enzymes. Optional deterative enzymes useful in the present invention may be included for a wide variety of fabric laundering purposes, including removal of protein-based, carbohydrate-based, or triglyceride-based stains, for example, and for the prevention of fugitive dye transfer. The enzymes to be incorporated include other proteases, amylases, lipases, cellulases, and peroxidases, as well as mixtures thereof. Other types of enzymes may also be included. They may be of any suitable origin, such as vegetable, animal, bacterial, fungal and yeast origin. However, their choice is governed by several factors such as pH-activity and/or stability optima, thermostability, stability versus active detergents, builders and so on. In this respect bacterial or fungal enzymes are preferred, such as bacterial amylases and proteases, and fungal cellulases.

Enzymes are normally incorporated at levels sufficient to provide up to about 50 mg by weight, more typically about 0.01 mg to about 10 mg, of active enzyme per gram of detergent composition. Stated otherwise, an effective amount of the optional enzymes employed in the present invention will typically comprise at least about 0.001%, preferably from about 0.001% to about 5%, more preferably from about 0.001% to about 1%, most preferably from about 0.01% to about 1%, by weight of detergent composition.

Suitable examples of optional proteases are the subtilisins which are obtained from particular strains of *B.subtilis* and *B.licheniformis*. Another suitable protease is a modified bacterial serine protease enzyme obtained from Bacillus subtilis or Bacillus licheniformis, having maximum activity throughout the pH range of 8-12, developed and sold

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by Novo Industries A/S under the registered trade mark ESPERASE. The preparation of this enzyme and analogous enzymes is described in British Patent Specification No. 1,243,784 of Novo. Other proteolytic enzymes that are commercially available include those sold under the trademarks ALCALASE and SAVINASE by Novo Industries A/S (Denmark) and MAXATASE by International Bio-Synthetics, Inc. (The Netherlands). Still other proteases include Protease A (see European Patent Application 130,756, published January 9, 1985) and Protease B (see European Patent Application 251,446, published January 7, 1988, and European Patent Application 130,756, Bott et al, published January 9, 1985), and what is called herein "Protease C", which is a triple variant of an alkaline serine protease from Bacillus in which tyrosine replaced valine at position 104, serine replaced asparagine at position 123, and alanine replaced threonine at position 274. Protease C is described in WO 91/06637, published May 16, 1991. Genetically modified variants, particularly of Protease C, are also included herein.

Amylases include, for example, α -amylases described in British Patent Specification No. 1,296,839 (Novo), RAPIDASETM, International Bio-Synthetics, Inc. and TERMAMYLTM, Novo Industries.

The cellulases usable in the present invention include both bacterial or fungal cellulase. Preferably, they will have a pH optimum of between 5 and 9.5. Suitable cellulases are disclosed in U.S. Patent 4,435,307, Barbesgoard et al, issued March 6, 1984, which discloses fungal cellulase produced from *Humicola insolens* and *Humicola* strain DSM1800 or a cellulase 212-producing fungus belonging to the genus *Aeromonas*, and cellulase extracted from the hepatopancreas of a marine mollusk (*Dolabella Auricula Solander*). Suitable cellulases are also disclosed in GB-A-2.075.028; GB-A-2.095.275 and DE-OS-2.247.832.

Suitable lipase enzymes for detergent usage include those produced by microorganisms of the *Pseudomonas* group, such as *Pseudomonas stutzeri* ATCC 19.154, as disclosed in British Patent 1,372,034. See also lipases in Japanese Patent Application 53-20487, laid open to public inspection on February 24, 1978. This lipase is

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available from Amano Pharmaceutical Co. Ltd., Nagoya, Japan, under the trade mark Lipase P "Amano," hereinafter referred to as "Amano-P." Other commercial lipases include Amano-CES, lipases ex *Chromobacter viscosum*, e.g. *Chromobacter viscosum* var. *lipolyticum* NRRLB 3673, commercially available from Toyo Jozo Co., Tagata, Japan; and further *Chromobacter viscosum* lipases from U.S. Biochemical Corp., U.S.A. and Disoynth Co., The Netherlands, and lipases ex *Pseudomonas gladioli*. The LIPOLASE™ enzyme, derived from the fungus *Humicola lanuginosa* and expressed in *Aspergillus oryzae* as host and commercially available from Novo (see also E.P. Patent 341,947) is a preferred lipase for use herein.

Peroxidase enzymes are used in combination with oxygen sources, e.g., percarbonate, perborate, persulfate, hydrogen peroxide, etc. They are used for "solution bleaching," i.e. to prevent transfer of dyes or pigments removed from sub-strates during wash operations to other substrates in the wash solution. Peroxidase enzymes are known in the art, and include, for example, horseradish peroxidase, ligninase, and haloperoxidase such as chloro- and bromo-peroxidase. Peroxidase-containing detergent compositions are disclosed, for example, in PCT International Application WO 89/099813, published October 19, 1989, by O. Kirk, assigned to Novo Industries A/S.

A wide range of enzyme materials and means for their incorporation into synthetic detergent granules is also disclosed in U.S. Patent 3,553,139, issued January 5, 1971 to McCarty et al. Enzymes are further disclosed in U.S. Patent 4,101,457, Place et al, issued July 18, 1978, and in U.S. Patent 4,507,219, Hughes, issued March 26, 1985, both. Enzyme materials useful for liquid detergent formulations, and their incorporation into such formulations, are disclosed in U.S. Patent 4,261,868, Hora et al, issued April 14, 1981. Enzymes for use in detergents can be stabilized by various techniques. Enzyme stabilization techniques are disclosed and exemplified in U.S. Patent 4,261,868, issued April 14, 1981 to Horn, et al, U.S. Patent 3,600,319, issued August 17, 1971 to Gedge, et al, and European Patent Application Publication No. 0199405, published October 29, 1986, Venegas. Enzyme stabilization systems

are also described, for example, in U.S. Patents 4,261,868, 3,600,319, and 3,519,570.

(ii) Enzyme Stabilizers

The enzymes employed herein can be stabilized by the presence of water-soluble sources of calcium ions in the finished compositions which provide calcium ions to the enzymes. Additional stability can be provided by the presence of various other art-disclosed stabilizers, especially borate species: see Severson, U.S. 4,537,706, cited above. Typical detergents, especially liquids, will comprise from about 1 to about 30, preferably from about 2 to about 20, more preferably from about 5 to about 15, and most preferably from about 8 to about 12, millimoles of calcium ion per liter of finished composition. This can vary somewhat, depending on the amount of enzyme present and its response to the calcium ions. The level of calcium ion should be selected so that there is always some minimum level available for the enzyme, after allowing for complexation with builders, fatty acids, etc., in the composition. Any water-soluble calcium salt can be used as the source of calcium ion, including, but not limited to, calcium chloride, calcium sulfate, calcium malate, calcium hydroxide, calcium formate, and calcium acetate. A small amount of calcium ion, generally from about 0.05 to about 0.4 millimoles per liter, is often also present in the composition due to calcium in the enzyme slurry and formula water. In solid detergent compositions the formulation may include a sufficient quantity of a water-soluble calcium ion source to provide such amounts in the laundry liquor. In the alternative, natural water hardness may suffice.

The compositions herein may also optionally, but preferably, contain various additional stabilizers including silicate coatings and, especially borate-type stabilizers. Typically, such stabilizers will be used at levels in the compositions from about 0.25% to about 10%, preferably from about 0.5% to about 5%, more preferably from about 0.75% to about 3%, by weight of boric acid or other borate compound capable of forming boric acid in the composition (calculated on the basis of boric acid). Boric acid is preferred, although other compounds such as boric oxide, borax and other alkali metal borates (e.g., sodium ~~ortho-~~, ~~meta-~~ and pyroborate, and sodium pentaborate) are suitable.

Substituted boric acids (e.g., phenylboronic acid, butane boronic acid, and p-bromo phenylboronic acid) can also be used in place of boric acid.

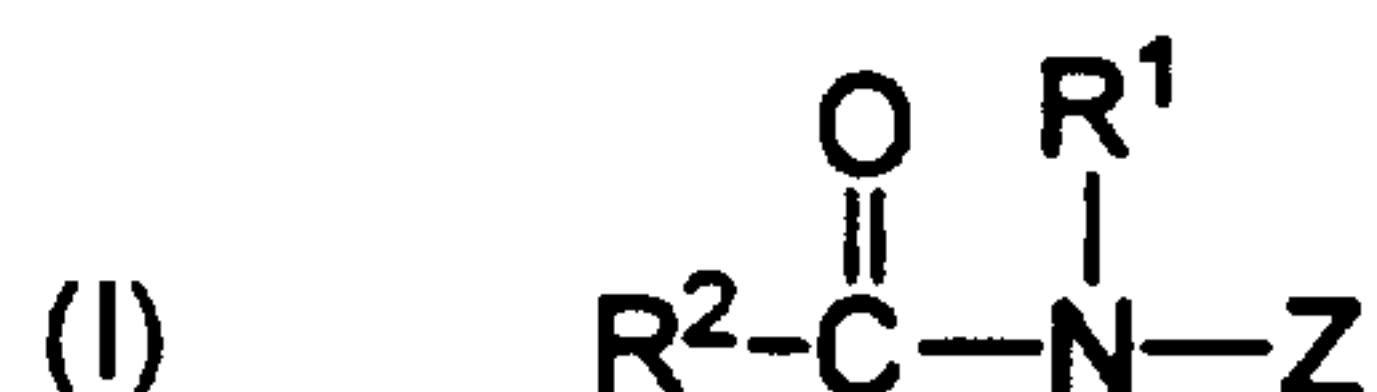
(iii) Deterasive Surfactant

5 The amount of deterasive surfactant included in the fully-formulated detergent compositions afforded by the present invention can vary from about 1% to about 99.8% depending upon the particular surfactants used and the effects desired. Preferably, the deterasive surfactants comprise from about 5% to about 80% by weight of the
10 detergent ingredients.

The deterasive surfactant can be nonionic, anionic, ampholytic, zwitterionic, or cationic. Mixtures of these surfactants can also be used. Preferred detergent compositions comprise anionic deterasive surfactants or mixtures of anionic surfactants with other surfactants,
15 especially nonionic surfactants.

Nonlimiting examples of surfactants useful herein include the conventional C₁₁-C₁₈ alkyl benzene sulfonates and primary, secondary, and random alkyl sulfates, the C₁₀-C₁₈ alkyl alkoxy sulfates, the C₁₀-C₁₈ alkyl polyglycosides and their corresponding
20 sulfated polyglycosides, C₁₂-C₁₈ alpha-sulfonated fatty acid esters, C₁₂-C₁₈ alkyl and alkyl phenol alkoxylates (especially ethoxylates and mixed ethoxy/propoxy), C₁₂-C₁₈ betaines and sulfobetaines ("sultaines"), C₁₀-C₁₈ amine oxides, C₈-C₂₄ sarcosinates (especially oleoyl sarcosinate) and the like. Other conventional useful surfactants
25 are listed in standard texts.

One particular class of adjunct nonionic surfactants especially useful herein comprises the polyhydroxy fatty acid amides of the formula:



30 wherein: R¹ is H, C₁-C₈ hydrocarbyl, 2-hydroxyethyl, 2-hydroxypropyl, or a mixture thereof, preferably C₁-C₄ alkyl, more preferably C₁ or C₂ alkyl, most preferably C₁ alkyl (i.e., methyl); and R² is a C₅-C₃₂ hydrocarbyl moiety, preferably straight chain C₇-C₁₉ alkyl or alkenyl, more preferably straight chain C₉-C₁₇ alkyl or alkenyl, most preferably

straight chain C_{11} - C_{19} alkyl or alkenyl, or mixture thereof; and Z is a polyhydroxyhydrocarbyl moiety having a linear hydrocarbyl chain with at least 2 (in the case of glyceraldehyde) or at least 3 hydroxyls (in the case of other reducing sugars) directly connected to the chain, or an
5 alkoxyated derivative (preferably ethoxylated or propoxylated) thereof. Z preferably will be derived from a reducing sugar in a reductive amination reaction; more preferably Z is a glycityl moiety. Suitable reducing sugars include glucose, fructose, maltose, lactose, galactose, mannose, and xylose, as well as glyceraldehyde. As raw materials,
10 high dextrose corn syrup, high fructose corn syrup, and high maltose corn syrup can be utilized as well as the individual sugars listed above. These corn syrups may yield a mix of sugar components for Z. It should be understood that it is by no means intended to exclude other suitable raw materials. Z preferably will be selected from the group
15 consisting of $-CH_2-(CHOH)_n-CH_2OH$, $-CH(CH_2OH)-(CHOH)_{n-1}-CH_2OH$, $-CH_2-(CHOH)_2(CHOH)(CHOR')(CHOH)-CH_2OH$, where n is an integer from 1 to 5, inclusive, and R' is H or a cyclic mono- or poly-saccharide, and alkoxyated derivatives thereof. Most preferred are glycityls wherein n is 4, particularly $-CH_2-(CHOH)_4CH_2OH$.

20 In Formula (I), R^1 can be, for example, N-methyl, N-ethyl, N-propyl, N-isopropyl, N-butyl, N-isobutyl, N-2-hydroxy ethyl, or N-2-hydroxy propyl. For highest sudsing, R^1 is preferably methyl or hydroxyalkyl. If lower sudsing is desired, R^1 is preferably C_2 - C_8 alkyl, especially n-propyl, iso-propyl, n-butyl, iso-butyl, pentyl, hexyl and 2-
25 ethyl hexyl.

$R^2-CO-N<$ can be, for example, cocamide, stearamide, oleamide, lauramide, myristamide, capricamide, palmitamide, tallowamide, etc.

Another class of nonionic surfactants particularly useful in the present invention are condensates of ethylene oxide with a
30 hydrophobic moiety to provide a surfactant having an average hydrophilic-lipophilic balance (HLB) in the range from 5 to 17, preferably from 6 to 14, more preferably from 7 to 12. The hydrophobic (lipophilic) moiety may be aliphatic or aromatic in nature and the length of the polyoxyethylene group which is condensed with any particular
35 hydrophobic group can be readily adjusted to yield a water-soluble

compound having the desired degree of balance between hydrophilic and hydrophobic elements.

Especially preferred nonionic surfactants of this type are the C₉-C₁₅ primary alcohol ethoxylates containing 3-8 moles of ethylene oxide per mole of alcohol, particularly the C₁₄-C₁₅ primary alcohols containing 6-8 moles of ethylene oxide per mole of alcohol, the C₁₂-C₁₅ primary alcohols containing 3-5 moles of ethylene oxide per mole of alcohol, and mixtures thereof.

(iv) Detersive Builders

Optional detergent ingredients employed in the present invention contain inorganic and/or organic detersive builders to assist in mineral hardness control. If used, these builders comprise from about 5% to about 80% by weight of the detergent compositions.

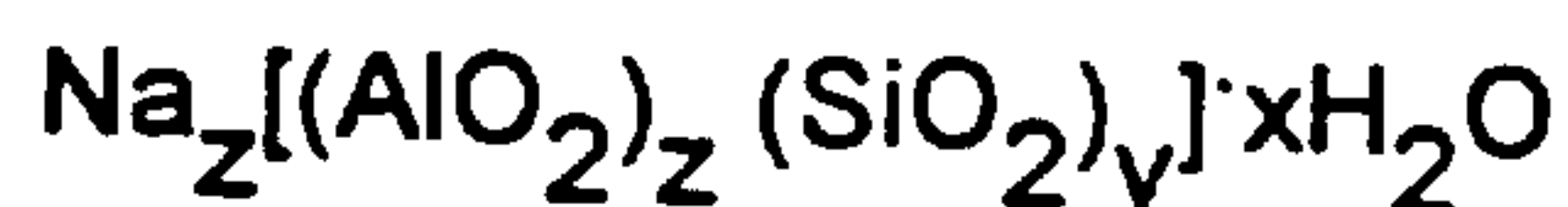
Inorganic detersive builders include, but are not limited to, the alkali metal, ammonium and alkanolammonium salts of polyphosphates (exemplified by the tri-polyphosphates, pyrophosphates, and glassy polymeric meta-phosphates), phosphonates, phytic acid, silicates, carbonates (including bicarbonates and sesqui-carbonates), sulphates, and aluminosilicates. However, nonphosphate builders are required in some locales.

Examples of silicate builders are the alkali metal silicates, particularly those having a SiO₂:Na₂O ratio in the range 1.6:1 to 3.2:1 and layered silicates, such as the layered sodium silicates described in U.S. Patent 4,664,839, issued May 12, 1987 to H. P. Rieck, available from Hoechst under the trademark "SKS"; SKS-6 is an especially preferred layered silicate builder.

Carbonate builders, especially a finely ground calcium carbonate with surface area greater than 10 m²/g, are preferred builders that can be used in granular compositions. The density of such alkali metal carbonate built detergents can be in the range of 450-850 g/l with the moisture content preferably below 4%.

Examples of carbonate builders are the alkaline earth and alkali metal carbonates as disclosed in German Patent Application No. 2,321,001 published on November 15, 1973.

Aluminosilicate builders are especially useful in the present invention. Preferred aluminosilicates are zeolite builders which have the formula:



5 wherein z and y are integers of at least 6, the molar ratio of z to y is in the range from 1.0 to about 0.5, and x is an integer from about 15 to about 264.

Useful aluminosilicate ion exchange materials are commercially available. These aluminosilicates can be crystalline or amorphous in
10 structure and can be naturally-occurring aluminosilicates or synthetically derived. Methods for producing aluminosilicate ion exchange materials are disclosed in U.S. Patent 3,985,669, Krummel, et al, issued October 12, 1976, and U.S. Patent 4,605,509, Corkill, et al, issued Aug. 12, 1986. Preferred synthetic crystalline aluminosilicate
15 ion exchange materials useful herein are available under the designations Zeolite A, Zeolite P (B) (including those disclosed in EPO 384,070), and Zeolite X. Preferably, the aluminosilicate has a particle size of about 0.1-10 microns in diameter.

Organic deterative builders suitable for the purposes of the
20 present invention include, but are not restricted to, a wide variety of polycarboxylate compounds, such as ether polycarboxylates, including oxydisuccinate, as disclosed in Berg, U.S. Patent 3,128,287, issued April 7, 1964, and Lamberti et al, U.S. Patent 3,635,830, issued January 18, 1972. See also "TMS/TDS" builders of U.S. Patent
25 4,663,071, issued to Bush et al, on May 5, 1987. Suitable ether polycarboxylates also include cyclic compounds, particularly alicyclic compounds, such as those described in U.S. Patents 3,923,679; 3,835,163; 4,158,635; 4,120,874 and 4,102,903.

Other useful deterative builders include the ether hydroxy-
30 polycarboxylates, copolymers of maleic anhydride with ethylene or vinyl methyl ether, 1, 3, 5-trihydroxy benzene-2, 4, 6-trisulphonic acid, and carboxymethyl-oxysuccinic acid, the various alkali metal, ammonium and substituted ammonium salts of polyacetic acids such as ethylenediamine tetraacetic acid and nitrilotriacetic acid, as well as
35 polycarboxylates such as mellitic acid, succinic acid, oxydisuccinic

acid, polymaleic acid, benzene 1,3,5-tricarboxylic acid, carboxymethyloxysuccinic acid, and soluble salts thereof.

5 Citrate builders, e.g., citric acid and soluble salts thereof (particularly sodium salt), are preferred polycarboxylate builders that can also be used in granular compositions, especially in combination with zeolite and/or layered silicate builders.

Also suitable in the detergent compositions of the present invention are the 3,3-dicarboxy-4-oxa-1,6-hexanedioates and the related compounds disclosed in U.S. Patent 4,566,984, Bush, issued
10 January 28, 1986.

In situations where phosphorus-based builders can be used, and especially in the formulation of bars used for hand-laundrying operations, the various alkali metal phosphates such as the well-known sodium tripolyphosphates, sodium pyrophosphate and sodium
15 orthophosphate can be used. Phosphonate builders such as ethane-1-hydroxy-1,1-diphosphonate and other known phosphonates (see, for example, U.S. Patents 3,159,581; 3,213,030; 3,422,021; 3,400,148 and 3,422,137) can also be used.

(v) Optional Detergent Adjuncts

20 As a preferred embodiment, the conventional detergent ingredients employed herein can be selected from typical detergent composition components such as detergent surfactants and detergent builders. Optionally, the detergent ingredients can include one or more other detergent adjuncts or other materials for assisting or enhancing
25 cleaning performance, treatment of the substrate to be cleaned, or to modify the aesthetics of the detergent composition. Usual detergent adjuncts of detergent compositions include the ingredients set forth in U.S. Pat. No. 3,936,537, Baskerville et al. Such adjuncts which can be included in detergent compositions employed in the present invention,
30 in their conventional art-established levels for use (generally from 0% to about 20% of the detergent ingredients, preferably from 0.5% to about 10%), include color speckles, suds boosters, suds suppressors, antitarnish and/or anticorrosion agents, soil-suspending agents, soil release agents, dyes, fillers, optical brighteners, germicides, alkalinity
35 sources, hydrotropes, antioxidants, perfumes, solvents, solubilizing agents, clay

soil removal/anti-redeposition agents, polymeric dispersing agents, processing aids, fabric softening components (e.g., smectite clays), dye transfer inhibiting agents (e.g., polyvinyl pyrrolidone), static control agents, etc.

5 Bleach systems optionally, but preferably, will also comprise a chelant which not only enhances bleach stability by scavenging heavy metal ions which tend to decompose bleaches, but also assists in the removal of polyphenolic stains such as tea stains, and the like. Various
10 chelants, including the aminophosphonates, available as DEQUEST from Monsanto, the nitrilotriacetates, the hydroxyethyl-ethylenediamine triacetates, and the like, are known for such use. Preferred biodegradable, non-phosphorus chelants include ethylene-diamine disuccinate ("EDDS"; see U.S. Patent 4,704,233, Hartman and Perkins), ethylenediamine-N,N'-diglutamate (EDDG) and 2-
15 hydroxypropylenediamine-N,N'-disuccinate (HPDDS) compounds. Such chelants can be used in their alkali or alkaline earth metal salts, typically at levels from about 0.1% to about 10% of the present compositions.

The present invention compositions are especially useful as
20 conventional laundry detergent compositions such as those typically found in granular detergents or laundry bars. U.S. Patent 3,178,370, Okenfuss, issued April 13, 1965, describes laundry detergent bars and processes for making them. Philippine Patent 13,778, Anderson, issued Sept. 23, 1980, describes synthetic detergent laundry bars.
25 Methods for making laundry detergent bars by various extrusion methods are well known in the art.

The following examples are given to further illustrate the present invention, but are not intended to be limiting thereof.

EXAMPLE 1

30 A granular detergent composition is prepared comprising the following ingredients.

	<u>Component</u>	<u>Weight %</u>
	C ₁₂ linear alkyl benzene sulfonate	22
	Phosphate (as sodium tripolyphosphate)	30
35	Sodium carbonate	14
	Sodium silicate	3

	Protease 12	0.3
	Sodium percarbonate	5
	Ethylenediamine disuccinate chelant (EDDS)	0.4
	Sodium sulfate	5.5
5	Nonanoyl caprolactam	5
	Filler* and water	Balance to 100%

*Can be selected from convenient materials such as CaCO_3 , talc, clay, silicates, and the like.

10 Aqueous crutcher mixes of heat and alkali stable components of the detergent compositions are prepared and spray-dried and the other ingredients are admixed so that they contain the ingredients tabulated at the levels shown.

15 In this example the Protease #'s 1-11 and 13-25 recited in Table III, among others including the additional proteases useful in the present invention described in Tables V and VI, are substituted for Protease #12, with substantially similar results. Also in this example, any combination of the proteases useful in the present invention recited in Tables III, V and VI among others, are substituted for Protease # 12 with substantially similar results.

20

EXAMPLE 2

A granular detergent composition is prepared comprising the following ingredients.

	<u>Component</u>	<u>Weight %</u>
	Anionic alkyl sulfate	7
25	Nonionic surfactant	5
	Zeolite (0.1-10 micron)	10
	Trisodium citrate	2
	SKS-6 silicate builder	10
	Acrylate maleate polymer	4
30	Nonanoyl caprolactam	5
	Sodium percarbonate*	15
	Sodium carbonate	5
	Ethylenediamine disuccinate chelant (EDDS)	0.4
	Suds suppressor	2
35	Protease 12	0.3
	Lipase	0.3

Soil release agent

0.2

Minors, filler** and water

Balance to 100%

*Average particle size of 400 to 1200 microns.

5 **Can be selected from convenient materials such as CaCO_3 , talc, clay, silicates, and the like.

Aqueous crutcher mixes of heat and alkali stable components of the detergent composition are prepared and spray-dried, and the other ingredients are admixed so that they contain the ingredients tabulated at the levels shown.

10 In this example the Protease #'s 1-11 and 13-25 recited in Table III, among others including the additional proteases useful in the present invention described in Tables V and VI, are substituted for Protease #12, with substantially similar results. Also in this example, any combination of the proteases useful in the present invention recited in Tables III, V
15 and VI among others, are substituted for Protease # 12 with substantially similar results.

EXAMPLE 3

A detergent composition is prepared by a procedure identical to that of Example 2, with the exceptions that 15% of a 1:1:1 mixture of
20 benzoyl caprolactam, nonanoyl caprolactam and (6-Nonanamidocaproyl)oxybenzenesulfonate is substituted for the nonanoyl caprolactam and the amount of sodium percarbonate is 30%.

In this example the Protease #'s 1-11 and 13-25 recited in Table III, among others including the additional proteases useful in the present
25 invention described in Tables V and VI, are substituted for Protease #12, with substantially similar results. Also in this example, any combination of the proteases useful in the present invention recited in Tables III, V and VI among others, are substituted for Protease # 12 with substantially similar results.

30

EXAMPLE 4

A detergent composition is prepared by a procedure identical to that of Example 1, with the exceptions that 20% of a 1:1 mixture of benzoyl caprolactam and (6-Nonanamidocaproyl)oxybenzenesulfonate is substituted for the nonanoyl caprolactam, the amount of sodium
35 percarbonate is 20%, and the amount of phosphate is 0%.

In this example the Protease #'s 1-11 and 13-25 recited in Table III, among others including the additional proteases useful in the present invention described in Tables V and VI, are substituted for Protease #12, with substantially similar results. Also in this example, any combination
5 of the proteases useful in the present invention recited in Tables III, V and VI among others, are substituted for Protease # 12 with substantially similar results.

EXAMPLE 5

A detergent composition is prepared by a procedure identical to
10 that of Example 2, with the single exception that an equivalent amount of a benzoxazin-type activator is substituted for the nonanoyl caprolactam.

In this example the Protease #'s 1-11 and 13-25 recited in Table III, among others including the additional proteases useful in the present
15 invention described in Tables V and VI, are substituted for Protease #12, with substantially similar results. Also in this example, any combination of the proteases useful in the present invention recited in Tables III, V and VI among others, are substituted for Protease # 12 with substantially similar results.

20

EXAMPLE 6

A detergent composition is prepared by a procedure identical to that of Example 2, with the exceptions that 10% of a 1:1 mixture of a benzoxazin-type activator and tetraacetyl ethylene diamine is substituted for the nonanoyl caprolactam and the amount of sodium
25 percarbonate is 25%.

In this example the Protease #'s 1-11 and 13-25 recited in Table III, among others including the additional proteases useful in the present invention described in Tables V and VI, are substituted for Protease #12, with substantially similar results. Also in this example, any combination
30 of the proteases useful in the present invention recited in Tables III, V and VI among others, are substituted for Protease # 12 with substantially similar results.

EXAMPLE 7

A laundry bar suitable for hand-washing soiled fabrics is prepared
35 by standard extrusion processes and comprises the following:

Component

Weight %

	C ₁₂ linear alkyl benzene sulfonate	30
	Phosphate (as sodium tripolyphosphate)	7
	Sodium carbonate	25
	Sodium pyrophosphate	7
5	Coconut monoethanolamide	2
	Zeolite A (0.1-10 micron)	5
	Carboxymethylcellulose	0.2
	Polyacrylate (m.w. 1400)	0.2
	(6-Nonanamidocaproyl)oxybenzenesulfonate	5
10	Sodium percarbonate	5
	Brightener, perfume	0.2
	Protease 12	0.3**
	Lipase	0.3
	CaSO ₄	1
15	MgSO ₄	1
	Water	4
	Filler*	Balance to 100%

*Can be selected from convenient materials such as CaCO₃, talc, clay, silicates, and the like.

20 **Denotes mg of active enzyme per gram of composition.

The detergent laundry bars are processed in conventional soap or detergent bar making equipment as commonly used in the art.

25 In this example the Protease #'s 1-11 and 13-25 recited in Table III, among others including the additional proteases useful in the present invention described in Tables V and VI, are substituted for Protease #12, with substantially similar results. Also in this example, any combination of the proteases useful in the present invention recited in Tables III, V and VI among others, are substituted for Protease # 12 with substantially similar results.

30

EXAMPLE 8

A detergent composition is prepared by a procedure identical to that of Example 7, with the single exception that an equivalent amount of benzoyl caprolactam is substituted for the (6-Nonanamidocaproyl)oxybenzenesulfonate.

35

In this example the Protease #'s 1-11 and 13-25 recited in Table III, among others including the additional proteases useful in the present

invention described in Tables V and VI, are substituted for Protease #12, with substantially similar results. Also in this example, any combination of the proteases useful in the present invention recited in Tables III, V and VI among others, are substituted for Protease # 12 with substantially similar results.

EXAMPLE 9

A detergent composition is prepared by a procedure identical to that of Example 7, with the single exception that an equivalent amount of nonanoyl caprolactam is substituted for the (6-Nonanamidocaproyl)oxybenzenesulfonate.

In this example the Protease #'s 1-11 and 13-25 recited in Table III, among others including the additional proteases useful in the present invention described in Tables V and VI, are substituted for Protease #12, with substantially similar results. Also in this example, any combination of the proteases useful in the present invention recited in Tables III, V and VI among others, are substituted for Protease # 12 with substantially similar results.

EXAMPLE 10

A granular detergent composition is prepared comprising the following ingredients.

	<u>Component</u>	<u>Weight %</u>
	Anionic alkyl sulfate	7
	Nonionic surfactant	5
	Zeolite (0.1-10 micron)	10
25	Trisodium citrate	2
	SKS-6 silicate builder	10
	Acrylate maleate polymer	4
	Nonanoyl caprolactam	5
	Sodium percarbonate*	15
30	Sodium carbonate	5
	Ethylenediamine disuccinate chelant (EDDS)	0.4
	Suds suppressor	2
	Protease 12	0.5
	Soil release agent	0.2
35	Minors, filler** and water	Balance to 100%

*Average particle size of 400 to 1200 microns.

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**Can be selected from convenient materials such as CaCO_3 , talc, clay, silicates, and the like.

5 Aqueous crutcher mixes of heat and alkali stable components of the detergent composition are prepared and spray-dried, and the other ingredients are admixed so that they contain the ingredients tabulated at the levels shown.

10 In this example the Protease #'s 1-11 and 13-25 recited in Table III, among others including the additional proteases useful in the present invention described in Tables V and VI, are substituted for Protease #12, with substantially similar results. Also in this example, any combination of the proteases useful in the present invention recited in Tables III, V and VI among others, are substituted for Protease # 12 with substantially similar results.

EXAMPLE 11

15 A detergent composition is prepared by a procedure identical to that of Example 10, with the single exception that an equivalent amount of benzoyl caprolactam is substituted for the nonanoyl caprolactam.

20 In this example the Protease #'s 1-11 and 13-25 recited in Table III, among others including the additional proteases useful in the present invention described in Tables V and VI, are substituted for Protease #12, with substantially similar results. Also in this example, any combination of the proteases useful in the present invention recited in Tables III, V and VI among others, are substituted for Protease # 12 with substantially similar results.

EXAMPLE 12

25 A detergent composition is prepared by a procedure identical to that of Example 10, with the exceptions that 15%, by weight, of (6-Nonanamido-caproyl)oxybenzenesulfonate is substituted for the nonanoyl caprolactam and the amount of sodium percarbonate is 30%.

In this example the Protease #'s 1-11 and 13-25 recited in Table III, among others including the additional proteases useful in the present invention described in Tables V and VI, are substituted for Protease #12, with substantially similar results. Also in this example, any combination
5 of the proteases useful in the present invention recited in Tables III, V and VI among others, are substituted for Protease # 12 with substantially similar results.

EXAMPLE 13

A detergent composition is prepared by a procedure identical to
10 that of Example 10, with the exceptions that 15%, by weight, of a benzoxazin-type activator is substituted for the nonanoyl caprolactam and the amount of sodium percarbonate is 30%.

In this example the Protease #'s 1-11 and 13-25 recited in Table III, among others including the additional proteases useful in the present
15 invention described in Tables V and VI, are substituted for Protease #12, with substantially similar results. Also in this example, any combination of the proteases useful in the present invention recited in Tables III, V and VI among others, are substituted for Protease # 12 with substantially similar results.

EXAMPLE 14

Wash Performance Test

The wash performance of the variants useful in the present invention compositions is evaluated by measuring the removal of stain from EMPA 116 (blood/milk/carbon black on cotton) cloth swatches
25 (Testfabrics, Inc., Middlesex, NJ 07030).

Six EMPA 116 swatches, cut to 3 X 4-1/2 inches with pinked edges, are placed in each pot of a Model 7243S Terg-O-Tometer (United States Testing Co., Inc., Hoboken, NJ) containing 1000 ml of water, 15 gpg hardness ($\text{Ca}^{++}:\text{Mg}^{++}::3:1::\text{w:w}$), 7 g of detergent, and enzyme as
30 appropriate. The detergent base is WFK1 detergent from wfk - Testgewebe GmbH, Adlerstrasse 42, Postfach 13 07 62, D-47759 Krefeld, Germany:

Component	% of Final Formulation
Zeolite A	25%
Sodium sulfate	25%
Soda Ash	10%

Linear alkylbenzenesulfonate	8.8%
Alcohol ethoxylate (7-8 EO)	4.5%
Sodium soap	3%
Sodium silicate (SiO ₂ :Na ₂ O::3.3:1)	3%

To this base detergent, the following additions are made:

Component	% of Final Formulation
Sodium perborate monohydrate	13%
Copolymer (Sokalan CP5)	4%
TAED (Mykon ATC Green)	3%
Enzyme	0.5%
Brightener (Tinopal AMS-GX)	0.2%

5 Sodium perborate monohydrate is obtained from Degussa Corporation, Ridgefield-Park, NJ 07660. Sokalan CP5 is obtained from BASF Corporation, Parsippany, NJ 07054. Mykon ATC Green (TAED, tetraacetythylenediamine) is obtained from Warwick International, Limited, Mostyn, Holywell, Clwyd CH8 9HE, England. Tinopal AMS GX is
10 obtained from Ciba-Geigy Corporation, Greensboro, NC 27419.

Six EMPA 116 swatches are washed in detergent with enzyme for 30 min at 60°C and are subsequently rinsed twice for 5 minutes each time in 1000 ml water. Enzymes are added at final concentrations of 0.05 to 1 ppm for standard curves, and 0.25 ppm for routine analyses.

15 Swatches are dried and pressed, and the reflectance from the swatches is measured using the L value on the L*a*b* scale of a Minolta Chroma Meter, Model CR-200 (Minolta Corporation, Ramsey, NJ 07446). Performance is reported as a percentage of the performance of *B. lentus* (GG36) protease and is calculated by dividing the amount of *B. lentus* (GG36) protease by the amount of variant protease that is needed to
20 provide the same stain removal performance X 100. The data are shown in Table VII.

Table VII

Enzyme	Wash Performance
<i>B. lentus</i> subtilisin	100
N76D	310
N76D/S103A	230
N76D/V104I	130
N76D/V107V	160
N76D/S99D/S101R	370
N76D/S99D/S103A	290
N76D/S101R/S103A	130
N76D/S101R/V104I	300

N76D/S103A/V104I	320
N76D/S103G/V104I	160
N76D/S103A/V104F	210
N76D/S103A/V104N	110
N76D/S103A/V104T	170
N76D/V104I/I107V	210
N76D/S99D/S101R/S103A	220
N76D/S99D/S101R/V104I	140
N76D/S101G/S103A/V104I	170
N76D/S101R/S103A/V104I	150
N76D/S103A/V104I/S105A	170
N76D/S103A/V104T/I107A	120
N76D/S103A/V104T/I107L	110
N76D/S103A/V104I/L126F	110
N76D/S103A/V104I/S128G	280
N76D/S103A/V104I/L135I	160
N76D/S103A/V104I/L135V	160
N76D/S103A/V104I/D197E	170
N76D/S103A/V104I/N204A	160
N76D/S103A/V104I/N204G	150
N76D/S103A/V104I/P210I	470
N76D/S103A/V104I/M222A	100
N76D/S103A/V104I/T260P	280
N76D/S103A/V104I/S265N	190

SEQUENCE LISTING

(1) GENERAL INFORMATION:

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- (iii) NUMBER OF SEQUENCES: 15
- (iv) CORRESPONDENCE ADDRESS:
 - (A) ADDRESSEE: The Procter & Gamble Company
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 - (C) CITY: Cincinnati
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 - (A) MEDIUM TYPE: Diskette
 - (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: PatentIn Release #1.0, Version #1.25
- (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER:
 - (B) FILING DATE: 13-OCT-1994
 - (C) CLASSIFICATION:
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(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 19 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GAAGCTGCAA CTCGTAAA

19

(2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 18 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GCTGCTCTAG ACAATTCG

18

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 39 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GTATTAGGGG CGGACGGTCG AGGCGCCATC AGCTCGATT

39

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 33 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

TCAGGTTCGG TCTCGAGCGT TGCCCAAGGA TTG

33

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 22 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CACGTTGCTA GCTTGAGTTT AG

22

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1497 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GGTCTACTAA AATATTATTC CATACTATAC AATTAATACA CAGAATAATC TGTCTATTGG

60

TTATTCTGCA AATGAAAAAA AGGAGAGGAT AAAGAGTGAG AGGCAAAAAA GTATGGATCA 120
GTTTGCTGTT TGCTTTAGCG TTAATCTTTA CGATGGCGTT CGGCAGCACA TCCTCTGCCC 180
AGGCGGCAGG GAAATCAAAC GGGGAAAAGA AATATATTGT CGGGTTTAAA CAGACAATGA 240
GCACGATGAG CGCCGCTAAG AAGAAAGATG TCATTTCTGA AAAAGGCGGG AAAGTGCAAA 300
AGCAATTCAA ATATGTAGAC GCAGCTTCAG TCACATTAAA CGAAAAAGCT GTAAAAGAAT 360
TGAAAAAGA CCCGAGCGTC GCTTACGTTG AAGAAGATCA CGTAGCACAT GCGTACGCGC 420
AGTCCGTGCC TTACGGCGTA TCACAAATTA AAGCCCCTGC TCTGCACTCT CAAGGCTACA 480
CTGGATCAAA TGTTAAAGTA GCGGTTATCG ACAGCGGTAT CGATTCTTCT CATCCTGATT 540
TAAAGGTAGC AAGCGGAGCC AGCATGGTTC CTTCTGAAAC AAATCCTTTC CAAGACAACA 600
ACTCTCACGG AACTCACGTT GCCGGCACAG TTGCGGCTCT TAATAACTCA ATCGGTGTAT 660
TAGGCGTTGC GCCAAGCGCA TCACTTTACG CTGTAAAAGT TCTCGGTGCT GACGGTTCGG 720
GCCAATACAG CTGGATCATT AACGGAATCG AGTGGGCGAT CGCAAACAAT ATGGACGTTA 780
TTAACATGAG CCTCGGCGGA CCTTCTGGTT CTGCTGCTTT AAAAGCGGCA GTTGATAAAG 840
CCGTTGCATC CGGCGTCGTA GTCGTTGCGG CAGCCGGTAA CGAAGGCACT TCCGGCAGCT 900
CAAGCACAGT GGGCTACCCT GGTAAATACC CTTCTGTCAT TGCAGTAGGC GCTGTTGACA 960
GCAGCAACCA AAGAGCATCT TTCTCAAGCG TAGGACCTGA GCTTGATGTC ATGGCACCTG 1020
GCGTATCTAT CCAAAGCACG CTTCTGGAA ACAATACGG GCGGTACAAC GGTACGTCAA 1080
TGGCATCTCC GCACGTTGCC GGAGCGGCTG CTTTGATTCT TTCTAAGCAC CCGAACTGGA 1140
CAAACACTCA AGTCCGCAGC AGTTTAGAAA ACACCACTAC AAAACTTGGT GATTCTTTGT 1200
ACTATGGAAA AGGGCTGATC AACGTACAAG CGGCAGCTCA GTAAACATA AAAAACCGGC 1260
CTTGCCCCCG CCGGTTTTTT ATTATTTTTC TTCCTCCGCA TGTTCAATCC GCTCCATAAT 1320
CGACGGATGG CTCCCTCTGA AAATTTTAAC GAGAAACGGC GGGTTGACCC GGCTCAGTCC 1380
CGTAACGGCC AACTCCTGAA ACGTCTCAAT CGCCGCTTCC CGGTTTCCGG TCAGCTCAAT 1440
GCCATAACGG TCGGCGGCGT TTCCTGATA CCGGGAGACG GCATTCTGTA TCGGATC 1497

(2) INFORMATION FOR SEQ ID NO:7:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 275 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Ala Gln Ser Val Pro Tyr Gly Val Ser Gln Ile Lys Ala Pro Ala Leu
1 5 10 15
His Ser Gln Gly Tyr Thr Gly Ser Asn Val Lys Val Ala Val Ile Asp
20 25 30
Ser Gly Ile Asp Ser Ser His Pro Asp Leu Lys Val Ala Gly Gly Ala
35 40 45

Ser Met Val Pro Ser Glu Thr Asn Pro Phe Gln Asp Asn Asn Ser His
 50 55 60
 Gly Thr His Val Ala Gly Thr Val Ala Ala Leu Asn Asn Ser Ile Gly
 65 70 75 80
 Val Leu Gly Val Ala Pro Ser Ala Ser Leu Tyr Ala Val Lys Val Leu
 85 90 95
 Gly Ala Asp Gly Ser Gly Gln Tyr Ser Trp Ile Ile Asn Gly Ile Glu
 100 105 110
 Trp Ala Ile Ala Asn Asn Met Asp Val Ile Asn Met Ser Leu Gly Gly
 115 120 125
 Pro Ser Gly Ser Ala Ala Leu Lys Ala Ala Val Asp Lys Ala Val Ala
 130 135 140
 Ser Gly Val Val Val Val Ala Ala Ala Gly Asn Glu Gly Thr Ser Gly
 145 150 155 160
 Ser Ser Ser Thr Val Gly Tyr Pro Gly Lys Tyr Pro Ser Val Ile Ala
 165 170 175
 Val Gly Ala Val Asp Ser Ser Asn Gln Arg Ala Ser Phe Ser Ser Val
 180 185 190
 Gly Pro Glu Leu Asp Val Met Ala Pro Gly Val Ser Ile Gln Ser Thr
 195 200 205
 Leu Pro Gly Asn Lys Tyr Gly Ala Tyr Asn Gly Thr Ser Met Ala Ser
 210 215 220
 Pro His Val Ala Gly Ala Ala Ala Leu Ile Leu Ser Lys His Pro Asn
 225 230 235 240
 Trp Thr Asn Thr Gln Val Arg Ser Ser Leu Glu Asn Thr Thr Thr Lys
 245 250 255
 Leu Gly Asp Ser Phe Tyr Tyr Gly Lys Gly Leu Ile Asn Val Gln Ala
 260 265 270
 Ala Ala Gln
 275

(2) INFORMATION FOR SEQ ID NO:8:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 275 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Ala Gln Ser Val Pro Tyr Gly Ile Ser Gln Ile Lys Ala Pro Ala Leu
 1 5 10 15
 His Ser Gln Gly Tyr Thr Gly Ser Asn Val Lys Val Ala Val Ile Asp
 20 25 30
 Ser Gly Ile Asp Ser Ser His Pro Asp Leu Asn Val Arg Gly Gly Ala
 35 40 45
 Ser Phe Val Pro Ser Glu Thr Asn Pro Tyr Gln Asp Gly Ser Ser His
 50 55 60

Gly Thr His Val Ala Gly Thr Ile Ala Ala Leu Asn Asn Ser Ile Gly
 65 70 75 80
 Val Leu Gly Val Ser Pro Ser Ala Ser Leu Tyr Ala Val Lys Val Leu
 85 90 95
 Asp Ser Thr Gly Ser Gly Gln Tyr Ser Trp Ile Ile Asn Gly Ile Glu
 100 105 110
 Trp Ala Ile Ser Asn Asn Met Asp Val Ile Asn Met Ser Leu Gly Gly
 115 120 125
 Pro Thr Gly Ser Thr Ala Leu Lys Thr Val Val Asp Lys Ala Val Ser
 130 135 140
 Ser Gly Ile Val Val Ala Ala Ala Ala Gly Asn Glu Gly Ser Ser Gly
 145 150 155 160
 Ser Thr Ser Thr Val Gly Tyr Pro Ala Lys Tyr Pro Ser Thr Ile Ala
 165 170 175
 Val Gly Ala Val Asn Ser Ser Asn Gln Arg Ala Ser Phe Ser Ser Ala
 180 185 190
 Gly Ser Glu Leu Asp Val Met Ala Pro Gly Val Ser Ile Gln Ser Thr
 195 200 205
 Leu Pro Gly Gly Thr Tyr Gly Ala Tyr Asn Gly Thr Ser Met Ala Thr
 210 215 220
 Pro His Val Ala Gly Ala Ala Ala Leu Ile Leu Ser Lys His Pro Thr
 225 230 235 240
 Trp Thr Asn Ala Gln Val Arg Asp Arg Leu Glu Ser Thr Ala Thr Tyr
 245 250 255
 Leu Gly Asn Ser Phe Tyr Tyr Gly Lys Gly Leu Ile Asn Val Gln Ala
 260 265 270
 Ala Ala Gln
 275

(2) INFORMATION FOR SEQ ID NO:9:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 274 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Ala Gln Thr Val Pro Tyr Gly Ile Pro Leu Ile Lys Ala Asp Lys Val
 1 5 10 15
 Gln Ala Gln Gly Phe Lys Gly Ala Asn Val Lys Val Ala Val Leu Asp
 20 25 30
 Thr Gly Ile Gln Ala Ser His Pro Asp Leu Asn Val Val Gly Gly Ala
 35 40 45
 Ser Phe Val Ala Gly Glu Ala Tyr Asn Thr Asp Gly Asn Gly His Gly
 50 55 60
 Thr His Val Ala Gly Thr Val Ala Ala Leu Asp Asn Thr Thr Gly Val
 65 70 75 80

[illegible]

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 269 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Ala	Gln	Ser	Val	Pro	Trp	Gly	Ile	Ser	Arg	Val	Gln	Ala	Pro	Ala	Ala	1	5	10	15
His	Asn	Arg	Gly	Leu	Thr	Gly	Ser	Gly	Val	Lys	Val	Ala	Val	Leu	Asp	20	25	30	
Thr	Gly	Ile	Ser	Thr	His	Pro	Asp	Leu	Asn	Ile	Arg	Gly	Gly	Ala	Ser	35	40	45	
Phe	Val	Pro	Gly	Glu	Pro	Ser	Thr	Gln	Asp	Gly	Asn	Gly	His	Gly	Thr	50	55	60	
His	Val	Ala	Gly	Thr	Ile	Ala	Ala	Leu	Asn	Asn	Ser	Ile	Gly	Val	Leu	65	70	75	80
Gly	Val	Ala	Pro	Ser	Ala	Glu	Leu	Tyr	Ala	Val	Lys	Val	Leu	Gly	Ala	85	90	95	

Ser Gly Ser Gly Ser Val Ser Ser Ile Ala Gln Gly Leu Glu Trp Ala
 100 105 110
 Gly Asn Asn Gly Met His Val Ala Asn Leu Ser Leu Gly Ser Pro Ser
 115 120 125
 Pro Ser Ala Thr Leu Glu Gln Ala Val Asn Ser Ala Thr Ser Arg Gly
 130 135 140
 Val Leu Val Val Ala Ala Ser Gly Asn Ser Gly Ala Gly Ser Ile Ser
 145 150 155 160
 Tyr Pro Ala Arg Tyr Ala Asn Ala Met Ala Val Gly Ala Thr Asp Gln
 165 170 175
 Asn Asn Asn Arg Ala Ser Phe Ser Gln Tyr Gly Ala Gly Leu Asp Ile
 180 185 190
 Val Ala Pro Gly Val Asn Val Gln Ser Thr Tyr Pro Gly Ser Thr Tyr
 195 200 205
 Ala Ser Leu Asn Gly Thr Ser Met Ala Thr Pro His Val Ala Gly Ala
 210 215 220
 Ala Ala Leu Val Lys Gln Lys Asn Pro Ser Trp Ser Asn Val Gln Ile
 225 230 235 240
 Arg Asn His Leu Lys Asn Thr Ala Thr Ser Leu Gly Ser Thr Asn Leu
 245 250 255
 Tyr Gly Ser Gly Leu Val Asn Ala Glu Ala Ala Thr Arg
 260 265

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1140 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

ATGAAGAAAC CGTTGGGGAA AATTGTCGCA AGCACC GCAC TACTCATTTC TGTTGCTTTT 60
 AGTTCATCGA TCGCATCGGC TGCTGAAGAA GCAAAAGAAA AATATTTAAT TGGCTTTAAT 120
 GAGCAGGAAG CTGTCAGTGA GTTTGTAGAA CAAGTAGAGG CAAATGACGA GGTCGCCATT 180
 CTCTCTGAGG AAGAGGAAGT CGAAATTGAA TTGCTTCATG AATTTGAAAC GATTCCTGTT 240
 TTATCCGTTG AGTTAAGCCC AGAAGATGTG GACGCGCTTG AACTCGATCC AGCGATTCTT 300
 TATATTGAAG AGGATGCAGA AGTAACGACA ATGGCGCAAT CAGTGCCATG GGGAATTAGC 360
 CGTGTGCAAG CCCAGCTGC CCATAACCGT GGATTGACAG GTTCTGGTGT AAAAGTTGCT 420
 GTCCTCGATA CAGGTATTTT CACTCATCCA GACTTAAATA TTCGTGGTGG CGCTAGCTTT 480
 GTACCAGGGG AACCATCCAC TCAAGATGGG AATGGGCATG GCACGCATGT GGCCGGGACG 540
 ATTGCTGCTT TAAACAATTC GATTGGCGTT CTTGGCGTAG CGCCGAGCGC GGAACATAC 600
 GCTGTAAAG TATTAGGGGC GAGCGGTTCA GGTTCGGTCA GCTCGATTGC CCAAGGATTG 660
 GAATGGGCAG GGAACAATGG CATGCACGTT GCTAATTTGA GTTTAGGAAG CCCTTCGCCA 720
 AGTGCCACAC TTGAGCAAGC TGTTAATAGC GCGACTTCTA GAGGCGTTCT TGTTGTAGCG 780

GCATCTGGGA ATTCAGGTGC AGGCTCAATC AGCTATCCGG CCCGTTATGC GAACGCAATG 840
GCAGTCGGAG CTACTGACCA AAACAACAAC CGCGCCAGCT TTTCACAGTA TGGCGCAGGG 900
CTTGACATTG TCGCACCAGG TGTAACGTG CAGAGCACAT ACCCAGGTTC AACGTATGCC 960
AGCTTAAACG GTACATCGAT GGCTACTCCT CATGTTGCAG GTGCAGCAGC CCTTGTTAAA 1020
CAAAAGAACC CATCTTGGTC CAATGTACAA ATCCGCAATC ATCTAAAGAA TACGGCAACG 1080
AGCTTAGGAA GCACGAACTT GTATGGAAGC GGACTTGTCA ATGCAGAAGC GGCAACACGC 1140

(2) INFORMATION FOR SEQ ID NO:12:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1140 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

ATGAAGAAAC CGTTGGGGAA AATTGTCGCA AGCACCGCAC TACTCATTTT TGTGCTTTT 60
AGTTCATCGA TCGCATCGGC TGCTGAAGAA GCAAAGAAA AATATTTAAT TGGCTTTAAT 120
GAGCAGGAAG CTGTCAGTGA GTTTGTAGAA CAAGTAGAGG CAAATGACGA GGTCGCCATT 180
CTCTCTGAGG AAGAGGAAGT CGAAATTGAA TTGCTTCATG AATTTGAAAC GATTCCTGTT 240
TTATCCGTTG AGTTAAGCCC AGAAGATGTG GACGCGCTTG AACTCGATCC AGCGATTCT 300
TATATTGAAG AGGATGCAGA AGTAACGACA ATGGCGCAAT CAGTGCCATG GGAATTAGC 360
CGTGTGCAAG CCCAGCTGC CCATAACCGT GGATTGACAG GTTCTGGTGT AAAAGTTGCT 420
GTCCTCGATA CAGGTATTTT CACTCATCCA GACTTAAATA TTCGTGGTGG CGCTAGCTTT 480
GTACCAGGGG AACCATCCAC TCAAGATGGG AATGGGCATG GCACGCATGT GGCCGGGACG 540
ATTGCTGCTT TAGACAACTC GATTGGCGTT CTTGGCGTAG CGCCGAGCGC GGAAGTATAC 600
GCTGTAAAG TATTAGGGGC GAGCGGTTCA GGCGCCATCA GCTCGATTGC CCAAGGATTG 660
GAATGGGCAG GGAACAATGG CATGCACGTT GCTAATTGA GTTTAGGAAG CCCTTCGCCA 720
AGTGCCACAC TTGAGCAAGC TGTTAATAGC GCGACTTCTA GAGGCGTTCT TGTGTAGCG 780
GCATCTGGGA ATTCAGGTGC AGGCTCAATC AGCTATCCGG CCCGTTATGC GAACGCAATG 840
GCAGTCGGAG CTACTGACCA AAACAACAAC CGCGCCAGCT TTTCACAGTA TGGCGCAGGG 900
CTTGACATTG TCGCACCAGG TGTAACGTG CAGAGCACAT ACCCAGGTTC AACGTATGCC 960
AGCTTAAACG GTACATCGAT GGCTACTCCT CATGTTGCAG GTGCAGCAGC CCTTGTTAAA 1020
CAAAAGAACC CATCTTGGTC CAATGTACAA ATCCGCAATC ATCTAAAGAA TACGGCAACG 1080
AGCTTAGGAA GCACGAACTT GTATGGAAGC GGACTTGTCA ATGCAGAAGC GGCAACACGC 1140

(2) INFORMATION FOR SEQ ID NO:13:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid

- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

TATGCCAGCC ACAACGGTAC TTCGATGGCT

30

(2) INFORMATION FOR SEQ ID NO:14:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 31 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

CACAGTTGCG GCTCTAGATA ACTCAATCGG T

31

(2) INFORMATION FOR SEQ ID NO:15:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 33 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

GCTGACGGTT CCGGCGCTAT TAGTTGGATC ATT

33

What is claimed is:

1. A bleaching composition comprising:
 - (a) from about 0.0001% to about 10% by weight of the bleaching composition of a protease enzyme which is a carbonyl hydrolase variant having an amino acid sequence not found in nature, which is derived from a precursor carbonyl hydrolase comprising a substitution of a different amino acid for a plurality of amino acid residues at a position in said precursor carbonyl hydrolase equivalent to position +76 in *Bacillus amyloliquefaciens* subtilisin, in combination with one or more amino acid residue positions equivalent to those selected from the group consisting of +99, +101, +103, +104, +107, +123, +27, +105, +109, +126, +128, +135, +156, +166, +195, +197, +204, +206, +210, +216, +217, +218, +222, +260, +265, and/or +274 in *Bacillus amyloliquefaciens* subtilisin; provided that:
 - i) when said carbonyl hydrolase variant includes a substitution of amino acid residues at positions equivalent to +76 and +195, there is also a substitution of an amino acid residue at one or more amino acid residue positions other than amino acid residue positions equivalent to positions +99; and
 - ii) when said carbonyl hydrolase variant includes a substitution of amino acid residues at positions equivalent to +76 and +156, there is also a substitution of an amino acid residue at one or more amino acid residue positions other than amino acid residue positions equivalent to positions +99; and
 - (b) a bleaching agent which either is an organic peroxyacid or is a combination of a bleach activator and a peroxygen compound capable of yielding hydrogen peroxide that can react with the activator to form an organic peroxyacid in situ in a bleaching solution formed from the composition; and (c) one or more cleaning composition materials compatible with the protease enzyme and bleaching agent.

2. The bleaching compositions according to Claim 1 wherein the cleaning composition materials are selected from the group consisting of surfactants, solvents, buffers, enzymes, soil release agents, clay soil removal agents, dispersing agents, brighteners, suds suppressors, fabric softeners, suds boosters, enzyme stabilizers, builders, dyes, perfumes, and mixtures thereof.
3. The bleaching compositions according to Claim 2 in a form useful as a fabric cleaning composition comprising at least one surfactant.
4. The bleaching compositions according to Claim 3 comprising at least about 5% surfactant selected from the group consisting of alkyl benzene sulfonates, primary alkyl sulfates, secondary alkyl sulfates, alkyl alkoxy sulfates, alkyl alkoxy carboxylates, alkyl polyglycosides and their corresponding sulfated polyglycosides, alpha-sulfonated fatty acid esters,

alkyl and alkyl phenol alkoxylates, betaines and sulfobetaines, amine oxides, N-methyl glucamides, nonionic primary alcohol ethoxylates, nonionic primary alcohol mixed ethoxy/propoxy, and mixtures thereof.

5. The bleaching compositions according to Claim 4 further comprising at least about 5% builder selected from the group consisting of zeolites, polycarboxylates, layered silicates, phosphates, and mixtures thereof.
6. The bleaching compositions according to Claim 4 in the form of a concentrated granular fabric cleaning composition comprising at least about 30% surfactant.
7. The bleaching compositions according to Claim 1 wherein the cleaning composition materials comprise at least one enzyme selected from the group consisting of cellulases, lipases, amylases, other proteases, peroxidases and mixtures thereof.
8. The bleaching compositions according to Claim 1 in a form useful as a liquid fabric cleaning composition comprising at least one solvent and at least one surfactant.
9. The bleaching compositions according to Claim 8 comprising water and at least 5% surfactant.
10. The bleaching compositions according to Claim 1 wherein the cleaning composition materials comprise at least one fabric softener.
11. The cleaning compositions according to Claim 1 wherein the precursor carbonyl hydrolase for the protease enzyme is a subtilisin, and the protease enzyme is a subtilisin variant wherein a combination of substitutions is made at the positions equivalent to 76/99, 76/101, 76/103, 76/104, 76/107, 76/123, 76/99/101, 76/99/103, 76/99/104, 76/101/103, 76/101/104, 76/103/104, 76/104/107, 76/104/123, 76/107/123, 76/99/101/103, 76/99/101/104, 76/99/103/104, 76/101/103/104, 76/103/104/123, 76/104/107/123, 76/99/101/103/104, 76/99/103/104/123,

76/99/101/103/104/123, 76/103/104/126, 76/103/104/135,
 10 76/103/104/197, 76/103/104/222, 76/103/104/260, 76/103/104/265,
 76/103/104/126/265, 27/76/104/123/274, 27/76/104/109/123/274,
 27/76/104/123/218/274, 27/76/104/123, 27/76/104/107/123,
 27/76/104/109/123, 27/76/104/109/123/218/274, 27/76/104/123/197,
 27/76/104/123/204, 27/76/104/123/206, 27/76/104/123/216,
 15 27/76/104/123/218, 27/76/104/123/260, 27/76/104/123/195/197,
 27/76/104/123/195/218, 27/76/104/123/197/218, 27/76/104/123/204/218,
 27/76/104/123/206/218, 27/76/104/123/218/260,
 27/76/104/123/195/197/218, 76/103/104/217, 76/103/104/156,
 76/103/104/166, 76/103/104/105, 76/101/103/104, 76/103/104/128,
 20 76/103/104/210, 76/103/104/107, 76/103/104/204, 76/217,
 76/103/104/156/166 and 76/103/104/128.

12. The cleaning compositions according to Claim 1 wherein the
 protease enzyme is a subtilisin variant selected from the group consisting
 of 76/99, 76/101, 76/103, 76/104, 76/107, 76/123, 76/99/101, 76/99/103,
 76/99/104, 76/101/103, 76/101/104, 76/103/104, 76/104/107, 76/104/123,
 5 76/107/123, 76/99/101/103, 76/99/101/104, 76/99/103/104,
 76/101/103/104, 76/103/104/123, 76/104/107/123, 76/99/101/103/104,
 76/99/103/104/123, 76/99/101/103/104/123, 76/103/104/128;
 76/103/104/260; 76/103/104/265; 76/103/104/197; 76/103/104/105;
 76/103/104/135; 76/103/104/126; 76/103/104/107; 76/103/104/210;
 10 76/103/104/126/265; and/or 76/103/104/222.

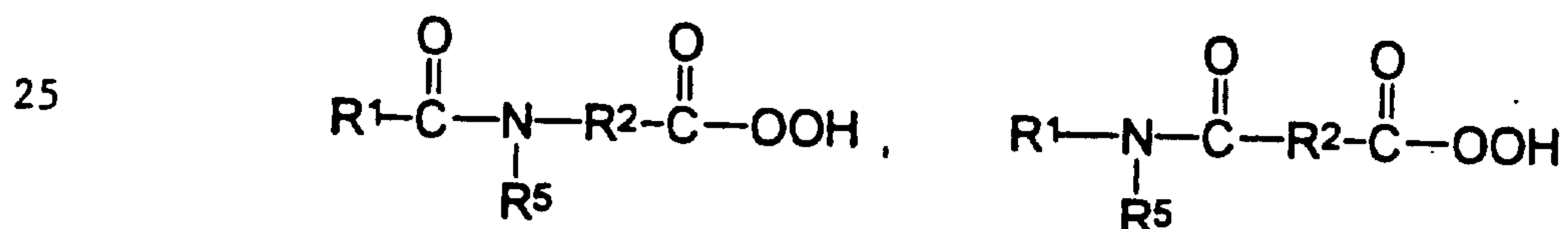
13. A fabric cleaning composition comprising:

(a) from about 0.0001% to about 10% by weight of the fabric
 cleaning composition of a protease enzyme which is a carbonyl hydrolase
 variant having an amino acid sequence not found in nature, which is derived
 5 from a subtilisin precursor carbonyl hydrolase, and wherein the protease
 enzyme is a subtilisin variant selected from N76D/S99D; N76D/S101R;
 N76D/S103A; N76D/V104I; N76D/I107V; N76D/N123S; N76D/S99D/S101R;
 N76D/S99D/S103A; N76D/S99D/V104I; N76D/S101R/S103A; N76D/S101R/
 V104I; N76D/S103A/V104I; N76D/V104I/I107V; N76D/V104Y/I107V;
 10 N76D/V104I/N123S; N76D/I107V/N123S; N76D/S99D/S101R/S103A;
 N76D/S99D/S101R/V104I; N76D/S99D/S103A/V104I;

N76D/S101R/S103AV104I; N76D/S103AV104I/N123S;
 N76D/V104I/I107V/N123S; N76D/S99D/S101R/S103AV104I;
 N76D/S99D/S103AV104I/N123S;
 15 N76D/S99D/S101R/S103AV104I/N123S; N76D/S103AV104I/S128G;
 N76D/S103AV104I/T260P; N76D/S103AV104I/S265N;
 N76D/S103AV104I/D197E; N76D/S103AV104I/S105A;
 N76D/S103AV104I/L135I; N76D/S103AV104I/L126F;
 N76D/S103AV104T/L107T; N76D/S103AV104I/L126F/S265N, and
 20 N76D/S103AV104I/M222A and mixtures thereof; and

(b) from about 0.5% to about 20% of a bleaching agent selected from the group consisting of:

(i) an organic peroxyacid of the formula:



wherein R^1 is an alkyl, aryl, or alkaryl group containing from about 1 to about 14 carbon atoms, R^2 is an alkylene, arylene or alkarylene group containing from about 1 to about 14 carbon atoms, and R^5 is H or an alkyl, aryl, or alkaryl group containing from about 1 to about 10 carbon atoms, and
 30

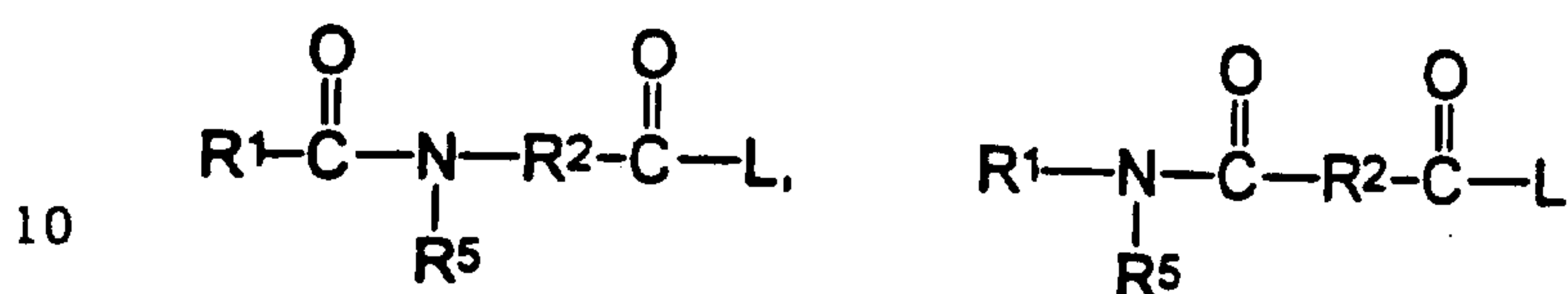
(ii) a combination of a bleach activator and a peroxygen compound capable of yielding hydrogen peroxide that can react with the activator to form an organic peroxyacid in situ in a bleaching solution formed from the composition, wherein said bleach activator has the general formula:
 35



wherein R is an alkyl group containing from about 5 to about 18 carbon atoms wherein the longest linear alkyl chain extending from and including the carbonyl carbon contains from about 6 to about 10 carbon atoms and L is a leaving group, the conjugate acid of which has a pKa in the range of from about 4 to about 13; and
 45

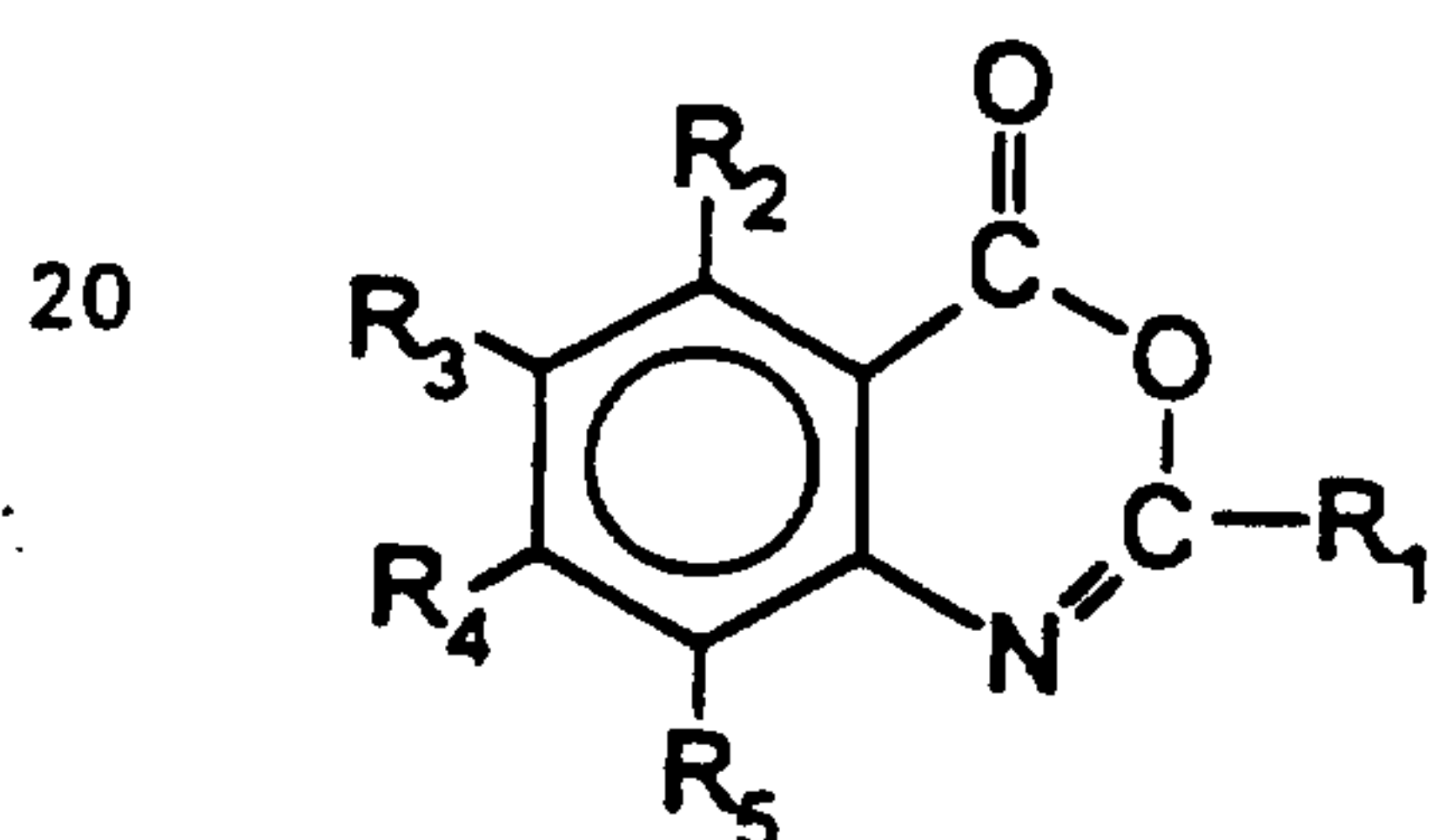
(c) one or more cleaning composition materials compatible with the protease enzyme and bleaching agent comprising at least about 5% surfactant and at least about 5% builder, by weight of the composition.

14. The fabric cleaning composition according to Claim 13 further comprising cleaning composition materials selected from the group consisting of solvents, buffers, enzymes, soil release agents, clay soil removal agents, dispersing agents, brighteners, suds suppressors, fabric softeners, suds boosters, enzyme stabilizers, dyes, perfumes, and mixtures thereof.
15. The fabric cleaning composition according to Claim 13 further comprising at least one enzyme selected from the group consisting of cellulases, lipases, amylases, other proteases, peroxidases, and mixtures thereof.
16. The fabric cleaning composition according to Claim 13 in the form of a liquid, granule or bar.
17. The fabric cleaning composition according to Claim 13 wherein the protease enzyme is a subtilisin variant selected from N76D/S99D, N76D/V104I, N76D/S99D/V104I, N76D/S103A/V104I, N76D/V104I/I107V, N76D/V104Y/I107V, N76D/S101R/S103A/V104I, N76D/S99D/S101R/S103A/V104I, N76D/S101R/V104I, and mixtures thereof.
18. The fabric cleaning composition according to Claim 13 wherein the protease enzyme is a Bacillus subtilisin variant selected from N76D/V104I, N76D/S103A/V104I, and mixtures thereof.
19. The fabric cleaning compositions according to Claim 13 comprising a bleaching agent comprising at least about 0.1% by weight of a peroxygen bleaching compound capable of yielding hydrogen peroxide in an aqueous liquor and at least 0.1% by weight of one or more bleach activators, wherein said bleach activators are members selected from the group consisting of:
- a) a bleach activator of the general formula:



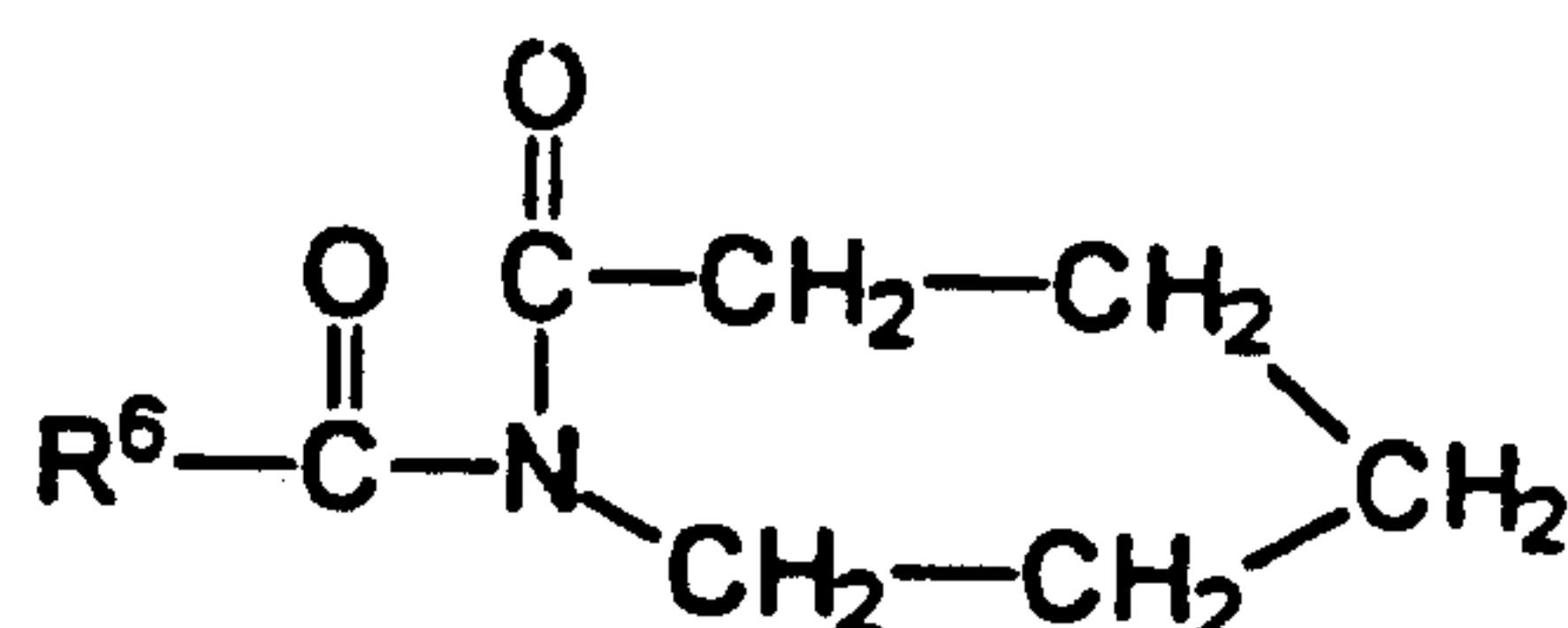
15 or mixtures thereof, wherein R^1 is an alkyl, aryl, or alkaryl group containing from about 1 to about 14 carbon atoms, R^2 is an alkylene, arylene or alkarylene group containing from about 1 to about 14 carbon atoms, R^5 is H or an alkyl, aryl, or alkaryl group containing from about 1 to about 10 carbon atoms, and L is a leaving group;

b) a benzoxazin-type bleach activator of the formula:



25 wherein R_1 is H, alkyl, alkaryl, aryl, arylalkyl, and wherein R_2 , R_3 , R_4 , and R_5 may be the same or different substituents selected from H, halogen, alkyl, alkenyl, aryl, hydroxyl, alkoxy, amino, alkylamino, $-\text{COOR}_6$, wherein R_6 is H or an alkyl group and carbonyl functions;

30 c) a N-acyl caprolactam bleach activator of the formula:



35 wherein R^6 is H or an alkyl, aryl, alkoxyaryl, or alkaryl group containing from 1 to 12 carbons; and

d) mixtures of a), b) and c).

20. The fabric cleaning composition according to Claim 13 wherein the corresponding carboxylic acid of the organic peroxyacid bleaching agent has a Hydrophilic-Lipophilic Balance value within the range of from about 3 to about 6.5.

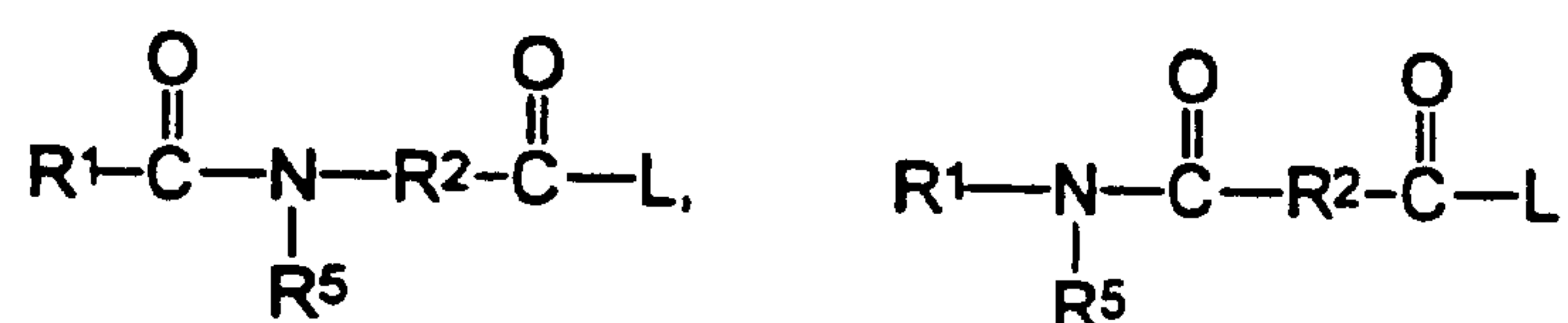
21. The fabric cleaning composition according to Claim 20 wherein the ratio of mg protease enzyme per 100 grams of composition to ppm theoretical Available O₂ of the organic peroxyacid is within the range of from about 1:1 to about 20:1.

5 22. A fabric cleaning composition comprising:

(a) from about 0.0001% to about 10% protease enzyme which is an N76D/S103A/V104I subtilisin variant derived from *Bacillus lentus* subtilisin;

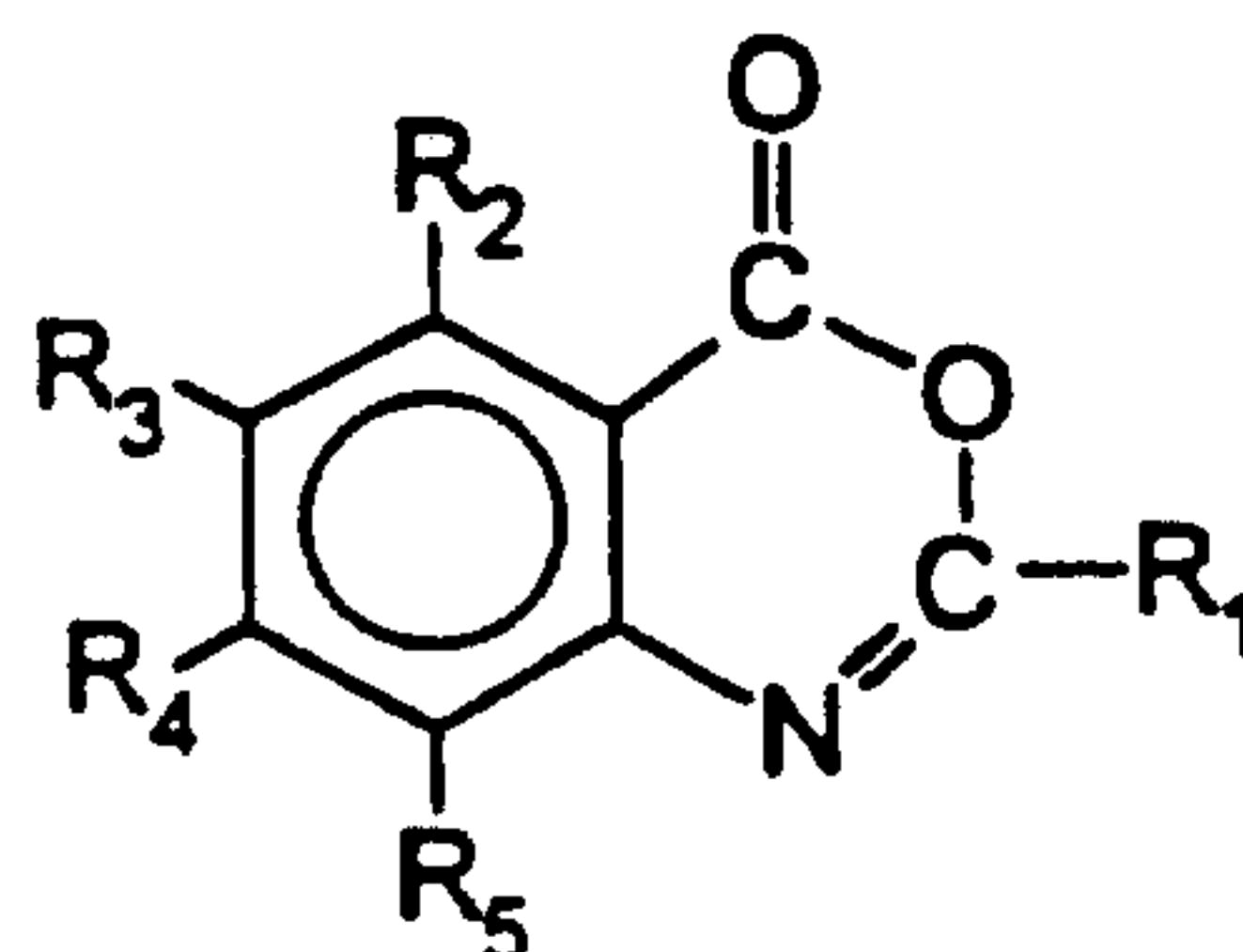
10 (b) from about 0.5% to about 20% of a bleaching system comprising at least about 0.1% by weight of a peroxygen bleaching compound capable of yielding hydrogen peroxide in an aqueous liquor and at least 0.1% by weight of one or more bleach activators, wherein said bleach activators are members selected from the group consisting of:

i) a bleach activator of the general formula:



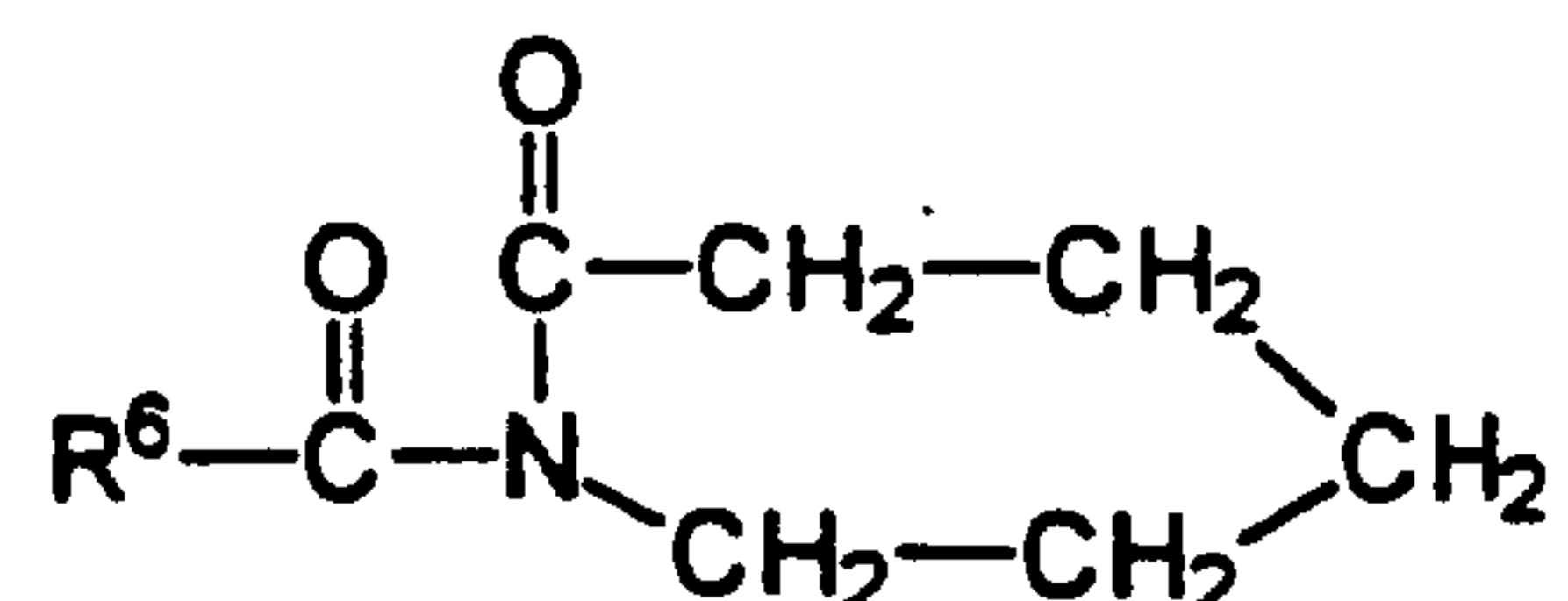
15 or mixtures thereof, wherein R¹ is an alkyl, aryl, or alkaryl group containing from about 1 to about 14 carbon atoms, R² is an alkylene, arylene or alkarylene group containing from about 1 to about 14 carbon atoms, R⁵ is H or an alkyl, aryl, or alkaryl group containing from about 1 to about 10 carbon atoms, and L is a leaving group;

20 ii) a benzoxazin-type bleach activator of the formula:



wherein R₁ is H, alkyl, alkaryl, aryl, arylalkyl, and wherein R₂, R₃, R₄, and R₅ may be the same or different substituents selected from H, halogen, alkyl, alkenyl, aryl, hydroxyl, alkoxy, amino, alkylamino, -COOR₆, wherein R₆ is H or an alkyl group and carbonyl functions;

- 5 iii) a N-acyl caprolactam bleach activator of the formula:



wherein R⁶ is H or an alkyl, aryl, alkoxyaryl, or alkaryl group containing from 1 to 12 carbons; and

- iv) mixtures of i), ii) and iii);
- (c) at least about 5% surfactant;
- (d) at least about 5% builder; and
- (e) optionally, one or more cleaning composition materials compatible with the protease enzyme and bleaching system selected from the group consisting of solvents, buffers, enzymes, soil release agents, clay soil removal agents, dispersing agents, brighteners, suds suppressors, fabric softeners, suds boosters, enzyme stabilizers, dyes, perfumes, and mixtures thereof.

20 23. The fabric cleaning composition according to Claim 22 wherein the surfactant is selected from the group consisting of alkyl benzene sulfonates, primary alkyl sulfates, secondary alkyl sulfates, alkyl alkoxy sulfates, alkyl alkoxy carboxylates, alkyl polyglycosides and their corresponding sulfated polyglycosides, alpha-sulfonated fatty acid esters, alkyl and alkyl phenol alkoxyates, betaines and sulfobetaines, amine oxides, N-methyl glucamides, nonionic primary alcohol ethoxylates, nonionic primary/alcohol mixed

ethoxy/propoxy, and mixtures thereof; and wherein further the builder is selected from the group consisting of zeolites, polycarboxylates, layered silicates, phosphates, and mixtures thereof.

5 24. A composition according to Claim 23 further comprising at least about 0.001%, by weight of said cleaning composition, of at least one enzyme selected from the group consisting of proteases, amylases, lipases, cellulases, peroxidases and mixtures thereof.

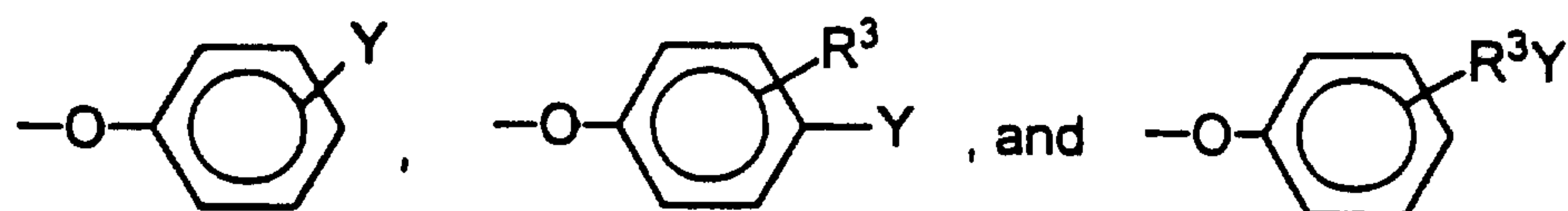
10 25. A composition according to Claim 22 wherein said bleach activator is selected from the group consisting of benzoyl caprolactam, nonanoyl caprolactam, (6-nonanamidocaproyl)oxybenzenesulfonate, and mixtures thereof.

15 26. A composition according to Claim 22 wherein the peroxygen bleaching compound is selected from the group consisting of sodium perborate monohydrate, sodium perborate tetrahydrate, sodium pyrophosphate peroxyhydrate, urea peroxyhydrate, sodium percarbonate, sodium peroxide and mixtures thereof.

27. A composition according to Claim 26 wherein the molar ratio of hydrogen peroxide to bleach activator is greater than about 1.0.

20 28. A composition according to Claim 22 wherein R^1 is an alkyl group containing from about 6 to about 12 carbon atoms, R^2 contains from about 1 to about 8 carbon atoms, and R^5 is H or methyl.

25 29. A composition according to Claim 22 wherein R^1 is an alkyl group containing from about 7 to about 10 carbon atoms and R^2 contains from about 4 to about 5 carbon atoms, and wherein L is selected from the group consisting of:



wherein R^3 is an alkyl chain containing from about 1 to about 8 carbon atoms, and Y is $\text{—SO}_3\text{—M}^+$ or $\text{—CO}_2\text{—M}^+$ wherein M is sodium or potassium.

30. A composition according to Claim 22 comprising an N-acyl caprolactam selected from the group consisting of benzoyl caprolactam, octanoyl caprolactam, nonanoyl caprolactam, 3,5,5-trimethylhexanoyl caprolactam, decanoyl caprolactam, undecenoyl caprolactam, and mixtures thereof.

31. A method for cleaning fabric, said method comprising contacting a fabric in need of cleaning with a wash solution containing a composition according to Claim 1.

32. A method for cleaning fabric, said method comprising contacting a fabric in need of cleaning with a wash solution containing an effective amount of a composition according to Claim 22.





His Bal Ala His Ala Tyr Ala Gln Ser Val Pro Tyr Gly Val Ser Gln Ile Lys Ala Pro Ala Leu His Ser Gln	10
399 CAC GTA GCA CAT GCG TAC GCG CAG TCC GTG CCT TAC GGC GTA TCA CAA ATT AAA GCC CCT GCT CTG CAC TCT CAA	
Gly Tyr Thr Gly Ser Asn Val Lys Val Ala Val Ile Asp Ser Gly Ile Asp Ser Ser His Pro Asn Leu Ivs Val	20 30 40
474 GGC TAC ACT GGA TCA AAT GTT AAA GTA GCG GTT ATC GAC AGC GGT ATC GAT TCT TCT CAT CCT GAT TTA AAG GTA	
Ala Gly Gly Ala Ser Met Val Pro Ser Glu Thr Asn Pro Phe Gln Asp Asn Ser His Glv Thr His Val Ala	50 60 Asp
549 GCA AGC GGA GCC AGC ATG GTT CCT TCT TCT GAA ACA AAT CCT TTC CAA GAC AAC AAC TCT CAC GGA ACT CAC GTT GCC	
Gly Thr Val Ala Ala Leu Asn Ser Ile Gly Val Leu Gly Val Ala Pro Ser Ala Ser Leu Tvr Ala Val Lvs	70 80 Ser Ala 90
624 GGC ACA GTT GCG GCT CTT AAT AAC TCA ATC GGT GTA TTA GGC GTT GCG CCA AGC GCA TCA CTT TAC GCT GTA AAA	
Val Leu Gly Ala Asp Gly Ser Gly Gln Tyr Ser Trp Ile Ile Asn Gly Ile Glu Trp Ala Ile Ala Asn Asn Met	110
699 GTT CTC GGT GCT GAC GGT TCC GGC CAA TAC AGC TGG ATC ATT AAC GGA ATC GAG TGG GCG ATC GCA AAC AAT ATG	
Asp Ala 100	120 130 140
774 Val Ile Asn Met Ser Leu Gly Gly Pro Ser Gly Ser Ala Ala Leu Lvs Ala Ala Val Asp Lvs Ala Val Ala	
774 GAC GTT ATT AAC ATG AGC CTC GGC GGA CCT TCT GGT TCT GCT GCT TTA AAA GCG GCA GTT GAT AAA GCC GTT GCA	
Ser Gly Val Val Val Ala Ala Ala Gly Asn Glu Gly Thr Ser Gly Ser Ser Thr Val Gly Tvr Pro Glv	150 160
849 TCC GGC GTC GTA GTC GTT GCG GCA GCC GGT AAC GAA GGC ACT TCC GGC AGC TCA AGC ACA GTG GGC TAC CCT GGT	

FIG-1B

170
Lvs Tyr Pro Ser Val Ile Ala Val Glv Ala Val Asp Ser Ser Asn Gln Arp Ala Ser Phe Ser Ser Vsl Glv Pro
924 AAA TAC CCT TCT GTC ATT GCA GTA GGC GCT GGT GAC AGC AGC AAC AAC GCA TCT TTC TCA AGC GTA GGA CCT

180
190
200
Glu Leu Asp Val Met Ala Pro Gly Val Ser Ile Gln Ser Thr Leu Pro Glv Asn Lvs Tyr Gly Ala Tyr Asn Glv
999 GAG CTT GAT GTC ATG GCA CCT GGC GTA TCT ATC CAA AGC AGC CTT CCT GGA AAC AAA TAC GGG GCG TAC AAC GGT

210
220
230
Thr Ser Met Ala Ser Pro His Val Ala Gly Aal Ala Ala Leu Ile Leu Ser Lvs His Pro Asn Trp Thr Asn Thr
1074 ACG TCA ATG GCA TCT CCG CAC GGT GCC GGA GCG GCT GCT TTG ATT CTT TCT AAG CAC CCG AAC TGG ACA AAC ACT

240
250 Gln
Gln Val Aro Ser Ser Leu Glu Asn Thr Thr Thr Lvs Leu Gly Asp Ser Phe Tyr Tyr Glv Lvs Glv Leu Ile Asn
1149 CAA GTC CGC AGC AGT TTA GAA AAC ACC ACT ACA AAA CTT GGT GAT TCT TTG TAC TAT GGA AAA GGG CTG ATC AAC

260
270
275
Val Gln Ala Ala Ala Gln DC
1224 GTA CAA GCG GCA GCT CAG TAA AACATAAAACCGGCTTGCCCGGGTTTTATTATTTTCTCTCCGCATGTTCAATCCGCTCC

TERM

1316 ATAATCGACGGATGGCTCCCTCTGAAAATTTTAACGAGAAACGGGGTTGACCCGGCTCAGTCCCGTAACGGCCAACTCCTGAAACGTCATCGCCG

1416 CTTCCTGGTTCCGGTCAGCTCAATGCCATAACGGTCGGCGGCGTTTTCTGTATACGGGAGACGGCATTTCGTAATCGGATC

FIG.-1C

CONSERVED RESIDUES IN SUBTILISINS FROM
BACILLUS AMYLOLIQUEFACIENS

```

1      10      20
A Q S V P . G . . . . A P A . H . . G

21      30      40
. T G S . V K V A V . D . G . . . . H P

41      50      60
D L . . . G G A S . V P . . . . . Q D

61      70      80
. N . H G T H V A G T . A A L N N S I G

81      90      100
V L G V A P S A . L Y A V K V L G A . G

101     110     120
S G . . S . L . . G . E W A . N . . .

121     130     140
V . N . S L G . P S . S . . . . . A .

141     150     160
. . . . . G V . V V A A . G N . G . .

161     170     180
. . . . . Y P . . Y . . . . A V G A .

181     190     200
D . . N . . A S F S . . G . . L D . . A

201     210     220
P G V . . Q S T . P G . . Y . . . N G T

221     230     240
S M A . P H V A G A A A L . . . K . .

241     250     260
W . . . Q . R . . L . N T . . . L G .

261     270
. . Y G . G L . N . . A A . .

```

FIG. 2

Comparison of subtilisin sequences from:

- B.amyloliquefaciens*
- B.subtilis*
- B.licheniformis*
- B.lentus*

01	A	A	A	A						10	Q	I	K	A	P	A	L	H	S	Q	G	Y	T	G	S	N	V	K	V	A	V	I	D	S	G	I	D	S	S	H	P	
	A	Q	S	V	P	Y	G	V	S	Q	I	K	A	P	A	L	H	S	Q	G	Y	T	G	S	N	V	K	V	A	V	I	D	S	G	I	D	S	S	H	P		
	A	Q	T	V	P	Y	G	I	S	Q	I	K	A	P	A	L	H	S	Q	G	Y	T	G	S	N	V	K	V	A	V	I	D	S	G	I	D	S	S	H	P		
	A	Q	S	V	P	W	G	I	S	R	V	Q	A	P	A	A	H	N	R	G	L	T	G	S	G	V	K	V	A	V	I	D	S	G	I	S	T	*	H	P		
41	D	L	K	V	A	G	G	A	S	M	V	P	S	E	T	N	P	F	Q	D	N	N	S	H	G	T	H	V	A	G	T	V	A	A	L	N	N	S	I	G		
	D	L	N	V	R	G	G	A	S	F	V	P	S	E	T	N	P	Y	Q	D	G	N	S	H	G	T	H	V	A	G	T	V	A	A	L	N	N	S	I	G		
	D	L	N	V	V	G	G	A	S	F	V	P	S	E	T	N	P	*	T	D	G	N	S	H	G	T	H	V	A	G	T	V	A	A	L	N	N	S	I	G		
	D	L	N	I	R	G	G	A	S	F	V	P	S	E	T	Q	D	G	N	G	N	G	H	G	T	H	V	A	G	T	V	A	A	L	N	N	S	I	G			
81	V	L	G	V	A	P	S	A	S	L	Y	A	V	K	V	L	G	A	D	G	S	G	Q	Y	S	W	I	I	N	G	I	E	W	A	I	A	N	N	M	D		
	V	L	G	V	S	P	S	A	S	L	Y	A	V	K	V	L	D	S	T	G	S	G	Q	Y	S	W	I	I	N	G	I	E	W	A	I	A	N	N	M	D		
	V	L	G	V	A	P	S	V	S	L	Y	A	V	K	V	L	N	S	S	G	S	G	S	Y	S	G	I	I	V	S	G	I	E	W	A	I	A	N	N	M	D	
	V	L	G	V	A	P	S	A	E	L	Y	A	V	K	V	L	G	A	S	G	S	G	S	V	S	I	A	Q	G	L	E	W	A	A	T	N	G	M	H			
121	V	I	N	M	S	L	G	G	P	S	G	S	A	A	L	K	A	A	V	D	K	A	V	A	S	G	V	V	V	V	A	A	A	A	A	A	S	G	S	G		
	V	I	N	M	S	L	G	G	P	T	G	S	T	A	L	K	T	V	V	D	K	A	V	S	S	G	I	V	V	V	V	A	A	A	A	A	A	S	G	S	G	
	V	I	N	M	S	L	G	G	A	S	G	S	T	A	M	K	Q	A	V	D	N	A	Y	A	R	G	V	V	V	V	V	A	A	A	A	A	A	S	G	S	G	
	V	A	N	L	S	L	G	S	P	S	P	S	A	T	L	E	Q	A	V	N	S	A	T	S	R	G	V	L	V	V	V	V	A	A	A	A	A	A	S	G	S	G

FIG 3/A

FIG 3/B

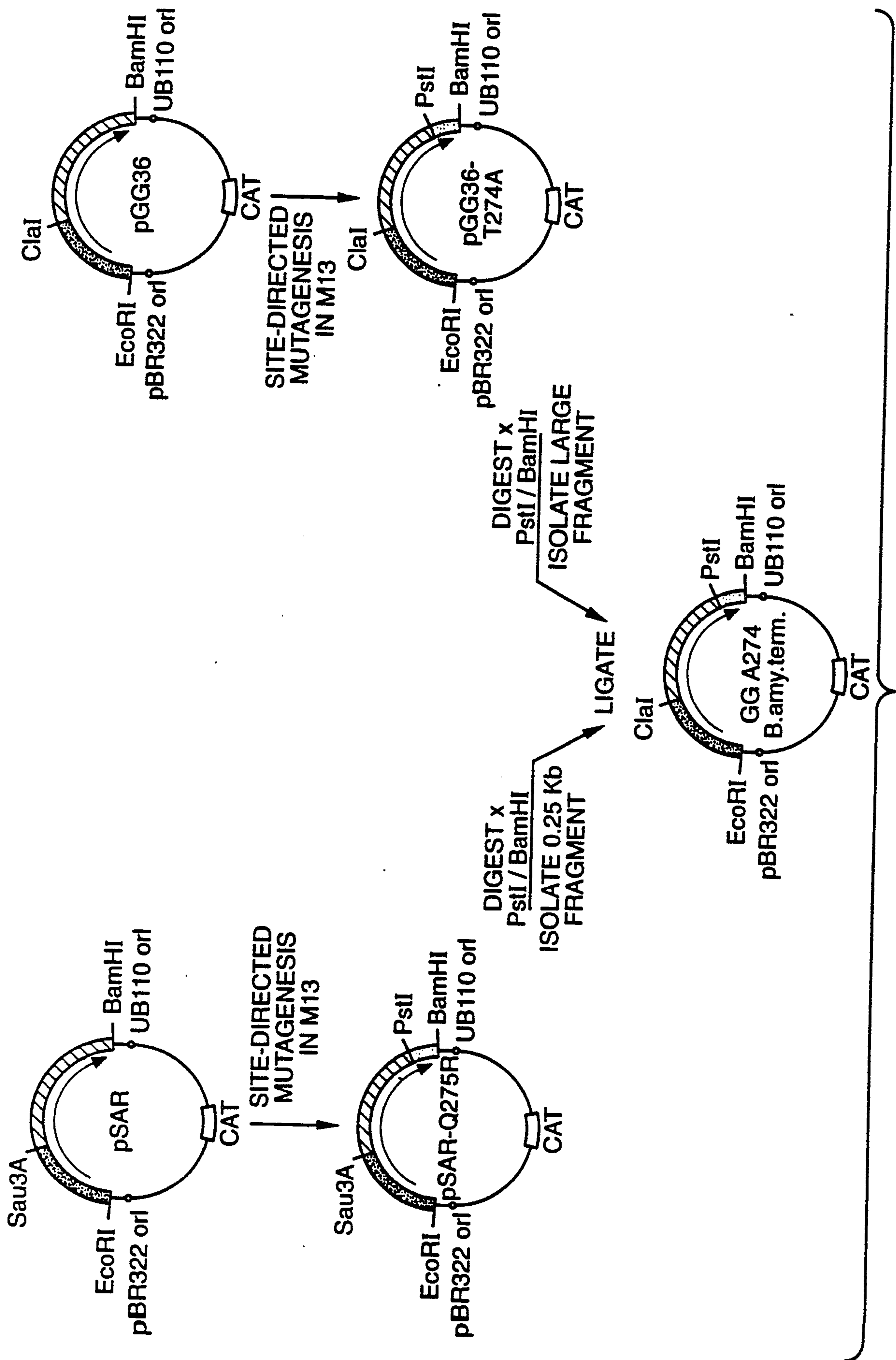


FIG. 4

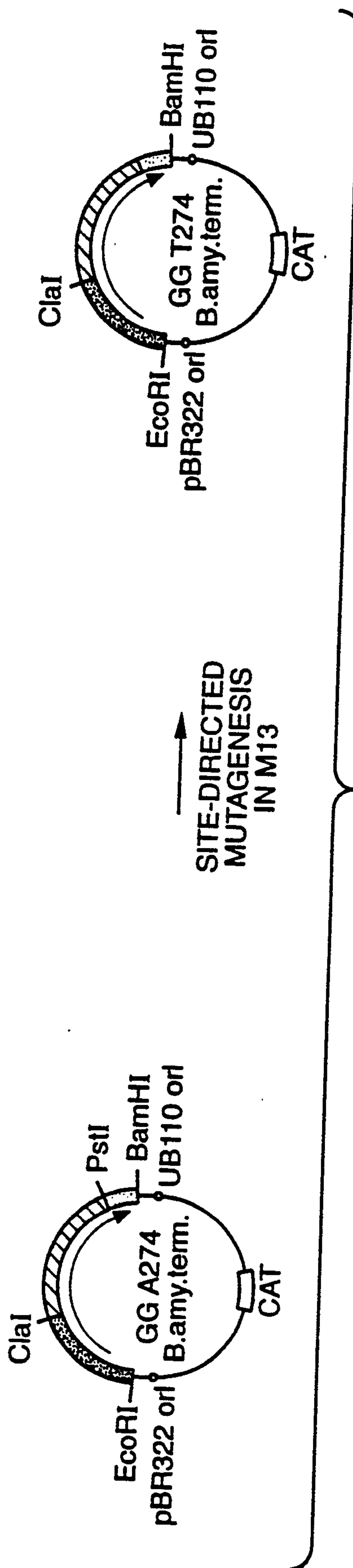


FIG. 5

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10 30 50
ATGAAGAAACCGTTGGGGAAAATTGTCGCAAGCACCGCACTACTCATTCTGTTGCTTTT
MetLysLysProLeuGlyLysIleValAlaSerThrAlaLeuLeuIleSerValAlaPhe

70 90 110
AGTTCATCGATCGCATCGGCTGCTGAAGAAGCAAAAGAAAAATATTTAATTGGCTTTAAT
SerSerSerIleAlaSerAlaAlaGluGluAlaLysGluLysTyrLeuIleGlyPheAsn

130 150 170
GAGCAGGAAGCTGTCAGTGAGTTTGTAGAACAAGTAGAGGCAAATGACGAGGTCGCCATT
GluGlnGluAlaValSerGluPheValGluGlnValGluAlaAsnAspGluValAlaIle

190 210 230
CTCTCTGAGGAAGAGGAAGTCGAAATTGAATTGCTTCATGAATTTGAAACGATTCCTGTT
LeuSerGluGluGluGluValGluIleGluLeuLeuHisGluPheGluThrIleProVal

250 270 290
TTATCCGTTGAGTTAAGCCCAGAAGATGTGGACGCGCTTGAACCTCGATCCAGCGATTCT
LeuSerValGluLeuSerProGluAspValAspAlaLeuGluLeuAspProAlaIleSer

310 330 350
TATATTGAAGAGGATGCAGAAGTAACGACAATGGCGCAATCAGTGCCATGGGGAATTAGC
TyrIleGluGluAspAlaGluValThrThrMetAlaGlnSerValProTrpGlyIleSer

370 390 410
CGTGTGCAAGCCCCAGCTGCCCATAACCGTGGATTGACAGGTTCTGGTGTAAAAGTTGCT
ArgValGlnAlaProAlaAlaHisAsnArgGlyLeuThrGlySerGlyValLysValAla

430 450 470
GTCCTCGATACAGGTATTTCCACTCATCCAGACTTAAATATTCGTGGTGGCGCTAGCTTT
ValLeuAspThrGlyIleSerThrHisProAspLeuAsnIleArgGlyGlyAlaSerPhe

490 510 530
GTACCAGGGGAACCATCCACTCAAGATGGGAATGGGCATGGCACGCATGTGGCCGGGACG
ValProGlyGluProSerThrGlnAspGlyAsnGlyHisGlyThrHisValAlaGlyThr

550 570 590
ATTGCTGCTTTAAACAATTGCGATTGGCGTTCTTGGCGTAGCGCCGAGCGCGGAACCTATAC
IleAlaAlaLeuAsnAsnSerIleGlyValLeuGlyValAlaProSerAlaGluLeuTyr

610 630 650
GCTGTAAAGTATTAGGGGCGAGCGGTTTCAGGTTTCGGTCAGCTCGATTGCCCAAGGATTG
AlaValLysValLeuGlyAlaSerGlySerGlySerValSerSerIleAlaGlnGlyLeu

670 690 710
GAATGGGCAGGGAACAATGGCATGCACGTTGCTAATTTGAGTTTAGGAAGCCCTTCGCCA
GluTrpAlaGlyAsnAsnGlyMetHisValAlaAlaAsnLeuSerLeuGlySerProSerPro

730 750 770
AGTGCCCACTTGAGCAAGCTGTTAATAGCGCGACTTCTAGAGGCGTTCTTGTGTAGCG
SerAlaThrLeuGluGlnAlaValAsnSerAlaThrSerArgGlyValLeuValValAla

790 810 830
GCATCTGGGAATTCAGGTGCAGGCTCAATCAGCTATCCGGCCCGTTATGCGAACGCAATG
AlaSerGlyAsnSerGlyAlaGlySerIleSerTyrProAlaArgTyrAlaAsnAlaMet

850 870 890
GCAGTCGGAGCTACTGACCAAACAACAACCGCGCCAGCTTTTCACAGTATGGCGCAGGG
AlaValGlyAlaThrAspGlnAsnAsnAsnArgAlaSerPheSerGlnTyrGlyAlaGly

FIG. 6A

910 930 950
CTTGACATTGTCGCACCAGGTGTAAACGTGCAGAGCACATACCCAGGTTCAACGTATGCC
LeuAspIleValAlaProGlyValAsnValGlnSerThrTyrProGlySerThrTyrAla

970 990 1010
AGCTTAAACGGTACATCGATGGCTACTCCTCATGTTGCAGGTGCAGCAGCCCTTGTTAA
SerLeuAsnGlyThrSerMetAlaThrProHisValAlaGlyAlaAlaAlaLeuValLys

1030 1050 1070
CAAAGAACCCATCTTGGTCCAATGTACAAATCCGCAATCATCTAAAGAATACGGCAACG
GlnLysAsnProSerTrpSerAsnValGlnIleArgAsnHisLeuLysAsnThrAlaThr

1090 1110 1130
AGCTTAGGAAGCACGAACTTGTATGGAAGCGGACTTGTCAATGCAGAAGCGGCAACACGC
SerLeuGlySerThrAsnLeuTyrGlySerGlyLeuValAsnAlaGluAlaAlaThrArg

FIG. 6B

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10 30 50
ATGAAGAAACCGTTGGGGAAAATTGTGCGCAAGCACCGCACTACTCATTCTGTGCTTTT
MetLysLysProLeuGlyLysIleValAlaSerThrAlaLeuLeuIleSerValAlaPhe

70 90 110
AGTTCATCGATCGCATCGGCTGCTGAAGAAGCAAAAGAAAAATATTTAATTGGCTTTAAT
SerSerSerIleAlaSerAlaAlaGluGluAlaLysGluLysTyrLeuIleGlyPheAsn

130 150 170
GAGCAGGAAGCTGTCAGTGAGTTTGTAGAACAGTAGAGGCAAATGACGAGGTCGCCATT
GluGlnGluAlaValSerGluPheValGluGlnValGluAlaAsnAspGluValAlaIle

190 210 230
CTCTCTGAGGAAGAGGAAGTCGAAATTGAATTGCTTCATGAATTTGAAACGATTCTGTT
LeuSerGluGluGluGluValGluIleGluLeuLeuHisGluPheGluThrIleProVal

250 270 290
TTATCCGTTGAGTTAAGCCCAGAAGATGTGGACCGCTTGAACCTCGATCCAGCGATTCT
LeuSerValGluLeuSerProGluAspValAspAlaLeuGluLeuAspProAlaIleSer

310 330 350
TATATTGAAGAGGATGCAGAAGTAACGACAATGGCGCAATCAGTGCCATGGGGAATTAGC
TyrIleGluGluAspAlaGluValThrThrMetAlaGlnSerValProTrpGlyIleSer

370 390 410
CGTGTGCAAGCCCCAGCTGCCCATACCGTGGATTGACAGGTTCTGGTGTAAGTTGCT
ArgValGlnAlaProAlaAlaHisAsnArgGlyLeuThrGlySerGlyValLysValAla

430 450 470
GTCCTCGATACAGGTATTTCCACTCATCCAGACTTAAATATTCGTGGTGGCGCTAGCTTT
ValLeuAspThrGlyIleSerThrHisProAspLeuAsnIleArgGlyGlyAlaSerPhe

490 510 530
GTACCAGGGGAACCATCCACTCAAGATGGGAATGGGCATGGCACGCATGTGGCCGGGACG
ValProGlyGluProSerThrGlnAspGlyAsnGlyHisGlyThrHisValAlaGlyThr

550 570 590
ATTGCTGCTTTAGACAACCTCGATTGGCGTTCTTGGCGTAGCGCCGAGCGCGGAACCTATAC
IleAlaAlaLeuAspAsnSerIleGlyValLeuGlyValAlaProSerAlaGluLeuTyr

610 630 650
GCTGTAAAGTATTAGGGGCGAGCGGTTTCAGGCGCCATCAGCTCGATTGCCCAAGGATTG
AlaValLysValLeuGlyAlaSerGlySerGlyAlaIleSerSerIleAlaGlnGlyLeu

670 690 710
GAATGGGCAGGGAACAATGGCATGCACGTTGCTAATTTGAGTTTAGGAAGCCCTTCGCCA
GluTrpAlaGlyAsnAsnGlyMetHisValAlaAsnLeuSerLeuGlySerProSerPro

730 750 770
AGTGCCACACTTGAGCAAGCTGTTAATAGCGCGACTTCTAGAGGCGTTCTTGTTGTAGCG
SerAlaThrLeuGluGlnAlaValAsnSerAlaThrSerArgGlyValLeuValValAla

790 810 830
GCATCTGGGAATTCAGGTGCAGGCTCAATCAGCTATCCGGCCCGTTATGCCAAGCAATG
AlaSerGlyAsnSerGlyAlaGlySerIleSerTyrProAlaArgTyrAlaAsnAlaMet

850 870 890
GCAGTCGGAGCTACTGACCAAACAACAACCGCGCCAGCTTTTCACAGTATGGCGCAGGG
AlaValGlyAlaThrAspGlnAsnAsnAsnArgAlaSerPheSerGlnTyrGlyAlaGly

FIG. 7A

CTTGACATTGTCGCACCAGGTGTAAACGTGCAGAGCACATACCCAGGTTCAACGTATGCC
LeuAspIleValAlaProGlyValAsnValGlnSerThrTyrProGlySerThrTyrAla
930 950
AGCTTAAACGGTACATCGATGGCTACTCCTCATGTTGCAGGTGCAGCAGCCCTTGTTAAA
SerLeuAsnGlyThrSerMetAlaThrProHisValAlaGlyAlaAlaAlaLeuValLys
970 990 1010
CAAAGAACCCATCTTGGTCCAATGTACAAATCCGCAATCATCTAAAGAATACGGCAACG
GlnLysAsnProSerTrpSerAsnValGlnIleArgAsnHisLeuLysAsnThrAlaThr
1030 1050 1070
AGCTTAGGAAGCACGAACTTGTATGGAAGCGGACTTGTCAATGCAGAAGCGGCAACACGC
SerLeuGlySerThrAsnLeuTyrGlySerGlyLeuValAsnAlaGluAlaAlaThrArg
1090 1110 1130

FIG. 7B

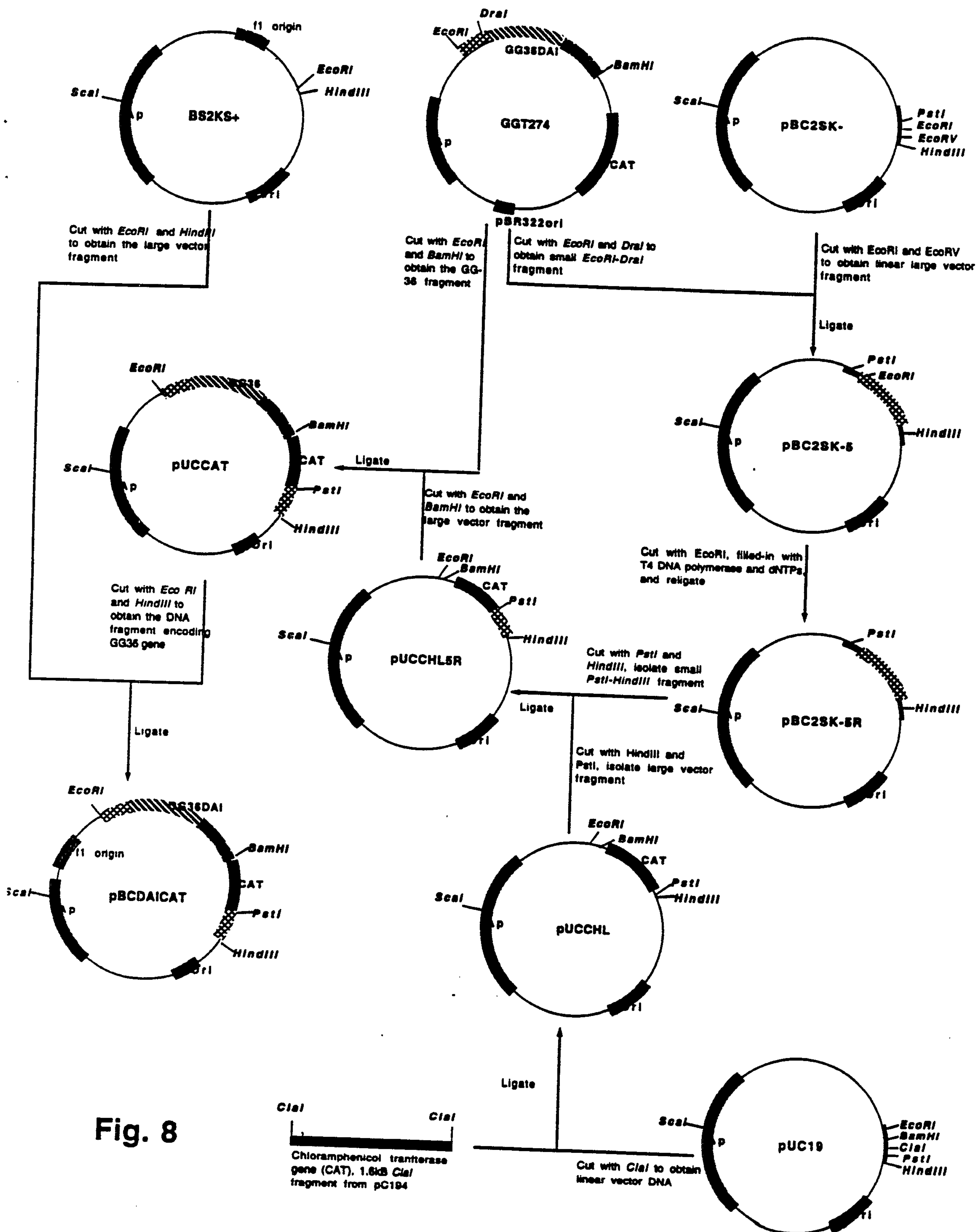


Fig. 8

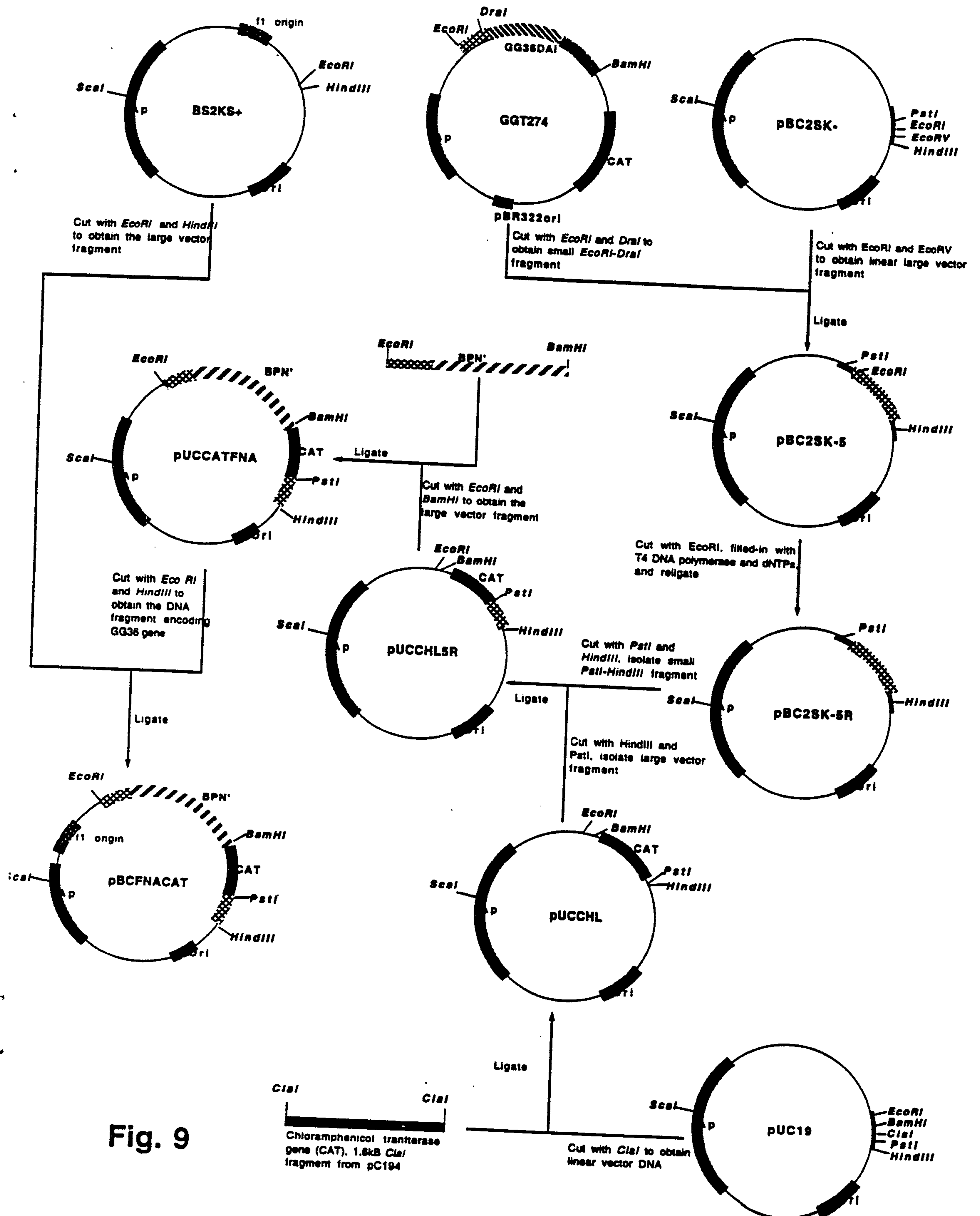


Fig. 9