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[54] **METHOD FOR CONJUGATING TO POLYAMINO COMPOUNDS EMPLOYING HALOACYL GROUPS AND COMPOSITIONS PREPARED THEREBY**

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[52] **U.S. Cl.** ..... **435/188;** 435/177; 435/7; 260/112 R; 424/12; 260/112 B; 260/112.5 R; 260/112.7
[58] **Field of Search** ..... 195/63, 68, DIG. 11; 260/112 R; 424/12; 435/7, 177, 174, 188

[56] **References Cited**

U.S. PATENT DOCUMENTS

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[57] **ABSTRACT**

Methods and compositions are provided for conjugating a wide variety of compounds, particularly polyfunctional compounds, having a mercapto group, either naturally present or synthetically introduced, to a polyamino compound, particularly a polypeptide (including proteins). The method employs a haloalkylcarbonyl compound, which is conjugated to one or more of the amino groups under mild acylating conditions. This is followed by combining the acylated polyamino compound with a mercapto containing compound, whereby the halogen is displaced by the sulfur of the mercapto group to form a stable thioether linkage. The resulting conjugates, depending on the compounds involved, can find uses in immunoassays, as hapten-antigen conjugates for the production of antibodies, and as ligand analog enzyme conjugates for use as reagents in controlling the distribution of substitution of a mercapto compound to a polyamino compound.

14 Claims, No Drawings

METHOD FOR CONJUGATING TO POLYAMINO COMPOUNDS EMPLOYING HALOACYL GROUPS AND COMPOSITIONS PREPARED THEREBY

BACKGROUND OF THE INVENTION

1. Field of the Invention

There is a continuously expanding interest in being able to conjugate, frequently selectively, a compound to another compound which is polyfunctional. Where both compounds are polyfunctional, the problem of conjugation is exacerbated, if one does not wish all of the functional groups to participate in the reaction. Also, the need to functionalize a polyfunctional compound for conjugating to a polyamino compound will frequently require the introduction of protective groups for alcohols and amines, so that the reactive functionality does not polymerize the compound to be conjugated.

One area of particular interest is the conjugation of a wide variety of haptens and antigens to polypeptides (including proteins), particularly where conjugation is to occur at available amino groups. In preparing antibodies for use in competitive protein binding assays, where the analyte of interest is haptenic, it is generally necessary to conjugate the hapten to an antigen, normally a protein. Where the analyte has a plurality of functionalities which can react with the active functionality to be used for conjugating to the polypeptide, it becomes necessary to introduce removable protective groups to prevent polymerization of the analyte. After conjugation, it is usually difficult to efficiently remove the protective groups.

Where the conjugate is to be used for the preparation of antibodies, the resulting antibodies not only recognize the analyte of interest, but the analyte having the protective groups. This may result in substantially reducing the specificity of the antibody composition for the analyte of interest.

One class of competitive protein binding assays involves the use of enzymes as a label. It is necessary to conjugate the analyte of interest to the enzyme. It is desirable that certain reactive site positions on the enzyme be preferentially conjugated as compared to other reactive site positions. A method which would provide the ability to discriminate to even a partial degree is desirable.

In addition, to have an enzyme which has been modified, whereby the same sites will be conjugated to analytes, regardless of the particular analyte, can provide a number of advantages. For example, in one of the assays which employs an enzyme as a label, it is desirable that the enzyme retain a substantial proportion of its initial activity after conjugation, but when antibody or other receptor is bound to the analytes conjugated to the enzyme, the enzymatic activity is substantially reduced. The fewer the analytes necessary to conjugate to the antibody to obtain the desired degree of reduction is enzymatic activity upon the binding of antibody or other receptor to the conjugated analyte, the more sensitive will be the assay response.

In addition, where a universal reagent can be employed for conjugation, greatly increased experience can be obtained in the handling of the compounds, the reacting of the compounds, as well as the subsequent handling and treatment after conjugation. This can provide for great efficiencies in synthesizing and subsequent formulation.

DESCRIPTION OF THE PRIOR ART

Kato, et al, Eur. J. Biochem. 62, 285 (1976), discloses the use of maleic anhydride with a polyamino compound to provide one or more maleimide groups, followed by the addition of a compound with a mercapto group to add to the double bond of the maleimide. See also, Lee and Kenny, Clinical Chem. 21,967(1975).

SUMMARY OF THE INVENTION

Methods and compositions are provided for combining a polyfunctional compound having a plurality of reactive primary and/or secondary amino functionalities with a second compound having a mercapto functionality, usually polyfunctional, having functionalities reactive to acyl groups e.g. hydroxylic and amino. The polyamino compound is initially reacted with a linking compound having an active halogen or pseudohalogen and a non-oxo carbonyl functionality for reacting with at least one of the amino functionalities. The mercapto compound is then added to the halo or pseudohalo containing polyamino compound for substitution of the halo groups to provide a thioether linked conjugate of the mercapto compound with the polyamino compound.

The method finds particular use in the preparation of polypeptide and protein conjugates for preparing antigens, enzyme conjugates for immunoassays, fluorescent labeling of polypeptides and proteins and the like. By employing the subject method, one can obtain a consistent pattern of substitution, the conjugation can be carried out under extremely mild conditions and some control of the positions of substitution can be achieved.

DESCRIPTION OF THE SPECIFIC EMBODIMENTS

The method of the subject invention involves the labeling as a first step of a poly(amino acid) compound, normally a polypeptide or protein, with a compound having an α-halo or α-pseudohaloalkylcarbonyl functionality and a non-oxo carbonyl functionality (including the nitrogen analog, imido, and sulfur analog, thiono), which may be the same or different from the carbonyl of the α-halo or α-pseudohaloalkylcarbonyl functionality. The reaction is carried out in a normally aqueous medium under mild pH conditions, generally at 9.5 or below, so as to form an amide (including the nitrogen and sulfur analogs, imidine and thioamide respectively). The product may then be purified under conventional conditions and the halo or pseudohalo substituted by a mercaptan under mild conditions in an aqueous solution at moderate pH, normally basic pH. The product may then be worked up and isolated.

The compounds prepared in accordance with this invention have many uses, for example haptens or antigens may be conjugated to labels, such as fluorescers and enzymes, and the resulting compounds employed in immunoassays for the determination of such haptens and antigens. In addition, haptens may be conjugated to antigens to be used for the production of antibodies, which may also serve as reagents in immunoassays.

The subject method provides a means for preparing derivatives of polyamino compounds, which have a limited number of active sites. The distribution of these active sites may be retained substantially constant, so that when conjugating haptens and antigens to the poly-

amino compounds, substitution will be relatively uniform, regardless of the particular compound which is conjugated.

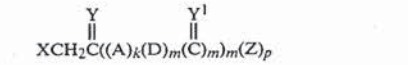
In addition, it will normally be found that due to the position of the halo substituent on the polyamino compound, the halo compounds may have varying activities. One can then distinguish between the varying activities, by employing two different mercaptan reagents, the first reagent being added in a sufficient amount to react with all or substantially all of the more reactive halogen. In this manner, the mercaptan compound of interest may be directed either to the more or less reactive sites. Also, the subject method provides for synthetic convenience, for so far as the polyamino compound, the same compound may be repetitively prepared, regardless of the compound to which it is to be conjugated.

MATERIALS

The materials which are employed in the subject invention are the active halogen or pseudohalogen compound, the polyamino polyfunctional compound to which the halo or pseudohalo compound is conjugated, and the mercaptan which is employed for substitution on the halogen or pseudohalogen.

The first compounds to be considered will be the halo or pseudohalo compounds. These compounds will normally be of from 2 to 20, more usually of from 2 to 16 carbon atoms, and preferably of from about 2 to 12 carbon atoms. Other than the halo or pseudohalo group, the compound will normally have at least two heteroatoms, and may have as many as 20 heteroatoms, more usually having from about 2 to 12 heteroatoms, and preferably from about 2 to 8 heteroatoms. The heteroatoms will normally be oxygen, nitrogen and sulfur or any appropriate counterion for a charged species. Oxygen will normally be present as in nitro, oxo or ether (an ester includes oxo and ether oxygens); nitrogen will be present as in nitro, amido, or bonded solely to carbon, e.g. tertiary amine; and sulfur will be present as thiono or thioether. The compounds will of necessity include aliphatic groups, but may also include alicyclic, aromatic, and heterocyclic groups.

For the most part, the compounds used for conjugation to the amino functionalized compounds will have the following formula:



wherein:

X—Cl,Br,CH_3SO_3 (mesylate), preferably Br;

Y and Y^1—O, NH, S, preferably O;

A—NH, O, preferably NH;

D—chain of from 1 to 9, usually 1 to 6 atoms in the chain, having a total number of atoms other than hydrogen of from 1 to 12, usually 1 to 10, preferably 1 to 6, which may be C, O, N and S, usually C, O and N, wherein: O is present as oxo or ether, particularly non-oxo carbonyl; N is present as amido or bonded solely to carbon and may be present as terminal nitrogen doubly bonded to (CY^1) where Y^1 is S to form isothiocyanate; and S is present as thiono or thioether; preferably hydrocarbon to form a hydrocarbylene group which may be aliphatic, alicyclic, aromatic or combinations thereof, preferably aliphatic, which may be aliphatically saturated or unsaturated having from 0 to 1 site of unsaturation i.e. ethylenic and acetylenic, preferably saturated and may be straight or branched chain, preferably straight chain;

Z - OV or OCO-alkyl, wherein alkyl is of from 1 to 6, usually 1 to 4 carbon atoms and V is hydrogen, p-nitrophenyl, N-oxy succinimide, or when Y is NH and m is zero, or Y^1 is NH and m is 1, alkyl of from 1 to 6 carbon atoms.

k, m and p—zero or 1, wherein p is zero when D and (CY^1) form an isothiocyanate group

The preferred halo compounds of this invention will have the following formula:

wherein:

Y^2 and Y^3—O, NH, preferably O

D^1—alkylene of from 1 to 8, usually 1 to 4 carbon atoms

Z^1—the same as Z, usually OH or N-oxy succinimide

Illustrative compounds include

N-bromoacetyl glycine, N-bromoacetyl valine, N-bromoacetyl 4-aminobutyric acid, N-bromoacetyl 3-aminopropionic acid, p-chloroacetylbenzoic acid, p-bromoacetylphenylacetic acid, N-bromoacetyl 4-aminocrotonic acid, their p-nitrophenyl esters, their N-succinimidyl esters, p-chloroacetylphenyl isothiocyanate, and methyl N-bromoacetyl glycinimidate.

The next group of compounds to be considered, are the polyamino functionalized compounds, which are primarily polypeptides and proteins, but may also include polyglucosamines and nucleic acids. These compounds may be included in combinations or assemblages which include bacteria, viruses, chromosomes, genes, mitochondria, nuclei, cell membranes and the like.

For the most part, the compounds will have a molecular weight of at least about 5,000, more usually at least about 10,000. In the poly(amino acid) category (includes polypeptides and proteins), the poly(amino acids) of interest will generally be from about 5,000 to 5,000,000 molecular weight, more usually from about 20,000 to 1,000,000 molecular weight. In this category, hormones of interest will generally range from about 5,000 to 60,000 molecular weight. Enzymes of interest will generally range from about 10,000 to 300,000 molecular weight. Immunoglobulins and portions thereof e.g. Fab fragments and Bence-Jones proteins, will generally range from about 23,000 to 1,000,000, with the immunoglobulins generally ranging from 150,000 to 1,000,000.

The wide variety of proteins may be considered as to the family of proteins having similar structural features, proteins having particular biological functions, proteins related to specific microorganisms, particularly disease causing microorganisms, etc.

The following are classes of proteins related by structure:

protamines
histones
albumins
globulins
scleroproteins
Phosphoproteins
mucoproteins
chromoproteins
lipoproteins

nucleoproteins
glycoproteins
unclassified proteins, e.g. somatotropin, prolactin, insulin, pepsin

A number of proteins found in the human plasma are important clinically and include:

Prealbumin
Albumin
α_1-Lipoprotein
α_1-Acid glycoprotein
α_1-Antitrypsin
α_1Glycoprotein
Transcortin
4.6S-Postalbumin
Tryptophan-poor
α_1-glycoprotein
α_1X-Glycoprotein
Thyroxin-binding globulin
Inter-α-trypsin-inhibitor
Gc-globulin
(Gc 1-1)
(Gc 2-1)
(Gc 2-2)
Haptoglobin
(Hp 1-1)
(Hp 2-1)
(Hp 2-2)
Ceruloplasmin
Cholinesterase
α_2-Lipoprotein(s)
α_2-Macroglobulin
α_2-HS-glycoprotein
Zn-α_2-glycoprotein
α_2-Neuramino-glycoprotein
Erythropoietin
β-lipoprotein
Transferrin
Hemopexin
Fibrinogen
Plasminogen
β_2-glycoprotein I
β_2-glycoprotein II
Immunoglobulin G
(IgG) or γG-globulin
Mol. formula:
$\gamma_2\kappa_2$ or $\gamma_2\lambda_2$
Immunoglobulin A (IgA) or γA-globulin
Mol. formula:
$(\alpha_2\kappa_2)^n$ or $(\alpha_2\lambda_2)^n$
Immunoglobulin M
(IgM) or γM-globulin
Mol. formula:
$(\mu_2\kappa_2)^5$ or $(\mu_2\lambda_2)^5$
Immunoglobulin D(IgD) or γD-Globulin (γD)
Mol. formula:
$(\delta_2\kappa_2)$ or $(\delta_2\lambda_2)$
Immunoglobulin E (IgE) or γE-Globulin (γE)
Mol. formula:
$(\epsilon_2\kappa_2)$ or $(\epsilon_2\lambda_2)$
Free K and γ light chains
Complement factors:
C'1
C'1q
C'1r
C'1s
C'2
C'3
β_1A
α_2 D
C'4
C'5
C'6
C'7
C'8
C'9

Important blood clotting factors include:

BLOOD CLOTTING FACTORS

International designation	Name
I	Fibrinogen
II	Prothrombin
IIa	Thrombin
III	Tissue thromboplastin
V and VI	Proaccelerin, accelerator globulin
VII	Proconvertin
VIII	Antihemophilic globulin (AHG)
IX	Christmas factor, plasma thromboplastin component (PTC)
X	Stuart-Prower factor, autoprothrombin III
XI	Plasma thromboplastin antecedent (PTA)
XII	Hagemann factor
XIII	Fibrin-stabilizing factor

Important protein hormones include:

Peptide and Protein Hormones
Parathyroid hormone (parathromone)
Thyrocalcitonin
Insulin
Glucagon
Relaxin
Erythropoietin
Melanotropin (melanocyte-stimulating hormone; intermedin)
Somatotropin (growth hormone)
Corticotropin (adrenocorticotropic hormone)
Thyrotropin
Follicle-stimulating hormone
Luteinizing hormone (interstitial cell-stimulating hormone)
Luteomammotropic hormone (luteotropin, prolactin)
Gonadotropin (chorionic gonadotropin)
Tissue Hormones
Secretin
Gastrin
Angiotensin I and II
Bradykinin
Human placental lactogen
Peptide Hormones from the Neurohypophysis
Oxytocin
Vasopressin
Releasing factors (RF)
CRF, LRF, TRF, Somatotropin-RF, GRF, FSH-RF, PIF, MIF

The microorganisms which are assayed may be intact, lysed, ground or otherwise fragmented, and the resulting composition or portion, e.g. by extraction, assayed. Microorganisms of interest include:

Corynebacteria
Corynebacterium diptheriae
Pneumococci
Diplococcus pneumoniae
Streptococci
Streptococcus pyogenes
Streptococcus salivarus

Staphylococci
Staphylococcus aureus
Staphylococcus albus
Neisseriae
Neisseria meningitidis
Neisseria gonorrheae

Enterobacteriaciae	
Escherichia coli, *Aerobacter aerogenes*, *Klebsiella pneumoniae*	The coliform bacteria
Salmonella typhosa, *Salmonella choleraesuis*, *Salmonella typhimurium*	The Salmonellae
Shigella dysenteriae, *Shigella schmitzii*, *Shigella arabinotarda*, *Shigella flexneri*, *Shigella boydii*, *Shigella Sonnei*	The Shigellae
Other enteric bacilli	
Proteus vulgaris, *Proteus mirabilis*, *Proteus morgani*	Proteus species
Pseudomonas aeruginosa	
Alcaligenes faecalis	
Vibrio cholerae	

Hemophilus-Bordetella group
Hemophilus influenzae,
H. ducreyi
H. hemophilus
H. aegypticus
H. paraiufluenzae
Bordetella pertussis
Pasteurellae
Pasteurella pestis
Pasteurella tulareusis
Brucellae
Brucella melitensis
Brucella abortus
Brucella suis
Aerobic Spore-forming Bacilli
Bacillus anthracis
Bacillus subtilis
Bacillus megaterium
Bacillus cereus
Anaerobic Spore-forming Bacilli
Clostridium botulinum
Clostridium tetani
Clostridium perfringens
Clostridium novyi
Clostridium septicum
Clostridium histolyticum
Clostridium tertium
Clostridium bifermentans
Clostridium sporogenes
Mycobacteria
Mycobacterium tuberculosis hominis
Mycobacterium bovis
Mycobacterium avium
Mycobacterium leprae
Mycobacterium paratuberculosis
Actinomycetes (fungus-like bacteria)
Actinomyces israelii
Actinomyces bovis
Actinomyces naeslundii
Nocardia asteroides
Nocardia brasiliensis
The Spirochetes
Treponema pallidum
Treponema pertenue
Treponema carateum
Borrelia recurrentis
Leptospira icterohemorrhagiae
Leptospira canicola
Spirillum minus
Streptobacillus moniliformis
Mycoplasmas
Mycoplasma pneumoniae
Other pathogens
Listeria monocytogenes
Erysipelothrix rhusiopathiae
Streptobacillus moniliformis
Donvania granulomatis
Bartonella bacilliformis
Rickettsiae (bacteria-like parasites)
Rickettsia prowazekii
Rickettsia mooseri
Rickettsia rickettsii
Rickettsia conori
Rickettsia australis
Rickettsia sibiricus
Rickettsia akari
Rickettsia tsutsugamushi
Rickettsia burnetii
Rickettsia quintana
Chlamydia (unclassifiable parasites bacterial/viral)
Chlamydia agents (naming uncertain)
Fungi
Cryptococcus neoformans
Blastomyces dermatidis
Histoplasma capsulatum
Coccidioides immitis
Paracoccidioides brasiliensis
Candida albicans
Aspergillus fumigatus
Mucor corymbifer (Absidia corymbifera)

Rhizopus oryzae, *Rhizopus arrhizus*, *Rhizopus nigricans*	Phycomycetes

Sporotrichum schenkii
Fonsecaea pedrosoi
Fonsecaea compacta
Fonsecaea dermatitidis
Cladosporium carrionii
Phialophora verrucosa
Aspergillus nidulans
Madurella mycetomi
Madurella grisea
Allescheria boydii
Phialosphora jeanselmei
Microsporum gypseum
Trichophyton mentagrophytes
Keratinomyces ajelloi
Microsporum canis
Trichophyton rubrum
Microsporum andouini
Viruses
Adenoviruses
Herpes viruses
Herpes simplex
Varicella (Chicken pox)
Herpes Zoster (Shingles)
Virus B

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