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CA 2187346 C 2010/06/29

(11)(21) 2 187 346

(12) BREVET CANADIEN
CANADIAN PATENT

(13) C

(86) Date de dépôt PCT/PCT Filing Date: 1995/04/07
(87) Date publication PCT/PCT Publication Date: 1995/10/19
(45) Date de délivrance/Issue Date: 2010/06/29
(85) Entrée phase nationale/National Entry: 1996/10/07
(86) N° demande PCT/PCT Application No.: US 1995/004404
(87) N° publication PCT/PCT Publication No.: 1995/027723
(30) Priorités/Priorities: 1994/04/08 (US08/224,831); 1995/03/16 (US08/406,192); 1995/03/16 (US08/406,191); 1995/03/16 (US08/406,194)

(51) Cl.Int./Int.Cl. *C07K 14/47* (2006.01), *A61K 31/70* (2006.01), *A61K 38/16* (2006.01), *A61K 47/48* (2006.01), *C07H 23/00* (2006.01), *G01N 33/82* (2006.01)

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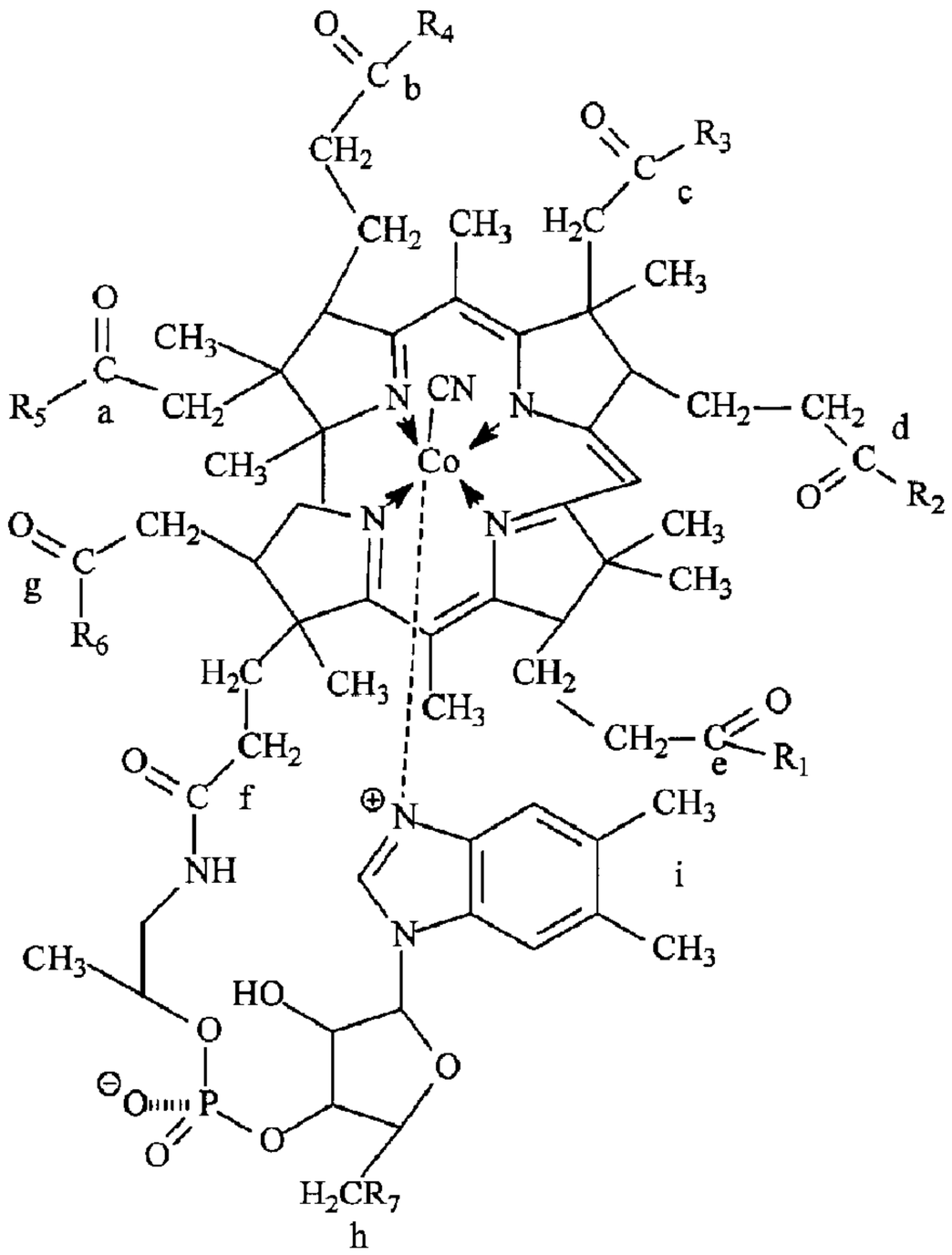
(54) Titre : AGENTS MODULATEURS DE RECEPTEURS ET PROCEDE ASSOCIES
(54) Title: RECEPTOR MODULATING AGENTS AND METHODS RELATING THERETO



Structure I

(57) Abrégé/Abstract:
Receptor modulating agents capable of modulating cell surface receptors by affecting the cell surface receptor trafficking pathway. The receptor modulating agents are comprised of a covalently bound rerouting moiety and targeting moiety.

2187346

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C07H 23/00, G01N 33/82, A61K 31/68 | **A1**

(11) International Publication Number: **WO 95/27723**

(43) International Publication Date: 19 October 1995 (19.10.95)

(21) International Application Number: PCT/US95/04404

(22) International Filing Date: 7 April 1995 (07.04.95)

(30) Priority Data:

08/224,831	8 April 1994 (08.04.94)	US
08/406,191	16 March 1995 (16.03.95)	US
08/406,192	16 March 1995 (16.03.95)	US
08/406,194	16 March 1995 (16.03.95)	US

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(81) Designated States: AU, CA, JP, KR, NO, NZ, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

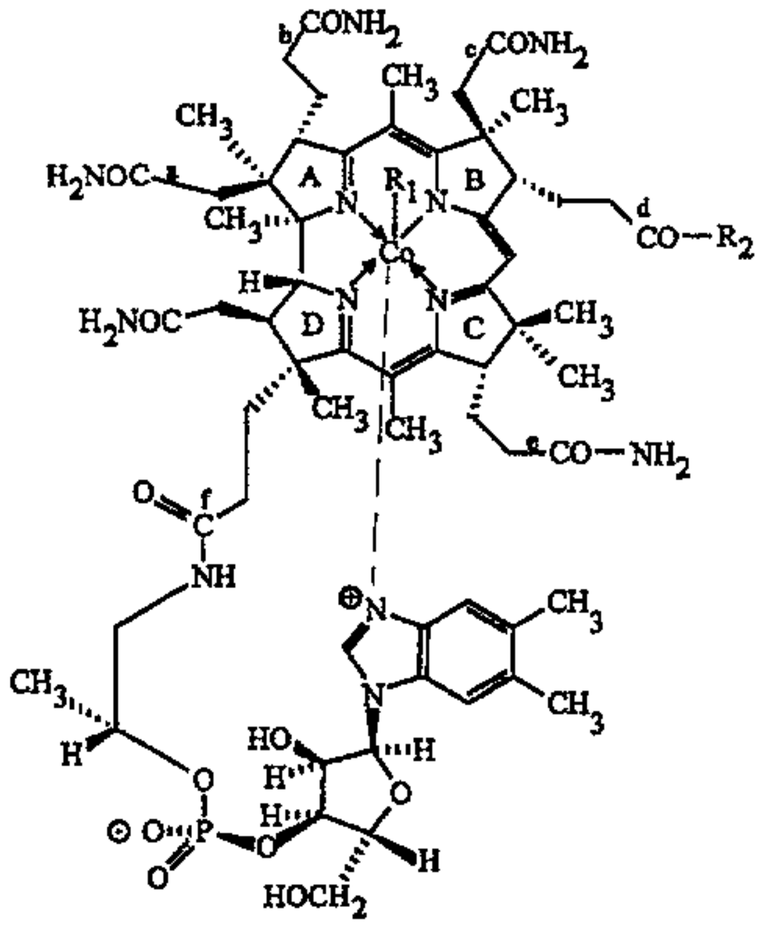
Published

With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: RECEPTOR MODULATING AGENTS AND METHODS RELATING THERETO

(57) Abstract

Receptor modulating agents capable of modulating cell surface receptors by affecting the cell surface receptor trafficking pathway. The receptor modulating agents are comprised of a covalently bound rerouting moiety and targeting moiety.



R_1 = CN ; R_2 = NH_2 (Cyanocobalamin)
R_1 = CN ; R_2 = OH (Cyanocobalamin -(3)-free acid)
R_1 = CN ; R_2 = HN-CH_2-CH_2-CH_2-CO_2H (GABA adduct)
R_1 = CN ; R_2 = GABA - Peptide (where GABA = linker)
R_1 = CN ; R_2 = Peptide
R_1 = CN ; R_2 = HN-(linker)-tyramine-^{125}I
R_1 = CN ; R_2 = HN-(linker)-lysosomotropic agent
R_1 = CN ; R_2 = HN-(linker)-X-linking agent
R_1 = CN ; R_2 = HN-(linker)-biotin
R_1 = CN ; R_2 = NH-$(CH_2)_{12}NH_2$

Description

RECEPTOR MODULATING AGENTS AND METHODS RELATING THERETO

Technical Field

The present invention is generally directed to receptor modulating agents which modulate cell surface receptors and, more specifically, to receptor modulating agents which bind to cell surface receptors and affect the receptor trafficking pathway and methods related thereto.

Background of the Invention

Cell surface receptors constitute a class of proteins which are responsible for receptor-mediated endocytosis of specific ligands. Basically, the receptors serve as escorts for ligand delivery to intracellular destinations.

Ligand delivery is generally achieved through coated regions on the plasma membrane called "coated pits." These pits continually invaginate and pinch off, forming "coated vesicles" in the cytoplasm. Coated pits and vesicles provide a pathway for receptor mediated endocytosis of specific ligands. The ligands that bind to specific cell surface receptors are internalized via coated pits, enabling cells to ingest large numbers of specific ligands without taking in correspondingly large volume of extracellular fluid. The internalized coated vesicles may or may not lose their coats and bind with other vesicles to form larger vesicles called "endosomes." In the endosome the ligand and the receptor are separated or "sorted." Endosomes which sort ligands and receptors are known as "compartment of uncoupling of receptor and ligand" or "CURL."

Endosomes may fuse with primary lysosomes, where their contents are digested, or they may be delivered to other intracellular destinations. The receptor proteins are generally not digested, but are rather recycled to the cell membrane surface through a process called "exocytosis," or transferred to early or late endosomes via multivesicular bodies. The entire pathway is referred to as the "receptor trafficking pathway."

Some receptors deliver their ligand directly to the cytoplasm or other specific intracellular locations. Perhaps one of the most studied receptor trafficking pathways is that of iron transport. In this pathway, a serum carrier protein, transferrin, binds iron and transports it to transferrin receptors on the plasma membrane surface.

After binding and internalization, via coated pits, the resulting vesicle combines first with early endosomes and then with late endosomes. This process results in the gradual drop in pH in the vesicle. The drop in pH causes the transferrin carrier protein to lose its affinity to iron. When this occurs, the iron translocates through the membrane of the vesicle and joins the intracellular pool of enzymes. The transferrin receptor may then recycle to the cell surface where it may repeat the process.

Other receptors may deliver their ligand directly to the lysosomes for digestion. For example, the epidermal growth factor ("EGF") receptor delivers its ligand directly to a lysosome for degradation (Prog. Histochem. Cytochem. 26:39-48,1992). The EGF receptor may recycle to the cell surface depending on its state of phosphorylation (Cancer Treat. Rep. 61:139-160, 1992; J. Cell. Biol. 116:321-330, 1992).

A single receptor may utilize more than one receptor trafficking pathway within the same cell. For example in polarized cells, such as specialized transport epithelia cells, membrane trafficking is distinct between apical and basal sides of the cell (Sem. Cell. Biol. 2:387-396, 1991). Moreover, non-polarized epithelia cells may simultaneously follow two separate sorting pathways.

The control or regulation of cell surface receptors may be achieved by a variety of techniques. Regulation of cell surface receptors may be accomplished, at a very basic level, by the binding of naturally occurring ligands. As discussed above, receptor binding of a ligand will generally trigger the internalization of the ligand-receptor complex. Such internalization may desensitize the cell to further ligand binding. (J. Immunol. 150:3161-9, 1993; Mol. Endocrinol. 6:2090-102, 1992; J. Cell. Physiol. 154:281-8, 1993; Receptor 1:13-32, 1990-91; Biochem. J. 288:55-61, 1992; J. Immunol. 148:2709-11, 1992; J. Cell. Physiol. 148:24-34, 1991). This type of regulation, however, is transient in nature and does not result in diminution of biologic response.

Regulation of cell surface receptors may also be accomplished by administration of receptor antagonists or agonists. Receptor antagonists are organic protein or peptide ligands generally derived through empirical structure-function studies, or through the use of detailed knowledge of ligand and receptor interaction. Essentially, an antagonist may constitute any molecule with similar binding activity to a natural ligand, but incapable of producing the biological response normally induced by the natural ligand. Thus, the antagonist competitively blocks receptor activity. With a competitive antagonist, the regulation of receptor activity is dependent upon both the antagonist's affinity for the receptor, as well as its extracellular concentration over time.

Receptor agonists are protein or peptide ligands derived in a similar manner as antagonists. Essentially, an agonist may constitute any molecule which binds to the receptor in a manner superior to that of the natural ligand.

One receptor of particular interest is the vitamin B_{12} receptor. As has been demonstrated in experimental in vitro data, pre-clinical animal models, and patient studies, vitamin B_{12} is a co-enzyme necessary in cell division, as well as cellular metabolism, in proliferating normal and neoplastic cells. Insufficient vitamin B_{12} causes cellular division to be held in abeyance and ultimately may result in apoptosis. The nutrient is generally derived from dietary intake and is transported throughout the body complexed to transport proteins. The complex of transport protein and vitamin B_{12} is recognized by a cellular receptor which internalizes the complex and releases the vitamin intracellularly. The overall process has been reviewed in GUT 31:59, 1991. Vitamin B_{12} is taken in through the diet. Binding proteins in the saliva (R-binder) and gut (intrinsic factor-(IF)) complex vitamin B_{12} after release from endogenous binding proteins by action of enzymes and low pH in the stomach. Vitamin B_{12} is transferred across the intestinal epithelium in a receptor specific fashion to transcobalamin II (TcII). The vitamin B_{12}/transcobalamin II complex is then transported throughout the body and recognized by receptors present on dividing cells, internalized and released within the cell where it is utilized by certain enzymes as a co-factor.

The high affinity receptor in dividing tissues or cells responsible for internalization of vitamin B_{12} recognizes transcobalamin II complexed with vitamin B_{12}. The vitamin B_{12}/TcII receptor recognizes only the vitamin B_{12}/TcII complex and not the serum transport protein or the vitamin alone. The receptor is undetectable on non-dividing cells; the mechanism for supplying non-dividing cells with vitamin B_{12} is poorly understood. However, it is known that more vitamin B_{12} is required during cell division than during metabolism, and that the vitamin B_{12}/TcII receptor is the only high affinity means for cellular uptake of vitamin B_{12} during cell division. When stimulated to divide, cells demonstrate transient expression of this receptor leading to vitamin B_{12} uptake which precedes actual DNA synthesis (J. Lab. Clin. Med. 103:70, 1984). Vitamin B_{12} receptor levels may be measured by binding of ^{57}Co-vitamin B_{12} complexed to transcobalamin II (present in serum) on replicate cultures grown in chemically defined medium without serum. No receptor mediated uptake occurs in the absence of carrier protein.

Dividing cells, induced to differentiate, lose receptor expression and no longer take up vitamin B_{12}. More importantly, leukemic cells, deprived of vitamin B_{12}, will stop dividing and die (Acta Haemat. 81:61, 1989). In a typical experiment,

leukemic cell cultures were deprived of serum for 3 days, and then supplemented either with serum (a source of vitamin B_{12}) or a non-metabolizable analogue of vitamin B_{12} and cultured up to five days. Cell cultures supplemented with vitamin B_{12} continued to grow, whereas those deprived of the active nutrient stopped growing and die.

Based on these observations, it has been suggested that whole body deprivation of vitamin B_{12} may be useful in the treatment of cancer or other disorders characterized by uncontrolled growth of cells. Moreover, because of the critical role played by vitamin B_{12}-containing enzymes in cell division, it is believed that vitamin B_{12} deprivation may be used in combination with chemotherapeutic drugs which inhibit cellular replication. For example, when vitamin B_{12} depletion was combined with methotrexate, the two modalities together were more efficient in depleting folate levels in leukemic cells than either alone (FASEB J. 4:1450, 1990; Arch. Biochem. Biophys. 270:729, 1989; Leukemia Research 15:165, 1991). Folates are precursors in the production of DNA and proteins. In typical experiments, cultures of leukemic cells were exposed to nitrous oxide for several hours to convert the active form of endogenous vitamin B_{12} to an inactive form. Replicate cultures were then left without further treatment, or additionally treated with methotrexate. Cellular folate levels were measured three days later. Cells treated with the combination (*i.e.*, both methotrexate and inactive vitamin B_{12}) showed a more striking decrease in cellular folate levels than with either of the two approaches alone. This combination also results in a higher cell kill in vitro. When this approach was applied to the treatment of highly aggressive leukemia/lymphoma in animal models (Am. J. Haematol. 34:128,1990; Anticancer Res. 6:737, 1986; Cancer Chemother. Pharmacol. 17:114, 1986; Br. J. Cancer 50:793, 1984), additive or synergy of anti-tumor action was observed, resulting in prolonged remissions and cures.

A key finding in the experiments described above was that short-term (hours to days), whole body depletion of vitamin B_{12} can act synergistically with chemotherapeutic drugs (such as methotrexate and 5-FU) to inhibit tumor growth and treat animals with leukemia/lymphoma. Despite synergistic anti-tumor activity, there was no toxicity attributable to the short-term vitamin B_{12} depletion for proliferating normal cells. This combination therapy was demonstrated in multiple animal models. Observations in patients have indicated that long-term (months to years) vitamin B_{12} depletion is required to produce significant normal tissue toxicity. Even in those cases, subsequent infusion of vitamin B_{12} can readily reverse symptomology (Br. J. Cancer 5:810, 1989).

Because of the promise of this therapeutic approach, various methods have been sought to efficiently and controllably perform a temporary depletion of vitamin B_{12}. Such methods, however, affect all of the body's stores of vitamin B_{12}. They include dietary restriction, high doses of vitamin B_{12} analogues (non-metabolizable-competitive antagonists which act as enzyme inhibitors), and nitrous oxide (transformation of vitamin B_{12} to inactivate form). These different methods have been used in culture systems and in animals to deplete vitamin B_{12}. The most efficient and the most utilized method has been the inhalation of nitrous oxide (laughing gas). Animals are maintained typically under an atmosphere of 50% to 70% of nitrous oxide for periods from a few hours to a few days, causing the conversion of endogenous vitamin B_{12} into an inactive form. This methodology has been utilized in combination with drugs for therapy of leukemia/lymphoma. A further method for vitamin B_{12} depletion involves infusion of a non-metabolizable analogue of vitamin B_{12} which essentially dilutes out the active form. This form of therapy is not specific for dividing cells but affects liver dependent metabolic processes. Another approach includes restricting the dietary intake of vitamin B_{12}. This method, however, requires very long periods of dietary restriction and is offset by hepatic storage of vitamin B_{12}. All of these methods suffer from problems of specificity, since they affect both vitamin B_{12}-dependent growth as well as basal metabolism, and therefore are not particularly suited to the development of anti-proliferative pharmaceutical products.

In view of the biological importance of cell surface receptors, receptor-controlling agents have emerged as a class of pharmaceutical drugs. Moreover, with the advent of genetic engineering for the isolation and amplification of genes for cell surface receptors, as well as computer programs to model the interactions between ligands and receptors (*i.e.*, "rational" drug design), the production of receptor-controlling drugs has been significantly enhanced.

To date, many months or even years of scientific research, as well as significant financial resources, are required to produce new receptor antagonists or agonists. To speed up this process, new screening technologies have been developed which utilize peptide or antibody recombinant libraries (*see*, *e.g.*, Gene 73:305, 1988; Proc. Nat. Acad. Sci. (USA) 87:6378, 1990; Biochromatography 5:22, 1990; Protein Engineering 3:641, 1989). While library screening does not require the same degree of knowledge of a specific receptor/ligand system, it does involve an intensive screening effort utilizing functional receptor-specific assays. Moreover, the initial compounds identified by such screening programs are generally only precursors to the development of therapeutic products through more typical structure-functional assessments.

While antagonists and agonists are generally capable of regulating a biological response, the surface receptors which bind such ligands are continually being re-expressed on the cell surface. Thus, effective regulation by antagonists or agonists must rely on a relatively high and sustained serum concentration in order to bind the new surface receptors continually being expressed on the cell surface.

Accordingly, there is a need in the art for agents which bind cell surface receptors and thus regulate biological responses associated therewith, and which further effect normal cellular trafficking of the bound receptor. There is also a need in the art for agents which, when bound by a cell surface receptor and internalized, promote retention of the receptor within the cell. Moreover, there exists a need for methods relating to the administration of such agents to regulate a biological response. The present invention fulfills these needs and provides further related advantages.

Summary of the Invention

Briefly stated, the present invention provides receptor modulating agents which are capable of affecting a receptor trafficking pathway of the cell. Receptor modulating agents of the present invention are comprised of a rerouting moiety coupled to a targeting moiety.

Suitable targeting moieties include, by way of example, a vitamin B_{12} molecule or any one of several proteins and peptides.

Suitable rerouting moieties include, by way of example, lysosomotropic moieties, such as gentamycin, kanamycin, neomycin, and streptomycin; intracellular polymerizing moieties, such as dipeptide esters and leucine zippers; peptide sorting sequences, such as endoplasmic reticulum retention peptides, golgi retention peptides, lysosomal retention peptides, organism specific retention peptides and clathrin-binding peptides; conditional membrane binding peptides, such as charged glutamate, aspartate, and histidine; and bi- or multi-valent receptor cross-linking moieties.

In a preferred embodiment of the present invention, a receptor modulating agent, is comprised of a vitamin B_{12} molecule coupled to a rerouting moiety by a linker. Generally, the linker is at least 4 atoms in length, typically, the linker is about 6 to 20 atoms in length and preferably, the linker is 12 atoms in length. Suitable linkers include linkers which include an amino group, such as diaminoalkyl, diaminoalkylaryl, diaminoheteroalkyl, diaminoheteroalkylaryl and diaminoalkanes. Preferably, the linker is $-NH(CH_2)_xNH-$ wherein x = 2-20 or $-NH(CH_2)_yCO-$, wherein y = 3-12. In one embodiment the linker is a trifunctional linker.

In a preferred embodiment of this aspect of the present invention. a B_{12} molecule is coupled to a rerouting moiety at a *b*-, *d*- or *e*- coupling site. In a particularly preferred embodiment of the present invention, a B_{12} molecule is coupled to a rerouting moiety at a *d*- or *e*- coupling site. In another embodiment, the B_{12} molecule is coupled to a rerouting moiety at a ribose coupling site. In yet another embodiment, the receptor modulating agent is bound to transcobalamin.

Receptor modulating agents of the present invention may act by affecting a receptor trafficking pathway in any one of several ways, including, by redirecting an agent/receptor complex; by cross-linking one or more cell surface receptors; by anchoring a cell surface receptor in the membrane; and by retaining a receptor in an endosome.

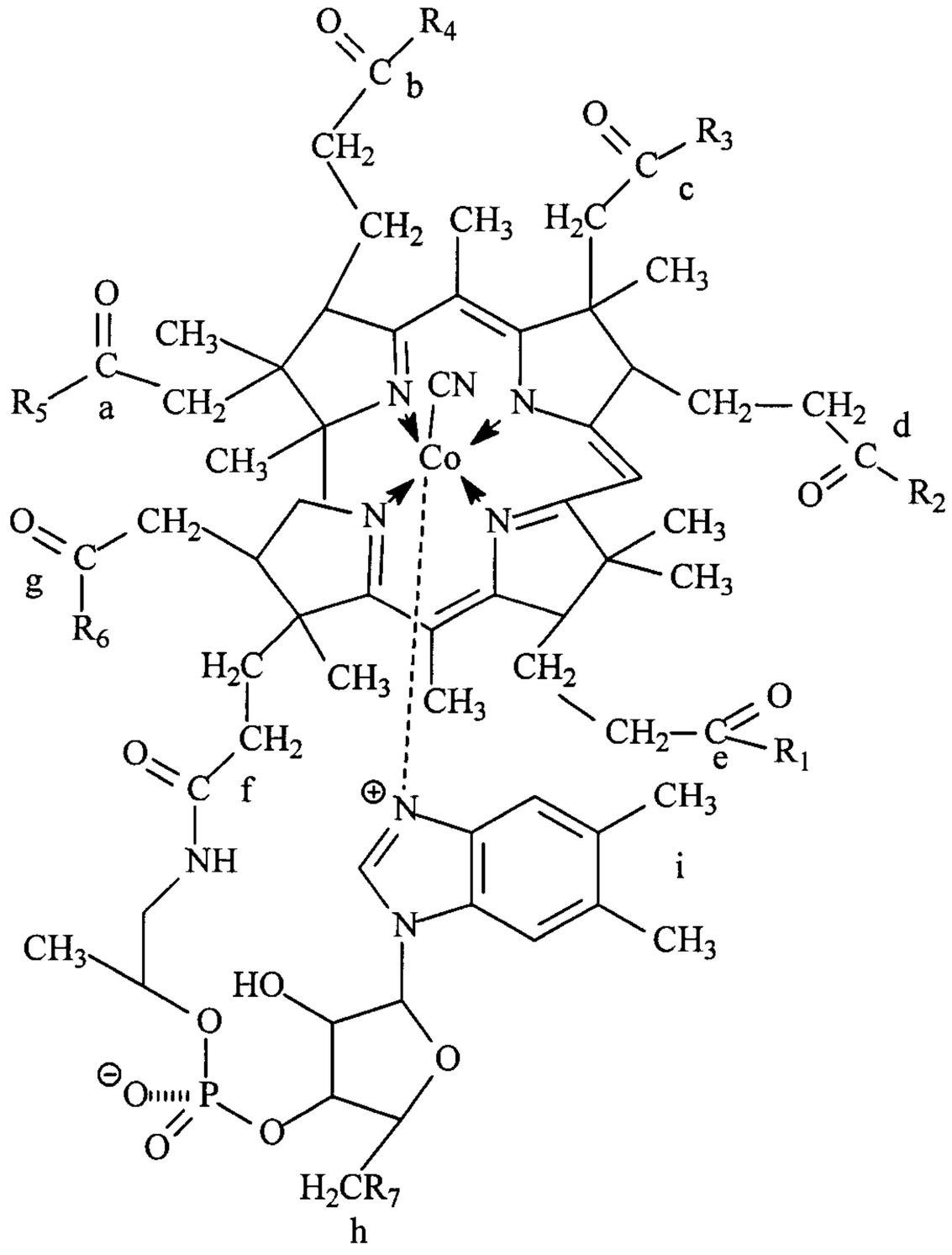
Another aspect of the present invention includes a vitamin B_{12} dimer comprising a first and a second vitamin B_{12} molecule coupled through a coupling site independently selected from the group consisting of coupling sites *a-g*, coupling sites *h*, and coupling sites *i*. In a preferred embodiment, the B_{12} molecule coupled through an *e*- or *d*- coupling site.

In another embodiment, B_{12} molecules are coupled by a linker. Generally, the linker is at least 4 atoms in length, typically, the linker is about 10 to 55 atoms in length and preferably, the linker is 35 to 45 atoms in length. In a preferred embodiment, the linker is a trifunctional linker. Suitable linkers include linkers which include an amino group, such as diaminoalkyl, diaminoalkylaryl, diaminoheteroalkyl, diaminoheteroalkylaryl and diaminoalkanes. Preferably, the linker is $-NH(CH_2)_xNH-$ wherein x = 2-20 or $-NH(CH_2)_yCO-$, wherein y = 3-12.

In another aspect of this embodiment, a vitamin B_{12} dimer is coupled to at least one transcobalamin II molecule. In yet another aspect of this embodiment, at least one of said first and said second vitamin B_{12} molecules of the dimer is a vitamin B_{12} derivative.

In another aspect, the invention provides a receptor modulating agent, comprising a vitamin B_{12} molecule coupled to a rerouting moiety by a linker, wherein said vitamin B_{12} molecule binds transcobalamin II, and wherein said rerouting moiety is selected from the group consisting of: lysosomotrophic moieties; intracellular polymerizing moieties; peptide sorting sequences; conditional membrane binding peptides; and bi- or multi-valent receptor cross-linking moieties.

In another aspect, the invention provides a vitamin B_{12} dimer comprising a first and second vitamin B_{12} molecule coupled through a coupling site independently selected from the group consisting of coupling sites *a-g*, coupling site h, and coupling site i, as shown in Structure I:



Structure I

wherein at least one of R_1, R_2, R_3, R_4, R_5, R_6, and R_7 is a linker wherein said first and second vitamin B_{12} molecules are coupled through at least one linker, and wherein said first and second vitamin B_{12} molecules bind transcobalamin II.

In another aspect, the invention provides use of a receptor modulating agent for modulating a vitamin B_{12} receptor, in a warm-blooded animal, said receptor modulating agent comprising a vitamin B_{12} molecule coupled to a rerouting moiety by a linker, wherein said vitamin B12 molecule binds transcobalamin II, and wherein said rerouting moiety is selected from the group consisting of: lysosomotrophic moieties; intracellular polymerizing moieties; peptide sorting sequences; conditional membrane binding peptides; and bi- or multi-valent receptor cross-linking moieties.

In another aspect, the invention provides a vitamin B_{12} molecule/biotin conjugate comprising a vitamin B_{12} molecule coupled by a linker to a biotin molecule through a coupling site on said vitamin B_{12} molecule selected from the group consisting of *b*-, *d*-, and *e*- as shown in Structure I:

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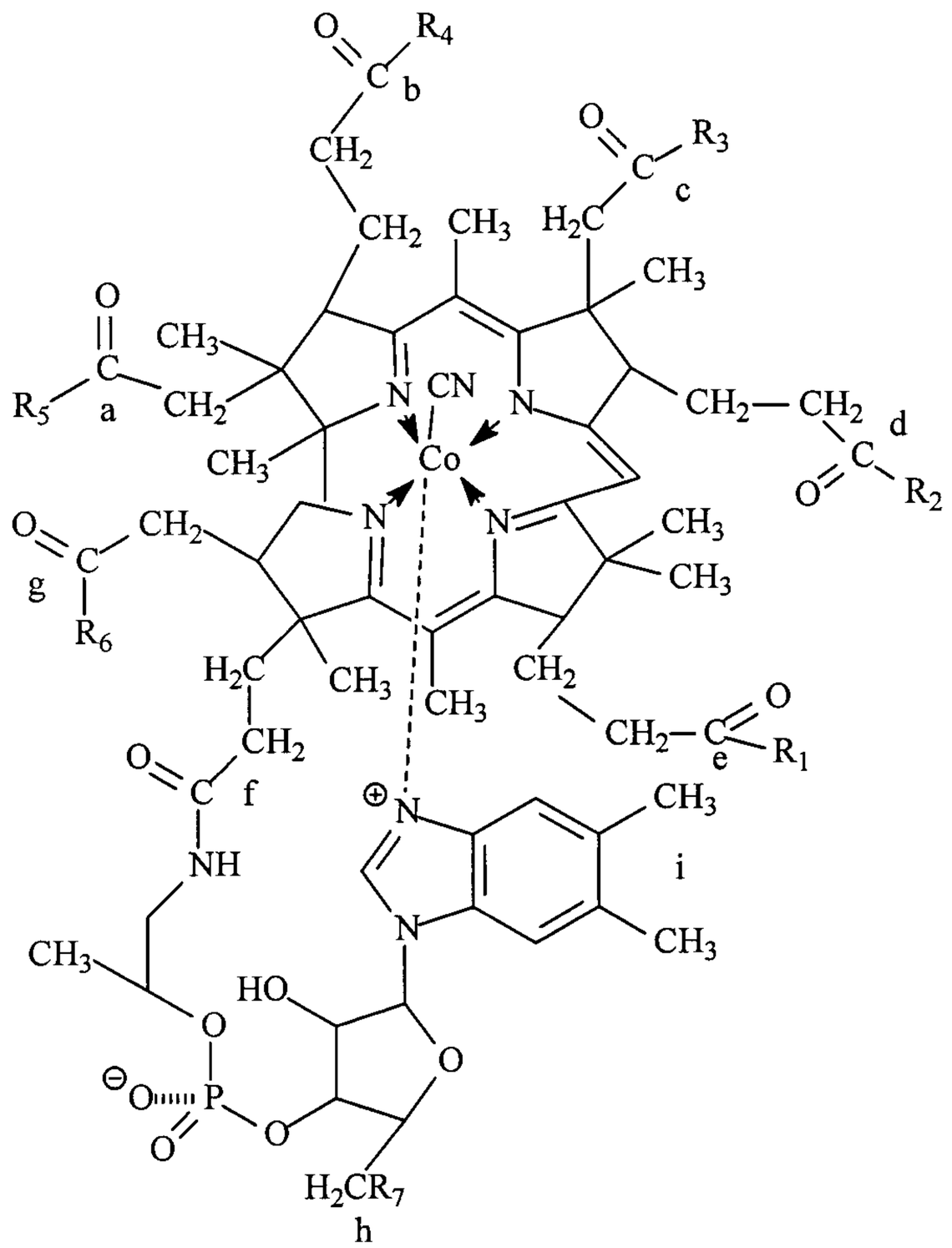


Structure I

wherein at least one of R_1, R_2, R_3, R_4, R_5, R_6, and R_7 is a linker and ribose coupling sites, wherein said vitamin B_{12} molecule binds transcobalamin II.

In another aspect, the invention provides a complex comprising a conjugate as described above bound to transcobalamin II.

In another aspect, the invention provides a kit comprising a conjugate as described above and instructions for using said conjugate for determining the presence or amount of transcobalamin in a sample.

In another aspect, the invention provides a pharmaceutical composition, comprising a conjugate as described above and a suitable pharmaceutical carrier or diluent.

These and other aspects of the present invention will become evident upon reference to the following detailed description and attached drawings. In addition, various references are set forth below which describe certain procedures or compositions in more detail.

Brief Description of the Drawings

Figure 1 is a schematic illustrating a mechanism of action of a receptor modulating agent of the present invention. A healthy receptor will internalize when bound by the appropriate ligand, release the ligand within the cell and then recycle to

the cell surface. Receptor modulating agents of the present invention impede the receptor trafficking pathway by inhibiting the recycling of receptors to the cell surface. Essentially, the targeting moiety on receptor modulating agents bind the receptor and the rerouting moiety redirects the receptor/receptor modulating agent complex to other points within the cell, where it may be retained or degraded. (Not shown in this schematic are receptors synthesized de novo).

Figures 2-5 are formulae representing families of antibiotics which act as rerouting moieties. The preferred reactive groups for coupling with a targeting moiety are indicated. These rerouting moieties facilitate retention of the receptor/receptor modulating agent complex through protonation of the complex, eventually delivering it to lysosomes for degradation.

Figure 2 illustrates formulae representing the gentamycin, sisomicin, and netilmicin families of antibiotics.

Figure 3 illustrates formulae representing the kanomycin, tobramycin, and amikacin families of antibiotics.

Figure 4 illustrates formulae representing the neomycin, paromomycin, ribostamycin, and butirosin families of antibiotics.

Figure 5 illustrates formulae representing the streptomycin family of antibiotics.

Figure 6 illustrates formulae representing substituted aminoquinolines (*e.g.*, chloroquine) substituted aminoacridines (*e.g.*, quinacrine), and substituted aminonapthalines (*e.g.*, dansyl cadaverine), all of which are representative rerouting moieties of the present invention. These rerouting moieties impede the receptor trafficking pathway through protonation and intracellular retention.

Figure 7 illustrates formulae representing glycosylation inhibitors, all of which are representative rerouting moieties of the present invention. These sugars may be conjugated to targeting moieties using linkages typical of oligomeric carbohydrate chains. The resulting receptor modulating agent is recognized by internal glycosyl transferases, subject to intracellular retention, and, ultimately, degradation in the lysosomes.

Figure 8 illustrates a formula representing a vitamin B_{12} (cyanocobalamin) molecule and identifies a preferred coupling site suitable for use in the present invention for derivatization and conjugation.

Figure 9 is a schematic depicting a representative reaction scheme for the synthesis of a vitamin B_{12}-GABA adduct.

Figure 10a is a schematic depicting a representative reaction scheme for the synthesis of a vitamin B_{12} derivative comprising a vitamin B_{12} molecule with a diaminododecane linker arm coupled to any one of coupling sites *d*-, *e*-, or *b*-.

Figure 10b is a schematic depicting a representative reaction scheme for coupling a succinic anhydride to a vitamin B_{12} diaminododecane adduct in preparation for coupling the adduct to a rerouting moiety, or other molecule, with an amino reaction site.

Figure 11 is a schematic depicting a representative reaction scheme for the synthesis of a vitamin B_{12} derivative comprising a vitamin B_{12} molecule and a diaminododecane linker arm coupled to a ribose coupling site.

Figure 12 is a schematic depicting a representative reaction scheme for coupling vitamin B_{12} or a vitamin B_{12}-GABA adduct to amikacin.

Figure 13 is a schematic depicting a representative reaction scheme for coupling vitamin B_{12} or a vitamin B_{12}-GABA adduct to streptomycin.

Figure 14 is a schematic depicting a representative reaction scheme for coupling a vitamin B_{12} carboxylate derivative or a vitamin B_{12}-GABA adduct to acridine.

Figure 15 is a schematic depicting a representative reaction scheme for the synthesis of a bivalent receptor modulating agent, a vitamin B_{12} dimer, using a trifunctional linker. The trifunctional linker allows for coupling with additional compounds (*e.g.*, R-NH_2) such as, by way of example, aminoglucosides (Figures 2-5), aminoacridines (Figure 6), glycosylation inhibitors (Figure 7), and biotin.

Figure 16 is a schematic depicting a representative reaction scheme for the synthesis of a vitamin B_{12} dimer using a homobifunctional or homotrifunctional cross-linking reagent.

Figure 17 is a schematic depicting a representative reaction scheme for the synthesis of a vitamin B_{12} dimer using a heterobifunctional cross-linker.

Figures 18-21 are schematics depicting representative reaction schemes for the synthesis of various receptor modulating agents generally comprised of a rerouting moiety, designated by the reactive group and R, selected from those represented in Figures 2-7, and a vitamin B_{12} molecule or derivative thereof as a targeting moiety.

Figure 22 is a graph illustrating the binding curve of Transcobalamin II to the cyanocobalamin monocarboxylic acids produced in Example 1. AD = Cyanocobalamin **(1)**; AL = Cyanocobalamin *b*-monocarboxylic acid **(2)**; AM =

Cyanocobalamin *e*-monocarboxylic acid **(3)**; and AN= Cyanocobalamin *d*-monocarboxylic acid **(4)**.

Figure 23 is a graph illustrating the binding curve of Transcobalamin II to the cyanocobalamin diaminododecane adducts produced in Example 3 and 4. AH = Cyanocobalamin *b*-monocarboxylic acid conjugate diaminododecane **(7)**; AI = Cyanocobalamin *e*-monocarboxylic acid conjugate diaminododecane **(8)**; AJ = Cyanocobalamin *d*-monocarboxylic acid conjugate diaminododecane **(9)**; AK = Cobalamin *e*-monocarboxylic acid conjugate diaminododecane, and AE = Cyanocobalamin ribose-succinate **(11)**.

Figure 24 is a graph illustrating the binding curve of Transcobalamin II to a series of vitamin B_{12} dimers. Dimer X = *b*-acid dimer with isophthaloyl dichloride **(36)**; Dimer Y = *e*-acid dimer with isophthaloyl dichloride **(37)**; dimer Z = *d*-acid dimer with isophthaloyl dichloride **(38)**; Dimer A= *b*-acid Dimer with *p*-iodo benzoyl isophthaloyl dichloride **(58)**; Dimer B = *e*-acid Dimer with *p*-iodo benzoyl isophthaloyl dichloride **(59)**; and Dimer C = *d*-acid Dimer with *p*-iodo benzoyl isophthaloyl dichloride **(60)**. These dimers were prepared as set forth in the Examples below. (*see* Examples 13 and 16.)

Figure 25 is a graph illustrating the binding curve of Transcobalamin II to a series of biotinylated vitamin B_{12} molecules. AA = Cyanocobalamin *b*-monocarboxylic acid conjugate diaminododecane and biotin **(17)**; AB = Cyanocobalamin *e*-monocarboxylic acid conjugate diaminododecane and biotin **(18)**; AC = Cyanocobalamin *d*-monocarboxylic acid conjugate diaminododecane and biotin **(19)**; AF = Cyanocobalamin ribose-succinate conjugate diaminododecane **(13)**; and AG = Cyanocobalamin ribose-succinate conjugate diaminododecane and biotin **(20)**. These biotinylated molecules were prepared as set forth in Examples below. (*see* Example 8.)

Detailed Description of the Invention

The present invention is generally directed to a receptor modulating agent which is capable of binding to a cell surface receptor to form a receptor modulating agent/receptor complex ("agent/receptor complex"). The binding of a suitable receptor modulating agent to a cell surface receptor generally results in invagination of the agent/receptor complex into the cell into the vesicular system in the same manner as the natural ligand. However, once internalized, or as part of the internalization process, a receptor modulating agent of the present invention affects the receptor trafficking pathway by effectively impeding, preventing, or delaying the

receptor from recycling to the surface, thus depriving the cell of receptors able to engage in binding its natural ligand and triggering related biological responses.

Within the context of the present invention, "affecting the receptor trafficking pathway" refers to impeding the receptor trafficking pathway in such a manner so as to affect biological response. This would include trapping, delaying, retaining, re-directing, or degrading the cell surface receptor. A "receptor modulating agent" is comprised of at least one targeting moiety covalently attached to at least one rerouting moiety. A "targeting moiety," as described in detail below, is a moiety capable of specifically binding to a cell surface receptor to yield an agent/receptor complex and, in a preferred embodiment, has an affinity for the cell surface receptor of within 100-fold, and more preferably, within 10-fold, of the affinity of the natural ligand for the receptor. A preferred targeting moiety is a vitamin B_{12} molecule. In contrast, a "rerouting moiety" is a moiety which redirects an agent/receptor complex, resulting in prolonged retention, degradation, and/or modulation of the receptor within the interior of a cell or on the cell surface, including, by way of example, retaining the receptor in the cell membrane or directing the receptor to a lysosome within the cell. Suitable rerouting moieties are described in detail below.

A targeting moiety is coupled to a rerouting moiety to yield the receptor modulating agent by any suitable means known in the art, including direct covalent linkage of an appropriate chemical linker or through a very tight association in non-covalent attachment. By way of example for the latter, in one embodiment, coupling is accomplished through the combination of an avidin or streptavidin conjugate with a vitamin B_{12}/biotin conjugate. Coupling of the targeting moiety and the rerouting moiety should be of a nature which resists cleavage by the enzymatic and low pH conditions normally encountered within the internal portion of the cell, including endosomes and lysosomes. Suitable linkers are noted below. The ability to resist cleavage may be detected by any means known in the art, including exposing the receptor modulating agent to enzymes at low pH and measuring release of the targeting or rerouting moiety using techniques known in the art.

Coupling of a targeting moiety and a rerouting moiety should not significantly hinder the ability of the targeting moiety to specifically bind the cell surface receptor. The receptor modulating agent may also include additional moieties, so long as they do not interfere with either the targeting or the rerouting moieties. For example, such moieties may be coupled to the receptor modulating agent through the use of a trifunctional linker or they may be coupled to a rerouting or targeting moiety. Optimal attachment of the two moieties may be determined by comparing the affinity of

binding of the receptor modulating agent with free targeting moiety in assays of inhibition of binding.

These, and other suitable techniques, are described in detail in Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, 1989.

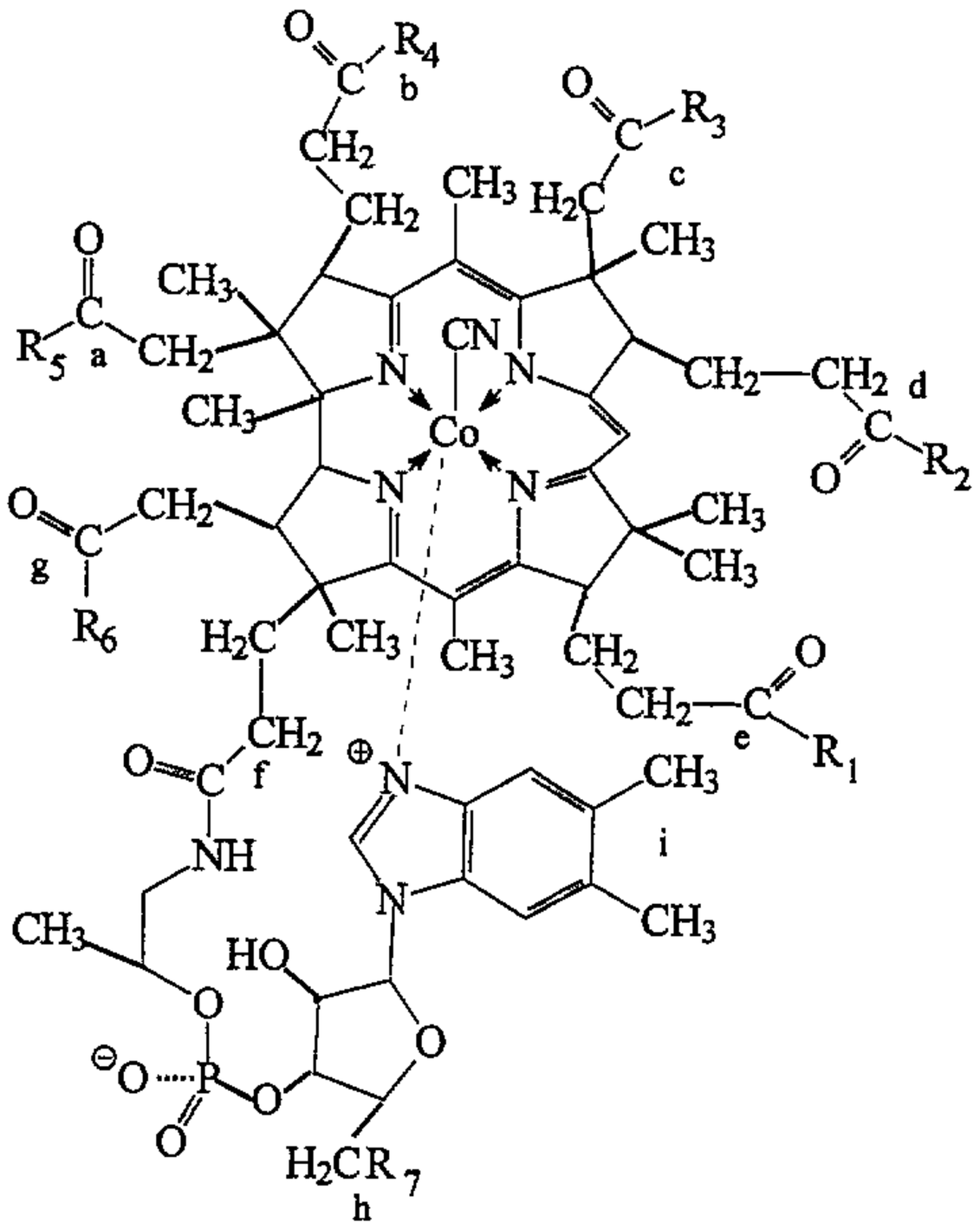
Coupling of a targeting moiety and a rerouting moiety should also not significantly affect the ability of the rerouting moiety to retain or delay the agent/receptor complex within the cell. This may be empirically determined by any one of several methods known in the art, including using labeling techniques to compare intracellular retention of the targeting moiety versus that of the receptor modulating agent as exemplified below.

As noted above, targeting moieties of a receptor modulating agent include any moiety which specifically binds to a cell surface receptor. Suitable targeting moieties include proteins and peptides. Representative examples of suitable targeting moieties include peptides such as bombesin, gastrin-releasing peptide, cell adhesion peptides, substance P, neuromedin-B, neuromedin-C and metenkephalin; hormones, including EGF, alpha- and beta-TGF, estradiol, neurotensin, melanocyte stimulating hormone, follicle stimulating hormone, luteinizing hormone, and human growth hormone; proteins corresponding to ligands for known cell surface receptors, including low density lipoproteins, transferrin and insulin; fibrinolytic enzymes; and biological response modifiers, including interleukin, interferon, erythropoietin and colony stimulating factor also constitute targeting moieties of this invention. Moreover, analogs of the above targeting moieties that retain the ability to specifically bind to a cell surface receptor are suitable targeting moieties. Essentially, any analog having about the same affinity as a targeting moiety, herein specified, could be used in synthesis of receptor modulating agents.

In a preferred embodiment, a targeting moiety is a vitamin B_{12} molecule. Vitamin B_{12} is an essential nutrient for dividing cells. By inhibiting its uptake, the growth of dividing cells can be halted. The cell surface receptor for vitamin B_{12} is the transcobalamin II/vitamin B_{12} ("TcII/B_{12}") receptor, which is characterized by a high affinity for the carrier protein, transcobalamin II (TcII), when complexed with vitamin B_{12} ("TcII/B_{12} complex"). The TcII/B_{12} receptor does not recognize vitamin B_{12} alone, but does recognize the carrier protein TcII with reduced affinity when not complexed with vitamin B_{12}. In many respects, this receptor system is similar to that for transferrin/iron in that the goal of the receptor system is to deliver vitamin B_{12} into cells such that it can be utilized by enzymes involved in DNA synthesis. Within the context of the present invention, the term "vitamin B_{12}" refers to the class of

compounds known as cobalamins and derivatives thereof, including, by way of example, cyanocobalamin. The term "vitamin B_{12}" is used interchangeably with the term cyanocobalamin.

Suitable vitamin B_{12} molecules includes any vitamin B_{12} capable of coupling to another molecule while maintaining its ability to form a TcII/B_{12} complex. A preferred vitamin B_{12} targeting moiety is generally comprised of a vitamin B_{12} molecule, such as a cyanocobalamin, and a linker, described in detail below. The linker may be coupled to any one of several sites on a vitamin B_{12} molecule, including potential carboxyl coupling sites *a*- through *g*-, an alcohol (ribose) coupling site ("coupling site *h*") or a benzimidazole coupling site ("coupling site *i*.") (*See* structure I below.) Preferably, a linker is coupled to coupling sites *b*-, *d*- or *e*- on a vitamin B_{12} molecule. Even more preferably, a linker is coupled to coupling site *d*- or *e*-. This embodiment of the present invention includes compounds represented by the following formula:



STRUCTURE I

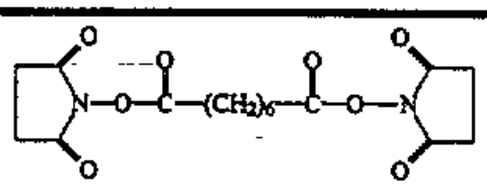
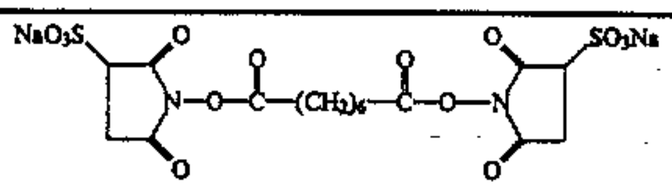
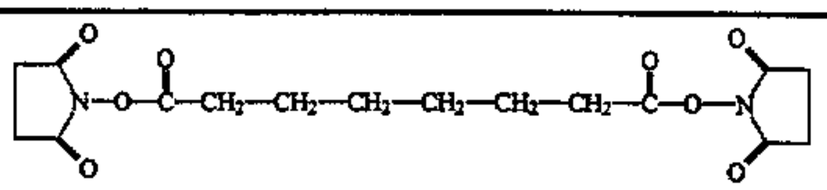
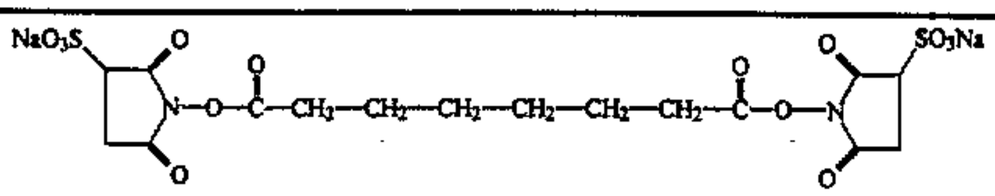
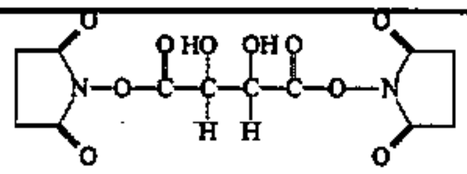
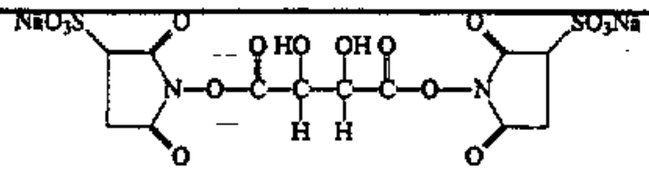
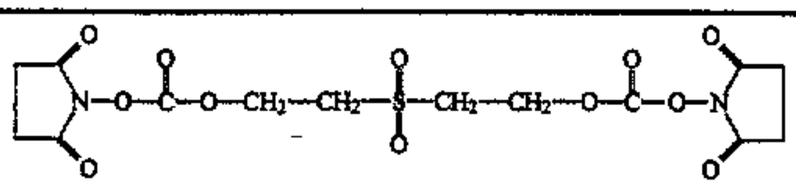
wherein at least one of R_1, R_2, R_3, R_4, R_5, R_6, and R_7 is a linker. One of ordinary skill in the art will appreciate that a number of other coupling sites on the vitamin B_{12}

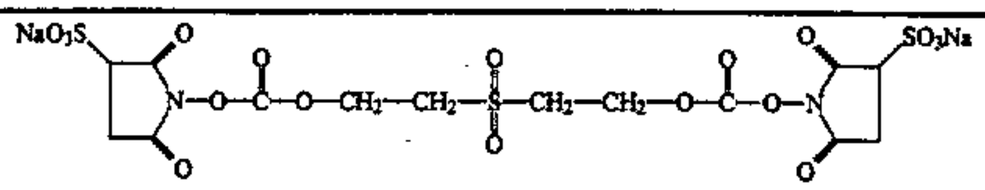
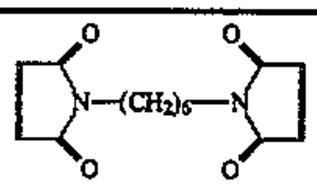
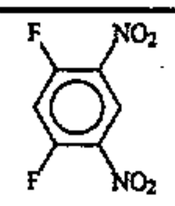
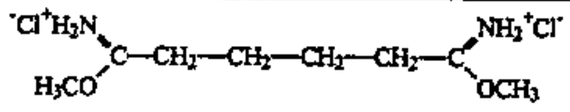
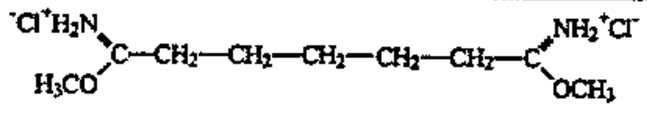
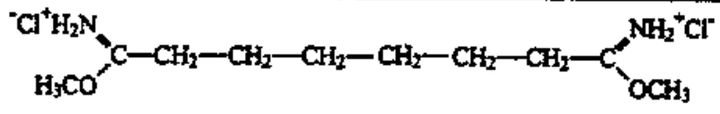
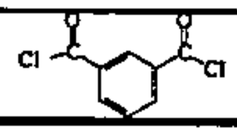
molecule may be chemically altered without affecting coupling of the molecule with a linker or TcII. Coupling sites which are not occupied by a linker may have a variety of chemical moieties attached thereto, including an amino, secondary amino, tertiary amino, hydroxy, lower alkyl, lower alkoxy, alkoxyalkyl, alkoxyalkoxy, cycloalkylalkoxy, and thioalkyl groups.

In a preferred embodiment, R_1, R_2 or R_4 is a linker and the remaining R groups are $-NH_2$, with the exception of R_7, which is preferably -OH. In an especially preferred embodiment, R_2 is a linker, R_1, R_3-R_6 are $-NH_2$ and R_7 is -OH.

In another preferred embodiment, R_7 is a linker and R_1-R_6 are $-NH_2$.

TABLE 1
HOMOBIFUNCTIONAL LINKERS

	disuccinimidyl suberate (DSS)*
	bis(sulfosuccinimidyl) suberate (BS^3)*
	disuccinimidyl suberate (DSS)*
	bis(sulfosuccinimidyl) suberate (BS^3)*
	disuccinimidyl tartarate (DST)*
	disulfosuccinimidyl tartarate (Sulfo-DST)*
	bis[2-(succinimidooxycarbonyloxy)ethyl]sulfone BSOCOES)*

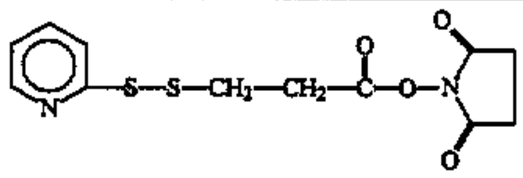
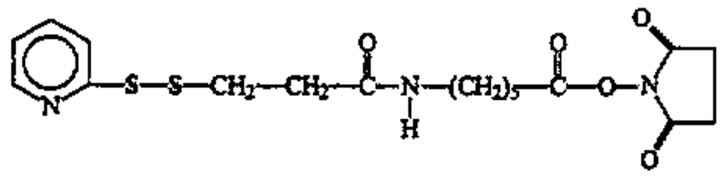
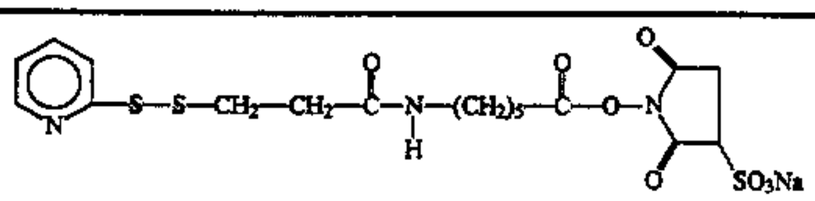
	bis[2-(sulfosuccinimidooxycarbonyloxy)ethyl]sulfone (Sulfo-BSOCOES)*
	bismaleimidohexane (BMH)*
	1,5-Difluoro-2,4-dinitrobenzene (DFDNB)*
	dimethyl adipimidate-2 HCl (DMA)*
	dimethyl pimelimidate-2 HCl (DMP)*
	dimethyl subevimidate-2 HCl (DMS)*
	isophthaloyl dichloride**

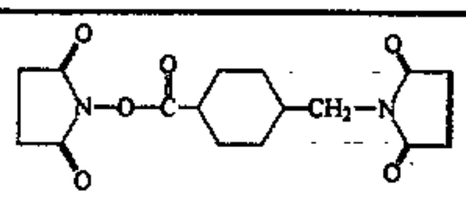
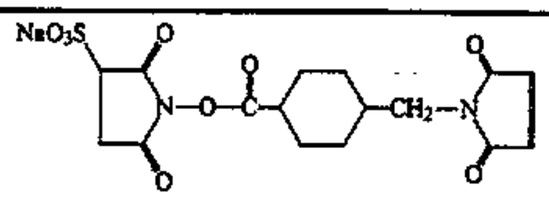
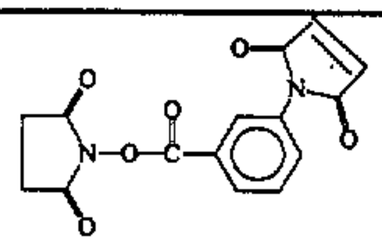
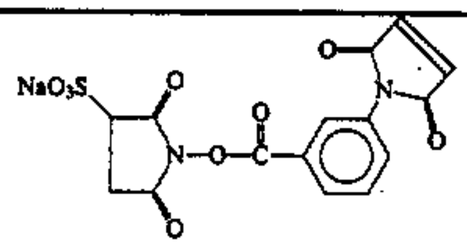
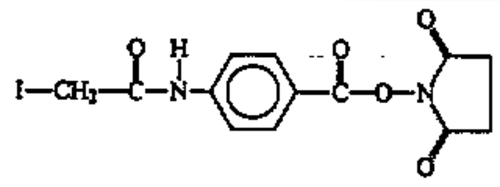
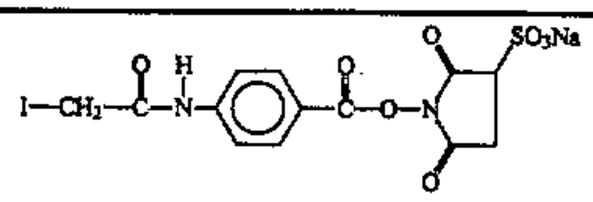
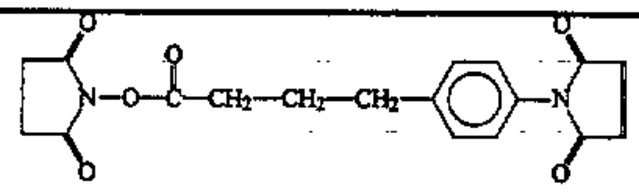
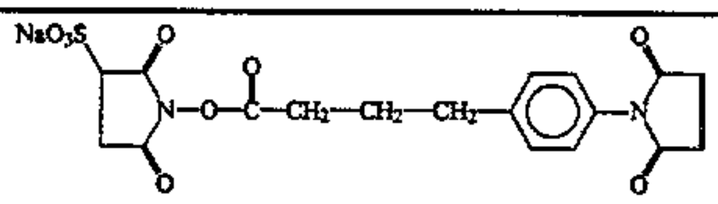
*Pierce Chemical, Co., Rockford, Illinois

**Aldrich Chemical Co., Milwaukee, Wisconsin

TABLE 2

HETEROBIFUNCTIONAL LINKERS

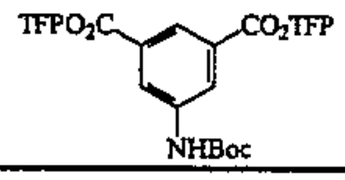
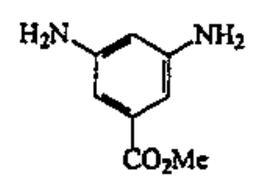
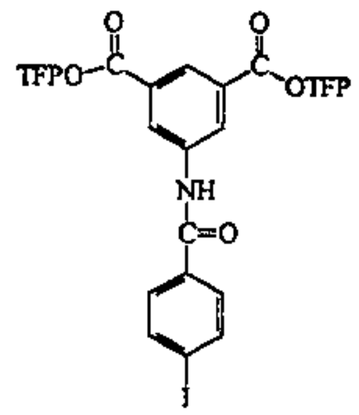
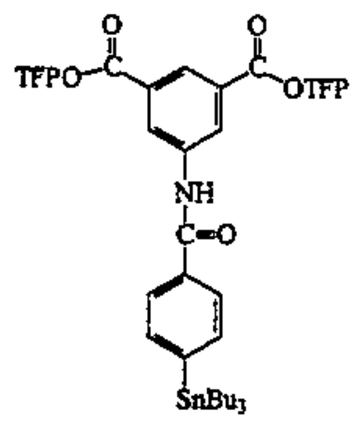
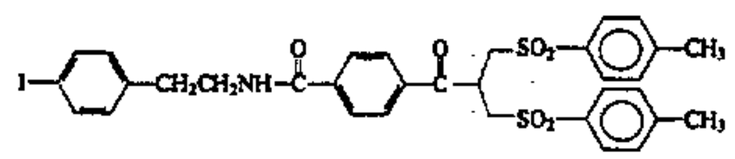
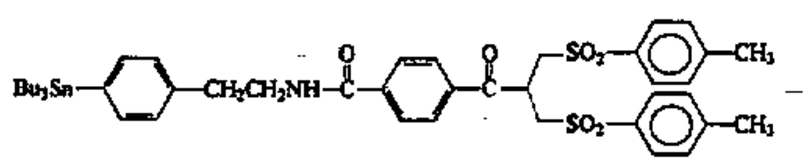
	N-succinimidyl-3-(2-pyridyldithio)propionate (SPDP)*
	succinimidyl 6[3(2-pyridyldithio) propionamido] hexanoate (LC-SPDP)*
	sulfosuccinimidyl 6-[3-(2-pyridyldithio) propionamido] hexanoate (Sulfo-LC-SPDP)*

	succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (SMCC)*
	sulfosuccinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (Sulfo-SMCC)*
	m-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS)*
	m-maleimidobenzoyl-N-hydroxysulfosuccinimide ester (Sulfo-MBS)*
	N-succinimidyl(4-iodoacetyl)aminobenzoate (SIAB)*
	sulfosuccinimidyl(4-iodoacetyl)aminobenzoate (Sulfo-SIAB)*
	succinimidyl-4-(*p*-maleimidophenyl)butyrate (SMPB)*
	sulfosuccinimidyl-4-(*p*-maleimidophenyl)butyrate (Sulfo-SMPB)*

*Pierce Chemical, Co., Rockford, Illinois

TABLE 3

TRIFUNCTIONAL LINKERS

	Derived from 5-amino isophthalic* acid - unreported synthesis (D.S. Wilbur, D.K. Hamlin, University of Washington)
	Derived from 3,5-diaminovbenzoic acid* - unreported synthesis
	5-(*p*-iodobenzoyl)amino-1,3-isophthaloyl ditetra-fluorophenyl ester - unreported synthesis (D.S. Wilbur, D.K. Hamlin, University of Washington)
	5(*p*-tri-N-butylisomylbenzoyl)-amino-1,3-isophthaloyl ditchtrafluorophenyl ester - unreported synthesis (D.S. Wilbur, D.K. Hamlin, University of Washington)
	D.S. Wilbur et al., Bioconjugate Chem. 5(3):220-235, 1994.
	D.S. Wilbur et al., Bioconjugate Chem. 5(3):220-235, 1994.

*Aldrich Chemical Co., Milwaukee, Wisconsin

Suitable linkers include any one of several linkers, preferably containing at least two coupling or reactive groups, allowing the linker to bind to both vitamin B_{12} and a rerouting moiety. In the context of the present invention, the terms "coupling group" and "reactive group" are used interchangeably. By way of example, a linker may be homobifunctional, heterobifunctional, homotrifunctional, or heterotrifunctional. Homobifunctional agents may facilitate cross-linking, or dimerization of vitamin B_{12}

molecules in a single step, hence a coupling reaction using these agents should be performed with an excess of homobifunctional agents, unless dimerization is the desired result, as in the synthesis of dimers described in detail below.

Suitable homobifunctional agents include those listed in Table 1, as well as those described in detail below. Heterobifunctional agents facilitate cross-linking in a stepwise method, allowing more than one linker to be incorporated and a variety of targeting agents such as vitamin B_{12} molecules to be linked. Suitable heterobifunctional agents include those listed in Table 2 as well as those described in detail below. Homo- and hetero- trifunctional linkers are coupled to a rerouting moiety and a vitamin B_{12} molecule as described above, with the additional advantage of a third coupling site on the linker. One of ordinary skill in the art will appreciate that this allows for any number of different molecules to couple with the rerouting moiety, including, by way of example, markers, such as radiolabeled and fluorescent molecules; proteins and peptides, such as antibodies; and conjugating molecules, such as biotin. Suitable trifunctional linkers are listed in Table 3. Homobifunctional, heterobifunctional, homotrifunctional, and heterotrifunctional linkers are commercially available.

Suitable linkers are generally relatively linear molecules greater than 4 atoms in length, typically between 6 and 30 atoms in length, and preferably are 8 to 20 atoms in length. In a particularly preferred embodiment, the linker is a linear molecule of 12 atoms in length. In the context of the present invention, the term "atom" refers to a chemical element such as, by way of example, C, N, O, or S. The ranges provided above are based on the relatively linear accounting of the linker. One of ordinary skill in the art will appreciate that a linker may be linear, branched, and even contain cyclical elements.

Coupling or reactive groups include any functional group capable of coupling a linker to a vitamin B_{12} molecule. Suitable coupling groups include, nucleophilic and electrophilic functional groups. Suitable nucleophilic groups include hydroxy groups, amino groups, and thio groups. Suitable electrophilic groups include carboxylic acid groups and carboxylic acid derivatives including acid halides, acid anhydrides, and active esters such as NHS esters.

Suitable homobifunctional linkers include, by way of example, diaminoalkanes, such as those represented by the formula $NH_2(CH_2)_xNH_2$, wherein x = 2-20. A preferred linker is a diaminododecane. Suitable heterobifunctional linkers include those represented by the formula $NH_2(CH_2)_yCOOH$, wherein y = 3-12. Those

of ordinary skill in the art will appreciate that a protecting group may be necessary when utilizing a heterobifunctional group.

A linker may be coupled to the preferred *b*-, *d*- or *e*- coupling sites (*see* Structure I above) by any one of several suitable means, including, by way of example, activating a vitamin B_{12} molecule by hydrolyzing its propionamide groups to produce monocarboxylates, purifying the resulting monocarboxylates, and coupling a linker to a selected coupling site. Hydrolysis of the coupling sites may be accomplished by exposing vitamin B_{12} to aqueous acid for a period of time and under suitable conditions to hydrolyze the desired propionamide groups. Preferably, hydrolysis is performed by exposure of the amide to dilute aqueous acid for a period of about 6 to 12 days, typically about 9 to 11 days, and most preferably about 10 days at room temperature. Suitable aqueous acids include, by way of example, 0.1N hydrochloric acid, 0.5N phosphoric acid or 0.5N sulfuric acid.

Purification of *b*-, *d*- and *e*- monocarboxylates can be accomplished by any one of several means, including column chromatography, such as gel permeation chromatography, adsorption chromatography, partition chromatography, ion exchange chromatography, and reverse phase chromatography. Preferably, column chromatography is preparative reverse phase liquid chromatography. These techniques are described in detail in Lim, HPLC of Small Molecules, IRL Press, Washington, D.C., 1986. Purification of monocarboxylates by preparative liquid chromatography (LC) should be accomplished at a very slow flow rate. For example, LC purification may be conducted at a flow rate of 0.15 mL/min. on a 5 μm, 4.6 X 250 mm propylamine column (RAININ microsorb-MV amino column) eluting with 58 μM pyridine acetate, pH 4.4 in H_2O : THF (96 : 4) solution. Even more preferably, the coupling reaction is monitored using analytical high pressure liquid chromatography (HPLC). Reverse-phase HPLC chromatography is preferably carried out using an analytical version of above-noted propylamine column using a gradient solvent system at a flow rate of 1 mL/min. Within the context of the present invention, the *d*- isomer is identified as the longest retained peak (third), the *e*- isomer is identified as the second retained peak, and the *b*- isomer is identified as the shortest retained peak (first) eluted from the LC column. The *d*- isomer may also be identified as that vitamin B_{12} derivative demonstrating the greatest biological activity as noted below.

A ribose coupling site (coupling site *h*, *see* structure I) may be activated by any one of several suitable means including, activating a hydroxyl group at coupling site *h* by reaction with a suitable reagent (*e.g.*, succinic anhydride), to yield a ribose derivative which bears a reactive group (*e.g.*, a carboxylate group). This technique is

described in detail in Toraya, Bioinorg. Chem. 4:245-255, 1975. Separation and purification of the activated molecule may be accomplished on a C18 column as noted below. Once coupling site *h* has been activated, a linker may be coupled to this site in the same manner as described below.

After activating the vitamin B_{12} molecule at a selected coupling site, linkers may be coupled to a vitamin B_{12} molecule to form a vitamin B_{12} linker adduct using any one of several means, including, by way of example, an amide forming reaction, employing an amine group on the linker and a carboxylate coupling site on a vitamin B_{12} molecule. Alternatively, a linker may be coupled to a vitamin B_{12} molecule through an amide forming reaction, employing a carboxylate group on the linker and an amino group on a B_{12} molecule. The amide forming reaction may include the use of a coupling agent. Suitable coupling agents include carbodiimide coupling agents, such as, by way of example, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), 1-benzyl-3-(3-dimethylaminopropyl) carbodiimide (BDC), 1-cyclohexyl-3-(2-morpholinyl-4-ethyl)carbodiimide (CMC), and 1,3-dicyclohexylcarbodiimide (DCC). Preferably, the coupling agent is water soluble. Even more preferably, the coupling agent is EDC.

Alternatively, the amide forming reaction coupling the linker to a B_{12} molecule may employ a reactive carboxylic acid group and an amine. Suitable reactive carboxylic acid groups include carboxylic acid derivatives which yield an amide upon reaction with an amine. Such reactive groups include, by way of example, any reactive carboxylic acid derivative, including, by way of example, carboxylic acid halides, such as acid chlorides and bromides; carboxylic acid anhydrides, such as acetic anhydrides and trifluoroacetic anhydrides; esters, such as p-nitrophenyl esters and N-hydroxysuccinimide esters. Such techniques are described in detail in Bodanszky, Principles of Peptide Synthesis, Springer Verlag, Berlin, 1984.

Although coupling of a linker through a cyano coupling site is possible it is not preferred, due to the instability of linkers coupled to this site. Dolphin, D., [205] Methods Enzymol. 18C:34-52, 1971. Additionally, a linker may be coupled to a benzimidazole (coupling site *i*, *see* Structure I) using techniques described in detail in Jacobsen, Anal. Biochem. 113:164-171, 1981.

Vitamin B_{12} linker adducts may be separated and purified using any suitable means, including column chromatography, such as gel permeation chromatography, adsorption chromatography, partition chromatography, ion exchange chromatography, and reverse phase chromatography. Preferably, column

chromatography is preparative LC. These techniques are described in detail in Lim, HPLC of Small Molecules, IRL Press, Washington, D.C., 1986.

As noted above, the vitamin B_{12} receptor modulating agents of the present invention must be capable of binding transcobalamin II. The ability of a receptor modulating agent to bind TcII may be ascertained using any one of several means known in the art, including competitive binding assays with the receptor modulating agent competing with native vitamin B_{12}.

Rerouting moieties of the present invention include any moiety which is capable of affecting the receptor trafficking pathway. This characteristic can be assessed by employing a receptor modulating agent having a radiolabeled targeting moiety and following its path through the cell. This is accomplished using techniques known in the art, including using radiolabeled, biotinylated, or FITC labeled targeting moiety followed by binding assays, ELISA, or flow cytometry. A preferred receptor modulating agent is one which results in the removal of the highest percent of receptor for the longest period of time.

Suitable rerouting moieties of this invention do not significantly detract from the selectivity of the targeting moiety. Whether a rerouting moiety detracts from the selectivity of a targeting moiety may be determined by any one of several methods known in the art, including comparing binding of the receptor modulating agent on receptor positive and receptor negative cells, as assessed by ELISA, flow cytometry, or other binding assays.

Rerouting moieties cause the retention/degradation of an agent/receptor complex within at least one cell type, but not necessarily in all cells. In like fashion, a rerouting moiety causes retention of an agent/receptor complex in some cells, but not necessarily other agent/receptor complexes in other cells. Different rerouting moieties may also distinguish between receptor species, for example, as in polarized epithelium where the same receptor may independently traffic on the apical, basal, or basolateral sides of the cell. To determine if a particular rerouting moiety is suitable, a rerouting moiety is covalently attached to the targeting moiety, and the resulting receptor modulating agent is compared for receptor modulation on different receptor-bearing cells using binding or functional assays known in the art.

Suitable rerouting moieties of this invention may be categorized into five different functional classes: (1) lysosmotropic moieties; (2) intracellular polymerizing moieties; (3) protein sorting signals or sequences; (4) conditional membrane binding peptides; and (5) bi- or multi-valent receptor cross linking moieties. While such rerouting moieties may have different functional mechanisms of action, all promote

retention of the agent/receptor complex within the intracellular vesicular system. All of these classes of rerouting moieties will impart the ability to affect the receptor trafficking pathway.

In one aspect of the present invention, a first functional class of rerouting moieties, lysosomotropic moieties, are disclosed. Within the context of the present invention, the term "lysosomotropic moieties" refers to moieties which route the agent/receptor complex to the lysosomes. Numerous suitable lysosomotropic moieties are known, and are reviewed in Biochem. Pharmacol. 23:2495-2531, 1974.

A preferred lysosomotropic moiety includes an aminoglycoside antibiotic marked by the characteristic ability to accumulate in lysosomes after intracellular protonation. Intracellular protonation occurs in the increasingly acidic conditions which occur during the transfer from early to late endosomes and, finally, to the lysosome. Strong positive charges prohibit the lysosomotropic moiety from leaving the membrane-enclosed vesicles, thus trapping the agent/receptor complex in the vessel.

Aminoglycoside antibiotics are similar in structure, but are divided into structurally related families of compounds based upon the sugar units. Each of the families of aminoglycoside antibiotics, as well as representative members thereof, are set forth in Figures 2-5. These families include gentamycin, kanamycin, neomycin and streptomycin. The gentamycin family includes gentamycin C_1, gentamycin C_2, gentamycin C_{1a}, sisomicin and netilmicin; the kanamycin family includes kanamycin A, tobramycin and amikacin; the neomycin family includes neomycin B, paromomycin, ribostamycin and bytirosin B; and the streptomycin family includes streptomycin A and streptomycin B.

In a particularly preferred embodiment of the present invention, the rerouting moiety is gentamycin, which accumulates in lysosomes in concentration as much as 300 fold that of the extracellular concentration (J. Pharmacol. Exp. Ther. 255:867-74, 1990; Ren. Fail. 14:351-7, 1992).

Suitable aminoglycosides have reactive amine groups capable of being coupled through peptide or other chemical linkers. Thus, a targeting moiety may be readily attached via covalent linkage to these rerouting moieties using any one of several techniques known in the art to form covalent bonds, for example, using thioether, disulfide, ether, ester and peptide bonds. Since many of the aminoglycoside antibiotics have several amines which could be derivatized in a conjugation procedure, a primary amine contained in these compounds can be selectively reacted to favor covalently attachment to the targeting moiety through this amine (*see* amine indicated with arrow in Figures 2-4). With regard to streptomycin, covalent attachment to the

targeting moiety may be accomplished by converting the aldehyde moiety indicated in Figure 5 to an amine, and attaching to the targeting moiety using carbodiimide or other suitable activated carboxylic acid. Aminoglycosides are water soluble and do not readily bind to other proteins, and thus do not impart non-specific binding to a receptor modulating agent.

Particularly preferred aminoglycosides include those which allow for preferential derivation of a selected amine. Specifically, preferred aminoglycosides include those compounds to which protective groups can be added to various nitrogen atoms thereof and, subsequently, selectively deprotected to yield a single free amine. The free amine can be further derivatized, for example, by addition of a peptide linker or covalently attached directly to the targeting moiety. These rerouting moieties include ribostamycin (*see* Figure 4), kanamycin (*see* Figure 3), amikacin, and streptomycin. Ribostamycin is particularly preferred, due to its relative low toxicity and its derivatization chemistry, allowing an acyl migration reaction to be effected on a hydroxyl protected ribostamycin to yield a single amine adduct. Kanamycin may also be used in a selective protection/acylation reaction; Amikacin is commercially available in a form which allows attachment without deprotecting its amines or alcohol groups; and streptomycin can also be readily derivatized by protonating guanidinium groups under physiologic conditions to provide the polycations necessary for cellular or lysosomal retention.

In another aspect of the present invention, non-aminoglycoside lysosomotropic compounds which may accumulate after intracellular protonation are also suitable rerouting moieties (*see* Figure 6). Suitable non-aminoglycoside compounds exhibiting this characteristic are known in the art, a series of aminoacridine and amino quinoline dyes, typified by cholquinine and quinacrine; a group of amino naphthalenes, typified by dansyl cadaverine; and derivatives thereof. Such dyes are characterized by cellular retention and low toxicity. All of these compounds have characteristic sites for covalent attachment to a targeting moiety via the nitrogen indicated in Figure 6 and may be attached thereto as described above.

Another aspect of the present invention utilizes a lysosomotropic peptide subject to charge modification under intracellular conditions is employed as a rerouting moiety. Once charge-modified, the rerouting peptide acts to retain an agent/receptor complex in the intracellular vesicular system until membrane flow delivers it to the lysosome for degradation. Preferably, these peptides are capable of being phosphorylated by intracellular protein kinases. When phosphorylated by the intracellular enzymes, such peptides would be highly anionic.

Charge-based retention can be an inherent property of the rerouting peptide or can be imparted by intracellular modification. Intracellular modification may be accomplished by any of several means known in the art, including phosphorylation of certain residues of some receptors (*e.g.*, the EGF receptor) may cause intracellular rerouting (Cancer Treat. Res. 61:139-160, 1992; J. Cell. Biol. 116:321-30, 1992).

The rerouting peptides may be covalently attached to a targeting moiety by any means, including, for example, covalently linking the peptide directly to the targeting moiety, or by use of an appropriate linker moiety, such as G-G-G, which may be derivatized and covalently attached to the targeting moiety.

Preferred rerouting peptides include protein kinase-substrate peptides that incorporate serine. These peptides are particularly preferred for enhancement of receptor rerouting in tumor target cells, which have increased levels of protein kinase activity for serines or tyrosines. Increased levels of kinase activity within tumor cells may be attributed to the presence of oncogene products, such as H-ras, on the cytoplasmic side of tumor cell plasma membranes (C.I.B.A. Found. Symp. 164:208-18, 1992).

Suitable rerouting peptides also include protein kinase substrates and peptides that possess a single positive charge. The latter type of rerouting peptide may form an ion pair with a "glutamate-like" residue of an attached or closely associated residue(s) of the receptor. Particularly preferred rerouting peptides may be derived, using technologies known in the art, from the proteins and the amino acid sequences identified in Table 4.

TABLE 4
REROUTING PEPTIDES

PEPTIDE SOURCE	AMINO ACID SEQUENCE
EGF receptor	DVVDADEYLIPQ
EGF fragment	CMHIESLDSYTC
Phosphorylase kinase	RTKRSGSVYEPLKI
Protein kinase C pseudosubstrate	RFARK-GALRQKNV
Myelin basic protein	S/T-XAA-K/R (where XAA is an uncharged residue)
Kemptide	RGYALG or RGYSLG
Glycogen synthetase	PLSRTLSVAA

Transferrin receptor	FSLAR
III histone	ASGSFKL
Casein kinase II substrate	AAAAAAASEEE or AAAAAAASDDD
Insulin receptor auto-phosphorylation substrate	DIYETDYYR
calmodulin-dependent protein kinase II	Waxman and Arenowski Biochem. 32(11):2923-30, 1993
Neurogranin	Chen et al., Biochem. 32(4):1032-9, 1993
MARCKS	Heemskerk et al., Biochem. Biophys. Res. Commun. 190(1):236-41, 1993
Glycogen synthase	Marais et al., FEBS Letters 277:151-5, 1990
Ribosomal protein S6	Munro et al., Biochem. Biophys. Acta 1054:225-30, 1990
Co-polymers which serve as substrates for protein kinase A, C, P	Abdel-Ghony et al., Proc. Nat'l. Acad. Sci. 86:1761-5, 1989; Abdel-Ghony et al., Proc. Nat'l. Acad. Sci. 85:1408-11, 1988
Serine-threonine kinases	Abdel-Ghony et al., Proc. Nat'l. Acad. Sci. 86:1761-5, 1989; Abdel-Ghony et al., Proc. Nat'l. Acad. Sci. 85:1408-11, 1988

In another aspect of the present invention, the rerouting moiety is a lysosomotropic amino acid ester which, in high concentration, can cause the lysis of granule containing cells, such as NK cells, cytolytic T cells and monocytes. The concentration must generally be maintained below 100 mM to avoid lysis. Suitable lysosomotropic amino acid esters and their sources are presented in Table 5.

TABLE 5
LYSOSOMOTROPIC AMINO ACID ESTERS

Leu-O-Me	Res. Immunol. 143:893-901, 1992 Eur. J. Immunol. 23:562-5, 1993 Intl. Arch. Aller. & Immunol. 100:56-59, 1993 Cell. Immunol. 139:281-91, 1992 Exp. Pathol. 42:121-7, 1991

Iso-leu-O-Me	Res. Immunol. 143:893-901, 1992
L-Val-O-Me	J. Immunol. 134:786-93, 1985
Phe-O-Me	J. Immunol. 148:3950-7, 1992 Blood 79:964-71, 1992
Phe-, Ala-, Met-, Trp-, Cys-, Try-, Asp-, & Glu-O-Me	Int. J. Immunopharmacol. 13:401-9, 1991

The lysosomotropic amino acid esters identified in Table 5 can be used to retain the agent/receptor complex in lysosomes after intracellular cleavage of the ester. In one embodiment, such amino acid esters may be utilized as the C-terminal portion of a larger peptide containing a linker sequence and/or a phosphorylation substrate sequence, and with suitable residues, such as cysteine, for covalent attachment to a targeting moiety, such as a sequence encoding a peptide or protein ligand for a given cell surface receptor.

In another embodiment of the present invention, a second functional class of rerouting moieties is disclosed. This class includes peptides which undergo polymerization within endosomes or lysosomes, inhibiting their passage through intracellular membranes.

Intracellular polymerizing compounds can be incorporated into a larger peptide containing the targeting moiety and a linker. Suitable peptides include the dipeptide ester referenced in Table 5 (*i.e.*, L-Leucyl-L-Leucine-O-Me). When transported into cells, these dipeptide esters preferentially accumulate in lysosomes and secondary granules of cytotoxic cells. These dipeptides also undergo self-association and polymerization, which results in trapping at low concentrations, and membrane rupture at higher concentrations.

TABLE 6

POLYMERIZING DI-PEPTIDE ESTER: L-LEUCYL-L-LEUCINE-O-ME

J. Invest. Dermat. 99:805-825, 1992
J. Clin. Invest. 84:1947-56, 1989
Transpl. 53:1334-40, 1992
J. Immunol. 138:51-7, 1987
J. Immunol. 148:3950-7, 1992

J. Immunol. 136:1038-48, 1986
Cryobiology 29:165-74, 1992
Acta. Biochem Biophys. Hung 24:299-311,1989
Blood 79:964-71, 1992
Blood 78:2131-8, 1991
J. Immunol. 139:2137-42, 1987
J. Exp. Med. 172:183-194, 1990
J. Clin. Invest. 78:1415-20, 1986
PNAS 87:83-7, 1990
J. Immunol. 137:1399-406, 1986
PNAS 82:2468-72, 1985

Suitable intracellular polymerizing compounds also include peptides that can self-associate into alpha-helical structures termed "leucine zippers". In the context of this invention, such structures may be used to form intracellular polymers that are incapable of exiting intracellular vesicles. Such sequences can be selected by observing self association of the compounds in solution, and the formation of polymers capable of binding to DNA. Suitable peptide sequences that can self-associate into alpha helical structures are presented in Table 7.

TABLE 7

LEUCINE ZIPPERS

Boc(t-butoxycarbonyl)-Aib(alpha-aminoisobutyryl) Glu(OB$_n$l)-(benzoyl ester)-Leu-Aib-Ala-Leu-Aib-Ala-
Boc-Aib-Leu-Aib-Aib-Leu-Leu-Aib-Leu-Aib-O-Me Proteins 12:324-30, 1992 Lys(Z)(benzyloxy-carbonyl)-Aib-O-Me PNAS 87:7921-5, 1990
GELEELLKHLKELLKGER Biochem. 31:1579-84, 1992

In another embodiment of the present invention, a third functional class of rerouting moieties is disclosed. This class includes moieties that can be recognized by intracellular receptors. Such sequences are identified by their ability to stop movement of endogenously synthesized proteins to the cell surface. Suitable peptides include certain peptide sequences (such as sorting or signal sequences) associated with the trafficking of endogenously synthesized proteins (Cur. Opin. Cell. Biol. 3:634-41, 1991). Such peptide sequences, when covalently attached to the C-terminus of an exogenously added targeting moiety, result in the retention of the agent/receptor complexes in the endoplasmic reticulum ("ER"), Golgi apparatus, or lysosomes.

Such peptide sequences are recognized by intracellular receptors, examples of which include both mammalian and bacterial versions of ER receptors described in detail in J. Cell. Biol. 120:325-8, 1993; Embo. J. 11:4187-95, 1992; Nature 348:162-3, 1990. Further exemplary peptide sequences and variants thereof (shown in parentheses) that can be recognized by intracellular receptors are set forth in Table 8, Sections A and B.

Certain signal sequences may be preferred for retention by one type of organism versus another type. For example, REDLK is a preferred sequence recognized by prokaryotic cells and to a lesser degree by eukaryotic cells (*see* Table 8, section C). Thus, employing this sequence as the rerouting moiety, receptor modulating agents can be constructed to selectively inhibit a receptor-mediated process in bacteria, while having little effect on mammalian cells.

TABLE 8

PEPTIDE SEQUENCES WHICH BIND INTRACELLULAR RECEPTORS

A. Endoplasmic Reticulum or Golgi Retention Peptides	
1. KDEL (DKEL, RDEL, KNEL, SDEL, KEEL, QDEL, KEDL, KDEL)	J. Biol. Chem. 265:5952-5, 1990 Biochem. Biophys. Res. Commun. 172:1384-91, 1990 J. Virol. 65:3938-42, 1991 Exp. Cell Res. 197:119-24, 1991 Growth Factors 5:243-53, 1991 J. Biol. Chem. 267(10):7022-6, 1992 J. Biol. Chem. 267:10631-7, 1992 J. Cell. Biol. 118:795-811, 1992 J. Cell. Biol. 119:85-97, 1992 Exp. Cell. Res. 203:1-4, 1992 P.N.A.S. 90:2695-9, 1993 Mol. Biochem Parasitol 48:47-58, 1991 Embo J. 4:2345-55, 1992 J. Biol. Chem. 266:14277-82, 1991 Mol. Cell Biol. 11:4036-44, 1991
2. HDEL (HVEL, HNEL, HTEL, TEHT, DDEL, HIEL)	J. Biol. Chem. 268:7728-32, 1993 Mol. Biochem Parasitol 57:193-202, 1993 J. Cell SCI 102:261-71, 1992 Eur J. Biochem. 206:801-6, 1992 J. Biol. Chem. 266:20498-503, 1991
3. ADEL	Embo J. 11:1583-91, 1992
4. REDLK	J. Biol. Chem. 266:17376-81, 1991
5. SEKDEL	Growth Factors 5:243-53, 1991
6. KTEL	J. Virol. 66:4951-6, 1992
B. Lysosomal Retention Peptides	
1. KFERQ	Trends Biochem SCI 15:305-9, 1990
2. Tyrosine-containing polypeptides	J. Cell Biol. 111:955-66, 1990
C. ORGANISM-SPECIFIC RETENTION PEPTIDES	
1. REDLK	J. Biol. Chem. 266:17376-17381, 1991

D. CLATHRIN-BINDING PEPTIDES (INTERNALIZATION SIGNALS)	
1. LLAV	J. Cell. Biol. 199:249-57, 1992
2. YKYSKV	J. Cell. Biol. 199:249-57, 1992 Embo. J. 7:3331-6, 1988
3. PPGYE	Cell 67:1203-9, 1991 Curr. Opin. Cell Biol. 3:1062, 1991

A further class of peptide sequences of this invention, termed "internalization signals," function by binding to clathrin, both in the coated pits, as well as those intracellular vesicles which maintain a clathrin coat. Representative examples of such clathrin-binding peptides (CBP) are disclosed in Table 8, section D. The CBP binds clathrin in the coated pits initially located on the cell surface causing retention of the targeting moiety to which it is conjugated.

A further class of moieties capable of recognizing intracellular receptors includes carbohydrates. Suitable carbohydrates include any carbohydrate which is capable of binding to intracellular carbohydrate (CHO) receptors but not cell surface CHO receptors. Such carbohydrates include: mannose-6-phosphate and glucose-6-phosphate. Suitable carbohydrate moieties include those which bind to the insulin-like growth factor II/mannose-6-phosphate (IGF II/M6P) receptor, include analogs of mannose-6-phosphate, as well as other phosphorylated saccharides (Carbohydrate Res. 213:37-46, 1991; FEBS Lett. 262:142-4, 1990).

The affinity of the rerouting moiety can be varied by changes in the chemical nature of the phosphorylated saccharides (J. Biol. Chem. 264:7970-5, 1989; J. Biol. Chem. 264:7962-9, 1989) (monosaccharides bind with the lowest affinity, while di- or tri-saccharides bind with increasingly higher affinity). Clustering of phosphorylated saccharides on protein carriers can dramatically increase affinity to the intracellular receptor.

Synthesis of various oligosaccharides are reviewed in Sem. Cell. Biol. 2:319-326, 1991. Although, mannose-6-phosphate receptor expression is primarily intracellular, expression also occurs on cell surfaces. Thus, in the context of the present invention, covalent attachment of a targeting moiety with a carbohydrate which binds the mannose-6-phosphate receptor should be constructed so as to give at least 100-fold difference in binding affinity between the targeting moiety and the rerouting moiety. For example, a vitamin B_{12}/transcobalamin II receptor targeting moiety, in this case vitamin B_{12}, would have a binding affinity for the carrier protein, transcobalamin II

(TcII), of $\geq 10^{-10}$ M and an affinity for the IGF II/M-6-P receptor of 10^{-8} M or less. This will maintain the specificity of the vitamin B_{12} binding (via TcII), while allowing transfer of the receptor modulating agent from serum M-6-P soluble receptor to cell surface receptor.

In addition to IGF II/M-6-P receptor moieties, other carbohydrate-based rerouting moieties also promote retention of the modulating agent/receptor complex in the ER or Golgi complex. Such moieties are based on the recognition by various glycosyl transferases of carbohydrate moieties, either as a natural substrate or as an inhibitor. Such moieties are reviewed in Sem. Cell. Biol. 2:289-308, 1991. For example, saccharide recognition moieties include penultimate sugars, such as glucose and N-acetyl glucosamine (which are natural substrates). More preferred, however, are glycosylation inhibitors which are recognized by glycosyl transferases, but cannot serve to append further carbohydrate residues on growing chains (Sem. Cell. Biol. 2:309-318, 1991) (*see* Figure 7).

In yet another embodiment of the present invention, a fourth functional class of rerouting moieties is disclosed. This class is generally comprised of rerouting moieties which anchor the receptor to the cell membrane. By way of example, this class includes membrane-binding peptides that exhibit conditional pH-dependent membrane binding. Such peptides exhibit α-helical character in acid but not neutral pH solutions. When a conditional membrane-binding peptide assumes a helical conformation at an acidic pH, it acquires the property of amphiphilicity, (*e.g.*, it has both hydrophobic and hydrophilic interfaces). More specifically, within a pH range of approximately 5.0-5.5, such a peptide forms an alpha-helical, amphiphilic structure that facilitates insertion of the peptide into a target membrane. An alpha helix-induced acidic pH environment may be found, for example, in the low pH environment present within cellular endosomes or lysosomes. In aqueous solution at physiological pH, a conditional, membrane-binding peptide is unfolded (due to strong charge repulsion among charged amino acid side chains) and is unable to interact with membranes.

Suitable conditional membrane-binding peptide sequences include the charged amino acids glutamate, aspartate, and histidine. A preferred conditional membrane-binding peptide includes those with a high percentage of helix-forming residues, such as glutamate, methionine, alanine, and leucine. Further, conditional membrane-binding peptide sequences include ionizable residues having pKas within the range of pH 5-7, so that a sufficiently uncharged membrane-binding domain will be present within the peptide at pH 5 to allow insertion into the target cell membrane. Conditional membrane-binding peptides can be incorporated through covalent bonds to

a chemical or peptide targeting moiety or synthesized as an entire peptide sequence including a linker and peptide targeting moiety.

A particularly preferred conditional membrane-binding peptide is aa1-aa2-aa3-EAALA(EALA)$_4$-EALEALAA-amide, which represents a modification of a published peptide sequence (Biochemistry 26:2964, 1987). Within this peptide sequence, the first amino acid residue (aa1) is preferably a unique residue such as cysteine or lysine, that facilitates chemical conjugation of the conditional membrane-binding peptide to a targeting protein. The peptide can also be incorporated into a fusion protein with a protein or peptide targeting moiety (*see* Example 7). Amino acid residues 2-3 (*i.e.*, aa2-aa3) may be selected to modulate the affinity of the translocating peptide for different membranes. For instance, if both residues 2 and 3 are lysine or arginine, the peptide will have the capacity to bind to membranes or patches of lipids having a negative surface charge. If residues 2-3 are neutral amino acids, the peptide will insert into neutral membranes.

Yet another preferred conditional membrane-binding peptide can be derived from sequences of apo-lipoprotein A-1 and B; peptide toxins such as melittin, bombolittin, delta hemolysin and the pardaxins; antibiotic peptides, such as alamethicin; peptide hormones, such as calcitonin, corticotrophin releasing factor, beta endorphin, glucagon, parathyroid hormone, and pancreatic polypeptide. Such peptides normally bind membranes at physiologic pH but through attachment of substituents the peptides can be enhanced in their ability to form alpha-helices at acidic pH and reduced in their membrane-binding at physiologic pH. An example of such a modified peptide having pH-dependent membrane binding at acidic pH is fully succinylated melittin. In this example, a peptide (melittin) that normally binds to membranes at physiological pH is converted to a pH-dependent peptide through succinylation of lysines. Upon succinylation, the peptide displays an amphipathic character only at acidic pHs.

Insertion of a conditional membrane-binding peptide into a target cell membrane is enhanced through stabilization of the amphiphilic alpha helix. Helix stabilization may be achieved: (1) by adding repeating "EALA" units to form a longer peptide; (2) by placing an amide at the C-terminus of the peptide, in order to counteract the helical dipole; (3) by polymerizing the peptide; (4) by substituting a natural helix-former for one or more of the stacked glutamates; or (5) by attaching the peptide to a targeting moiety through use of a longer linker, in order to provide sufficient distance between the membrane binding peptide and the targeting moiety for the peptide to contact and interact with the target cell intracellular membranes.

In yet another embodiment of the present invention, a fifth functional class of rerouting moieties is disclosed. In this context, the rerouting moiety merely functions as a modulating agent in that the moiety disables the receptors by crosslinking the same. This class includes bi- or multi-valent receptor crosslinking moieties formed from monovalent binding targeting moieties. Cross-linking of receptors in some receptor systems is sufficient to cause a rerouting of cell surface receptors to lysosomes for degradation, rather than their normal pathway of receptor recycling. The synthesis of a bivalent receptor modulating agent is exemplified in greater detail in the examples below.

A preferred cross-linking receptor modulating agent is a vitamin B_{12} dimer. In this embodiment, each vitamin B_{12} molecule acts as a targeting agent and a rerouting agent; cross-linking the B_{12} dimer will cross-link the vitamin B_{12} receptors, thus impeding the receptor trafficking pathway. A preferred vitamin B_{12} dimer is generally comprised of two vitamin B_{12} molecules, such as cyanocobalamin, coupled by one or more linkers through coupling sites independently selected from *a-g*, *h* (ribose), and *i* (benzimidazole). Preferably, cross-linking occurs between *d-* or *e-* coupling sites on both molecules. The dimer must be capable of forming a B_{12}/TcII complex. As noted above, this characteristic may be assayed using any one of several techniques known in the art, including competitive binding assays.

A vitamin B_{12} may be coupled to a second vitamin B_{12} molecule in the same manner as described in detail for conjugation of rerouting moieties to vitamin B_{12} targeting moieties. As noted above, dimers may be synthesized using one or more linkers of various lengths and any combination of homobifunctional, heterobifunctional, homotrifunctional, or heterotrifunctional linkers. As noted above, the use of a trifunctional linker allows for coupling with any number of additional moieties.

In selecting a linker for dimer synthesis, it should be noted that the total number of atoms comprising the linker between the vitamin B_{12} molecules should generally be greater than 10 atoms, typically be in the range of 30 to 55 atoms and, preferably be 45. As noted above, one of ordinary skill in the art will appreciate that although the number of atoms is calculated relative to a <u>linear</u> chain of atoms, <u>linear</u> chain, branched chain, and cyclical chain linkers or combinations thereof would be suitable. Hence, the structure of the atom chain in a linker would include, by way of example, alkyl, heteroalky, alkylaryl, and heteroalkyl aryl.

By way of example, a dimer may be synthesized by combining two different vitamin B_{12} linker adducts in the presence of a coupling agent. The linkers

couple and dimers may then be separated and purified using the same methods outlined above.

Alternatively, activated vitamin B_{12} may simply be combined with a homobifunctional or homotrifunctional linker (Tables 1 and 3). Preferably, in this embodiment, the ratio of vitamin B_{12} to linker should be in the range of 2:1. Preferably, a 1:1 ratio is used in preparation of mixed dimers (*e.g.*, *b*- and *e*-acid derivatives) or mixed ligands (*e.g.*, B_{12} and hormone). Dimers may be separated and purified as noted above.

In still another alternative, vitamin B_{12} linker adducts, synthesized as described, above may be coupled by a third linker. The third linker, a "cross-linker," serves to bridge the linkers on the vitamin B_{12} linker adducts. Suitable cross-linkers include those noted in Tables 1, 2, and 3.

Polymerization of peptides may be accomplished by placing a cysteine residue at each end of a peptide, followed by oxidation using dissolved oxygen or other mild oxidizing agent, such as oxidized glutathione. The average length of a polymerized peptide may be controlled by varying the polymerization reaction conditions.

The amino acid sequence of any of the peptides of this invention may be selected to include all L-amino acids or all D-amino acids having a side chain pK_a from 5.0 to 9.0. D-amino acids may be advantageously used to form non-proteolyzable peptides, since the D-amino acids are not metabolized within the cell. Further, the peptides of the present invention may include a combination of L- and D-amino acids, wherein D-amino acids are substituted for L-amino acids on either side of a proteolytic cleavage site. Yet another preferred noncleavable peptide incorporates peptide bond analogs that are not susceptible to proteolytic cleavage by cellular enzymes.

As discussed above, the receptor modulating agents of this invention comprise a targeting moiety coupled to the rerouting moiety. The rerouting moieties identified above may be covalently attached to the targeting moiety by any one of several techniques known in the art, including (a) by chemical modifications such as a disulfide formation, thioether formation, amide formation or a reduced or non-reduced Schiff's base, (b) by direct peptide bond formation as in a fusion protein, or (c) by use of a chemical and peptide linker. Suitable peptide linkers in this regard correspond to two or more amino acid residues that allow the rerouting peptide to assume its active conformation independent of its interaction with the targeting moiety, and which allows sufficient distance for rerouting moiety access to, for example, intracellular membranes from the peptide attachment site on the targeting moiety.

In one embodiment, a rerouting moiety may be conjugated to a vitamin B_{12} targeting moiety by any one of several means, including, by way of example, coupling a rerouting moiety to a reactive group on a vitamin B_{12} linker adduct; coupling a vitamin B_{12} to a reactive group on a rerouting moiety linker adduct or an appropriate side chain thereof; coupling a vitamin B_{12} linker adduct to a rerouting moiety linker adduct or an appropriate side chain thereof; coupling a rerouting moiety/biotin binding protein conjugate to a vitamin B_{12}/biotin conjugate; or coupling a rerouting moiety biotin conjugate to a vitamin B_{12}/biotin binding protein conjugate.

Coupling of a rerouting moiety to a vitamin B_{12} linker adduct, or a vitamin B_{12} to a rerouting moiety linker adduct, may be accomplished using the same techniques noted above for coupling a vitamin B_{12} molecule with a linker. The only critical consideration of this aspect of the invention is that the total linker length must be sufficient to avoid steric hindrance. Preferably, the total linker length is at least 6 atoms.

Coupling of a rerouting moiety/biotin binding protein conjugate to a vitamin B_{12}/biotin conjugate may be accomplished using any one of several means described in detail in Avidin-Biotin Chemistry: A Handbook, ed. D. Savage, Pierce Chemical Co., 1992. Briefly, a biotin binding protein conjugate is prepared using a rerouting moiety or, as in a second embodiment, a vitamin B_{12} molecule. Suitable biotin binding proteins include avidin or streptavidin. In some circumstances, a linker may be utilized to distance the molecules. For example, when coupling a vitamin B_{12} to an avidin, a linker of at least 6 atoms is preferred.

A biotin conjugate is prepared using a vitamin B_{12} molecule or, as in a second embodiment, a rerouting moiety. By way of example, a vitamin B_{12} molecule is combined with an NHS ester of biotin. Preferably, the vitamin B_{12} molecule is a vitamin B_{12} linker adduct as described above. Even more preferably, the vitamin B_{12} molecule is a vitamin B_{12} linker adduct characterized by a 12 atom linear linker coupled to the *d*- or *e*- coupling site.

Once formulated, coupling between the biotin conjugates and biotin binding protein conjugates is easily accomplished by combining the complementing conjugates, *i.e.*, a vitamin B_{12}/biotin conjugate with a rerouting moiety/avidin conjugate.

In another aspect of the present invention, a B_{12}/biotin conjugate is utilized to couple a vitamin B_{12} to any number of compounds through biotin binding protein conjugates. Using a vitamin B_{12}/biotin conjugate, any compound which is capable of coupling a biotin binding protein may be coupled to a vitamin B_{12} and

thereby internalized into cells expressing the vitamin B_{12} receptor. Such compounds include, in addition to the rerouting moieties described in detail below, hormones, enzymes, antibodies or fragments thereof, markers, or therapeutics. Coupling any of these compounds to a biotin binding protein, such as avidin or streptavidin, may be accomplished using techniques described in detail in Avidin-Biotin Chemistry: A Handbook, ed. D. Savage, Pierce Chemical Co., 1992.

In one aspect of this embodiment, a vitamin B_{12}/biotin conjugate is coupled to a therapeutic/avidin conjugate directed at neoplastic disorders. Neoplastic disorder therapeutics which may be coupled to a vitamin B_{12}/biotin conjugate through avidin include doxorubicin, daunorubicin, etoposide, teniposide, vinblastine, vincristin, cyclophophamide, cisplatin and nucleoside antimetabolites such as arabinosylcytosine, arabinosyladenine and fludarabine.

In another aspect of this embodiment, a vitamin B_{12}/biotin conjugate is coupled to a marker conjugated with a biotin binding protein. Suitable markers include, by way of example, fluorescent molecules or radiolabeled molecules. This combination may be utilized as a detection system incorporated into a screening device to identify patients with low receptor bearing cells or in the evaluation of receptor up-regulation, for example, following treatment of patients for any one of a wide variety of receptor modulation disorders.

In another aspect of this embodiment, a vitamin B_{12}/biotin conjugate is coupled to a radioisotope conjugated to a biotin binding protein. Suitable radioisotopes include, any high energy emitting radioisotopes capable of conjugating a biotin binding protein. This combination may be utilized as a targeted radiodiagnostic or radiotherapeutic.

In yet another aspect of this embodiment, a vitamin B_{12}/biotin conjugate is used to immobilize vitamin B_{12} to a solid matrix or avidin-coated substrate. By way of example, this would enable one to isolate TcII, TcII receptors, and evaluate coupling sites on the Vitamin B_{12}.

The receptor modulating agents of this invention regulate receptor-dependent biological responses through alterations in the receptor trafficking pathway. As illustrated in Figure 1, with specific reference to the receptor for vitamin B_{12}, cell surface receptors are often associated with clathrin-coated pits. When bound by the receptor modulating agent of the present invention, the coated pits invaginate to form vesicles. The vesicles are then directed by the rerouting agent to lysosomes for receptor degradation or delivered to endosomes where the rerouting agent securely binds or

delays the agent/receptor complex. Thus, the receptor modulating agents can incapacitate the receptors normally undergoing recycling.

Newly synthesized receptors will eventually replace the internalized receptor on the cell surface. However, this process is far more time consuming than recycling--many cells require hours or days to achieve maximal receptor re-expression. Continued exposure of the cell to the receptor modulating agents will exhaust the intracellular receptor pools. Thus, by modulating a plasma membrane receptor, re-expression of the receptor can be substantially delayed, thereby regulating a biological response associated with that receptor for a prolonged period of time.

Biological activity of receptor modulating agents of the present invention may be ascertained in vitro by any one of several means known in the art including, competition binding assays or cell proliferation studies. These techniques are described in detail in Laboratory Techniques in Biochemistry and Molecular Biology: An Introduction to Radioimmunoassay and Related Techniques, 3rd Edition, ed. Burdon and van Knippenberg, Elsevier, 1987. By way of example, a receptor modulating agent may be cultured with a suitable cell line, such as K562 cells (ATCC CCL 243), under conditions representing in vivo conditions. Such conditions would include the provision of a human source of TcII (such as human serum), vitamin B_{12}, and, preferably by careful removal by chromatography, of all TcII from other medium supplements such that proliferation is solely dependent on a known amount of exogenous TcII. Cell cultures deprived of vitamin B_{12} gradually lose their proliferative capacity, eventually resulting in cell death. Biological activity may be evaluated in vivo using techniques described in detail in Shieh et al., J. Immunol. 152(2):859-866, 1994 in which human tumor cell lines are injected into nude mice, followed by therapy with receptor modulating agents. Next, tumor cells are removed, single cell suspensions prepared and TcII cell surface receptor density may be evaluated by flow cytometry and biotinylated vitamin B_{12} and avidin FITC.

The receptor modulating agent of the present invention may be administered in a therapeutically effective amount to treat a variety of disorders characterized in which control of the disease process or symptoms can be achieved by modulation of one or more receptor systems and the associated biological responses. Such disorders include neoplastic disorders, autoimmune diseases, rheumatic arthritis, cardiovascular disease, and neurodegenerative diseases.

Common to many non-neoplastic disease processes is a stage in which the disease process itself, or its symptoms, can be halted or ameliorated by the use of an anti-proliferative agent such as vitamin B_{12}/TcII receptor modulating agents. These

commonly recognized stages include a sensitization or elicitation phase in which immune cells responsible for the disease become turned on by antigen specific or non-specific means, followed by a proliferative phase in which the immune cells expand in number, and finally a symptomatic phase in which the expanded immune cells create tissue damage directly or indirectly. Neoplastic disorders include, by way of example, leukemia, sarcoma, myeloma, carcinoma, neuroma, melanoma, cancers of the breast, lung, liver, brain, colon, cervix, prostrate, Hodgkin's disease, and non-Hodgkin's lymphoma. Because of this, anti-proliferative chemotherapeutic drugs are commonly utilized in the treatment of many diseases other than cancer, but are limited in use to life threatening situations due to their associated toxicity. Anti-proliferative agents, such as the ones of the present invention (with little of the direct toxicity of chemotherapeutic drugs), may be used more widely. More specifically, the vitamin B_{12} receptor modulating agents of the present invention are not destructive to plasma membrane processes (*e.g.*, ion transport). In addition, the anti-proliferative activity is reversible by administration of vitamin B_{12}. Furthermore, the agents of this invention may not be mutagenic, teratogenic, or carcinogenic since they act at the level of the plasma membrane, and not at the level of the nucleus, and DNA by intercalation or cross-linking (as many chemotherapeutic drugs act).

An understanding of the pharmaceutical applications for B_{12}/TcII receptor modulating agents requires a knowledge of the cell types targeted by such therapy. To this end, various pharmaceutical applications are disclosed in Table 9 below.

TABLE 9

TARGET CELLS FOR VITAMIN B_{12} RECEPTOR MODULATING AGENTS

TARGET CELL	OTHER PROLIFERATION ASSOCIATED MARKERS	POTENTIAL PHARMACEUTICAL APPLICATIONS
Activated T-Cell	IL-2 receptor Transferrin Receptor Insulin Receptor Class II Histocompatibility Antigens	Graft versus Host Disease Organ Transplants Auto-Immune Diseases Asthma Crohn's Disease
Tumor Cells	Tumor Assoc. Ags. Ki67 Transferrin Receptor	Tumor Therapy (alone and in combination with chemotherapeutic drugs)

Bone Marrow Stem Cells	CD-34 Transferrin Receptor Class II Histocompatibility Antigens IL-1, IL-3 Receptors	Allogeneic Bone Marrow Transplants Reduction in Toxicity of Chemotherapy
Proliferating Fibroblasts	Thy 1.1 Transferrin Receptor Insulin & Insulin-like Growth-Factor Receptors Fibroblast Growth-Factor Receptor	Inhibition of Adhesions, Scarring Scleroderma
Proliferating Epithelium or Epidermal (Keratinocytes)	EGF Receptor Proto-Oncogenes	Psoriasis

Proliferating and activated T-cells can cause a wide variety of diseases ranging from the chronic inflammation of Crohn's disease to more acute organ graft rejection. In all of these diseases, the T-cell may serve a central pathogenic role or a more accessory role. Anti-proliferative chemotherapeutic drugs serve to reduce symptomotology and in some cases lead to long-term remission. Similarly, proliferating fibroblasts and epithelial cells may give rise to diseases characterized by cell overgrowth. Vitamin B_{12} receptor modulating agents may be used to replace or used in combination with existing chemotherapeutic regimens in these diseases. An important aspect of the use of anti-proliferative vitamin B_{12} receptor modulating agents in these diseases is not to apply it so aggressively or with improper timing such that normal healing (adhesions, scarring) or cell renewal (psoriasis) processes are also inhibited. As such, low doses of receptor modulating agents may be used during healing and higher doses once healing is completed. Alternatively, receptor modulating agents may not be administered at all until after healing is completed.

As previously mentioned, B_{12}/TcII receptor modulating agents can be used to deprive neoplastic cells of vitamin B_{12}. It has already been shown that sufficient deprivation leads to the death of rapidly proliferating lymphoid neoplasms such as leukemia and lymphoma. Moreover, short term treatment to reduce cellular availability of this nutrient, combined with existing chemotherapeutic agents, markedly improves therapeutic efficacy.

For solid tumors, vitamin B_{12} depletion may induce cytostasis and differentiation as well as cell death. Thus, B_{12}/TcII receptor modulating agents may be used to induce differentiation in hormonally responsive solid tumors. An increase in the number of cells expressing a differentiated phenotype should translate into an increase in expression of hormone receptors. The hormone receptor status of tumors, such as breast and prostrate cancer, are directly correlated with their response to hormonal therapy. Accordingly, B_{12}/TcII receptor modulating agents can be used to increase the number of receptor positive tumor cells or increase receptor density in order to enhance efficacy of subsequent hormonal therapy.

Vitamin B_{12} receptor modulating agents may affect both replicating neoplastic and normal cells. However, bone marrow progenitors demonstrate differential sensitivity or response. Thus, B_{12} receptor modulating agents can be used to modulate sensitivity of bone marrow progenitors so as to enhance their resistance to the toxic effects of chemotherapeutic agents. Such chemotherapeutic drugs act primarily on replicating cells, with non-replicating cells being much less sensitive. Decreasing the sensitivity of progenitors to toxic drugs would increase the bone marrow reserves and enhance subsequent response to colony stimulating factors, and enable higher doses of chemotherapy or reduce the interval to reconstitution. It should also be recognized that such positive effects on bone marrow progenitors, as a natural consequence of B_{12} receptor therapy for cancer, is an additional mechanism by which the therapeutic index of chemotherapeutic drugs other than 5-FU and methotrexate can be improved.

In a variety of autoimmune diseases, graft versus host disease, ectopic allergy, and organ transplantation, an initial 'induction' phase, in which the patient becomes sensitized to self or allo-antigens, is followed by a "proliferative" phase in which forbidden or unregulated clones of B- or T-cells are expanded. It has long been known that treatment with anti-proliferative, chemotherapeutic drugs following induction can inhibit expansion of forbidden clones, inhibit progression of disease, and restore a stable state of tolerance.

Inflammation is an application for which antibodies are already being utilized in clinical trials. The primary emphasis has been on inhibiting the early manifestations of inflammation by inhibiting recruitment or binding of inflammatory cells to vascular endothelium of injured tissue. It also well recognized that proliferation of cells at the site of inflammation contributes to the pathology and tissue destruction of both acute as well as chronic inflammation. To this end, anti-proliferative, chemotherapeutic drugs have been widely used to inhibit sequelae of inflammation.

Methotrexate is one such drug commonly used to treat symptoms associated with rheumatoid arthritis. The drug acts to reduce both localized (*e.g.*, synovium) and generalized inflammation associated with disease progression. Methotrexate acts synergistically with vitamin B_{12} depletion in therapy of leukemia. B_{12} receptor modulating agents can therefore be combined with methotrexate to enhance efficacy in rheumatoid arthritis. Other methotrexate applications include treating destructive inflammation associated with chronic heart disease and colitis.

Surgery, radiation or chemotherapy to the abdomen is often complicated by the development of tissue adhesions. These represent a considerable clinical problem because they lead to bowel blockage and require surgical intervention. Peritoneal adhesions arise as a result of proliferation of the cells of the peritoneal membrane lining the abdomen. A non-toxic means of interfering with such proliferation could lead to restoration of these normal cells to homeostatic control mechanisms and thereby inhibition of adhesion formation. A similar process of benign proliferation and subsequent scarring is a complication of retinal surgery. Direct instillation of a small molecule analog of an antibody receptor antagonist could prevent such disabling complications.

The term "treatment" as used within the context of the present invention, refers to reducing or alleviating symptoms in a subject, preventing symptoms from worsening or progressing, inhibition or elimination of the causative agent, or prevention of the infection or disorder in a subject who is free therefrom. Thus, for example, treatment of infection includes destruction of the infecting agent, inhibition of or interference with its growth or maturation, neutralization of its pathological effects and the like. A disorder is "treated" by partially or wholly remedying the deficiency which causes the deficiency or which makes it more severe.

The receptor modulating agents of the present invention are administered in a therapeutically effective dose. A therapeutically effective dose may be determined by in vitro experiment followed by in vivo studies.

Pharmaceutical compositions containing the receptor modulating agents in an admixture with a pharmaceutical carrier or diluent can be prepared according to conventional pharmaceutical compounding techniques. The carrier may take a wide variety of forms depending on the form of preparation desired for administration (*e.g.*, intravenous, oral topical, aerosol, suppository, parenteral or spinal injection). Preferably, administration is via stereotactical injection.

The following examples are offered by way of illustration, not limitation.

EXAMPLES

In summary, the examples which follow disclose the synthesis of several receptor modulating agents of this invention utilizing different functional classes of rerouting moieties. More specifically, a series of examples are presented which employ vitamin B_{12} as a targeting moiety in a receptor modulating agent.

All chemicals purchased from commercial sources were analytical grade or better and were used without further purification unless noted. Isophthaloyl dichloride was purchased from Lancaster Synthesis Inc. (Windham, NH). All other reagents were obtained from Aldrich Chemical Co. (Milwaukee, WI). Solvents for HPLC analysis were obtained as HPLC grade and were filtered (0.2 μm) prior to use. Ion exchange chromatography was conducted with 200-400 mesh strongly basic anion 2% cross-linking Dowex*-1-chloride (Aldrich Chemical Co). Amberlite* XAD-2 nonionic polymeric adsorbent and octadecyl functionalized silica gel for column chromatography were obtained from Aldrich Chemical Co.

^{1}H NMR were obtained on Bruker AC-500 (500 MHz) instrument. The chemical shifts are expressed as ppm (δ) using tetramethylsilane as internal reference. IR data were obtained on a Perkin-Elmer 1420 infrared spectrophotometer. UV data were obtained on a Perkin-Elmer Lambda 2 UV/V is spectrophotometer. Mass spectral data were obtained on a VG 7070H mass spectrometer using fast atom bombardment (FAB).

HPLC separations of compounds were obtained on Hewlett-Packard quaternary 1050 gradient pumping system with a UV detector. Analysis of the HPLC data were obtained on a Hewlett-Packard HPLC Chemstation software.

HPLC for Monomers: HPLC separations were conducted at a flow rate of 1 mL/min. on a 5 mm, 4.6 250 mm NH_2 column (RAININ microsorb-MV amino column) eluting with 58 mM pyridine acetate, pH 4.4 in H_2O : THF (96 : 4) solution. Retention times were: **1**= 4.3 min; **2** = 6.5 min; **3** = 8.0 min; **4** = 8.8 min; **5** = 10.9 min; **6** = 2.3 min; **7** = 2.3 min; **8** = 3.0 min; **9** = 2.9 min; **10** = 2.9 min; **13** = 3.4 min.

Reverse-phase HPLC chromatography was carried out using a Hewlett-Packard Lichrospher* 100 RP-18 (5 mm, 125 X 4 mm) C-18 column using a gradient solvent system at a flow rate of 1 mL/min. Solvent A in the gradient was methanol. Solvent B was H_2O. Starting from an 40% A, the gradient was increased to 100% A over 10 min. The gradient was then brought back to 40% A over a 5 min period. Retention times under these conditions for biotin conjugates were: **17** = 7.1 min; **18** = 7.2 min; **19** = 6.9 min; **20** = 6.4 min.

*Trade-mark

Preparative LC was conducted to separate the mixture of monocarboxylic acids using RAININ Rabbit-plus peristaltic pumping system with a DYNAMAX (model UV-1) UV-visible absorbance detector at a flow rate of 0.15 mL/min. ID column (Alltech, 150 psi), (1000 mm X 25 mm) packed with aminopropyl silica (40-63 mm) was used.

HPLC for Dimers: For dimers **36, 37,** and **38** solvent A in the gradient was methanol. Solvent B was H_2O. The gradient was held at the starting mixture of 70% A for 2 min, then the percentage of A was linearly increased to 100% over the next 10 min. The gradient was held at 100% A for 20 min. Retention times under these conditions for dimers were: **36** = 8.7 min; **37** = 9.0 min; **38** = 8.9 min. For dimers **58-60** and **64-66** Solvent A in the gradient was methanol. Solvent B was aqueous 1% acetic acid. The gradient was begun at 40% A and was held at that composition for 2 min, then the percentage of A was linearly increased to 100% over the next 10 min. Retention times for the compounds examined under these conditions were: **58** = 14.0 min; **59** = 14.1 min; **60** = 13.9 min; **64** = 8.7 min; **65** = 8.6 min; **66** = 9.0 min.

EXAMPLE 1

PREPARATION AND PURIFICATION OF CYANOCOBALAMIN MONOCARBOXYLATES: MODIFICATION ON THE CORRIN RING

This example serves to demonstrate the hydrolysis of *b*-, *d*- and *e*-propionamide sites on a vitamin B_{12} molecule using dilute acid in preparation for coupling of a linker to the sites. Importantly, the hydrolysis of the *b*-, *d*- and *e*-propionamides is selective over the hydrolysis of *a*-, *c*- and *g*-acetamides, or the *f*-amide in the heterocyclic chain connecting the benzimidazole. An optimal yield of monocarboxylate to di- and tri-carboxylate derivatives was obtained at room temperature in 0.1 N HCl over a 10 day period. The non-hydrolyzed vitamin B_{12} and the di- and tri-carboxylates produced were readily isolated from the desired monocarboxylates by preparative liquid chromatography.

Specifically, cyanocobalamin **(1)** (3.7 mmol, 5 g) was dissolved in 500 mL of 0.1 N HCl and stirred at room temperature for 10 days under argon atmosphere. The solution was then neutralized with 6 N NaOH and the cobamides were desalted by extraction into phenol and applied to a 200 g (60 x 4 cm, 200-400 mesh) Dowex Cl^- x 2 column (acetate form; prepared by washing with saturated sodium acetate until it was free from Cl^-, then washing with 200 mL water). The column was eluted with water to

remove unreacted cyanocobalamin and then eluted with 0.04 M sodium acetate (pH 4.67).

The first fraction of the elution contained three monocarboxylic acids. These were desalted by extraction into 100 mL of 90% (w/w) phenol, twice with 25 mL and once with 10 mL of phenol. Three volumes of ethyl ether (3 x 160 mL) and 1 volume of acetone (160 mL) were added to the combined phenol extracts. Monocarboxylic acids were removed from the organic phase by extraction with water (2 x 100 mL). The combined aqueous phases were extracted twice with 20 mL of ether to remove residual phenol. The aqueous solution of monocarboxylic acids was evaporated to dryness. Yield: 2.5 g (50%).

The mixture of three acids (0.350 g) was then applied to a 200 g (1000 mm x 25 mm) column of aminopropyl coated silica (40-63 mm) and was eluted with 58 mM pyridine acetate pH 4.4 in H_2O : THF (96 : 4); the elute was collected with an automatic fraction collector. The first eluted acid was found to be *b*-monocarboxylic acid **(2)**, the second eluted acid was *e*-monocarboxylic acid **(3)** and the third eluted acid was *d*-monocarboxylic acid **(4)**. The acid fractions were desalted by phenol extraction. The solids obtained were crystallized from aqueous acetone.

b-acid **(2)**: yield 0.122 g (35%), mp 267-270°C with decomposition, ^1H NMR (MeOH-d_4, δ) 0.43 (s, 3H, C-20 CH_3); 1.00 (m, 2H); 1.18 (s, 3H, C-46 CH_3); 1.24 (d, 3H, Pr_3 CH_3); 1.36 (br s, 9H, C-47 CH_3, C-54 CH_3); 1.4 (s, 3H, C-25 CH_3); 1.9 (d, 7H, C-36 CH_3, C-30 CH_2, C-48 CH_2); 2.26 (d, 6H, B10 & B11, CH_3); 2.36 (d, 2H, C-26 CH_2); 2.57 (s, 10H, C-35 CH_3, C-31 CH_2, C-37 CH_2, C-53 CH_3); 2.8 (m, 2H, C-60 CH_2); 3.3 (m, 3H, C-8H, C-13H); 3.6 (m, 2H, Pr_1 CH_2); 3.7 (d, 1H, R_5); 3.9 (d, 1H, R_5); 4.0 (m, 1H, R_4); 4.12 (d, 1H, C-19); 4.17 (s, 1H, C-3); 4.3 (m, 1H, R_2); 4.5 (m, 1H); 4.7 (m, 1H, R_3); 6.0 (s, 1H, C-10); 6.2 (s,1H, R_1); 6.5 (s,1H, B4); 7.1 (s, 1H, B2); 7.2 (s, 1H, B7). MS (FAB^+): m/e 1357 (M^+ +1). IR (KBr): 3400, 3200, 2950, 2060, 1660, 1570, 1490, 1060 cm^{-1}. UV (MeOH): λ360 (ε23441)

e-acid **(3)**: yield 0.168 g (48%), mp 245-250° C with decomposition, ^1H NMR (MeOH-d_4, δ) 0.43 (s, 3H, C-20 CH_3); 1.01 (m, 2H); 1.15 (s, 3H, C-46 CH_3); 1.23 (d, 3H, Pr_3 CH_3); 1.36 (br s, 9H, C-47 CH_3, C-54 CH_3); 1.4 (s, 3H, C-25 CH_3); 1.83 (s, 4H, C-55 CH_2); 1.93 (m, 6H, C-36 CH_3, C-30 CH_2, C-48 CH_2); 2.22 (d, 6H, B10 & B11 CH_3); 2.35 (s, 3H,C-26 CH_2); 2.5 (d, 13H, C-35 CH_3, C-31 CH_2, C-37 CH_2, C-53 CH_3); 2.9 (m, 1H, C-60 H); 3.2 (m, 1H, C-13H); 3.4 (m, 1H, C-8 H); 3.6 (d, 1H, Pr1 CH); 3.7 (d, 1H); 3.9 (d, 1H); 4.0 (m, 2H); 4.1 (d, 1H); 4.2 (m, 2H); 4.6 (m, 1H); 6.0 (s, 1H, C-10); 6.3 (d, 1H, R1); 6.5 (s, 1H, B4); 7.0 (s, 1H, B2); 7.2 (s,

1H, B7). MS (FAB^+): m/e 1357 (M^+ +1). IR (KBr): 3400, 3200, 2950, 2060, 1660, 1570, 1490, 1060 cm^{-1}. UV (MeOH): λ360 (ε21 842)]

d-acid **(4)**: yield 0.060 g (17%), mp > 300° C, ^1H NMR (MeOH-d_4, δ) 0.43 (s, 3H, C-20 CH_3); 1.04 (m, 2H); 1.15 (s, 3H, C-46 CH_3); 1.25 (d, 3H, Pr_3 CH_3); 1.36 (br s, 9H, C-47 CH_3, C-54 CH_3); 1.4 (s, 3H, C-25 CH_3); 1.85 (s, 4H); 2.01 (s, 6H); 2.23 (d, 8H, B10 & B11 CH_3); 2.38 (d, 3H, C-26 CH_2); 2.53 (d, 13H, C-36 CH_3, C-30 CH_2, C-48 CH_2); 2.6 (m, 5H); 2.9 (m, 1H, C-60 H); 3.3 (d, 1H, C-13H); 3.4 (m, 1H, C-8 H); 3.6 (d, 1H, Pr_1 CH); 3.7 (d, 1H); 3.9 (d, 1H); 4.0 (m, 2H); 4.1 (d, 1H); 4.3 (m, 2H); 6.0 (s, 1H, C-10); 6.3 (d, 1H, R1); 6.5 (s, 1H, B4); 7.1 (s, 1H, B2); 7.2 (s, 1H, B7); UV (MeOH): λ360 (ε22 127). MS (FAB^+): m/e 1357 (M^+ +1). IR (KBr): 3400, 3200, 2950, 2060, 1660, 1570, 1490, 1060 cm^{-1}.

EXAMPLE 2

CYANOCOBALAMIN MODIFIED ON RIBOSE: SUCCINATE CONJUGATE (5)

This example serves to demonstrate the activation of the ribose coupling site coupling site *h* (*see* structure I) with succinic anhydide. Cyanocobalamin **(1)** (0.15 mmoL, 200 mg) was dissolved in 40 mL of dimethylsulfoxide (DMSO) containing 8 g (80 mmoL) of succinic anhydride and 6.4 mL of pyridine. After 14-16 h at room temperature, the excess of succinic anhydride was destroyed by adding 500 mL of water and keeping the pH of the reaction mixture at 6 with 10% KOH. KCN was then added at a final concentration of 0.01 M and the pH of the solution was readjusted to 6 with 3 N HCl. After 1 h the cyanocobalamin components were desalted by phenol extraction and applied to a 100 g of Dowex Cl^- (60 x 2.5 cm) column (acetate form, 200-400 mesh). The cyanocobalamin was eluted with water. Succinate conjugate **(5)** was eluted with NaOAc (0.04 M, pH 4.67) which yielded 180 mg (85 %) after isolation. The O2',O5'-disuccinyl derivative remained absorbed on the column under these conditions. mp 208-210° C with decomposition.

^1H NMR (D_2O-d_4, δ): 0.43 (s, 3H, C-20 CH_3); 0.95 (m, 2H); 1.15 (s, 3H); 1.2 (d, 3H); 1.35 (d, 7H); 1.4 (s, 3H); 1.8 (s, 3H); 1.9 (s, 12H); 2.2 (d, 6H); 2.36 (d, 2H); 2.5 (d, 10H); 2.6-2.7 (m, 7H); 3.0 (m, 1H); 3.3 (d, 1H); 3.37 (m, 1H); 3.5 (d, 1H); 4.0 (d. 1H); 4.18 (m, 2H); 4.25 (m, 3H); 4.54 (d, 1H); 6.0 (s, 1H); 6.3 (d, 1H); 6.4 (s, 1H); 7.0 (s, 1H); 7.2 (s, 1H). MS (FAB^+): m/e 1455 (M^+ +1). IR (KBr): 3400, 3200, 2950, 2060, 1660, 1570, 1490, 1060 cm^{-1}; UV (MeOH): λ360 (ε 26041).

EXAMPLE 3

COUPLING OF CYANOCOBALAMIN MONOCARBOXYLIC ACIDS WITH 1,12-DIAMINODODECANE: REACTION WITHOUT SODIUM CYANIDE

This example serves to demonstrate the coupling of a linker to a cyanocobalamin monocarboxylate. Coupling of the monocarboxylates **(2, 3, 4)** with diaminododecane was first attempted using N-ethyl-N'-dimethylamino-propyl-carbodiimide hydrochloride (EDC) in H_2O according to Yamada and Hogenkamp, J. Biol. Chem. 247, 6266-6270, 1972. However, the products obtained did not have a reactive amino group. Alteration of the reaction conditions by changing the reaction mixture to DMF/H_2O and adding NaCN/N-hydroxysuccinimide (*see* Example 4) to the reaction mixture gave the desired diaminododecane adducts.

A mixture of cyanocobalamin monocarboxylic acid (0.370 mmoL, 500 mg) and 1,12-diaminododecane (3.6 g) in 100 mL H_2O was adjusted to pH 6 with 1 N HCl. The solution was then treated with N-ethyl-N'-dimethylamino-propyl-carbodiimide-hydrochloride (EDC) (726 mg) and stirred at room temperature for 22 h. In 5 intervals of 6 to 14 h, 650 mg of EDC was added to the reaction mixture. After a total reaction time of 4 days (HPLC monitoring) the solution was evaporated to dryness, the residue was digested with 100 mL of acetone and the solvent was decanted. The solid residue was dissolved in 50 mL of water and applied to an 175 g Amberlite XAD-2 (60 x 4 cm) column. Contaminates were washed from the column with 1L water, then the crude product was eluted with 500 mL of methanol. The solution was evaporated to dryness, the residue was dissolved in 25 mL of water and was applied to a 100g Dowex Cl^- (60 x 2.5 cm) column (acetate form, 200-400 mesh). The final product was eluted using 250 mL of water, thereby leaving non-converted acid bound to the column, which was later eluted with 0.04 mol/L sodium acetate buffer pH 4.67. The fraction containing the final product was evaporated to dryness.

The mass spectral value obtained indicated that HCN was lost from the desired product. Further, ^1H NMR data suggested that some protons were being affected by the cobalt. Thus, this reaction was conducted with NaCN (Example 4) to drive the equilibrium towards retention of Co-CN. N-hydroxy succinimide was also added to facilitate the coupling reaction.

e-acid adduct **(6)**: Yield: 222 mg (40%). mp 172-174° C with decomposition. ^1H NMR (MeOH-d_4, δ): 0.43 (m, 3H, C-20 CH_3); 1.06 (t, 4H, C-46 CH_3); 1.16 (m, 5H); 1.2 (m, 5H); 1.33 (m, 7H); 1.43 (s, 3H); 1.68 (m, 4H); 1.86 (m,

5H); 2.2 (m, 8H); 2.3 (m, 6H); 2.4 (m, 10H); 2.55 (m, 10H); 2.8 (m, 4H); 3.1 (m, 6H); 3.3 (m, 5H); 3.6 (m, 2H); 3.7 (m, 2H); 3.8 (m, 1H); 4.0 (m, 1H); 4.1 (m, 1H); 4.16 (m, 1H); 4.3 (m, 1H); 4.48 (m, 1H); 4.6 (m, 1H); 6.0 (d 1H, C-10); 6.2 (m, 1H, R1); 6.5 (m, 1H, B4); 7.1 (m, 1H, B2); 7.2 (m, 1H, B7). MS (FAB^+): m/e 1512. IR (KBr): 3400, 3200, 2950, 1660, 1570, 1490, 1060 cm^{-1}. UV (MeOH): λ360 (ε21 877).

d-acid adduct ***(7)***: yield: 225 mg (45%), mp 195-198° C with decomposition. ^1H NMR (MeOH-d_4, δ): 0.43 (m, 3H, C-20 CH_3); 1.09 (m, 7H); 1.14 (m, 6H); 1.2 (m, 10H); 1.27 (m, 10H); 1.33 (m, 6H); 1.5 (m, 3H); 1.77 (s, 3H); 2.2 (m, 8H); 2.26 (s, 2H); 2.5 (m, 10H); 2.7 (m, 5H); 3.0 (m, 2H); 3.1 (m, 2H); 3.2 (m, 3H); 3.5 (m, 2H); 3.6 (m, 1H); 3.8 (m, 1H); 3.9 (m, 1H); 4.0 (m, 1H); 4.1 (m, 1H); 4.2 (m, 1H); 4.4 (m, 1H); 4.6 (m, 1H); 6.0 (d 1H, C-10); 6.1 (m, 1H, R_1); 6.4 (m, 1H, B4); 7.0 (m, 1H, B2); 7.1 (m, 1H, B7); MS (FAB^+): m/e 1512, IR (KBr): 3400, 3200, 2950, 1660, 1570, 1490, 1060 cm^{-1}; UV (MeOH): λ360 (ε22 680).

EXAMPLE 4

COUPLING OF CYANOCOBALAMIN MONOCARBOXYLIC ACIDS WITH 1,12-DIAMINODODECANE: REACTION CONTAINING SODIUM CYANIDE

Cyanocobalamin monocarboxylic acid **(2, 3, 4)** (0.370 mmoL, 500 mg) and N-hydroxysuccinimide (1.48 mmoL, 170 mg) were dissolved in a mixture of DMF : H_2O (1:1) (18.4 mL) and 363 mg of NaCN was added. 1,12-Diaminododecane was dissolved in a mixture of DMF : H_2O (1:1) (18.4 mL) and the pH was adjusted to 6 with 1 N HCl. The diaminododecane solution was then added in one portion to the cyanocobalamin solution. EDC (285 mg) was added and the pH of the solution was readjusted to 5.5. The reaction mixture was then stirred overnight in the dark at room temperature. In 5 intervals of 6-14 h, 170 mg of N-hydroxysuccinimide and 285 mg of EDC were added to the solution, readjusting the pH value 5.5 each time. After a total reaction time of 4 days (reaction followed by HPLC), the solution was evaporated to dryness. The residue was digested with 100 mL of acetone and the solvent was decanted. The solid residue was dissolved in 50 mL of H_2O and applied to an 200 g Amberlite XAD-2 (60 x 4 cm) column. The column was eluted with 1 L water to remove undesired materials, then the desired product was eluted with 500 mL methanol. The solution was evaporated to dryness, the residue was dissolved in 25 mL of water and was applied to a 100 g Dowex Cl^- (60 x 2.5 cm) column (acetate form, 200-400 mesh). The desired product was eluted from the column with 250 mL water, leaving any non-reacted acid bound to the column. This was followed by elution with 0.04

mol/L sodium acetate buffer pH 4.7. The fractions containing the final product were evaporated to dryness.

b-isomer **(8)**: yield 410 mg (82%), mp 172-174° C with decomposition. ^{1}H NMR (MeOH-d_4, δ) 0.43 (s, 3H, C-20 CH_3); 1.18 (s, 4H); 1.3 (m, 13H); 1.39 (m, 13H); 1.45 (s, 5H); 1.6 (m, 4H); 1.72 (m, 2H); 1.9 (s, 6H); 2.25 (d, 6H, B10 & B11 CH_3); 2.35 (m, 5H); 2.56 (m, 5H); 2.8-3.0 (m, 8H); 3.15 (m, 4H); 3.3 (m, 2H); 3.4 (m, 2H); 3.6 (m, 1H); 3.68 (m, 1H); 3.75 (m, 1H); 3.9 (d, 1H); 4.07 (m, 1H); 4.12 (d, 1H); 4.2 (br s, 1H); 4.3 (m, 1H); 4.47 (m, 1H); 4.7 (m, 1H); 6.0 (s, 1H, C-10); 6.2 (d,1H, R_1); 6.5 (s,1H, B4); 7.1 (s, 1H, B2); 7.2 (s, 1H, B7); MS (FAB^+): m/e 1539 (M^+ +1). IR (KBr): 3400, 3200, 2950, 2060, 1660, 1570, 1490, 1060 cm^{-1}. UV (MeOH): λ360 (ε15409).

e-isomer **(9)**: yield: 430 mg (86%), mp 175-180° C with decomposition, ^{1}H NMR (MeOH-d_4, δ) 0.43 (s, 3H, C-20 CH_3); 1.17 (s, 4H, C-46 CH_3); 1.22 (d, 4H, Pr_3 CH_3); 1.29 (s, 24H); 1.36 (br s, 6H); 1.4 (s, 6H); 1.6 (m, 3H); 1.87 (s, 8H); 2.05 (m, 2H); 2.25 (s, 6H, B10 & B11 CH_3); 2.36 (m, 3H); 2.55 (d, 10H); 2.8 (s, 4H); 3.06 (t, 2H); 3.1 (m, 3H); 3.3 (s, 1H); 3.34 (m, 1H); 3.4 (m, 1H); 3.58 (m, 1H); 3.65 (m, 1H); 3.75 (d, 1H); 3.9 (d, 1H); 4.0 (m, 1H); 4.1 (d, 1H); 4.16 (m, 1H); 4.3 (m, 2H); 4.48 (m, 2H); 4.6 (m, 1H); 6.0 (s, 1H, C-10); 6.3 (d, 1H, R1); 6.5 (s, 1H, B4); 7.0 (s, 1H, B2); 7.2 (s, 1H, B7); MS (FAB^+): m/e 1539 (M^+ +1). IR (KBr): 3400, 3200, 2950, 2060, 1660, 1570, 1490, 1060 cm^{-1}. UV (MeOH): λ360 (ε16 720)

d-isomer **(10)**: yield: 400 mg (80%), mp 174-178° C with decomposition, ^{1}H NMR (MeOH-d_4, δ) 0.43 (s, 3H, C-20 CH_3); 1.07 (m, 3H, C-46 CH_3); 1.2 (d, 4H, Pr_3 CH_3); 1.27 (m, 15H); 1.35 (br s, 9H); 1.42 (s, 3H); 1.53 (m, 2H); 1.6 (m, 4H); 1.86 (s, 4H); 2.25 (d, 6H, B10 & B11 CH_3); 2.5 (d, 10H); 2.8 (s, 3H); 2.9 (m, 6H); 3.15 (m, 3H); 3.2 (m, 4H); 3.4 (m, 3H); 3.6 (d, 1H); 3.75 (d, 1H); 3.96 (d, 1H); 4.08 (m, 2H); 4.19 (m, 1H); 4.3 (m, 2H); 4.65 (m, 1H); 6.0 (s, 1H, C-10); 6.3 (d, 1H, R_1); 6.5 (s, 1H, B4); 7.1 (s, 1H, B2); 7.2 (s, 1H, B7); UV (MeOH): λ360 (ε17 665). MS (FAB^+): m/e 1539 (M^+ +1). IR (KBr): 3400, 3200, 2950, 2060, 1660, 1570, 1490, 1060 cm^{-1}.

EXAMPLE 5

COUPLING OF CYANOCOBALAMIN MONOCARBOXYLIC ACIDS WITH GAMMA-AMINOBUTYRIC ACID (GABA)

This example serves to demonstrate the coupling of a gamma-aminobutyric acid (GABA) linker to a vitamin B_{12} molecule. This reaction scheme is represented in Figure 9.

Gamma-aminobutyric acid (GABA) *tert*-butyl ester **(11)** (1 mmol) and cyanocobalamin monocarboxylates **(2, 3, 4)** (0.1 mmol.) are mixed in 20 mL H_2O and sufficient 0.1 N HCl is added to adjust to pH to 6.0. N-ethyl-N^1-dimethylaminopropylcarbodiimide hydrochloride (EDC) (0.5 mmol) is added to the solution. The reaction mixture is stirred at room temperature for 24 hours and then the mixture is dried under vacuum. This reaction mixture is treated with TFA to remove the *tert*-butyl ester. A cyanocobalamin-GABA adduct **(12)** was purified. Reverse-phase HPLC chromatography is carried out as described above. A cyanocobalamin-GABA adduct **(12)** can be further activated with a carbodiimide and coupled to a moiety as described below.

EXAMPLE 6

CYANOCOBALAMIN MODIFIED ON RIBOSE: SUCCINATE-DIAMINODODECANE CONJUGATE (13)

Cyanocobalamin-Ribose-Succinate **(5)** (0.370 mmoL, 538 mg) and N-hydroxylsuccinimide (1.48 mmoL, 170 mg) were dissolved in a mixture of DMF : H_2O (1:1) (18.4 mL) and 363 mg of NaCN was added. This reaction scheme is represented in Figure 11. 1,12-Diaminododecane was taken in a mixture of DMF : H_2O (1:1) (18.4 mL), pH was adjusted to 6 with 1N HCl. The diaminododecane solution was then added in a portion to the cyanocobalamin solution. EDC (285 mg) was added, the pH of the solution was readjusted to 5.5 and the reaction mix. was stirred overnight in the dark at room temperature. In 5 intervals of 6 to 14 h 170 mg of N-hydroxysuccinimide and 285 mg of EDC was added to the solution, readjusting the pH 5.5 each time. After a total reaction time of 4 days (HPLC monitored) the solution was evaporated to dryness, the residue was digested with 100 mL of acetone and the solvent was decanted. The solid residue was dissolved in 50 mL of H_2O and applied to an 200 g Amberlite XAD-2 (60 x 4 cm) column. Contaminates were washed from the column with 1 L water and then the crude product was eluted with 500 mL methanol. The solution was

evaporated to dryness, the residue was dissolved in 25 mL of water and was applied to a 100 g Dowex Cl^- (60 x 2.5 cm) column (acetate form, 200-400 mesh). The final product was eluted using 250 mL water, thereby leaving non-converted acid bound to the column, which was later eluted with 0.04 mol/L sodium acetate buffer pH 4.7. The fraction containing the final product **(13)** was evaporated to dryness. Yield : 425 mg (70%), mp 185-187° C with decomposition.

^1H NMR (MeOH-d_4, δ): 0.43 (s, 3H, C-20 CH_3); 1.15 (s, 3H); 1.2 (d, 3H); 1.3 (s, 27H); 1.4 (m, 3H); 1.55 (m, 6H); 1.85 (m, 12H); 2.2 (d, 6H); 2.3 (d, 6H); 2.5 (d, 10H); 2.8 (m, 10H); 3.0 (t, 3H); 3.1 (t, 3H); 3.2 (s, 6H); 3.3 (m, 4H); 3.58 (m, 2H); 3.6 (d, 1H); 4.1 (d. 1H); 4.2 (m, 2H); 4.3 (m, 1H); 4.4 (d, 1H); 6.0 (s, 1H); 6.2 (d, 1H); 6.5 (s, 1H); 7.1 (s, 1H); 7.2 (s, 1H). MS (FAB^+): m/e 1638 (M^+). IR (KBr): 3400, 3200, 2950, 2060, 1660, 1570, 1490, 1060 cm^{-1}; UV (MeOH): λ360.

EXAMPLE 7

MODIFICATION OF CYANOCOBALAMIN MONOCARBOXYLIC ACIDS CONJUGATED WITH 1,12-DIAMINODODECANE: REACTION WITH SUCCINIC ANHYDRIDE

This example serves to demonstrate modification of an amino terminus linking moiety to a carboxylate terminus. Such a modification may be necessary for conjugating amino containing rerouting agents (*e.g.*, aminosugars) to cyanocobalamin derivatives containing a linker.

Cyanocobalamin carboxylic acid diaminododecane conjugate **(8, 9, 10)** (0.138 mmoL, 200 mg) was dissolved in 40 mL of dimethylsulfoxide (DMSO) containing 8 g (80 mmoL) of succinic anhydride and 6.4 mL of pyridine. After 14-16 h at room temperature, the excess of succinic anhydride was destroyed by adding 500 mL of water and keeping the pH of the reaction mixture at 6 with 10% KOH. KCN was then added at a final concentration of 0.01 M and the pH of the solution was readjusted to 6 with 3 N HCl. After 1 h the cyanocobalamin components were desalted by phenol extraction. The residue was digested with 100 mL of acetone and the solvent was decanted. It was dissolved in 40 mL of H_2O. 1N NaOH (2 mL) was added to it and the reaction was stirred at room temperature for 15-20 min. It was then neutralized with 1N HCl and the cyanocobalamin components **(14, 15, 16)** were desalted by phenol extraction. Yield: 80 mg (40%); mp 190-198° C with decomposition.

^1H NMR (MeOH-d_4, δ): 0.43 (s, 3H, C-20 CH_3); 1.17 (s, 4H, C-46 CH_3); 1.23 (d, 4H, Pr_3 CH_3); 1.29 (s, 24H); 1.36 (br s, 6H); 1.4 (s, 6H); 1.87 (s, 4H); 2.05 (m, 2H); 2.25 (s, 6H, B10 & B11 CH_3); 2.35 (m, 3H); 2.4 (m, 5H); 2.55 (d,

10H); 2.7 (s, 5H); 2.8 (m, 2H); 3.1 (m, 6H); 3.3 (s, 6H); 3.4 (m, 1H); 3.65 (m, 2H); 3.75 (d, 1H); 3.9 (d, 1H); 4.0 (m, 1H); 4.1 (d, 1H); 4.16 (m, 1H); 4.3 (m, 1H); 4.48 (m, 1H); 4.6 (m, 2H); 6.0 (s, 1H, C-10); 6.3 (d, 1H, R_1); 6.5 (s, 1H, B4); 7.1 (s, 1H, B2); 7.2 (s, 1H, B7). MS (FAB^+): m/e 1639 (M^+). IR (KBr): 3400, 3200, 2950, 2060, 1660, 1570, 1490, 1060 cm^{-1}. UV (MeOH): λ360 (ε 22 564).

EXAMPLE 8

CYANOCOBALAMIN MODIFIED ON MONOCARBOXYLIC ACID: DIAMINODODECANE-BIOTIN CONJUGATES

This example serves to demonstrate coupling a vitamin B_{12} derivative and biotin. Biotin conjugates **(17, 18, 19)** were obtained by reaction of activated cyanocobalamin monocarboxylic acid diaminododecane **(14)**, **(15)**, and **(16)** with the NHS ester of biotin (Sigma Chemical Co.).

To a solution of cyanocobalamin monocarboxylic acid diaminododecane conjugate **(14, 15, 16)** (300 mg, 0.195 mmoL) in DMF (35 mL), was added triethylamine (0.027 mL, 0.195 mmoL). N-Hydroxysuccinimidobiotin (100 mg, 0.295 mmoL) was then added over a period of 10-15 min and evaporated to dryness. The solid residue was dissolved in 20 mL of water and applied to an 75 g of Dowex Cl^- (40 x 2 cm) (acetate form, 200-400 mesh) column. The product was eluted using 250 mL of water. It was then evaporated to dryness, the residue was dissolved in a 10 mL of methanol - water (7:3 v/v) and the solution was applied to a reverse phase C-18 column (500 mm x 25 mm, Alltech, 150 psi) which was developed with the same solvent. RAININ Rabbit-plus peristaltic pumping system was used with a DYNAMAX (model UV-1) UV visible absorbance detector. The eluate was collected with an automatic fraction collector. The fractions containing the final product (HPLC monitored) were evaporated to dryness.

b-isomer **(17)**: yield 159 mg (53%), mp 210-212° C with decomposition, ^1H NMR (MeOH-d_4, δ): 0.43 (s, 3H, C-20 CH_3); 1.18 (s, 4H); 1.3 (m, 13H); 1.39 (m, 13H); 1.45 (s, 5H); 1.6 (m, 4H); 1.72 (m, 2H); 1.9 (s, 6H); 2.2 (d, 8H, B10 & B11 CH_3); 2.6 (d, 12H); 2.7 (m, 3H); 2.8-3.0 (m, 8H); 3.1 (m, 3H); 3.2 (m, 2H); 3.4 (s, 1H); 3.6 (m, 2H); 3.68 (d, 1H); 3.75 (m, 1H); 3.9 (d, 1H); 4.07 (m, 1H); 4.12 (d, 1H); 4.2 (s, 1H); 4.3 (m, 1H); 4.47 (m, 1H); 4.7 (m, 1H); 6.0 (s, 1H, C-10); 6.2 (d,1H, R1); 6.5 (s,1H, B4); 7.1 (s, 1H, B2); 7.2 (s, 1H, B7); MS (FAB^+): m/e 1764 (M^+). IR (KBr): 3400, 3200, 2950, 2060, 1660, 1570, 1490, 1060 cm^{-1}. UV (MeOH): λ360 (ε23 746).

Anal. Calcd. for $C_{85}H_{127}N_{17}O_{16}CoPS•11H_2O$: C, 51.98; H, 7.59; N, 12.13. Found: C, 51.91; H, 7.81; N, 12.31.

e-isomer **(18)**: yield 174 mg (58%), mp 222-224° C with decomposition, ^1H NMR (MeOH-d_4, δ): 0.43 (s, 3H, C-20 CH_3); 1.17 (s, 4H, C-46 CH_3); 1.22 (d, 4H, Pr_3 CH_3); 1.29 (s, 24H); 1.36 (br s, 6H); 1.4 (s, 6H); 1.6 (m, 4H); 1.72 (m, 2H); 1.87 (s, 4H); 2.17 (m, 3H); 2.25 (s, 6H, B10 & B11 CH_3); 2.36 (m, 3H); 2.55 (d, 10H); 2.64 (m, 2H); 2.8 (s, 4H); 2.97 (s, 4H); 3.1 (m, 3H); 3.3 (m, 1H); 3.4 (m, 1H); 3.58 (m, 1H); 3.65 (m, 1H); 3.75 (d, 1H); 3.9 (d, 1H); 4.0 (m, 1H); 4.1 (d, 1H); 4.16 (m, 1H); 4.3 (m, 2H); 4.48 (m, 2H); 4.6 (m, 1H); 6.0 (s, 1H, C-10); 6.3 (d, 1H, R1); 6.5 (s, 1H, B4); 7.0 (s, 1H, B2); 7.2 (s, 1H, B7); MS (FAB^+): m/e 1764 (M^+). IR (KBr): 3400, 3200, 2950, 2060, 1660, 1570, 1490, 1060 cm^{-1}. UV (MeOH): λ360 (ε24 441).

Anal. Calcd. for $C_{85}H_{127}N_{17}O_{16}CoPS•9H_2O$ (13): C, 52.96; H, 7.53; N, 12.35. Found: C, 52.85; H, 7.55; N, 12.30.

d-isomer **(19)**: yield 165 mg (55%), mp 216-218° C with decomposition, ^1H NMR (MeOH-d_4, δ): 0.43 (s, 3H, C-20 CH_3); 1.16 (s, 3H, C-46 CH_3); 1.2 (d, 4H, Pr_3 CH_3); 1.28 (s, 15H); 1.35 (br s, 9H); 1.42 (s, 3H); 1.53 (m, 2H); 1.6 (m, 4H); 1.72 (m, 2H); 1.86 (s, 6H); 2.16 (m, 3H); 2.02 (m, 4H); 2.25 (d, 6H, B10 & B11 CH_3); 2.5 (d, 10H); 2.7 (d, 1H); 2.8 (m, 5H); 3.1 (m, 6H); 3.2 (m, 3H); 3.4 (m, 1H); 3.57 (m, 1H); 3.6 (d, 1H); 3.7 (d, 1H); 3.9 (d, 1H); 4.0 (m, 1H); 4.11 (d, 1H); 4.17 (m, 1H); 4.3 (m, 2H); 4.4 (m, 2H); 4.6 (m, 1H); 6.0 (s, 1H, C-10); 6.3 (d, 1H, R1); 6.5 (s, 1H, B4); 7.1 (s, 1H, B2); 7.2 (s, 1H, B7); MS (FAB^+): m/e 1764 (M^+); IR (KBr): 3400, 3200, 2950, 2060, 1660, 1570, 1490, 1060 cm^{-1}; UV (MeOH): λ360 (ε29 824).

Anal. Calcd for $C_{85}H_{127}N_{17}O_{16}CoPS•10H_2O$: C, 52.46; H, 7.56; N, 12.24. Found: C, 52.27; H, 7.56; N, 12.34.

EXAMPLE 9

CYANOCOBALAMIN MODIFIED ON RIBOSE:

SUCCINATE-DIAMINODODECANE-BIOTIN CONJUGATE (20)

This example serves to demonstrate the conjugation of the ribose-linked diaminododecane adduct **(13)** with biotin to produce a cyanocobalamin biotin conjugate **(20)**.

To a solution of (**11**) (300 mg, 0.183 mmoL) in DMF (35 mL), triethylamine (0.025 mL, 0.183 mmoL) was added. N-hydroxysuccinimidobiotin (100

mg, 0.295 mmoL) was added over a period of 10-15 min. and then evaporated to dryness. The solid residue was dissolved in 20 mL of water and adjusted to pH 10 with 1N NaOH and applied to an 75 g Dowex Cl^- (40 x 2 cm) (200-400 mesh) column. The water fraction was discarded. The product was then eluted with 0.1N NH_4OAc and was desalted by phenol extraction. The residue was dissolved in a 10 mL of methanol - water (7:3 v/v) and the solution was applied to a reverse phase column (octadecyl) which was developed with the same solvent. The fractions containing the final product **(20)** (HPLC monitored) were evaporated to dryness. Yield 135 mg (45 %), mp 198-205 ° C with decomposition.

^1H NMR (MeOH-d_4, δ): 0.43 (s, 3H, C-20 CH_3); 1.15 (s, 3H); 1.2 (d, 3H); 1.3 (s, 27H); 1.36 (m, 6H); 1.4 (m, 3H); 1.6 (m, 4H); 1.7 (m, 2H); 1.85 (m, 12H); 2.0 (d, 3H); 2.17 (m, 3H); 2.2 (d, 6H); 2.3 (d, 6H); 2.5 (d, 10H); 2.64 (m, 2H); 2.8 (m, 10H); 3.1 (m, 6H); 3.25 (m, 6H); 3.58 (m, 2H); 4.0 (m, 1H); 4.1 (m, 1H); 4.16 (m, 1H); 4.4 (m, 1H); 4.6 (s, 2H); 4.7 (m, 1H); 6.0 (s, 1H); 6.2 (d, 1H); 6.5 (s, 1H); 7.1 (s, 1H); 7.2 (s, 1H). MS (FAB^+): m/e 1866 (M^+). IR (KBr): 3400, 3200, 2950, 2060, 1660, 1570, 1490, 1060 cm^{-1}. UV (MeOH): λ360 (ε28 434).

EXAMPLE 10

SYNTHESIS OF A CYANOCOBALAMIN/LYSOSOMOTROPIC COMPOUND (STREPTOMYCIN) RECEPTOR MODULATING AGENT

This example demonstrates coupling of streptomycin to a cyanocobalamin or cobalamin derivative. Streptomycin **(21)** is conjugated with cyanocobalamin monocarboxylate **(2, 3, 4)** or a diaminoalkylsuccinate derivative **(14, 15, 16)** through the use of an oxime coupled linking moiety (Figure 13). The linking group, ((3-aminopropyl)aminoxy)acetamide **(22)** is prepared by reaction of the N-hydroxysuccinimidyl ester of 1,1-dimethylethoxycarbonyl-aminooxyacetic acid **(23)** (J. Med. Chem. 36:1255-126, 1993) with an excess of diaminopropane in anhydrous THF. The linking group is separated from other compounds in the reaction mixture by preparative chromatography. The linker (1 g) is then mixed with streptomycin (0.5g) in 10 mL of H_2O containing sodium acetate. The aqueous solution is warmed in a H_2O bath for 10 minutes to yield a crude streptomycin-linker adduct **(25)** which may be purified by chromatography on acid washed alumina (J. Am. Chem. Soc. 68:1460, 1946). The aqueous solution containing the streptomycin linker adduct (0.15 mmol) is mixed with an aqueous solution of activated cyanocobalamin **(2, 3, 4)** (01. mmol) and EDC (0.5 mmol) is added. The reaction mixture is stirred at room temperature for 24

hours, then run over a reversed-phase preparative chromatography column for purification of the cyanocobalamin-streptomycin receptor modulating agent **(26)**.

EXAMPLE 11

SYNTHESIS OF A CYANOCOBALAMIN/LYSOSOMOTROPIC COMPOUND (ACRIDINE) RECEPTOR MODULATING AGENT

This example demonstrates the coupling of the vitamin B_{12} to acridine. Chloroquine, quinacrine and acridine are lysosomotropic dyes which are relatively non-toxic and concentrated as much as several hundred fold in lysosomes. Acridine derivatives may be covalently attached to a targeting moiety (such as cyanocobalamin) by the reaction scheme illustrated in Figure 14, method A, or similarly as described in method B. Both reaction schemes produce a cyanocobalamin-acridine conjugate.

Method A: A diamine side chain is first synthesized in a manner analogous to the side chain of quinacrine. Specifically, mono-phthaloyl protected 1,4-diaminobutane **(27)** is reacted with 6,9-dichloro-2-methoxyacridine **(28)** in phenol (J. Am. Chem. Soc. 66:1921-1924, 1944). The reaction mixture is then poured into an excess of 2 N NaOH and extracted with ether. The ether extract is washed with 1 M $NaHCO_3$, then H_2O, and dried over $MgSO_4$. The crude product is recrystallized from H_2O-alcohol. The phthaloyl protecting group is removed using anhydrous hydrazine in MeOH (Bioconjugate Chem. 2:435-440, 1991) to yield the aminoacridine, **(29)**. Aminoacridine **(29)** is then conjugated with vitamin B_{12} monocarboxylic acid **(2, 3, 4)** to yield a cyanocobalamin-acridine conjugate **(30)**.

Method B: Acridine derivative **(31)** (0.098 mmol, 0.045 g) was dissolved in 0.5 mL of trifluoroacetic acid. This solution was stirred at room temperature for 0.5 h. TFA was removed by aspirator vacuum. The residue was dissolved in 5 mL of acetonitrile and was neutralized by few drops of triethylamine. Acetonitrile was then removed by aspirator vacuum. The residue was dissolved in DMSO (10 mL) and cyanocobalamin carboxylic acid-diaminododecane-succinyl derivative **(15, 16, 17)** (0.098 mmol, 134 mg) was added followed by triethylamine (12 µL). The reaction mixture was then stirred at room temperature for 24 h. (HPLC monitored), and evaporated to dryness. The residue was digested with 100 mL of acetone and the solvent was decanted yielding a cyanocobalamin-acridine conjugate **(32)**. Yield: 120 mg (62%). mp 182-188 °C.

^1H NMR (MeOH-d_4, δ): 0.43 (s, 3H, C-20 CH_3); 1.17 (s, 4H, C-46 CH_3); 1.23 (d, 4H, Pr_3 CH_3); 1.29 (s, 24H); 1.36 (br s, 6H); 1.4 (s, 6H); 1.65 (m,

2H); 1.87 (s, 4H); 2.05 (m, 2H); 2.25 (s, 6H, B10 & B11 CH_3); 2.35 (m, 3H); 2.4 (d, 5H); 2.44 (d, 2H); 2.55 (d, 10H); 2.64 (s, 5H); 2.8-2.9 (m, 8H); 3.1-3.15 (m, 6H); 3.3 (s, 6H); 3.4 (m, 1H); 3.65 (m, 2H); 3.75 (d, 1H); 3.9 (d, 1H); 3.98 (s, 2H); 4.0 (m, 2H); 4.1 (d, 1H); 4.16 (m, 1H); 4.3 (m, 1H); 4.48 (m, 1H); 4.6 (m, 2H); 6.0 (s, 1H, C-10); 6.3 (d, 1H, R_1); 6.5 (s, 1H, B4); 7.1 (s, 1H, B2); 7.2 (s, 1H, B7); 7.3 (t, 1H); 7.4 (dd, 1H); 7.6 (dd, 1H); 7.7 (2dd, 2H); 7.8 (d, 1H); 7.9 (d, 1H); 8.4 (d, 1H).

EXAMPLE 12

SYNTHESIS OF A CYANOCOBALAMIN/LYSOSOMOTROPIC COMPOUND (AMIKACIN) RECEPTOR MODULATING AGENT

This example demonstrates conjugation of amikacin to a cyanocobalamin molecule to form a cyanocobalamin-amikacin conjugate. A reaction scheme for the conjugation is depicted in Figure 12. As noted above, chemical moieties that are retained subcellularly within lysosomes are termed lysosomotropic. Aminoglycosides are lysosomotropic compounds, and thus may be used as rerouting moieties of this invention. The primary long chain amine on the hydroxyaminobutyric acid side chain of the aminoglycoside, amikacin (*see* Figure 3), is preferentially reactive. Specifically, amikacin **(33)** (Sigma Chemical Co., St. Louis), is reacted with a vitamin B_{12} monocarboxylate **(2, 3, 4)** in the presence of EDC. A cyanocobalamin-amikacin conjugate **(34)** is then separated and purified by reverse-phase LC chromatography under conditions noted above.

EXAMPLE 13

CYANOCOBALAMIN MONOCARBOXYLIC ACID DIAMINODODECANE CONJUGATE DIMER: ISOPHTHALOYL DICHLORIDE CROSS-LINKING

This example demonstrates the production of a cyanocobalamin dimer suitable for use as a cross-linking receptor modulating agent. Cross-linking of receptors in some receptor systems is sufficient to cause a rerouting of cell surface receptors to lysosomes for degradation, rather than their normal pathway of receptor recycling.

To a solution of cyanocobalamin monocarboxylic acid diaminododecane conjugate **(8, 9, 10)** (0.192 mmol, 0.300 g) in DMF (30 mL), was added triethylamine (18 μL). Isophthaloyl dichloride **(35)** (0.096 mmol, 0.0195 g) was added over a period of 10-15 min. The reaction mixture was stirred at 55-60°C for 48 h (HPLC monitored) and evaporated to dryness. The solid residue was dissolved in 20 mL of methanol :

H_2O (7:3) and applied to a reverse phase C-18 column (500 mm x 25 mm, Alltech, 150 psi) which was developed with the same solvent. RAININ Rabbit-plus peristaltic pumping system was used with a DYNAMAX (model UV-1) UV visible absorbance detector; the elute was collected with an automatic fraction collector. The fractions containing the final product (HPLC monitored) were evaporated to dryness.

b-acid dimer **(36)**: yield 96 mg (30%), mp 217-220° C with decomposition, ^{1}H NMR (D_2O, δ) 0.43 (s, 6H, C-20 CH_3); 1.18 (s, 8H); 1.3 (m, 36H); 1.37 (m, 12H); 1.46 (s, 10H); 1.6 (m, 8H); 1.9 (d, 12H); 2.05 (m, 10H); 2.2 (d, 16H, B10 & B11 CH_3); 2.35 (m, 8H); 2.6 (d, 18H); 2.8-3.0 (m, 16H); 3.15 (m, 6H); 3.3 (s, 8H); 3.37 (m, 14H); 3.6 (m, 4H); 3.76 (m, 2H); 3.9 (d, 2H); 4.07 (m, 2H); 4.12 (m, 2H); 4.18 (m, 2H); 4.3 (m, 2H); 4.5 (m, 2H); 4.6 (s, 2H); 4.68 (m, 2H); 6.0 (s, 2H, 2C-10); 6.26 (d,2H, 2R1); 6.6 (s,2H, 2B4); 7.1 (s, 2H, 2B2); 7.25 (s, 2H, 2B7); 7.54 (t, 1H); 7.95 (d, 2H); 8.25 (s, 1H); MS (FAB^+): m/e 3208. IR (KBr): 3400, 3200, 2950, 2060, 1660, 1570, 1490, 1060 cm^{-1}; UV: λ360 (ε42 380).

e-acid dimer **(37)**: yield 121 mg (38%), mp 220-222° C with decomposition, ^{1}H NMR (D_2O, δ) 0.43 (s, 6H, C-20 CH_3); 1.17 (s, 8H); 1.22 (d, 13H); 1.29 (s, 45H); 1.36 (d, 22H); 1.44 (s, 10H); 1.6 (m, 8H); 1.87 (s, 8H); 2.04 (m, 10H); 2.25 (s, 12H, B10 & B11 CH_3); 2.36 (m, 8H); 2.55 (d, 20H); 2.8 (m, 8H); 3.15 (m, 8H); 3.29 (s, 10H); 3.36 (m, 14H); 3.6 (m, 4H); 3.73 (m, 2H); 3.9 (d, 2H); 4.07 (m, 2H); 4.12 (m, 2H); 4.16 (m, 2H); 4.3 (m, 2H); 4.5 (m, 2H); 4.6 (s, 2H); 4.66 (m, 2H); 6.0 (s, 2H, 2C-10); 6.26 (d,2H, 2R1); 6.6 (s,2H, 2B4); 7.1 (s, 2H, 2B2); 7.25 (s, 2H, 2B7); 7.54 (t, 1H); 7.93 (d, 2H); 8.25 (s, 1H); MS (FAB^+): m/e 3208. IR (KBr): 3400, 3200, 2950, 2060, 1660, 1570, 1490, 1060 cm^{-1}. UV (MeOH): λ360 (ε 33 854)

d-acid dimer **(38)**: yield 96 mg (30%), mp 225-228° C with decomposition, ^{1}H NMR (D_2O, δ) 0.43 (s, 6H, C-20 CH_3); 1.16 (s, 8H); 1.29 (m, 36H); 1.35 (d, 12H); 1.44 (s, 10H); 1.53 (m, 6H); 1.6 (m, 8H); 1.85 (s, 12H); 2.03 (m, 8H); 2.25 (d, 12H, B10 & B11 CH_3); 2.33 (m, 8H); 2.54 (d, 20H); 2.8 (m, 8H); 3.13 (m, 8H); 3.28 (s, 12H); 3.35 (m, 12H); 3.6 (m, 4H); 3.73 (m, 2H); 3.9 (d, 2H); 4.07 (m, 2H); 4.12 (m, 2H); 4.16 (m, 2H); 4.3 (m, 2H); 4.5 (m, 2H); 4.64 (m, 2H); 4.7 (s, 2H); 6.0 (s, 2H, 2C-10); 6.26 (d,2H, 2R1); 6.6 (s,2H, 2B4); 7.1 (s, 2H, 2B2); 7.25 (s, 2H, 2B7); 7.54 (t, 1H); 7.93 (d, 2H); 8.25 (s, 1H); MS (FAB^+): m/e 3208. IR (KBr): 3400, 3200, 2950, 2060, 1660, 1570, 1490, 1060 cm^{-1} UV (MeOH): λ360 (ε 31 747).

EXAMPLE 14

CYANOCOBALAMIN MONOCARBOXYLIC ACID DIAMINODODECANE CONJUGATE DIMER: ETAC CROSS-LINKING

This example serves to illustrate synthesis of a bivalent receptor modulating agent using a heterotrifunctional cross-linker. The reaction scheme for this synthesis is depicted in Figure 15. The heterotrifunctional cross-linker is formed an ETAC reagent (Bioconjugate Chem. 1:36-50, 1990; Bioconjugate Chem. 1:51-59, 1990; J. Am. Chem. Soc. 101:3097-3110, 1979). Bivalency, in addition to enhancing affinity of binding, also imparts the ability to cross-link neighboring receptors and trigger endocytosis. The bivalent "arms" of the agent may be lengthened with peptide or other linking molecules to enable simultaneous binding of both "arms". In the case of vitamin B_{12} this may be assessed by gel filtration. If the linkers allow simultaneous interaction, there will be 2 moles of TcII for every mole of ETAC dimer present in a single peak of 80,000 m.w. (versus 40,000 m.w. of monomeric TcII). Simultaneous binding of 2 moles of TcII will then have the potential for bivalent binding to cell surface receptor. This can be tested by comparing the affinity of monomer and dimer binding to receptor. While the bivalent agent can be synthesized to include any rerouting moiety of this invention which enhances lysosomal targeting and retention, the compound tyramine, useful for radio-labeling is disclosed for the purpose of illustration.

Referring to Figure 15, carboxy-ETAC **(39)** is prepared by the method of Liberatore et al. (Bioconjugate Chem. 1:1990). The carboxy-ETAC is converted to its acid chloride by reaction in thionyl chloride. Addition of amine **(40)** gives the amine-ETAC adduct **(41)**. Reaction of amine-ETAC (1 mmol) in CH_3CN with 1 M aqueous cysteamine (10 mmol) is conducted by stirring at room temperature for 24 h. This compound is reduced with $NaCNBH_3$ under acidic conditions. The crude amine-ETAC-cysteamine adduct **(42)** is purified by reverse-phase LC, using conditions noted above. A vitamin B_{12} monocarboxylate **(2, 3, 4)** is conjugated with tyramine-ETAC-cysteamine compound by reaction with EDC in H_2O. The resultant vitamin B_{12}-ETAC-tyramine dimer **(43)** is purified by reverse phase LC, using conditions described above.

EXAMPLE 15

CYANOCOBALAMIN MONOCARBOXYLIC ACID DIAMINODODECANE CONJUGATE DIMER: ISOPHTHLATE CROSS-LINKING WITH BIOTIN MOIETY

This example illustrates the synthesis of a bivalent receptor modulating agent which is additionally coupled to a biotin moiety **(44)**. Further modification can be obtained by coupling of this molecule with an avidin or streptavidin moiety.

Reaction Step A: Biotin (12.3 mmol, 3 g) was dissolved in warm (bath temperature 70°C) DMF (60 mL) under argon atmosphere. It was then cool to ambient temperature and DCC (13.5 mmol, 2.79 g) was added, followed by tetrafluorophenol (24.6 mmol, 4.08g). The reaction mixture was then cooled to 0°C and stirred for 0.5 h. It was then brought back to ambient temperature and stirred for another 4-5 h. The reaction mixture was filtered and the filtrate was evaporated to dryness. The precipitate was washed with acetonitrile (50 mL) and was filtered to yield 5 g (98%) of white solid **(45)**.

^{1}H NMR (DMSO, δ): 1.4 (m, 2H); 1.7 (m, 2H); 2.5 (t, 2H); 2.8 (t, 2H); 3.1 (m, 1H); 4.1 (m, 1H); 4.3 (m, 1H); 6.4 (d, 2H); 7.9 (m, 1H).

Reaction Step B: 6-Aminocaproic acid **(46)** (7.5 mmol, 0.99g) was dissolved in H_2O (75 mL). Triethylamine (0.5 mL) was added followed by a solution of TFP ester of Biotin (5 mmol, 1.96 g) in warm acetonitrile (300 mL). The reaction was stirred overnight at room temperature. It was then filtered, washed with H_2O (50 mL) and dried on high vacuum. Yield: 0.870 g (47%). The filtrate was evaporated to dryness. The residue was taken in boiling acetonitrile (75 mL) and was filtered, washed with hot acetonitrile. The solid **(47)** was dried on high vacuum to give 0.6 g, for a total yield of 1.47 g (79%).

^{1}H NMR (DMSO-d_6, δ): 1.2-1.6 (m, 8H); 2.0 (t, 2H); 2.2 (t, 2H); 2.5 (dd, 2H); 2.8 (dd, 2H); 3.1 (m, 3H); 4.1 (m, 1H); 4.3 (m, 1H); 6.4 (d, 2H); 7.7 (m, 1H).

Reaction Step C: Biotin conjugated caproic acid **(47)** (2.68 mmol, 1 g) was dissolved in DMSO (50 mL). Triethylamine (0.4 mL) was added followed by TFP acetate (4.02 mmol, 1.05 g). The reaction mixture was then stirred at room temperature for 15-20 min (HPLC monitored). It was then evaporated to dryness. The residue was washed with ether and dichloromethane and dried on high vacuum **(48)**. Yield: 1.24 g (89%).

^{1}H NMR (DMSO-d_6, δ): 1.2 (t, 2H); 1.3-1.7 (m, 5H); 2.1 (t, 2H); 2.6 (dd, 2H); 2.8 (m, 4H); 3.1 (m, 4H); 4.2 (m, 1H); 4.4 (m, 1H); 6.4 (d, 2H); 7.8 (t, 1H); 8.0 (m, 1H).

Reaction Step D: TFP ester of Biotin-caproic acid **(48)** (0.67 mmol, 0.35 g) was dissolved in DMF (40 mL). Triethylamine (80 μL) was added followed by aminoisophthalic acid (1.005 mmol, 0.182 g). The reaction was stirred at room temp. for 8 days (HPLC monitored) while adding triethylamine (80 μL) every after 24 h. It was then evaporated to dryness. The residue was then applied to a column of silica and was initially eluted with acetonitrile (450 mL). It was then eluted with methanol, 20 mL of fractions were collected, at the fraction 2 the solvent was changed to DMF. The fractions containing the final product (HPLC monitored) were evaporated to dryness **(49)** to yield 230 mg (65%).

^{1}H NMR (DMSO-d_6, δ): 1.3-1.7 (m, 8H); 2.1 (t, 2H); 2.3 (t, 2H); 2.6 (m, 2H); 2.8 (m, 2H); 3.1 (m, 3H); 4.1 (m, 1H); 4.3 (m, 1H); 6.4 (d, 2H); 7.8 (t, 1H); 8.1 (m, 1H); 8.46 (s, 2H).

Reaction Step E: Biotin-caproic acid-isophthalic acid **(49)** (0.376 mmol, 200 mg) was dissolved in DMF (30 mL) under argon atmosphere. TFP acetate (0.94 mmol, 241 mg) was added by double ended needle, followed by triethylamine (112 μL). The reaction was then stirred at room temp. for 24 h (HPLC monitored). It was then evaporated to dryness. The light brownish oil was taken in ether, solid was filtered and was washed with ether (50 mL) **(50)** to yield 250 mg (86%).

^{1}H NMR (DMSO-d_6, δ): 1.3-1.7 (m, 8H); 2.1 (t, 2H); 2.3 (t, 2H); 2.6 (m, 2H); 2.8 (m, 2H); 3.1 (m, 3H); 4.2 (m, 1H); 4.4 (m, 1H); 6.4 (d, 2H); 7.8 (t, 1H); 8.1 (m, 2H); 8.57 (s, 1H); 8.9 (s, 2H).

Reaction Step F: In a solution of cyanocobalamin carboxylic acid - diaminododecane conjugate **(8, 9, 10)** (0.130 mmol, 0.2 g) in a mixture of DMF : H_2O (3:1) (40 mL) triethylamine (12 μL) was added. DiTFP ester of biotin-caproic acid-isophthalic acid **(50)** (0.065 mmol, 0.050 g) was added over a period of 5-10 min. The reaction mixture was stirred at room temperature for 3 h (HPLC monitored). It was then evaporated to dryness. The residue was digested with 100 mL of acetone and the solvent was decanted to yield 230 mg (62%) **(51).** mp 195-198°C with decomposition.

60

EXAMPLE 16

CYANOCOBALAMIN MONOCARBOXYLIC ACID DIAMINODODECANE CONJUGATE DIMER: ISOPHTHALATE CROSS-LINKING WITH PARA-IODOBENZOYL MOIETY

This is an example of a bivalent receptor modulating agent which is also conjugated to a *para*-iodobenzoyl moiety.

Reaction Step A: A 5g (28 mmol) quantity of 5-aminoisophthalic acid **(52)** was dissolved in 30 mL 1N NaOH and placed in an ice/water bath. To the cold solution was added 7.5g (28 mmol) 4-iodobenzoyl chloride **(52)** in 60 mL of acetonitrile, dropwise. The thick white precipitate was then stirred for 10 minutes before removing the ice/water bath and allowing the mixture to stir an additional 10 minutes. The reaction mixture was adjusted to pH 4 with acetic acid and the resulting solid collected. This solid was then dissolved in 30 mL 1N NaOH and washed with ether (2 x 50 mL). The resulting aqueous solution was filtered and acidified to pH 4 with acetic acid. The white precipitate was the collected and dried on high vacuum to yield .6 g (99+%) of **(54)**. mp >300 ^{o}C; IR (Nujol, cm^{-1}) 3570(m), 3300(m), 1645, 1580(m), 1525(m), 760(m); ^{1}H NMR (DMSO-d_6, δ), 8.51 (2H, d, J = 0.7 Hz), 8.27 (1H, s), 7.94 (2H, d, J = 4.2 Hz), 7.84 (2H, d, J = 4.1 Hz).

Reaction Step B: A 5g (12.2 mmol) quantity of 5-[N-iodobenzoyl)amino]-isophthalic acid **(54)** was suspended in 100 mL anhydrous ethyl acetate. To this was added 12.5g (73 mmol) 2,3,5,6-tetrafluorophenol **(55)** followed by 5g (24.2 mmol) 1,3-dicyclohexylcarbodiimide. This suspension was then stirred at room temperature for 3 days before filtering off the solid and washing with an additional 20 mL of ethyl acetate. The filtrate was then evaporated to dryness. The resulting sticky white solid was suspended in 50 mL acetonitrile and stirred for 30 minutes. Filtering yielded 3.75g of white solid (43%) **(56)**. mp 250-251 ^{o}C; IR (Nujol, cm-1) 3220(m), 3060(m), 1750, 1655, 1520, 1485, 1330, 1195, 1110, 1085, 955(m), 945(m); ^{1}H NMR (DMSO-d_6, δ), 9.06 (2H, d, J = 0.7 Hz), 8.57 (1H, t, J = 1.4 Hz), 8.04 (2H, m), 7.94 (2H, d, J = 4.2 Hz), 7.81 (2H, d, J = 4.3 Hz).

Reaction Step C: To a solution of cyanocobalamin carboxylic acid - diaminododecane conjugate **(56)** (0.192 mmol, 0.3 g) in a mixture of DMF : H_2O (3:1) (40 mL) was added triethylamine (0.018 mL). To this solution, DiTFP ester of 5-[N-(p-Iodobenzoyl)amino]-Isophthalic acid **(57)**(0.096 mmol, 0.068 g) was added over a period of 5-10 min. The reaction mixture was stirred at room temperature for 4-5 h (HPLC monitored). It was then evaporated to dryness. The solid residue was dissolved in 20 mL of methanol : H_2O (8:2) and applied to a reverse phase C-18 column (500 mm

x 25 mm, Alltech, 150 psi) which was developed with the same solvent. RAININ Rabbit-plus peristaltic pumping system was used with a DYNAMAX (model UV-1) UV visible absorbance detector; the elute was collected with an automatic fraction collector. The fractions containing the final product (HPLC monitored) were evaporated to dryness.

b-acid dimer **(58)**: yield: 280 mg (76%), mp 230-233 °C with decomposition, ^{1}H NMR (D_2O, δ) 0.43 (s, 6H, C-20 CH_3); 1.19 (s, 8H); 1.3 (m, 36H); 1.37 (d, 12H); 1.46 (s, 10H); 1.63 (m, 8H); 1.87 (s, 12H); 2.05 (m, 10H); 2.27 (d, 16H, B10 & B11 CH_3); 2.35 (m, 8H); 2.6 (d, 18H); 2.8 (s, 8H); 3.0 (s, 10H); 3.15 (m, 8H); 3.3 (d, 8H); 3.37 (m, 14H); 3.6 (m, 2H); 3.68 (d, 2H); 3.76 (m, 2H); 3.9 (d, 2H); 4.07 (m, 2H); 4.12 (m, 2H); 4.18 (m, 2H); 4.3 (m, 2H); 4.5 (m, 2H); 4.64 (m, 4H); 6.0 (s, 2H, 2C-10); 6.26 (d, 2H, $2R_1$); 6.6 (s, 2H, 2B4); 7.1 (s, 2H, 2B2); 7.25 (s, 2H, 2B7); 7.7 (d, 2H); 7.9 (d, 2H); 7.99 (d, 1H); 8.28 (s, 2H); MS (FAB^+): m/e 3453. IR (KBr): 3400, 3200, 2950, 2060, 1660, 1570, 1490, 1060 cm^{-1}. UV (MeOH): λ360.6 (ε48 871)

e-acid dimer **(59)**: yield: 258 mg (70%), mp 285-290 °C with decomposition, ^{1}H NMR (D_2O, δ) 0.43 (s, 6H, C-20 CH_3); 1.17 (s, 8H); 1.22 (d, 13H); 1.29 (s, 45H); 1.36 (d, 22H); 1.44 (s, 10H); 1.6 (m, 8H); 1.86 (s, 12H); 2.04 (m, 10H); 2.25 (s, 12H, B10 & B11 CH_3); 2.36 (m, 8H); 2.55 (d, 20H); 2.83 (m, 8H); 3.15 (m, 8H); 3.29 (s, 10H); 3.36 (m, 8H); 3.58 (m, 2H); 3.65 (m, 2H); 3.75 (m, 2H); 3.9 (d, 2H); 4.06 (m, 2H); 4.12 (m, 2H); 4.16 (m, 2H); 4.3 (m, 2H); 4.5 (m, 2H); 4.57 (s, 2H); 4.65 (m, 2H); 6.0 (s, 2H, 2C-10); 6.26 (d, 2H, 2R1); 6.5 (s, 2H, 2B4); 7.1 (s, 2H, 2B2); 7.25 (s, 2H, 2B7); 7.7 (d, 2H); 7.89 (d, 2H); 7.98 (s, 1H); 8.26 (s, 2H); MS (FAB^+): m/e 3453. IR (KBr): 3400, 3200, 2950, 2060, 1660, 1570, 1490, 1060 cm^{-1}; UV (MeOH): λ360 (ε41 481).

d-acid dimer **(60)**: yield 265 mg (72%), mp 253-255 °C with decomposition, ^{1}H NMR (D_2O, δ) 0.43 (s, 6H, C-20 CH_3); 1.16 (s, 8H); 1.22 (d, 12H); 1.33 (m, 36H); 1.43 (s, 10H); 1.53 (m, 6H); 1.6 (m, 8H); 1.86 (s, 12H); 2.03 (m, 8H); 2.25 (d, 12H, B10 & B11 CH_3); 2.33 (m, 8H); 2.54 (d, 20H); 2.8 (s, 4H); 3.0 (s, 4H); 3.28 (s, 10H); 3.35 (m, 8H); 3.58 (m, 2H); 3.65 (m, 2H); 3.73 (m, 2H); 3.88 (d, 2H); 4.05 (m, 2H); 4.1 (m, 2H); 4.17 (m, 2H); 4.3 (m, 2H); 4.5 (m, 2H); 4.57 (s, 2H); 4.63 (m, 2H); 6.0 (s, 2H, 2C-10); 6.26 (d,2H, $2R_1$); 6.5 (s,2H, 2B4); 7.1 (s, 2H, 2B2); 7.25 (s, 2H, 2B7); 7.7 (d, 2H); 7.89 (d, 2H); 7.98 (s, 1H); 8.26 (s, 2H); MS (FAB^+): m/e 3453. IR (KBr): 3400, 3200, 2950, 2060, 1660, 1570, 1490, 1060 cm^{-1}; UV (MeOH): λ360 (ε48 245).

EXAMPLE 17

CYANOCOBALAMIN MONOCARBOXYLIC ACID DIAMINODODECANE CONJUGATE DIMER: ISOPHTAHATE CROSS-LINKING WITH PARA-(TRI-BUTYLSTANNYL)BENZOYL MOIETY

This is an example of a bivalent receptor modulating agent coupled to a *para*-tri-N-butyl stannyl moiety.

Reaction Step A: A 2 g (2.8 mmol) quantity of the diTFP ester of 5-[N-(p-Iodobenzoyl)amino]-Isophthalic acid **(57)** (as prepared above) was dissolved in 20 mL dry toluene under argon. To this was added 2.8 mL (5.5 mmol) of *bis*(tributyltin) **(61)** followed by 40 mg (0.04 mmol) tetrakis(triphenylphosphine)palladium **(62)**. The mixture was stirred at room temperature for 15 minutes before heating to 80°C for 2 h. Since the mixture only darkened slightly over the 2 h period, an additional 40 mg of palladium catalyst was added. Within 1 hour the mixture had turned black. After cooling to room temperature, the toluene was removed by rotary evaporation. The resulting black oil (containing solids), was then taken into 20 mL ethyl acetate and dried onto 10 g silica gel (via rotoevaporation). This solid was then added to a 250 g (40 x 3.5 cm) silica gel column and eluted initially with hexanes containing 5% acetic acid. After 600 mL, the solvent was changed to 90/10 hexanes/ethyl acetate (containing 5% acetic acid). Fractions 14-16 were combined and dried to yield 1.5 g (62%) of white solid **(62)**. mp 120-123 °C;

^{1}H NMR ($CDCl_3$, δ), 8.87 (2H, d, J = 0.7 Hz), 8.76 (1H, t, J = 1.6 Hz), 8.38 (1H, s), 7.84 (2H, d, J = 4.1 Hz), 7.62 (2H, d, J = 4.1 Hz), 7.07 (2H, m), 1.55 (6H, m), 1.36 (15H,m), 1.11 (6H,m), 0.89 (9H, t, J = 7.3 Hz); MS (FAB^+) M+H patterns calculated 870 (75.1%), 871 (52.9%), 872 (100%), 873 (41.0%), 874 (21.4%), found 870 (82.1%), 871 (55.1%), 872 (100%), 873 (42.1%), 874 (25.2%).

IR (Nujol, cm^{-1}) 1750, 1645, 1520, 1480(m), 1185, 1100, 1085.

Reaction Step B: In a solution of cyanocobalamin carboxylic acid - diaminododecane conjugate **(8, 9, 10)** (0.065 mmol, 0.1 g) in a mixture of DMF : H_2O (3:1) (40 mL) triethylamine (0.006 mL) was added. DiTFP ester of 5-[N-(p-tributyltin benzoyl) amino]-Isophthalic acid **(63)**(0.0325 mmol, 0.028 g) was added over a period of 5-10 min. The reaction mixture was stirred at room temperature for 12-14 h (HPLC monitored). It was then evaporated to dryness. The residue was digested with 100 mL of acetone and the solvent was decanted.

b-acid dimer **(64)**: yield: 90 mg (70%), mp 208-212 °C with decomposition, ^{1}H NMR (D_2O, δ) 0.43 (s, 6H, C-20 CH_3); 0.88 (t, 9H); 1.15 (t,

12H); 1.19 (s, 8H); 1.3 (m, 36H); 1.37 (d, 12H); 1.46 (s, 10H); 1.6 (m, 8H); 1.9 (s, 12H); 2.05 (m, 10H); 2.28 (d, 16H, B10 & B11 CH_3); 2.35 (m, 8H); 2.6 (d, 18H); 2.8-2.9 (m, 16H); 3.15 (m, 8H); 3.3 (s, 8H); 3.37 (m, 14H); 3.6 (m, 4H); 3.76 (m, 2H); 3.9 (d, 2H); 4.07 (m, 2H); 4.12 (m, 2H); 4.18 (m, 2H); 4.3 (m, 2H); 4.5 (m, 2H); 4.68 (m, 2H); 6.0 (s, 2H, 2C-10); 6.26 (d,2H, $2R_1$); 6.6 (s, 2H, 2B4); 7.1 (s, 2H, 2B2); 7.25 (d, 2H, 2B7); 7.6 (d, 2H); 7.9 (d, 2H); 7.99 (br s, 1H); 8.28 (br s, 2H); IR (KBr): 3400, 3200, 2950, 2060, 1660, 1570, 1490, 1060 cm^{-1}.

e-acid dimer **(65)**: yield: 93 mg (72%), mp >300 °C, ^1H NMR (D_2O, δ) 0.43 (s, 6H, C-20 CH_3); 0.88 (t, 9H); 1.12 (t, 12H); 1.17 (d, 8H); 1.22 (d, 13H); 1.29 (s, 45H); 1.36 (d, 22H); 1.44 (s, 10H); 1.6 (m, 8H); 1.87 (d, 12H); 2.04 (m, 10H); 2.25 (s, 12H, B10 & B11 CH_3); 2.36 (m, 8H); 2.55 (d, 20H); 2.8 (m, 8H); 3.15 (m, 8H); 3.29 (s, 10H); 3.36 (m, 14H); 3.6 (m, 4H); 3.73 (m, 2H); 3.9 (d, 2H); 4.07 (m, 2H); 4.12 (m, 2H); 4.16 (m, 2H); 4.3 (m, 2H); 4.5 (m, 2H); 4.66 (m, 2H); 6.0 (s, 2H, 2C-10); 6.26 (d,2H, $2R_1$); 6.6 (s,2H, 2B4); 7.1 (s, 2H, 2B2); 7.25 (s, 2H, 2B7); 7.6 (d, 2H); 7.9 (d, 2H); 7.98 (br s, 1H); 8.28 (br s, 2H); IR (KBr): 3400, 3200, 2950, 2060, 1660, 1570, 1490, 1060 cm^{-1}.

d-acid dimer **(66)**: yield: 100 mg (78%), mp 202-205 °C with decomposition, ^1H NMR (D_2O, δ) 0.43 (s, 6H, C-20 CH_3); 0.88 (t, 9H); 1.12 (t, 12H); 1.15 (s, 8H); 1.29 (m, 36H); 1.35 (d, 12H); 1.44 (s, 10H); 1.53 (m, 6H); 1.6 (m, 8H); 1.86 (d, 12H); 2.03 (m, 8H); 2.25 (d, 12H, B10 & B11 CH_3); 2.33 (m, 8H); 2.54 (d, 20H); 2.8 (m, 8H); 3.13 (m, 8H); 3.28 (s, 10H); 3.35 (m, 10H); 3.6 (m, 4H); 3.73 (m, 2H); 3.9 (d, 2H); 4.05 (m, 2H); 4.1 (m, 2H); 4.17 (m, 2H); 4.3 (m, 2H); 4.5 (m, 2H); 4.6 (m, 2H); 6.0 (s, 2H, 2C-10); 6.26 (d,2H, 2R1); 6.6 (s,2H, 2B4); 7.1 (s, 2H, 2B2); 7.25 (s, 2H, 2B7); 7.6 (d, 2H); 7.9 (d, 2H); 7.98 (br s, 1H); 8.28 (br s, 2H); IR (KBr): 3400, 3200, 2950, 2060, 1660, 1570, 1490, 1060 cm^{-1}.

EXAMPLE 18

EVALUATION OF THE ABILITY OF VITAMIN B_{12} RECEPTOR MODULATING AGENTS TO BIND TO TCII

This example serves to demonstrate a competitive binding assay suitable for evaluating the ability of vitamin B_{12} receptor modulating agents to bind TcII. Binding of the vitamin B_{12} derivatives to recombinant transcobalamin II was conducted in picomolar concentrations and the percent bound ascertained.

In this competitive binding assay, various B_{12} derivatives, including vitamin B_{12} receptor modulating agents, were evaluated for their ability to bind to TcII

relative to radiolabeled B_{12}. Varying concentrations of each derivative were incubated with immobilized TcII in the presence of a constant amount of radiolabeled B_{12}. After incubation for 20 minutes at 37° C, the free radiolabeled B_{12} was separated from the TcII bound tracer by removal of the supernatant. The radioactivity of the supernatant solution was then measured to determine the amount of free radiolabeled B_{12} present at the end of each competition. By measuring the amount of free radiolabeled B_{12} for each competition, the ability of each derivative to inhibit radiolabeled B_{12} binding was determined. A binding curve was then be constructed for each B_{12} derivative where the amount of radiolabeled B_{12} bound (% radiolabel bound) was correlated with the concentration of derivative present in the original mixture. The more effective the derivative is in binding to TcII, the lower the percent bound radiolabeled vitamin B_{12}.

Figure 22 illustrates the binding curve of Transcobalamin II to the cyanocobalamin monocarboxylic acids produced in Example 1. AD = Cyanocobalamin **(1)**; AL = Cyanocobalamin *b*-monocarboxylic acid **(2)**; AM = Cyanocobalamin *e*-monocarboxylic acid **(3)**; and AN= Cyanocobalamin *d*-monocarboxylic acid **(4)**. The *d*-carboxylate **(3)** appears to bind nearly as well as cyanocobalamin. Two samples of vitamin B_{12} were used, one as a known standard and the other as an unknown.

Figure 23 illustrates the binding curve of Transcobalamin II to the cyanocobalamin diaminododecane adducts **(8, 9, 10)** and succinate adduct **(13)** produced in Example 3 and 4 above. AH = Cyanocobalamin *b*-monocarboxylic acid conj Diaminododecane **(7)**; AI = Cyanocobalamin *e*-monocarboxylic acid conj Diaminododecane **(8)**; AJ = Cyanocobalamin *d*-monocarboxylic acid conj Diaminododecane **(9)**; AK = Cobalamin *e*-monocarboxylic acid conj Diaminododecane, and AE = Cyanocobalamin Ribose-Succinate **(11)**. The *b*-conjugate **(17)** has the least binding, whereas the *e*-conjugate **(18)** has intermediate binding, and the *d*-conjugate **(19)** binds quite well. The biotin conjugate attached to the ribose site **(13)** appears to bind very well, as does its precursor amino derivative **(12)**. The additional compound studied is of unknown structure, but may have the amine group coordinated with the cobalt atom as the mass spectrum indicates that it has the appropriate mass for **(7)** minus HCN. It is clear that this unknown compound is not likely to bind TcII.

Figure 24 illustrates the binding curve of Transcobalamin II to a series of vitamin B_{12} dimers. Dimer X = *b*-acid dimer with Isophthaloyl dichloride **(36)**; Dimer Y = *e*-acid dimer with Isophthaloyl dichloride **(37)**; dimer Z = *d*-acid dimer with Isophthaloyl dichloride **(38)**; Dimer A= *b*-acid Dimer with *p*-Iodo benzoyl Isophthaloyl dichloride **(58)**; Dimer B = *e*-acid Dimer with *p*-Iodo benzoyl Isophthaloyl dichloride **(59)**; and Dimer C = *d*-acid Dimer with *p*-Iodo benzoyl Isophthaloyl dichloride **(60)**.

Figure 25 illustrates the binding curve of Transcobalamin II to a series of biotinylated vitamin B_{12} molecules. AA = Cyanocobalamin *b*-monocarboxylic acid conj Diaminododecane and Biotin **(17)**; AB = Cyanocobalamin *e*-monocarboxylic acid conj Diaminododecane and Biotin **(18)**; AC = Cyanocobalamin *d*-monocarboxylic acid conj Diaminododecane and Biotin **(19)**; AF = Cyanocobalamin Ribose-Succinate conj Diaminododecane **(13)**; and AG = Cyanocobalamin Ribose-Succinate conj. Diaminododecane and Biotin **(20)**.

EXAMPLE 19

ASSAY FOR BIOLOGICAL ACTIVITY OF VITAMIN B_{12} RECEPTOR MODULATING AGENTS

This example serves to demonstrate the use of an assay to ascertain biological activity of the receptor modulating agents of the present invention.

Receptor down-modulation involves a comparison of treatment of a target cell line such as K562, each sample is treated with vitamin B_{12} or a vitamin B_{12} receptor modulating agent at 4°C for 24 hours. Following this period, cells of each sample are separated from a vitamin B_{12} or a vitamin B_{12} receptor modulating agent by centrifugation. The cells are then washed and resuspended in phosphate buffered saline containing 2 mM EDTA for a brief period of time not to exceed 15 minutes at 4°C. Then, the cells are washed again and returned to a tissue culture medium at 4°C. The tissue culture medium containing TcII and a radiolabeled TcII/B_{12} complex. The time course of TcII/B_{12} binding to the cell receptor is determined by measuring the percent radiolabel bound to the cell at 0, 15, 30, 60, 120, and 240 minutes. Those samples exposed to the vitamin B_{12} receptor modulating agents of the present invention show significantly reduced TcII/B_{12} complex binding compared to cells cultured in vitamin B_{12}. Trypsin treated cells reveal any nonspecific binding or uptake of the labeled vitamin B_{12} on or within the cell.

EXAMPLE 20

METHOD FOR ASSESSING BIOLOGICAL ACTIVITY OF A RECEPTOR MODULATING AGENT

This example serves to demonstrate a method suitable for assessing the biological activity of a receptor modulating agent of the present invention.

$0.2x10^6$ cells/ml K562 cells were cultured in RPMI medium modified by addition of 10 μM MeTHF, 2.7 nM vitamin B_{12} and 1% human serum. No folate was added. 10 μM *d*-diamimododecane adduct (7) was added and cultured over 9 days at 37°C. 10 μM vitamin B_{12} cultured under identical conditions as (7) was utilized as a control. The cultures were then independently assessed for proliferation and cell death by Trypan blue exclusion. The results are described in Table 10, below, in terms of the percent cell death.

Table 10

	Control	*d*-diaminododecane adduct (7)
Proliferation	98%	9 %
Cell Death	8 %	85 %

The receptor modulating agent, in this case *d*-diaminododecane adduct (7), clearly demonstrates the marked biological activity of the receptor modulating agent.

EXAMPLE 21

SYNTHESIS OF AN ANTI-INFLAMMATORY RECEPTOR MODULATING AGENT

The synthetic peptide f-met-leu-phe is equivalent to a bacterial cell wall constituent (Biochem. Soc. Trans. 19:1127-9, 1991; Agents Actions Suppl. 35:3-8, 1991; Agents Actions Suppl. 35:11-6, 1991; J Immunol. 146:975-80, 1991). This peptide is recognized by receptors on PMN which can respond by chemotaxis to sites of local inflammation along a gradient of the peptide. During inflammation, receptor expression can be dramatically increased by mobilizing receptor from intracellular pools. Non-specific methods used to abrogate this up-regulation also inhibit chemotaxis and presumably the anti-inflammatory reaction associated with local inflammation (J. Immunol. 145:2633-8, 1990). The synthesis of a receptor modulation agent useful as an inhibitor of early inflammation is described below.

The peptide f-met-leu-phe-$(gly)_3$-leu-O-Me is synthesized using tea-bag methodology or solid phase peptide synthesis procedures described by Merrifield et al. (Biochemistry 21:5020-31, 1982) and Houghten (Proc. Nat'l. Acad. Sci. (USA) 82:5131-35, 1985), or using a commercially available automated synthesizer, such as the Applied Biosystems 430 A peptide synthesizer. The peptide-amide is deprotected

in 45% trifluoroacetic acid-51% methylene chloride-2% ethanedithiol-2% anisole for 20 minutes, and cleaved from the 4-methylbenzhydrylamine resin using the Tam-Merrifield low-high HF procedure (J. P. Tam et al., J. Am. Chem. Soc. 105:6442-55, 1983). The peptide is then extracted from the resin using 0.1 M ammonium acetate buffer, pH 8, and is lyophilized. The crude peptide is purified using reverse phase HPLC on a Vydac* C-4 analytical column (The Separations Group, Hesperia, Calif.), and a linear gradient of 0.5-1.0%/min. from 100% acetonitrile + 0.1%v/v trifluoroacetate to 100% acetonitrile + 0.1% trifluoroacetate. The HPLC-purified peptide is analyzed by amino acid analysis (R. L. Heinriksen and S. C. Meredith, Anal. Biochem. 160:65-74, 1984) after gas phase hydrolysis (N. M. Meltzer et al., Anal. Biochem. 160:356-61, 1987). The sequence of the purified peptide may be confirmed by Edman degradation on a commercially available sequencer (R. M. Hewick et al., J. Biol. Chem. 15:7990-8005, 1981). The peptide amide is converted to an O-methyl ester (*i.e.*, f-met-leu-phe-$(gly)_3$-leu-O-Me) by treatment with dimethylformamide (5g/60 mL with 1.3 equivalents of $NaHCO_3$ in excess methyl iodide (4 equivalents). The mixture is stirred under argon gas at room temperature for 40 hours. If required, the peptide is extracted to dryness with 150 mL of ethyl acetate. The receptor for modulating agent is used to treat PMN, activated with GM-CSF (to increase expression of fMLP receptors). Loss of binding of biotinylated fMLP is compared on fMLP versus f-MLP receptor modulating agent treated cells.

EXAMPLE 22

SYNTHESIS OF A FUSION PROTEIN RECEPTOR MODULATING AGENT

An EGF receptor modulating agent containing a genetically engineered fusion protein is hereby described. Briefly, the C-terminus of a DNA sequence encoding EGF, or its receptor binding domain, is ligated by conventional procedures (*e.g.*, using T_4DNA ligase) to a DNA sequence corresponding to a GGG spacer. The C-terminus of the EGF-GGG DNA sequence is then fused to the N-terminus of a DNA sequence encoding the conditional, membrane binding peptide KGEAALA$(EALA)_4$-EALEALAA. Alternately, peptide-spacer DNA sequences may be synthesized *in vitro* using standard oligonucleotide synthesis procedures (*see, e.g.*, U.S. Pat. Nos. 4,500,707 and 4, 668,777). The recombinant EGF peptide DNA sequence is cloned in an *E. coli* expression vector using conventional procedures. *E. coli* strain HB101 is transformed with the fused recombinant DNA sequence and cultured to produce the EGF peptide. The fusion protein is purified form the transformed *E. coli* culture by standard methods.

*Trade-mark

including anti-EGF affinity chromatography. The fusion protein may be eluted from the affinity matrix using standard techniques, such as high salt, chaotropic agents, or high or low pH. Loss of EGF receptor is measured by flow cytometry and mouse monoclonal antibody to EGF receptor.

From the foregoing, it will be appreciated that, although specific embodiments of this invention have been described herein for purposes of illustration, various modifications may be made without deviating from the spirit and scope of the invention. Accordingly, the invention is not limited except by the appended claims.

CLAIMS:

1. A receptor modulating agent, comprising a vitamin B_{12} molecule coupled to a rerouting moiety by a linker, wherein said vitamin B_{12} molecule binds transcobalamin II, and wherein said rerouting moiety is selected from the group consisting of: lysosomotrophic moieties; intracellular polymerizing moieties; peptide sorting sequences; conditional membrane binding peptides; and bi- or multi-valent receptor cross-linking moieties.

2. The receptor modulating agent of claim 1 wherein said linker is at least 4 atoms in length.

3. The receptor modulating agent of claim 2 wherein said linker is 6 to 20 atoms in length.

4. The receptor modulating agent of claim 3 wherein said linker is 12 atoms in length.

5. The receptor modulating agent of claim 1 wherein said linker includes at least one amino group.

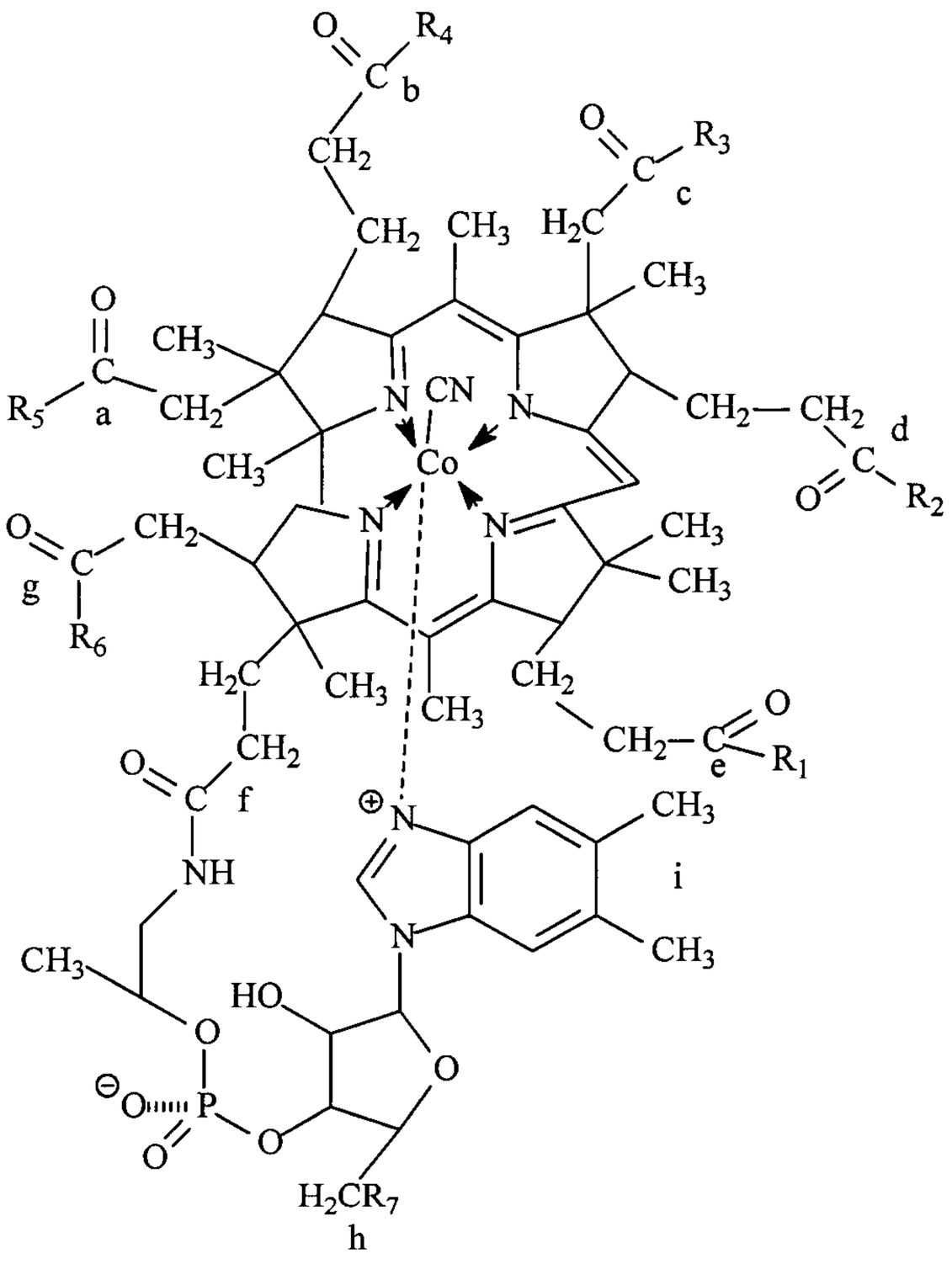
6. The receptor modulating agent of claim 5 wherein said linker additionally includes a group selected from the group consisting of sulfhydryls and carboxyls.

7. The receptor modulating agent of claim 5 wherein said linker is selected from the group consisting of diaminoalkyls, diaminoalkylaryls, diaminoheteroalkyls, diaminoheteroalkylaryls, and diaminoalkanes.

8. The receptor modulating agent of claim 5 wherein said linker is selected from the group consisting of $-NH(CH_2)_xNH-$ wherein x is an integer between 2-20.

9. The receptor modulating agent of claim 5 wherein said linker is selected from the group consisting of $-NH(CH_2)_yCO-$, wherein y is an integer between 3-12.

10. The receptor modulating agent of claim 1 wherein said linker is coupled to said rerouting moiety through a coupling site on said vitamin B_{12} molecule selected from the group consisting of *b*-, *d*-, and *e*- as shown in Structure I:



Structure I

wherein at least one of R_1, R_2, R_3, R_4, R_5, R_6, and R_7 is a linker.

11. The receptor modulating agent of claim 10 wherein said linker is coupled through a coupling site selected from the group consisting of *d*- and *e*- coupling sites as shown in Structure I.

12. The receptor modulating agent of claim 1 wherein said linker is coupled to a ribose coupling site on said vitamin B_{12} molecule.

13. The receptor modulating agent of claim 1 wherein said linker is a trifunctional linker.

14. The receptor modulating agent of claim 13 wherein a biotin molecule is coupled through a reactive site on said trifunctional linker.

15. The receptor modulating agent of claim 1 wherein said receptor modulating agent affects a receptor trafficking pathway by redirecting an agent/receptor complex.

16. The receptor modulating agent of claim 1 wherein said receptor modulating agent affects a receptor trafficking pathway by cross-linking one or more receptors.

17. The receptor modulating agent of claim 16 wherein said receptor modulating agent is a vitamin B_{12} dimer.

18. The receptor modulating agent as in claim 1 wherein said receptor modulating agent affects a receptor trafficking pathway by anchoring a receptor in a cell membrane.

19. The receptor modulating agent as in claim 1 wherein said receptor modulating agent affects a receptor trafficking pathway by retaining an agent/receptor complex in an endosome.

20. The receptor modulating agent as in claim 1 wherein said rerouting moiety is a lysosomotropic moiety selected from the group consisting of gentamycin, sisomicin,

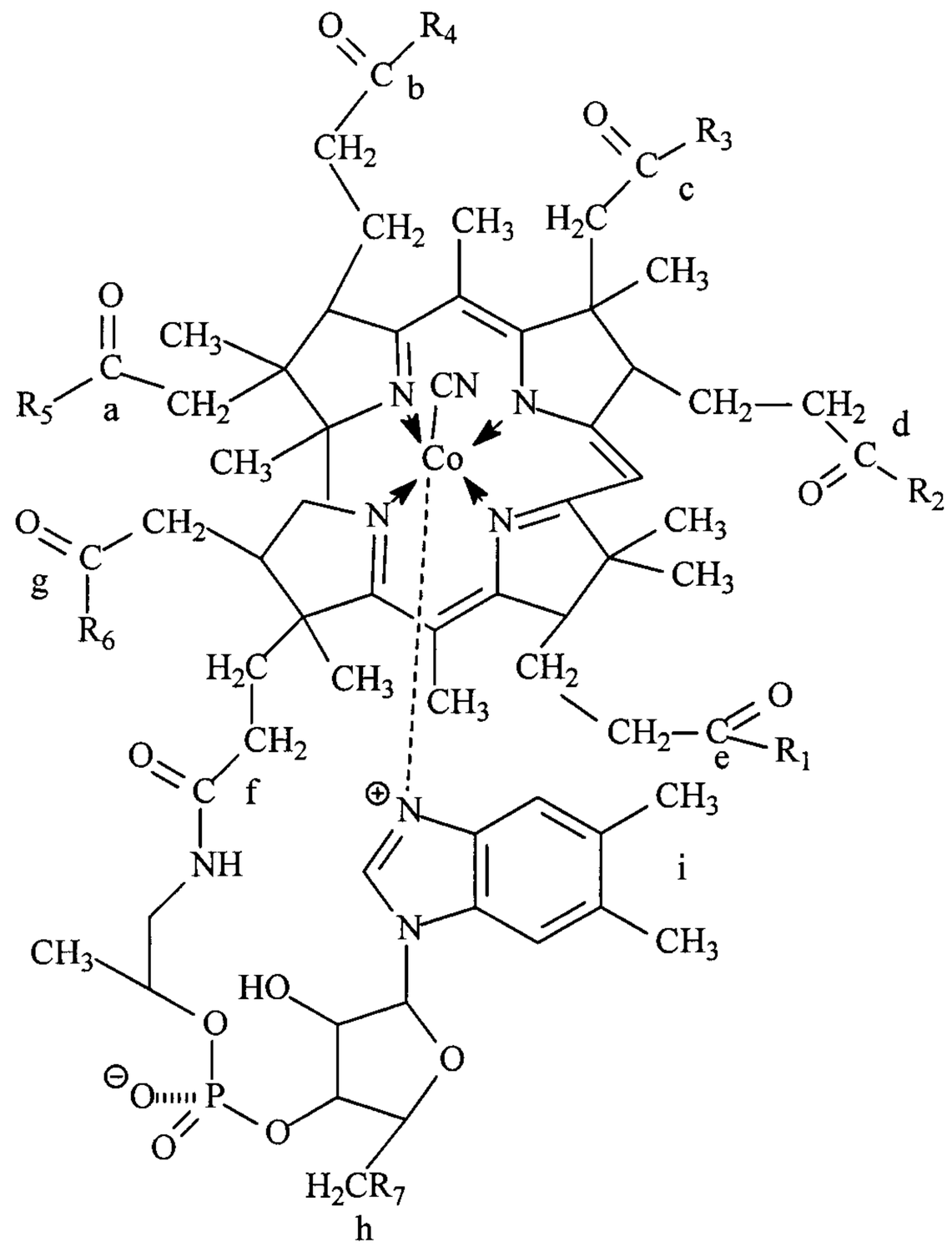
netilmicin, kanamycin, tobramycin, amikacin, neomycin, paromomycin, ribostamycin, butirosin, and streptomycin.

21. The receptor modulating agent as in claim 1 wherein said rerouting moiety is an intracellular polymerizing moiety selected from the group consisting of dipeptide esters and leucine zippers.

22. The receptor modulating agent as in claim 1 wherein said rerouting moiety is a peptide sorting sequence selected from the group consisting of endoplasmic reticulum retention peptides, golgi retention peptides, lysosomal retention peptides, organism specific retention peptides and clathrin-binding peptides.

23. The receptor modulating agent as in claim 1 wherein said rerouting moiety is a conditional membrane binding peptide comprising a charged amino acid selected from the group consisting of glutamate, aspartate, and histidine.

24. A vitamin B_{12} dimer comprising a first and second vitamin B_{12} molecule coupled through a coupling site independently selected from the group consisting of coupling sites *a*-*g*, coupling site h, and coupling site i, as shown in Structure I:



Structure I

wherein at least one of R_1, R_2, R_3, R_4, R_5, R_6, and R_7 is a linker wherein said first and second vitamin B_{12} molecules are coupled through at least one linker, and wherein said first and second vitamin B_{12} molecules bind transcobalamin II.

25. The dimer of claim 24 wherein said first and second vitamin B_{12} molecules are coupled through a coupling site independently selected from the group consisting of *d*- and *e*- coupling sites on said first and said second vitamin B_{12} molecule.

26. The dimer of claim 24 wherein at least one of said first and said second vitamin B_{12} molecules is cyanocobalamin.

27. The dimer of claim 24 wherein said first and second B_{12} molecules are coupled through at least one linker.

28. The dimer of claim 27 wherein said linker is at least 4 atoms in length.

29. The dimer of claim 28 wherein said linker is about 10 to 55 atoms in length.

30. The dimer of claim 29 wherein said linker is 35 to 45 atoms in length.

31. The dimer of claim 27 wherein said linker includes at least one amino group.

32. The dimer of claim 31 wherein said linker additionally includes a group selected from the group consisting of sulfhydryls and carboxyls.

33. The dimer of claim 31 wherein said linker is selected from the group consisting of diaminoalkyls, diaminoalkylaryls, diaminoheteroalkyls, diaminoheteroalkylaryls, and diaminoalkanes.

34. The dimer of claim 31 wherein said linker is selected from the group consisting of $-NH(CH_2)_xNH-$ wherein x is an integer between 2-20.

35. The dimer of claim 31 wherein said linker is selected from the group consisting of $-NH(CH_2)_yCO-$, wherein y is an integer between 3-12.

36. The dimer of claim 27 wherein said linker is a trifunctional linker.

37. Use of a receptor modulating agent for modulating a vitamin B_{12} receptor, in a warm-blooded animal, said receptor modulating agent comprising a vitamin B_{12} molecule coupled to a rerouting moiety by a linker, wherein said vitamin B12 molecule binds transcobalamin II, and wherein said rerouting moiety is selected from the group consisting of: lysosomotrophic moieties; intracellular polymerizing moieties; peptide sorting sequences; conditional membrane binding peptides; and bi- or multi-valent receptor cross-linking moieties.

38. The use of claim 37 wherein said B_{12} molecule is coupled to said rerouting moiety by a linker.

39. The use of claim 38 wherein said linker is at least 4 atoms in length.

40. The use of claim 39 wherein said linker is 6 to 20 atoms in length.

41. The use of claim 40 wherein said linker is 12 atoms in length.

42. The use of claim 38 wherein said linker includes at least one amino group.

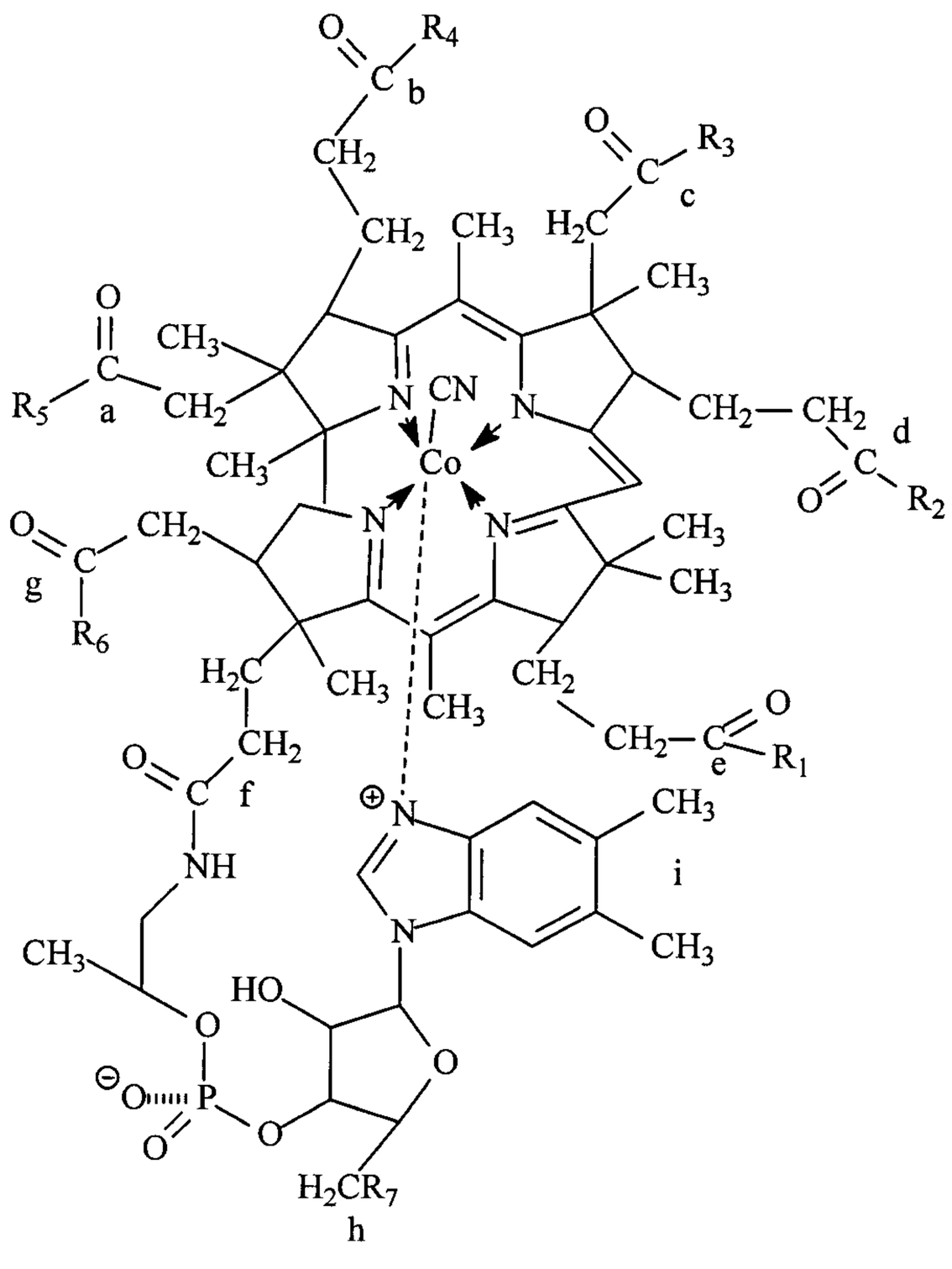
43. The use of claim 42 wherein said linker additionally includes a group selected from the group consisting of sulfhydryls and carboxyls.

44. The use of claim 42 wherein said linker is selected from the group consisting of diaminoalkyls, diaminoalkylaryls, diaminoheteroalkyls, diaminoheteroalkylaryls, and diaminoalkanes.

45. The use of claim 42, wherein said linker is selected from the group consisting of $-NH(CH_2)_xNH-$ wherein x is an integer between 2-20.

46. The use of claim 42, wherein said linker is selected from the group consisting of $-NH(CH_2)_yCO-$, wherein y is an integer between 3-12.

47. The use of claim 37 wherein said linker is coupled to said rerouting moiety through a coupling site on said vitamin B_{12} molecule selected from the group consisting of *b*-, *d*-, and *e*- as shown in Structure I:



Structure I

wherein at least one of R_1, R_2, R_3, R_4, R_5, R_6, and R_7 is a linker.

48. The use of claim 47 wherein said linker is coupled through a coupling site selected from the group consisting of *d*- and *e*- coupling sites as shown in Structure I.

49. The use of claim 38 wherein said linker is coupled to a ribose coupling site on said vitamin B_{12} molecule.

50. The use of claim 38 wherein said linker is a trifunctional linker.

51. The use of claim 37 wherein said receptor modulating agent affects a receptor trafficking pathway by redirecting an agent/receptor complex.

52. The use of claim 37 wherein said receptor modulating agent affects a receptor trafficking pathway by cross-linking one or more receptors.

53. The use of claim 52 wherein said receptor modulating agent is a vitamin B_{12} dimer.

54. The use of claim 37 wherein said receptor modulating agent affects a receptor trafficking pathway by anchoring a receptor in a cell membrane.

55. The use of claim 37 wherein said receptor modulating agent affects a receptor trafficking pathway by retaining an agent/receptor complex in an endosome.

56. The use of claim 37 wherein said rerouting moiety is a lysosomotropic moiety selected from the group consisting of gentamycin, sisomicin, netilmicin, kanamycin, tobramycin, amikacin, neomycin, paromomycin, ribostamycin, butirosin, and streptomycin.

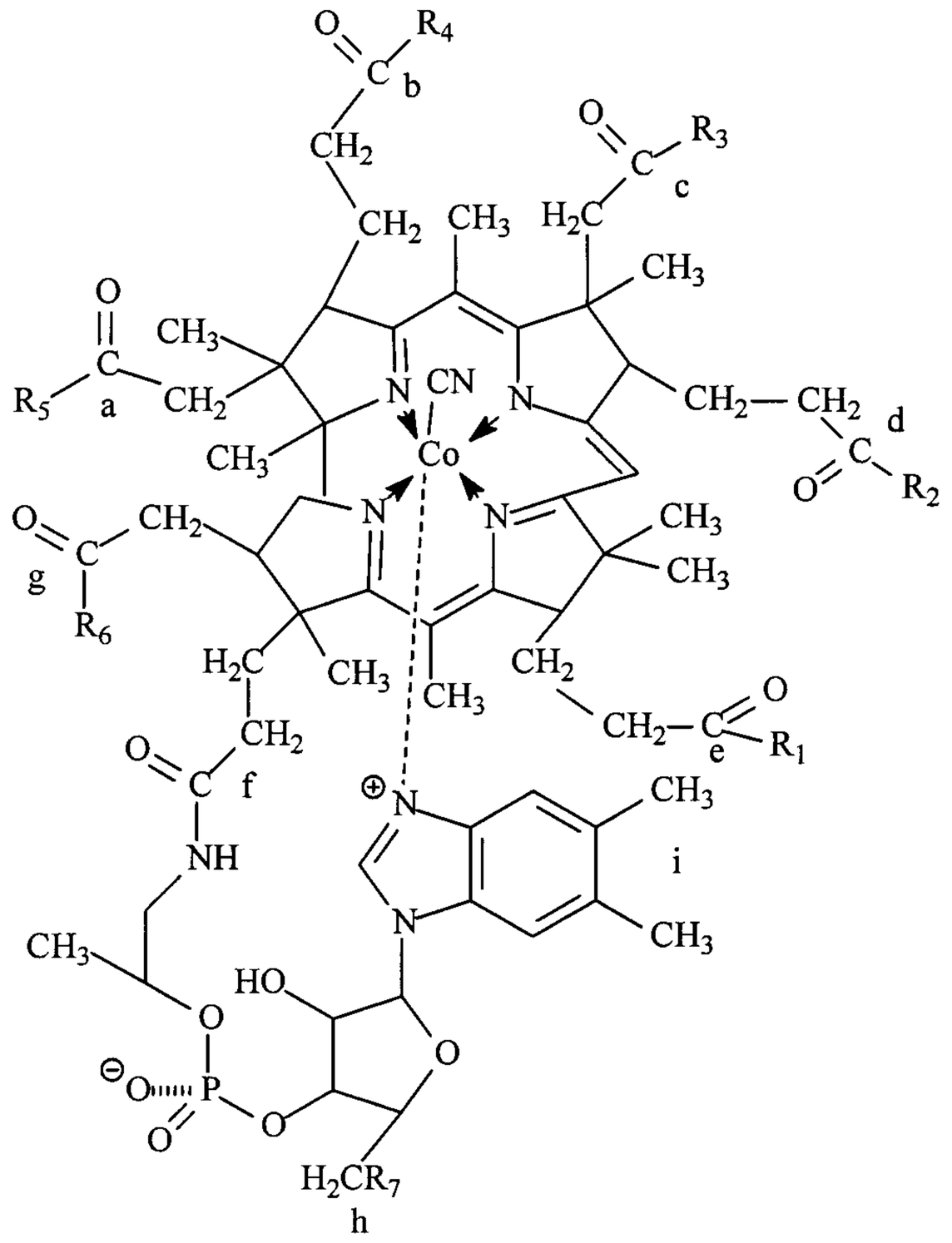
57. The use of claim 37 wherein said rerouting moiety is an intracellular polymerizing moiety selected from the group consisting of dipeptide esters and leucine zippers.

58. The use of claim 37 wherein said rerouting moiety is a peptide sorting sequence selected from the group consisting of endoplasmic reticulum retention peptides, golgi retention peptides, lysosomal retention peptides, organism specific retention peptides and clathrin-binding peptides.

59. The use of claim 50 wherein said rerouting moiety is a conditional membrane binding peptide comprising a charged amino acid selected from the group consisting of glutamate, aspartate, and histidine.

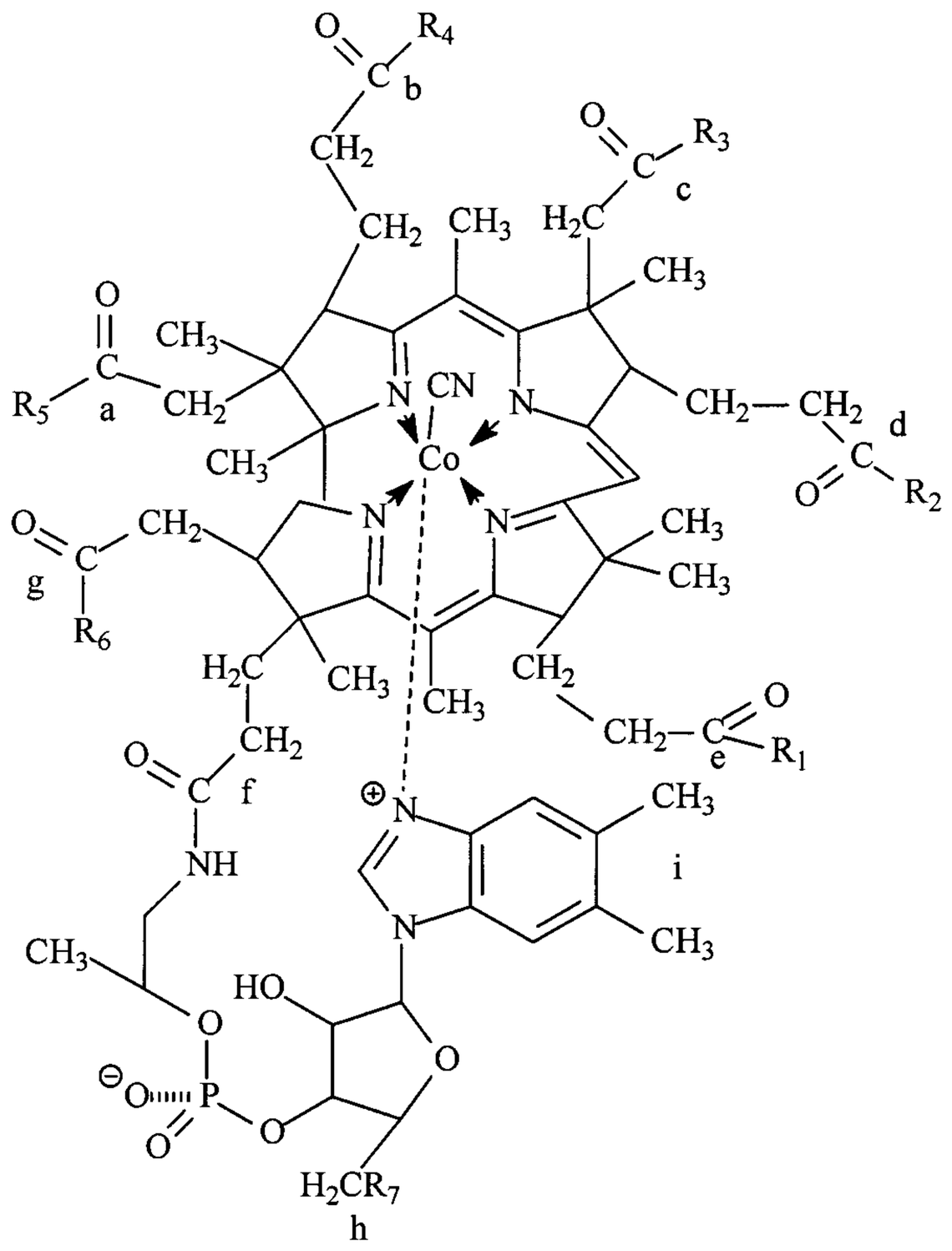
60. The use of claim 53 wherein said vitamin B_{12} dimer is comprised of a first and a second vitamin B_{12} molecule coupled through a coupling site independently selected from the group consisting of coupling sites *a-g*, coupling site h, and coupling site i as shown in Structure I:

69140-170



Structure I .

61. The use of claim 60 wherein said first and second vitamin B_{12} molecules are coupled through a coupling site independently selected from the group consisting of *d*- and *e*- coupling sites on said first and second vitamin B_{12} molecule as shown in Structure I:



Structure I

wherein at least one of R_1, R_2, R_3, R_4, R_5, R_6, and R_7 is a linker.

62. The use of claim 60 wherein at least one of said first and said second vitamin B_{12} molecules is cyanocobalamin.

63. The use of claim 62 wherein said first and second B_{12} molecules are coupled through at least one linker.

64. The use of claim 60 wherein said linker is at least 4 atoms in length.

65. The use of claim 64 wherein said linker is about 10 to 55 atoms in length.

66. The use of claim 65 wherein said linker is 35 to 45 atoms in length.

67. The use of claim 63 wherein said linker includes at least one amino group.

68. The use of claim 67 wherein said linker additionally includes a group selected from the group consisting of sulfhydryls and carboxyls.

69. The use of claim 67 wherein said linker is selected from the group consisting of diaminoalkyls, diaminoalkylaryls, diaminoheteroalkyls, diaminoheteroalkylaryls, and diaminoalkanes.

70. The use of claim 67 wherein said linker is selected from the group consisting of $-NH(CH_2)_xNH-$ wherein x is an integer between 2-20.

71. The use of claim 67 wherein said linker is selected from the group consisting of $-NH(CH_2)_yCO-$, wherein y is an integer between 3-12.

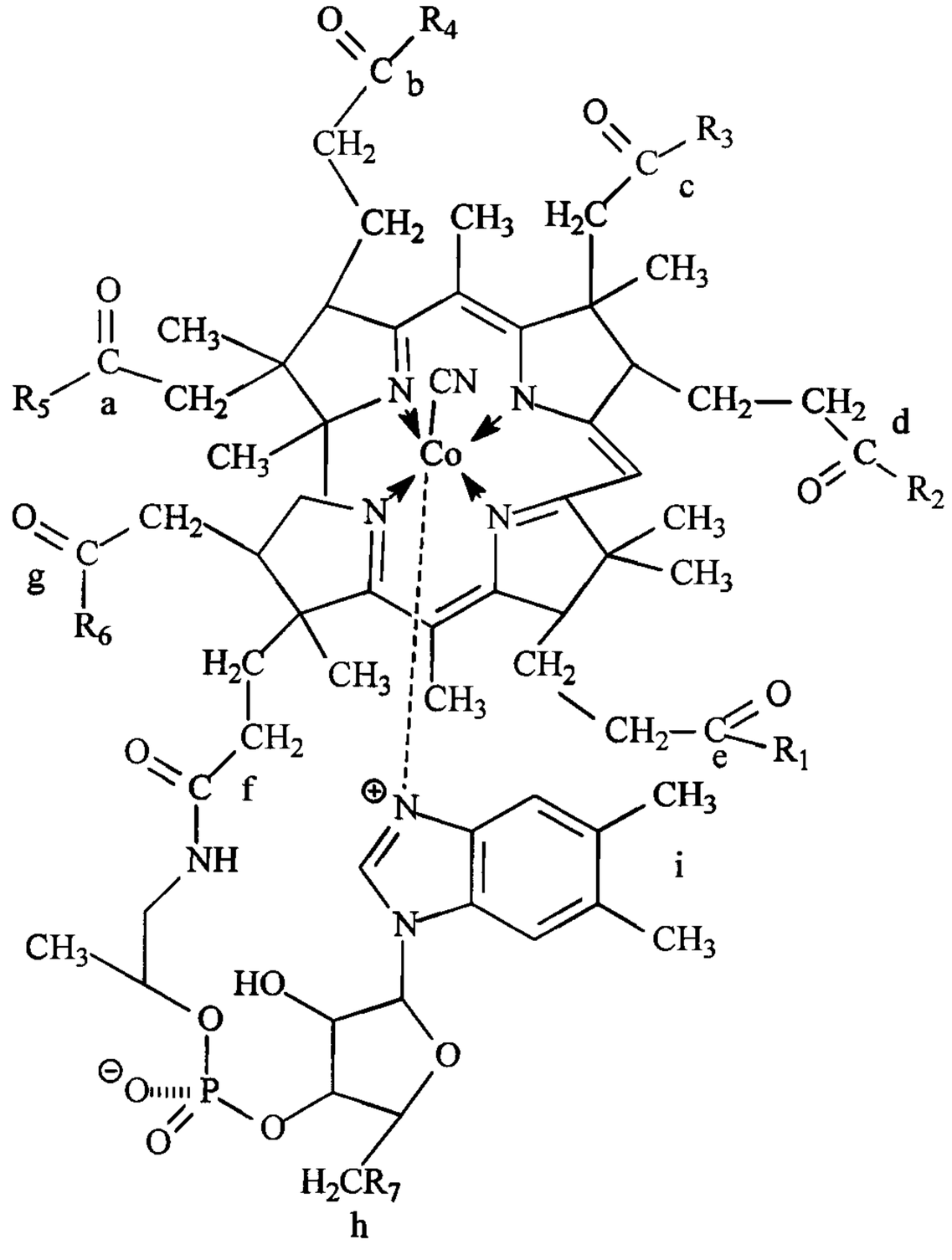
72. The use of claim 60 wherein said linker is a trifunctional linker.

73. The use of claim 72 wherein a reactive site on said trifunctional linker is coupled to a biotin molecule.

74. The use of claim 37 wherein said vitamin B_{12} receptor modulation is sufficient to treat a neoplastic disorder.

75. The use of claim 74 wherein said neoplastic disorder is selected from the group consisting of leukemia, sarcoma, myeloma, carcinoma, neuroma, melanoma, cancers of the lung, liver, breast, brain, colon, cervix, prostate, Hodgkin's disease, and non-Hodgkin's lymphoma.

76. A vitamin B_{12} molecule/biotin conjugate comprising a vitamin B_{12} molecule coupled by a linker to a biotin molecule through a coupling site on said vitamin B_{12} molecule selected from the group consisting of *b*-, *d*-, and *e*- as shown in Structure I:



Structure I

wherein at least one of R_1, R_2, R_3, R_4, R_5, R_6, and R_7 is a linker and ribose coupling sites, wherein said vitamin B_{12} molecule binds transcobalamin II.

77. The conjugate of claim 76 wherein said vitamin B_{12} molecule is cyanocobalamin.

78. The conjugate of claim 76 wherein said linker is at least 4 atoms in length.

79. The conjugate of claim 78 wherein said linker is 6 to 20 atoms in length.

80. The conjugate of claim 79 wherein said linker is 12 atoms in length.

81. The conjugate of claim 76 wherein said linker includes at least one amino group.

82. The conjugate of claim 81 wherein said linker additionally includes a group selected from the group consisting of sulfhydryls and carboxyls.

83. The conjugate of claim 81 wherein said linker is selected from the group consisting of diaminoalkyls, diaminoalkylaryls, diaminoheteroalkyls, diaminoheteroalkylaryls, and diaminoalkanes.

84. The conjugate of claim 81 wherein said linker is selected from the group consisting of $-NH(CH_2)_xNH-$ wherein x is an integer between 2-20.

85. The conjugate of claim 82 wherein said linker is selected from the group consisting of $-NH(CH_2)_yCO-$, wherein y is an integer between 3-12.

86. The conjugate of claim 76 wherein said linker is coupled to said vitamin B_{12} molecule through a coupling site on said vitamin B_{12} molecule selected from the group consisting of *b*-, *d*-, and *e*- as shown in Structure I.

87. The conjugate of claim 86 wherein said linker is coupled through a coupling site selected from the group consisting of *d*- and *e*- coupling sites on said vitamin B_{12} molecule as shown in Structure I.

88. The conjugate of claim 76 wherein said linker is coupled to a ribose coupling site on said vitamin B_{12} molecule.

89. The conjugate of claim 76 wherein said linker is a trifunctional linker.

90. The conjugate of claim 76 wherein said biotin is additionally coupled to a rerouting moiety.

91. The conjugate of claim 90 wherein said biotin is coupled to said rerouting moiety by a biotin binding protein.

92. The conjugate of claim 91 wherein said biotin binding protein is selected from the group consisting of avidin and streptavidin.

93. A complex comprising a conjugate according to any one of claims 76 to 92 bound to transcobalamin II.

94. A kit comprising a conjugate according to any one of claims 76 to 92 and instructions for using said conjugate for determining the presence or amount of transcobalamin in a sample.

95. A pharmaceutical composition, comprising a conjugate according to any one of claims 76 to 92 and a suitable pharmaceutical carrier or diluent.

SMART & BIGGAR

PATENT AGENTS

OTTAWA, CANADA

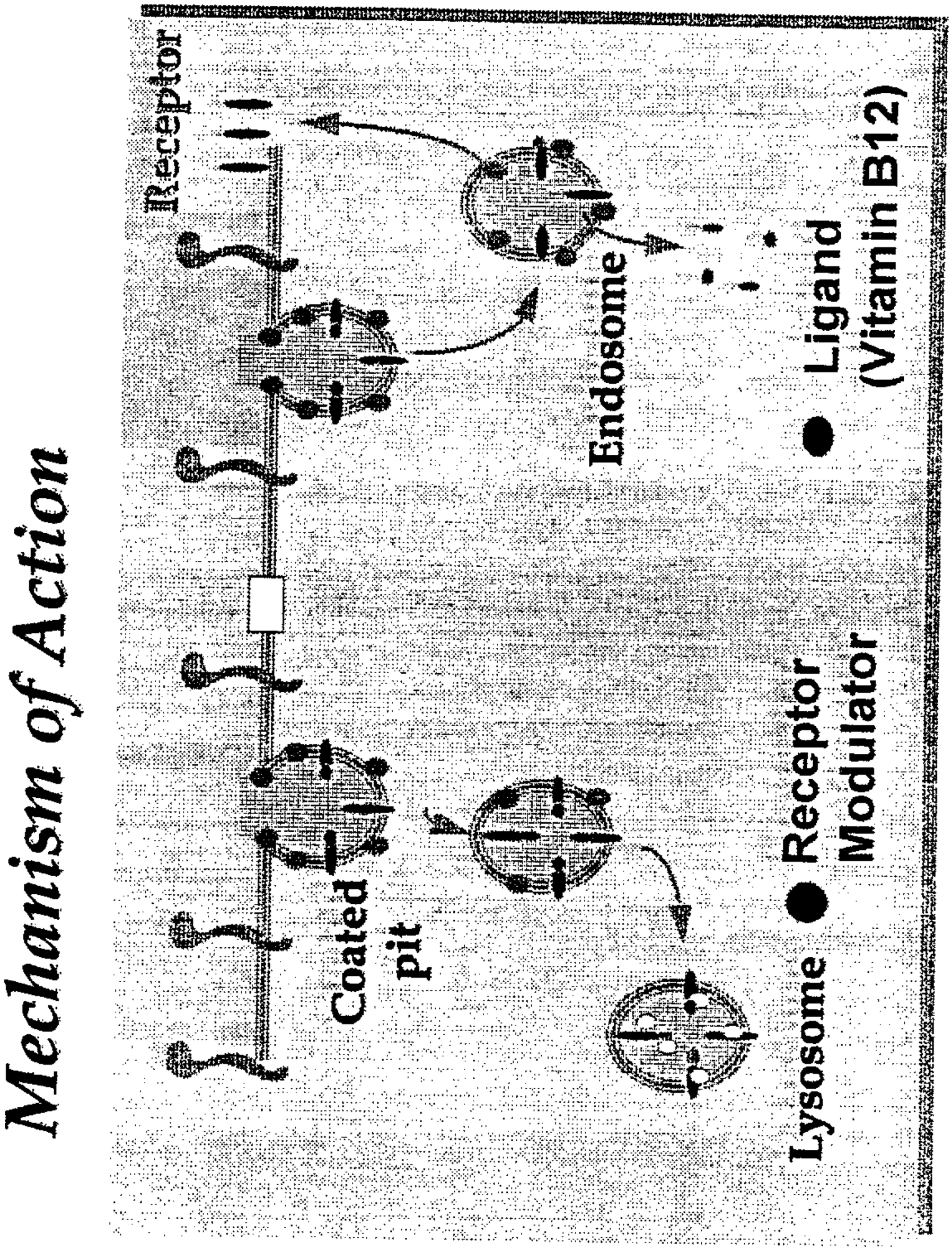
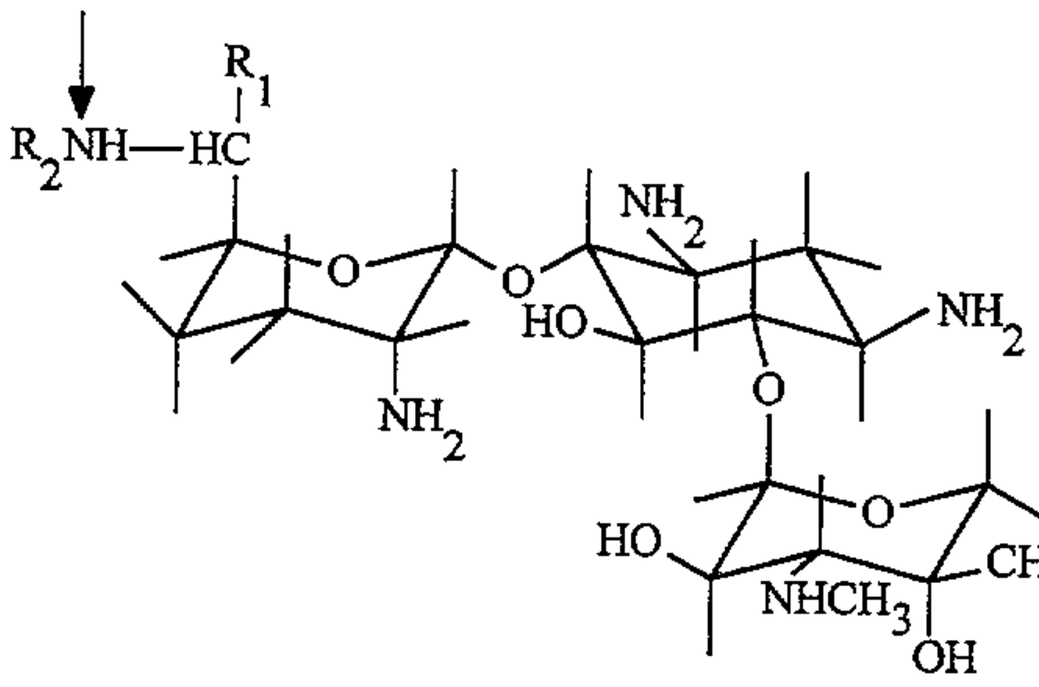
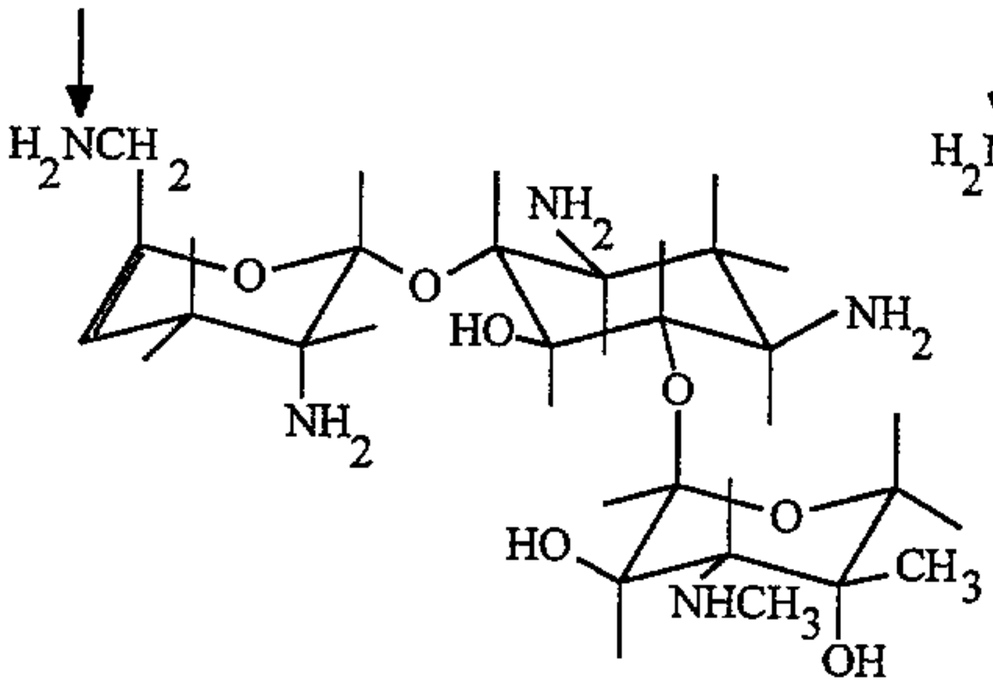

Mechanism of Action
Receptor
Endosome
Ligand
(Vitamin B12)
Coated
pit
Lysosome
Receptor
Modulator

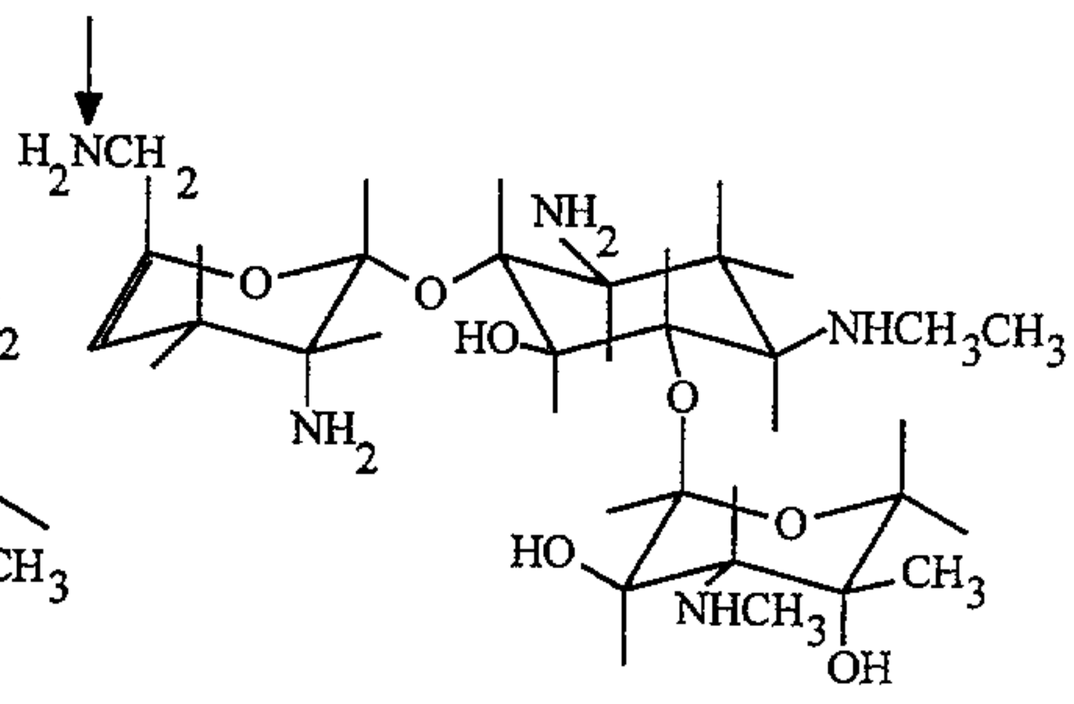
FIGURE 1



Gentamicin C_1 : $R_1 = R_2 = CH_3$
Gentamicin C_2 : $R_1 = CH_3$; $R_2 = H$
Gentamicin C_{1a}: $R_1 = R_2 = H$

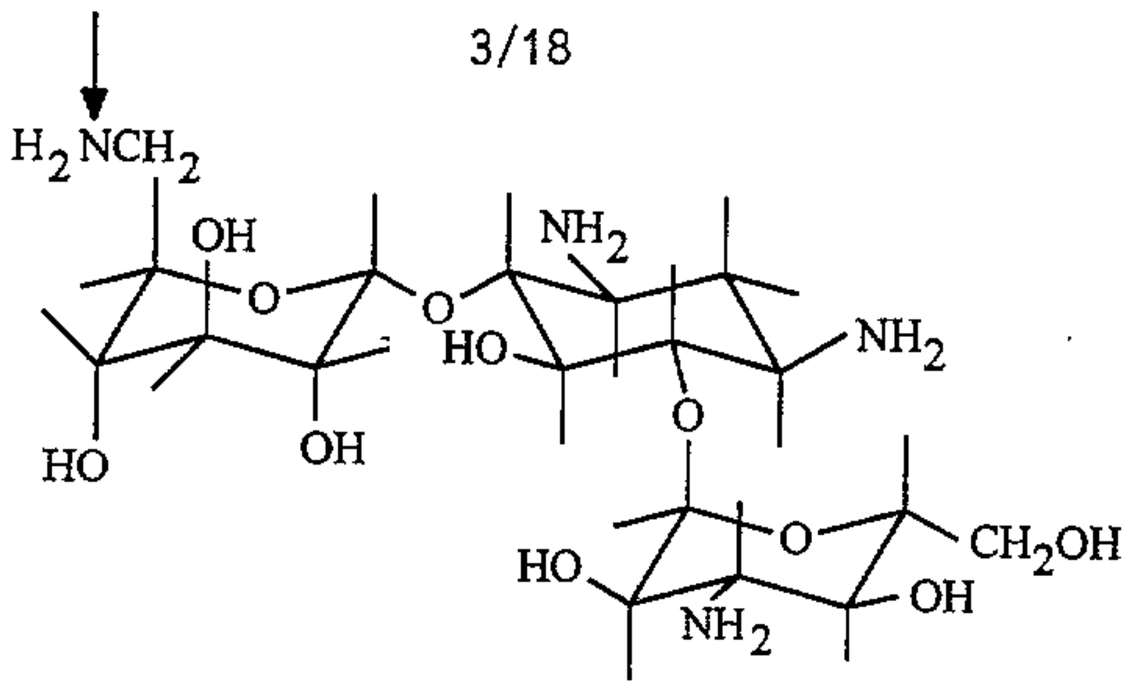


Sisomicin

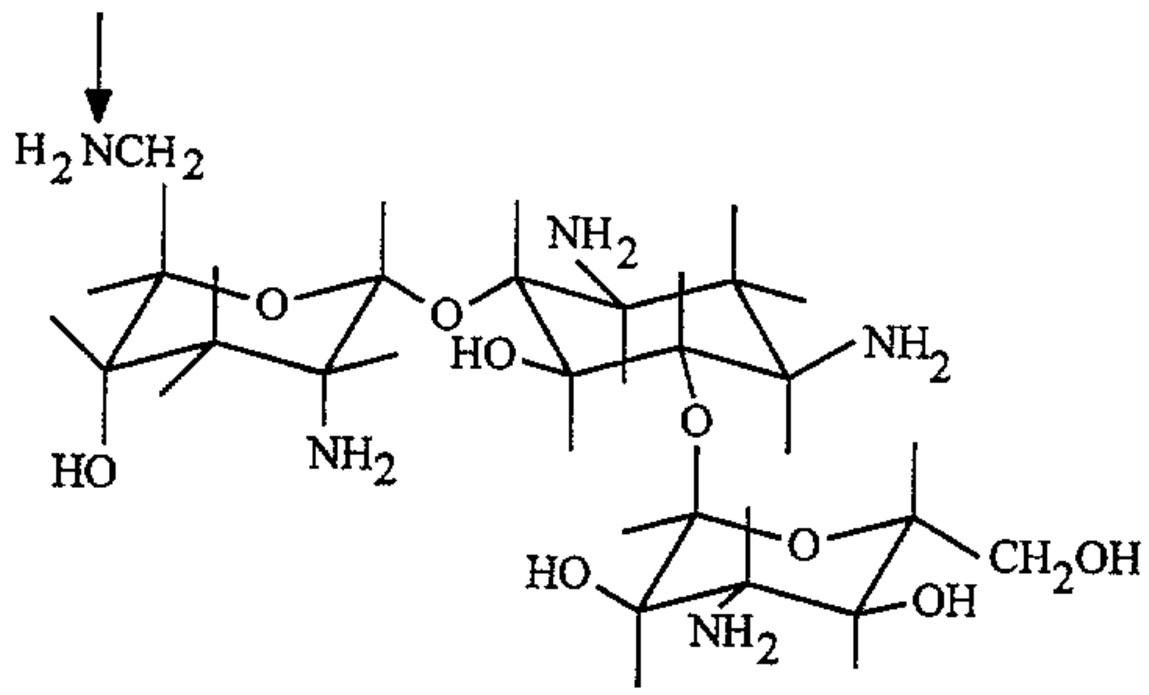


Netilmicin

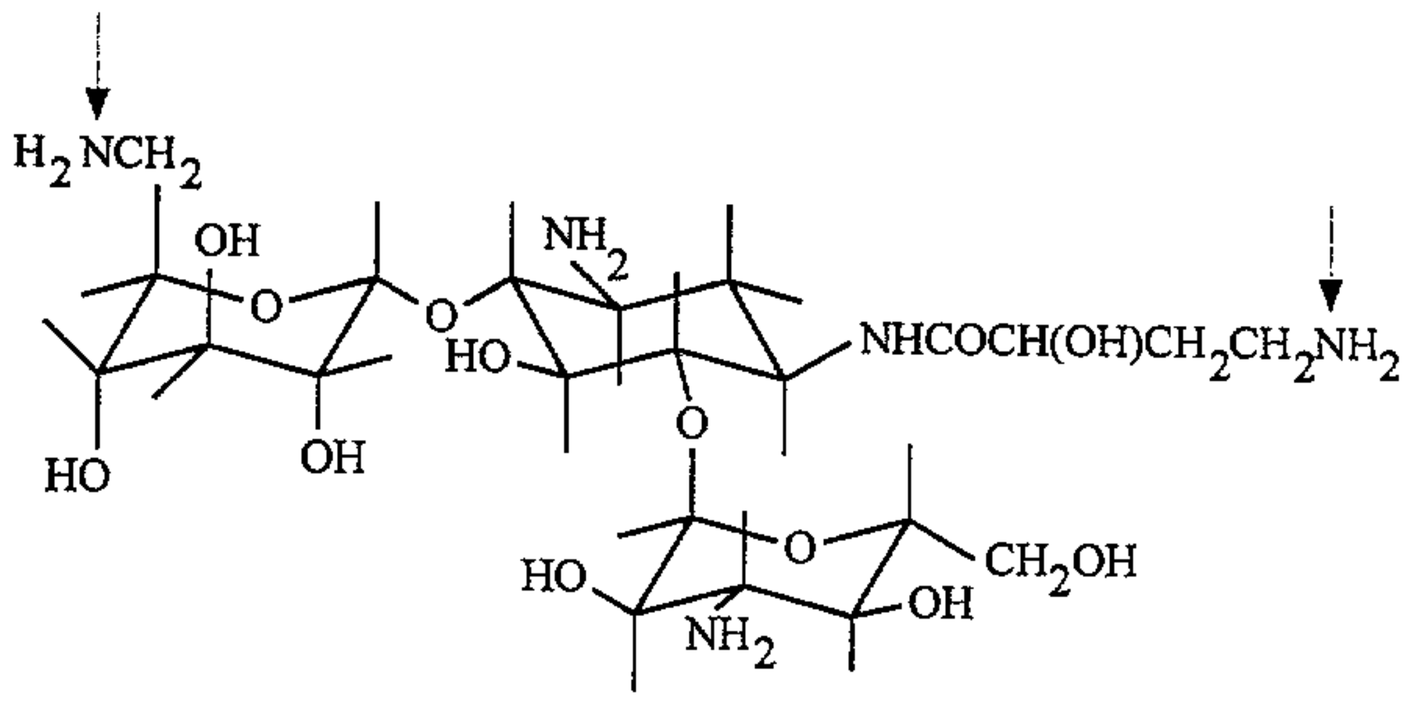
Fig. 2



Kanamycin A

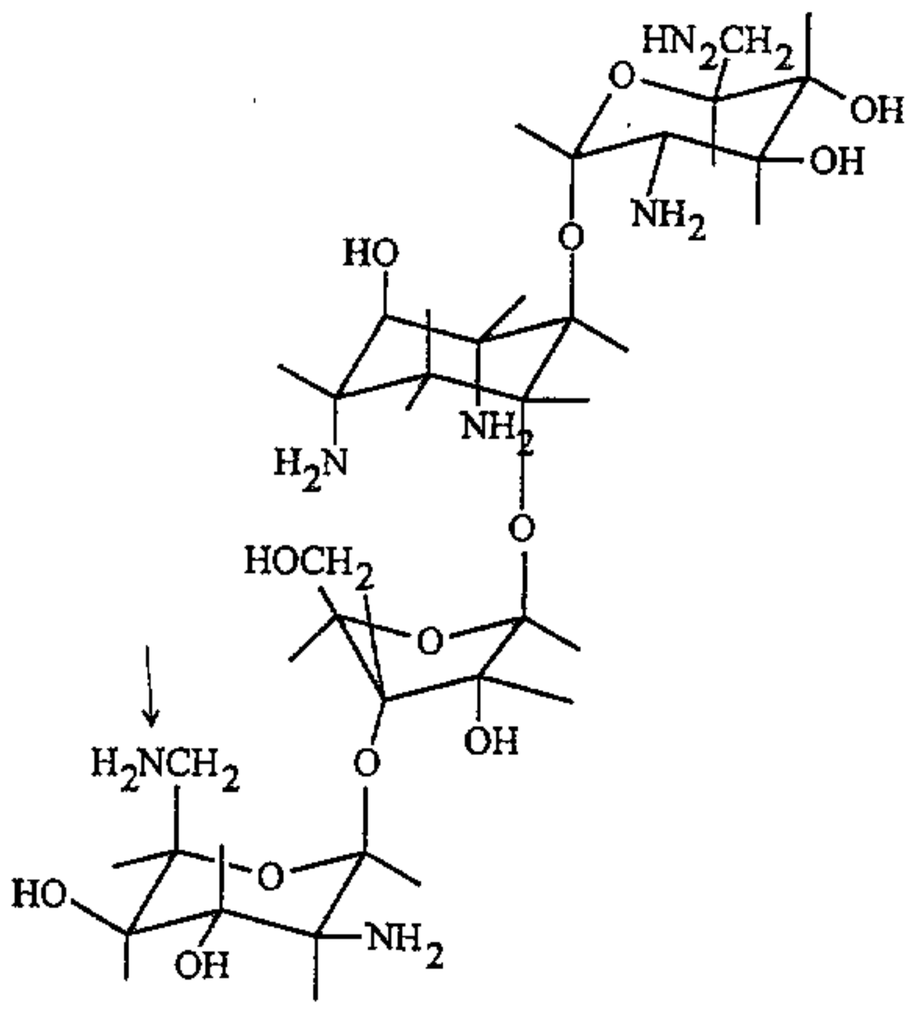


Tobramycin

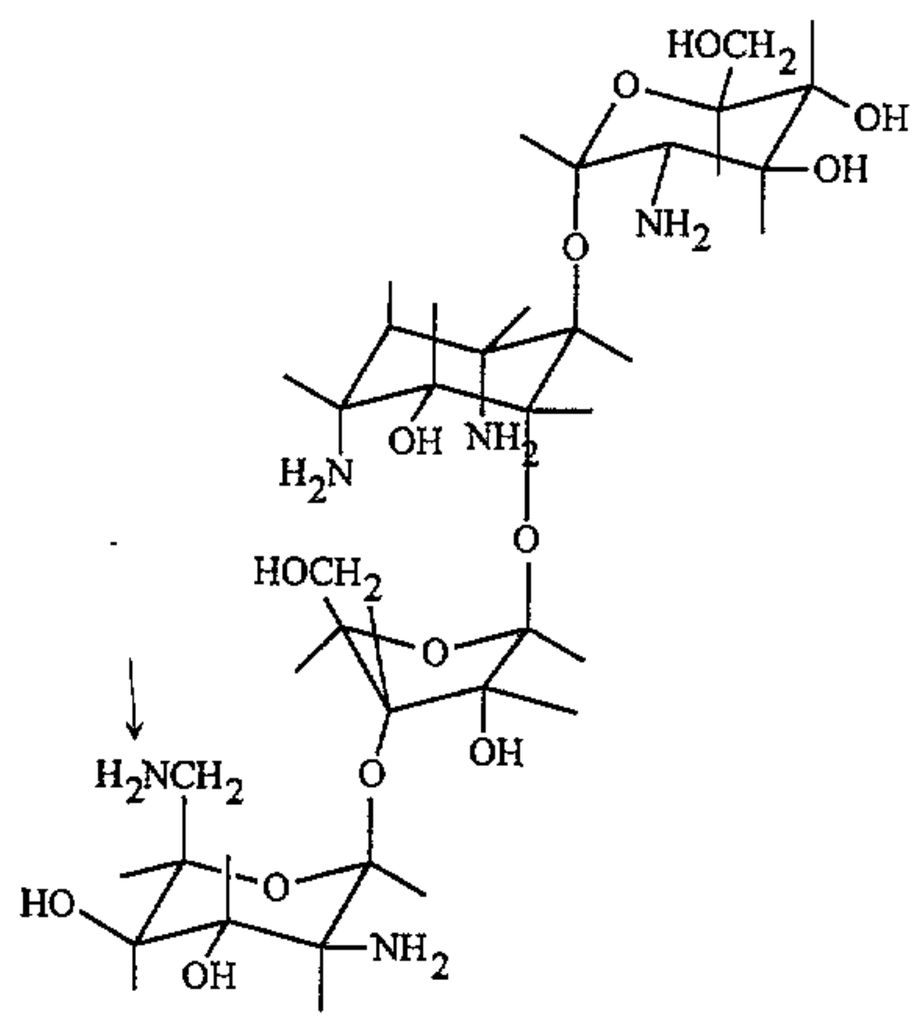


Amikacin

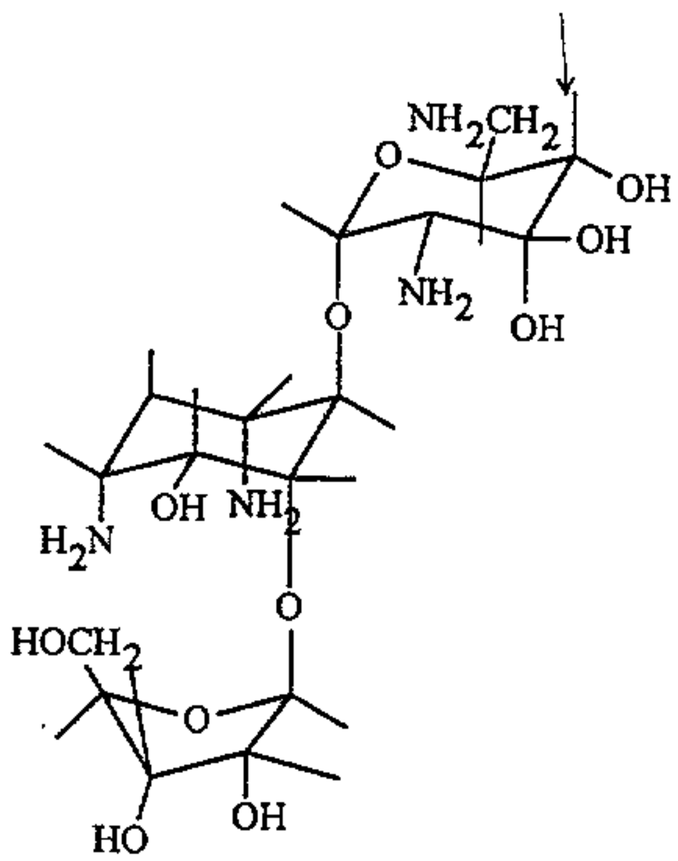
Fig. 3



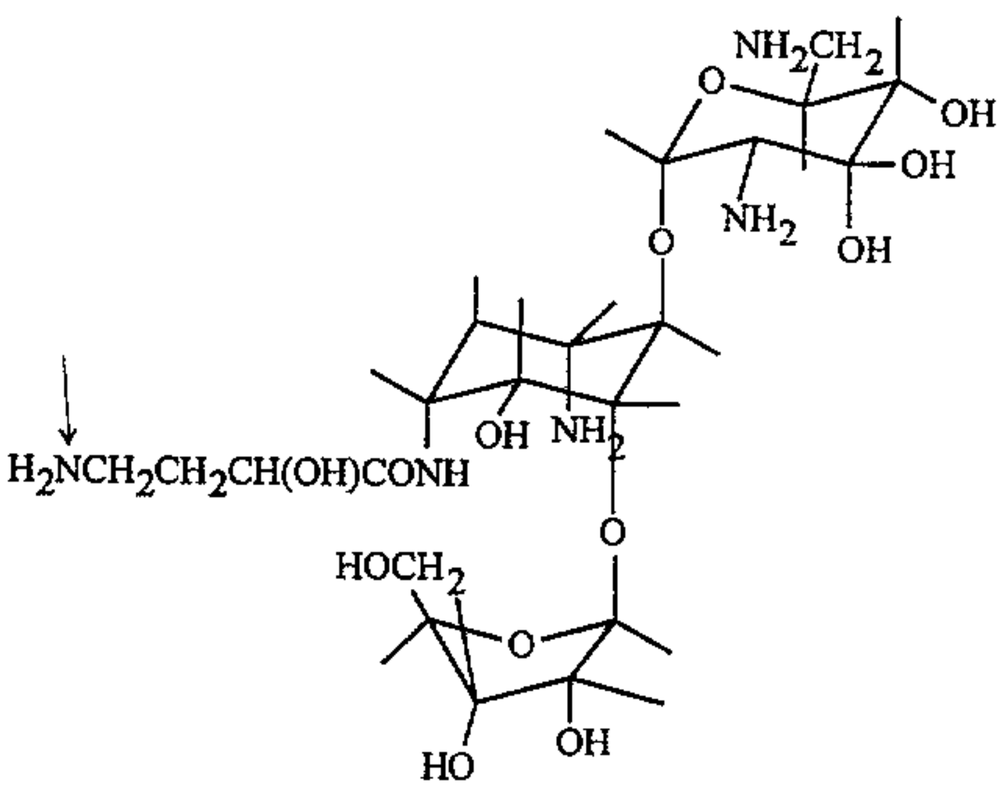
Neomycin B



Paromomycin



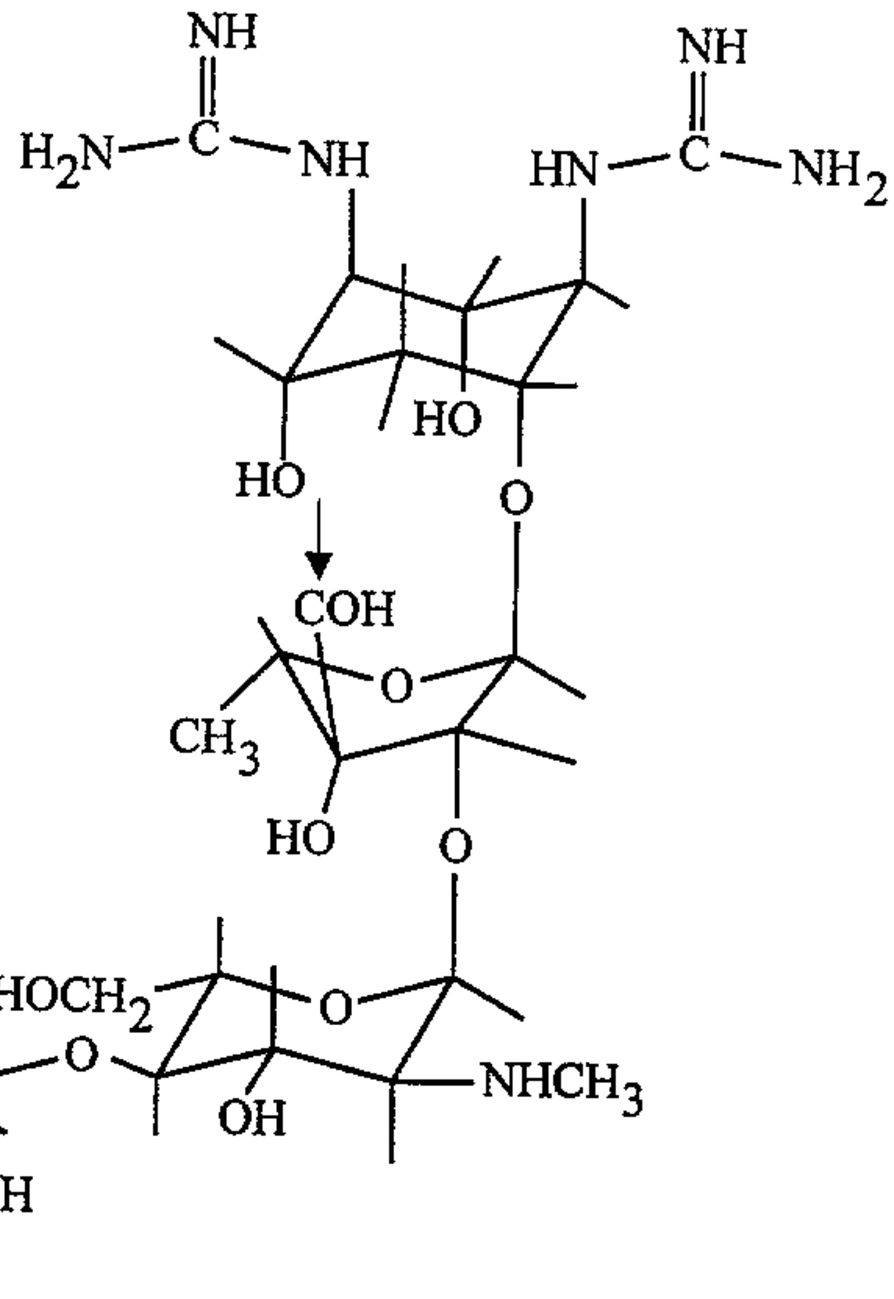
Ribostamycin



Butirosin B

Fig. 4

Streptomycin A



Streptomycin B

Fig. 5

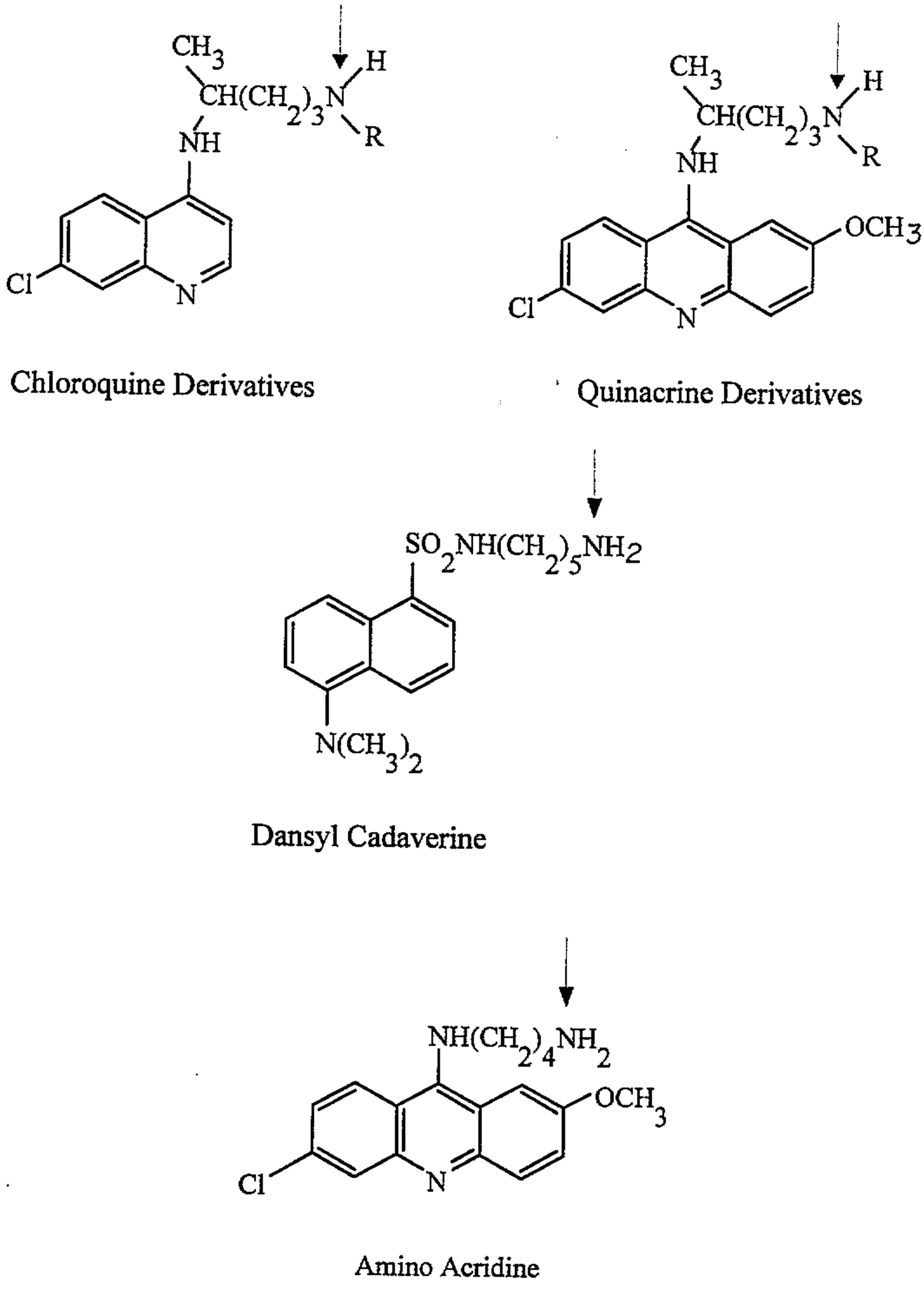

Chloroquine Derivatives
Quinacrine Derivatives
Dansyl Cadaverine
Amino Acridine

Fig. 6

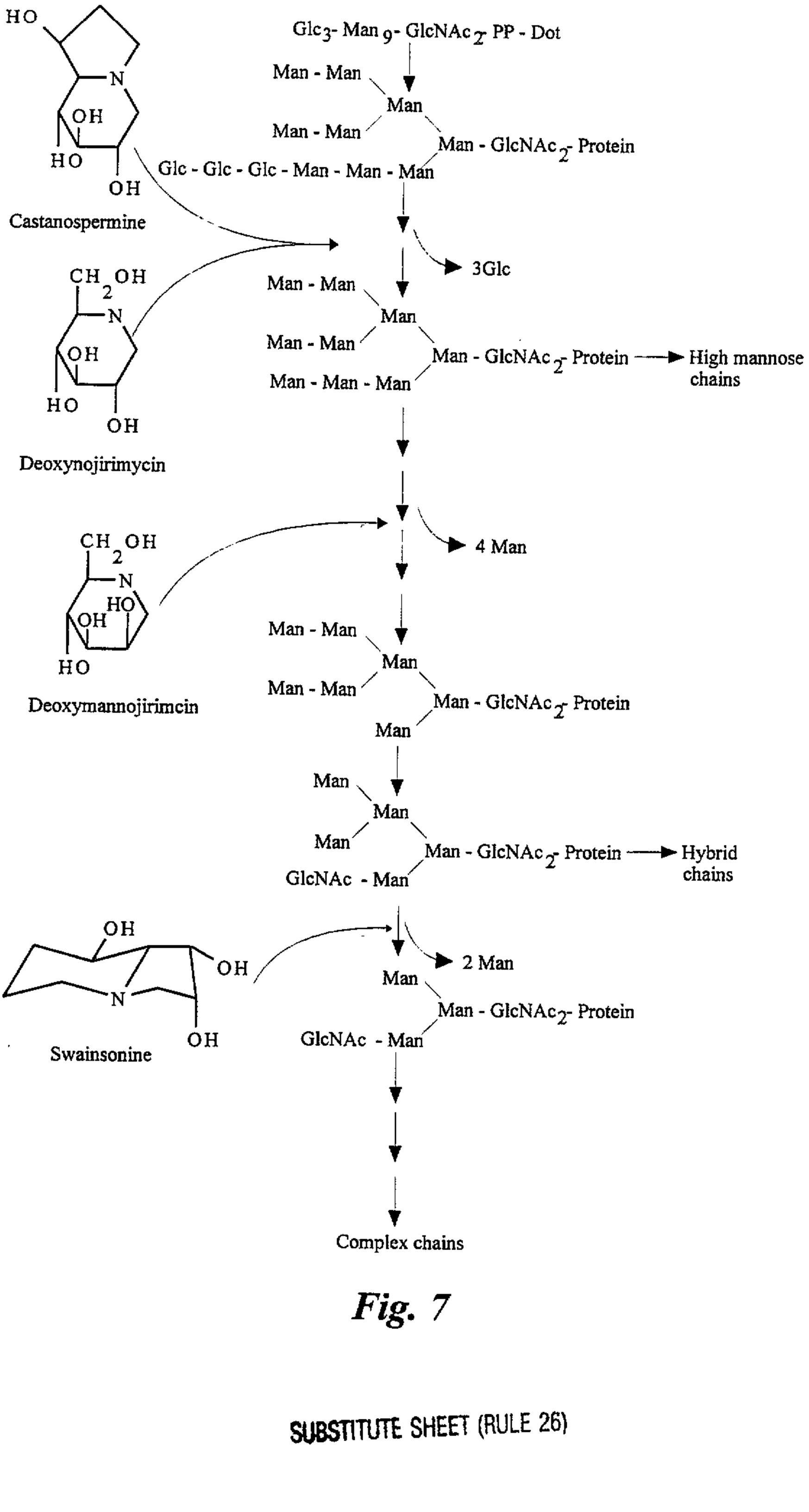
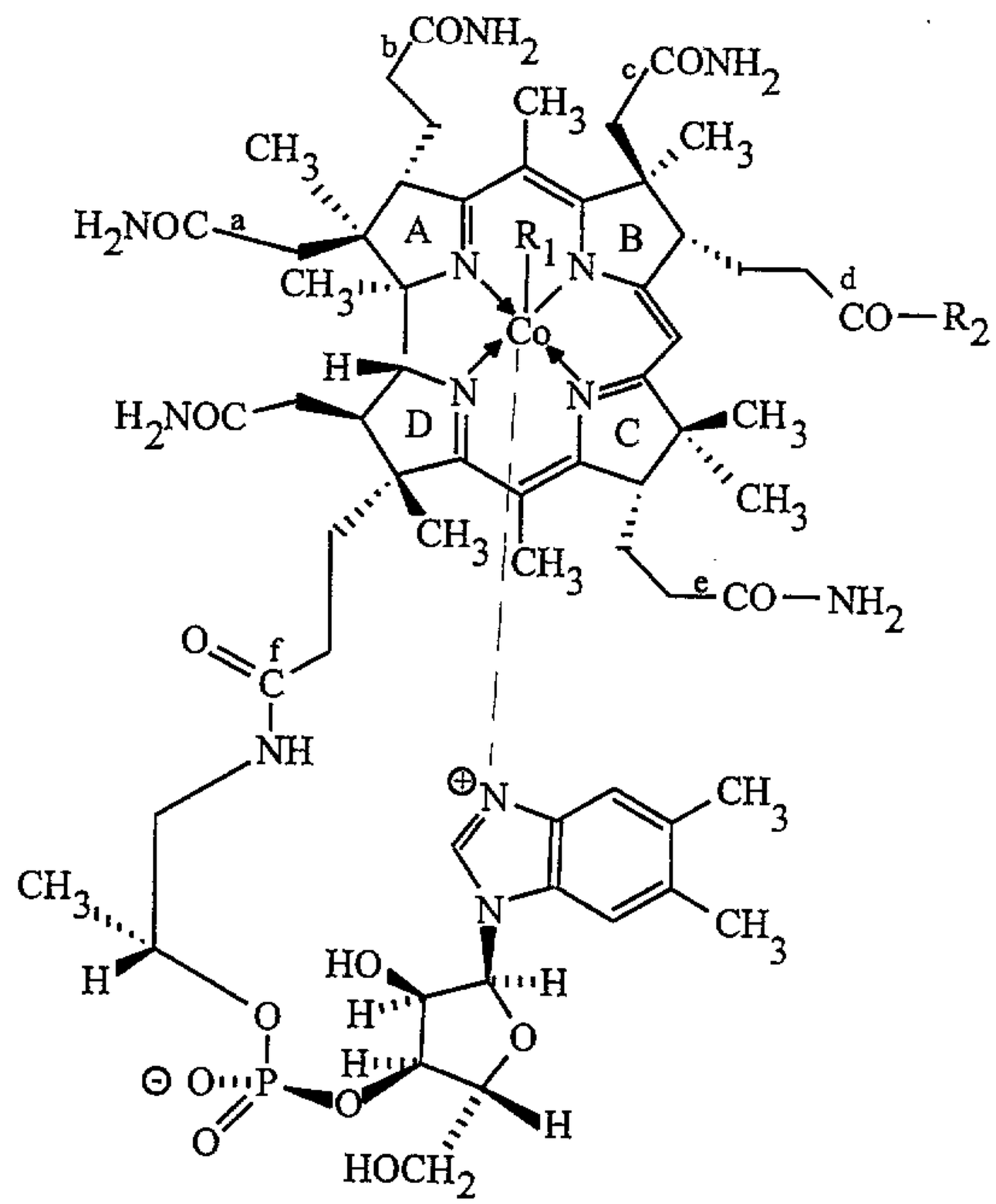

Glc3- Man 9- GlcNAc 2 PP - Dot
Man - Man
Man
Man - Man
Man - GlcNAc 2- Protein
Glc - Glc - Glc - Man - Man - Man
3Glc
Man - Man
Man
Man - Man
Man - GlcNAc 2- Protein
Man - Man - Man
High mannose chains
4 Man
Man - Man
Man
Man - Man
Man - GlcNAc 2- Protein
Man
Man
Man
Man - GlcNAc 2- Protein
GlcNAc - Man
Hybrid chains
2 Man
Man
Man - GlcNAc2- Protein
GlcNAc - Man
Complex chains
Castanospermine
Deoxynojirimycin
Deoxymannojirimcin
Swainsonine

Fig. 7



$R_1 = CN$; $R_2 = NH_2$ (Cyanocobalamin)
$R_1 = CN$; $R_2 = OH$ (Cyanocobalamin -(3)-free acid)
$R_1 = CN$; $R_2 = HN\text{-}CH_2\text{-}CH_2\text{-}CH_2\text{-}CO_2H$ (GABA adduct)
$R_1 = CN$; R_2 = GABA - Peptide (where GABA = linker)
$R_1 = CN$; R_2 = Peptide
$R_1 = CN$; R_2 = HN-(linker)-tyramine-^{125}I
$R_1 = CN$; R_2 = HN-(linker)-lysosomotropic agent
$R_1 = CN$; R_2 = HN-(linker)-X-linking agent
$R_1 = CN$; R_2 = HN-(linker)-biotin
$R_1 = CN$; $R_2 = NH\text{-}(CH_2)_{12}NH_2$

Fig. 8

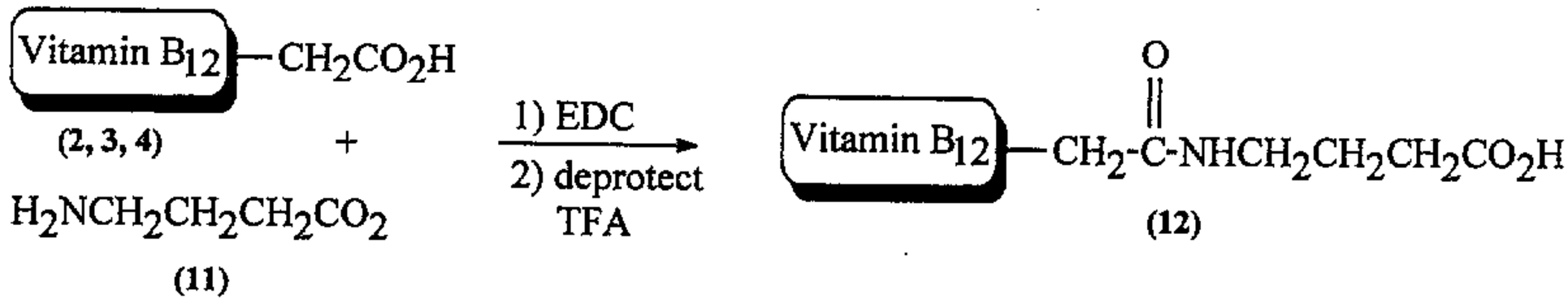


Fig. 9

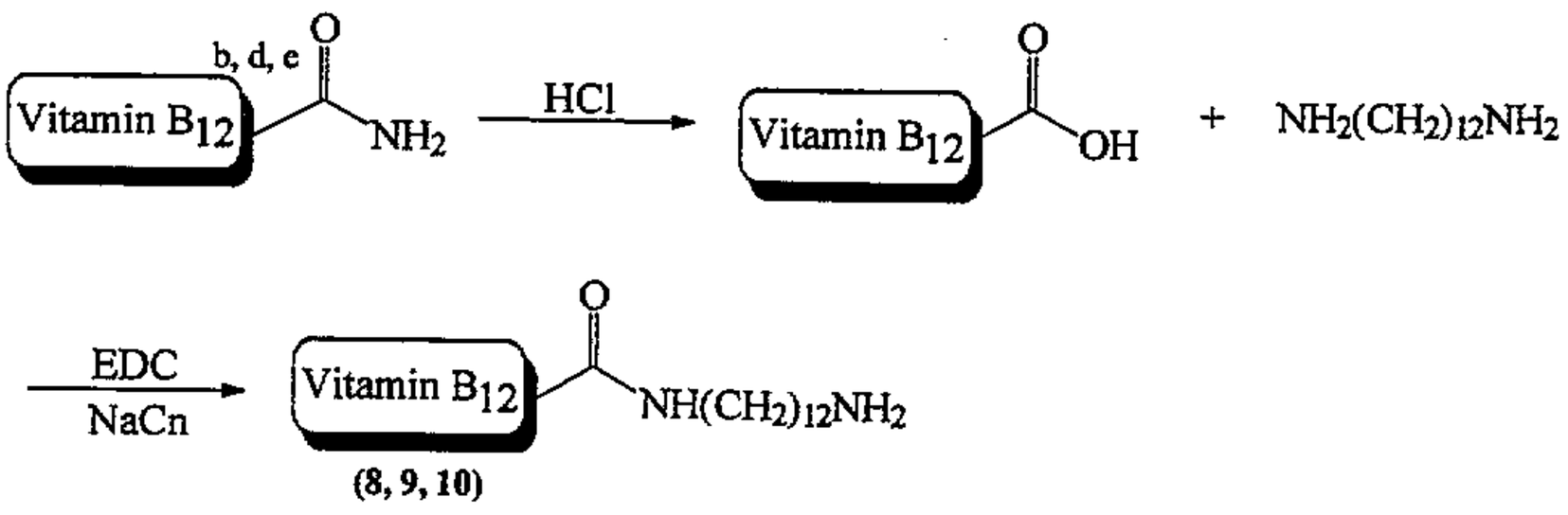


Fig. 10a

Fig. 10b

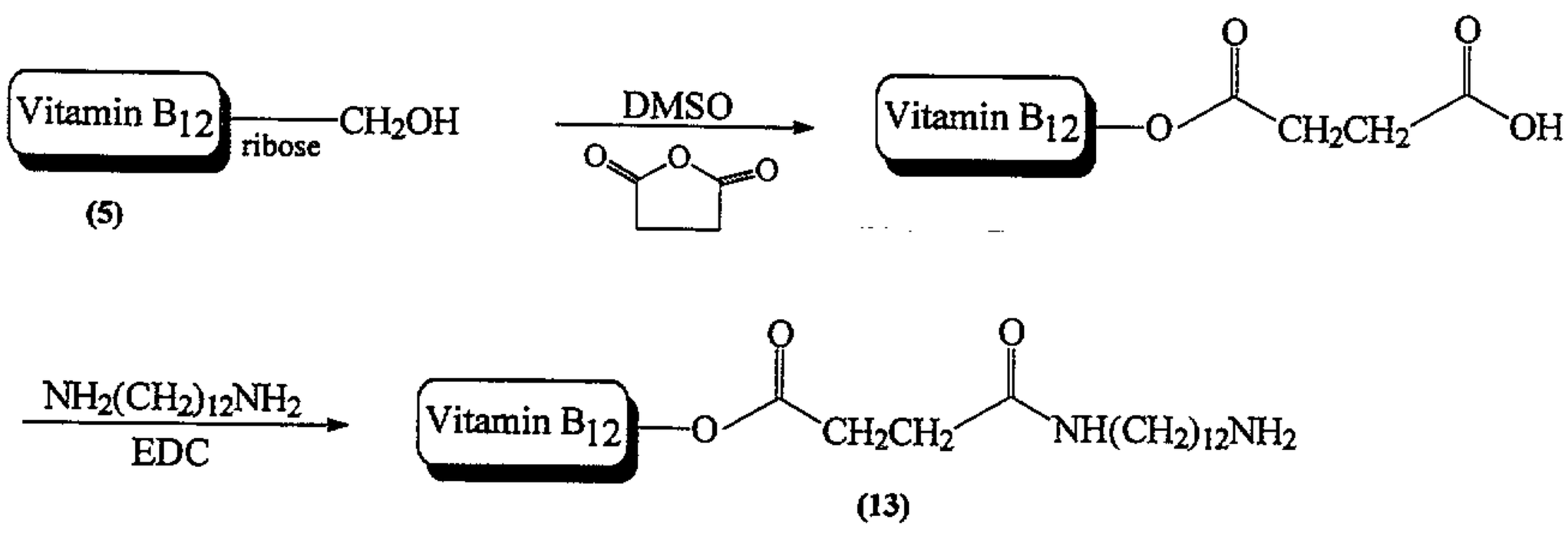


Fig. 11

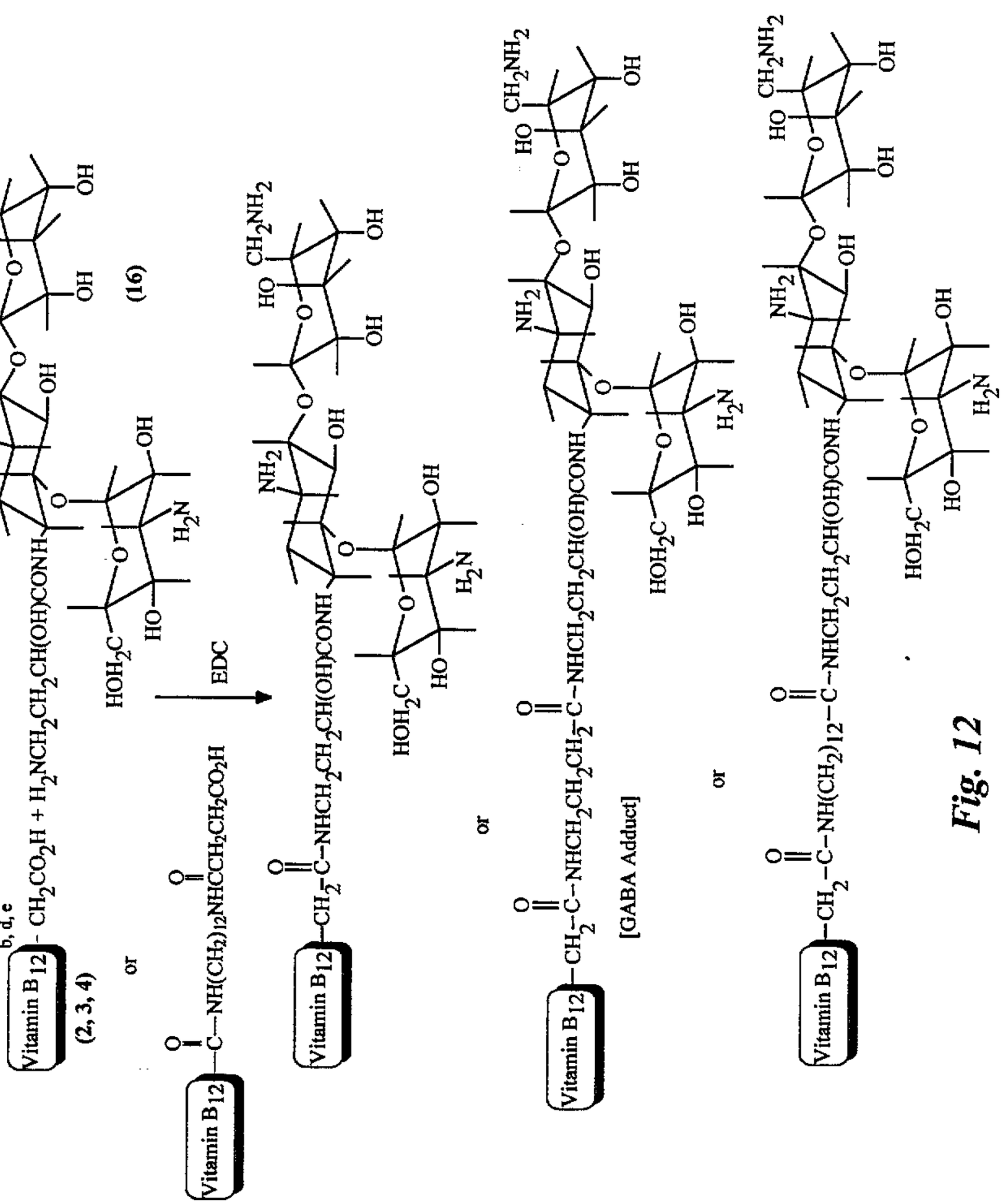

Vitamin B12
(2, 3, 4)
b, d, e
CH2CO2H + H2NCH2CH2CH(OH)CONH
(16)
or
HO
OH
NH2
O
H2N
HOH2C
EDC
C–NH(CH2)12NHCCH2CH2CO2H
[GABA Adduct]

Fig. 12

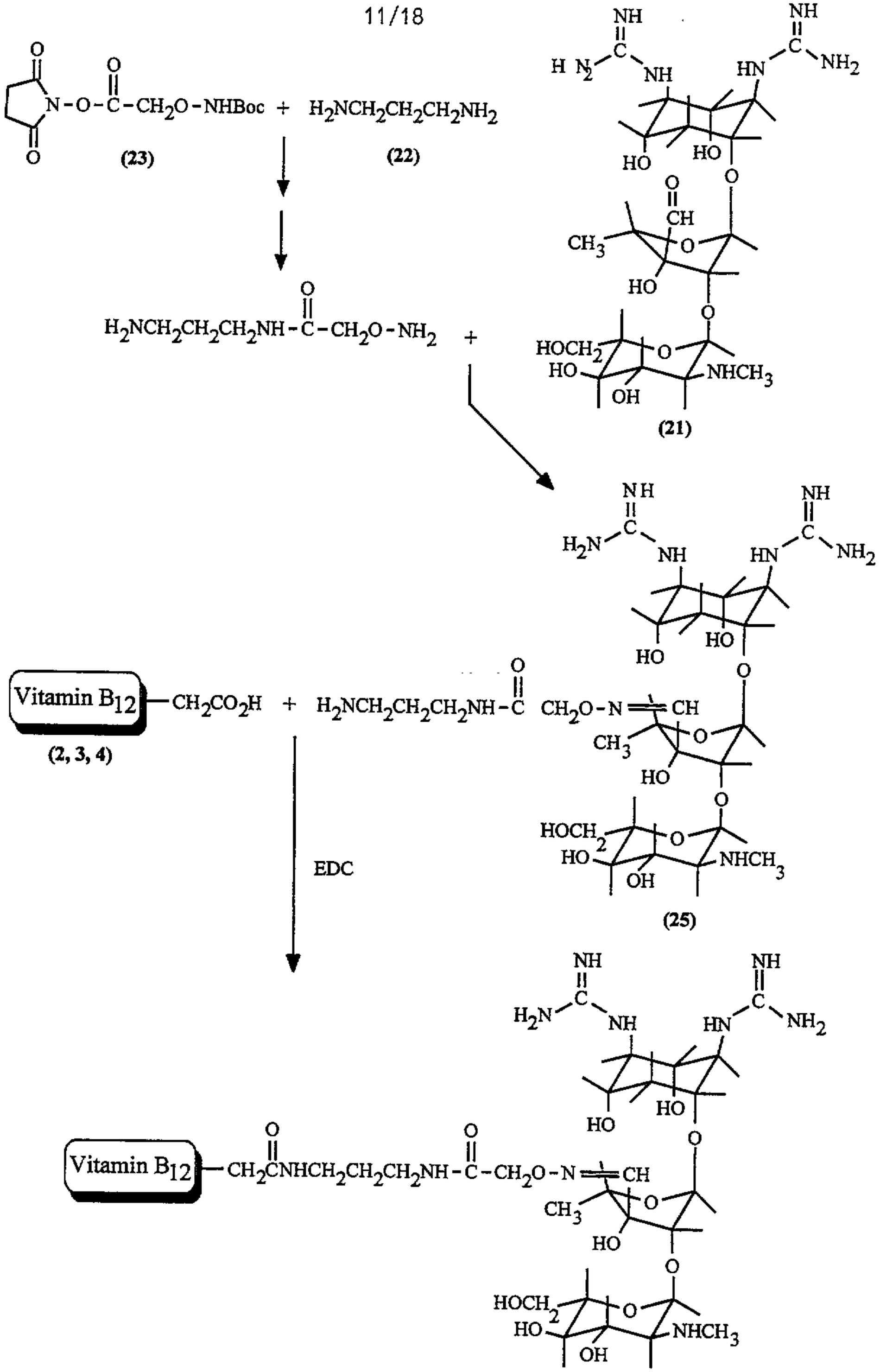


Fig. 13

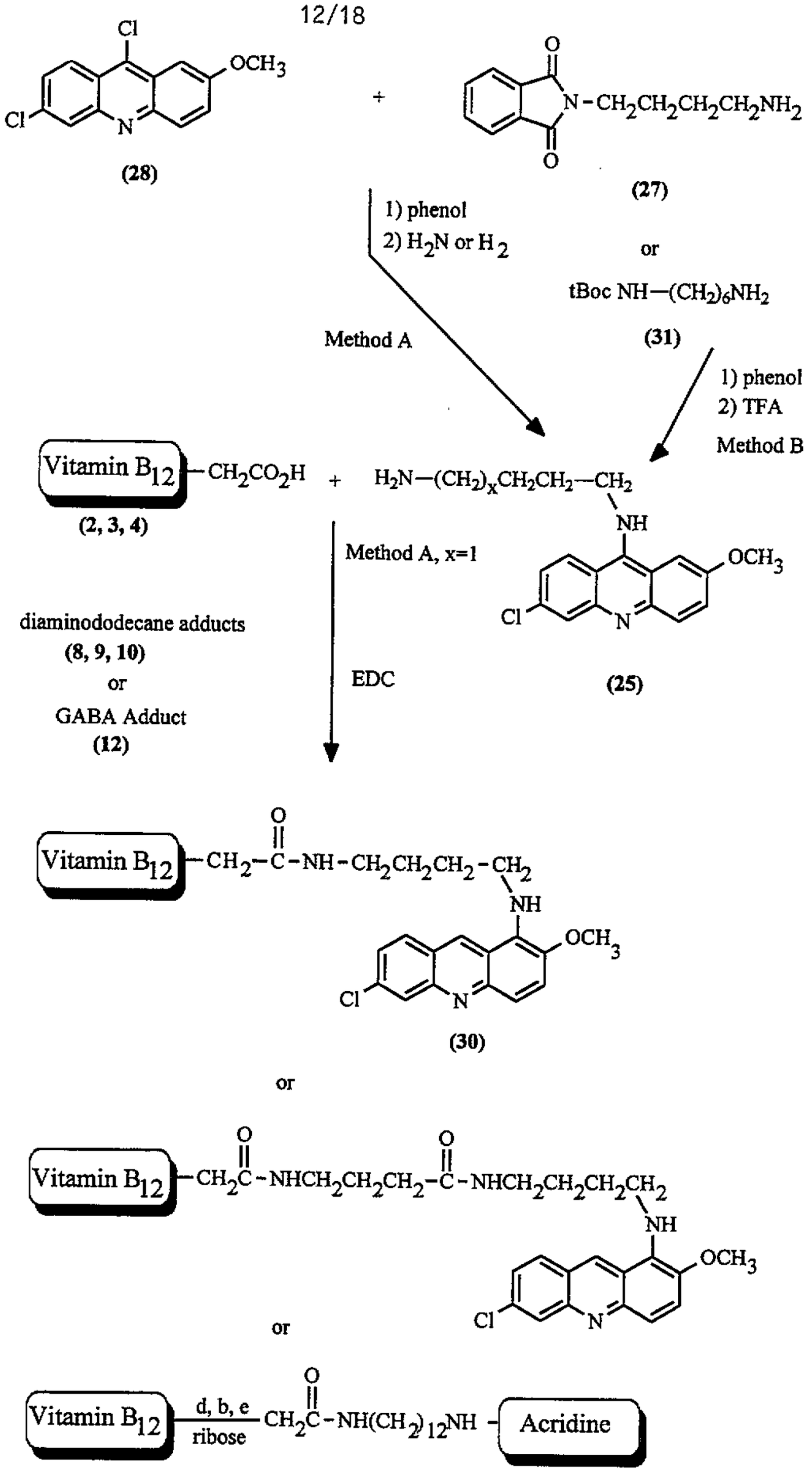

Cl
OCH3
Cl
N
(28)
+
O
N-CH2CH2CH2CH2NH2
O
(27)
1) phenol
2) H2N or H2
or
tBoc NH—(CH2)6NH2
(31)
Method A
1) phenol
2) TFA
Method B
Vitamin B12—CH2CO2H
(2, 3, 4)
+
H2N—(CH2)xCH2CH2—CH2
NH
Method A, x=1
OCH3
Cl
N
(25)
diaminododecane adducts
(8, 9, 10)
or
GABA Adduct
(12)
EDC
Vitamin B12—CH2—C—NH—CH2CH2CH2—CH2
O
NH
OCH3
Cl
N
(30)
or
Vitamin B12—CH2C—NHCH2CH2CH2—C—NHCH2CH2CH2CH2
O
O
NH
OCH3
Cl
N
or
Vitamin B12
d, b, e
ribose
CH2C—NH(CH2)12NH
O
Acridine

Fig. 14

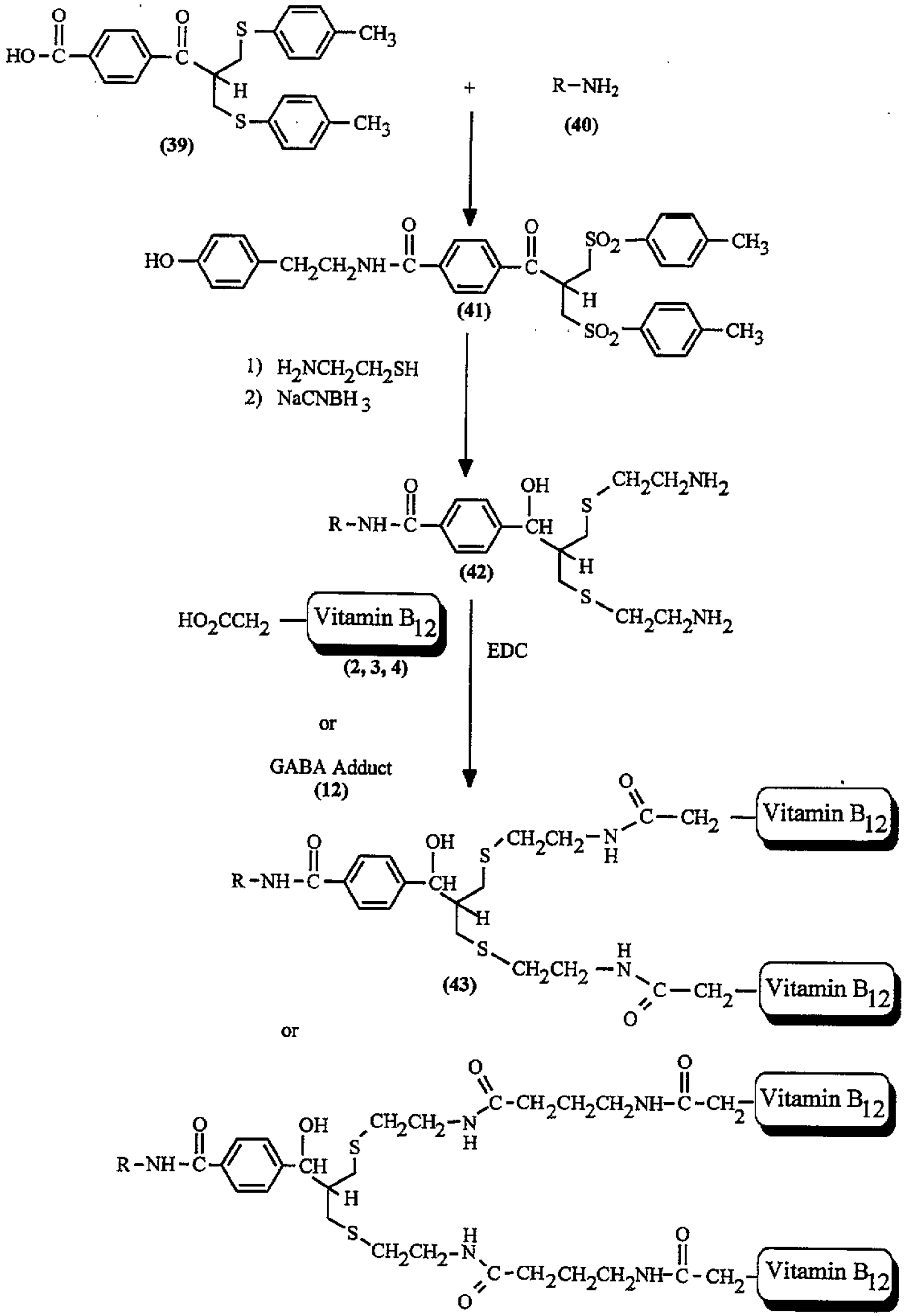
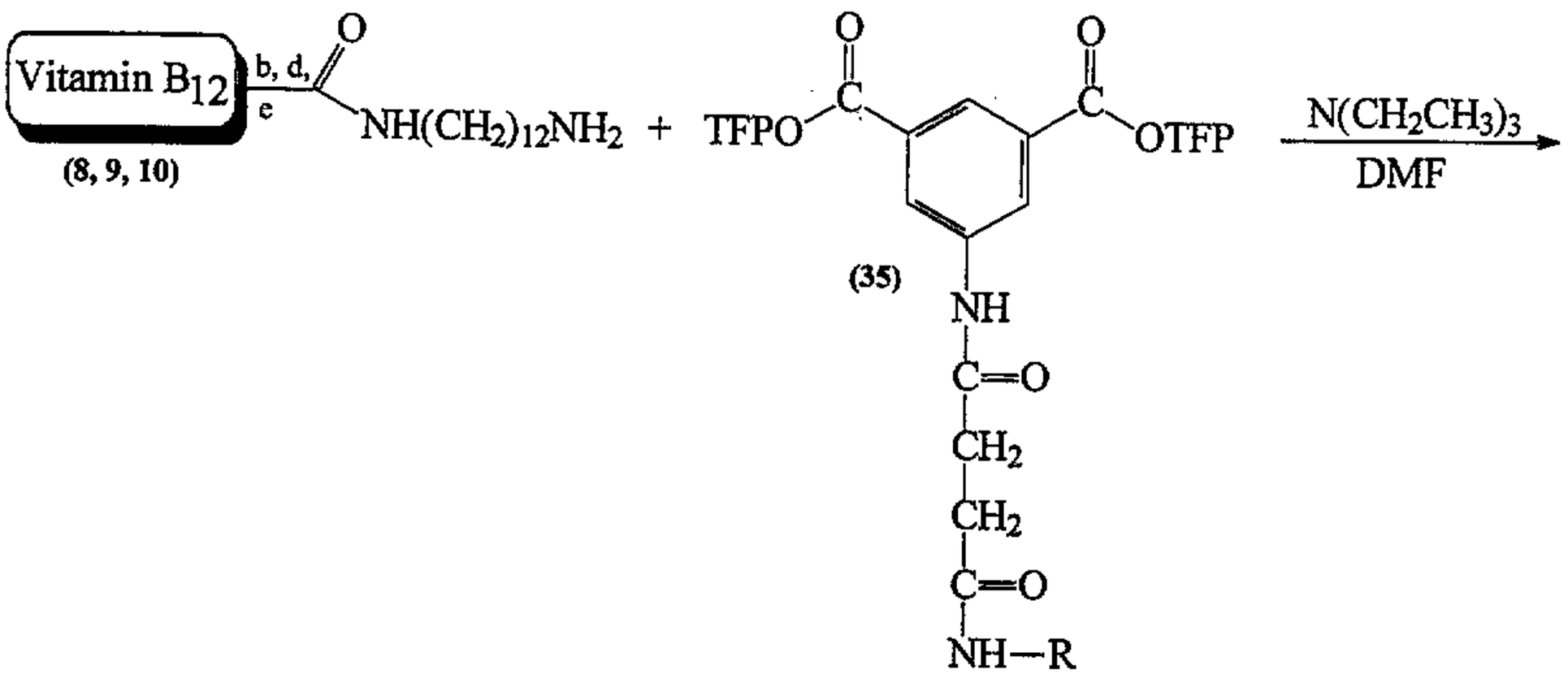

HO-C
O
C
O
S
CH3
H
S
CH3
(39)
+
R-NH2
(40)
HO
CH2CH2NH-C
O
C
O
SO2
CH3
H
SO2
CH3
(41)
1) H2NCH2CH2SH
2) NaCNBH3
R-NH-C
O
OH
CH
CH2CH2NH2
S
H
S
CH2CH2NH2
(42)
HO2CCH2
Vitamin B12
(2, 3, 4)
EDC
or
GABA Adduct
(12)
R-NH-C
O
OH
CH
S
CH2CH2-N
H
C-CH2
O
Vitamin B12
H
S
CH2CH2-N
H
C-CH2
O
Vitamin B12
(43)
or
R-NH-C
O
OH
CH
S
CH2CH2-N
H
C-CH2CH2CH2NH-C-CH2
O
O
Vitamin B12
H
S
CH2CH2-N
H
C-CH2CH2CH2NH-C-CH2
O
O
Vitamin B12

Fig. 15


Vitamin B12
b, d, e
O
NH(CH2)12NH2
(8, 9, 10)
+
TFPO
O
C
O
C
OTFP
N(CH2CH3)3
DMF
(35)
NH
C=O
CH2
CH2
C=O
NH—R

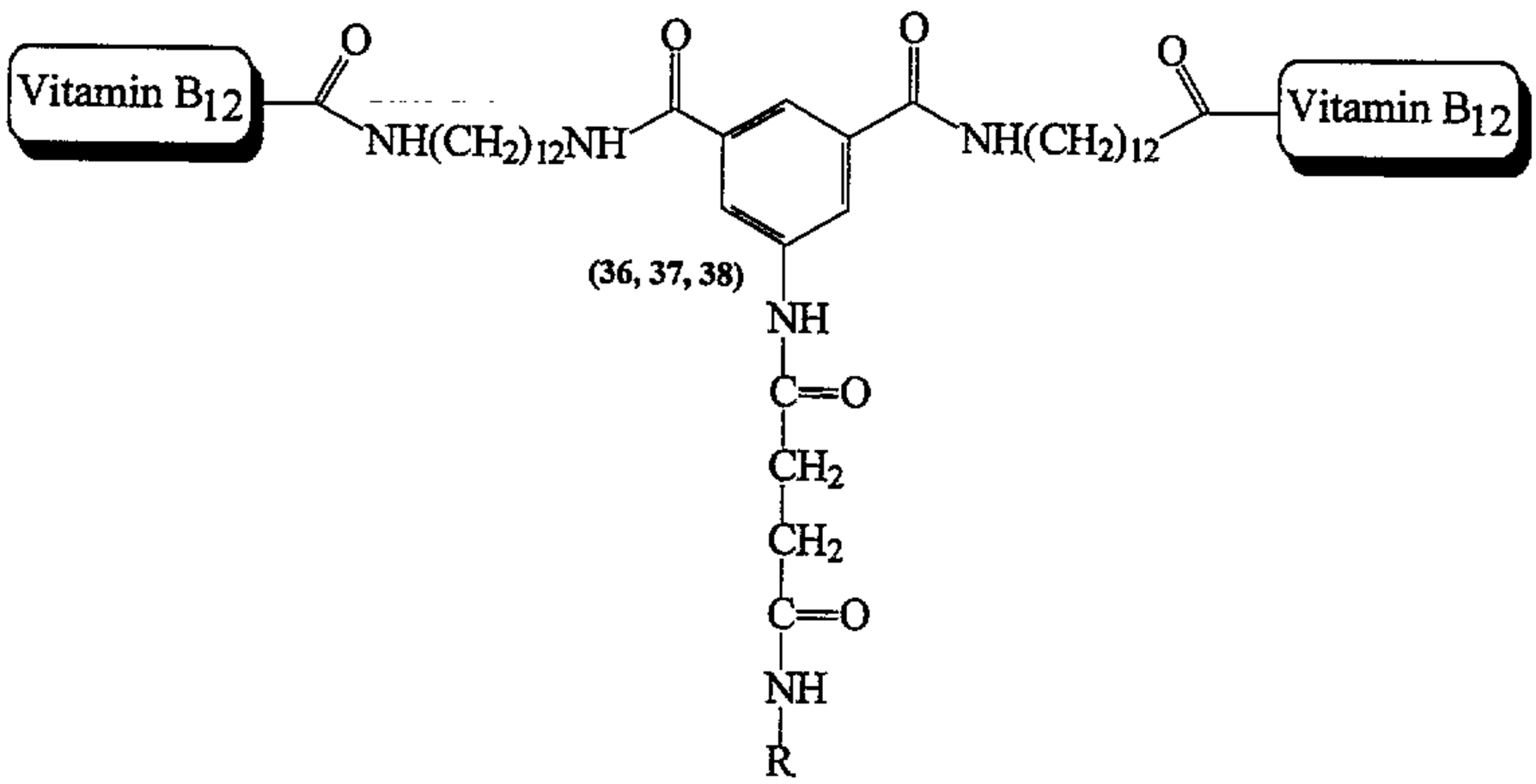
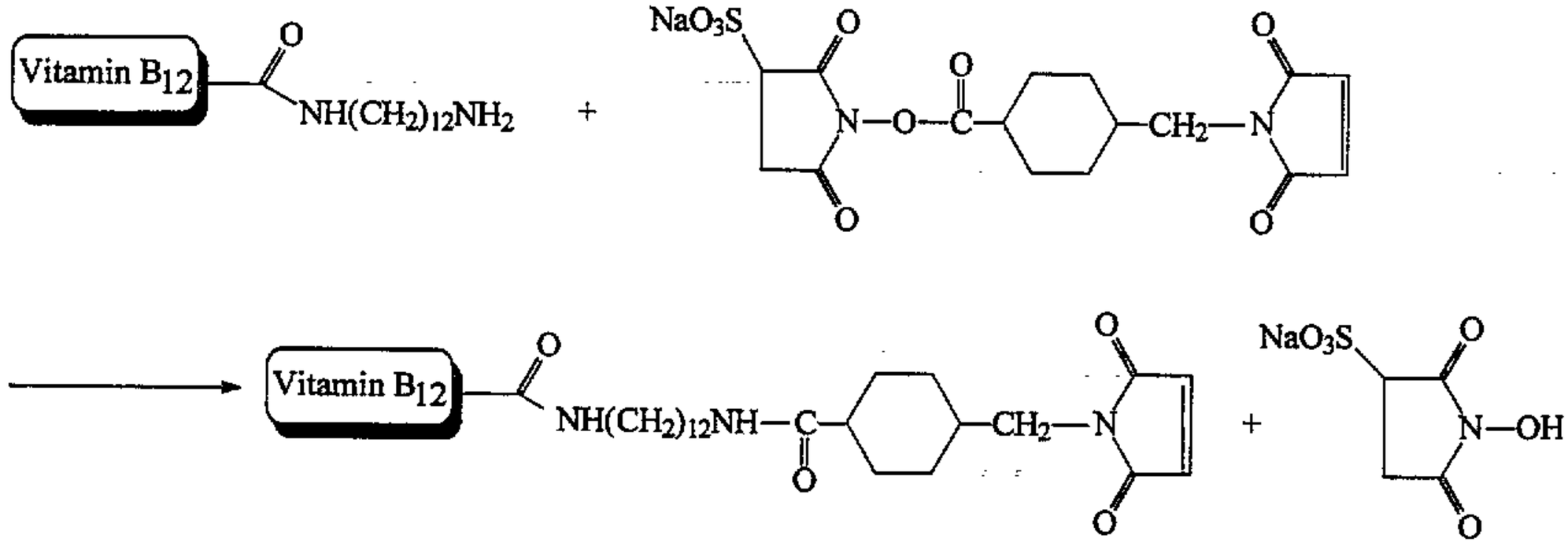
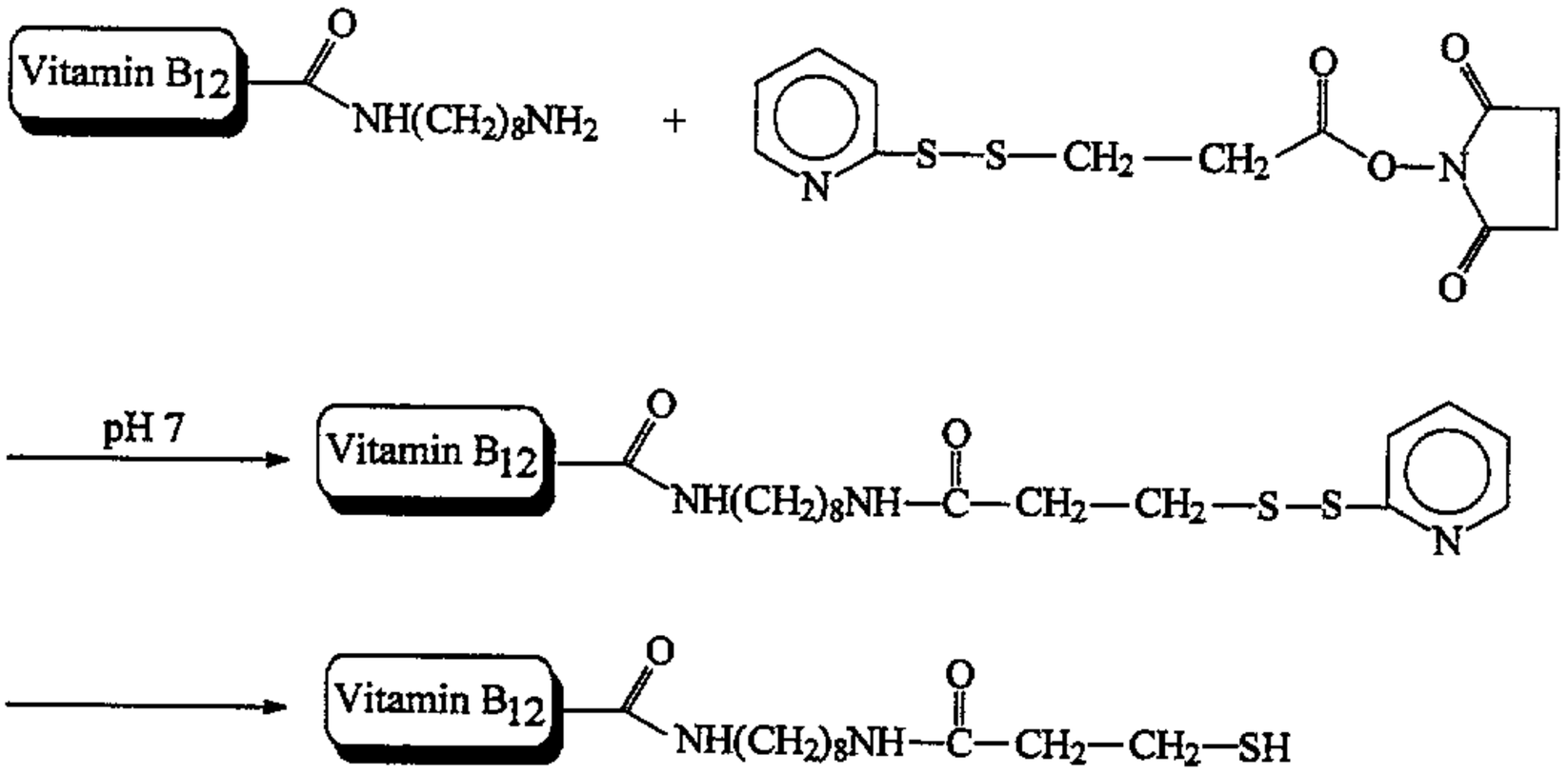

Vitamin B12
O
NH(CH2)12NH
O
O
NH(CH2)12
O
Vitamin B12
(36, 37, 38)
NH
C=O
CH2
CH2
C=O
NH
R

Fig. 16

A.


Vitamin B12
NH(CH2)12NH2
NaO3S
N—O—C
CH2—N
NH(CH2)12NH—C
NaO3S
N—OH

B.


Vitamin B12
NH(CH2)8NH2
S—S—CH2—CH2
pH 7
NH(CH2)8NH—C—CH2—CH2—S—S
Vitamin B12
NH(CH2)8NH—C—CH2—CH2—SH

C.

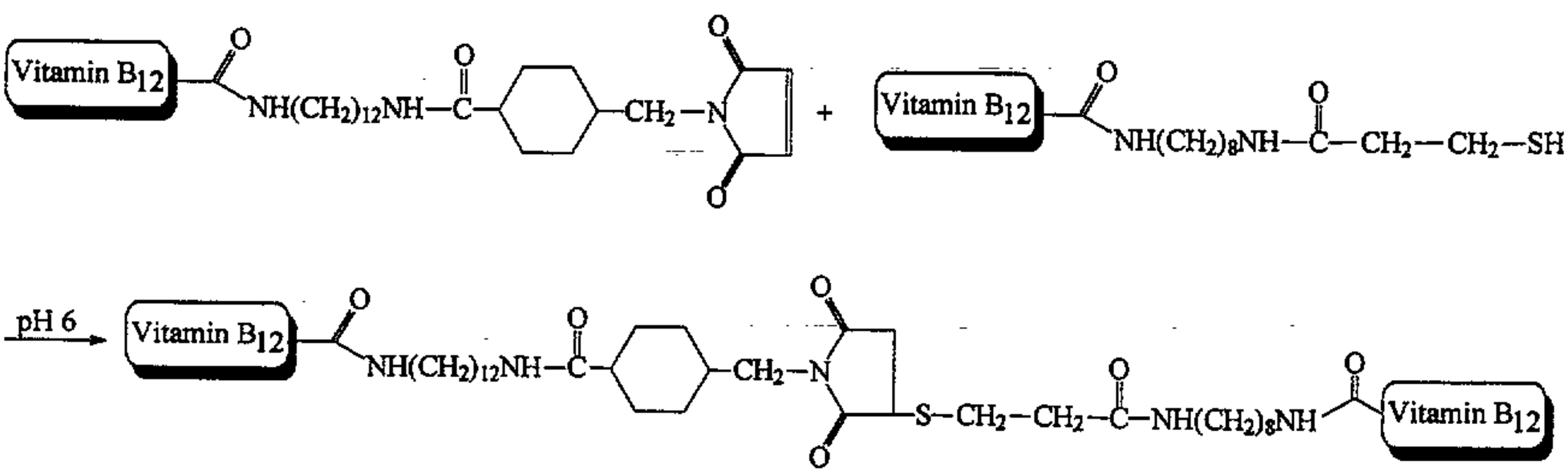

Vitamin B12
NH(CH2)12NH—C
CH2—N
Vitamin B12
NH(CH2)8NH—C—CH2—CH2—SH
pH 6
Vitamin B12
NH(CH2)12NH—C
CH2—N
S—CH2—CH2—C—NH(CH2)8NH
Vitamin B12

Fig. 17

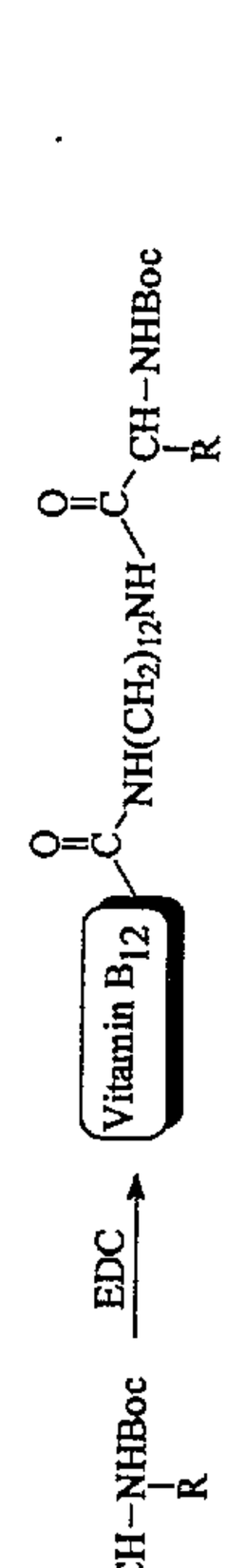


Fig. 18

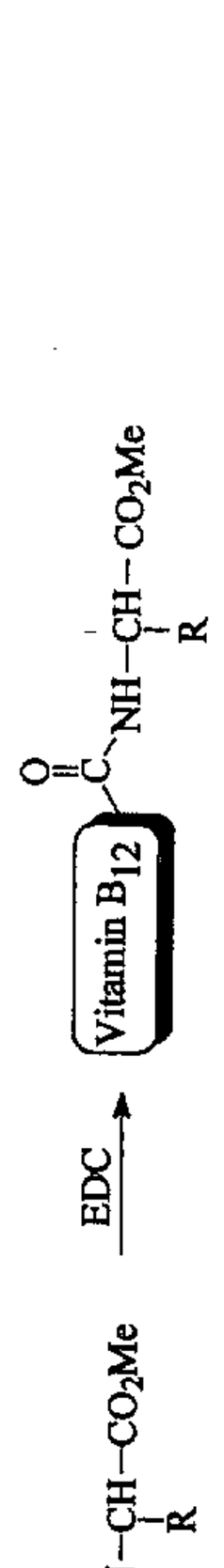


Fig. 19

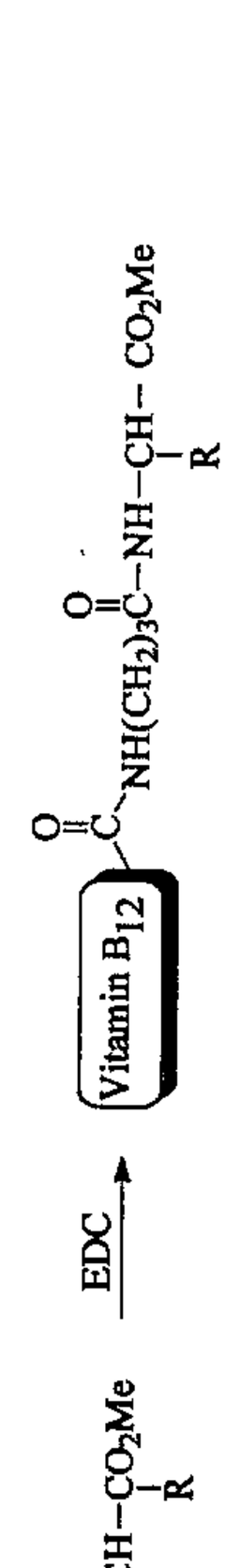


Fig. 20

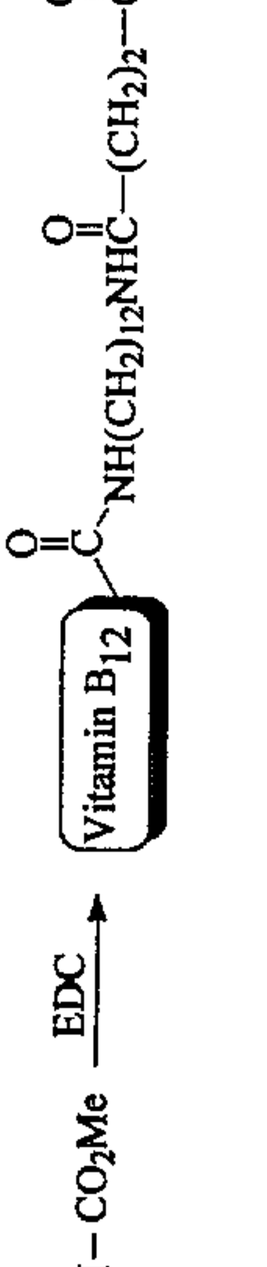


Fig. 21

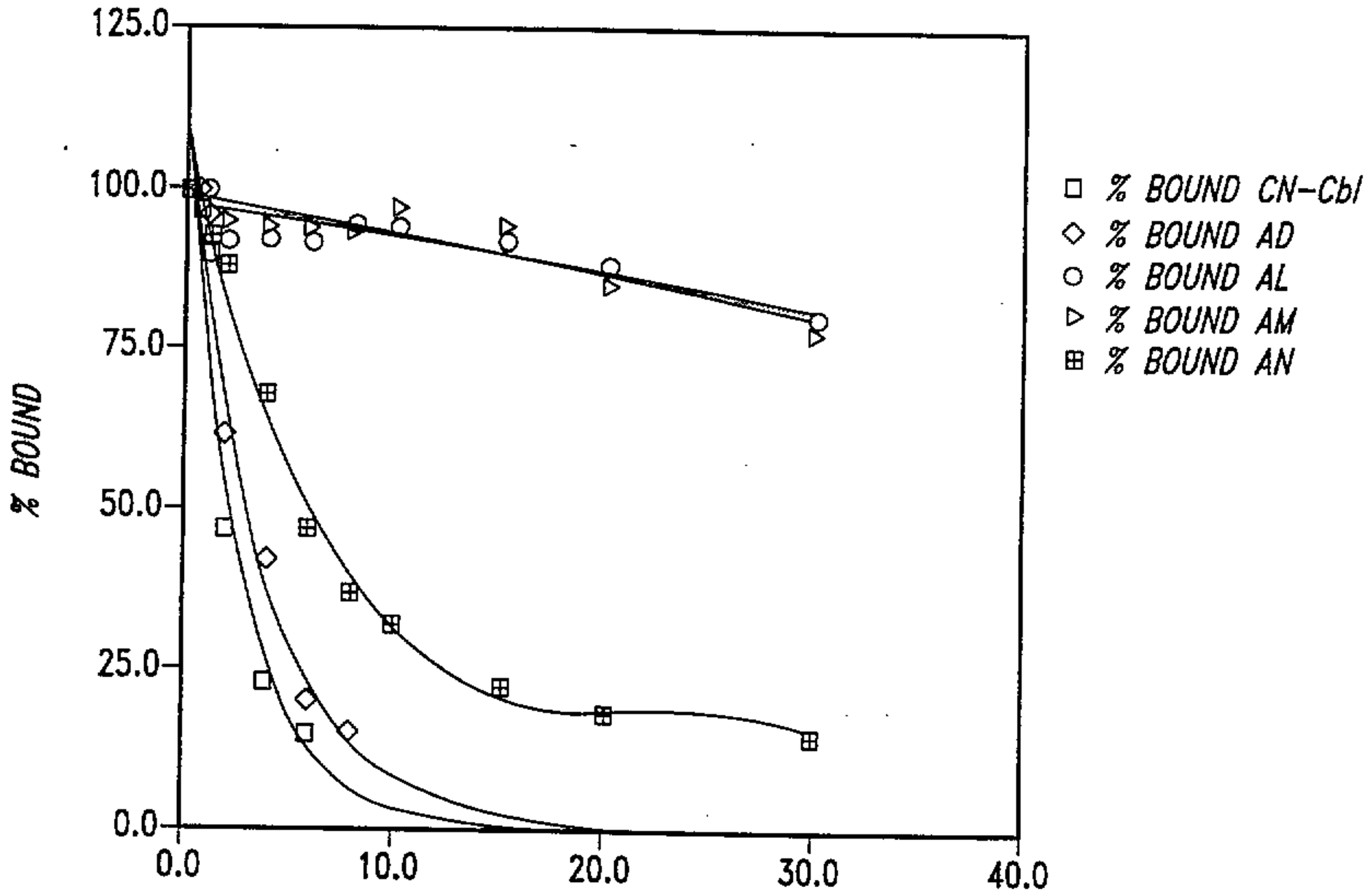

125.0
100.0
75.0
50.0
25.0
0.0
% BOUND
0.0
10.0
20.0
30.0
40.0
pmol
□ % BOUND CN-Cbl
◇ % BOUND AD
○ % BOUND AL
▷ % BOUND AM
⊞ % BOUND AN

Fig. 22

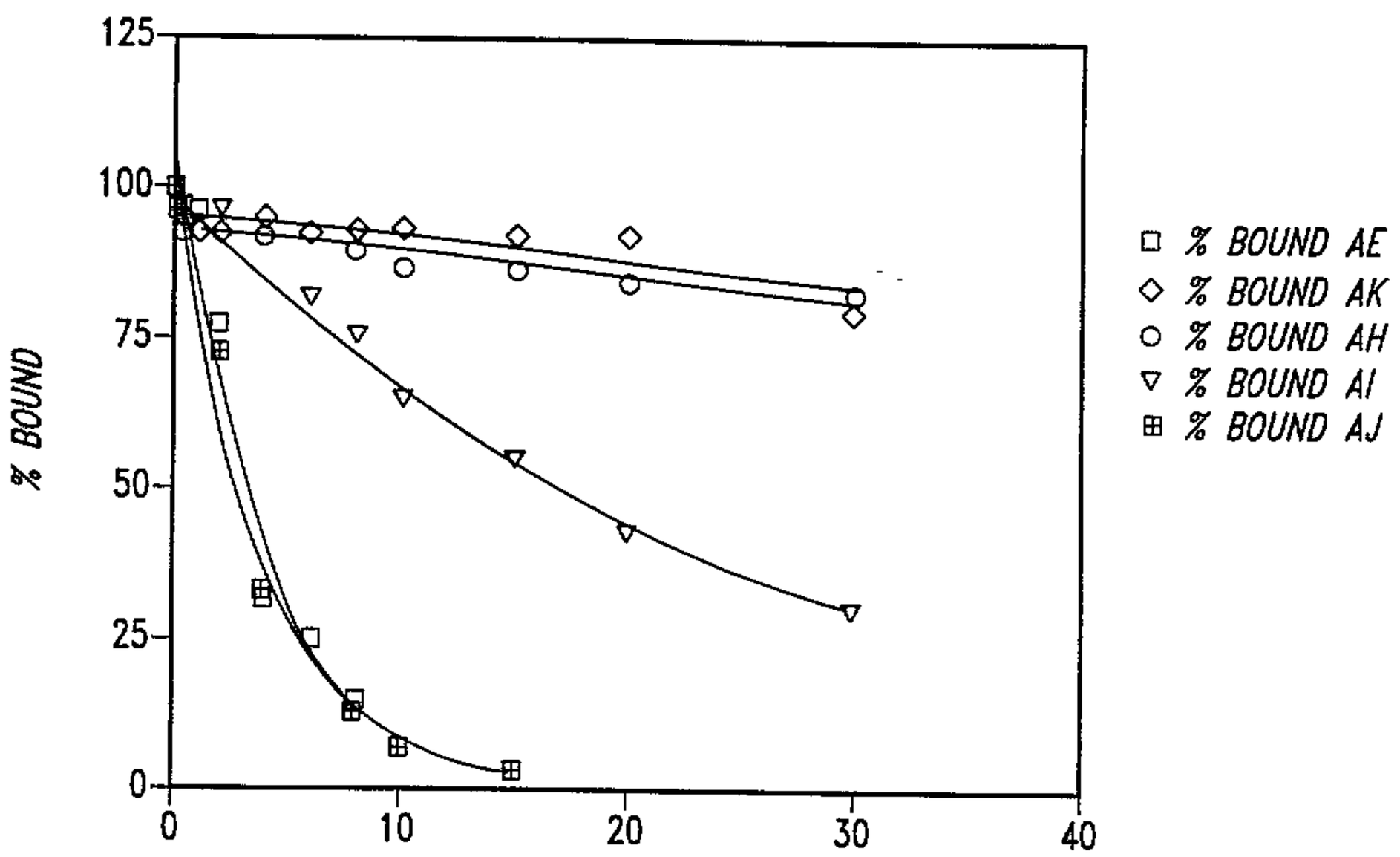
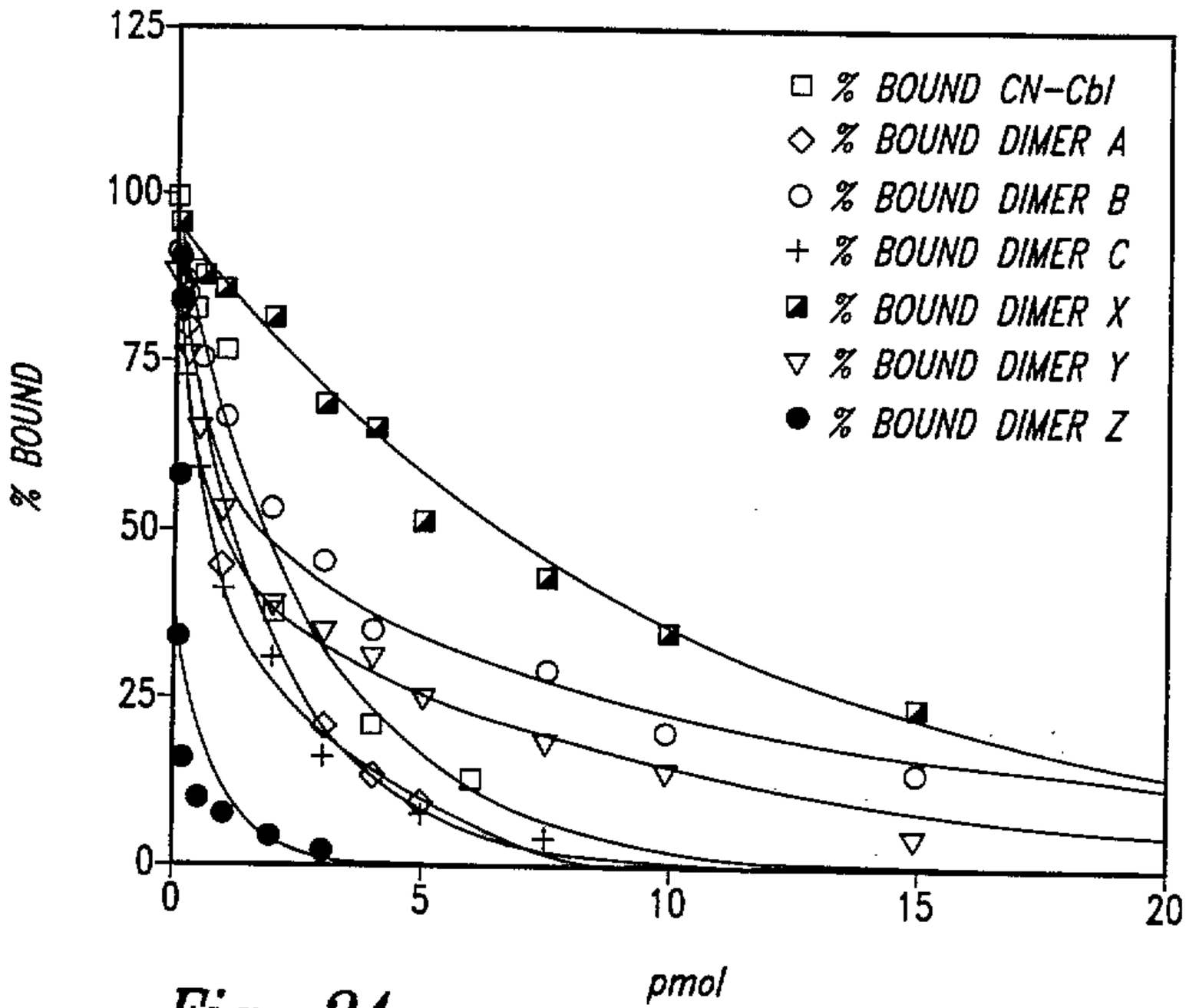

125
100
75
50
25
0
% BOUND
0
10
20
30
40
ANALOGUE pmol
□ % BOUND AE
◇ % BOUND AK
○ % BOUND AH
▽ % BOUND AI
⊞ % BOUND AJ

Fig. 23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100
75
50
25
0
0
5
10
15
20
% BOUND
pmol
□ % BOUND CN-CbI
◇ % BOUND DIMER A
○ % BOUND DIMER B
+ % BOUND DIMER C
◪ % BOUND DIMER X
▽ % BOUND DIMER Y
● % BOUND DIMER Z

Fig. 24

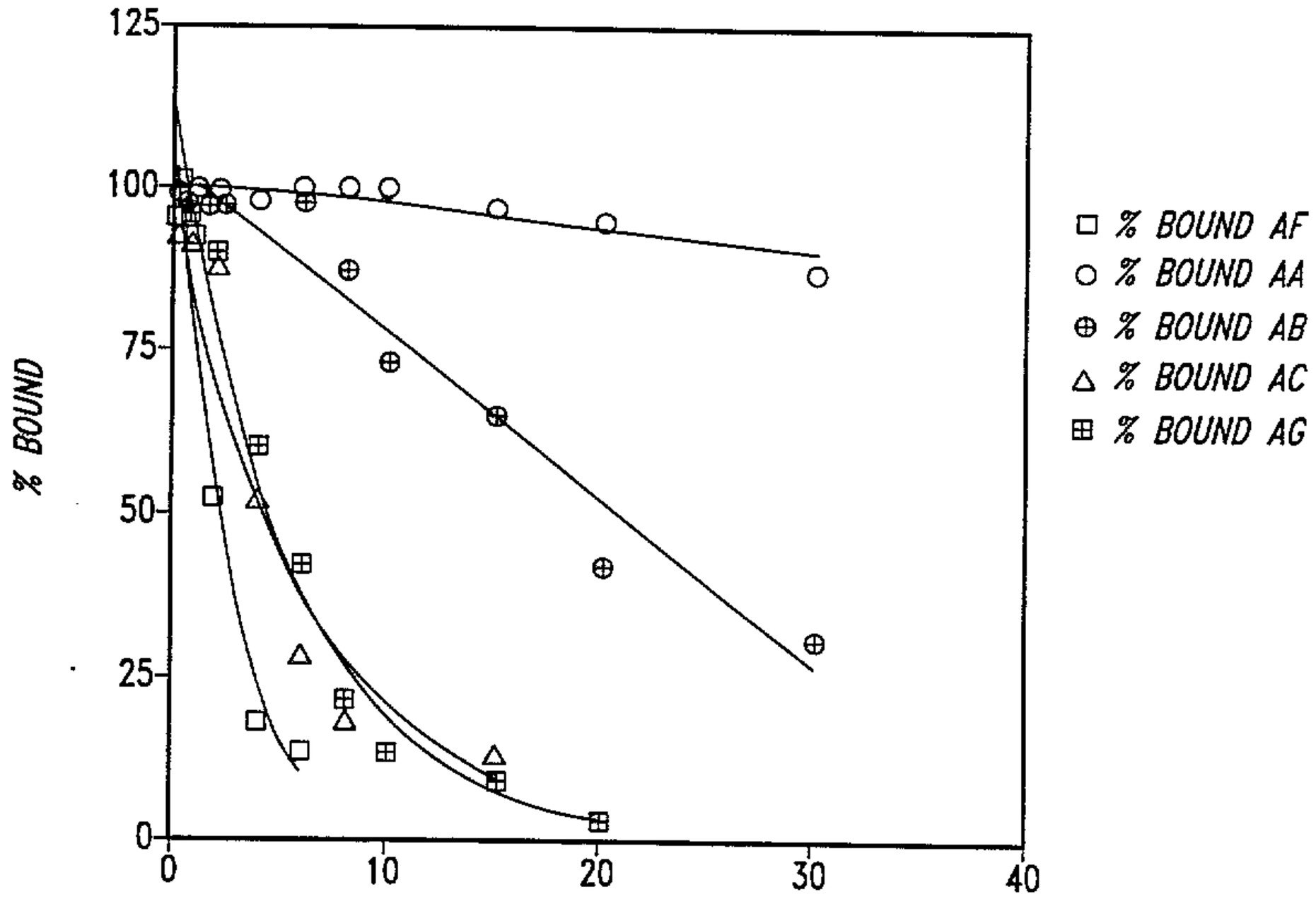
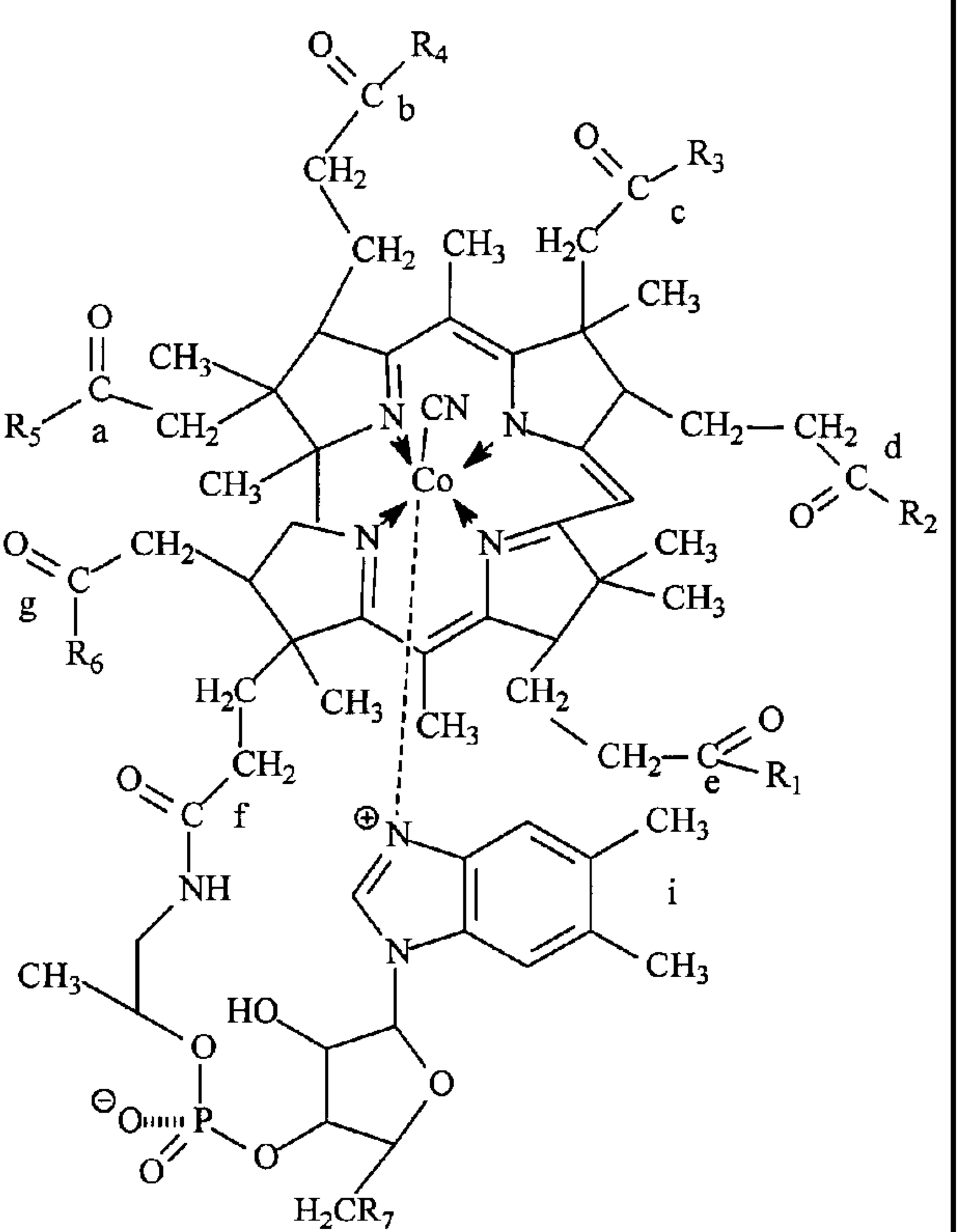

125
100
75
50
25
0
0
10
20
30
40
% BOUND
ANALOGUE pmol
□ % BOUND AF
○ % BOUND AA
⊕ % BOUND AB
△ % BOUND AC
⊞ % BOUND AG

Fig. 25



Structure I