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Preparation of enzyme - analyte conjugates containing linker arms

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Preparation of Enzyme - Analyte Conjugates Containing Linker Arms

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Title of Thesis ***Preparation of Enzyme - Analyte Conjugates Containing Linker Arms***

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Abstract

In this study, we are examining the effect of the length and polarity of peptide linker arms on the performance of enzyme - analyte conjugates in a reagent-limited heterogeneous immunoassay which utilizes a solid phase format. In our model system, our goal is to attach benzoylecgonine, the primary urinary metabolite of cocaine, to glucose oxidase by a series of peptide linker arms.

Peptide linker arms were synthesized using the fluorenylmethoxycarbonyl (Fmoc) solid phase peptide synthesis strategy described by Stewart and Young (1). We attached the BEC to the exposed amino - terminal end of the peptide linker arm by the free carboxyl group of BEC prior to removal of the peptide from the solid phase to avoid the requirement for isolation of the peptide before reacting with the analyte. The BEC-peptide will be activated by the mixed anhydride method (2) and coupled to glucose oxidase. The BEC - peptide can also be conjugated to the carbohydrate portion of glucose oxidase using peptides containing carboxy - terminal hydrazides.

The enzyme-analyte conjugates will be characterized immunochemically to determine the effect of the linker arm on conjugate performance in an immunoassay. We will focus mainly on the following three factors: binding of the conjugate to the antibody in the absence of benzoylecgonine; displacement of the bound enzyme - analyte conjugate by the physiological concentrations of the analyte; and non - specific binding.

It is expected that the enzyme analyte conjugate containing the linker arm system should give better binding of the antibody to the enzyme - analyte than the one without any linker arm. This system for binding an analyte to an enzyme through a peptide linker arm could be expanded to a variety of applications in the future.

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CHAPTER I

INTRODUCTION

In this study, we are examining the effect of the length and polarity of peptide linker arms on the performance of enzyme - analyte conjugate in a reagent-limited heterogeneous immunoassay which utilizes a solid phase format. In our model system, our goal is to attach benzoylecgonine (see fig.1), the primary urinary metabolite of cocaine, to glucose oxidase by a series of peptide linker arms (see fig. 2).

The concentration of small molecules in physiological fluids is often determined by a competitive immunoassay - an analytical procedure based on the reversible binding of an analyte to an antibody. The labeled analyte is mixed with the test solution containing an unknown amount of the analyte. The solution containing the labeled and unlabeled analyte reacts with a limited amount of antibody bound to a solid surface.

A variety of methods have been developed to measure either the bound or unbound forms of the labeled analytes. A homogenous assay approach does not require the separation of the bound and free labeled analyte because they can be directly distinguished from one another. The enzymatic activities of the label are changed because of the binding of the antibody to the enzyme-labeled

analyte. In most instances, binding of antibody to the enzyme - analyte conjugate inhibits the enzyme by limiting the substrate's access to the catalytic site. A good example is the gentamicin assay (4). The measurement of thyroxin (T_4) is based on the competition between serum T_4 and enzyme-labeled T_4 for the limited binding sites on the thyroxin antibody. The enzyme label, malate dehydrogenase, becomes inactive once it binds to T_4 because the active site is blocked by T_4 . The activity of the enzyme increases in the presence of T_4 antibody because T_4 binds to the antibody and therefore is displaced from the active sites (4).

On the other hand, a heterogeneous assay requires the separation of the bound and free labeled analyte. Some techniques, such as nonspecific absorbents, specific absorbents, chromatography, salt precipitation, and double antibody precipitation have been used to perform the separations. The concentration of the analyte then is inversely proportional to the signal produced by the labeled analyte that still binds to the antibody. In some assays, the labeled enzyme - analyte does not produce signals. Therefore, the concentration of analyte is determined by the free form of the labeled analyte.

Consequently, successful conjugation between analytes of interest (such as drugs, hormones, environmental toxins, cell surface antigens, and fragments of viral coat proteins) and reporter groups (such as enzymes, dyed or magnetic

particles, or colloidal gold) is essential to the development of new analytical methods, new therapeutic reagents, and many other areas of biomedical research. Because it will provide a great body of information which could not have been obtained by other methods.

The most commonly used signal generators for an immunoassay are radioisotopes, enzymes, and fluorophores. During recent years, competitive immunoassays based on enzymes and fluorescent labels have become more popular due to the increasing awareness of the inherent difficulties in generation and disposal of radioactive waste. Signal amplification by an enzyme label is achieved by rapid turnover of substrate to form colored, fluorescent, or luminescent products. The reactions can be photometrically monitored.

When using an enzyme label for small analytes such as drugs of abuse, the challenge is to insure that the attached analyte is not sterically hindered from interaction with antibodies. Usually, the analyte attached to an enzyme is so small that it may be buried within the enzyme and therefore cannot interact with antibodies (see fig. 3). Hence, the thought is that the conjugated analyte could be attached to the enzyme through a linker arm to be extended away from the surface of the enzymes. As a result, it could more effectively compete with the free analyte in solution.

Linker arms have been used in a wide variety of biological applications. They are often necessary to preserve the biological activity of two proteins that are cross - linked. This was demonstrated in cases of a carbohydrate linker arm that was used to conjugate two enzymes (5).The Chitin Lash was used to crosslink staphylococcal nuclease to ribonuclease A with retention of 75% and 78%, respectively, of the starting enzyme activities.

Different peptide linker arms have been used to influence the local environment of immobilized enzymes (6). In this study, polypeptide arms of known composition were quantitatively grafted to alcohol dehydrogenase. The properties of the microcavity of the gel, in which the enzyme is immobilized by multipoint covalent linkages, are fully determined by the linker arms. According to the article, the results obtained using the soluble preparation indicate that the chemical activation by itself (alcohol dehydrogenase) does not induce any modification of the activation energy of the reaction, whereas the grafting of a complete peptide induces a rise of this value for the two hydrophilic peptides. The grafting of only one amino acid (Alcohol dehydrogenase - Homocystine) induces a slightly lower value. This can be explained by the possible dynamic interactions between the two hydrophilic peptides and the outer layer of the enzyme. These interactions could stablize the outer layer of the protein . As a result, the transconformational mobility of the protein could be inhibited to a certain extent and that would lead to a higher activation energy of the reaction.

Peptide linker arms have also been found to affect the metabolism and biodistribution of antibody - analyte conjugates (7). Studer et al., compared two kinds of antibody-conjugated benzyl - EDTA: one had a peptide linker arm:(Ala - Leu - Ala - Leu) whereas the other conjugate did not. Digestion of the benzyl - EDTA with linker arm and without linker arm in *vivo* showed that the conjugate containing the linker arm was cleaved at the liver more rapidly by the liver protease cathepsin B1 (T1/2=6 h) than the other conjugate, which had 79% radioactivity still attached to the protein after 97h of exposures to cathepsin B1. Indeed, the result indicated that the rate of clearance by liver depends on the linker. Furthermore, the excretion rate of the conjugate containing the linker arm was faster than the one which lacked this feature.

Optimal linker arm lengths have been identified for improving the efficacy of inhibitors of human leukocyte elastase (8) and inhibitors of chloride - transporting systems in membranes (9). It was demonstrated that the human leukocyte elastase inhibitory potency of derivatives of benzisothiazolinone 1,1-dioxide (saccharin) N - acetylated with aliphatic and aromatic substituted acyl groups increased as a function of their carbon chain length. The result indicated that macromolecular conjugates were demonstrably inhibitory to red blood cell anion exchange when the ligands were appropriately coupled: inhibitory efficacy strongly depended on the chemical structure of the coupled ligand and the spacer length between the inhibitory moiety and the macromolecule.

Similarly, by increasing the length of the alkyl linker arms in derivatives of the β - adrenergic drug, isoproterenol, the potency of these derivatives was increased in an *in vitro* assay (10). Reitz et al., found that a benzyl carbonate derivative containing a branched, seven-carbon spacer group was 40 times more potent than isoproterenol in the *in vitro* assay.

Finally, linker arms have been used to improve the sensitivity of a quantitative assay for specific ribonucleic acid sequences (11). Improved hybridization behavior was noted by increasing the length of the alkyl spacer of the oligonucleotide-alkaline phosphatase conjugate. The enzyme moiety was separated from the nucleic acid by ethylene or hexene. A two - fold increase in signal - to - background ratio was observed for the detection of the probe having a six-carbon spacer between the oligonucleotide and the enzyme.

Peptide chains are chosen as linker arms because numerous possibilities of composition and properties (polar, non - polar, positively charged, negatively charged, flexible and rigid) can be achieved through the choice of amino acids and because solid phase peptide synthesis methods are well established .

Selection of appropriate enzyme for use as a label for the immunochemical reagents is very often empirical, and each has distinct advantages and disadvantages. Enzymes used as labels differ from radioisotopes in that the

binding reaction can modify their activity. Consequently, enzymes can be used as labels only when they satisfy the following criteria (4):

1. The enzymes must have a very high specific activity. The signal amplification seen with enzymes is related to the amount of substrate converted to product during the time of incubation. Enzymes with the highest specific activities are potentially capable of giving the greatest amplification. Assays using such enzymes are able to detect very low concentrations of analytes.
2. The labels must be stable during the assay and under refrigerated storage conditions.
3. The enzymes must not be present in the biological fluid or tissue sample that is to be analyzed. Contaminating label in the biological fluid would increase background, decrease the sensitivity, and give false positives.
4. The enzymes must retain most of their activity when attached to the antibody.

Glucose oxidase (GO[1]) is considered an ideal enzyme for biosensor development (12) and has been used as the enzyme label in steroid immunoassays (13). This enzyme has been used as the basis for glucose sensors in some glucose detection kits. It catalyzes the oxidation of β-D-glucose to glucono-δ-lactone and the reduction of molecular oxygen to hydrogen peroxide (equation 1).

$$\text{glucose} + O_2 \xrightarrow{\text{glucose oxidase}} \text{gluconic acid} + H_2O_2 \qquad \text{(equ. 1)}$$

[1] BEC, benzoylecgonine; DCC, dicyclohexylcarbodiimide; DCM, dichloromethane; DIEA, diisopropylethlamine; DMF, dimethylformamide; Fmoc, fluorenylmethyoxycarbonyl; GO, glucose oxidase; HOBt, N-hydroxybenzotriazole; NSB, non-specific binding.

The hydrogen peroxide produced from the glucose oxidase reaction is consumed by a peroxidase-dye indicator reaction in which the oxidized dye is colored, allowing the reaction to be photometrically monitored (equation 2).

$$H_2O_2 + \text{reduced dye} \xleftrightarrow{\text{peroxidase}} \text{oxidized dye} + H_2O \quad \text{(equ. 2)}$$

In our research, the substrate (reduced dye) 3,3',5,5'-tetramethylbenzidine will be oxidized by horseradish peroxidase. The reaction can be detected at 410 nm. Since a single molecule of enzyme can catalyze the turnover of many molecules of substrate, the sensitivity of the assay could be improved .

Glucose oxidase is selected from among other potential enzyme labels based on its excellent stability (14), particularly in lyophilized preparation. Lyophilized glucose oxidase is stable for 2 years at 0^0C. Additionally, there are 14 lysine residues per subunit (15) of glucose oxidase, most of which are likely to be found on the enzyme surface where they could be available as attachment sites for the BEC-peptide.

Our first approach is to couple the BEC - peptide to glucose oxidase by forming an amide bound between the carboxy end of the peptide linker arm and the amino terminal of the lysine residues on glucose oxidase (see fig. 4). Peptide linker arms were synthesized using the fluorenylmethoxycarbonyl (Fmoc) solid phase peptide synthesis strategy described by Stewart and Young (1). We attached the BEC to the exposed amino - terminal end of the peptide linker arm

attached the BEC to the exposed amino - terminal end of the peptide linker arm by the free carboxyl group of BEC prior to removal of the peptide from the solid phase to avoid the requirement for isolation of the peptide before reacting with the analyte (***method 1***).

The BEC - peptide can also be conjugated to the carbohydrate portion of glucose oxidase using peptides containing carboxy - terminal hydrazides (***method 2***, see fig. 5). BEC - peptide hydrazides can be prepared according to the methods of Ramage et al. (3). The site - directed immobilization of antibodies on hydrazide - containing solid supports following oxidation of their N - linked and O - linked carbohydrate moieties is well documented (16) The resulting vicinal dialdehydes will react with the BEC - peptide hydrazides to form stable hydrazone linkages.

Many factors need to be considered in the development of an immunoassay. Major factors are: sensitivity, accuracy, specificity, et al.. The enzyme-analyte conjugates we develop will be characterized immunochemically to determine the effect of the linker arm on conjugate performance in an immunoassay. We will focus mainly on the following three factors in assay performance which relate specifically to enzyme - analyte conjugates:

1. Binding of the conjugate to the antibody in the absence of benzoylecgonine. The concentration of free analyte in solution is inversely proportional to the

antibody. To be effective in an immunoassay, the conjugate must bind with sufficient affinity to the immobilized antibody to generate a colorimetric signal of approximately 1.5 to 2.0 absorbance units in the absence of the free analyte.

2. Displacement of the bound enzyme - analyte conjugate by the analyte. In a competitive assay, the labeled analyte will compete with the free analyte in physiological fluid. It is necessary to develop conjugates that can bind tightly enough to the antibody to generate a detectable signal. On the other hand, it should also be displaced by the analyte in a specified concentration range.
3. Non - specific binding (NSB) refers to the conjugates that are non - specifically bound to the tube in the absence of antibody. The immunoaffinity purification procedures described below should reduce NSB.

CHAPTER II

EXPERIMENTAL DESIGN

A. MATERIALS

1. Reagents

Glucose oxidase (*Aspergillus niger)* and horseradish peroxidase were from Sigma Chemical Co. (St. Louis, MO). Resins and Fmoc-amino acids were from Peptide International Inc. Benzoylecgonine was generously provided by the Research Technology Branch of the National Institute on Drug Abuse (Research Triangle Park, NC). All other reagents were of the highest quality commercially available:

Phenol, liquefied	J.T. Baker Inc.
Ethanol, absolute	Aaper Alcohol and Chemical Co.
Potassium cyanide	Sigma Chemical Co.
Pyridine	J.T. Baker Inc.
Ninhydrin	Aldrich Chemical Inc.
Fmoc-Gly	Peptides International Inc.
Fmoc-Phe	Peptides International Inc.
Fmoc-Gly-Wang resin	Peptides International Inc.
Boc-Gly-O-resin	Peptides International Inc.
Dichloromethane(DCM)	J.T. Baker Inc.
Dimethylformamide(DMF)	Fisher Chemical Co.
Isopropanol(iPrOH)	J.T. Baker Inc.
Piperidine	Sigma Chemical Co.

Diisopropylethylamine(DIEA)	Aldrich Chemical Inc.
Trifluoroacetic acid(TFA)	Sigma Chemical Co.
Dicyclohexylcarbodiimide(DCC)	Sigma Chemical Co.
Ethyl ether	E.M. Company
Hydrazine	Sigma Chemical Co.
N-hydroxybenzotriazole(HOBt)	Peptides International Inc.

2. Instruments

Syn -Thor 2000	Peptides International Inc.
HPLC	Autochrom Co.

B. METHODS

1. Synthesis of the Benzoylecgonine - Peptide Conjugate

Method 1 We used the Fmoc solid phase peptide synthesis strategy described by Stewart and Young (1) with some modifications (see figure 6). The first glycine residue was already attached to resin by its carboxyl end when purchased and subsequent amino acid residues were attached at the amino terminus of the growing peptide.

At each cycle, the Fmoc-amino group was deprotected by a 20% piperidine/ DCM treatment followed by several washing steps. Pre-formed symmetrical anhydrides (see figure 7) in DCM were mixed with the resin until monitoring showed complete reaction. Symmetrical anhydrides were chosen because of their high reactivities (2). They were prepared using 4 equivalents of protected amino acid and 2 equivalents of DCC in 50% DMF. The urea produced was removed by filtration. DIEA, a tertiary amine, was added (equivalent to the amount of peptide on the resin) 15 minutes after the coupling reaction had begun to accelerate the reaction. The coupling efficiency was checked during each cycle using the Kaiser test (17).

The Kaiser test involves the following reagents: 5 g of ninhydrin in 100 ml ethanol, 80 g of liquefied phenol in 20 ml ethanol, and 2 ml of 0.001 M aqueous potassium cyanide in 98 ml of pyridine. A few resin beads were removed from the mixing chamber, thoroughly washed with ethanol, and sampled with 2 drops of each reagent above for 5 minutes at 120^0C. Yellow solution and white beads indicated a complete coupling. Blue beads indicated an incomplete coupling.

The active ester method (see figure 8) was chosen to attach the BEC to the peptide - resin. BEC was preactivated with DCC and N - HOBt in high quality DMF to form the HOBt active ester. For HOBt active esters, 5 equiv. of BEC ,

DCC and HOBt were used. The filtrate was reacted with the peptide-resin until the Kaiser test gave a negative result.

At the end of the synthesis, the resin was shrunk by methanol or isopropanol treatment. The BEC-peptide-resin was dried under vacuum for at least 4 hours before cleavage. 82.5% trifluoroacetic acid aqueous solution was used to release the conjugate from the resin. The filtrate was brought to dryness and precipitated from cold ether. (For detailed synthesis protocol see Appendix A)

Method 2 (Peptides Containing Carboxy - Terminal Hydrazides) BEC - peptide hydrazides (see figure 6) were prepared by using Boc - Gly O - Resin. 50% TFA/DCM was used as deprotection reagent. The peptide -O - resin was synthesized following the Fmoc peptide synthesis strategy described above. The active ester coupling method was again used to attach BEC to peptide -resin. The peptide resin was then suspended in purified DMF (5 ml per g of resin) and anhydrous hydrazine (30 equiv. per equiv. of peptide). The mixture was stirred for 2 days at room temperature. Resin was removed by filtration and washed with DMF. The combined filtrate and washings were evaporated in vacuum and then were purified. (For detailed protocol see Appendix B)

2. Purification of BEC - peptide

Our initial attempts to purify the BEC - peptide involved cation exchange chromatography . The use of volatile pyridine acetate buffers (18) would enable us to lyophilize the isolated fractions to obtain salt-free BEC-peptide in anhydrous condition.

Two buffers were prepared, the pH 3.1 0.2N pyridine acetate buffer and the pH 5.0 2.0N pyridine acetate buffer. All reagents were degassed. The Dowex-50X2 resin was swollen in pH 3.1 buffer at a ratio of 1 volume of resin to 2 volumes of buffer. An HPLC pump, low pressure mixing valve and control module from the Autochrom Co. were connected to the column to control the flow rate (1 ml/min) and to run a gradient of the two buffers. The column was washed with 3 volumes of the pH 3.1 buffer before the sample was applied.

The separation was initiated with pH 3.1 buffer. Elution conditions were:

Initial	100% pH 3.1 buffer	1.0 ml/min	
10 min	100% pH 3.1 buffer	1.0 ml/min	linear gradient to
30 min	100% pH 5.0 buffer	1.0 ml/min	
40 min	100% pH 5.0 buffer	1.0 ml/min	linear gradient to
50 min	100% pH 3.1 buffer	1.0 ml/min	

Effluent fractions were collected every 30 seconds.

We attemped to analyze the purity of the crude sample by HPLC. Solution A (0.1% TFA in water) and solution B (0.1% TFA in acetonitrile) were prepared. Elution conditions were:

Initial	100% A	1.0 ml/min	
10 min	100% A	1.0 ml/min	linear gradient to
30 min	100% B	1.0 ml/min	
40 min	100% B	1.0 ml/min	linear gradient to
50 min	100% A	1.0 ml/min	
55 min	100% A	1.0 ml/min	

CHAPTER III

RESULTS & DISCUSSION

A. Synthesis of the Benzoylecgonine - Peptide Conjugate

The most commonly used test to monitor solid phase peptide synthesis is the Kaiser test. It is simple and rapid and applicable to all N-terminal residues (1). Any free primary amino group will be indicated by an intense blue color. Hence, it can be used to monitor both the deprotection and coupling. It is expected that after each attachment, the Kaiser test will give a negative result. The washing step in the Kaiser test is critical to avoid producing a false positive result (1). We performed the Kaiser test to monitor the coupling reaction. The result showed us that we successfully synthesized the gly - gly - gly - resin and attached BEC to the gly - gly - gly - resin. The crude yield of method 1 is 90%, method 2 is 300%.

When we used the standard method to prepare symmetrical anhydride using 2 equivalents of protected amino acid and 1 equivalent of DCC for each run, coupling results were checked at 3 hours, 4 hours, 5 hours, 6 hours , and 8 hours after the reaction, respectively. Unfortunately, coupling was incomplete after 8 hours. However, 30 minutes after we added the second equivalent of preformed anhydride to the reaction chamber, the Kaiser test gave us a negative result. To get a satisfactory coupling, we decided to use excess amino acid. The

anhydride was prepared using 4 equivalents of amino acid with 2 equivalents of DCC. Within an hour of the reaction, the Kaiser test indicated complete coupling.

Conjugation of the carboxylic acid group of the BEC with the amino terminus of the growing peptide was attempted. Initially, we tried to attach the BEC symmetrical anhydride molecules, which are the actual intermediates in coupling reactions, directly to the peptide - resin. This technique has been found to give the best results of all methods tried in many difficult sequences (1). Unfortunately, it failed. The reaction was monitored by the Kaiser test for 12 hours. Blue beads and dark blue solutions indicated an unsuccessful coupling. This could be attributed to the fact that BEC , which has a tertiary amino group, is a big molecule relative to glycine. The N - terminal of glycine could be sterically hindered from attacking the BEC symmetrical anhydride molecule. The second approach was an active ester method, wherein an HOBt ester of BEC was prepared using DCC coupling. Two hours after coupling, the Kaiser test indicated a successful coupling.

B. Purification of BEC - peptide

We tried to purify the BEC - gly - gly - gly by cation exchange chromatography. The results were inconclusive. The effluent fractions were examined by the ninhydrin identification spray. We did not expect to see any color change for the

fractions which had only BEC - gly - gly - gly residues. Unfortunately, all the fractions turned to purple, indicating the presence of some free amino groups. Further refinements of purification and analysis of the BEC - peptides are described below.

CHAPTER IV

Plans / Thoughts

A. Solid Phase Synthesis of the Benzoylecgonine - Peptide Conjugate with an enzyme cleavable linker arm

The BEC - peptide could also be cleaved from the resin by a protease. We will synthesize a peptide in which the first two residues represent a specific site for the enzyme cleavage. For example, a peptide which has a glycine and phenylalanine at its carboxy terminal end can be cleaved by chymotrypsin. The BEC - peptide would then be released into an aqueous solution, where direct coupling to GO could be attempted using a water soluble coupling reagent such as diisopropyl carbodiimide.

B. Conjugation of the Benzylecgonine - Peptide to the Enzyme

The purified BEC-peptide will be dissolved in an anhydrous organic solvent, activated with isobutyl chloroformate, then added to the enzyme solution in sodium phosphate,10 mmol/L, pH 7.4 (see fig.10). The BEC - peptide is not soluble in tetrahydrofuran, therefore, NMP is chosen using these criteria: (a) it must dissolve BEC - peptide; (b) it must not denature the enzyme; (c) it must have a freezing point below -25^{0}C, the temperature for the activation reaction.

C. Purification of BEC - peptide - GO by Immunoaffinity Chromatography

An antibody towards BEC will be immobilized on Affi - Gel Hz (Bio - Rad Laboratories) according to the manufacturer's specification. BEC - peptide - GO will be loaded onto the resin containing immobilized anti - BEC. Following a high salt wash step, the conjugate will be eluted with citrate buffer (10 mmol/L, pH 3.0).

D. Characterization of Enzyme - Analyte Conjugate

We will focus mainly on the following three factors in assay performance which relate specifically to enzyme - analyte conjugates:

1. Binding of the conjugate to the antibody in the absence of benzoylecgonine. First, the antibody will be immobilized on microtiter wells using published methods (19). The wells will be coated with polylysine, which binds irreversibly to their surface. The carbohydrate portion of the antibody will be oxidized with sodium periodate. The resulting aldehyde groups will react with the ε-amino groups of the lysine side-chains of the polylysine, thereby immobilizing the antibody. Then the conjugate will be incubated for 30 min. with immobilized antibody, the plates washed to remove unbound conjugate, substrate added for an hour and then the reaction will be quenched after 1

hour with 1N sulfuric acid. Absorbance at 405 nm of 1.5 to 2.0 units is acceptable.

2. Displacement of the bound enzyme - analyte conjugate by the analyte. We will then incubate the conjugate plus a series of BEC concentrations in a solid - phase immunoassay format. We will evaluate the data to determine the midpoint of the calibration curve and the level of non-specific binding.
3. Non - specific binding. A_{405} values at BEC concentrations two orders of magnitude greater than the mid point of the BEC calibration curve (A_{405} vs. log[BEC]) should be less than 1% of the signal in the absence of BEC.

Reference

1. Stewart J M, Young JD. In: *Solid phase peptide synthesis*. Pierce Chemical company, Rockford,IL1984
2. Brown et al.; *J.C.S. Perkin Trans.I,* 1983.
3. Ramage R, Irving SL, McInnes C. Tetrahedron Letters 1993; **34**: 6599-602
4. Kaplan L A , Pesce A. J. *Clinical chemistry* 1989;The C. V. Mosby Company, St. Louis, MS.
5. Guan K, Cecchini DJ, Giese RW. *Carbohydr Res* 1993; **246**: 205-17
6. Bille V, Plainshamp D, Lavielle S, Chassaing G, Remacle J, *Eur J Biochem* 1989; **180**: 41-7
7 Studer M, Kroger LA, DeNardo SJ, Kukis DL, Meares CF. *Bioconj Chem* 1992; *3*:424-9
8. Kerneur C, Hornebeck W, Robert L, Moczar E. *Biochem Pharmacol* 1993; **45**:1889-95
9. Eidelman O, Yani P, Englert HC, Lang HG, Greger R, Cabantchik ZL. *Am J Physiol* 1991; **260**: c1094-103
10. Reitz AB, Sonveaux RP, Verlander MS, Meimon KL, Hoffman BB, Akita Y, Castagnoli N, Goodman M. *J Med Chem* 1985; **28**: 634-42
11. Ishii JK, Ghosh SS. *Bioconj Chem* 1993; **4**: 34-41
12. Wilson R, Turner APF. *Biosensors & Bioelectronics* 1992; **7**:165-8
13. Hosoda H, Tsukamoto R, Shishidoo M, Takasaki W, Nambara T. *Chem Pharm Bull* 1987; **35**: 2856-61
14. Kellin D, Hartree EF. *Biochem J* 1948;**42**:221-9
15. Frederick KR, Tung J, Emerick RS, Masiarz FR, Chamberlain SH, Chamberlain SH, et al. *J Biol Chem* 1990;**265**:3793-802
16. O'Shannessy DJ, Hoffman WL. *Biotech App Biochem* 1987; **9**: 488-96
17. Kaiser ET. *Natural* 1985; **313**:788-98
18. Ohno, M. And Anfinsen, C.B. *J. Am. Chem. Soc.* 1967,**89**:5994-95
19. Schramm WS, Paek S-H, Kuo H-H, Yang T. *Anal Chim Acta* 1991; **248**:517-528

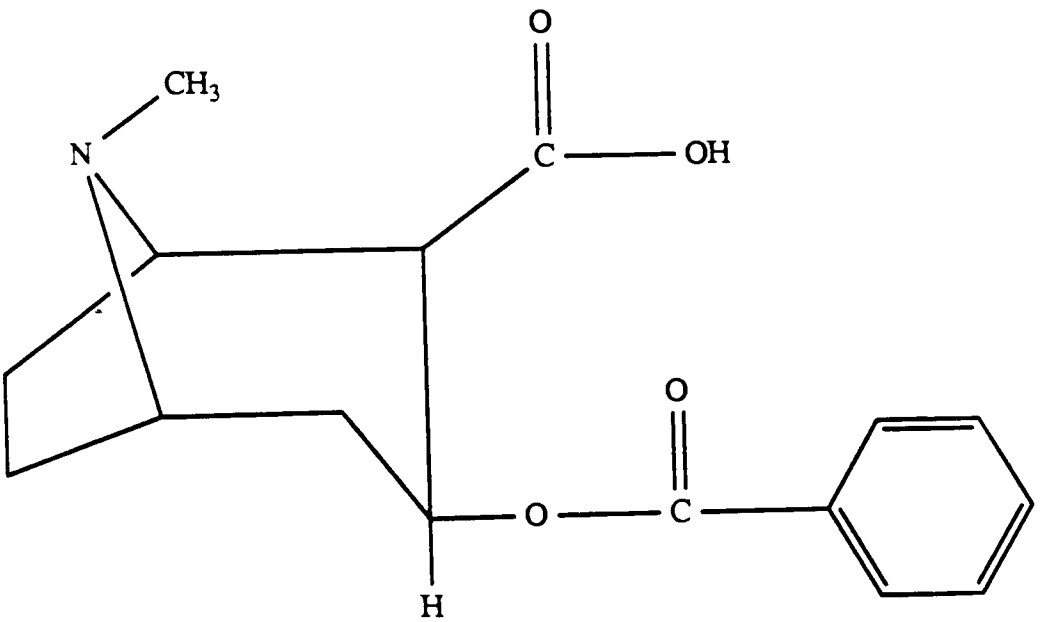

Fig. 1 Structure of BEC

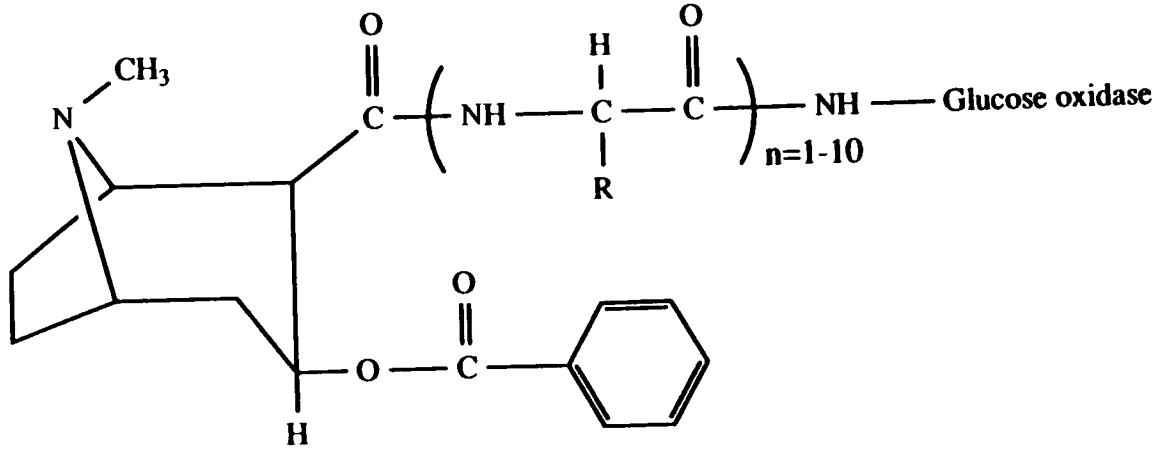

Fig. 2 Proposed BEC - GO Conjugates with Peptide Linker Arm

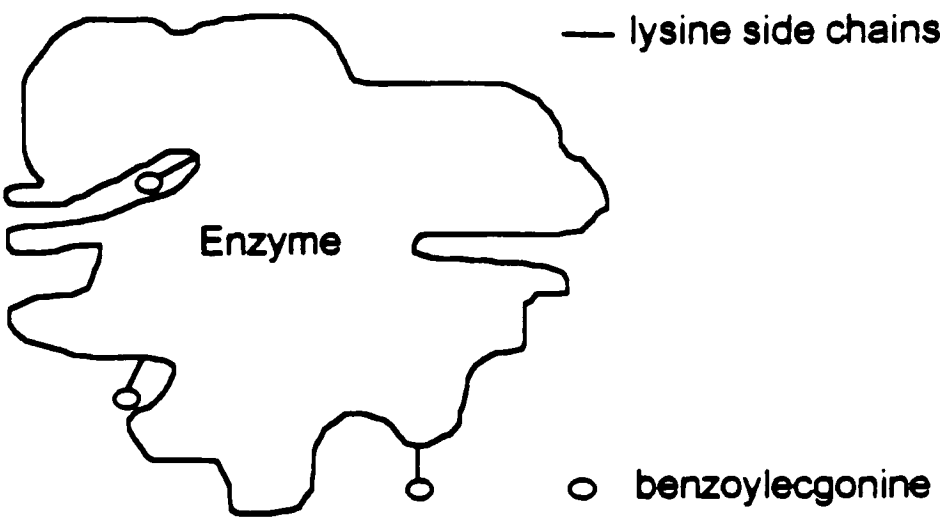

Fig. 3 Enzyme - Analyte Conjugate

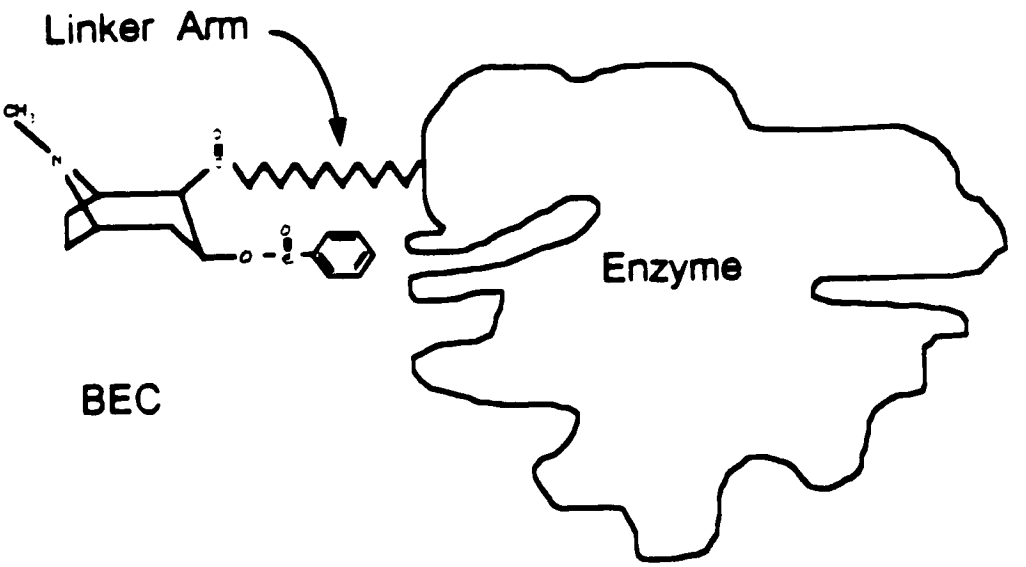

Fig. 4 Attaching BEC to Enzyme by Linker Arm

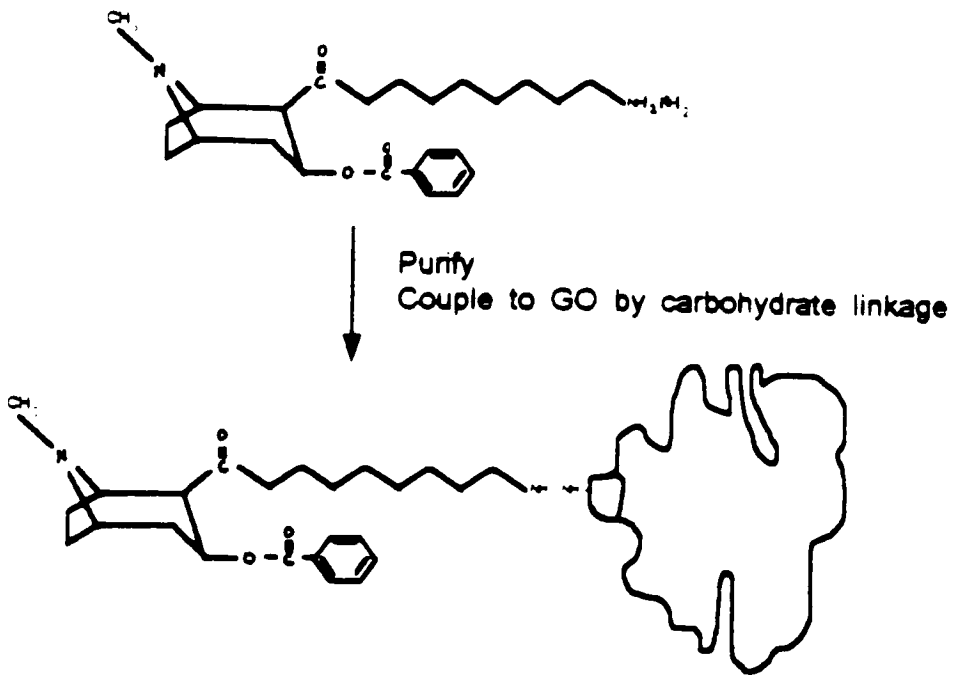

Fig. 5 Attaching BEC - peptide Hydrazide to enzyme

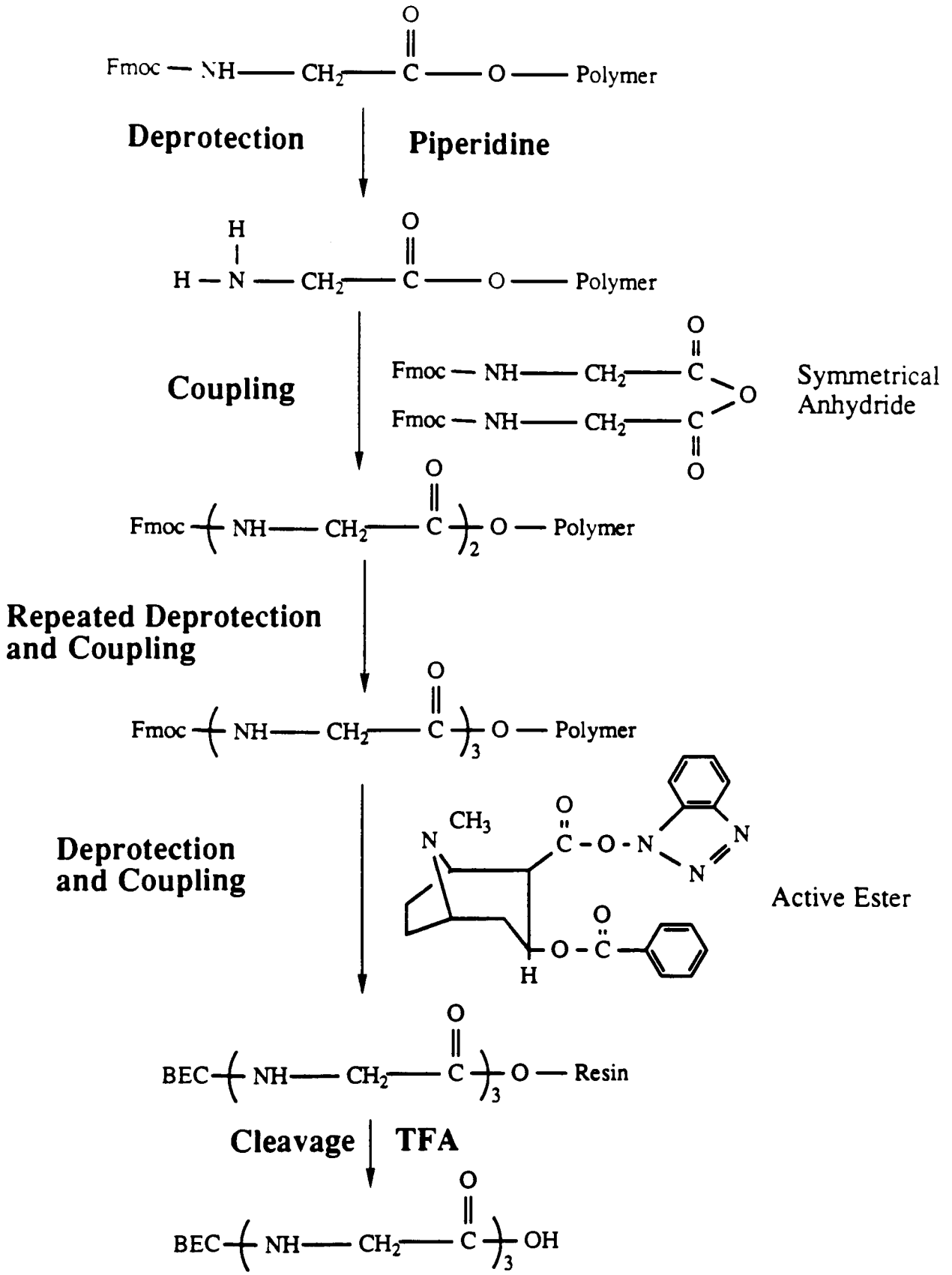

Fig. 6 ynthesis of BEC - gly - gly - gly (method 1)

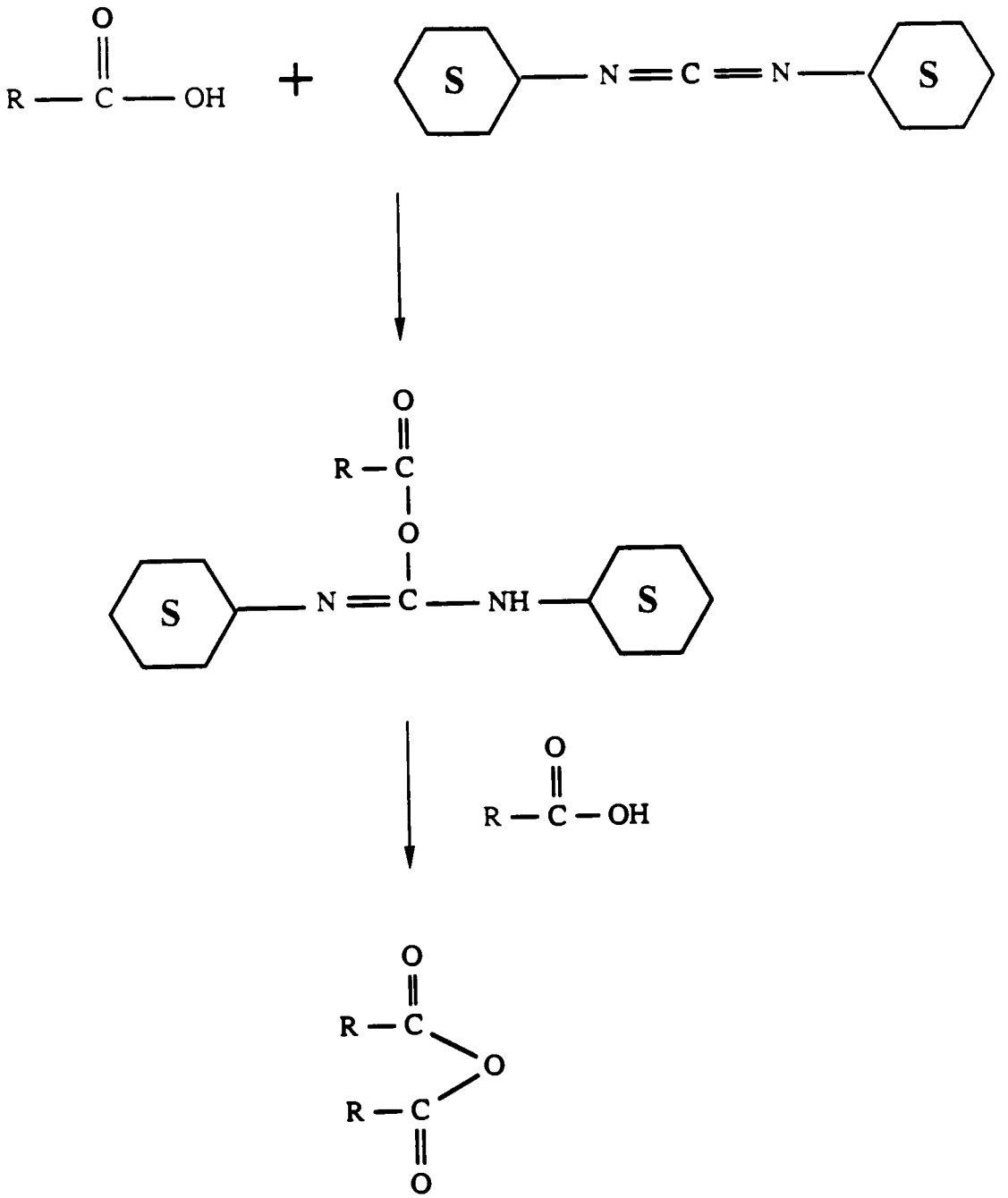

Fig. 7 Synthesis of Symmetrical Anhydride

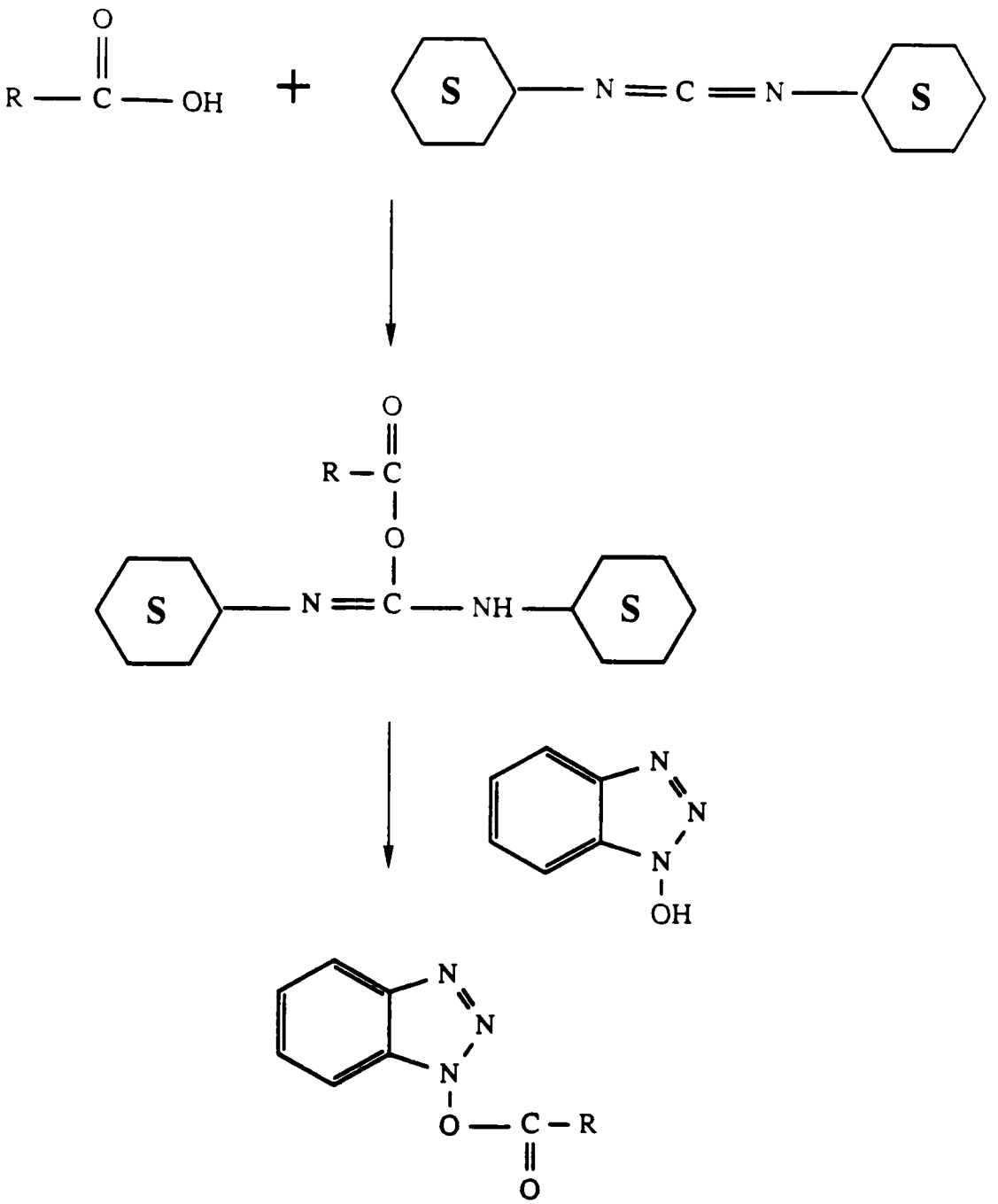

Fig. 8 Synthesis of HOBt Active Esters

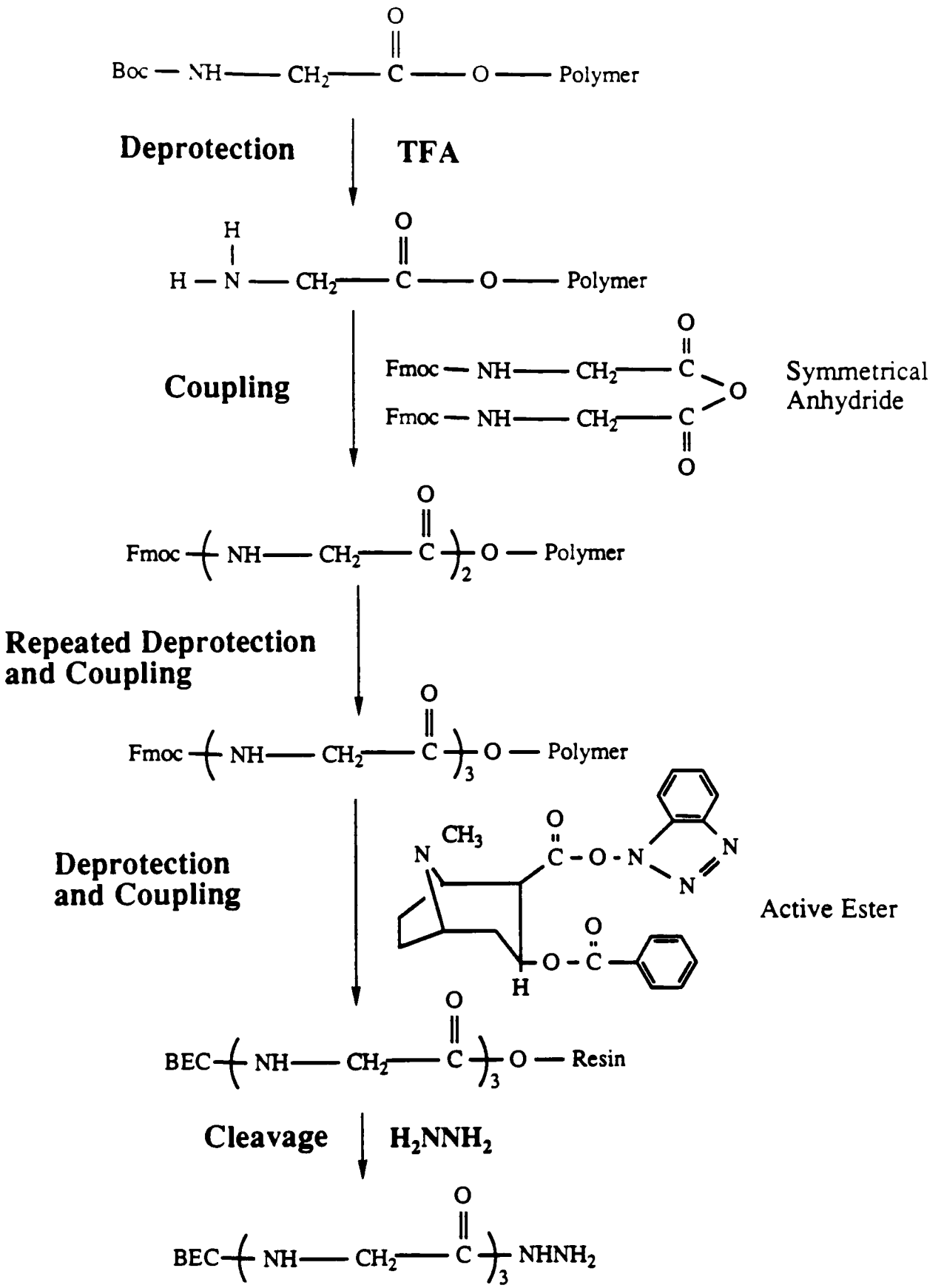

Fig. 9 Synthesis of BEC - gly - gly - gly (method 2)

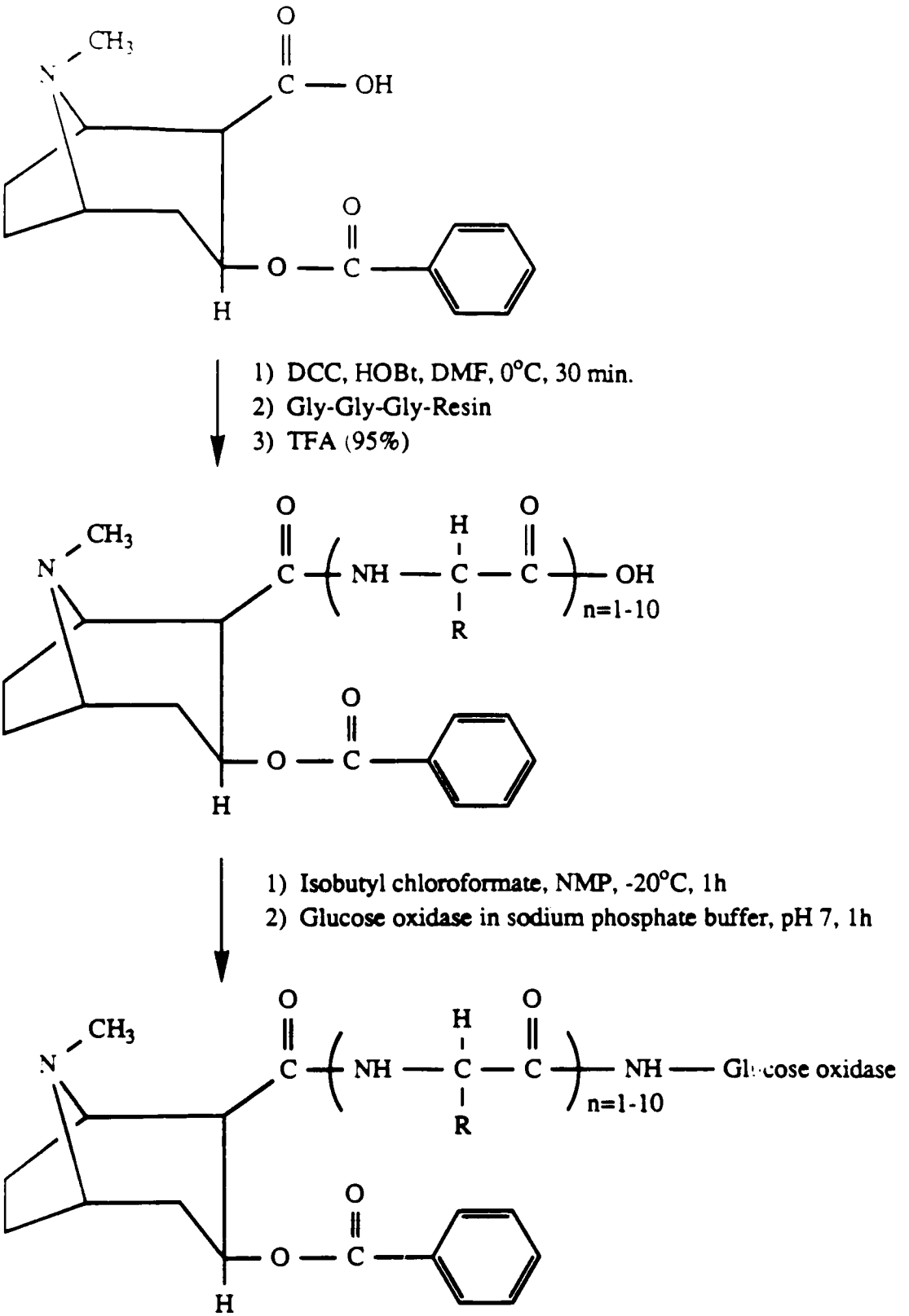

Fig. 10 Synthesis of BEC - GO Conjugate

Appendix A

Schedule A For BEC - Peptide Synthesis

(method 1: using Fmoc - gly - resin)

Wash 1	DCM wash, 2 x 30 ml x 2 min.
	DMF wash, 3 x 30 ml x 2 min.
Deprotect	30% (v/v) piperidine in DCM, 1 x 30 ml x 3 min., 1 x 30 ml x 7 min.
Wash 2	DMF wash, 3 x 30 ml x 1 min.
	DCM wash, 3 x 30 ml x 1 min.
Couple	Symmetrical anhydride of Fmoc - amino acid in 50% (v/v) DCM/DMF, 20 ml x 15 min.
	DIEA, 1 equivalent to the resin, 30 min. or until monitoring shows coupling complete
Recouple	Repeat steps above
	BEC HOBt active ester in DMF, 30 min. or until monitoring shows coupling complete
Wash 3	DCM wash, 3 x 30 ml x 2 min.
	Methanol wash, 2 x 30 ml x 7 min.
	Dry under high vacuum for 5 or more hours
Cleavage	50 % or 82.5% (v/v) TFA/water, 20 ml x 120 min.
Ether precipitation	Filter the cleavage mixture using a sintered glass funnel.
	Wash the resin several times with the cleavage mixture.
	Combine filtrates and bring them to dryness by rotary evaporation. Add cold ether to precipitate the crude sample.

Appendix B

Schedule B For BEC - Peptide Synthesis

(method 2: using Boc - gly - resin)

Wash 1	DCM wash, 2 x 30 ml x 2 min.
Deprotect	50% (v/v) TFA/DCM, 20 ml x 15 min.
Wash 2	DCM wash, 2 x 30 ml x 2 min.
	DMF wash, 3 x 30 ml x 2 min.
Couple	Symmetrical anhydride of Fmoc - amino acid in 50% (v/v) DCM/DMF, 20 ml x 15 min.
	DIEA, 1 equivalent to the resin, 30 min. or until monitoring shows coupling complete
Wash 3	DCM wash, 3 x 30 ml x 2 min.
	DMF wash, 3 x 30 ml x 2 min.
Deprotect	30% (v/v) piperidine in DCM, 1 x 30 ml x 3 min., 1 x 30 ml x 7 min.
Wash 4	DMF wash, 3 x 30 ml x 1 min.
	DCM wash, 3 x 30 ml x 1 min.
Recouple	Repeat steps ***Wash 1*** to ***Recouple*** in **Schedule A**
Wash	DCM wash, 3 x 30 ml x 2 min.
	DMF wash, 3 x 30 ml x 2 min.
	High quality DMF wash, 3 x 30 ml x 2 min.
Cleavage	Suspend resin in high quality DMF (5 ml per g of resin) with anhydrous hydrazine (30 equiv. of resin) for 2 days.
	Bring the filtrate to dryness by rotary evaporation.