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(54) Title: IMMUNOCHEMICALLY EQUIVALENT HIV DRUG ANALOGS

(57) **Abrégé/Abstract:**

HIV protease inhibitor analogs and non-nucleoside reverse transcriptase inhibitor analogs are provided that are immunochemically equivalent to the parent compound. These analog compounds are useful as calibrators and positive controls in assays for determining HIV protease inhibitors and non-nucleoside reverse transcriptase inhibitors in samples. Methods for preparing calibration curves using the analogs and test kits comprising the analogs are also provided.



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(57) Abstract: HIV protease inhibitor analogs and non-nucleoside reverse transcriptase inhibitor analogs are provided that are immunochemically equivalent to the parent compound. These analog compounds are useful as calibrators and positive controls in assays for determining HIV protease inhibitors and non-nucleoside reverse transcriptase inhibitors in samples. Methods for preparing calibration curves using the analogs and test kits comprising the analogs are also provided.



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IMMUNOCHEMICALLY EQUIVALENT HIV DRUG ANALOGS

Related applications

This application claims priority to US provisional application 60/863,442 filed October 30, 2006.

Field of the invention

5 The present invention pertains to the field of therapeutic drug monitoring, and in particular, to immunoassay methods for determining the presence or amount of an HIV protease inhibitor or a non-nucleoside reverse transcriptase inhibitor in a biological sample. More particularly, the present disclosure provides analogs of HIV protease inhibitors and non-nucleoside reverse transcriptase inhibitors that are useful as calibrators or as positive controls in immunoassays for measuring the amount of parent drug
10 present in a sample.

Background

The clinical care of patients having acquired immunodeficiency disease syndrome (AIDS) has been substantially improved by the introduction of HIV protease inhibitors and HIV reverse transcriptase inhibitors.

15 Currently a number of HIV protease inhibitors have been approved by the Food and Drug Administration (FDA) for treatment of AIDS patients, including amprenavir, fosamprenavir, atazanavir, tipranavir, indinavir, darunavir, lopinavir, nelfinavir, ritonavir, and saquinavir. Combination therapies involving HIV protease inhibitors and HIV reverse transcriptase inhibitors are the cornerstones of currently recommended therapies for HIV infection. HIV reverse transcriptase inhibitors fall into two general
20 classes depending upon whether or not they are nucleoside or non-nucleoside analogs. The nucleoside reverse transcriptase inhibitors (NRTIs) are prodrugs which are converted intracellularly to their active nucleotide forms. On the other hand, non-nucleoside reverse transcriptase inhibitors (NNRTIs) are active drug forms as administered to a patient. Currently three NNRTIs are approved by the Food and Drug Administration for treatment of AIDS patients: delavirdine, efavirenz, and nevirapine. Not all AIDS
25 patients show the same optimal response to a combination therapy regimen, and there can be a large variability in drug response between individual patients. Relationships between systemic exposure to protease inhibitors or NNRTIs and their antiviral effect have been supported by accumulating clinical information. When a combination therapeutic regimen is administered to a patient, potential

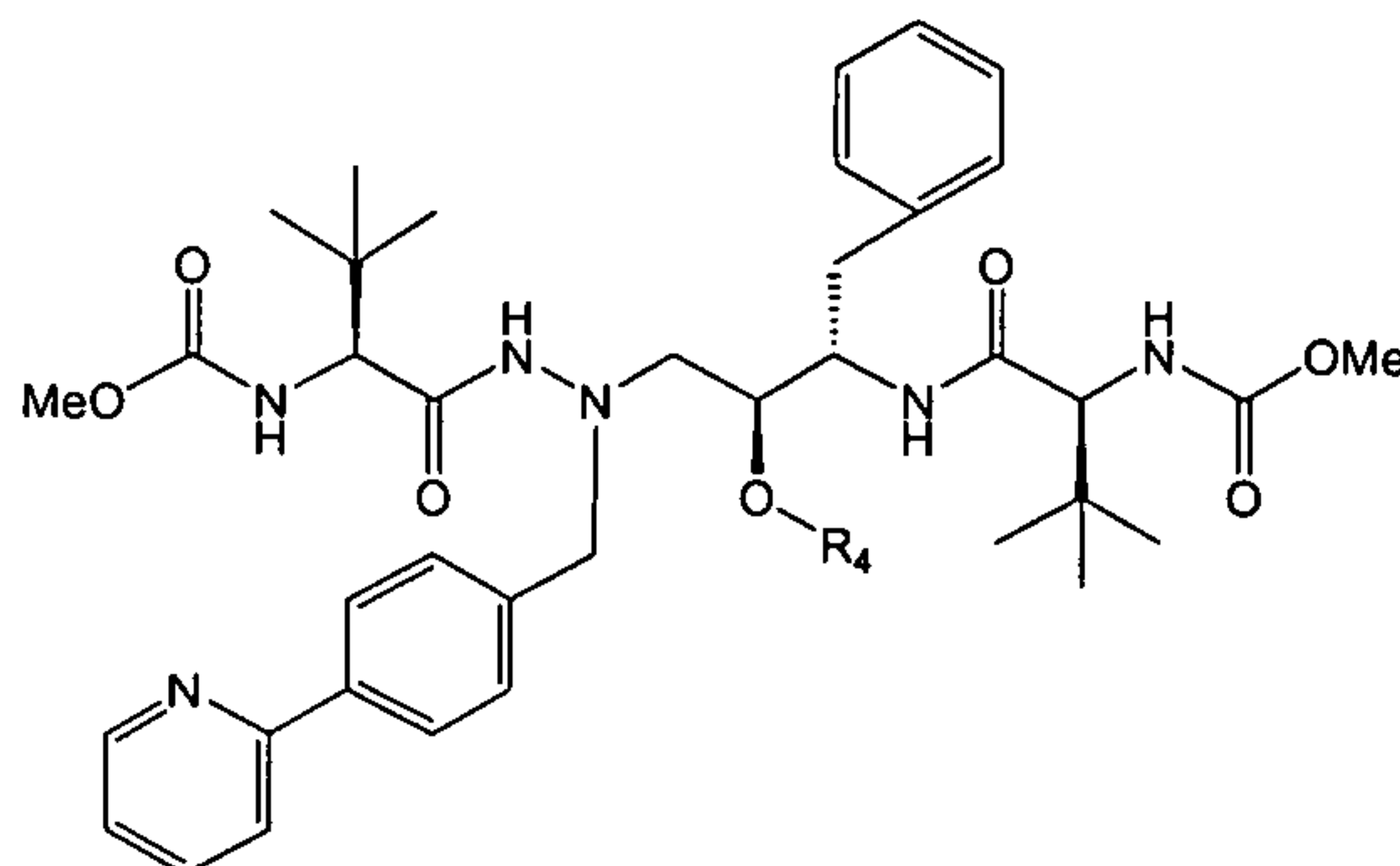
pharmacokinetic drug-drug interactions can improve or weaken the treatment. Patient compliance, which directly relates to maintenance of adequate drug levels, may also affect the outcome of the treatment.

It is thus desirable to measure concentrations of HIV protease inhibitors and NNRTIs in patients to ensure that drug exposure is sufficient to maintain antiviral activity in AIDS patients. Such quantitative
5 measurement methods need to be carefully calibrated and quality controlled using calibrators or reference standards comprising the parent drugs. This is especially true when the method of measurement is an immunoassay. However, it is a problem that such standards are often not commercially available or are not suitable for routine measurement. In the case of immunoassay methods for detecting and measuring HIV drugs, it has been surprisingly found that analogs of HIV protease inhibitors and NNRTIs can be
10 prepared that are immunochemically equivalent to the corresponding parent drugs and can be substituted for the parent drugs as calibrators and positive controls.

Summary of the invention

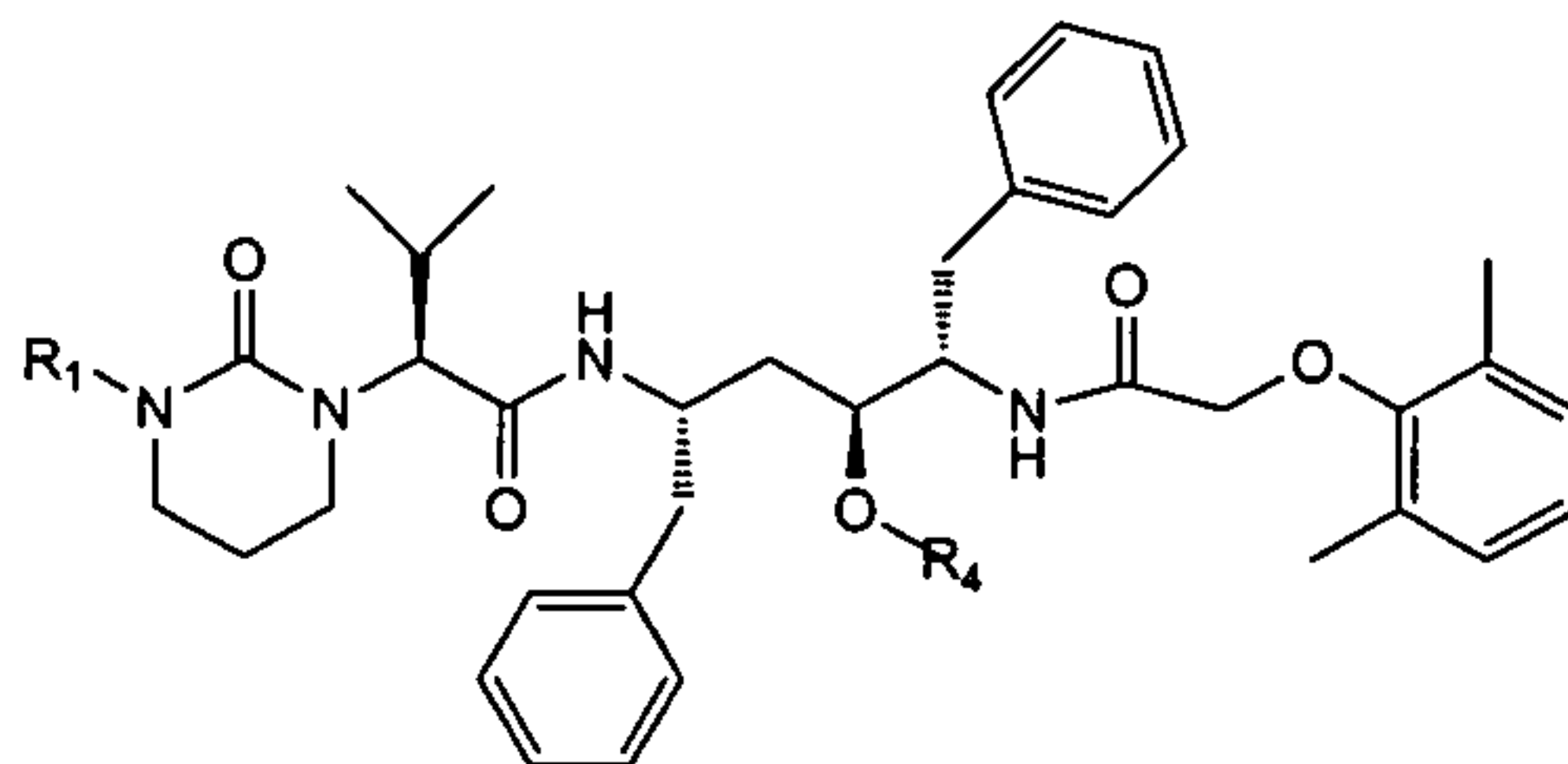
As disclosed herein, analogs of HIV protease inhibitors and NNRTIs are provided wherein the analogs exhibit binding affinities immunochemically equivalent to the parent drug for antibodies raised against
15 the parent HIV protease inhibitors and NNRTI compounds, respectively. Accordingly, a protease inhibitor analog of amprenavir, atazanavir, tipranavir, indinavir, darunavir, lopinavir, nelfinavir, ritonavir, or saquinavir is provided wherein the central hydroxyl group of the parent drug has been replaced with a group selected from $-O(C_1-C_{10} \text{ alkyl})$, $-OCONHR_3$, $-OCH_2COOR_3$, and $-OCH_2CONHR_3$ wherein R_3 is selected from the group consisting of H, C_1-C_6 alkyl, phenyl, heteroaryl, and $-(CH_2)_n-X$ wherein n is 1-6
20 and X is $COOR_5$ or $NH-CH_2\text{-phenyl-}COOR_5$ wherein R_5 is H or C_1-C_6 alkyl. Also accordingly, analogs of non-nucleoside reverse transcriptase inhibitors are provided wherein the analog is N-alkylated with C_1-C_6 alkyl or $(C_1-C_6 \text{ alkyl})OH$. Such HIV protease inhibitor and NNRTI analogs are immunochemically equivalent to the parent protease inhibitor and NNRTI compound, respectively.

More specifically, in one embodiment, an immunochemically equivalent analog of atazanavir (ATV) is
25 provided wherein the analog has the structure:



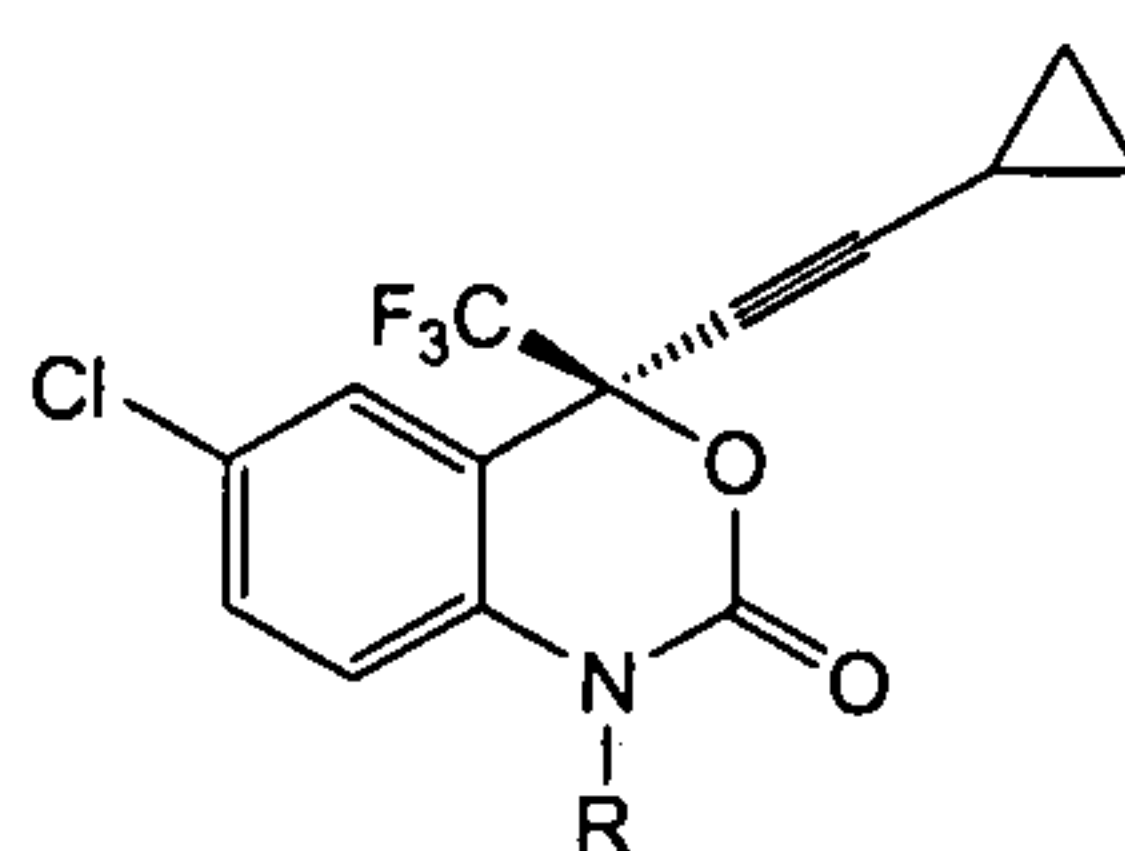
wherein R_4 is selected from the group consisting of C_1 - C_{10} alkyl, $-CH_2COOR_3$, $-CH_2CONHR_3$ and $-CONHR_3$, wherein R_3 is selected from the group consisting of H, C_1 - C_6 alkyl, phenyl, heteroaryl, and $-(CH_2)_n-X$ wherein n is 1-6 and X is $COOR_5$ or $NH-CH_2$ -phenyl- $COOR_5$ wherein R_5 is H or C_1 - C_6 alkyl.

- 5 In another specific embodiment, an immunochemically equivalent analog of lopinavir (LPV) is provided wherein the analog has the structure:



- 10 wherein R_1 is selected from the group consisting of H and $-CONHR_3$ and R_4 is selected from the group consisting of C_1 - C_{10} alkyl, $-CH_2COOR_3$, $-CH_2CONHR_3$ and $-CONHR_3$, wherein R_3 is selected from the group consisting of H, C_1 - C_6 alkyl, phenyl, heteroaryl, and $-(CH_2)_n-X$ wherein n is 1-6 and X is $COOR_5$ or $NH-CH_2$ -phenyl- $COOR_5$ wherein R_5 is H or C_1 - C_6 alkyl.

In another specific embodiment, an immunochemically equivalent analog of efavirenz (EFV) is provided wherein the analog has the structure:



- 15 wherein R is C_1 - C_6 alkyl or $(C_1$ - C_6 alkyl)OH.

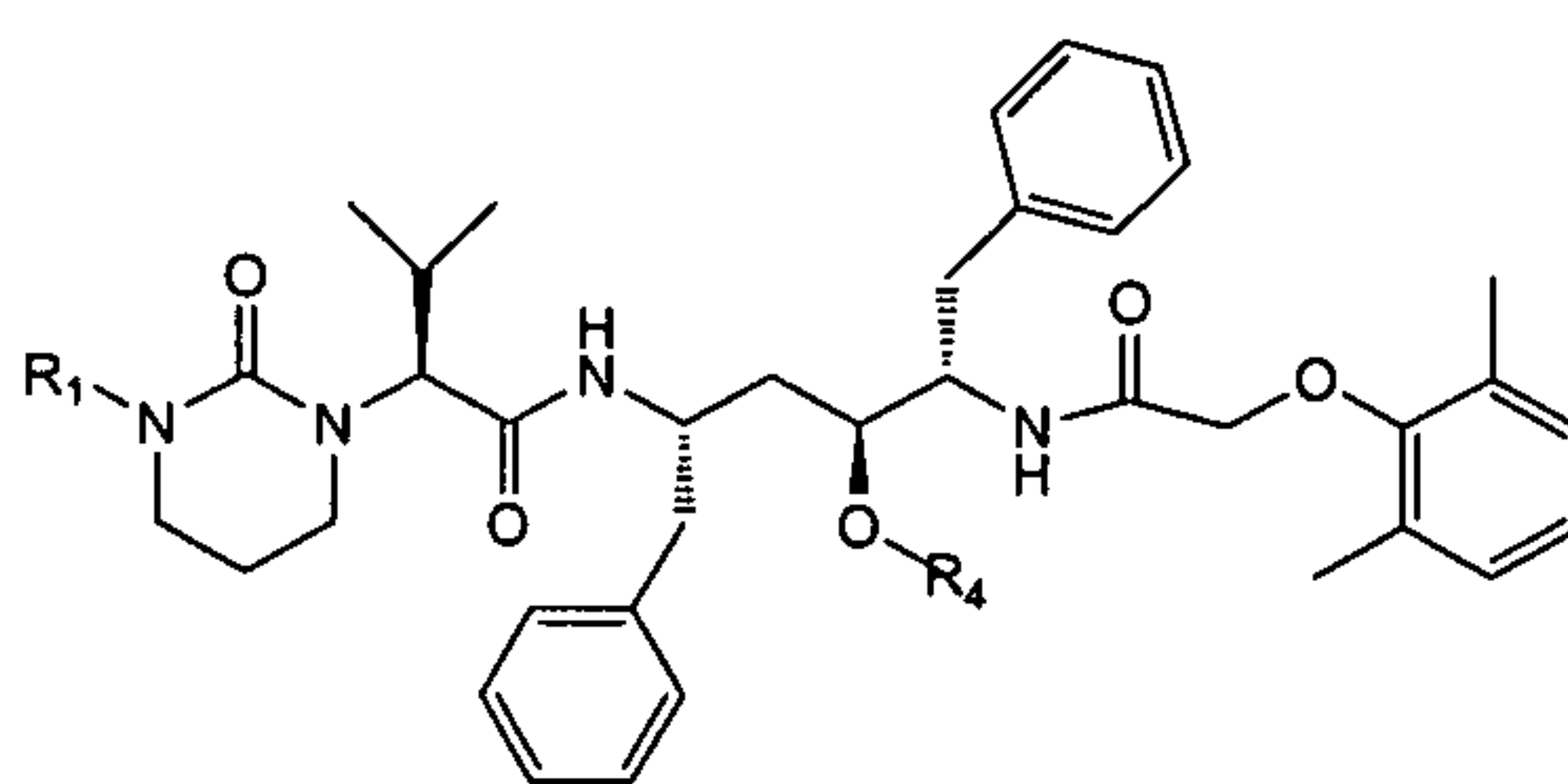
Methods are also provided for using the immunochemically equivalent analogs disclosed herein to establish or prepare a calibration curve for an HIV protease inhibitor or a non-nucleoside reverse transcriptase inhibitor for use in an immunoassay or as a positive control for use in immunochemically-based experiments. In accordance with one specific embodiment, a calibration curve for a protease inhibitor selected from the group consisting of amprenavir, atazanavir, tipranavir, indinavir, darunavir, lopinavir, nelfinavir, ritonavir, and saquinavir is prepared using the protease inhibitor analogs disclosed herein. The method of preparing such a calibration curve comprises preparing a series of samples comprising an immunochemically equivalent analog of the protease inhibitor, wherein the central hydroxyl group of the protease inhibitor has been replaced in the analog with a group selected from -O(C₁-C₁₀ alkyl), -OCONHR₃, -OCH₂CONHR₃, and -OCH₂COOR₃, wherein R₃ is selected from the group consisting of H, C₁-C₆ alkyl, phenyl, heteroaryl, and -(CH₂)_n-X wherein n is 1-6 and X is COOR₅ or NH-CH₂-phenyl-COOR₅ wherein R₅ is H or C₁-C₆ alkyl. Each sample is then contacted with a fixed amount of an antibody and a labeled conjugate of the HIV drug. A competition results between the labeled conjugate and the HIV drug analog for the limited number of antibody binding sites. After a specified reaction time, the amount of the labeled conjugate bound to the antibody is detected. (The skilled artisan will also recognize that, alternatively, the amount of labeled conjugate remaining free, i.e., unbound, can also be detected.) A calibration curve is then constructed which shows an inverse relationship between the signal from the labeled conjugate and the amount of drug analog present. Such calibration curves are substantially equivalent to curves generated from the parent drug.

In another specific embodiment, a method for establishing a calibration curve for the HIV non-nucleoside reverse transcriptase inhibitor efavirenz is provided. The method comprises the step of preparing a series of samples or set of calibrators comprising a range of concentrations of an immunochemically equivalent analog of said non-nucleoside reverse transcriptase inhibitor wherein the analog is N-alkylated with C₁-C₆ alkyl or (C₁-C₆ alkyl)OH. Each sample is then contacted with a fixed amount of an antibody and a conjugate comprising the NNRTI and a signal-generating moiety or label. A competition results between the labeled conjugate and the NNRTI drug analog for the limited number of antibody binding sites. After a specified reaction time, the labeled conjugate bound to the antibody is detected and measured. A calibration curve is then constructed which shows an inverse relationship between the signal from the labeled conjugate and the amount of drug analog present. Such calibration curves are substantially equivalent to curves generated using the parent drug.

Furthermore, test kits are provided for determining or measuring the amount of an HIV protease inhibitor or non-nucleoside reverse transcriptase inhibitor present in a sample. The kits in accordance with one embodiment comprise an antibody that specifically binds to an HIV protease inhibitor, and an

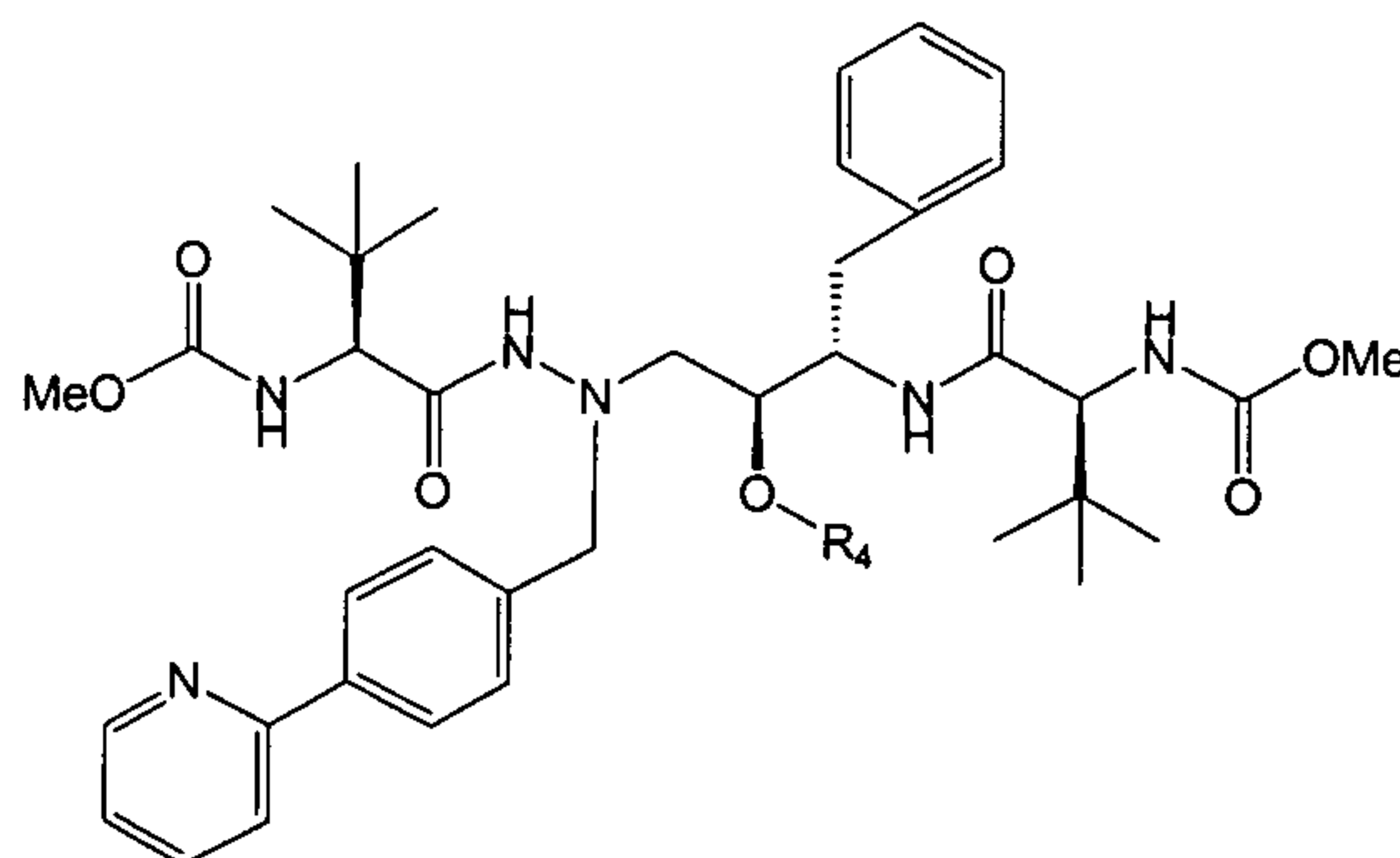
immunochemically equivalent analog of the protease inhibitor, wherein the central hydroxyl group of the protease inhibitor has been replaced in said analog with a group selected from the group consisting of -O(C₁-C₁₀ alkyl), -OCONHR₃, -OCH₂COOR₃, and -OCH₂CONHR₃, wherein R₃ is selected from the group consisting of H, C₁-C₆ alkyl, phenyl, heteroaryl, and -(CH₂)_n-X wherein n is 1-6 and X is COOR₅ or NH-CH₂-phenyl-COOR₅ wherein R₅ is H or C₁-C₆ alkyl.

More specifically, in one embodiment kits are provided for measuring lopinavir in a sample, the kit comprising an antibody that specifically binds lopinavir and an immunochemically equivalent lopinavir analog with the structure:



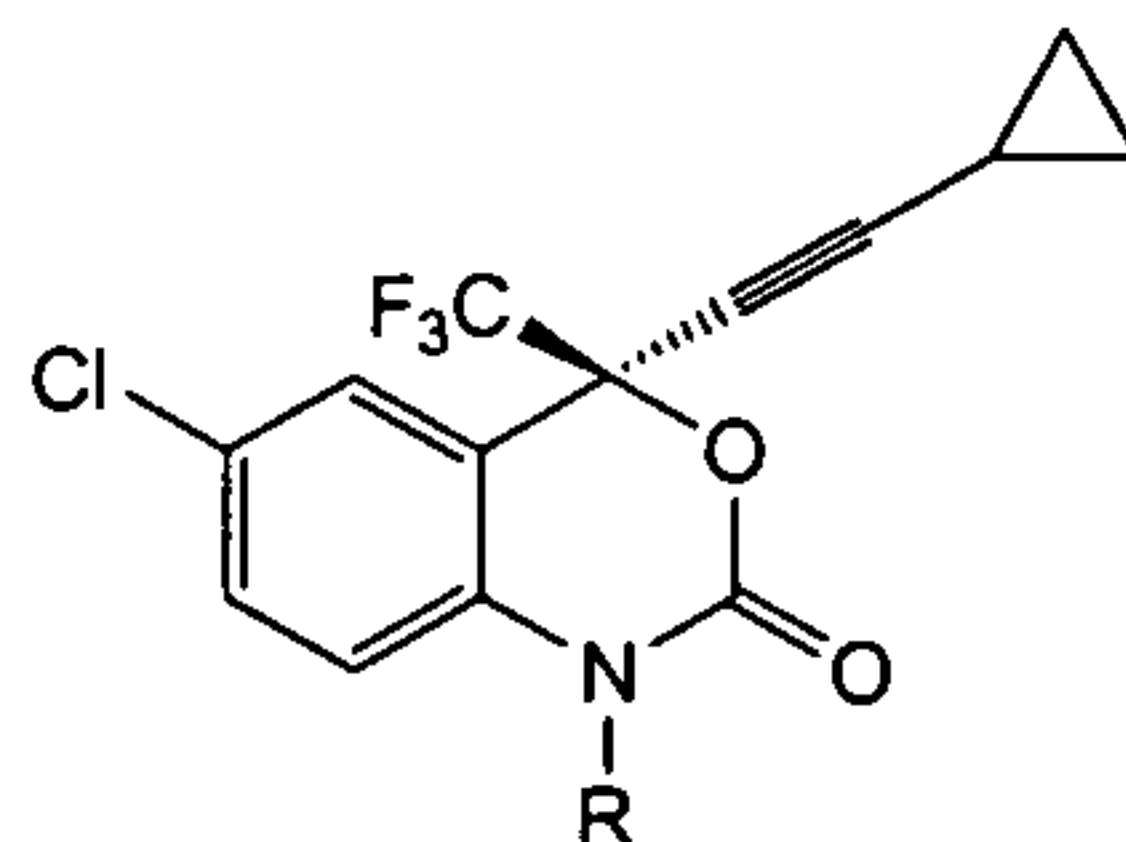
wherein R₁ is selected from the group consisting of H and -CONHR₃ and R₄ is selected from the group consisting of C₁-C₁₀ alkyl, -CH₂COOR₃, -CH₂CONHR₃ and -CONHR₃, wherein R₃ is selected from the group consisting of H, C₁-C₆ alkyl, phenyl, heteroaryl, and -(CH₂)_n-X wherein n is 1-6 and X is COOR₅ or NH-CH₂-phenyl-COOR₅ wherein R₅ is H or C₁-C₆ alkyl.

In another specific embodiment, a test kit is provided for measuring atazanavir in a biological sample, the kit comprising an antibody that specifically binds atazanavir and an immunochemically equivalent atazanavir analog having the structure:



wherein R₄ is selected from the group consisting of C₁-C₁₀ alkyl, -CH₂COOR₃, -CH₂CONHR₃ and -CONHR₃, wherein R₃ is selected from the group consisting of H, C₁-C₆ alkyl, phenyl, heteroaryl, and -(CH₂)_n-X wherein n is 1-6 and X is COOR₅ or NH-CH₂-phenyl-COOR₅ wherein R₅ is H or C₁-C₆ alkyl.

The kits in accordance with another specific embodiment comprise an antibody that specifically binds to the non-nucleoside reverse transcriptase inhibitor and an immunochemically equivalent analog of the non-nucleoside reverse transcriptase inhibitor wherein the analog is N-alkylated with C₁-C₆ alkyl or (C₁-C₆ alkyl)OH. In a specific embodiment, a kit is provided for measuring the amount of efavirenz in a sample
 5 comprising an antibody that specifically binds efavirenz and an efavirenz analog having the structure:



wherein R is C₁-C₆ alkyl or (C₁-C₆ alkyl)OH.

Brief description of the drawings

Figure 1 shows the structures for the various HIV protease inhibitor compounds discussed herein. An
 10 arrow points to the relevant central hydroxy group in each structure.

Figure 2 is a schematic representation of a synthetic scheme for the synthesis of lopinavir-O^c-methyl ether
 (2).

Figure 3 is a schematic representation of a synthetic scheme for the synthesis of lopinavir-O^c-
 (methoxycarbonylmethyl) ether (3).

Figure 4 is a schematic representation of a synthetic scheme for the synthesis of lopinavir-O^c-(N-*tert*-
 15 butylcarbamoylmethyl) ether (6).

Figure 5 is a schematic representation of a synthetic scheme for the synthesis of lopinavir-O^c carbamate
 (8).

Figure 6 is a schematic representation of a synthetic scheme for the synthesis of N-carbamoyl-lopinavir-
 20 O^c-carbamate (10).

Figure 7 is a schematic representation of a synthetic scheme for the synthesis of N-carbamoyl-lopinavir
 (13).

Figure 8 is a schematic representation of a synthetic scheme for the synthesis of N-alkylated efavirenz analogs 14, 15, 16, 16a, 17, and 17a.

Figure 9 is a schematic representation of a synthetic scheme for the synthesis of N-(hydroxypropyl)efavirenz (19) and of N-(hydroxyethyl)efavirenz (19a).

- 5 Figure 10 is a schematic representation of a synthetic scheme for the synthesis of atazanavir-O^c-ethylcarbamate (21), atazanavir-O^c-isopropylcarbamate (22), atazanavir-O^c-*tert*-butyl carbamate (23), atazanavir-O^c-carbamate (25), and atazanavir-O^c-(3-pyridyl)carbamate (41).

Figure 11 is a schematic representation of a synthetic scheme for the synthesis of atazanavir-O^c-methylether (26) and atazanavir-O^c-(hydroxyethyl)ether (28).

- 10 Figure 12a shows calibration curves for lopinavir antibody LPV 1.1.85 obtained using lopinavir and N-carbamoyl-lopinavir (13) as calibrators.

Figure 12b shows calibration curves for lopinavir antibody LPV 1.7.90 obtained using lopinavir and N-carbamoyl-lopinavir (13) as calibrators.

- 15 Figure 13 is a schematic representation of a synthetic scheme for the synthesis of atazanavir-O^c-carbonyl-aminobutyryl-aminomethylbenzoic acid NHS ester (33).

Figure 14 is a schematic representation of a synthetic scheme for the synthesis of 3-(2-iodo-acetamido)-propionic acid ethyl ester (34).

Figure 15 is a schematic representation of a synthetic scheme for the synthesis of 4-[3-(lopinavir-O^c)-acetamido]propionamido]methylbenzoic acid N-hydroxysuccinimide ester (40).

- 20 Figure 16 shows calibration curves for an atazanavir microparticle immunoassay using atazanavir and atazanavir-O^c-carbamate as calibrators.

Figure 17 shows a spiking recovery study of an atazanavir microparticle immunoassay using serum samples spiked with atazanavir and assayed against an atazanavir-O^c-carbamate calibration curve.

- 25 Figure 18 shows a spiking recovery study of a lopinavir microparticle immunoassay using serum samples spiked with lopinavir and assayed against an N-carbamoyl-lopinavir calibration curve.

Figure 19 is a schematic representation of a synthetic scheme for the synthesis of efavirenz aminodextran conjugate (45).

Detailed description of the invention

As used herein, the term "specific binding" refers to high avidity and/or high affinity binding between two
5 paired species including, for example, a ligand/target pair, an enzyme/substrate pair, a receptor/agonist pair, an antibody/antigen pair, and a lectin/carbohydrate pair. The binding interaction may be mediated by covalent or non-covalent interactions or a combination of covalent and non-covalent interactions.

As used herein, the term "antibody" refers to a polypeptide which is comprised of at least one binding
10 domain. An antibody binding domain is formed from the folding of variable domains of an antibody molecule to form three-dimensional binding spaces with an internal surface shape and charge distribution complementary to the features of an antigenic determinant of an antigen, which allows an immunological reaction with the antigen. Unless otherwise stated, a general reference to an antibody encompasses polyclonal as well as monoclonal antibodies. The term "antibody" also includes recombinant proteins comprising the binding domains, as well as fragments of antibodies, including Fab, Fab', F(ab)₂, and
15 F(ab')₂ fragments.

As used herein, the term "immunochemically equivalent" refers to two or more related compounds that bind to a single antibody with similar affinity, wherein the antibody was raised against, and specifically binds to, one member of an immunochemically equivalent group of compounds. More particularly, compounds that are immunochemically equivalent will cross-react with an antibody in an immunoassay
20 within a range of about 70% to about 130% of that achieved using the compound the antibody was raised against.

The term "C₁-C_n alkyl" represents a branched or linear alkyl group having from one to the specified number (n) of carbon atoms. Typical C₁-C₆ alkyl groups include, but are not limited to, methyl, ethyl, n-propyl, iso-propyl, butyl, iso-butyl, sec-butyl, tert-butyl, pentyl, hexyl, and the like.

25 The term "aryl" as used herein refers to a mono- or bicyclic carbocyclic ring system having one or two aromatic rings including, but not limited to, phenyl, naphthyl, tetrahydronaphthyl, indanyl, indenyl, and the like. Aryl groups (including bicyclic aryl groups) can be unsubstituted or substituted with one, two, or three substituents independently selected from lower alkyl, haloalkyl, alkoxy, amino, alkylamino, dialkylamino, hydroxyl, halo, and nitro. In addition, substituted aryl groups include tetrafluorophenyl and
30 pentafluorophenyl.

The term "heteroaryl" as used herein refers to a mono- or bicyclic ring system containing one or two aromatic rings and containing at least one nitrogen, oxygen, or sulfur atom in an aromatic ring. Heteroaryl groups (including bicyclic heteroaryl groups) can be unsubstituted or substituted with one, two, or three substituents independently selected from lower alkyl, haloalkyl, alkoxy, amino, alkylamino, dialkylamino, hydroxyl, halo, and nitro. Examples of heteroaryl groups include but are not limited to pyridine, pyrazine, pyrimidine, pyridazine, pyrazole, triazole, thiazole, isothiazole, benzothiazole, benzoxazole, thiadiazole, oxazole, pyrrole, imidazole, and isoxazole.

The term "heteroalkyl" as used herein refers to an alkyl chain that contains one or more nitrogen, oxygen, or sulfur atoms within the backbone of the alkyl chain.

It is noted that terms like "preferably", "commonly", and "typically" are not utilized herein to limit the scope of the claimed invention or to imply that certain features are critical, essential, or even important to the structure or function of the claimed invention. Rather, these terms are merely intended to highlight alternative or additional features that may or may not be utilized in a particular embodiment of the present invention.

For the purposes of describing and defining the present invention, it is noted that the term "substantially" is utilized herein to represent the inherent degree of uncertainty that may be attributed to any quantitative comparison, value, measurement, or other representation. The term "substantially" is also utilized herein to represent the degree by which a quantitative representation may vary from a stated reference without resulting in a change in the basic function of the subject matter at issue.

More particularly, the term "substantially equivalent", means that the result obtained for a particular sample in a set of calibrators, or the absorbance measured for a particular calibrator, or the curve generated from such result or absorbance measurement, is within the limits of accuracy of the assay. In particular, calibrators or standards prepared with an analog compound are considered to be substantially equivalent to calibrators or standards prepared using the parent drug if the slope of a spike and recovery curve obtained using the analog calibrators is within 10% of the ideal value, i.e., if the slope of a curve prepared using the parent drug is 1.0, then the slope of a spike and recovery curve prepared using analog calibrators should be 0.9 to 1.1.

As used herein, an "analog" of an HIV protease inhibitor or non-nucleoside reverse transcriptase inhibitor means a chemical compound which is derived from said HIV protease inhibitor or non-nucleoside reverse transcriptase inhibitor and which behaves substantially the same as the HIV protease inhibitor or non-

nucleoside reverse transcriptase inhibitor with respect to binding affinity of an antibody which specifically binds to said HIV protease inhibitor or non-nucleoside reverse transcriptase inhibitor.

The term “derivative” refers to a chemical compound or molecule made from a “parent” compound or molecule by one or more chemical reactions. In the context of the present invention, parent compounds
5 are HIV protease inhibitors and non-nucleoside reverse transcriptase inhibitors, and analogs are derivatives of the HIV protease inhibitors and non-nucleoside reverse transcriptase inhibitors, respectively.

As used herein, a “signal-generating moiety” is an identifying tag or label which, when attached to a carrier substance or molecule, can be used to detect an analyte such as an HIV protease inhibitor or a non-
10 nucleoside reverse transcriptase inhibitor. A label may be attached to its carrier substance directly or indirectly by means of a linking or bridging moiety. Examples of signal-generating moieties include enzymes such as β -galactosidase and peroxidase, fluorescent compounds such as rhodamine and fluorescein isothiocyanate (FITC), luminescent compounds such as dioxetanes and luciferin, radioactive isotopes such as ^{125}I , and microparticles.

15 Any sample that is reasonably suspected of containing an HIV protease inhibitor or non-nucleoside reverse transcriptase inhibitor of interest can be analyzed by the method of the present invention. The sample is typically an aqueous solution such as a body fluid from a host, for example, urine, whole blood, plasma, serum, saliva, semen, stool, sputum, cerebral spinal fluid, tears, mucus or the like, but preferably is plasma or serum. The sample can be pretreated if desired and can be prepared in any convenient
20 medium that does not interfere with the assay. An aqueous medium is preferred.

A “calibrator” means any standard or reference material containing a known amount of the HIV protease inhibitor or non-nucleoside reverse transcriptase inhibitor to be measured. A sample suspected of containing an HIV protease inhibitor or non-nucleoside reverse transcriptase inhibitor of interest and commonly a set of calibrators are assayed under similar conditions. Concentration of the HIV protease
25 inhibitor or non-nucleoside reverse transcriptase inhibitor in the sample is then determined by comparing the results obtained for the unknown sample with results obtained for a calibrator or set of calibrators. This is commonly done by constructing a calibration curve such as in Figures 12a and 12b.

A “positive control” is a sample containing a known amount of an HIV protease inhibitor or non-nucleoside reverse transcriptase inhibitor of interest. A sample suspected of containing an HIV protease
30 inhibitor or non-nucleoside reverse transcriptase inhibitor of interest and a positive control are assayed

under similar conditions, and the accuracy of the assay result obtained for the unknown sample is determined by the percent recovery observed in the positive control.

Abbreviations used herein for various HIV protease inhibitors are those that are now becoming the standard used in the field, e.g., ATV for atazanavir, LPV for lopinavir, and EFV for efavirenz. Certain
5 patent applications incorporated herein by reference may use older abbreviations for the same HIV protease inhibitors, e.g., ATZ for atazanavir, LOPIN for lopinavir, and EFA for efavirenz. However, the older and the newer abbreviations both refer to the same HIV protease inhibitor.

Another aspect of the present invention relates to test kits useful for establishing a calibration curve for an HIV protease inhibitor or non-nucleoside reverse transcriptase inhibitor and for performing an assay for
10 the determination of an HIV protease inhibitor or non-nucleoside reverse transcriptase inhibitor in a sample. Reagents useful in the methods of the invention can be conveniently provided in packaged combination, in the same or separate containers, in liquid or lyophilized form, so that the ratio of the reagents provides for substantial optimization of the method and assay. The reagents may each be in separate containers or various reagents can be combined in one or more containers depending on the
15 cross-reactivity and stability of the reagents. The test kits commonly include appropriate calibrators, controls, and instructions for use.

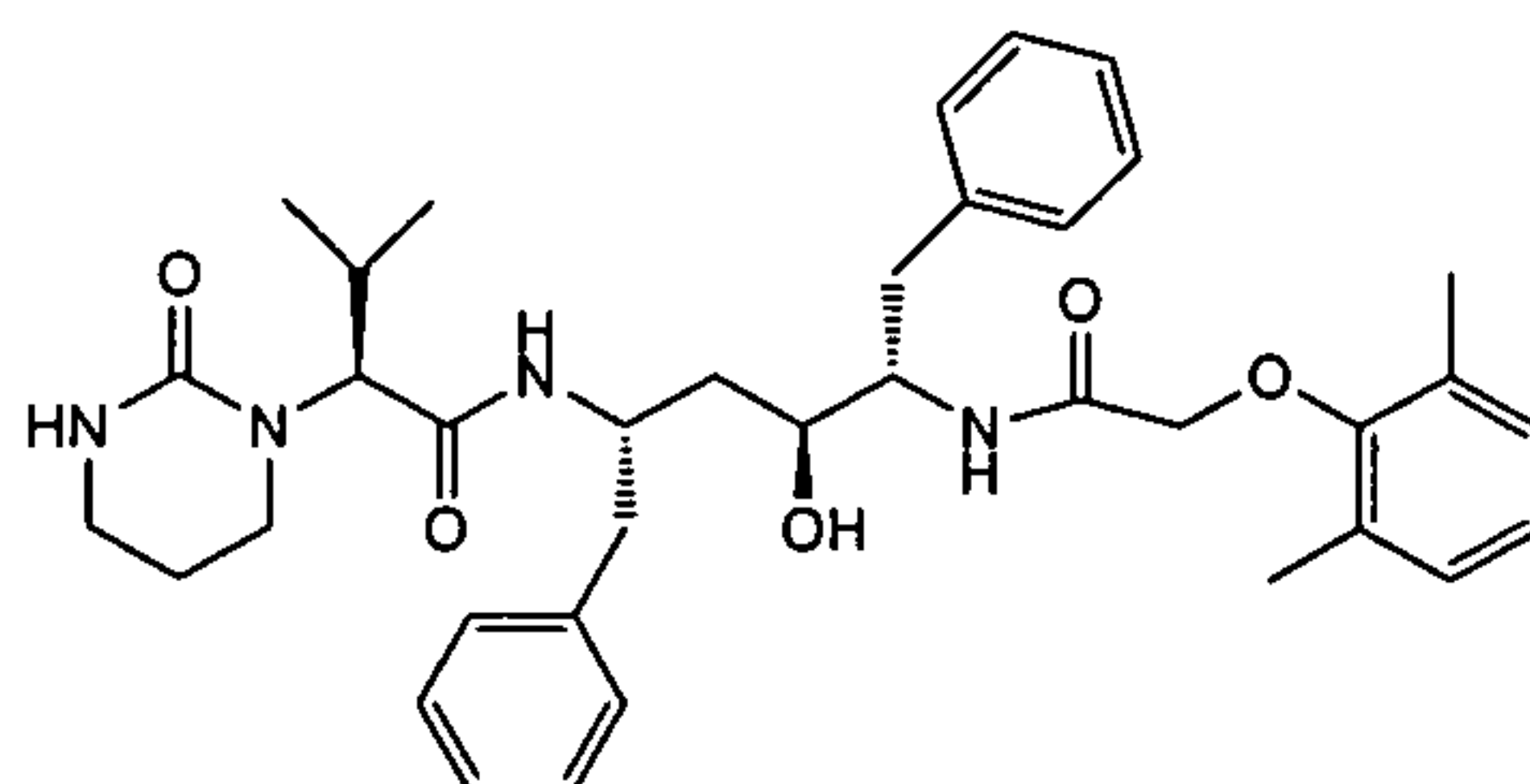
In accordance with one embodiment, analogs of HIV protease inhibitor drugs are provided that are immunochemically equivalent to the parent drug compound. More particularly, in one embodiment an HIV protease inhibitor analog is provided that is immunochemically equivalent to the parent protease
20 inhibitor such that it can be used as drug calibrator and/or positive control in antibody-based immunoassays. In one embodiment, an HIV protease inhibitor analog has a cross-reactivity in an immunoassay of about 80% to about 120% relative to that obtained with its parent compound. In a further embodiment, an HIV protease inhibitor analog has a cross-reactivity of about 90% to about 115% in an immunoassay relative to that obtained with its parent compound. In accordance with another embodiment,
25 an HIV protease inhibitor analog is provided wherein the protease inhibitor is selected from the group consisting of amprenavir, atazanavir, tipranavir, indinavir, darunavir, lopinavir, nelfinavir, ritonavir, and saquinavir, and wherein the central hydroxyl group of the protease inhibitor has been derivatized to form an ether or a carbamate.

In accordance with another embodiment, a protease inhibitor analog is provided comprising a protease
30 inhibitor selected from the group consisting of amprenavir, atazanavir, tipranavir, indinavir, darunavir, lopinavir, nelfinavir, ritonavir, and saquinavir wherein the inhibitor has been modified by replacing the

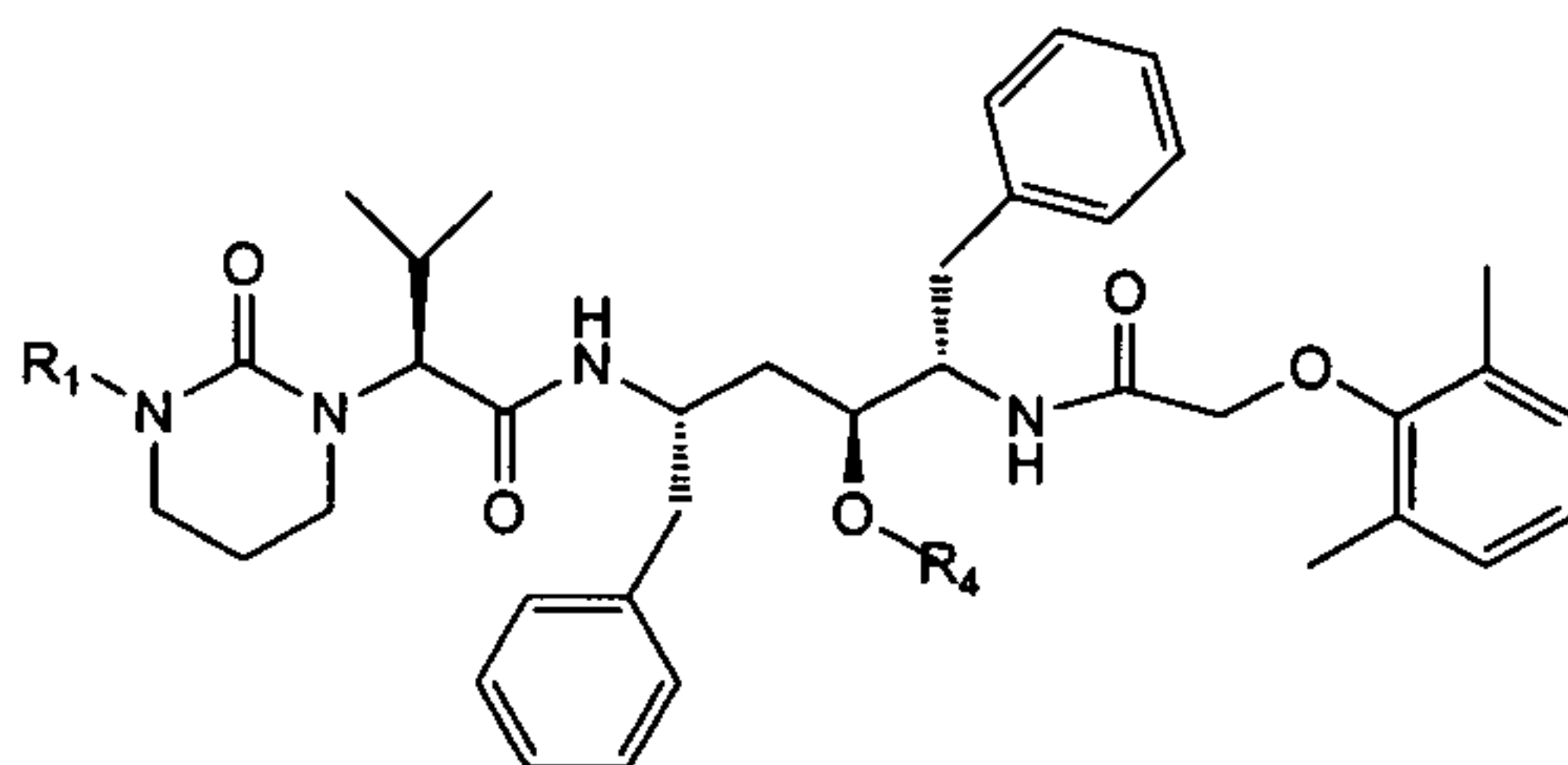
central hydroxyl group with $-O(C_1-C_{10} \text{ alkyl})$, $-\text{CH}_2\text{COOR}_3$, $-\text{CONHR}_3$, or $-\text{CH}_2\text{CONHR}_3$, wherein R_3 is selected from the group consisting of H, C_1-C_6 alkyl, phenyl, heteroaryl, and $-(\text{CH}_2)_n\text{-X}$ wherein n is 1-6 and X is COOR_5 or $\text{NH-CH}_2\text{-phenyl-COOR}_5$ wherein R_5 is H or C_1-C_6 alkyl.

In accordance with one embodiment, immunochemically equivalent analogs of lopinavir are provided.

5 The structure of lopinavir is:



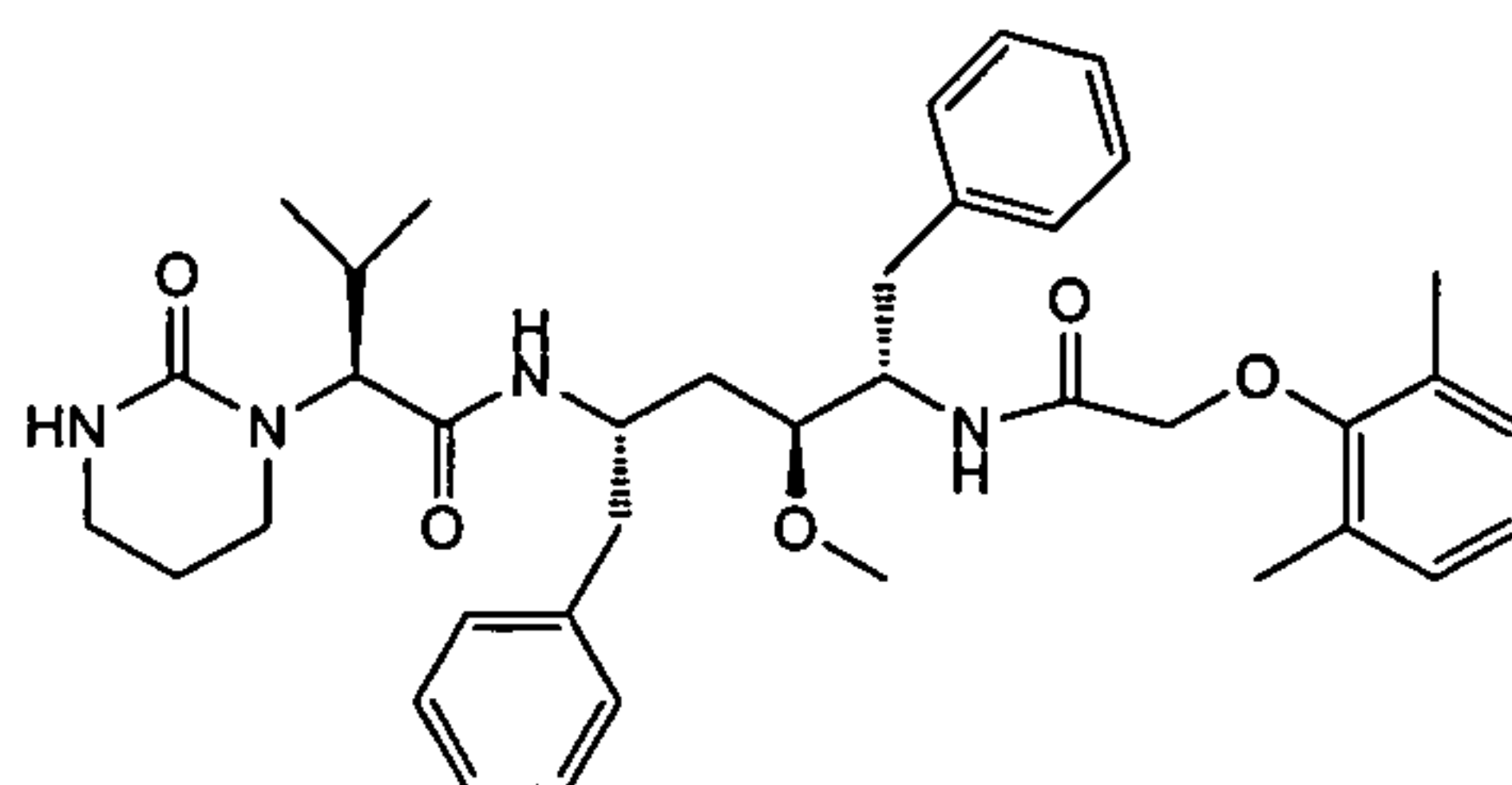
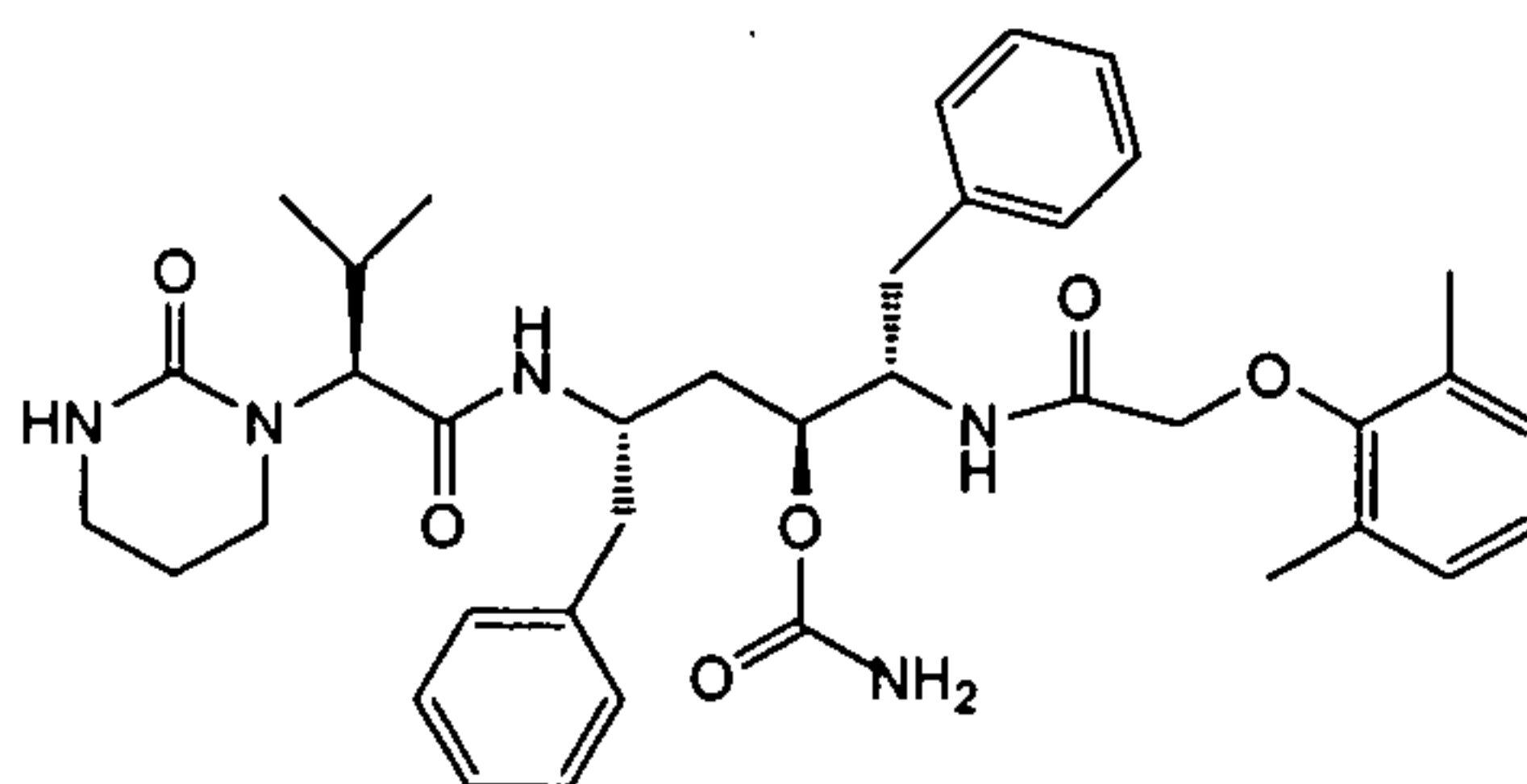
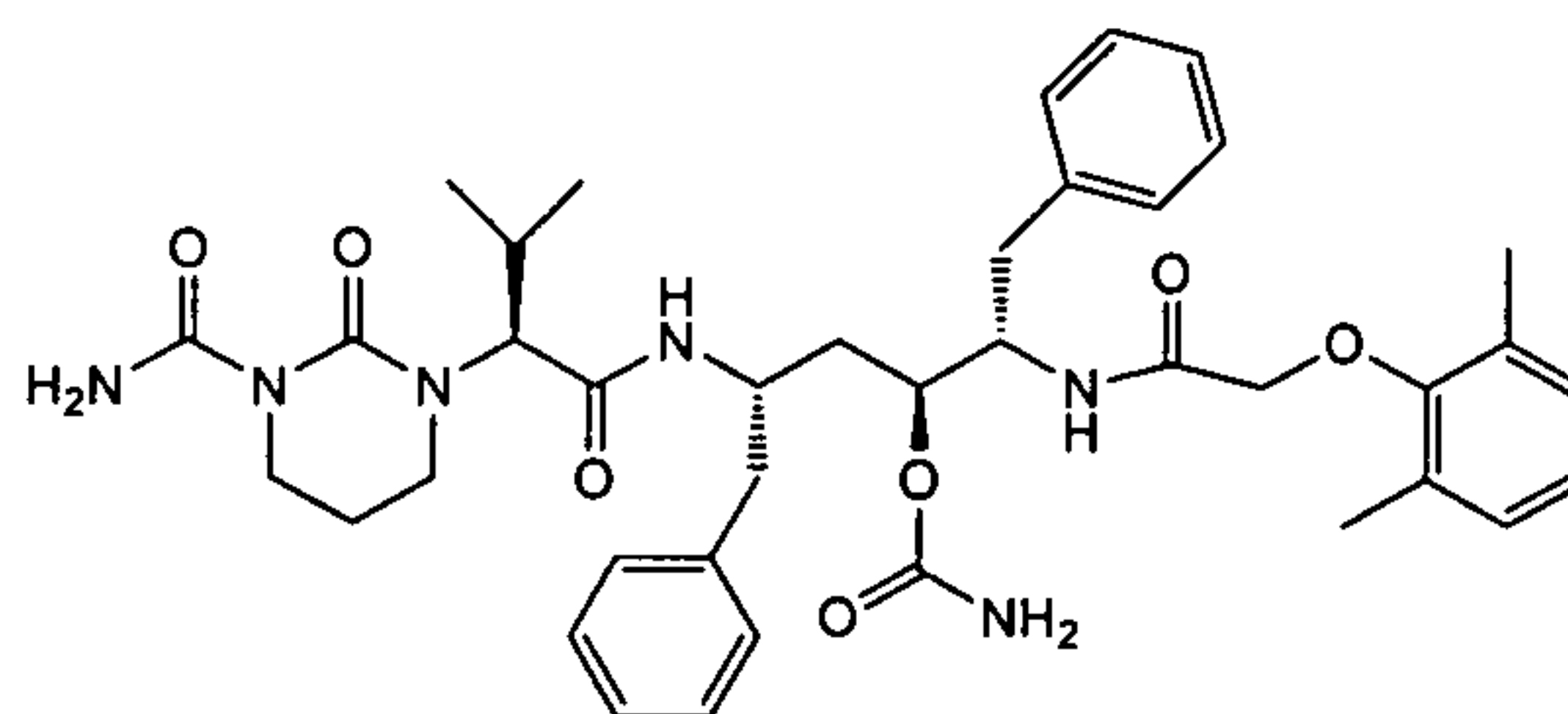
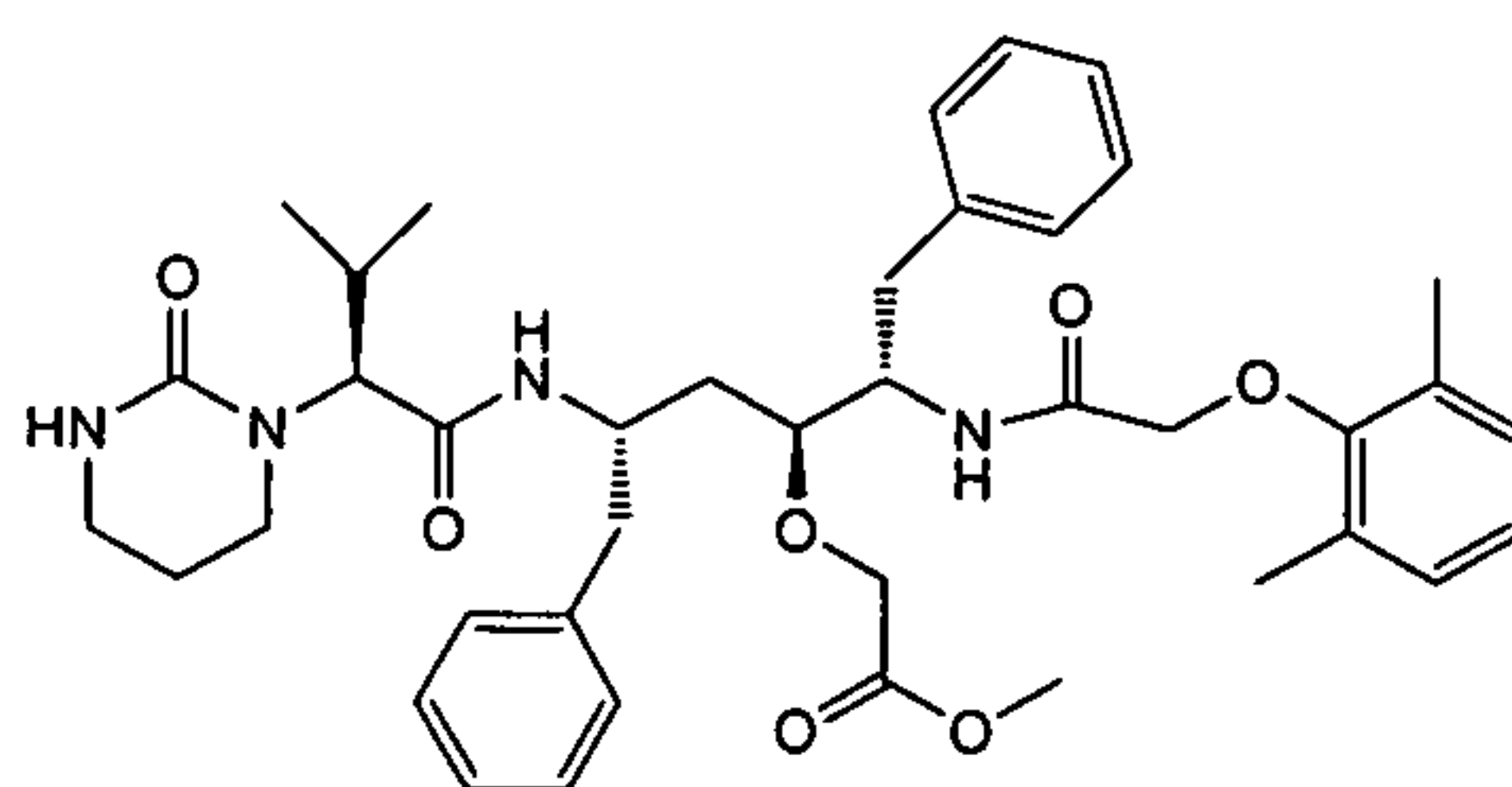
In accordance with one embodiment, an immunochemically equivalent analog of lopinavir is provided wherein the central hydroxyl group of lopinavir has been derivatized to form ethers or carbamates and/or an N-carbamate group has been added to the terminal cyclic urea. In accordance with one embodiment,
10 the immunochemically equivalent lopinavir analog has the structure:

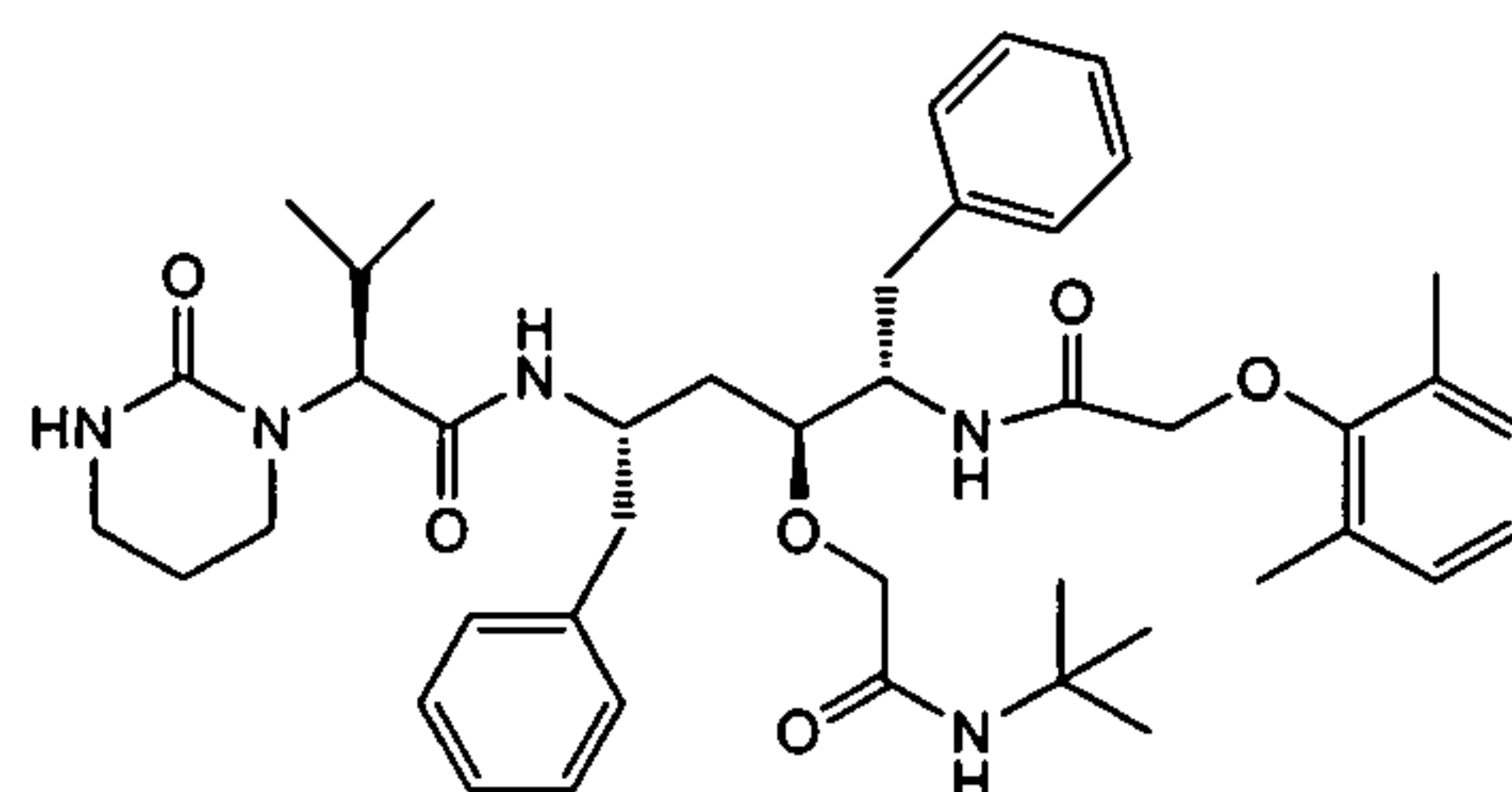
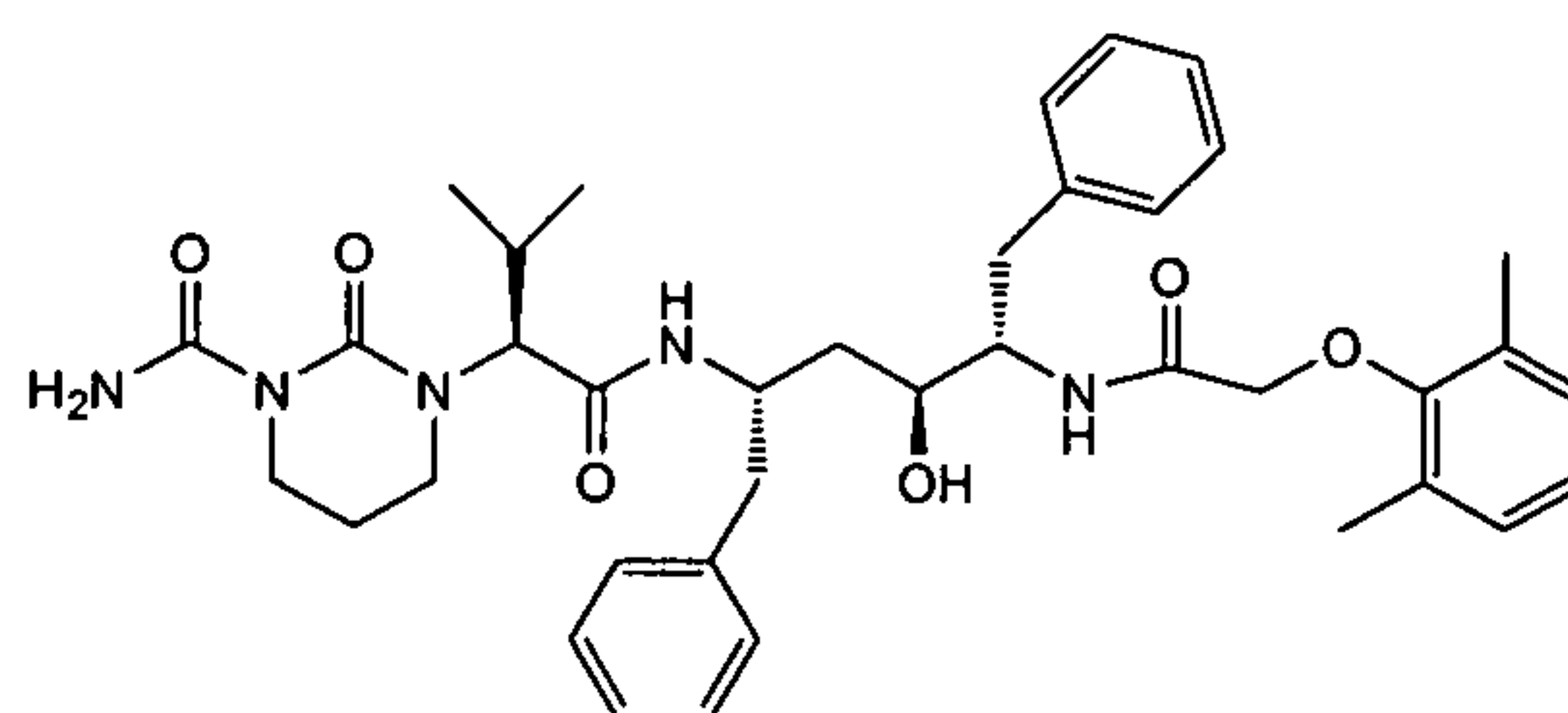


wherein R_1 is selected from the group consisting of H and $-\text{CONHR}_3$ and R_4 is selected from the group consisting of C_1-C_{10} alkyl, $-\text{CH}_2\text{COOR}_3$, $-\text{CH}_2\text{CONHR}_3$ and $-\text{CONHR}_3$, wherein R_3 is selected from the group consisting of H, C_1-C_6 alkyl, phenyl, heteroaryl, and $-(\text{CH}_2)_n\text{-X}$ wherein n is 1-6 and X is COOR_5 or
15 $\text{NH-CH}_2\text{-phenyl-COOR}_5$ wherein R_5 is H or C_1-C_6 alkyl.

Specific examples of lopinavir analogs suitable for use as a drug calibrator and/or positive control for antibody-based assays for lopinavir include the following compounds:

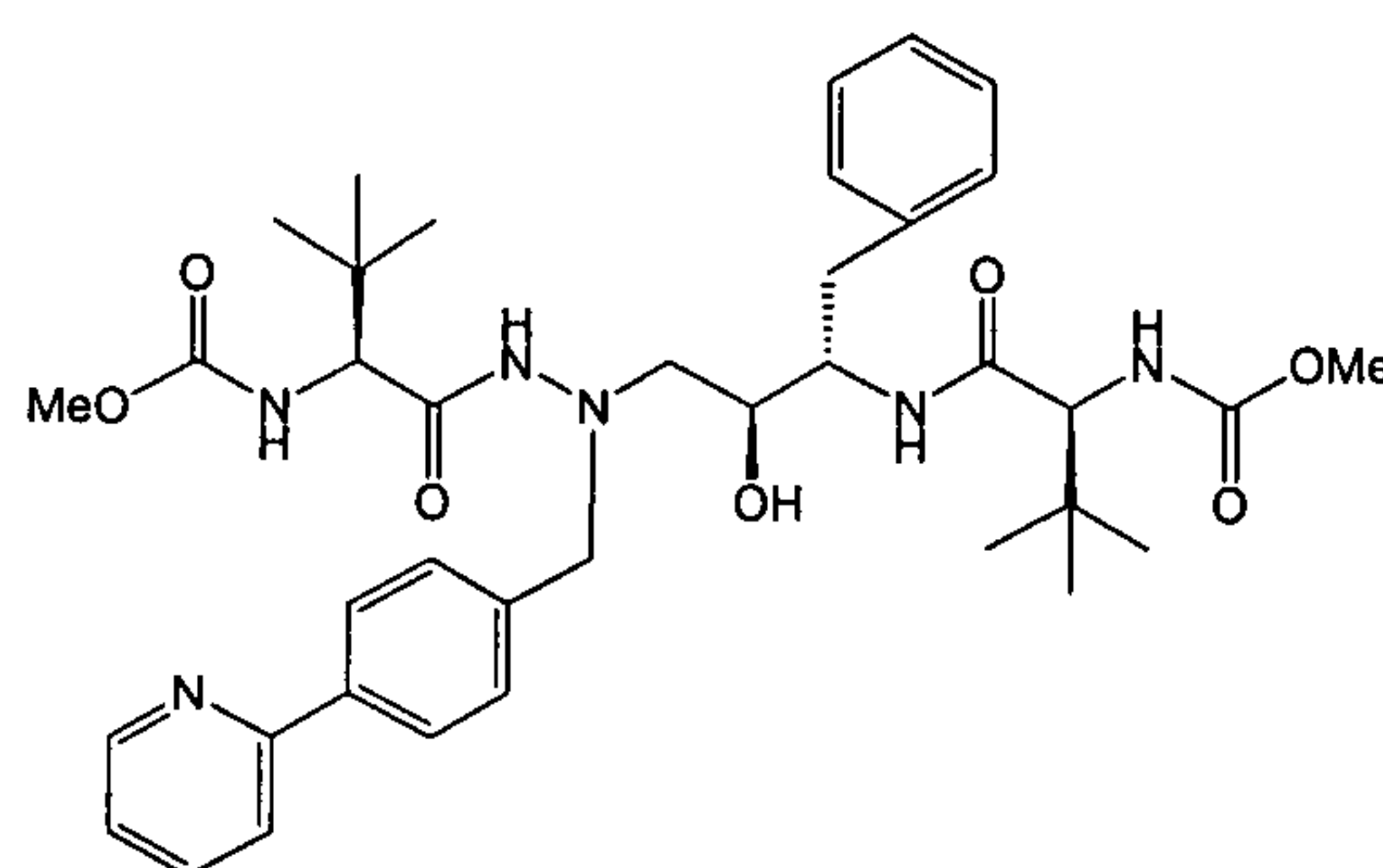
23814 WO

lopinavir-O^c-methylether (**2**)lopinavir-O^c-carbamate (**8**)N-carbamoyl-lopinavir-O^c-carbamate (**10**)lopinavir-O^c-(methoxycarbonylmethyl)ether (**3**)

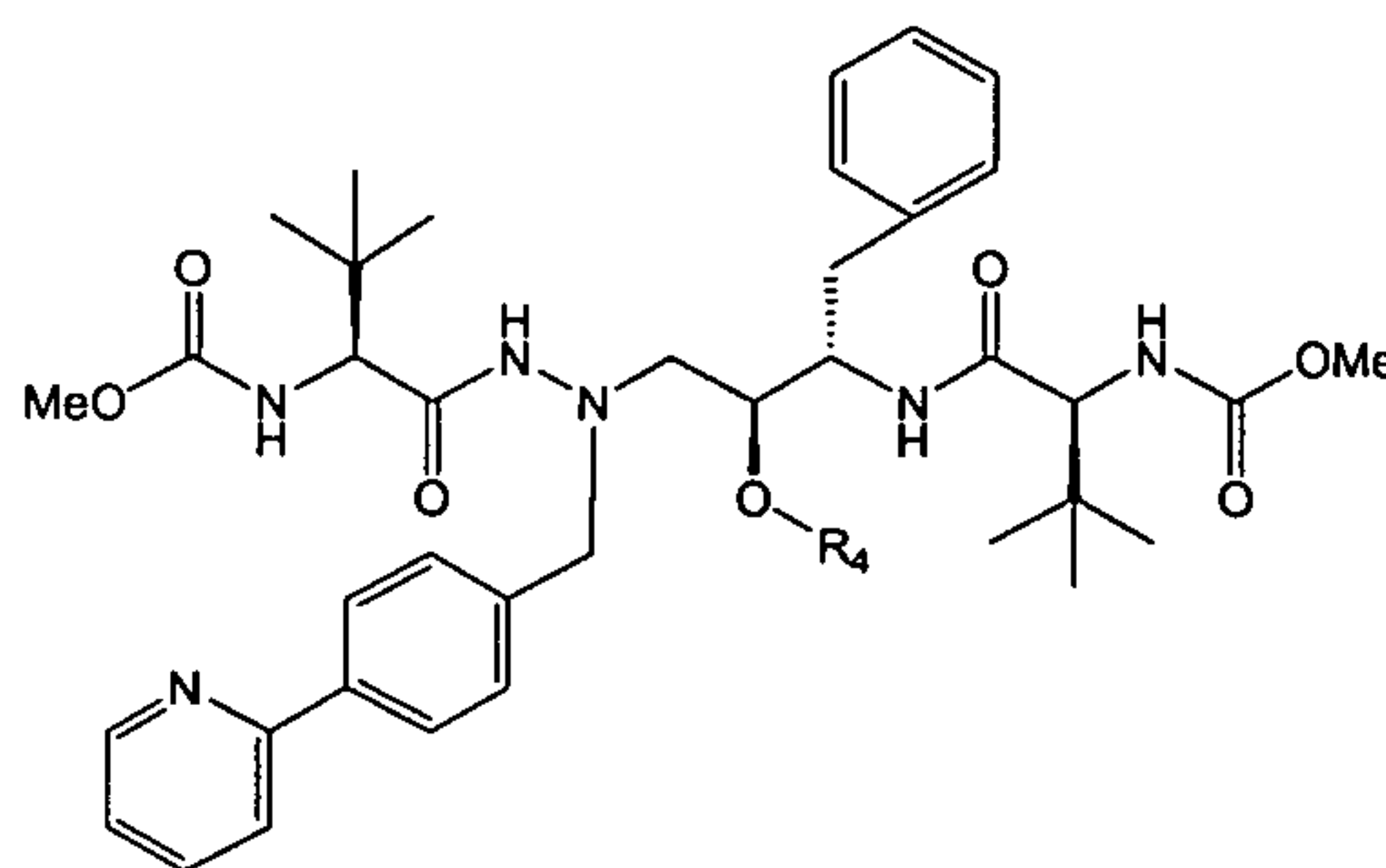
lopinavir-O^c-(N-*tert*-butylcarbamoylmethyl) ether (6)N-carbamoyl-lopinavir (13)

- 5 In accordance with one embodiment, immunochemically equivalent analogs of atazanavir are provided.

The structure of atazanavir is:

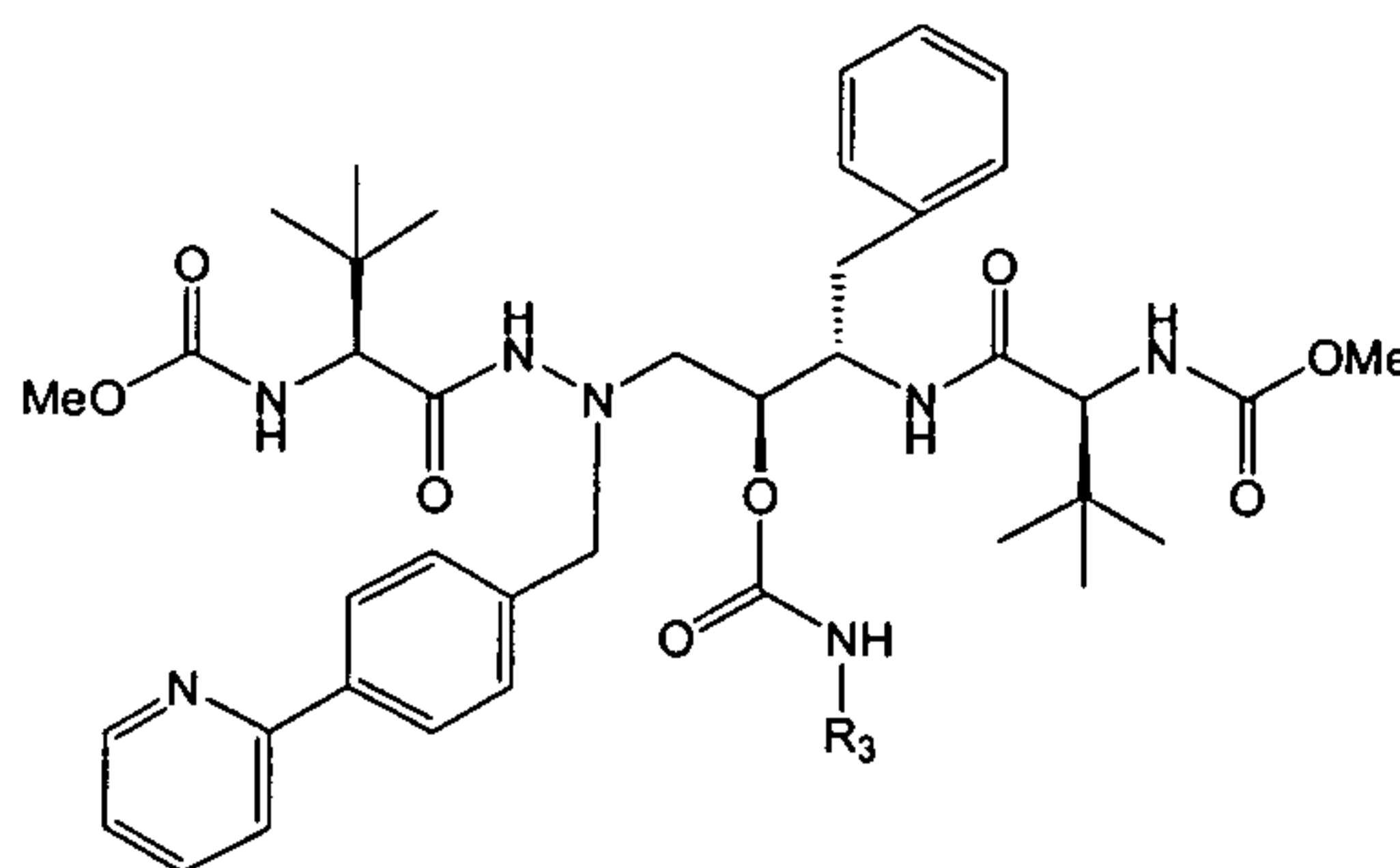


In accordance with one embodiment, an immunochemically equivalent analog of atazanavir is provided having the structure:



wherein R_4 is selected from the group consisting of C_1 - C_{10} alkyl, $-CH_2COOR_3$, $-CH_2CONHR_3$, and $-CONHR_3$, wherein R_3 is selected from the group consisting of H, C_1 - C_6 alkyl, phenyl, heteroaryl, and $-(CH_2)_n-X$ wherein n is 1-6 and X is $COOR_5$ or $NH-CH_2$ -phenyl- $COOR_5$ wherein R_5 is H or C_1 - C_6 alkyl. In another embodiment, R_4 is selected from the group consisting of $-CH_2CONHR_3$ and $-CONHR_3$, wherein R_3 is H or C_1 - C_4 alkyl. In another embodiment, R_4 is $-CONHR_3$, wherein R_3 is H or CH_2CH_3 .

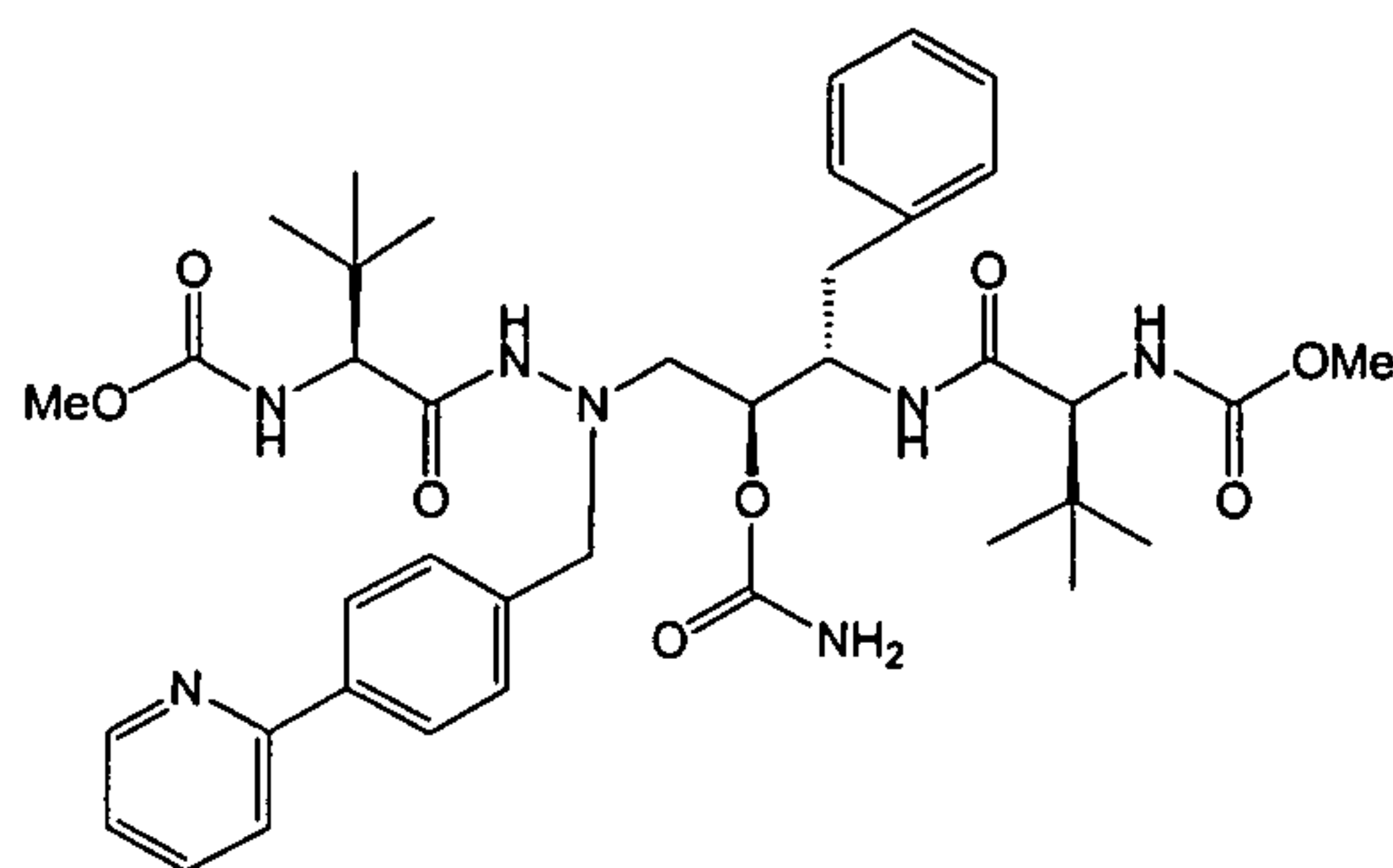
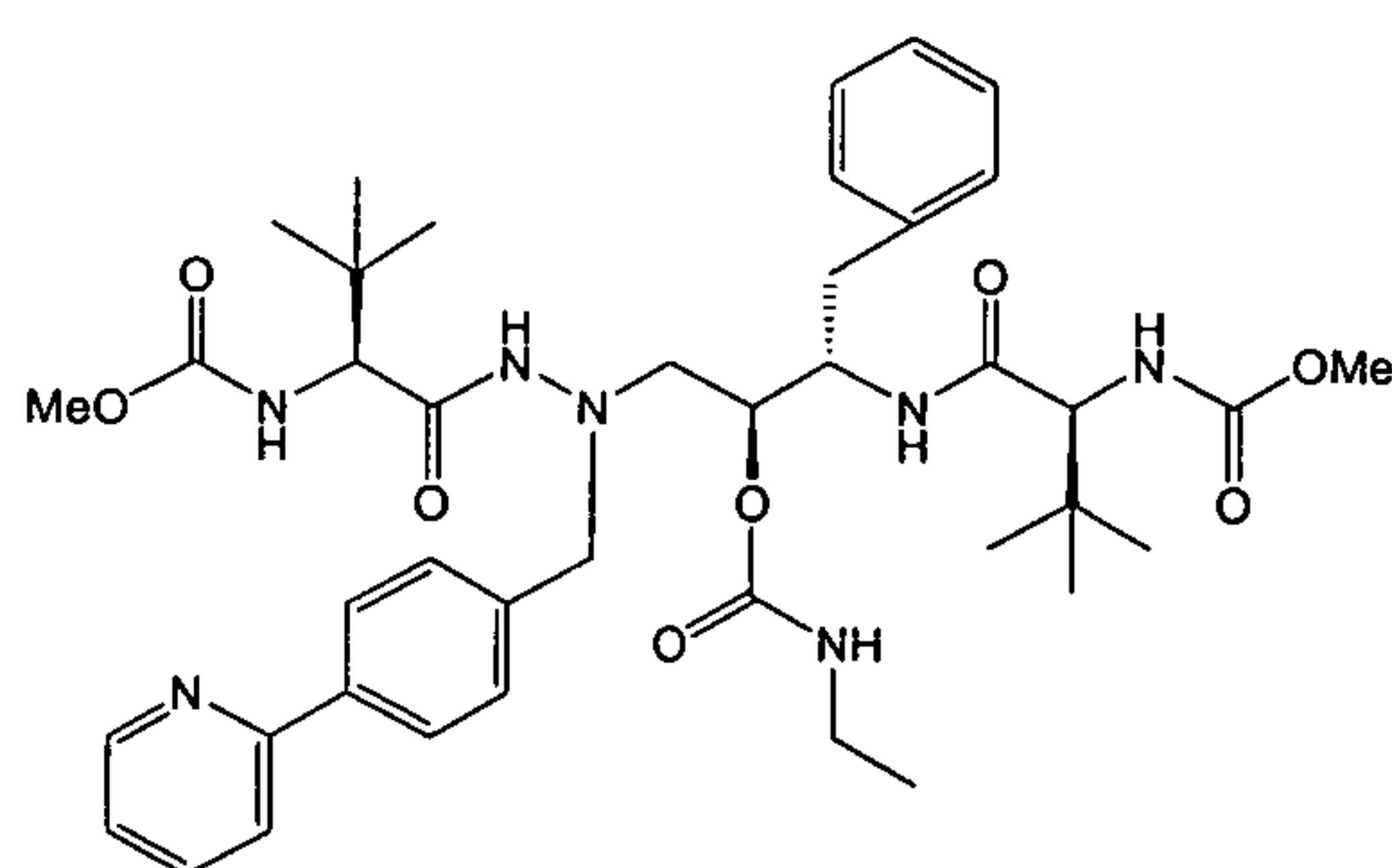
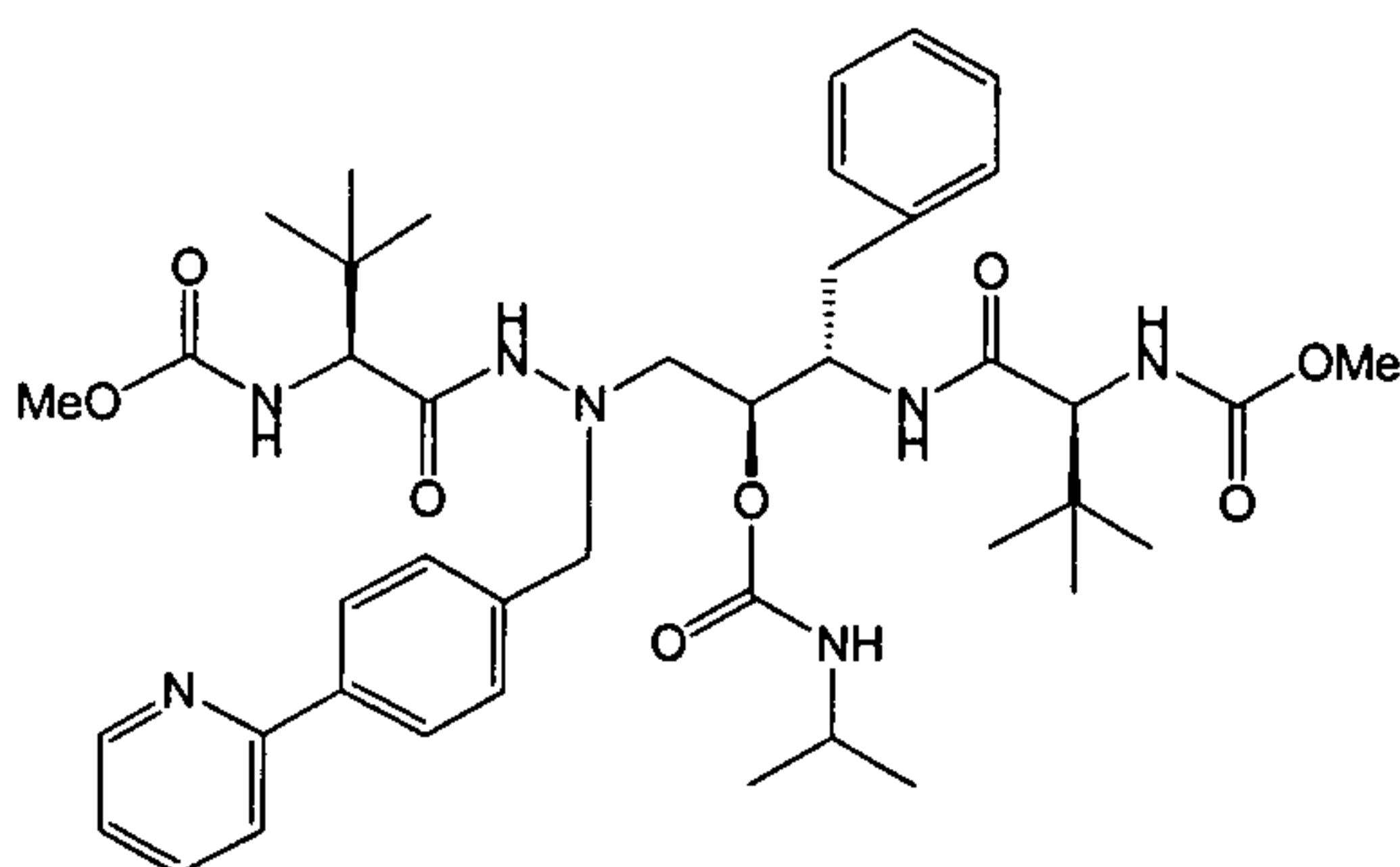
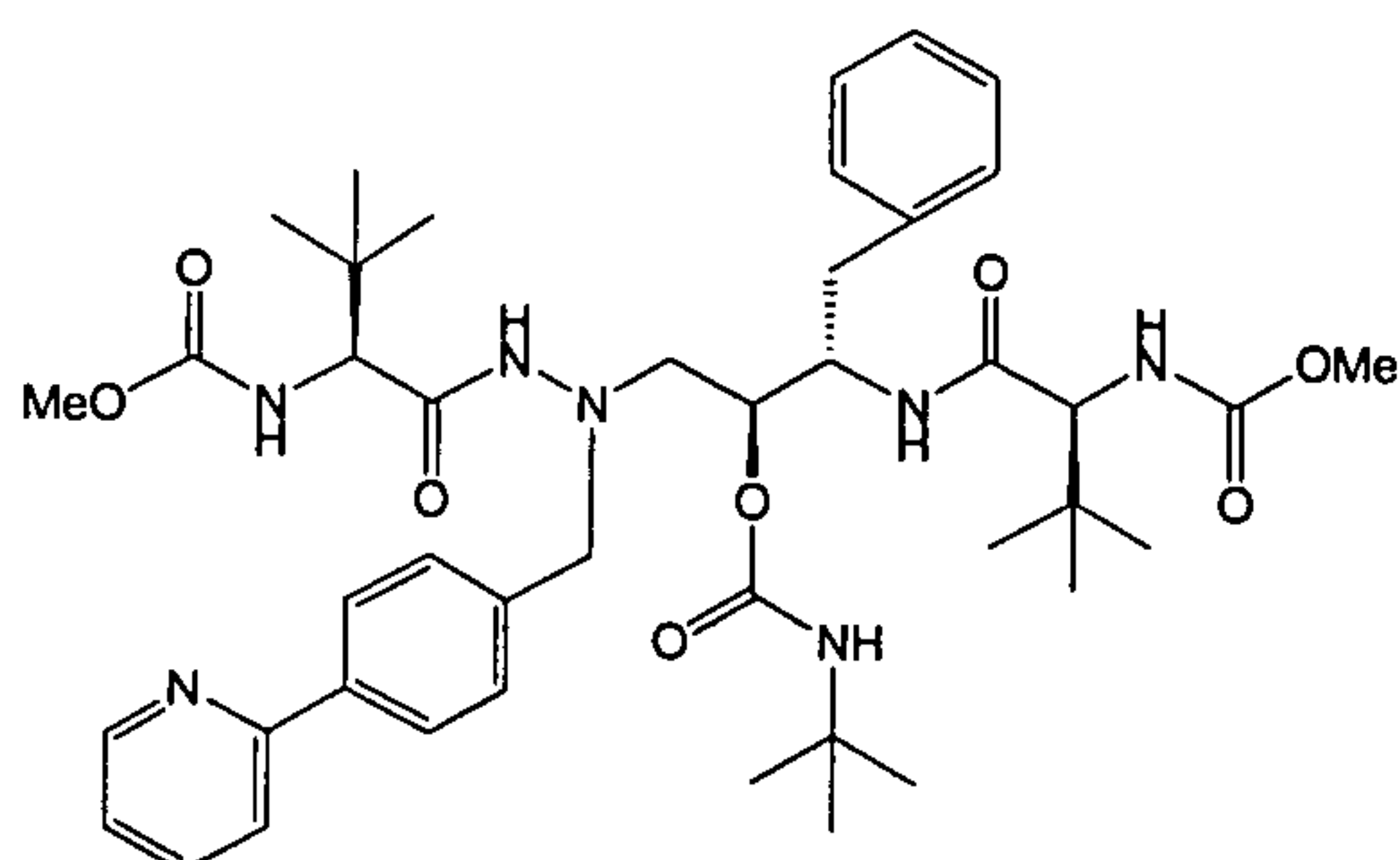
In accordance with one embodiment, an immunochemically equivalent analog of atazanavir is provided wherein the central hydroxyl group of atazanavir has been derivatized to form a carbamate. In accordance with one embodiment the atazanavir immunochemically equivalent analog has the structure:



wherein R_3 is selected from the group consisting of H, C_1 - C_6 alkyl, phenyl, heteroaryl, and $-(CH_2)_n-X$ wherein n is 1-6 and X is $COOR_5$ or $NH-CH_2$ -phenyl- $COOR_5$ wherein R_5 is H or C_1 - C_6 alkyl.

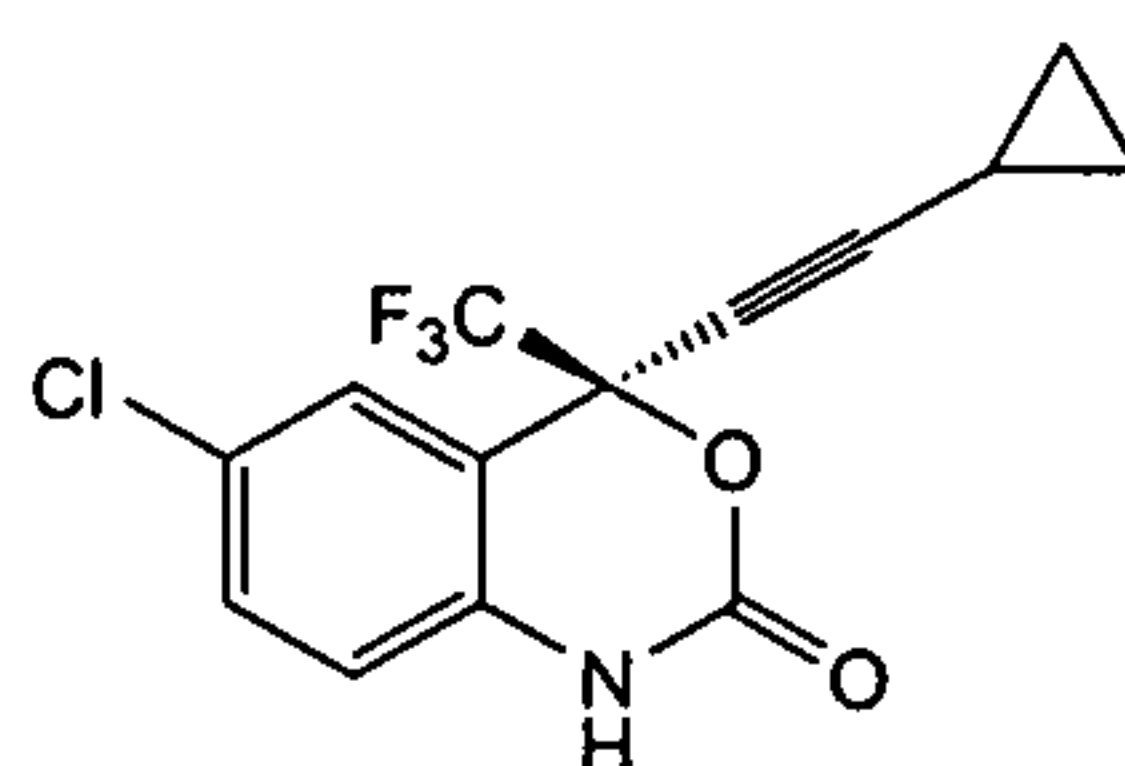
Specific examples of atazanavir analogs suitable for use as a calibrator or positive control in an antibody-based assay for determining atazanavir include the following compounds:

23814 WO

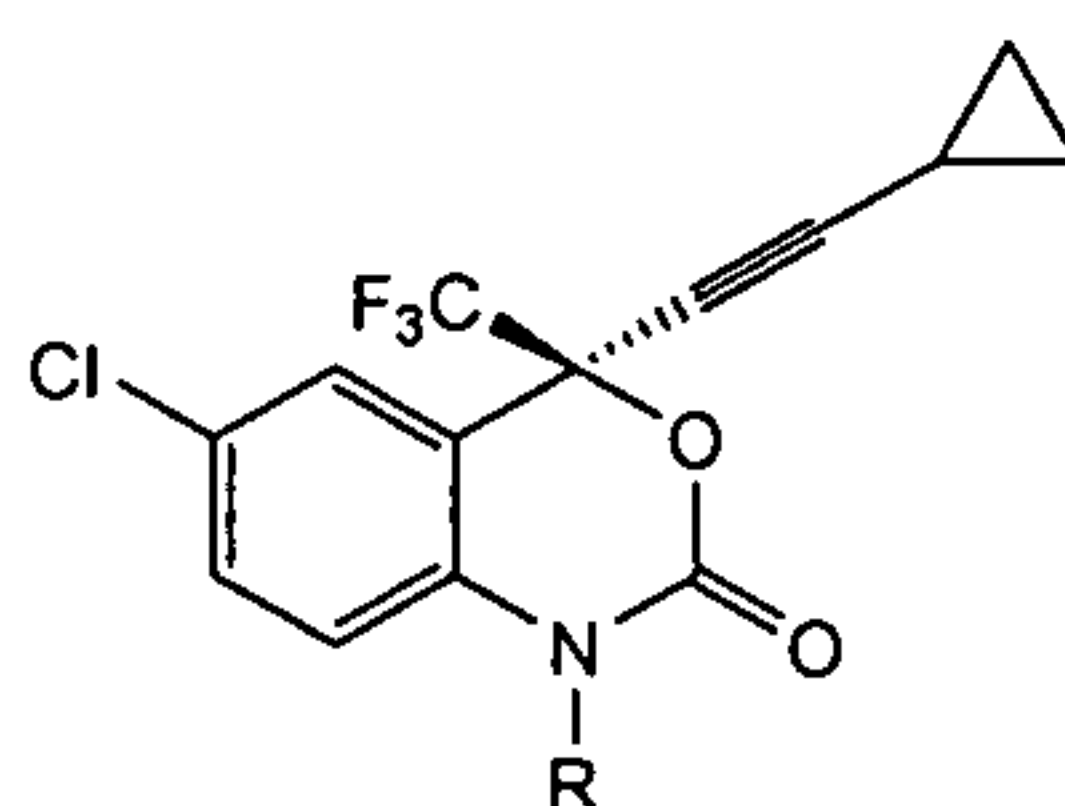
atazanavir-O^c-carbamate (**25**)atazanavir-O^c-ethylcarbamate (**21**)atazanavir-O^c-isopropylcarbamate (**22**)

atazanavir-O^c-*tert*butylcarbamate (**23**)

In accordance with one embodiment, immunochemically equivalent analogs of non-nucleoside reverse transcriptase inhibitors (NNRTIs) are provided. More particularly, in one embodiment an NNRTI analog is provided that is immunochemically equivalent to the parent NNRTI such that it can be used as a calibrator and/or positive control in antibody-based immunoassays for determining the NNRTI in a sample. In one embodiment, an NNRTI analog has a cross-reactivity of about 80% to about 120% in an immunoassay relative to that obtained with the parent NNRTI compound. In a further embodiment, an NNRTI analog has a cross-reactivity of about 90% to about 115% in an immunoassay relative to that obtained with the parent NNRTI. In accordance with one embodiment, the NNRTI analog is efavirenz. In accordance with one embodiment, N-alkylated analogs of efavirenz are provided. The structure of efavirenz is:



Specific examples of efavirenz analogs suitable for use as a drug calibrator and/or positive control in antibody-based assays for determining efavirenz in a sample include the following compounds:



R = CH₃ (**14**)

R = CH₂CH₃ (**15**)

R = CH₂CH₂CH₃ (**16**)

R = CH (CH₃)₂ (**16a**)

R = (CH₂)₃CH₃ (**17**)

R = CH₂CH(CH₃)₂ (**17a**)

R = (CH₂)₃OH (**19**)

R = (CH₂)₂OH (**19a**)

The present invention also encompasses test kits containing assay reagents for measuring HIV protease inhibitors and non-nucleoside reverse transcriptase inhibitors in samples. The kit may further include, in packaged combination, a variety of containers, e.g., vials, tubes, bottles, and the like containing reagents used to perform the assay. Preferably, the kits will also include instructions for use. In one embodiment, the test kit comprises an antibody that specifically binds to an HIV drug to be detected in a sample and an immunochemically equivalent analog of the HIV drug to be detected. In accordance with one embodiment, a kit comprises an antibody that specifically binds lopinavir and the lopinavir analog lopinavir-O^c-(N-*tert*-butylcarbamoylemethyl) ether (**6**). In another embodiment, a kit comprises a lopinavir antibody and the lopinavir analog N-carbamoyl-lopinavir (**13**). In accordance with one embodiment, a kit comprises an antibody that specifically binds atazanavir and the analog atazanavir-O^c-carbamate (**25**). In another embodiment, a kit comprises an antibody specific for atazanavir and the analog atazanavir-O^c-ethylcarbamate (**21**). Accordingly, the kits disclosed herein can be used to establish or generate a calibration curve for the HIV protease inhibitor or non-nucleoside reverse transcriptase inhibitor to be detected or measured using the drug analog supplied therein. The analog may or may not have the inhibitory activity of the parent drug; however, since the analog is immunochemically equivalent to the parent drug, the calibration curve established using the drug analog can be used to determine the concentration of the drug in a given sample based on the detected signal from the immunoassay.

These protease inhibitor and NNRTI analogs and derivatives may be synthesized using methods described below as well as with methods known to those who are skilled in the art. For lopinavir, in addition to a central hydroxyl group, the molecule also contains an acidic nitrogen atom on the cyclic tetrahydropyrimidinone moiety. Selective modification at the nitrogen requires protection of the central hydroxyl group. Many suitable protecting groups are well known in the art. See, for example, "Protective Groups in Organic Synthesis", 2nd edition, T. Greene and P. Wuts, Wiley-Interscience, 1991.

The central hydroxyl group can be protected as a silyl protecting group, e.g., a TBDMS (t-butyltrimethylsilyl) or TBDPS (t-butylphenylsilyl) group. An ester group can also be used to protect the central hydroxyl functionality of lopinavir, preferably an acetate group in the presence of a base, preferably pyridine (see compound **11** in Figure 7). Protection of the central hydroxyl group of lopinavir is carried out in a solvent such as dimethylformamide (DMF), preferably tetrahydrofuran (THF), for a time which typically ranges from 0.5 h to 7 days. After protection of the central hydroxyl group, the acidic nitrogen on the cyclic tetrahydropyrimidinone moiety can be modified using a protected isocyanate, most preferably trichloroacetyl isocyanate. Trichloroacetyl isothiocyanate can also be used to prepare a lopinavir N-thiocarbamoyl analog. Hydrolysis under basic conditions deprotects both the central

acetate group as well as the protected N-carbamoyl group to give the desired lopinavir N-carbamoyl analog (compound 13 in Figure 7).

In another embodiment, a di-substituted lopinavir analog, protected N-carbamoyl lopinavir O^c-carbamate has been prepared by reaction of lopinavir with an excess of protected isocyanate reagent, preferably
5 trichloroacetyl isocyanate. Hydrolysis under basic conditions, preferably using aqueous potassium carbonate, provides the desired N-carbamoyl lopinavir O^c-carbamate (compound 10 in Figure 6).

In another embodiment, lopinavir protected O^c-carbamate has been prepared by reaction of lopinavir with a limited amount of trichloroacetyl isocyanate. Hydrolysis under basic conditions, preferably using aqueous potassium carbonate, provides the desired lopinavir O^c-carbamate (compound 8 in Figure 5).

10 In another embodiment, the central hydroxyl group of lopinavir can be modified by an ether linkage. A variety of halo alkylating agents can be used to modify the central hydroxyl functionality of lopinavir under controlled conditions, e.g., using a limited amount of an alkylating agent in the presence of a limited amount of a base, preferably sodium hydride in a solvent, preferably DMF at a temperature ranging from 0°C to room temperature. See lopinavir analogs, compound 2 in Figure 2 and compound 3
15 in Figure 3.

Hydrolysis of an ester group of a lopinavir analog can be achieved under acidic or basic conditions, preferably under basic conditions, e.g., lithium hydroxide at 0°C to room temperature (Figure 4). The resulting acidic functionality can be activated by using a variety of activating agents, preferably 1-(3-(dimethylamino)propyl)-3-ethyl-carbodiimide (EDC) or N-hydroxysuccinimide. The resulting activated
20 ester can be reacted with an amine, preferably t-butylamine to give lopinavir t-butylamide analog (compound 6 in Figure 4).

Atazanavir also possesses the central hydroxyl group found in other protease inhibitors, but in atazanavir, the group has a reactivity that is influenced by its specific structural attributes such as, but not limited to, the sterically demanding phenyl-pyridyl moiety in close proximity to it. Substitution at the hydroxyl
25 group may be made to furnish alkylated analogs such as methyl, ethyl, or alkyl analogs. Such substitution may be carried out by reaction with halogenated alkyls, with or without other suitably protected functionalities present on the alkyl moiety, in the presence of catalysts or mediating reagents such as transition metal salts, e.g. rhodium salts or silver oxide, in a suitable solvent such as DMF, THF, and the like, at temperatures typically between room temperature and about 100°C. Alkylating agents such as
30 suitably protected α -haloacetates, alkyl azides, azidoacetates, azido alkanoates, diazoacetates, diazoalkanoates, alkyl tosylates, and the like may be considered, while other reagents will be suggested to

one skilled in the art. For example, reaction in a solvent such as DMF in the presence of silver oxide with an alkyl halide such as methyl iodide affords the O^c-methyl ether 26 as shown in Example 20; and reaction with a tetrahydropyran-protected bromoethanol affords the O^c-ethylene alcohol 28 as described in Example 21. Less preferably, bases such as metal hydrides may also be used to deprotonate the hydroxyl group followed by reaction with a suitable alkylating reagent such as an alkyl halide, azide, tosylates, and the like, with possible formation of side products or rearrangement products.

Another approach is to react the hydroxyl group of atazanavir or other protease inhibitor with an alkyl or aryl isocyanate or isothiocyanate to give simple analogs with a carbamate linkage at the position of the hydroxyl wherein the oxygen now comprises part of the carbamate linkage. Many suitable isocyanates or isothiocyanates may be used for this purpose such as C₁-C₁₀ alkyl, phenyl, and heteroaryl isocyanates or isothiocyanates. Preferably, C₁-C₄ alkyl isocyanates are used. As examples, reaction with ethylisocyanate, isopropylisocyanate, or *tert*-butylisocyanate affords the corresponding alkyl carbamates, as exemplified by compounds 21, 22 and 23 and as described in Examples 15, 16 and 17. Unsubstituted carbamates such as compound 25, for example, may be synthesized by reaction with a protected isocyanate such as, but not limited to, trichloroacetyl isocyanate followed by deprotection under mildly basic conditions, such as with carbonate in a water-alcohol mixture, to afford the "bare" carbamate. Alternatives such as reaction of the hydroxyl with phosgene to form the O^c-carbonyl chloride, followed by reaction with ammonia will give the same analog, and reaction with a phosgene equivalent such as nitrophenylchloroformate, followed by reaction with ammonia, may also be used.

Alternatively, substituted alkyl or aryl isocyanates or isothiocyanates may be used, such as has been described in US 7,157,561, the disclosure of which is herein incorporated by reference, to afford analogs and derivatives with a carbamate linkage at the central hydroxyl position bearing an alkyl or aryl linker out of the carbamate group with a functionality on it suitable for further elaboration. Preferably, C₁-C₁₀ alkyl, phenyl, and heteroaryl isocyanates or isothiocyanates are used, and more preferably, C₁-C₆ isocyanates, wherein the alkyl or aryl group carries another suitable functionality such as a protected carboxy, a protected amine, a protected thiol, or a maleimido group. Such reactions may be carried out in any suitable solvent such as DMF or THF, with or without the addition of bases such as tertiary amines such as triethylamine or diisopropylethylamine. Such reactions may be carried out at temperatures typically ranging from 0°C to 100°C, frequently from ambient temperature to 60°C. A non-limiting illustration of this approach is the synthesis of the carbamate-butyrate derivative (29) as described in Example 22 and its elaboration to the NHS ester (33) as described in Examples 23 through 26 and as shown in Figure 13. Other applications of this approach to synthesize equivalent compounds will be suggested to one skilled in the art.

Other carbamate formation equivalents may also be used. For example, reaction of the central hydroxyl of atazanavir with phosgene, carbonyl diimidazole, disuccinimidyl carbonate, or nitrophenylchloroformate will give an active intermediate which, on reaction with a suitable alkyl or aryl amine, will give the corresponding substituted carbamate. Other protease inhibitors would also be amenable to derivatization in this manner.

An activated derivative of lopinavir (40) was also prepared for conjugation to aminodextran. Since the lopinavir immunogen used to generate the lopinavir antibodies was modified at the central hydroxyl group of lopinavir (compound 6F in US 7,193,065, the disclosure of which is incorporated herein by reference), it was preferable to have the lopinavir label coming out of the same position. In lopinavir derivative 40, the central hydroxyl group of lopinavir was attached to a linking group using an ether linkage. An ether linkage, in general, is more stable than an ester or a carbamate linkage. The alkylation reaction of lopinavir was performed in the presence of a base, preferably sodium hydride, using a halo-alkylating agent at a temperature ranging from 0°C to room temperature, preferably in DMF. The halo-alkylating agent may contain C₁-C₁₀ atoms including one or more heteroatoms. Preferably, the alkylating agent is compound 34, which can be produced from iodoacetic acid (Figure 14). The resulting lopinavir acid derivative (35), after alkylation of lopinavir following hydrolysis, can be converted to an active ester group. Conversion of an acid derivative to an activated ester is known to those skilled in the art and can be accomplished, preferably by using a carbodiimide such as EDC·HCl and N-hydroxysuccinimide. The activated ester (37) can be extended further, such as by reaction with methyl aminomethylbenzoate (Figure 15) in the presence of a tertiary amine to give the lopinavir methyl ester derivative 38, which can be hydrolyzed under acidic or basic conditions. In this case, hydrolysis was performed by basic hydrolysis using aqueous lithium hydroxide in a mixture of THF and methanol. The lopinavir acid (39) can be converted to an active ester by using a carbodiimide and N-hydroxysuccinimide as described above. An active ester can also be prepared by using different activating agents and, in the present instance, preferably using O-(N-succinimidyl)-N,N,N',N'-tetramethyluronium tetrafluoroborate in the presence of a tertiary amine, preferably diisopropylethylamine, in THF to give the lopinavir derivative 40.

Stable synthetic precursors to activated hapten derivatives are also suitable for use as analogs. For example, stable synthetic precursors isolated in the course of preparing labeled conjugates may serve a dual purpose as analogs for drug calibration and quality control. These stable synthetic precursors include carboxylic acids such as compounds 30, 32, 35, and 39 as well as stable esters such as compounds 29 and 38.

Synthesis of efavirenz analogs involves direct alkylation of efavirenz. The alkylation may be performed by reaction with a haloalkyl reagent in which an alkali metal carbonate is used as a base in the presence of a phase transfer catalyst such as a crown ether. In one embodiment, potassium carbonate was used in combination with 18-crown-6. The reaction can be carried out in a solvent, e.g., DMF, at a temperature
5 range of room temperature to 150°C for 1 to 24 hours. (See compounds 14, 15, 16, and 17 in Figure 8.)

In another embodiment, a hydroxyalkyl efavirenz analog has been provided. Alkylation of efavirenz can be carried out using a haloalkylating reagent with protected hydroxyl functionality, preferably a silyl protecting group. The resulting silylated efavirenz intermediate can be deprotected, preferably in the presence of tetrabutylammonium fluoride in THF (compound 19, Figure 9).

10 In accordance with one embodiment of the present invention, a method for establishing or preparing a calibration curve for an HIV protease inhibitor is provided wherein the protease inhibitor is selected from the group consisting of amprenavir, atazanavir, tipranavir, indinavir, darunavir, lopinavir, nelfinavir, ritonavir, nelfinavir, and saquinavir. The method comprises the steps of preparing a set of calibrators comprising an immunochemically equivalent analog of said protease inhibitor, wherein the central
15 hydroxyl group of the protease inhibitor has been replaced in said analog with a group selected from -O(C₁-C₁₀ alkyl), -OCONHR₃, -OCH₂CONHR₃, and -OCH₂COOR₃, wherein R₃ is selected from the group consisting of H, C₁-C₆ alkyl, phenyl, heteroaryl, and -(CH₂)_n-X wherein n is 1-6 and X is COOR₅ or NH-CH₂-phenyl-COOR₅ wherein R₅ is H or C₁-C₆ alkyl, wherein each calibrator comprises one of a range of concentrations of the analog. A sample from each calibrator in the set is then contacted with an antibody
20 that specifically binds to said protease inhibitor analog. Each sample is then contacted with a fixed amount of an antibody specific for the analog parent and a conjugate comprising the HIV protease inhibitor and a signal-generating moiety. A competition results between the conjugate and the HIV drug analog in the sample for the limited number of antibody binding sites. In one preferred format, the conjugate is an aminodextran-analog conjugate and the antibodies are bound to microparticles. After a
25 specified reaction time, the amount of signal generated from reaction of the antibody with the conjugate is measured. In the preferred microparticle format, the signal is produced by a change of absorbance in the visible region due to particle agglutination. A calibration curve is then constructed, for example by plotting analog concentration along one axis of a graph and signal along the other axis, which will show an inverse relationship between the signal from the labeled conjugate and the amount of drug analog in
30 the sample. Such calibration curves are substantially equivalent to curves generated using the parent drug in place of the analog. The minimum number of samples in the set is two; however, additional samples are generally preferred to increase the number of data points, and thus accuracy of the curve so produced. Typically one of the sample concentrations is zero.

In a similar manner, a calibration curve can also be prepared for an HIV non-nucleoside reverse transcriptase inhibitor, including the NNRTI efavirenz. In this embodiment, a set of calibrators comprising a range of concentrations of an immunochemically equivalent analog of the non-nucleoside reverse transcriptase inhibitor of interest is provided, wherein the analog comprises said non-nucleoside reverse transcriptase inhibitor N-alkylated with C₁-C₆ alkyl or (C₁-C₆ alkyl)OH. A sample from each
5 calibrator in the set is then contacted with a fixed amount of an antibody specific for the non-nucleoside reverse transcriptase inhibitor and analog and a conjugate comprising the non-nucleoside reverse transcriptase inhibitor and a signal-generating moiety. A competition results between the conjugate and the non-nucleoside reverse transcriptase inhibitor analog in the sample for a limited number of antibody
10 binding sites. In a preferred assay format, the conjugate is an aminodextran-NNRTI conjugate, and the antibody is bound to microparticles. After a specified reaction time, the amount of signal generated from binding of the antibody with the conjugate is detected. In this microparticle format, the signal is due to a change of absorbance in the visible region due to particle agglutination, the change being detectable photometrically. A calibration curve is then constructed as previously described which shows an inverse
15 relationship between the signal from the labeled conjugate and the amount of drug analog present in the sample. Such calibration curves are substantially equivalent to curves generated from the parent drug.

In another embodiment, a conjugate of the HIV drug (protease inhibitor or NNRTI) is bound to a solid phase in a heterogeneous immunoassay format, e.g., enzyme-linked immunosorbent assay or ELISA format. A series of samples comprising varying amounts of an analog of the HIV drug are then contacted
20 with a fixed amount of antibody specific for the drug and the drug conjugate. A competition results between the conjugate and the HIV drug analog for the limited number of antibody binding sites. After a specified reaction time, the amount of antibody bound to the solid phase is detected using a secondary antibody labeled with a signal generating moiety, the secondary antibody specifically binding with the drug-specific antibody, thereby producing a detectable signal upon binding to the drug-specific antibody.
25 A calibration curve is then constructed as described above which shows an inverse relationship between the amount of antibody bound to the solid phase conjugate and the amount of drug analog present in solution. Such calibration curves are substantially equivalent to curves generated using the parent drug.

Specific embodiments

Reagents were obtained from Sigma-Aldrich Chemical Company unless otherwise stated. All solvents
30 were obtained from J.T. Baker and were of ACS grade or HPLC grade or better unless otherwise stated. Triethylamine was obtained from Fluka Chemical Co. Diisopropylethylamine (DIEA), dimethylformamide (DMF), and anhydrous dimethylsulfoxide (DMSO) were obtained from Aldrich

Chemical Co. Tetrahydrofuran (THF) was dried by boiling over and distillation from sodium/benzophenone under argon. Methylene chloride (CH_2Cl_2) was dried by boiling over and distillation from calcium hydride under argon. Column chromatography was performed using flash-grade silica gel from E.M. Science (Silica gel 60; 230-400 mesh ASTM) under a positive pressure of nitrogen.

5 Thin layer chromatography (TLC) was performed using silica gel plates obtained from E.M. Science (0.025 cm thickness). Mixed solvents are expressed as volume for volume percentages (e.g., 10% MeOH- CHCl_3 is chloroform containing 10% of methanol by volume). HPLC analyses were performed on an Agilent 1100 LC/MS system configured with a diode-array detector and a quaternary pump. The LC analyses were performed with a Vydac 218TP54 column (RP-C18; 300Å, 5μ) equipped with a
10 Phenomenex guard module (Phenomenex KJO-4282/C18 ODS 5μ) with the chromatographic stream ported post-column into the MS detector. The MSD utilized was run in ES+ mode (electrospray, positive mode). Unless otherwise stated, analytical runs were performed using a gradient of either 5% or 0% (at 0 minutes = start) to 100% (at 20 minutes) of 0.1% TFA/MeCN in 0.1% TFA/ H_2O with a flow of 1 mL/minutes. Preparative RP-HPLC was performed on a Varian Dynamax (Rainin) system employing two
15 SD1 titanium head 2000 psi pumps with a Varian Dynamax UV-C variable wavelength detector. Separations were carried out on modular Varian Dynamax radial compression columns with either a 250 x 21.4 mm (R00083221C; Microsorb 60-8, C18) column equipped with a guard module (R00083221G; C18, 8μ) or a 250 x 41.4 mm column (R00083241C; Microsorb 60-8, C18,) equipped with a guard module (R00083241G; C18, 8μ). ^1H -NMR spectra were obtained at 200 MHz on a Varian Gemini 2000
20 or at 400 MHz on a Varian XL-400 spectrometer, each equipped with a Sun/Sparc station.

Example 1: Synthesis of lopinavir- O^c -methyl ether (2)

To 16 mg (0.040 mmol) of sodium hydride (60% in oil) was added 3 mL of hexane and the supernatant was decanted off. To the residue, 3 mL of anhydrous THF (freshly distilled) was added followed by 50 mg (0.079 mmol) of lopinavir as a solid. A stock solution of 45 μL of iodomethane in 1 mL of THF was
25 made, and 45 μL (0.31 mmol) of this iodomethane solution in THF was added to the above reaction mixture at room temperature. The reaction mixture was allowed to stir at room temperature for 30 minutes, and 5 mL of 50 mM potassium phosphate (pH 7.5) was added and concentrated in a rotary evaporator to remove THF as much as possible. The aqueous residue was extracted with 6 x 10 mL of chloroform. The organic layers were combined, dried (anhydrous Na_2SO_4), and concentrated. The residue
30 was purified by preparative RP-HPLC using a gradient run consisting of water and acetonitrile containing 0.1% trifluoroacetic acid. Fractions containing the desired product were concentrated in a rotary

evaporator and lyophilized to give 7 mg (0.01 mmol, 14%) of lopinavir-O^c-methyl ether (2) as a white solid. LC/MS: M+H 643.3.

Example 2: Synthesis of lopinavir-O^c-(methoxycarbonylmethyl) ether (3)

To 112 mg (2.8 mmol) of sodium hydride (60% in oil) was added 10 mL of hexane, and the supernatant
5 was decanted off. To the residue, 10 mL of anhydrous THF (freshly distilled) was added followed by 200
mg (0.31 mmol) of lopinavir as a solid. The reaction mixture was allowed to stir at room temperature for
15 minutes, and 380 μ L (4.0 mmol) of methyl bromoacetate was added. The reaction mixture was
allowed to stir at room temperature for 2 hours, and the reaction was quenched with 20 mL of saturated
ammonium chloride solution. The resulting reaction mixture was concentrated in a rotary evaporator to
10 remove THF as much as possible, and the aqueous residue was extracted with 6 x 30 mL of chloroform.
The organic layers were combined, dried (anhydrous Na₂SO₄), and concentrated. The residue was purified
by preparative RP-HPLC using a gradient run consisting of water and acetonitrile containing 0.1%
trifluoroacetic acid. Fractions containing the desired product were concentrated in a rotary evaporator and
lyophilized to give 61 mg (0.087 mmol, 27%) of lopinavir-O^c-(methoxycarbonylmethyl) ether (3) as a
15 white solid. LC/MS: (M+H) 701.3.

Example 3: Synthesis of O^c lopinavir acetic acid (4)

To a suspension of 262 mg (6.5 mmol) of sodium hydride (60% in oil) in 5 mL of freshly distilled THF
was added a solution of 250 mg (0.39 mmol) of lopinavir in 5 mL of freshly distilled THF at room
temperature. The mixture was allowed to stir at room temperature for 1 hour. To the reaction mixture 80
20 μ L (0.84 mmol) of methyl bromoacetate was added, and the resulting reaction mixture was allowed to stir
at room temperature for 3 days. To the reaction mixture 5 mL of 50 mM potassium phosphate (pH 7.5)
was added followed by 100 mg (2.3 mmol) of lithium hydroxide monohydrate as a solid. The resulting
reaction mixture was allowed to stir at room temperature 18 hours and concentrated in a rotary
evaporator. The aqueous residue was acidified with dilute phosphoric acid to pH 5-6 and extracted with 5
25 x 50 mL of chloroform. The combined organic layers were dried (anhydrous Na₂SO₄) and concentrated to
give a white gummy solid. This was purified by preparative thin layer chromatography using 20%
methanol in chloroform to give 43 mg (0.068 mmol) of recovered lopinavir and 109 mg (0.16 mmol,
40%) of O^c lopinavir acetic acid (4) as a white solid, LC-MS: (M+H) 687.4.

Example 4: Synthesis of lopinavir-O^c-(N-tert-butylcarbamoylemethyl) ether (6)

To a solution of 259 mg (0.37 mmol) of lopinavir acetic acid (4) in 30 mL of freshly distilled dichloromethane (distilled over CaH₂) was added 152 mg (1.32 mmol) of N-hydroxysuccinimide and 253 mg (1.32 mmol) of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC·HCl). The
5 reaction mixture was allowed to stir at room temperature for 18 hours. The crude NHS ester (5) prepared *in situ* was used in the next step without isolation. To the above reaction mixture a solution of 70 µL (0.65 mmol) of t-butylamine in 930 µL of freshly distilled dichloromethane (distilled over CaH₂) was added, and the reaction allowed to stir at room temperature for 2 hours. To the reaction mixture 30 mL of water was added, and the organic layer was separated. The organic layer was washed with 30 mL of saturated
10 sodium bicarbonate followed by 30 mL of water, dried (anhydrous Na₂SO₄), and concentrated. The residue was purified by preparative RP-HPLC using a gradient run consisting of water and acetonitrile containing 0.1% trifluoroacetic acid. Fractions containing the desired product were concentrated in a rotary evaporator and lyophilized to give 137 mg (0.18 mmol, 49%) of lopinavir-O^c-(N-tert-butylcarbamoylemethyl) ether (6) as a white solid. LC/MS (M+H) 742.5.

15 Example 5: Synthesis of lopinavir-O^c-carbamate (8)

To a solution of 160 mg (0.25 mmol) of lopinavir in freshly distilled dichloromethane (distilled over CaH₂) cooled in an ice-bath was added 50 µL (0.42 mmol) of trichloroacetylisocyanate. The resulting reaction mixture was allowed to stir at 0°C for 4 hours and concentrated under reduced pressure to give crude a product mixture containing lopinavir-O^c-trichloroacetylcarbamate (7). This was used *in situ* in the
20 next step without further purification. To the crude 7 was added 720 mg (5.2 mmol) of potassium carbonate, 7 mL of methanol, and 2 mL water at 0°C. The resulting reaction mixture was allowed to stir at 0°C for 1 hour and at room temperature for 18 hours. This was concentrated under reduced pressure, and 150 mL of chloroform and 50 mL of water were added to the residue. The organic layer was separated, and the aqueous layer was extracted with 3 x 50 mL of chloroform. All the organic layers were
25 combined, dried (anhydrous Na₂SO₄), and concentrated under reduced pressure. The residue was purified by preparative RP-HPLC using a gradient run consisting of water and acetonitrile containing 0.1% trifluoroacetic acid. Fractions containing the desired product were concentrated on a rotary evaporator and lyophilized to give 130 mg (0.19 mmol, 76%) of lopinavir-O^c-carbamate (8) as a white solid, LC-MS: M+H 672.4.

Example 6: Synthesis of N-carbamoyl-lopinavir-O^c-carbamate (10)

To a solution of 200 mg (0.32 mmol) of lopinavir in 8 mL of freshly distilled dichloromethane (distilled over CaH₂), 1.08 g (7.8 mmol) potassium carbonate and 100 µL (0.83 mmol) of trichloroacetyl isocyanate were added. This was allowed to stir at 0°C for 3 hours and concentrated to give a crude reaction mixture containing N-(trichloroacetyl-amino-carbonyl)-lopinavir-O^c-trichloroacetylcarbamate (9). This was used *in situ* in the next step without further purification. To the residue 16 mL of methanol and 4 mL of water were added at 0°C. This was allowed to stir at 0°C for 1 hour and at room temperature for 18 hours. The resulting reaction mixture was concentrated under reduced pressure. To the residue 150 mL chloroform and 50 mL of water were added. The organic layer was separated and the aqueous layer was extracted with 3 x 50 mL of chloroform. All the organic layers were combined, dried (anhydrous Na₂SO₄), and concentrated under reduced pressure. The residue was purified by preparative RP-HPLC using a gradient run consisting of water and acetonitrile containing 0.1% trifluoroacetic acid. Fractions containing the desired product were concentrated on a rotary evaporator and lyophilized to give 145 mg (0.20 mmol, 83%) of N-carbamoyl-lopinavir-O^c-carbamate (10) as a white solid, LC/MS: M+H 715.3.

Example 7: Synthesis of O^c lopinavir acetate (11)

To 3.0 g (4.7 mmol) of lopinavir was added 210 mg (1.71 mmol) of 4-(dimethylamino) pyridine followed by 90 mL of freshly distilled THF (distilled over sodium & benzophenone). The reaction mixture was allowed to cool in an ice-bath, and 750 µL (9.2 mmol) of pyridine was added. This was followed by addition of 750 µL (10.5 mmol) of acetyl chloride. The reaction mixture was allowed to warm up to room temperature and stirred at room temperature for 72 hours. The reaction mixture turned cloudy. This was concentrated and purified by preparative RP-HPLC using a gradient run consisting of water and acetonitrile containing 0.1% trifluoroacetic acid. Fractions containing the desired product were concentrated on a rotary evaporator and lyophilized to give 2.48 g (3.69 mmol, 78%) of O^c-lopinavir acetate (11), LC-MS: (M+H) 671.3.

Example 8: Synthesis of N-carbamoyl lopinavir (13)

To 2.48 g (3.69 mmol) of lopinavir acetate (11) in 100 mL of THF at 0°C was added 1 mL (8.39 mmol) of trichloroacetyl isocyanate. The mixture was allowed to stir at 4°C for 18 hours. The solution turned light yellow in color. This was concentrated on a rotary evaporator, and an off-white solid was obtained. The reaction was monitored by LC/MS and the product identified as N-(trichloroacetylcarbamoyl)-lopinavir-O^c-acetate (12) as the desired intermediate product by LC/MS (M+H 858.3). To the reaction mixture 160 mL of methanol and 40 mL of water were added followed by 4.8 g (36 mmol) of potassium

carbonate. The mixture was allowed to stir at room temperature for 4 hours and a white slurry was obtained. This was concentrated under reduced pressure, and 400 mL of water was added. The resulting reaction mixture was extracted with 4 x 400 mL of ethyl acetate. The organic layers were combined, dried (Na_2SO_4), and concentrated to give a white solid. This was purified by silica gel column chromatography by eluting with ethyl acetate to give a white solid. ^1H NMR indicated the presence of ethyl acetate even after being dried under high vacuum. All of the material was dissolved in 1:1 water/acetonitrile and lyophilized to give 1.8 g (2.6 mmol, 72%) of N-carbamoyl lopinavir (**13**) as a white solid, LC/MS: (M+H) 672.3.

Example 9: Synthesis of N-methyl efavirenz (**14**)

To a solution of 40 mg (0.13 mmol) of efavirenz in 2 mL of anhydrous DMF was added 33 mg (0.24 mmol) of anhydrous potassium carbonate, 1 mg (3.78×10^{-3} mmol) of 18-crown-6, and 16 μL (0.25 mmol) of iodomethane. The reaction mixture was heated to 80°C for 1.5 hours, then cooled and concentrated under reduced pressure. To the residue 10 mL of dichloromethane was added and the mixture filtered. The filtrate was transferred to a separatory funnel and washed with 2 x 5 mL of water. The organic layer was dried (anhydrous Na_2SO_4) and concentrated under reduced pressure. The residue was purified by preparative RP-HPLC using a gradient run consisting of water and acetonitrile containing 0.1% trifluoroacetic acid. Fractions containing the desired product were concentrated on a rotary evaporator and lyophilized to give 30 mg (0.090 mmol, 73%) of N-methyl efavirenz (**14**), LC-MS: (M+H) 330.0.

Example 10: Synthesis of N-ethyl efavirenz (**15**)

To 1.1 g (3.49 mmol) of efavirenz was added 40 mL of anhydrous DMF, 40 mg (0.15 mmol) of 18-crown-6, 2.65 g (19.2 mmol) of anhydrous potassium carbonate, 210 mg (1.40 mmol) of sodium iodide and 2.6 mL (34.7 mmol) of bromoethane. The reaction mixture was allowed to stir at 125°C for 2 hours. The color of the reaction mixture turned orange with white solids at the bottom of the reaction flask. The reaction mixture was allowed to cool to room temperature and filtered. The filtrate was concentrated under reduced pressure. To the residue 200 mL of chloroform was added, washed with 2 x 100 mL of water, dried (MgSO_4), and concentrated under reduced pressure to give 1.3 g of orange oil. This was purified by preparative RP-HPLC using a gradient run consisting of water and acetonitrile containing 0.1% trifluoroacetic acid. Fractions containing the desired product were concentrated on a rotary evaporator and lyophilized to give 884 mg (2.57 mmol, 74%) of N-ethyl efavirenz (**15**) as a white powder, LC-MS: M+H 344.0.

Example 11: Synthesis of N-propyl efavirenz (16)

To 60 mg (0.19 mmol) of efavirenz was added 2 mL of anhydrous DMF followed by 50 mg (0.36 mmol) of anhydrous potassium carbonate, 10 mg (0.06 mmol) of sodium iodide, 39 mg (0.31 mmol) of 1-bromopropane, and 2 mg (7.5×10^{-3} mmol) of 18-crown-6. The resulting reaction mixture was allowed to stir at 125°C for 1 hour and then cooled to room temperature. This was filtered, and the filtrate was concentrated under reduced pressure. To the residue 50 mL of chloroform was added, and the organic layer was washed with 2 x 25 mL of water, dried (anhydrous Na₂SO₄), and concentrated under reduced pressure. The residue was purified by silica gel column chromatography using 10% ethyl acetate in hexane as eluent followed by 40% ethyl acetate in hexane to give 32 mg (0.089 mmol, 49%) of N-propyl efavirenz (16) as a white solid, LC-MS (M+H) 358.0.

Example 12: Synthesis of N-butyl efavirenz (17)

To a solution of 40 mg (0.12 mmol) of efavirenz in 2 mL of anhydrous DMF was added 300 mg (2.1 mmol) of anhydrous potassium carbonate, 1 mg (3.78×10^{-3} mmol) of 18-crown-6, and 68 µL (0.62 mmol) of 1-bromobutane. The mixture was heated to 60°C for 4 hours and then to 120°C for 1 hour. The resulting reaction mixture was concentrated under reduced pressure, 10 mL of dichloromethane was added, and the mixture was filtered. The filtrate was transferred to a separatory funnel and washed with 2 x 5 mL of water. The organic layer was dried (anhydrous Na₂SO₄) and concentrated under reduced pressure. The residue was purified by preparative RP-HPLC using a gradient run consisting of water and acetonitrile containing 0.1% trifluoroacetic acid. Fractions containing the desired product were concentrated on a rotary evaporator and lyophilized to give 33 mg (0.088 mmol, 70%) of N-butyl efavirenz (17) as a colorless oil, LC/MS (M+H) 372.0.

Example 13: Synthesis of N-(tert-butyldimethylsilyloxypropyl) efavirenz (18)

To a solution of 50 mg (0.16 mmol) of efavirenz in 5 mL of distilled acetone was added 48 mg (0.19 mmol) of (3-bromopropoxy)-tert-butyl-dimethylsilane, 32 mg (0.23 mmol) potassium carbonate, 3 mg (0.011 mmol) 18-crown-6, 100 µL of anhydrous DMF, and 10 mg (0.06 mmol) sodium iodide. The reaction mixture was allowed to reflux for 18 hours, and the reaction progress was monitored by LC/MS, which indicated only a trace of desired product with unreacted starting efavirenz. The reaction mixture was concentrated under reduced pressure, and 2 mL of anhydrous DMF was added followed by 35 mg (0.25 mmol) of anhydrous potassium carbonate and 40 mg (0.15 mmol) of (3-bromopropoxy)-tert-butyl-dimethylsilane. The resulting reaction mixture was allowed to heat at 125°C for 2 hours, cooled to room temperature, and filtered. The residue was washed with 50 mL of ethyl acetate. The combined filtrate was

concentrated, 50 mL of chloroform was added, and the organic phase washed with 25 mL of water. The organic layer was dried (anhydrous Na₂SO₄) and concentrated under reduced pressure to give a gummy solid. This was purified by silica gel column chromatography using 20% ethyl acetate in hexane to give 49 mg (0.10 mmol, 63%) of N-(*tert*-butyldimethylsilyloxypropyl) efavirenz (**18**) as a white semisolid,

5 LC-MS: M+H 488.0

Example 14: Synthesis of N-hydroxypropyl efavirenz (**19**)

To 43 mg (0.13 mmol) of N-(*tert*-butyldimethylsilyloxypropyl)efavirenz (**18**) was added 100 µL of freshly distilled THF followed by 500 µL (0.49 mmol) of 1M tetrabutylammonium fluoride. The resulting reaction mixture was allowed to stir at room temperature for 18 hours and concentrated under reduced
10 pressure. To the residue 50 mL of chloroform was added and the organic layer was washed with 2 x 25 mL of water. The organic layer was dried (anhydrous Na₂SO₄) and concentrated under reduced pressure. The residue was purified by silica gel column chromatography using 30% hexane in ethyl acetate. The product isolated indicated some impurities and was further purified by preparative RP-HPLC using a gradient run consisting of water and acetonitrile containing 0.1% trifluoroacetic acid. Fractions containing
15 the desired product were concentrated on a rotary evaporator and lyophilized to give 30 mg (0.080 mmol, 93%) of N-hydroxypropyl efavirenz (**19**) as a white powder, LC-MS: M+H 374.0.

Example 15: Synthesis of atazanavir-O^c-ethylcarbamate (**21**)

To a solution of 102 mg of atazanavir (**20**) in 8.1 mL of dry dimethylformamide (DMF) under argon was added 22.5 µL (2 eq) of ethyl isocyanide (Sigma-Aldrich) and the reaction stirred at room temperature.
20 After ~29 hours an additional 11 µL of ethyl isocyanate was added the next day, and stirring continued overnight. Analysis by LC/MS indicated the reaction was complete. Solvent and volatile material were removed under vacuum (high vacuum rotovap), the residue redissolved in 1:1 acetonitrile (MeCN)/water (H₂O), filtered, and the filtrate purified by preparative RP-HPLC, eluting with a 0.1% trifluoroacetic acid (TFA)/MeCN in 0.1% TFA/water gradient. The product fractions were collected, MeCN removed at room
25 temperature (rotovap) and the residue frozen and lyophilized to give 77 mg of the product (**21**) as the partial TFA salt and as a fluffy white solid. LC/MS: M+H (Parent) 776.3; HR-MS: Calc M+H (Parent) 776.4342, Observed 776.4330; ¹H-NMR: Compatible; Microanalysis (MA): agreed with C₄₁H₅₇N₇O₈·0.33CF₃COOH.

Example 16: Synthesis of atazanavir-O^c-isopropylcarbamate (22)

To a solution of 52 mg of atazanavir (20) in 2 mL of dry DMF under argon was added 14.2 μ L (~1.1 eq) of DIEA followed by 8.6 μ L (~1.2 eq) of isopropyl isocyanate (Sigma-Aldrich) and the reaction stirred overnight at room temperature. An additional 3 μ L of DIEA and 2 μ L of isopropyl isocyanate were added the next day, and stirring continued overnight. Analysis by LC/MS indicated reaction was complete. Solvent and volatile material were removed under vacuum (high vacuum rotovap), the residue redissolved in 1:1 acetonitrile (MeCN)/water (H₂O), filtered, and the filtrate purified by preparative RP-HPLC, eluting with a 0.1% trifluoroacetic acid (TFA)/MeCN in 0.1% TFA/water gradient. The product fractions were collected, MeCN removed at room temperature (rotovap) and the residue frozen and lyophilized to give 53 mg of the product (22) as the partial TFA salt and as a fluffy white solid. LC/MS: M+H (Parent) 790.5; HR-MS: Calc M+H 790.4498, Observed 790.4495; ¹H-NMR: Compatible; Microanalysis (MA): agreed with C₄₂H₅₉N₇O₈·1.25CF₃COOH.

Example 17: Synthesis of atazanavir-O^c-tert-butylcarbamate (23)

To a solution of 57 mg of atazanavir (20) in 0.75 mL of dry DMF under argon was added 28.5 μ L (~2 eq) of DIEA followed by 95.5 μ L (~10 eq) of *tert*-butyl isocyanate (Sigma-Aldrich) and the reaction stirred at room temperature for 4 hours. Analysis by LC/MS indicated reaction was complete. Solvent and volatile material were removed under vacuum (high vacuum rotovap). The residue was redissolved in 1:1 acetonitrile (MeCN)/water (H₂O), combined with the residue from a similar second reaction (with 53 mg of atazanavir, 26.5 μ L of DIEA, and 89 μ L of *tert*-butyl isocyanate), filtered and the filtrate purified by preparative RP-HPLC, eluting with a 0.1% trifluoroacetic acid (TFA)/MeCN in 0.1% TFA/water gradient. The product fractions were collected, MeCN removed at room temperature (rotovap) and the residue frozen and lyophilized to give 118 mg of the product (23) as the partial TFA salt and as a fluffy white solid. LC/MS: M+H (Parent) 804.4; HR-MS: Calc M+H (Parent) 804.4655, Observed 804.4646; ¹H-NMR: Compatible; Microanalysis (MA): agreed with C₄₃H₆₁N₇O₈·1.4CF₃COOH.

Example 18: Synthesis of atazanavir-O^c-trichloroacetylcarbamate (24)

To a solution of 52 mg of atazanavir (20) in 1 mL of dry DMF under argon was added 70 μ L (~8 eq) of trichloroacetyl isocyanate (Sigma-Aldrich) and the reaction stirred at room temperature for ~1 hour. Analysis by LC/MS indicated reaction was essentially complete. Solvent and volatile material were removed under vacuum (high vacuum rotovap). The residue was redissolved in 1:1 acetonitrile (MeCN)/water (H₂O), filtered and the filtrate purified by preparative RP-HPLC, eluting with a 0.1%

trifluoroacetic acid (TFA)/MeCN in 0.1% TFA/water gradient. The center cut of the product fraction was collected, MeCN removed at room temperature (rotovap), and the residue frozen and lyophilized to give 52 mg of the product (**24**) as a fluffy white solid, assigned as the TFA salt. LC/MS: M+H (Parent) 892.2 (three chlorines isotope pattern); ¹H-NMR: Compatible.

5 **Example 19: Synthesis of atazanavir-O^c-carbamate (**25**)**

To a solution of 1.0 g of atazanavir (**20**) in 20 mL of dry DMF under argon was added 1.345 mL (~8 eq) of trichloroacetylisocyanate (Sigma-Aldrich) and the reaction stirred at room temperature for ~1 hour. Analysis by LC/MS indicated reaction was essentially complete. Solvent and volatile material were removed under vacuum (high vacuum rotovap) and the residue dried further under high vacuum to give
10 crude **24**. All of the crude **24** was redissolved in 58 mL of methanol (MeOH), the solution diluted with 14.5 mL of water followed by the addition of 4.96 g (>25 eq) of potassium carbonate. The slightly heterogeneous reaction mixture was stirred vigorously under argon for ~4 hours. MeOH was removed under reduced pressure (rotovap), the aqueous residue diluted with 50 mL of water, and the mixture extracted with ethyl acetate (EtOAc) (2 x 150 mL). The extracts were dried with sodium sulfate (Na₂SO₄),
15 filtered, and evaporated to give crude product which was redissolved in a small volume of EtOAc and purified by column chromatography on flash-grade silica gel, eluting with EtOAc. The product fractions (analysis by TLC) were combined and evaporated to give 888 mg of fairly clean product (**25**) as the free base and as a white solid still containing EtOAc. This material was redissolved in 5:1 MeCN:H₂O and repurified by preparative RP-HPLC, eluting with a 0.1% trifluoroacetic acid (TFA)/MeCN in 0.1%
20 TFA/water gradient. The main cuts of the product peak were combined, MeCN removed (rotovap), and the residue frozen and lyophilized to give 870 mg of **25** as a white solid and as the TFA salt hydrate. LC/MS: M+H (Parent) 748.3; HR-MS: Calc M+H 748.4029, Observed 748.4031; ¹H-NMR: Compatible; Microanalysis (MA): agreed with C₃₉H₅₃N₇O₈·1.5CF₃COOH·1.5H₂O.

Example 20: Synthesis of atazanavir-O^c-methyl ether (26**)**

25 To a solution of 10 mg of atazanavir (**20**) in 0.8 mL of dry DMF in a small reaction vial with a Teflon-lined screw cap (Wheaton Science Products) was added 10 mg of silver (I) oxide followed by 10 µL (~11.5 eq) of iodomethane (Sigma-Aldrich), the vial capped, and the reaction stirred at room temperature for 2 days. An additional 21 mg of silver (I) oxide was added, the vial recapped, and stirring continued for a further day. Analysis by LC/MS indicated essential consumption of atazanavir with the appearance of
30 multiple product peaks, with the major peak having the expected molecular ion and UV spectrum for the O^c-methyl ether. The reaction was diluted with a little DMF and filtered from silver salts. Solvent and

volatile material were removed from the filtrate under vacuum (high vacuum rotovap), the residue redissolved in MeCN/water, filtered, and the filtrate purified by preparative RP-HPLC, eluting with a 0.1% trifluoroacetic acid (TFA)/MeCN in 0.1% TFA/water gradient. The center cuts of the most predominant product peak fractions were collected, MeCN removed at room temperature (rotovap), and the residue frozen and lyophilized to give material which was repurified by semi-preparative C18 RP-HPLC (Vydac 218TP510; 250 x 10 mm, 300Å, 5μ), also eluting with a 0.1% trifluoroacetic acid (TFA)/MeCN in 0.1%TFA/water gradient. The product fraction was collected, MeCN removed at room temperature (rotovap), and the residue frozen and lyophilized to give a small amount of the product 26 as an amorphous solid. LC/MS: M+H (Parent) 719.3; UV spectrum same as that for atazanavir.

10 **Example 21: Synthesis of atazanavir-O^c-(2' -hydroxyethyl)ether (28)**

To a solution of 10 mg of atazanavir in 0.8 mL of dry DMF in a small reaction vial was added 10 mg of silver (I) oxide followed by 23 mL (~11.5 eq) of 2-(2-bromoethoxy)-tetrahydro-2H-pyran (Sigma-Aldrich). After stirring at room temperature for two days, a further 30 mg of silver (I) oxide was added together with 30 mg of sodium iodide, the vial recapped, and the thick slurry stirred at room temperature for an additional two days. LC/MS indicated consumption of atazanavir with the appearance of three main product peaks (plus others), with the middle main peak having the expected M+H of 833.3 and UV for the tetrahydropyran derivative (27). The reaction mixture was diluted with DMF and filtered (0.45m) [slow] from silver and sodium salts. The filtrate was diluted further with methylene chloride and refiltered (0.45μ). The filtrate was evaporated to dryness under reduced pressure. The residue was redissolved in 1:1 MeCN/water and purified by preparative RP-HPLC eluting with a 0.1% trifluoroacetic acid (TFA)/MeCN in 0.1%TFA/water gradient. The desired peak was collected, MeCN removed (rotovap), and the residue frozen and lyophilized to give 1.2 mg of amorphous semi-solid. This material was redissolved in 2 mL of 0.1%TFA in 1:1 MeCN/water and allowed to stand for 5 hours at room temperature, after which LC/MS indicated essentially complete conversion to 28. The solution was concentrated and purified by semi-preparative C18 RP-HPLC (Vydac 218TP510; 250 x 10 mm, 300Å, 5μ), also eluting with a 0.1% trifluoroacetic acid (TFA)/MeCN in 0.1%TFA/water gradient. The product fraction was collected, MeCN removed at room temperature (rotovap), and the residue frozen and lyophilized to give 1.1 mg of the product (28) as an amorphous solid. LC/MS: M+H (Parent) 749.2; UV spectrum same as that for atazanavir.

Example 22: Synthesis of 4-[(atazanavir-O^c)-carbonylamino]butyric acid ethyl ester (29)

To a solution of 97.4 mg of atazanavir (20) in 2.5 mL of dry DMF was added 55 μ L (2.7 equiv.) of ethyl 4-isocyanatobutyrate (Sigma-Aldrich) followed by 23 μ L (1.2 equiv.) of anhydrous triethylamine (TEA). The solution was stirred under argon and heated overnight with an oil bath held at approximately 60°C.

5 The reaction was then cooled to room temperature, DMF removed under vacuum (high vacuum rotovap), and the residue purified by preparative RP-HPLC, eluting with a 0.1% trifluoroacetic acid (TFA)/MeCN in 0.1% TFA/water gradient. The main cuts of the product peak were combined, MeCN removed (rotovap) and the residue frozen and lyophilized to give 116 mg of the product (29) as an amorphous, slightly sticky solid, and as the TFA salt hydrate. LC/MS: M+H (Parent) 862.5; HR-MS: Calc M+H
10 862.4709, Observed 862.4708; ¹H-NMR: Compatible; Microanalysis (MA): agreed with C₄₅H₆₃N₇O₁₀·2CF₃COOH·2H₂O.

Example 23: Synthesis of 4-[(atazanavir-O^c)-carbonylamino]butyric acid (30)

To a solution of 40.6 mg of 29 in 1.25 mL of MeOH and 1.25 mL of water was added 21.2 mg (10.7 equiv.) of lithium hydroxide monohydrate, and the clear reaction solution was stirred at room temperature
15 under argon for approximately 2 hours. Analysis by LC/MS indicated the hydrolysis was complete. This was combined with another identically complete reaction of 86.4 mg of 29 with 42.2 mg of LiOH·H₂O in 2.5 mL of MeOH and 2.5 mL of water. The reaction mixture was neutralized with 1N HCl to about pH 6 to 7, and solvents were removed on a rotovap. The residue was redissolved in MeCN/water and purified
20 by preparative RP-HPLC, eluting with a 0.1% trifluoroacetic acid (TFA)/MeCN in 0.1% TFA/water gradient. The main cuts of the product peak were combined, MeCN removed (rotovap), and the residue frozen and lyophilized to give 124 mg of the product (30) as a white solid and as the TFA salt. LC/MS: M+H (Parent) 834.4; HR-MS: Calc M+H 834.4396, Observed 834.4386; ¹H-NMR: Compatible.

Example 24: Synthesis of 4-[(atazanavir-O^c)-carbonylamino]butyric acid N-hydroxysuccinimide ester (31)

25 To a stirring solution/suspension of 123.4 mg of 30 in 9 mL of freshly distilled dry methylene chloride under argon was added 91.5 mg (6.1 equiv.) of N-hydroxysuccinimide (NHS) followed by 151.9 mg (6.1 equiv.) of 1-[(3-dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDC·HCl), and the clear reaction was stirred at room temperature for approximately 2 hours. Analysis by LC/MS showed a single atazanavir product peak with the desired M+H of ^{m/z} 931.5. The reaction was diluted with distilled
30 CH₂Cl₂ and washed sequentially but quickly with 0.1N HCl (x 1), 0.1N NaOH (x 1), sat. brine (x 1), the

organic phase dried (Na_2SO_4), filtered, and evaporated (rotovap) to give crude but fairly clean NHS ester (**31**). The material was used immediately in the synthesis of **32** (Example 25).

In another run, reaction of 34.5 mg of **30** in 2 mL of CH_2Cl_2 with 27.3 mg of NHS and 42.4 mg of EDC·HCl gave, by LC/MS analysis, the same product peak with M+H of m/z 931.5. Solvent was removed (rotovap) and the residue redissolved in 2:1 MeCN/water and purified directly by preparative RP-HPLC, eluting with a 0.1% trifluoroacetic acid (TFA)/MeCN in 0.1% TFA/water gradient. The main cut of the product peak was immediately frozen (dry ice/acetone), MeCN largely removed (sublimation onto the dry ice/acetone cold finger of the high vacuum rotovap), and the frozen residue lyophilized at $<20^\circ\text{C}$ to give 23.3 mg of purified NHS ester (**31**) as a white solid and as the TFA salt. LC/MS: M+H (Parent) 931.5; HR-MS: Calc M+H 931.4560, Observed 931.4553; $^1\text{H-NMR}$: Compatible.

Example 25: Synthesis of 4-[4-[(atazanavir- O^6)-carbonylamino]butyramido)]methylbenzoic acid (32**)**

A solution of 22.1 mg of 4-(aminomethyl)benzoic acid (Sigma-Aldrich) in 1.5 mL of water (heated for dissolution, then cooled to room temperature) was adjusted to pH ~ 10 with a small volume of 0.1N NaOH. This solution was then added to a solution of all of the crude NHS ester (**31**) from Example 24, first paragraph, in 11 mL of freshly distilled dry tetrahydrofuran (THF) and the reaction mixture stirred at room temperature overnight under argon. Analysis by LC/MS indicated consumption of NHS ester and formation of the desired product, M+H at m/z 967.5. The product mixture was neutralized with a little 0.1N HCl and evaporated to dryness (high vac rotovap). The residue was taken up in 10% MeOH in ethyl acetate (EtOAc) and applied to the top of a silica gel column which was then eluted with a gradient of 10% to 20% MeOH in EtOAc. The product fractions were combined and evaporated (rotovap), the residue redissolved in dry CH_2Cl_2 , filtered, and re-evaporated (rotovap) to give 79 mg of the product (**32**) as a white powder. LC/MS: M+H 967.5; HR-MS: Calc M+H 967.4924, Observed 967.4921; $^1\text{H-NMR}$: Compatible.

Example 26: Synthesis of 4-[4-[(atazanavir- O^6)-carbonylamino]butyramido)]methylbenzoic acid N-hydroxysuccinimide ester (33**)**

To a solution of 62 mg of the benzoic acid product (**32**) in 4.5 mL of freshly distilled dry CH_2Cl_2 was added 9.0 mg (1.12 equiv.) of NHS followed by 15.4 mg (1.15 equiv.) of EDC·HCl and the reaction stirred at room temperature under argon. LC/MS analysis after ~ 4 hours indicated the reaction was complete. Solvent was removed (rotovap). The residue was redissolved in 2:1 MeCN/water and purified by preparative RP-HPLC, eluting with a 0.1% trifluoroacetic acid (TFA)/MeCN in 0.1% TFA/water

gradient. The main cut of the product peak was immediately frozen (dry ice/acetone), MeCN largely removed (sublimation onto the dry ice/acetone cold finger of the high vacuum rotovap), and the frozen residue lyophilized at <20°C to give 40.2 mg of purified NHS ester (**33**) as a white solid and as the TFA salt. LC/MS: M+H (Parent) 1064.5; ¹H-NMR: Compatible.

5 **Example 27: Synthesis of 3-(2-iodo-acetamido)-propionic acid ethyl ester (**34**)**

A solution of 7.4 g (39.7 mmol) of iodoacetic acid in 90 mL of distilled dichloromethane (distilled over CaH₂) was cooled to 0°C. To the reaction mixture 4.4 mL (39.9 mmol) of N-methylmorpholine was added followed by 5.18 mL (39.9 mmol) of isobutylchloroformate. The reaction mixture was allowed to stir at 0°C for 20 minutes. The reaction mixture was clear when N-methylmorpholine was added but
10 turned dark yellow after the addition of isobutylchloroformate.

To the above reaction mixture, a mixed solution of β-alanine ethyl ester hydrochloride (6.14 g, 39.9 mmol, Sigma-Aldrich) in 60 mL of dichloromethane and 4.4 mL (39.9 mmol) of N-methylmorpholine was added over a period of 5 minutes, and the reaction mixture was allowed to stir at 0°C for 30 minutes. The reaction mixture was allowed to warm up to room temperature for 18 hours. The reaction mixture
15 turned red in color. The reaction mixture was transferred into a separatory funnel, and the organic layer was washed with 2 x 75 mL of 20% citric acid, 2 x 75 mL of saturated NaHCO₃, and 1 x 50 mL of saturated aqueous sodium chloride solution, dried (MgSO₄), and filtered. To the filtrate 3 g of activated charcoal was added and filtered through Celite (10 g). The resulting filtrate appeared light yellow in color. This was concentrated under reduced pressure to give a light yellow oil. To this oil 2 x 20 mL of hexane
20 was added, the mixture swirled and allowed to settle, and the hexane layer was removed each time with a Pasteur pipette. To the resulting oil was added 40 mL of distilled acetone (distilled over anhydrous potassium carbonate) and 15 g (100 mmol) of sodium iodide. The reaction mixture was allowed to stir at room temperature for 18 hours and then filtered. The filtrate was concentrated, and a yellow oil was obtained. This was dissolved in 90 mL of dichloromethane. The organic layer was washed with 30 mL of
25 water, 30 mL of 2% solution of sodium thiosulfate, 30 mL of water, and 30 mL of saturated aqueous sodium chloride solution to give 8.7 g (30.5 mmol, 77%) of the product (**34**) as a pale yellow oil, which upon storage at 4°C solidified to a light yellow solid. LC-MS (M+Na): 307.9.

Example 28: Synthesis of 3-[(lopinavir-O⁶)-acetamido]-propionic acid (35**)**

To 500 mg (0.79 mmol) of lopinavir was added 10 mL of anhydrous toluene and the mixture concentrated
30 on a rotary evaporator. This process was repeated once. To the residue was added 4 mL of freshly distilled THF. In another flask was added 130 mg (3.25 mmol) of sodium hydride (60% in oil) and 4 mL

of freshly distilled THF. To this stirring suspension of sodium hydride in THF, the solution of lopinavir in THF (prepared above) was added dropwise, and the reaction mixture was allowed to stir at room temperature for 2 hours. A solution of the iodoacetamido linker (**34**) (250 mg, 0.87 mmol) in 4 mL of freshly distilled THF was added to the reaction mixture, and the reaction allowed to stir at room temperature for 3 hours. The reaction flask was cooled in ice, and the reaction was quenched with 10 mL of 50 mM potassium phosphate (pH 7.5) and allowed to stir at room temperature for 30 minutes. To the resulting mixture 4 mL of water was added followed by 250 mg (5.95 mmol) of LiOH·H₂O, and the mixture was allowed to stir at 4°C for 18 hours. The reaction mixture was concentrated on a rotary evaporator. The pH of the residue was adjusted to 3-4, and the resulting aqueous mixture extracted with 6 x 50 mL of ethyl acetate. The organic layers were combined, dried (anhydrous Na₂SO₄), and concentrated on a rotary evaporator to give a pale yellow oil. LC/MS indicated the presence of product. This oil was dissolved in 3:1 acetonitrile/water and purified by preparative RP-HPLC to give 286 mg (0.37 mmol, 36%) of lopinavir acid derivative **35** and 83 mg of recovered lopinavir starting material. Yield of the lopinavir acid derivative **35** was 53% based on recovered starting lopinavir. LC/MS (M+H): 758.3.

Example 29: Synthesis of 4-[3-(lopinavir-O^c)-acetamido]-propionamido]methylbenzoic acid methyl ester (38**)**

A solution of 255 mg (0.33 mmol) of lopinavir acid (**35**) in 19 mL of dichloromethane was prepared. To this magnetically stirred reaction mixture was added 264 mg (1.37 mmol) of 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDC·HCl) followed by 163 mg (1.4 mmol) of N-hydroxysuccinimide. The reaction mixture was allowed to stir at room temperature for 18 hours. LC/MS analysis indicated product formation, (M+H) at ^{m/z} 855.3. This activated ester (**37**) was used in the next step without isolation.

To a magnetically stirred solution of 210 mg (1.04 mmol) of methyl 4-(aminomethyl)benzoate hydrochloride (Sigma-Aldrich) in 5.6 mL of anhydrous DMF was added 0.76 mL (5.4 mmol) of triethylamine followed by the lopinavir aliphatic NHS ester (**37**) solution prepared *in situ* from above. The reaction mixture was allowed to stir at room temperature for 18 hours, then concentrated on a rotary evaporator and then under high vacuum. The residue was dissolved in 35 mL of water and extracted with 4 x 100 mL of ethyl acetate. The combined organic layer was dried (Na₂SO₄) and concentrated. The residue was dissolved in 2:1 acetonitrile/water and purified by preparative RP-HPLC to give 254 mg (0.28 mmol, 83%) of **38** as a white solid. LC/MS (M+H): 905.3.

Example 30: Synthesis of 4-[3-(lopinavir-O^c)-acetamido]-propionamido]methylbenzoic acid (39)

A solution of lopinavir methyl ester (38, 302 mg, 0.33 mmol) in 10 mL of methanol and 10 mL of freshly distilled THF was prepared. To this was added a solution of 22 mg (0.52 mmol) of lithium hydroxide monohydrate in 22 mL of water. The reaction mixture was allowed to stir at room temperature for 3 days. The resulting solution was concentrated under reduced pressure and the pH adjusted to 3-4. White solids were observed to be formed. The aqueous mixture was extracted with 4 x 60 mL of ethyl acetate. The organic layers were combined, dried (Na₂SO₄) and concentrated. The residue was dissolved in 2:1 acetonitrile/water and purified by preparative RP-HPLC to give 245 mg (0.27 mmol, 82%) of lopinavir benzoic acid derivative 39 as a white solid. LC/MS (M+H): 891.3.

Example 31: Synthesis of 4-[3-(lopinavir-O^c)-acetamido]propinamido]methylbenzoic acid N-hydroxysuccinimide ester (40)

To a solution of 128 mg (0.14 mmol) of lopinavir acid 39 in 6.8 mL of freshly distilled THF (distilled over sodium and benzophenone) was added 130 mg (0.43 mmol) of O-(N-succinimidyl)-N,N,N',N'-tetramethyluronium tetrafluoroborate (Sigma-Aldrich) and 75 µL (0.42 mmol) of diisopropylethylamine.

The reaction mixture was allowed to stir at room temperature for 3 hours and then concentrated under reduced pressure. To the residue 5 mL of water was added, and the aqueous layer was extracted with 4 x 10 mL of ethyl acetate. The organic layers were combined, dried (anhydrous Na₂SO₄), and concentrated under reduced pressure. The residue was dissolved in 2:1 acetonitrile/water and purified by preparative RP-HPLC to give 102 mg (0.103 mmol, 72%) of lopinavir derivative 40 as a white solid. LC/MS (M+H): 988.3.

Example 32: Synthesis of atazanavir-O^c-(3-pyridyl)carbamate (41)

To a solution of 100 mg of atazanavir (20) in 1.0 mL of dry DMF was added 50 µL (just over 2 eq) of DIEA followed by 45 mg (~2.65 eq) of 3-isocyanatopyridine (Oakwood Products Inc., West Columbia, SC 29172; material used as received) and the reaction stirred at room temperature overnight in a capped flask. An additional 125 mg of 3-isocyanatopyridine (~7.35 eq; total = 10 eq) was added, the reaction mixture warmed with a heat gun to effect dissolution of reagent and the reaction allowed to stir overnight. Analysis by LC/MS indicated an ~1:1 mixture of product and starting material. Solvent and volatile material were removed under vacuum (high vacuum rotovap). The residue was redissolved in 1:1 acetonitrile (MeCN)/water (H₂O), filtered and the filtrate purified by preparative RP-HPLC, eluting with a 0.1% trifluoroacetic acid (TFA)/MeCN in 0.1% TFA/water gradient. The product fractions were collected, MeCN removed at room temperature (rotovap) and the residue frozen and lyophilized to give

the product **41** assigned as the partial TFA salt and as a white solid. LC/MS: $t_R \sim 10.9$ min, M+H (Parent) 825.4; $^1\text{H-NMR}$: Compatible.

Example 33: Synthesis of 4-(6-chloro-4-cyclopropylethynyl-2-oxo-4-trifluoromethyl-4H-benzo-[d][1,3]oxazin-1-yl)-[4-butyramidomethyl] benzoic acid (43**)**

5 The efavirenz derivative **42** was prepared according to the procedure described in US 2004/0214251, the disclosure of which is herein incorporated by reference. To 91 mg (0.60 mmol) of aminomethylbenzoic acid was added 3 mL of water, 650 μL of 1N NaOH followed by 10 mL of freshly distilled THF. To this reaction mixture was added a solution of 282 mg (0.56 mmol) of efavirenz NHS ester (**42**) in 7 mL of freshly distilled THF at room temperature. The pH of the reaction was maintained at 9 for a period of 2 h
10 and concentrated to remove THF as much as possible. To the residue 150 mL of water was added, and the pH of the resulting solution was adjusted to pH 5. This was extracted with 2 x 100 mL of chloroform. The organic layers were combined, dried (anhydrous Na_2SO_4) and concentrated. The residue was purified by preparative RP-HPLC using a gradient run consisting of water and acetonitrile containing 0.1% trifluoroacetic acid. Fractions containing the desired product were concentrated on a rotary evaporator and
15 lyophilized to give 74 mg (0.14 mmol, 24 %) of efavirenz NHS ester (**43**) as a white powder, LC-MS (M+H) 535.2.

Example 34: Synthesis of 4-(6-chloro-4-cyclopropylethynyl-2-oxo-4-trifluoromethyl-4H-benzo-[d][1,3]oxazin-1-yl)-[4-butyramidomethyl]benzoic acid N-hydroxysuccinimide ester (44**)**

To a solution of 63 mg (0.12 mmol) of efavirenz derivative **43** in 6 mL of dichloromethane were added 47
20 mg (0.25 mmol) of 1-ethyl-3(3-dimethylaminopropyl)carbodiimide, hydrochloride followed by 21 mg (0.18 mmol) of N-hydroxysuccinimide. The reaction mixture was allowed to stir at room temperature for 18 h and 40 mL of dichloromethane was added. The organic layer was washed with 15 mL of water and 15 mL of saturated sodium bicarbonate followed by 15 mL of water. This was dried (anhydrous Na_2SO_4) and concentrated. The residue was purified by preparative RP-HPLC using a gradient run consisting of
25 water and acetonitrile containing 0.1% trifluoroacetic acid. Fractions containing the desired product were concentrated on a rotary evaporator and lyophilized to give 70 mg (0.11 mmol, 94 %) of efavirenz NHS ester (**44**) as a white powder, LC-MS (M+H) 632.0.

Example 35: Efavirenz-aminodextran conjugate (45**)**

Aminodextran (MW 40,000) was prepared according to a procedure described in US 6,653,456, the
30 content of which is incorporated herein by reference. To 65 mg of aminodextran was added 4.5 mL of

DMSO at room temperature. The mixture was allowed to stir at room temperature until all aminodextran went into solution. To the reaction mixture 19.5 μ L (0.138 mmol) of triethylamine was added. 24.6 mg (0.037 mmol) of the efavirenz derivative **44**, 24.6 mg (0.037 mmol) was dissolved in 3 mL of anhydrous DMSO, and 1 ml of this solution was added dropwise to the stirred aminodextran solution. The mixture
5 was allowed to stir at room temperature for 44 hours and was transferred into SPECTRAPOR dialysis tubing (Spectrum Medical Industries, mw cut-off 2000) and dialyzed (each dialysis using 1 L volume) according to the following schedule (1 L volume, at least 8 h each) at room temperature: 80% DMSO, 60% DMSO, 40% DMSO, and 20% DMSO in deionized water followed by deionized water. The solution was taken out of the dialysis tubing and lyophilized to give 60 mg of efavirenz -dextran conjugate **45** as a
10 white powder.

Example 36: Synthesis of N-(tert-butyldimethylsilyloxyethyl) efavirenz (18a**)**

To a solution of 300 mg (0.95 mmol) of efavirenz in 4 mL of anhydrous DMF were added 408 μ L (1.88 mmol) of (2-bromoethoxy)-tert-butyl-dimethylsilane, 275 mg (1.98 mmol) potassium carbonate, 5 mg of 18-crown-6, and 50 mg (0.33 mmol) sodium iodide. The residue was washed with 2 x 5 mL of ethyl
15 acetate and concentrated. The same reaction was repeated with 100 mg efavirenz. The crude products from both batches were combined and dissolved in 150 mL of ethyl acetate. The organic layer was washed with 3 x 50 mL of water, dried (anhydrous Na₂SO₄) and concentrated. The residue was purified by silica gel column chromatography using 20% ethyl acetate in hexane to give 600 mg (1.22 mmol, 97 %) of N-(tert-butyldimethylsilyloxyethyl) efavirenz (**18a**) as colorless gum.

Example 37: Synthesis of N-hydroxyethyl efavirenz (19a**)**

To 380 mg (0.80 mmol) of N-(tert-butyldimethylsilyloxyethyl)efavirenz (**18a**) in 3 mL of was added 3 mL of freshly distilled THF followed by 800 μ L of 1 M tetrabutylammonium fluoride. The resulting reaction mixture was allowed to stir at room temperature for 18 hours and concentrated under reduced pressure. The residue was purified by silica gel column chromatography using 50% hexane in ethyl
25 acetate. The isolated product indicated some impurities and was further purified by preparative RP-HPLC using a gradient run consisting of water and acetonitrile containing 0.1% trifluoroacetic acid. Fractions containing the desired product were concentrated on a rotary evaporator and lyophilized to give 180 mg (0.50 mmol, 63%) of N-hydroxyethyl efavirenz (**19a**) as a white powder, LC-MS (M+H) 360.0.

Example 38: Synthesis of N-isopropyl efavirenz (16a)

To 300 mg (0.95 mmol) of efavirenz was added 5 mL of anhydrous DMF followed by 263 mg (1.95 mmol) of anhydrous potassium carbonate, 60 mg (0.040 mmol) of sodium iodide, 178 μ L (1.90 mmol) of 2-bromopropane, and 5 mg of 18-crown-6. The resulting reaction mixture was allowed to stir at 120°C for 2 hours and then cooled to room temperature. The reaction mixture was filtered, and the filtrate was concentrated under reduced pressure. The same reaction was repeated with 100 mg efavirenz. The crude products from both batches were combined and purified by preparative RP-HPLC using a gradient run consisting of water and acetonitrile containing 0.1% trifluoroacetic acid. Fractions containing the desired product were concentrated on a rotary evaporator and lyophilized to give 295 mg (0.82 mmol, 87 %) N-isopropyl efavirenz (16a), LC-MS (M+H) 358.0.

Example 39: Synthesis of N-isobutyl efavirenz (17a)

To a solution of 300 mg (0.95 mmol) of efavirenz in 5 mL of anhydrous DMF was added 272 mg (1.96 mmol) of anhydrous potassium carbonate, 5 mg of 18-crown-6, and 225 μ L (1.94 mmol) of 2-bromo-2-methylpropane. The mixture was heated to 120°C for 2 hours, cooled to room temperature and filtered. The residue was washed with 2 x 5 mL of ethyl acetate. All the filtrates were combined and concentrated under reduced pressure. The same reaction was repeated with 100 mg efavirenz. The crude product from both batches was combined, and the residue was purified by preparative RP-HPLC using a gradient run consisting of water and acetonitrile containing 0.1% trifluoroacetic acid. Fractions containing the desired product were concentrated on a rotary evaporator and lyophilized to give 270 mg (0.72 mmol, 57%) of N-isobutyl efavirenz (17a), LC/MS (M+H) 372.0.

Example 40: Development of antibodies to lopinavir

Antibodies to lopinavir were developed as described in US 7,193,065.

Example 41: Preparation of lopinavir aminodextran conjugate

One hundred mg of aminodextran (AMD) was dissolved in 10 mL of anhydrous dimethylsulfoxide (DMSO). To this solution 30 μ L of triethylamine was added. The aminodextran solution was stirred continuously. A solution of 19.8 mg of lopinavir derivative 40 in anhydrous DMSO (1 mL) was added slowly to the aminodextran solution, and the reaction mixture was stirred for 48 hours at room temperature. After 48 hours, the entire solution was transferred to dialysis tubing (MW cut-off = 2000) and dialyzed against DMSO:water at room temperature for over 5 days with decreasing amounts of

DMSO. After the final dialysis against water, the lopinavir-aminodextran conjugate (LPV-AMD) was lyophilized to dryness. The LPV:AMD stoichiometry was determined by UV-Vis spectroscopy using $\epsilon_{250} = 16,495 \text{ M}^{-1}\text{cm}^{-1}$ and assuming that AMD did not contribute significantly to the UV absorbance at that wavelength. The ratio of LPV to AMD in the LPV-AMD conjugate was found to be 3.75.

5 **Example 42: Preparation of lopinavir antibody microparticles**

Five monoclonal antibodies to lopinavir (LPV Abs), 1.1.85, 1.12.0, 1.7.90, 1.50.0 and 1.39.0, were screened during the development of the immunoassay. Covalent attachment of monoclonal lopinavir antibodies to latex microparticles was performed as described below.

One percent latex microparticles (number of carboxy groups = 0.21 mmol/g latex, Seradyne Inc.) were washed free of the original storage solution by washing with 50 mM 2-morpholino-ethanesulfonic acid (MES), pH 5.5. The desired volume of 1% microparticles in 50 mM MES, pH 5.5 was measured out and the microparticles activated by the addition of *N*-hydroxysulfosuccinimide (sulfo-NHS) followed immediately by the addition of *N*-ethyl-*N'*-(3-dimethyl-aminopropyl)carbodiimide hydrochloride (EDC·HCl). Both sulfo-NHS and EDC were added such that the number of moles of each reagent was 10 times that of the number of carboxylates present on the surface of the microparticles. After stirring for 2 hours at room temperature, the mixture was centrifuged (15,000 rpm, 4°C, 45-60 minutes), and the supernatant decanted. The activated microparticles were resuspended to a concentration of 1% by sonication in 50 mM 3-morpholinopropanesulfonic acid (MOPS), pH 6.4. Monodispersion after sonication was confirmed by light scattering measurements in a COBAS MIRA analyzer.

A solution of lopinavir monoclonal antibodies in 50 mM MOPS buffer, pH 6.4, was added to the resuspended microparticles such that there was 10 µg antibody/mg latex. The antibody-latex mixture was stirred for 2 hours at room temperature. After 2 hours, a solution of 50 mg/mL of BSA in 50 mM MOPS, pH 6.4, was added to the latex mixture (2.5 mg BSA/mg latex). After 2 hours, a solution of 840 mM 2-aminoethoxyethanol in 50 mM MOPS containing 0.09% (w/v) NaN_3 , pH 8.5, was added to the latex mixture. After stirring overnight, the mixture was centrifuged (15,000 rpm, 4 °C, 45-60 minutes), and the latex resuspended by sonication in storage buffer (50 mM MOPS, pH 7.2, 0.1% bovine serum albumin (BSA), 0.09% azide). This was followed by another centrifugation and sonication to give, finally, 1% latex, which was stored at 2-8°C in storage buffer (50 mM MOPS, pH 7.2, 0.1% BSA, 0.09% azide).

Example 43: Cross-reactivity of lopinavir analogs to lopinavir antibodies

Assays were performed on Roche/Hitachi 917 clinical chemistry analyzers (Roche Diagnostics GmbH) at 37°C. The reaction mixtures (296 µL) contained 3 µL of a lopinavir calibrator or sample as described below, 0.05% (w/v) microparticles from Example 34, 0.4 µg/mL LPV-AMD conjugate, 0.6-0.8% (w/v) polyacrylic acid (PAA), 50 mM KSCN, 5 mM triethanolamine, 1.25 mM ethylenediaminetetraacetic acid, 0.5% (w/v) BSA, 0.09% (w/v) NaN₃, 90 mM piperazine-1,4-bis(2-ethanesulfonic acid), 25 mM MOPS, pH 7.3. Assays were monitored spectrophotometrically by following agglutination of the microparticles at a wavelength of 600 nm.

Reagents were calibrated against a multi-analyte set of calibrators that contained lopinavir at concentrations of 0, 0.8, 1.6, 3.2, 6.4, and 12.8 µg/mL in human serum for therapeutic drug monitoring (TDM serum) (Valley Biomedical Products and Services, Inc., Winchester, VA), containing 0.1% (w/v) *D*-α-tocopheryl polyethylene glycol-1000 succinate (TPGS, Eastman Chemical Co). Ethanolic stock solutions of lopinavir, lopinavir M-1 metabolite, and each of the lopinavir analogs were prepared gravimetrically. Samples for the assay were prepared by spiking each drug from the ethanolic stock solutions into TDM serum to concentrations of 0.8, 1.6, 3.2, 6.4, and 12.8 µg/mL. Each sample was assayed in triplicate. Cross-reactivities of the analogs were calculated relative to lopinavir. The results obtained are shown in Table 1 below.

Alternatively, the lopinavir immunoassay reagents with lopinavir antibodies LPV 1.1.85 and LPV 1.7.90 were calibrated against calibrators that contained only the N-carbamoyl-lopinavir analog (**13**). The calibration curves for lopinavir antibodies LPV 1.1.85 and LPV 1.7.90 obtained when lopinavir or N-carbamoyl-lopinavir (**13**) was used as calibrator are shown in Figures 12a and 12b. Table 1 below shows the cross-reactivity of lopinavir analogs and metabolite to lopinavir antibodies relative to lopinavir. Values listed are percents.

Table 1

	LPV 1.1.85	LPV 1.12.0	LPV 1.7.90	LPV 1.39.0	LPV 1.50.0
Lopinavir	100	100	100	100	100
Lopinavir M1 metabolite	100	90	130	90	95
Lopinavir-Oc-methylether (2)	73	50	40	45	45
Lopinavir-Oc-(methoxycarbonylmethyl) ether (3)	130	130	150	130	130
Lopinavir-Oc-(N-tert- butylcarbamoylemethyl) ether (6)	65	110	215	105	105
Lopinavir-Oc-carbamate (8)	550	60	170	60	60
N-carbamoyl-lopinavir-Oc-carbamate (10)	500	75	150	65	75
N-Carbamoyl-lopinavir (13)	95	130	100	130	125

Example 44: Development of antibodies to atazanavir

Antibodies to atazanavir were developed as described in patent application publication US 2005/0064517.

Example 45: Preparation of atazanavir-aminodextran conjugate

5 Aminodextran (AMD) was synthesized as described in US 6,653,456, the disclosure of which is incorporated herein by reference, and contained 6 amino groups per mole with a molecular weight of ca. 40,000. ATV-N-hydroxysuccinimide (NHS) ester derivative **33** was synthesized as described in Example 26. ATV-AMD conjugate was prepared by reaction of an 8- to 16-fold excess of ATV-NHS ester derivative **33** with AMD in anhydrous dimethylsulfoxide (DMSO) in the presence of triethylamine (0.3
10 μ L TEA/1 mg AMD). The reaction mixture was stirred at room temperature for 23 hours and then dialyzed against DMSO - H₂O mixtures containing progressively lower DMSO contents. The final, aqueous solution was lyophilized to dryness and the ATV:AMD stoichiometry determined by UV-Vis spectroscopy using $\epsilon_{250} = 16,495 \text{ M}^{-1}\text{cm}^{-1}$ and assuming that AMD does not contribute significantly to the UV absorbance at that wavelength. Lyophilization was performed on a Virtis Freezemobile 25ES freeze-
15 drier. UV-Visible (UV-Vis) spectra were recorded on a Cary 50 Bio spectrophotometer (Varian).

Example 46: Preparation of atazanavir antibody microparticles

Carboxylated polystyrene microparticles (Part No. 83000520100390) were obtained from Seradyn Inc. (Indianapolis, IN) as a 10% (w/v) aqueous suspension. The mean microparticle diameter was ca. 200 nm, and the surface charge density of carboxyl groups was 0.29 mmol/g (microparticles). Centrifugation was performed on a DuPont Sorvall RC-5B centrifuge at 4°C. The microparticles were washed extensively prior to use by at least three cycles of centrifugation and resuspension in water followed by 50 mM 2-morpholinoethanesulfonic acid (MES), pH 5.5. Resuspension of chilled microparticles was effected by 20 to 120 s bursts of sonication using a Homogenizer 4710 Series sonicator (Cole-Parmer) at 25 to 45% output power. Determination of microparticle monodispersities and concentrations was performed on a COBAS MIRA analyzer by light scattering at multiple wavelengths. The washed and resuspended microparticles were stored in 50 mM MES, pH 5.5, at 4°C until required.

ATV antibodies (from clones 5.1, 14.3, 28.3, 36.3, 62.1.1, 71.1.1) were covalently attached to microparticle surfaces as follows. To 180 mL of 1.00% (w/v) microparticles in 50 mM MES pH 5.5, was added 22.55 mL of 50 mg/mL *N*-hydroxysulfosuccinimide in the same buffer, followed by 20.04 mL of 50 mg/mL aqueous *N*-ethyl-*N'*-(3-dimethyl-aminopropyl)carbodiimide hydrochloride. The mixture was stirred at room temperature for 1 hour before centrifugation (15,000 rpm, 4°C, 45 minutes). The microparticles were resuspended in 50 mM 3-morpholinopropanesulfonic acid (MOPS), 0.09% (w/v) NaN₃, pH 6.4 (Buffer A) to 1.0% (w/v), and divided into 10 mL aliquots. To each aliquot was added 2 mL of a solution containing 0.25 to 1.5 mg of an ATV Ab in Buffer A. After stirring at room temperature for 1.5 hours, 5 mL of 50 mg/mL bovine serum albumin (BSA) in Buffer A was added and the mixture stirred at room temperature for a further 1.5 hours before addition of 6.9 mL of 840 mM 2-aminoethoxyethanol in 50 mM MOPS, 0.09% (w/v) NaN₃, pH 8.5. The mixture was stirred overnight at room temperature and then subjected to centrifugation (15,000 rpm, 4°C, 45 minutes). The microparticles were resuspended in 50 mM MOPS, 0.1% (w/v) BSA, 0.09% (w/v) NaN₃, pH 7.5 (Buffer B) and collected by centrifugation (15,000 rpm, 4°C, 45 minutes). The pellets were resuspended in Buffer B to 1.0% (w/v) and stored at 4°C until required.

Example 47: Cross-reactivity of atazanavir analogs to atazanavir antibodies

Stock solutions of atazanavir parent drug and analogs were prepared in EtOH and the concentration of each compound verified using $\epsilon_{250} = 14,520 \text{ M}^{-1} \text{ cm}^{-1}$ (free base). The EtOH stocks were used to prepare 0.10 to 3.35 $\mu\text{g/mL}$ (free base) samples of the compounds in purchased human serum for therapeutic drug monitoring (TDM) (Valley Biomedical Products and Services, Inc., Winchester, VA), containing 0.1%

(w/v) *D*- α -tocopheryl polyethylene glycol-1000 succinate (TPGS) (Eastman Chemical Co., Kingsport, TN). A set of calibrators containing 0.00, 0.15, 0.33, 0.75, 1.50, and 3.00 $\mu\text{g/mL}$ of atazanavir parent drug was made in similar fashion from an independently prepared ethanolic stock. Polyacrylic acid (PAA, molecular weight 225,000) was purchased from Polysciences Inc. (Warrington, PA). Assays were

5 performed on a Roche/Hitachi 917 clinical chemistry analyzer at 37°C. The reaction mixtures (296 μL) contained 3 μL of an ATV calibrator, atazanavir parent drug or atazanavir analog prepared as described above, 0.05% (w/v) sensitized microparticles, 0.05-0.15 $\mu\text{g/mL}$ ATV-AMD conjugate, 0.5-1.0% (w/v) PAA, 50 mM KSCN, 5 mM triethanolamine, 1.25 mM ethylenediaminetetraacetic acid, 0.1% (w/v) BSA, 0.09% (w/v) NaN_3 , 90 mM piperazine-1,4-bis(2-ethanesulfonic acid), and 25 mM MOPS, pH 7.3. Assays

10 were monitored spectrophotometrically by following the agglutination of microparticles at a wavelength of 600 nm. Cross-reactivities were determined by comparing the response of the analog to that of an equivalent concentration of parent drug. The cross-reactivity of atazanavir and atazanavir analogs to each antibody is shown in Table 2 and is derived from at least three independent sets of measurements. Cross-reactivity of the analogs was calculated relative to the parent drug, atazanavir. Values listed are percents.

15 Table 2

	ATV 5.1	ATV 14.3	ATV 28.3	ATV 36.3	ATV 62.1.1	ATV 71.1.1
Atazanavir (<u>20</u>)	100	100	100	100	100	100
Atazanavir-Oc-carbamate (<u>25</u>)	113	122	121	119	129	112
Atazanavir-Oc-ethylcarbamate (<u>21</u>)	171	102	127	101	143	150
Atazanavir-Oc-isopropylcarbamate (<u>22</u>)	157	79	124	82	139	149
Atazanavir-Oc-tertbutylcarbamate (<u>23</u>)	126	71	117	76	120	118

Example 48: Microparticle immunoassay calibration curves using atazanavir or atazanavir-Oc-carbamate (25)

Calibrators of either atazanavir or atazanavir-O^c-carbamate (25) in human serum for TDM containing 0.1

20 % (w/v) TPGS were prepared as described in Example 47. The free base concentrations of atazanavir or atazanavir-O^c-carbamate were 0.00, 0.15, 0.33, 0.75, 1.50, and 3.00 $\mu\text{g/mL}$. Assays were performed as

described in Example 47 using microparticles sensitized with antibody ATV 5.1, yielding the calibration curves shown in Figure 16.

The nearly superimposable calibration curves obtained with either compound confirm the cross-reactivity data of atazanavir-O^c-carbamate to antibody ATV 5.1 in Table 2, and provide additional assurance that
5 using either atazanavir or atazanavir-O^c-carbamate as calibrators for an atazanavir TDM immunoassay will yield similar results when assaying clinical samples containing atazanavir.

Example 49: Atazanavir microparticle immunoassay spiking recovery study using atazanavir-O^c-carbamate calibrators

Individual serum samples from 10 healthy blood donors were spiked with atazanavir from an ethanolic
10 stock solution to give 55 discrete serum samples containing atazanavir concentrations ranging from 0.04 to 3.00 µgml⁻¹. Each sample was quantitated by atazanavir microparticle immunoassay against an atazanavir-O^c-carbamate calibration curve obtained as described in Example 47. The assays were performed as described in Example 47 using microparticles sensitized with antibody ATV 5.1 except that the final reaction mixture additionally contained 0.07 % (w/v) BRIJ-35 (Croda Uniqema Inc.). A plot of
15 the measured concentrations of atazanavir against the spiked concentrations is shown in Figure 17 along with a linear regression analysis of the data.

The linear regression analysis in which both the slope (0.91) and correlation coefficient (0.994) fall within the required ranges (0.90 - 1.10 and > 0.90 respectively), confirm the suitability of using atazanavir-O^c-carbamate as calibrators for quantitating atazanavir in human plasma or serum.

20 Example 50: Lopinavir microparticle immunoassay spiking recovery study using N-carbamoyl-lopinavir (13) calibrators

Individual serum samples from 10 healthy blood donors were spiked with lopinavir from an ethanolic stock solution to give 55 discrete serum samples containing lopinavir concentrations ranging from 0.21 to 12.80 µgml⁻¹. Each sample was quantitated by lopinavir microparticle immunoassay against an N-
25 carbamoyl-lopinavir (13) calibration curve obtained as described in Example 43. The assays were run as described in Example 43 using microparticles sensitized with antibody LPV 1.7.90 except that the final reaction mixture additionally contained 0.08 % (w/v) BRIJ-35. A plot of the measured concentrations of lopinavir against the spiked concentrations is shown in Figure 18 along with a linear regression analysis of the data.

The linear regression analysis in which both the slope (1.00) and correlation coefficient (0.994) fall within the required ranges (0.90 - 1.10 and > 0.90 respectively), confirm the suitability of using N-carbamoyl-lopinavir as calibrators for quantitating lopinavir in human plasma or serum.

Example 51: Development of antibodies to efavirenz.

5 Female Balb/c mice of 16 weeks of age were immunized with efavirenz-KLH immunogen, 46. The preparation of efavirenz-KLH immunogen 46 has been described in EP1470 825 A1, the disclosure of which is incorporated herein by reference. Initial immunizations were of 100 µg of immunogen emulsified in Freund's Complete Adjuvant, given via intraperitoneal route. Approximately three week intervals were allowed to pass between immunizations. All subsequent immunizations were of the same
10 dosage emulsified in Freund's Incomplete Adjuvant, also via intraperitoneal injection. A total of four immunizations were given.

Fusion

Four days following the last immunization, the mouse was killed by cervical dislocation and the spleen harvested. The spleen was processed by grinding between two sterile frosted glass slides in warmed cell
15 culture media. The liberated cells were taken up into a sterile 15 mL centrifuge tube and large fragments allowed to settle for 1 to 2 minutes. The suspended cells were pipetted into a separate sterile 50 mL centrifuge tube and a sample taken for counting. Myeloma cells of the F0 strain (ATCC) were added at a ratio of 1 myeloma cell to 5 splenocytes and the resulting mixture was centrifuged to settle the cells into a compact pellet. Fusion comprised adding myeloma cells (1/5 the number of lymphocytes), washing *via*
20 centrifugation, resuspension in serum-free warm Iscove's Modified Dulbecco's Media (IMDM, Irvine Scientific), and re-centrifugation. The centrifuge tubes containing the resulting pellets were gently tapped to loosen the cells, then 1 mL of warmed PEG/DMSO solution (Sigma Chemicals) was slowly added with gentle mixing. The cells were kept warm for 1.5 minutes, after which pre-warmed serum-free IMDM was added at the rates of 1 mL/minutes, 2 mL/minutes, 4 mL/minutes, and 10mL/minutes, then the tube was
25 filled to 50 mL, sealed, and incubated for 15 minutes. The cell suspensions were centrifuged, the supernatant decanted, and IMDM containing 10% fetal calf serum (Summit Biologicals) was added. The cells were centrifuged once again and resuspended in complete cloning medium. This contained IMDM, 10% FCS, 10% Condimed H1 (Roche Molecular Systems), 4 mM glutamine, 50 µM 2-mercaptoethanol, 40 µM ethanolamine, and pen/strep antibiotics (all from Sigma). The cells were suspended at a density of
30 4×10^5 lymphocytes/mL, distributed 100 µL/well into sterile 96-well microculture plates, and incubated at 37°C in 5% CO₂ for 24 hours. The next day, 100 µL of HMT selective medium (cloning medium +

1:25 HMT supplement from Sigma Chemicals) was added. On the 6th day of incubation, approximately 150 μ L of media was drawn from each well using a sterile 8-place manifold connected to a light vacuum source. One hundred fifty microliters of HT media was then added. This contained cloning medium + 1:50 HT supplement (Sigma Chemicals). The plates were returned to the incubator and inspected daily for signs of growth. When growth was judged sufficient, wells were screened for antibody production via ELISA.

ELISA screening

Microtiter plates were coated with 100 μ L efavirenz-BSA conjugate 47 in 0.1 M carbonate buffer, pH 9.5, for 1 hour at 37°C (humidified). The preparation of efavirenz-BSA conjugate 47 has been described in EP1470 825 A1. The plates were then emptied and filled with a post-coat solution consisting of Tris buffer, 1% gelatin hydrolysate, 2% sucrose, and 0.17% TWEEN 20 (all reagents were from Sigma Chemicals). The plates were incubated for an additional 1 hour at 37°C (humidified) after which they were washed with phosphate buffered saline containing 0.1% TWEEN 20. The plates were then filled briefly with a 2% sucrose solution in 0.15M Tris, pH 7.2-7.4, then emptied and allowed to air dry at room temperature. When dried, the plates were packed in ZIPLOC bags containing several desiccant pillows, sealed, and stored at 4°C until use.

When the growing clones were judged ready for testing, 25 μ L of supernatant from the wells were taken and transferred to 96 well flexible plates. Culture medium was added to each well to provide a 1:10 dilution of the media sample. Twenty-five microliters of PBS-TWEEN were added to each well of one coated microplate, and 25 microliters of an 800 ng/mL solution of chiral efavirenz in PBS-TWEEN were added to the wells of another coated plate. Twenty-five microliters of the diluted media samples were transferred to each of the coated plates. The plates were incubated covered for 1 hour at 37°C, then washed with PBS-TWEEN. The wells were then filled with 100 μ L of goat anti-mouse IgG-HRP conjugate (Zymed Labs) diluted in PBS-TWEEN and the plates re-incubated for 1 hour. The plates were then washed again, and 100 μ L of K-BLUE substrate (Neogen Corp) was added to each well. Color was allowed to develop for 5 to 15 minutes, the reaction being stopped by the addition of 100 μ L of 1 N HCl. Color was read via a microplate reader at 450 nm and results collected by computer for analysis. The criteria for selection was binding to the efavirenz-BSA conjugate-coated wells which received the PBS-TWEEN, and reduced binding to the coated wells containing free efavirenz drug.

Example 52: Preparation of efavirenz antibody microparticles

Carboxylated polystyrene microparticles (Part No. 83000520100390) were obtained from Seradyn Inc. (Indianapolis, IN) as a 10% (w/v) aqueous suspension. The mean microparticle diameter was ca. 200 nm, and the surface charge density of carboxyl groups was 0.21 mmol/g (microparticles). Centrifugation was performed on a DuPont Sorvall RC-5B centrifuge at 4°C. The microparticles were washed extensively prior to use by at least three cycles of centrifugation and resuspension in water followed by 50 mM 2-morpholinoethanesulfonic acid (MES), pH 5.5. Resuspension of chilled microparticles was effected by 20 to 120 s bursts of sonication using a Homogenizer 4710 Series sonicator (Cole-Parmer) at 25 to 45% output power. Determination of microparticle monodispersities and concentrations was performed on a COBAS MIRA analyzer by light scattering at multiple wavelengths. The washed and resuspended microparticles were stored in 50 mM MES, pH 5.5, at 4°C until required.

Efavirenz (EFV) antibodies (from clones 129.1.1.1, 137.1.1.1, 142.1.1.1.1, 143.1, and 149.1.3) were covalently attached to microparticle surfaces as follows. To 140 mL of 1.00% (w/v) microparticles in 50 mM MES pH 5.5, was added 12.7 mL of 50 mg/mL *N*-hydroxysulfosuccinimide in the same buffer, followed by 11.29 mL of 50 mg/mL aqueous *N*-ethyl-*N'*-(3-dimethyl-aminopropyl)carbodiimide hydrochloride. The mixture was stirred at room temperature for 1 hour before centrifugation (15,000 rpm, 4°C, 50 minutes). The microparticles were resuspended in 50 mM 3-morpholinopropanesulfonic acid (MOPS), 0.09% (w/v) NaN₃, pH 6.4 (Buffer A) to 1.00% (w/v), and divided into 10 mL aliquots. To each aliquot was added 2 mL of a solution containing 0.25 to 1.5 mg of an EFV Ab in Buffer A. After stirring at room temperature for 1.5 hours, 5 mL of 50 mg/mL bovine serum albumin (BSA) in Buffer A was added and the mixture stirred at room temperature for a further 1.5 hours before addition of 5.0 mL of 840 mM 2-aminoethoxyethanol in 50 mM MOPS, 0.09% (w/v) NaN₃, pH 8.5. The mixture was stirred overnight at room temperature and then subjected to centrifugation (15,000 rpm, 4°C, 50 minutes). The microparticles were resuspended in 50 mM MOPS, 1.0% (w/v) BSA, 0.09% (w/v) NaN₃, pH 7.2 (Buffer B) and collected by centrifugation (15,000 rpm, 4°C, 50 minutes). The pellets were resuspended in Buffer B to 1.00% (w/v) and stored at 4°C until required.

Example 53: Cross-reactivity of efavirenz analogs to efavirenz antibodies

Stock solutions of efavirenz parent drug and analogs were prepared in EtOH and the concentration of each compound verified using $\epsilon_{243} = 14,806 \text{ M}^{-1}\text{cm}^{-1}$ (free base). The EtOH stocks were used to prepare 1.00 to 7.00 µg/mL (free base) samples of the compounds in purchased human serum for therapeutic drug monitoring (TDM) (Valley Biomedical Products and Services, Inc., Winchester, VA), containing 0.1%

(w/v) *D*- α -tocopheryl polyethylene glycol-1000 succinate (TPGS) (Eastman Chemical Co., Kingsport, TN). A set of calibrators containing 0.00, 0.60, 1.20, 2.40, 4.80, and 9.60 μ g/mL of efavirenz parent drug was made in similar fashion from an independently prepared ethanolic stock. Assays were performed on a Roche/Hitachi 917 clinical chemistry analyzer at 37°C. The reaction mixtures (296 μ L) contained 3 μ L of an efavirenz calibrator, efavirenz parent drug or efavirenz analog prepared as described above, 0.05% (w/v) sensitized microparticles, 0.10-0.20 μ g/mL EFV-aminodextran conjugate **45**, 0.7-1.1% (w/v) polyacrylic acid (PAA, molecular weight 225,000, purchased from Polysciences Inc. (Warrington, PA)), 50 mM KSCN, 5 mM triethanolamine, 1.25 mM ethylenediaminetetraacetic acid, 1.0% (w/v) BSA, 0.09% (w/v) NaN₃, 90 mM piperazine-1,4-bis(2-ethanesulfonic acid), 25 mM MOPS, pH 7.2, and a surfactant as indicated in Table 3. Assays were monitored spectrophotometrically by following the agglutination of microparticles at a wavelength of 600 nm. Cross-reactivities were determined by comparing the response of the analog to that of an equivalent concentration of parent drug. The cross-reactivity of efavirenz and efavirenz analogs to each antibody is shown in Table 3.

Table 3

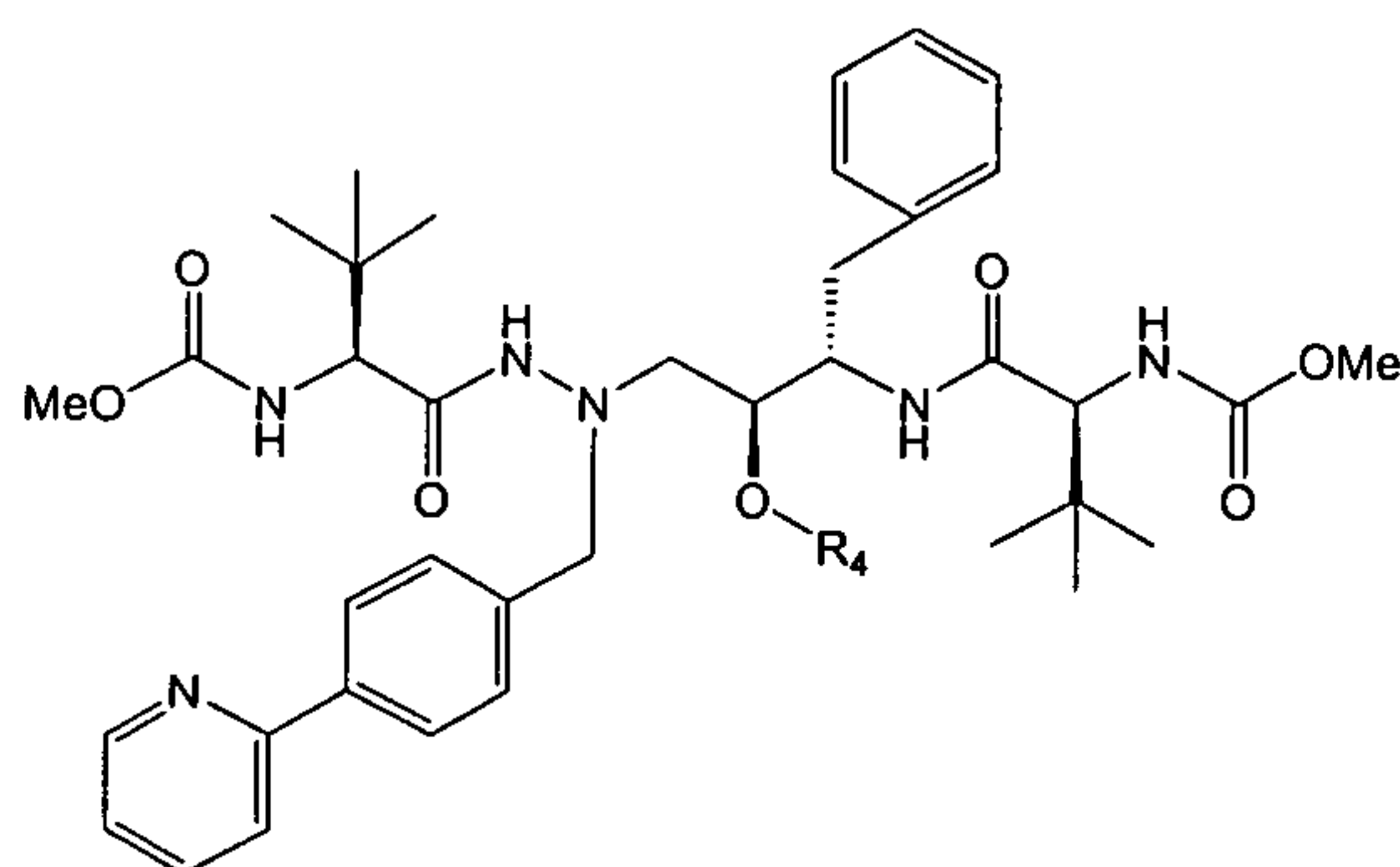
	EFV 129.1.1.1	EFV 137.1.1.1	EFV 142.1.1.1.1	EFV 143.1	EFV 149.1.3
Efavirenz	100	100	100	100	100
N-methyl efavirenz [†] (14)	13	129	116	21	151
N-ethyl efavirenz [†] (15)	15	58	49	N/D	53
N-isopropyl efavirenz [†] (16a)	N/D	58	48	7	41
N-propyl efavirenz [†] (16)	28	46	38	33	42
N-isobutyl efavirenz [†] (17a)	N/D	40	30	19	25
N-butyl efavirenz [†] (17)	43	38	33	39	33
N-hydroxyethyl efavirenz [†] (19a)	N/D	101	92	18	89
N-hydroxypropyl efavirenz [†] (19)	14	82	79	17	89

[†] The assay contains 0.05 % (w/v) BRIJ-35 for each Ab with the exception of Ab 143.1 where [BRIJ-35] = 0.1 % (w/v)

[‡] The assay surfactant concentrations are: Abs 137.1.1.1 and 142.1.1.1.1 ~ 0.05 % (w/v) TPGS, Ab 143.1 ~ 0.1 % (w/v) BRIJ-35, Ab 149.1.3 ~ 0.05 % (w/v) Pluronic-F127

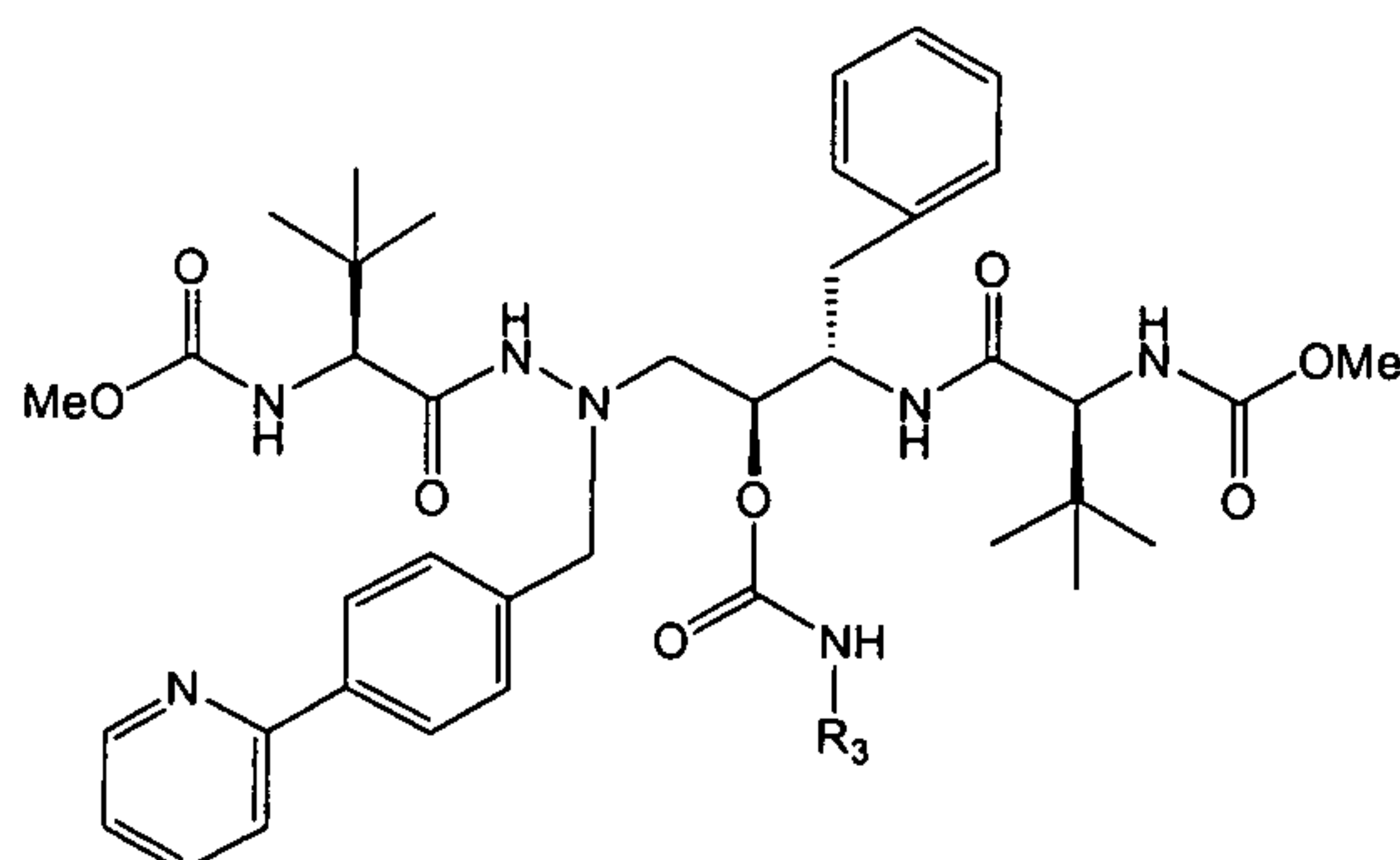
What is claimed is:

1. A compound comprising an HIV protease inhibitor selected from the group consisting of amprenavir, atazanavir, tipranavir, indinavir, darunavir, lopinavir, nelfinavir, ritonavir, and saquinavir, wherein the central hydroxyl group of the protease inhibitor is replaced with a group selected from the group consisting of $-O(C_1-C_{10} \text{ alkyl})$, $-OCONHR_3$, $-OCH_2COOR_3$, and $-OCH_2CONHR_3$, wherein R_3 is selected from the group consisting of H, C_1-C_6 alkyl, phenyl, heteroaryl, and $-(CH_2)_n-X$ wherein n is 1-6 and X is $COOR_5$ or $NH-CH_2\text{-phenyl-}COOR_5$ wherein R_5 is H or C_1-C_6 alkyl.
2. A compound having a structure



wherein R_4 is selected from the group consisting of C_1-C_{10} alkyl, $-CH_2COOR_3$, $-CH_2CONHR_3$, and $-CONHR_3$, wherein R_3 is selected from the group consisting of H, C_1-C_6 alkyl, phenyl, heteroaryl, and $-(CH_2)_n-X$ wherein n is 1-6 and X is $COOR_5$ or $NH-CH_2\text{-phenyl-}COOR_5$ wherein R_5 is H or C_1-C_6 alkyl.

3. A compound having a structure

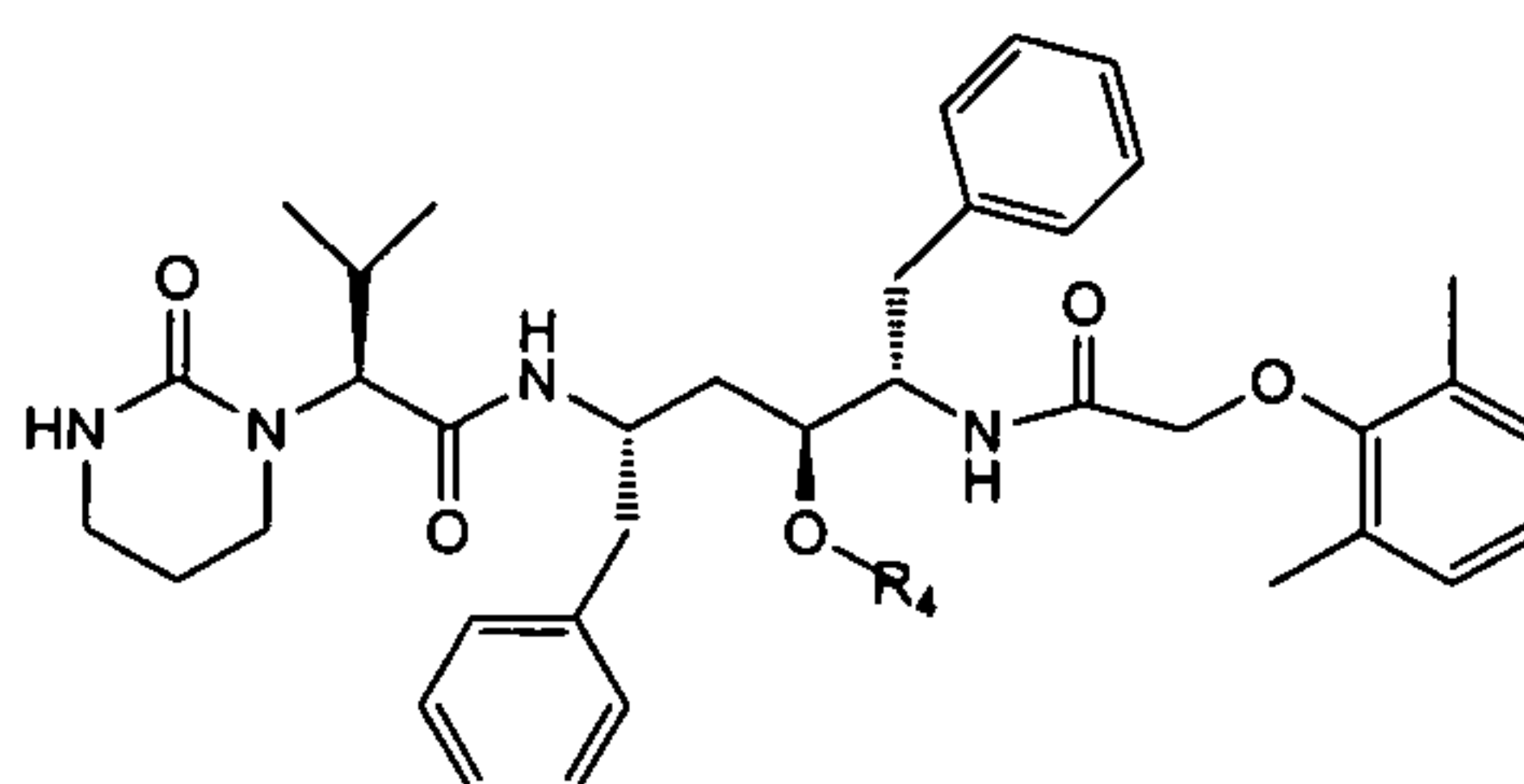


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wherein R_3 is selected from the group consisting of H, C_1 - C_6 alkyl, phenyl, heteroaryl, and $-(CH_2)_n-X$ wherein n is 1-6 and X is $COOR_5$ or $NH-CH_2$ -phenyl- $COOR_5$ wherein R_5 is H or C_1 - C_6 alkyl.

4. The compound of claim 3 wherein R_3 is H or C_1 - C_4 alkyl.

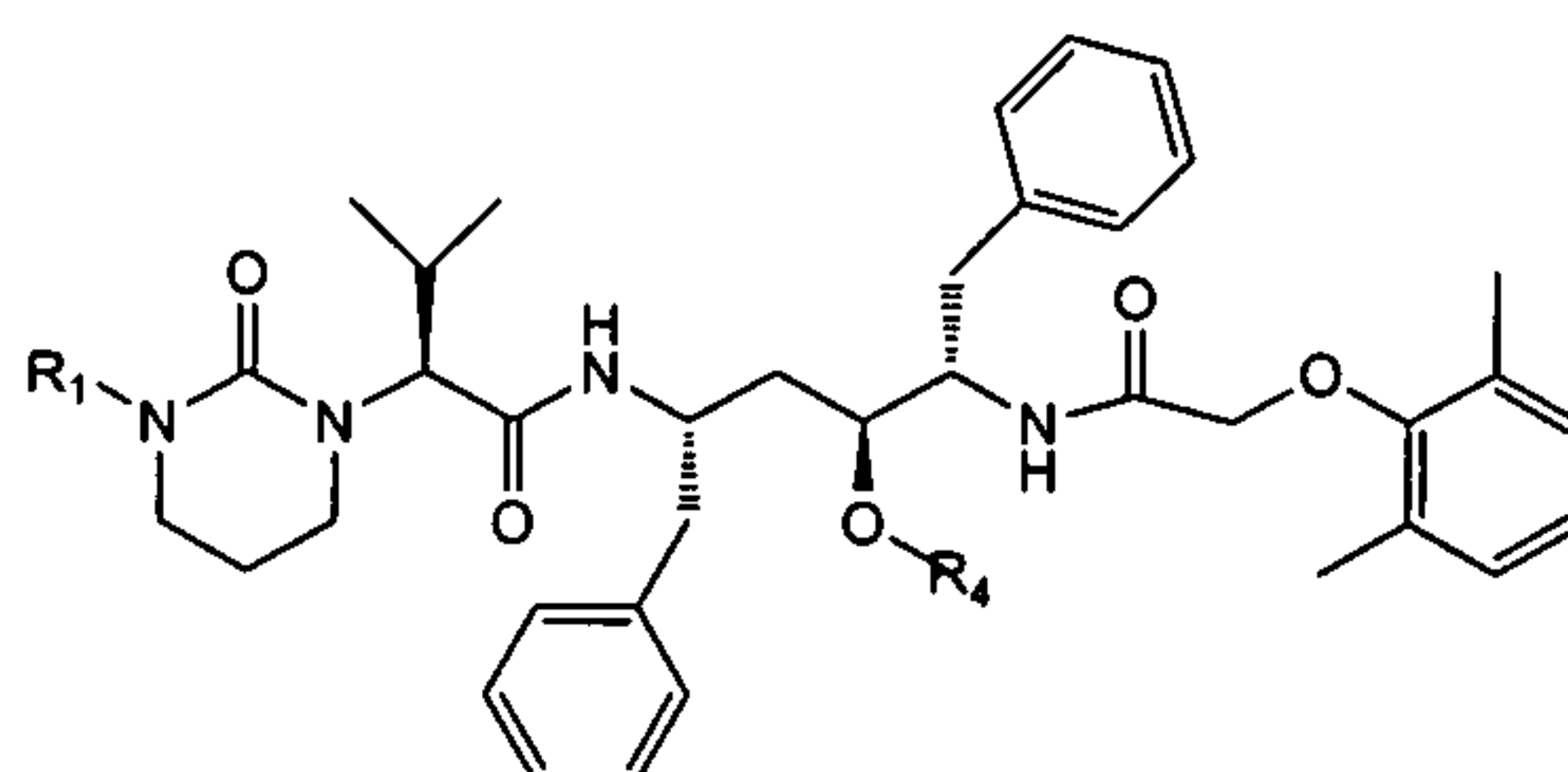
5. A compound having a structure



wherein R_4 is selected from the group consisting of $-(C_1-C_{10}$ alkyl), $-CONHR_3$, $-CH_2COOR_3$, and $-CH_2CONHR_3$, wherein R_3 is selected from the group consisting of H, C_1 - C_6 alkyl, phenyl, heteroaryl, and $-(CH_2)_n-X$ wherein n is 1-6 and X is $COOR_5$ or $NH-CH_2$ -phenyl- $COOR_5$ wherein R_5 is H or C_1 - C_6 alkyl.

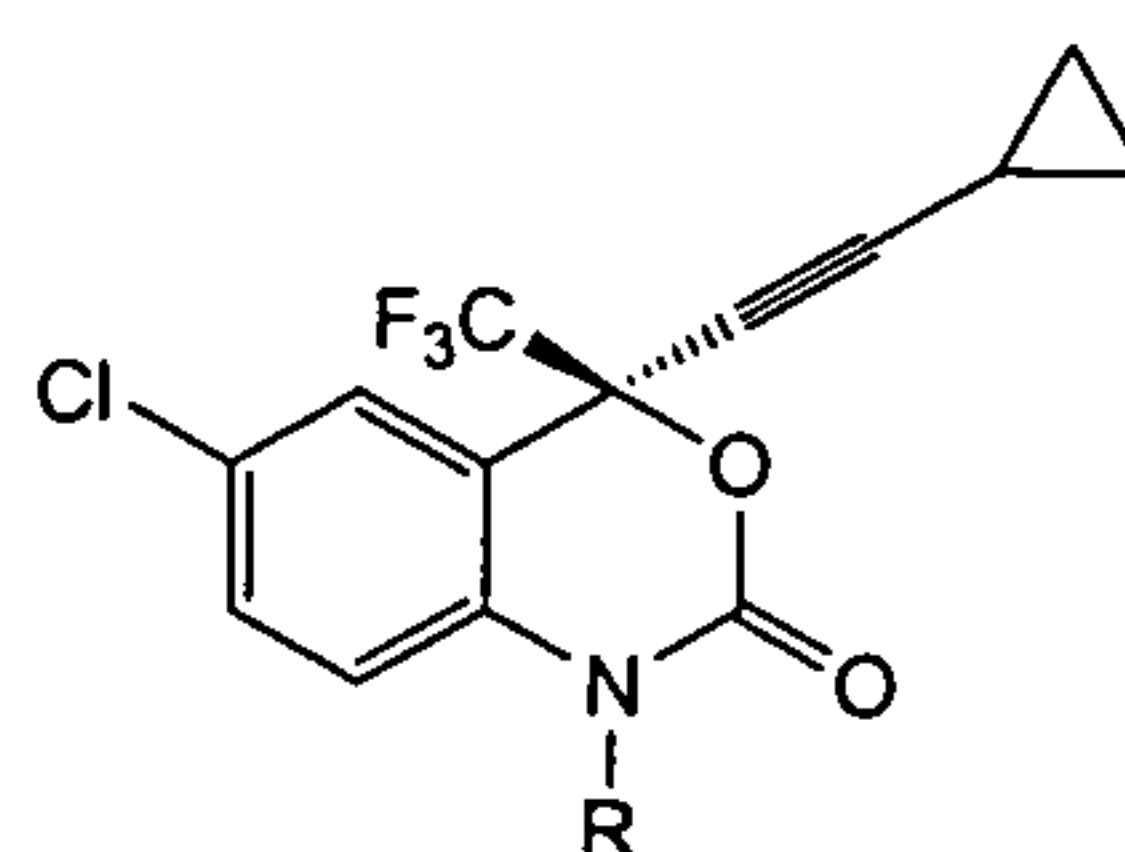
6. The compound of claim 5 wherein R_4 is $-CONHR_3$ or $-CH_2COOR_3$ and R_3 is selected from the group consisting of H, C_1 - C_6 alkyl, phenyl, heteroaryl, and $-(CH_2)_n-X$ wherein n is 1-6 and X is $COOR_5$ or $NH-CH_2$ -phenyl- $COOR_5$ wherein R_5 is H or C_1 - C_6 alkyl.

7. A compound having a structure



wherein R_1 is selected from the group consisting of H and $-CONHR_3$, and R_4 is selected from the group consisting of C_1 - C_{10} alkyl, $-CH_2COOR_3$, $-CH_2CONHR_3$ and $-CONHR_3$, wherein R_3 is selected from the group consisting of H, C_1 - C_6 alkyl, phenyl, heteroaryl, and $-(CH_2)_n-X$ wherein n is 1-6 and X is $COOR_5$ or $NH-CH_2$ -phenyl- $COOR_5$ wherein R_5 is H or C_1 - C_6 alkyl.

8. The compound of claim 7 wherein R_1 is H, R_4 is selected from the group consisting of $-\text{CH}_2\text{COOR}_3$, $-\text{CH}_2\text{CONHR}_3$, and $-\text{CONHR}_3$, and R_3 is H or $\text{C}_1\text{-C}_6$ alkyl.
9. The compound of claim 7 wherein R_1 is H, R_4 is $-\text{CONHR}_3$ or $-\text{CH}_2\text{COOR}_3$, and R_3 is $\text{C}_1\text{-C}_4$ alkyl.
10. The compound of claim 7 wherein R_1 is $-\text{CONHR}_3$, R_4 is H, and R_3 is H or $\text{C}_1\text{-C}_6$ alkyl.
11. The compound of claim 7 wherein R_1 is $-\text{CONHR}_3$, R_4 is $-\text{CONHR}_2$, and R_3 and R_2 are each $\text{C}_1\text{-C}_4$ alkyl.
12. A compound having a structure



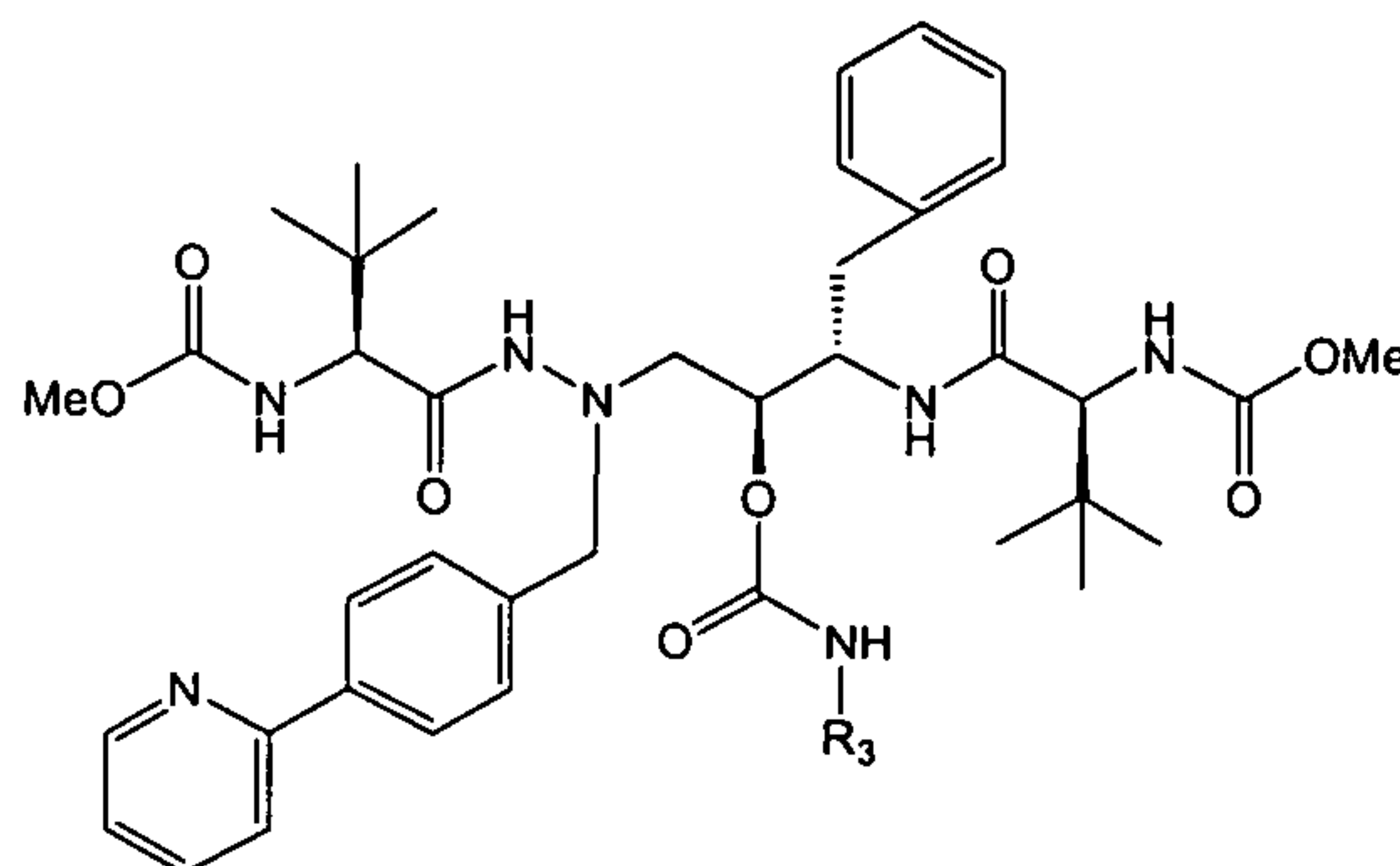
wherein R is $\text{C}_1\text{-C}_6$ alkyl or $(\text{C}_1\text{-C}_6 \text{ alkyl})\text{OH}$.

13. A test kit for determining the amount of a protease inhibitor in a sample, wherein the protease inhibitor is selected from the group consisting of amprenavir, atazanavir, tipranavir, indinavir, darunavir, lopinavir, nelfinavir, ritonavir and saquinavir, present in a sample, said kit comprising in packaged combination:

an antibody that specifically binds to said protease inhibitor,

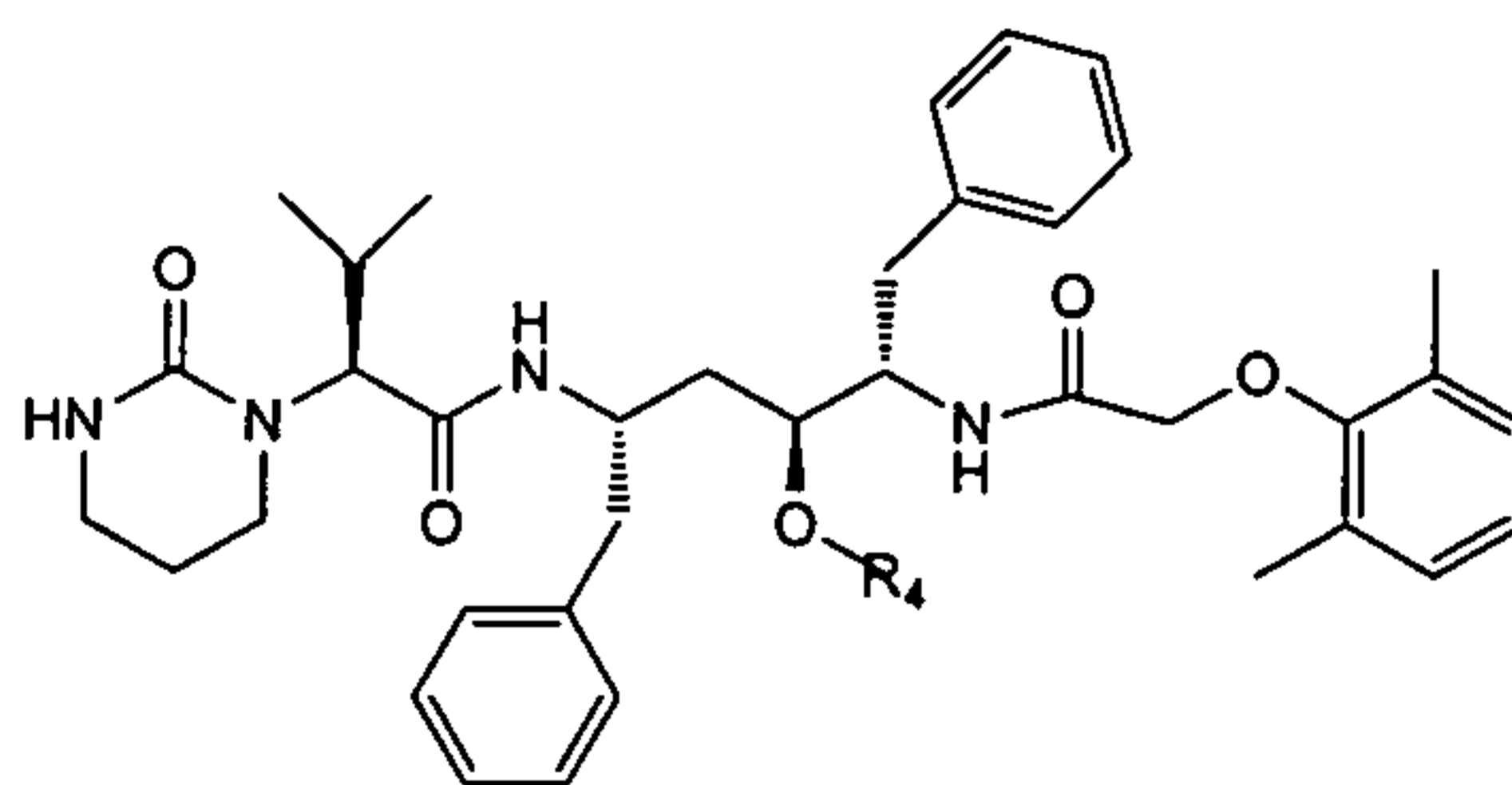
an analog of said protease inhibitor, wherein the central hydroxyl group of the protease inhibitor has been replaced with a group selected from the group consisting of $-\text{O}(\text{C}_1\text{-C}_{10} \text{ alkyl})$, $-\text{OCONHR}_3$, $-\text{OCH}_2\text{COOR}_3$, and $-\text{OCH}_2\text{CONHR}_3$, wherein R_3 is selected from the group consisting of H, $\text{C}_1\text{-C}_6$ alkyl, phenyl, heteroaryl, and $-(\text{CH}_2)_n\text{-X}$ wherein n is 1-6 and X is COOR_5 or $\text{NH-CH}_2\text{-phenyl-COOR}_5$ wherein R_5 is H or $\text{C}_1\text{-C}_6$ alkyl, and wherein the analog is immunochemically equivalent to the protease inhibitor, and

instructions for determining the amount of the protease inhibitor in the sample.
14. The test kit of claim 13 wherein the protease inhibitor is atazanavir and the analog has a structure



wherein R_3 is selected from the group consisting of H, C_1 - C_6 alkyl, phenyl, heteroaryl, and $-(CH_2)_n-X$ wherein n is 1-6 and X is $COOR_5$ or $NH-CH_2$ -phenyl- $COOR_5$ wherein R_5 is H or C_1 - C_6 alkyl.

15. The test kit of claim 14 wherein R_3 is H or C_1 - C_4 alkyl.
16. The test kit of claim 13 wherein the protease inhibitor is lopinavir and the analog has a structure



wherein R_4 is $-CONHR_3$ or $-CH_2COOR_3$ and R_3 is selected from the group consisting of H, C_1 - C_6 alkyl, phenyl, heteroaryl, and $-(CH_2)_n-X$ wherein n is 1-6 and X is $COOR_5$ or $NH-CH_2$ -phenyl- $COOR_5$ wherein R_5 is H or C_1 - C_6 alkyl.

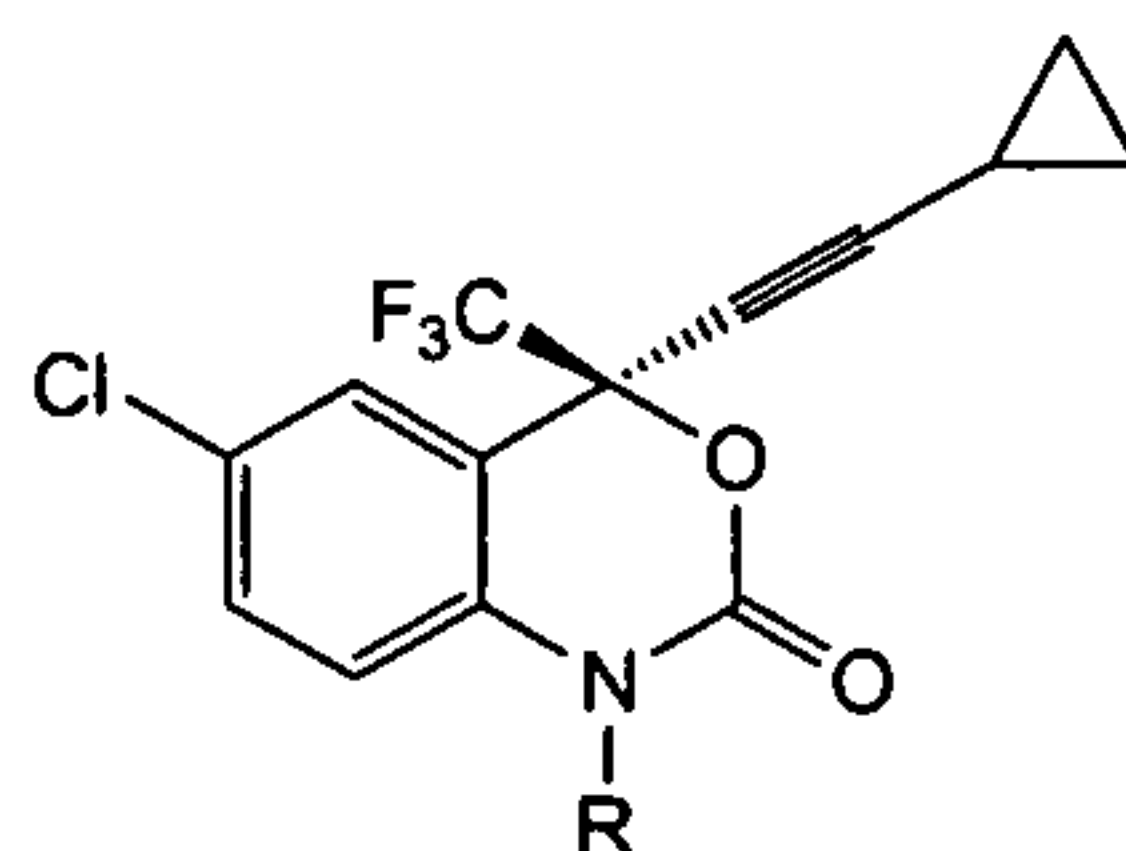
17. A test kit for determining the amount of efavirenz in a sample, said kit comprising in packaged combination:

an antibody that specifically binds to efavirenz,

an analog of efavirenz comprising an efavirenz derivative that has been N-alkylated with C_1 - C_6 alkyl or $(C_1$ - C_6 alkyl)OH, and wherein the analog is immunochemically equivalent to efavirenz, and

instructions for determining the amount of efavirenz in the sample.

18. The test kit of claim 17 wherein the analog has a structure



wherein R is C₁-C₆ alkyl or (C₁-C₆ alkyl)OH.

19. A method for preparing a calibration curve for an HIV protease inhibitor selected from the group consisting of amprenavir, atazanavir, tipranavir, indinavir, darunavir, lopinavir, nelfinavir, ritonavir, and saquinavir, said method comprising the steps of:

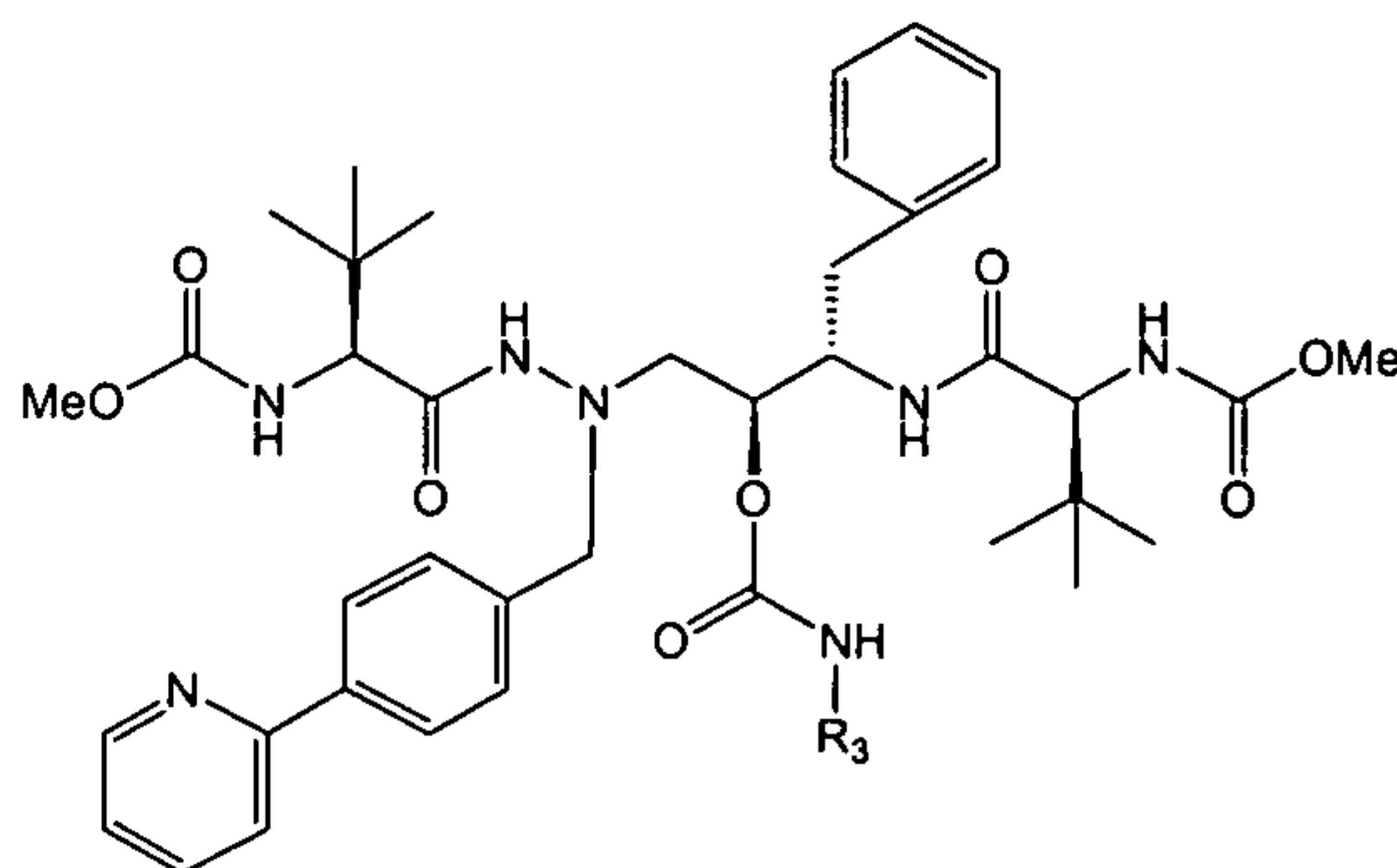
providing a set of calibrators, each calibrator containing one of a range of concentrations of an immunochemically equivalent analog of said protease inhibitor, said analog comprising a derivative of said protease inhibitor wherein the central hydroxyl group of the protease inhibitor has been replaced with a group selected from the group consisting of -O(C₁-C₁₀ alkyl), -OCONHR₃, -OCH₂CONHR₃, and -OCH₂COOR₃, wherein R₃ is selected from the group consisting of H, C₁-C₆ alkyl, phenyl, heteroaryl, and -(CH₂)_n-X wherein n is 1-6 and X is COOR₅ or NH-CH₂-phenyl-COOR₅ wherein R₅ is H or C₁-C₆ alkyl,

contacting each of the calibrators with a monoclonal antibody that specifically binds to said protease inhibitor and analog, and a conjugate comprising the protease inhibitor and a moiety that generates a signal upon binding of the antibody to the analog,

measuring the signal generated by each calibrator, and

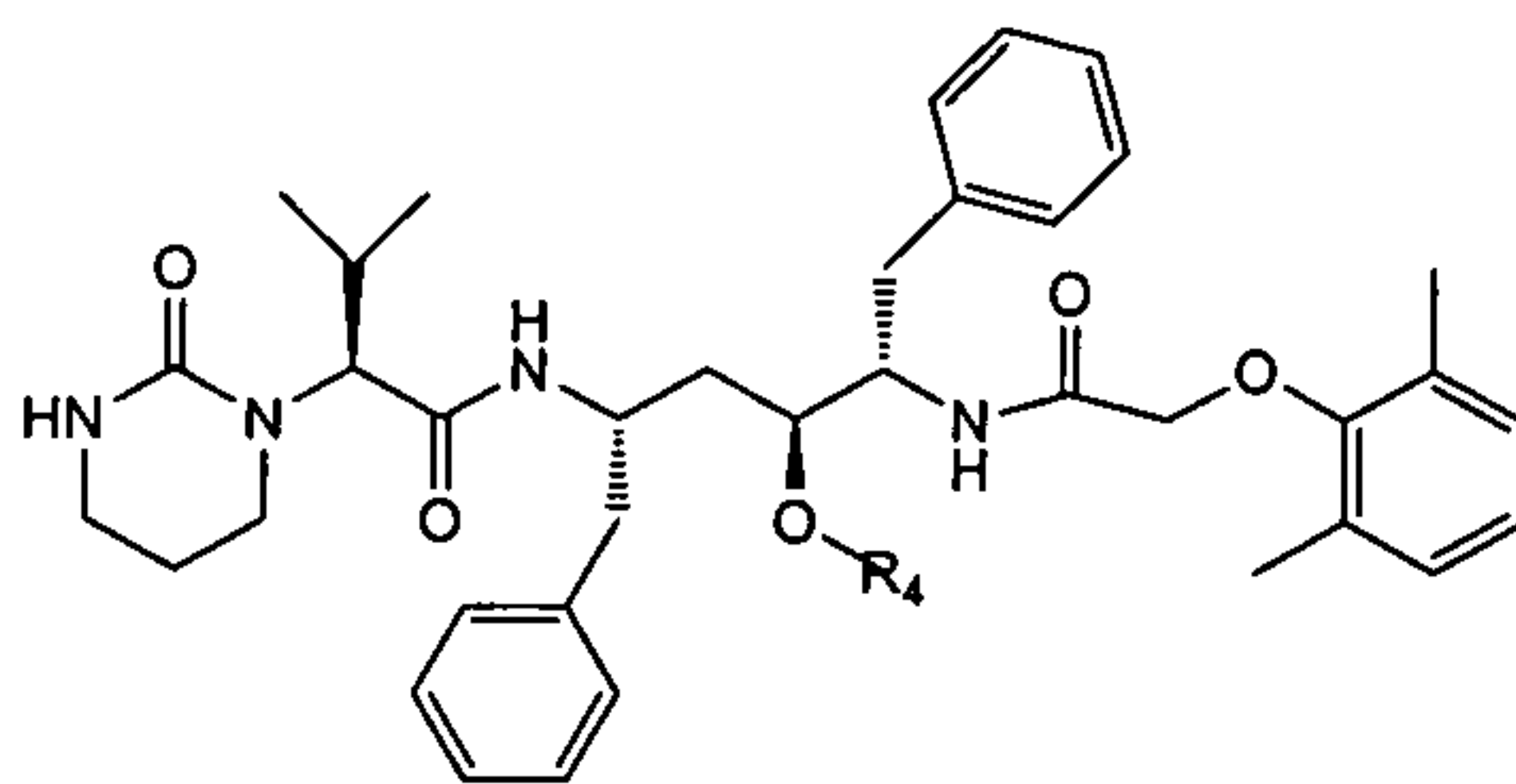
establishing a calibration curve by plotting concentration of the analog versus signal measured for each calibrator.

20. The method of claim 19 wherein the protease inhibitor is atazanavir and the analog has a structure



wherein R₃ is selected from the group consisting of H, C₁-C₆ alkyl, phenyl, heteroaryl, and - (CH₂)_n-X wherein n is 1-6 and X is COOR₅ or NH-CH₂-phenyl-COOR₅ wherein R₅ is H or C₁-C₆ alkyl.

21. The method of claim 20 wherein R₃ is H or C₁-C₄ alkyl.
22. The method of claim 19 wherein the protease inhibitor is lopinavir and the analog has a structure



wherein R₄ is -CONHR₃ or -CH₂COOR₃ and R₃ is selected from the group consisting of H, C₁-C₆ alkyl, phenyl, heteroaryl, and -(CH₂)_n-X wherein n is 1-6 and X is COOR₅ or NH-CH₂-phenyl-COOR₅ wherein R₅ is H or C₁-C₆ alkyl.

23. A method for establishing a calibration curve for efavirenz, said method comprising the steps of:

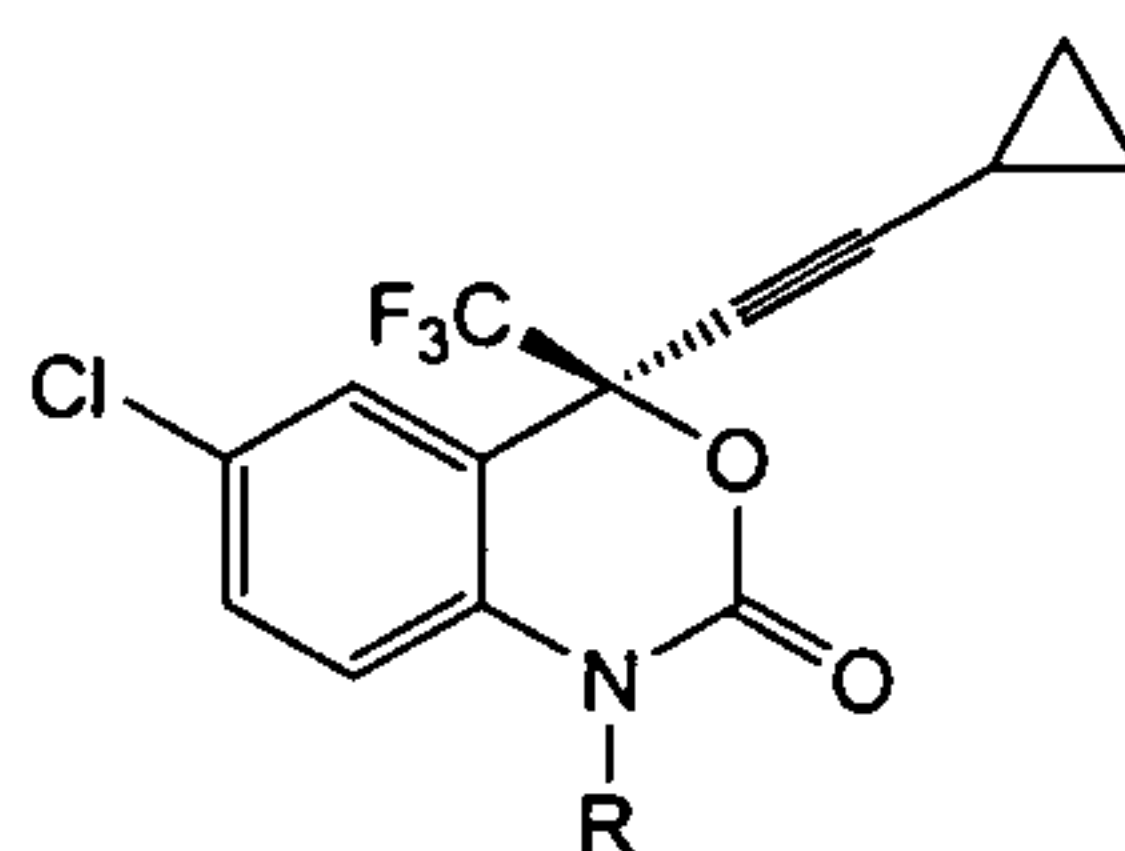
providing a set of calibrators, each calibrator containing one of a range of concentrations of an immunochemically equivalent analog of efavirenz, the analog comprising a derivative of efavirenz N-alkylated with C₁-C₆ alkyl or (C₁-C₆ alkyl)OH,

contacting each of the calibrators with a monoclonal antibody that specifically binds to efavirenz and the analog, and a conjugate comprising efavirenz and a moiety that generates a signal upon binding of the antibody to the analog,

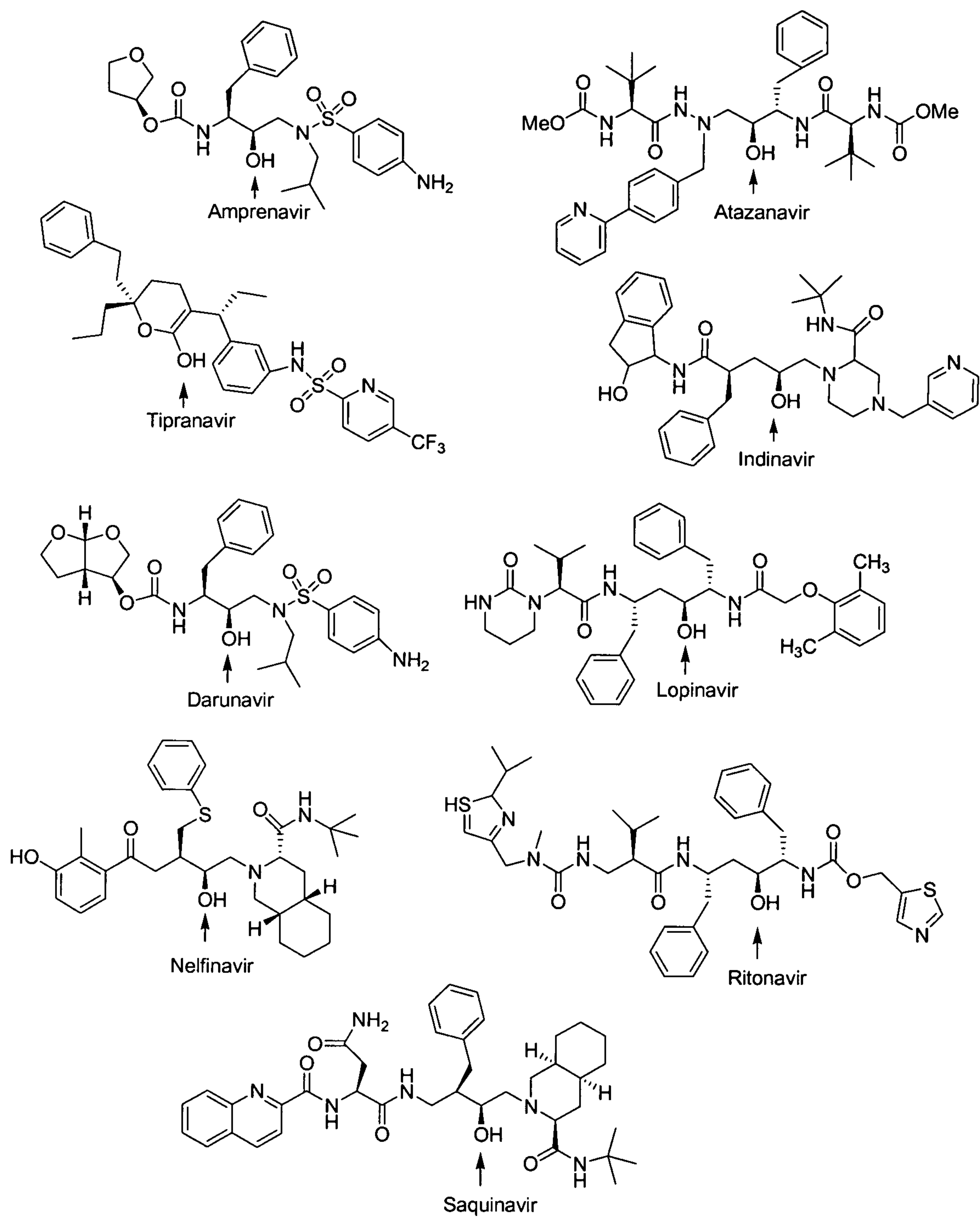
measuring the signal generated by each calibrator, and

establishing a calibration curve by plotting concentration of the analog versus signal measured for each calibrator.

24. The method of claim 23 wherein the analog has a structure



wherein R is C₁-C₆ alkyl or (C₁-C₆ alkyl)OH.

**Figure 1**

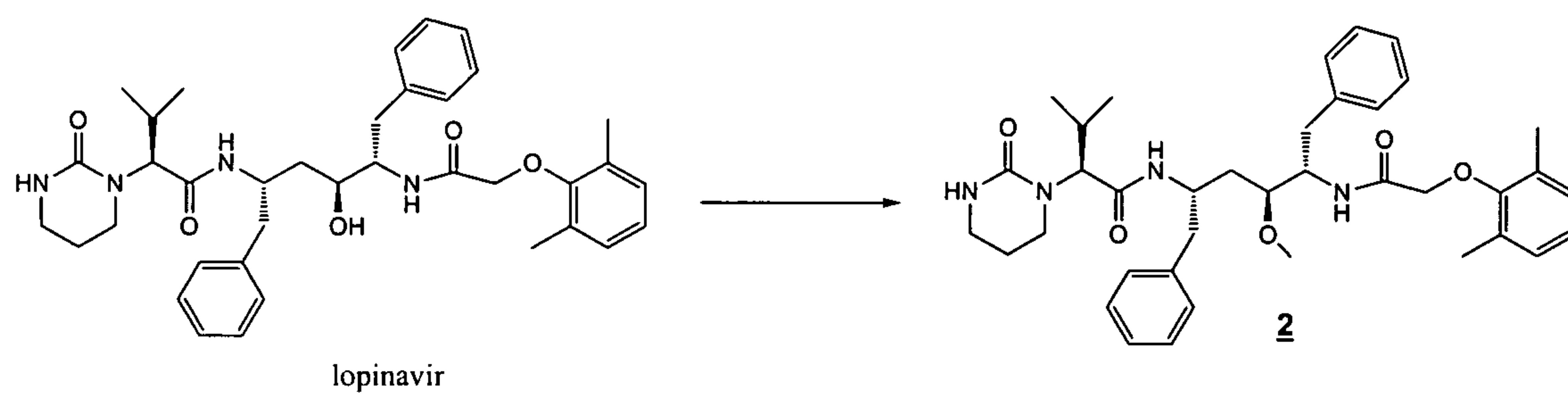


Figure 2

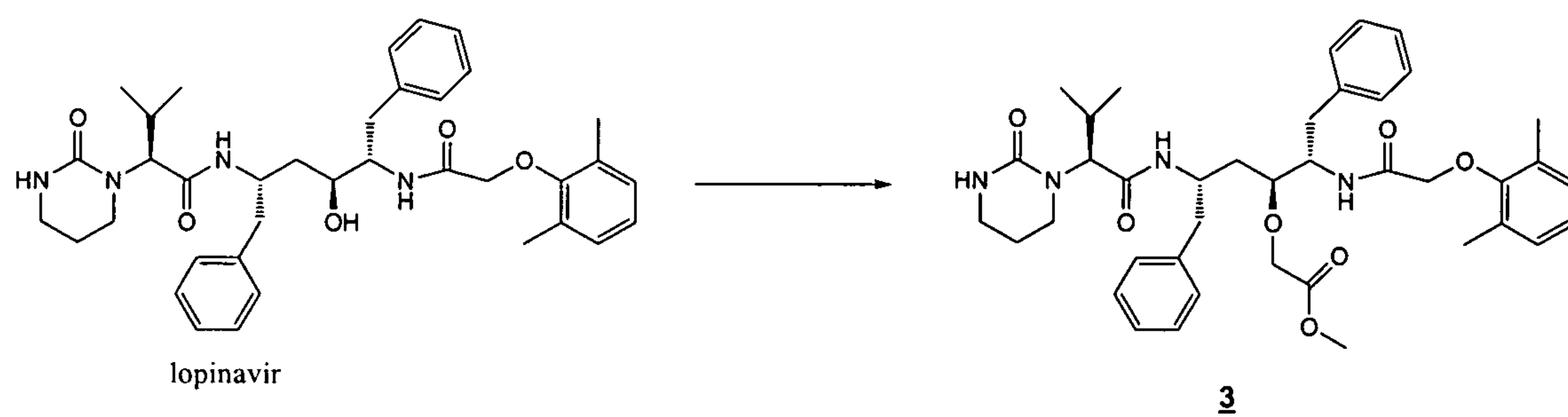


Figure 3

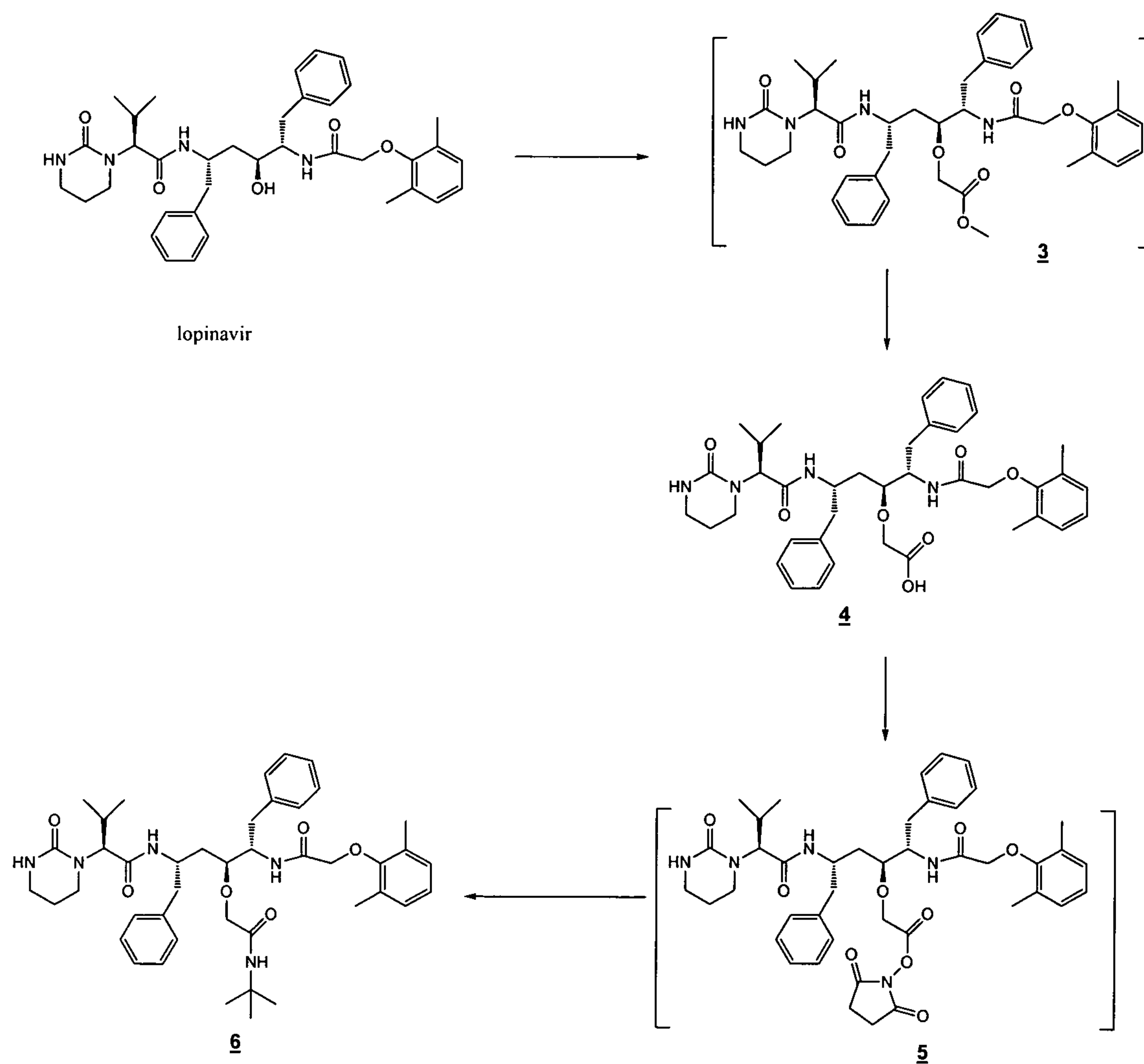


Figure 4

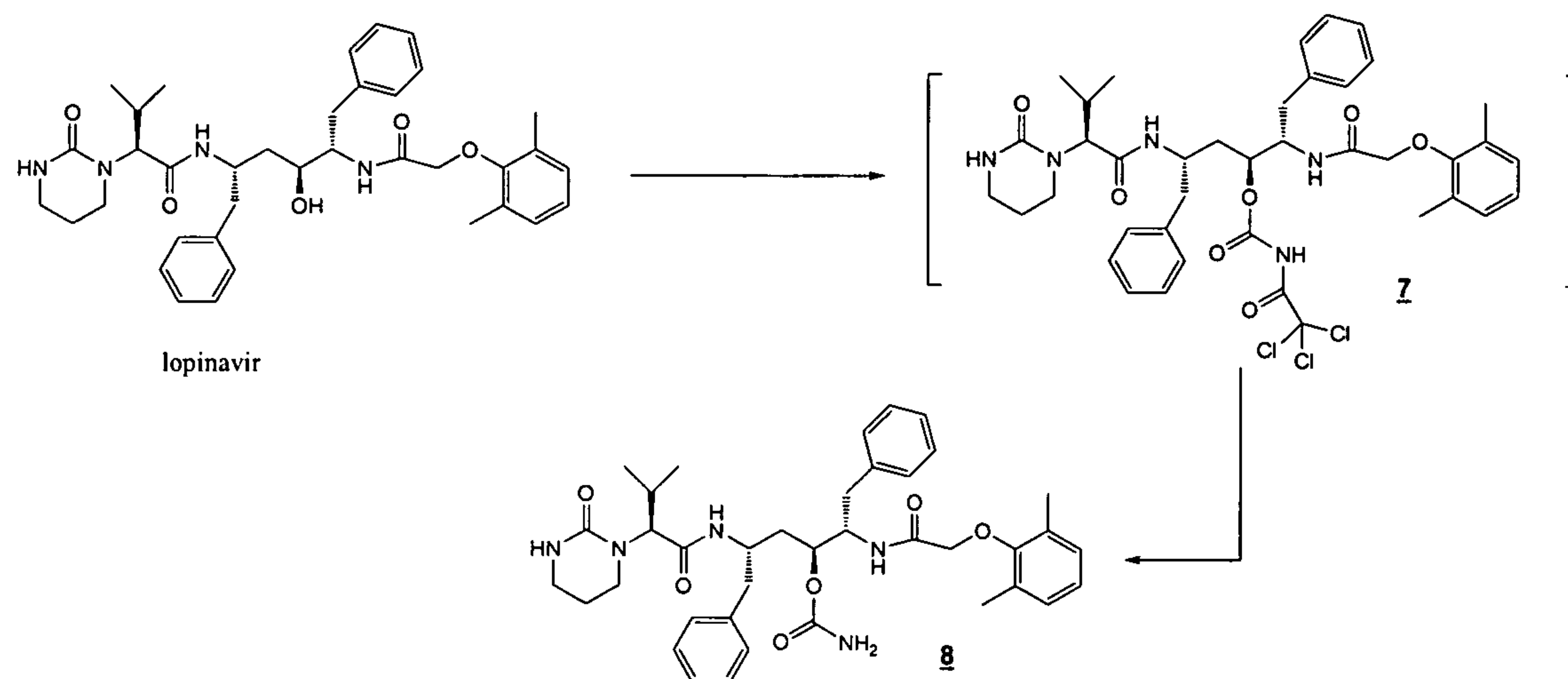


Figure 5

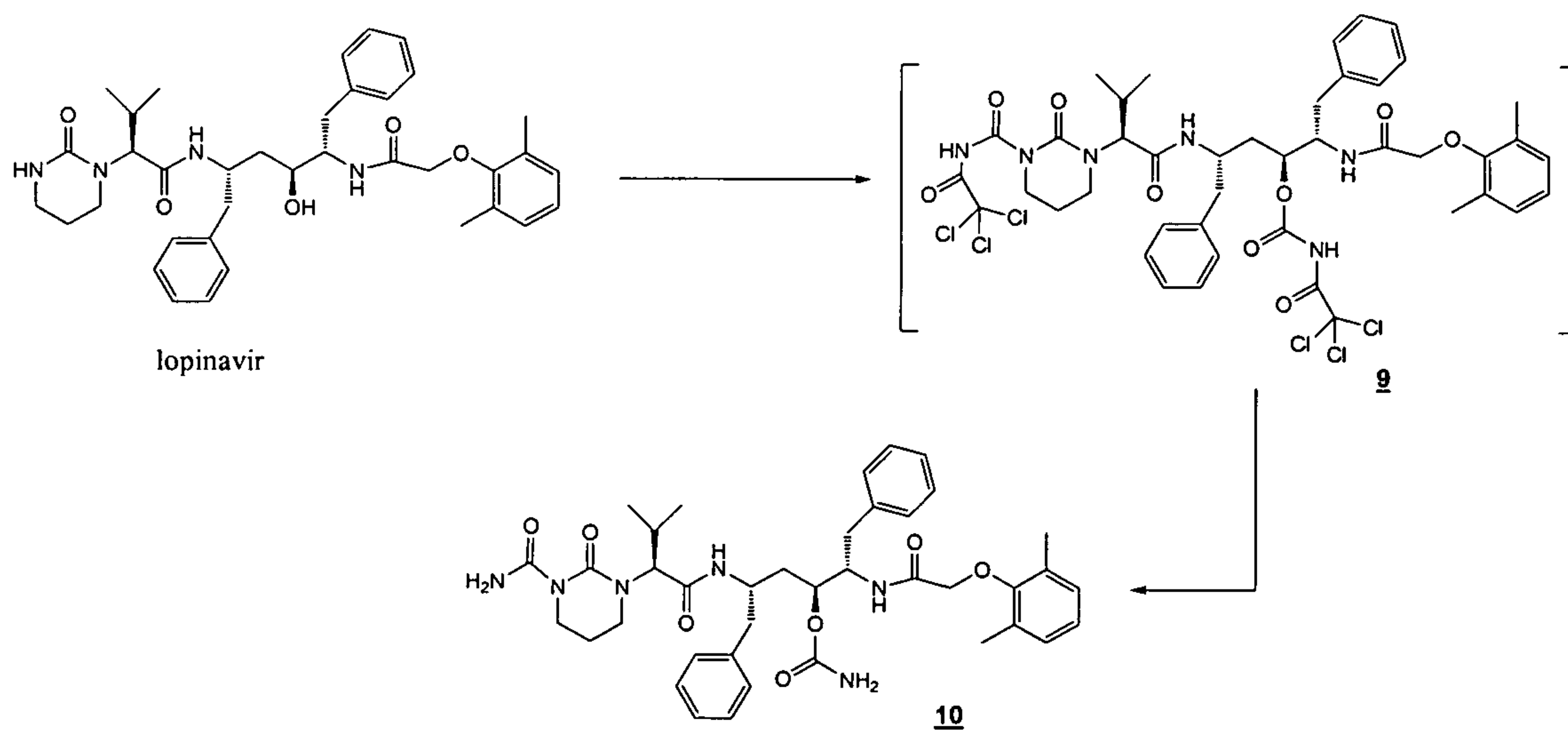


Figure 6

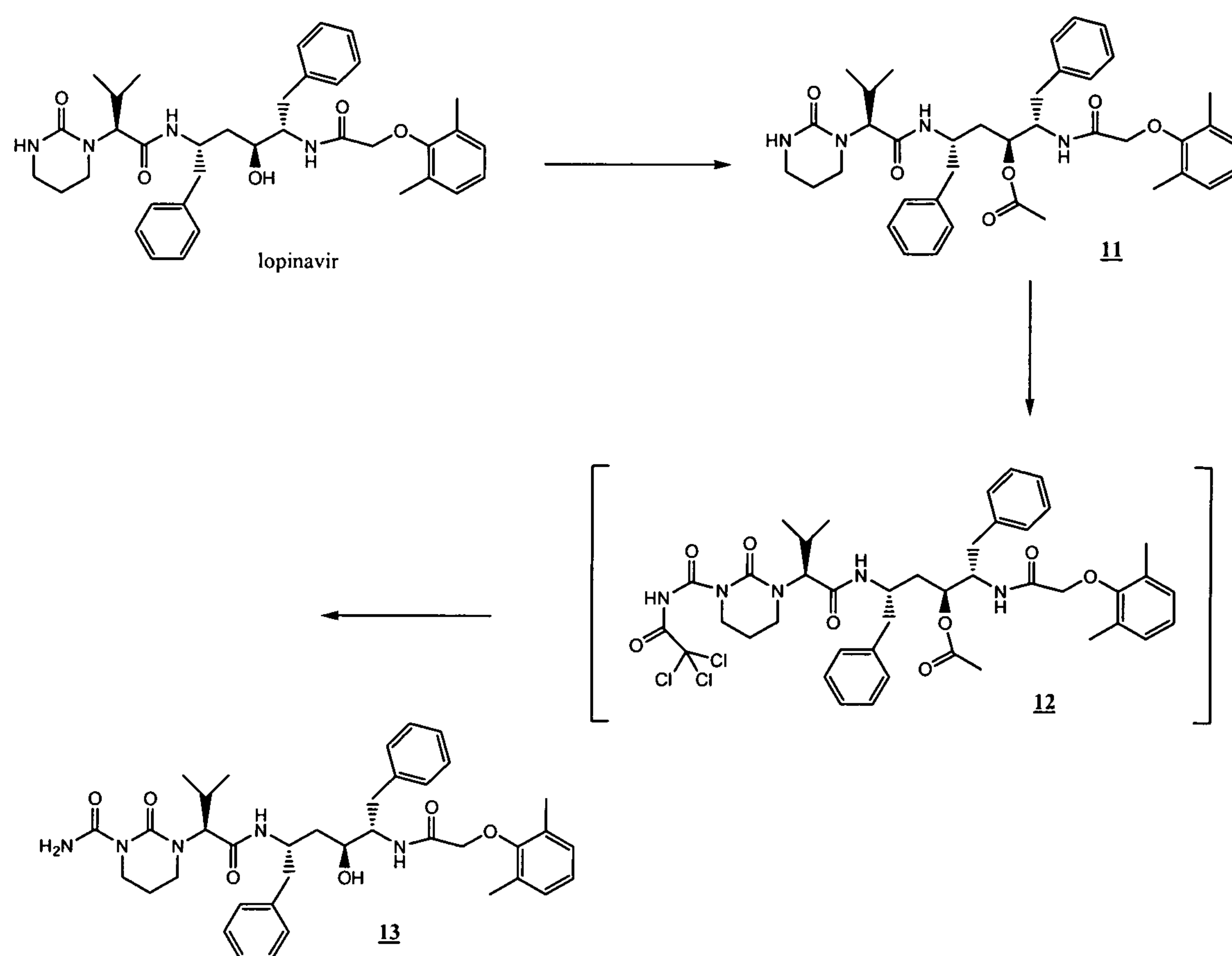


Figure 7

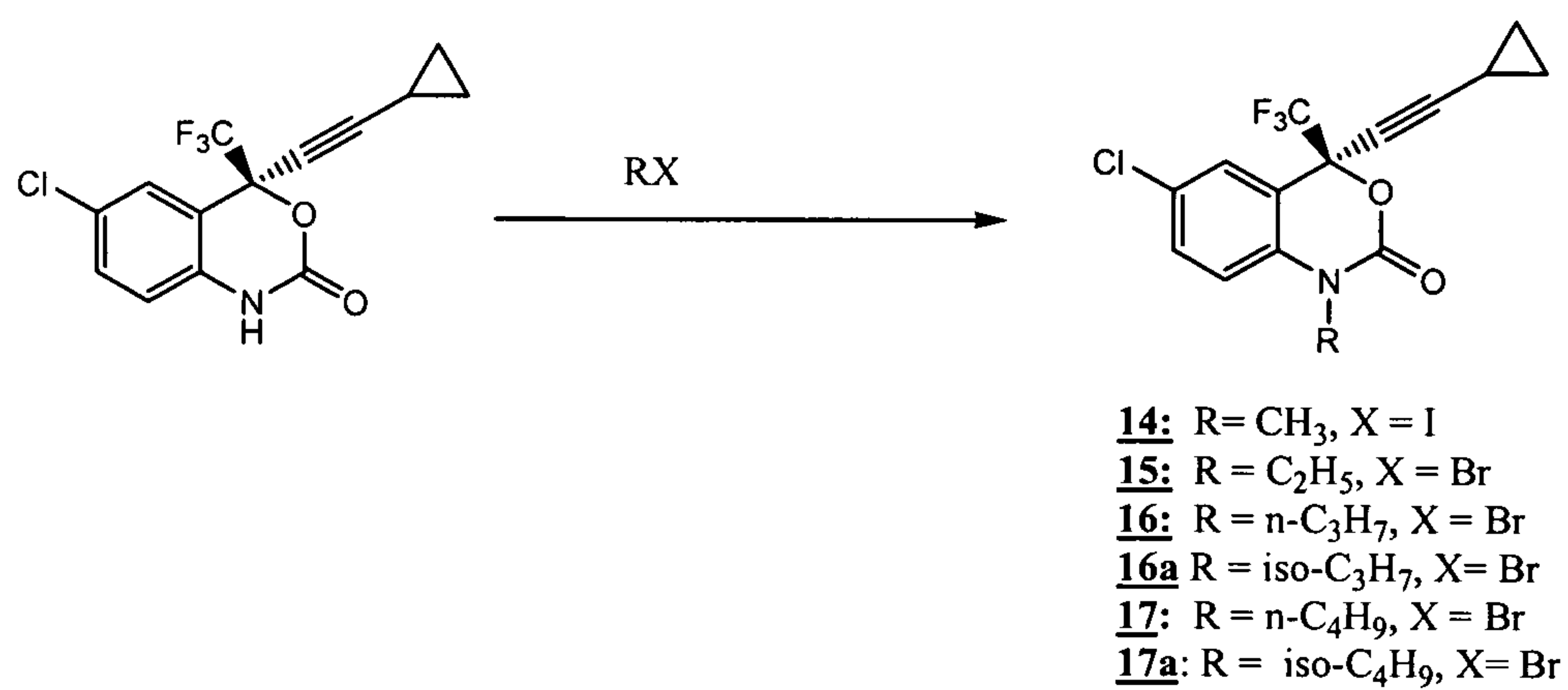


Figure 8

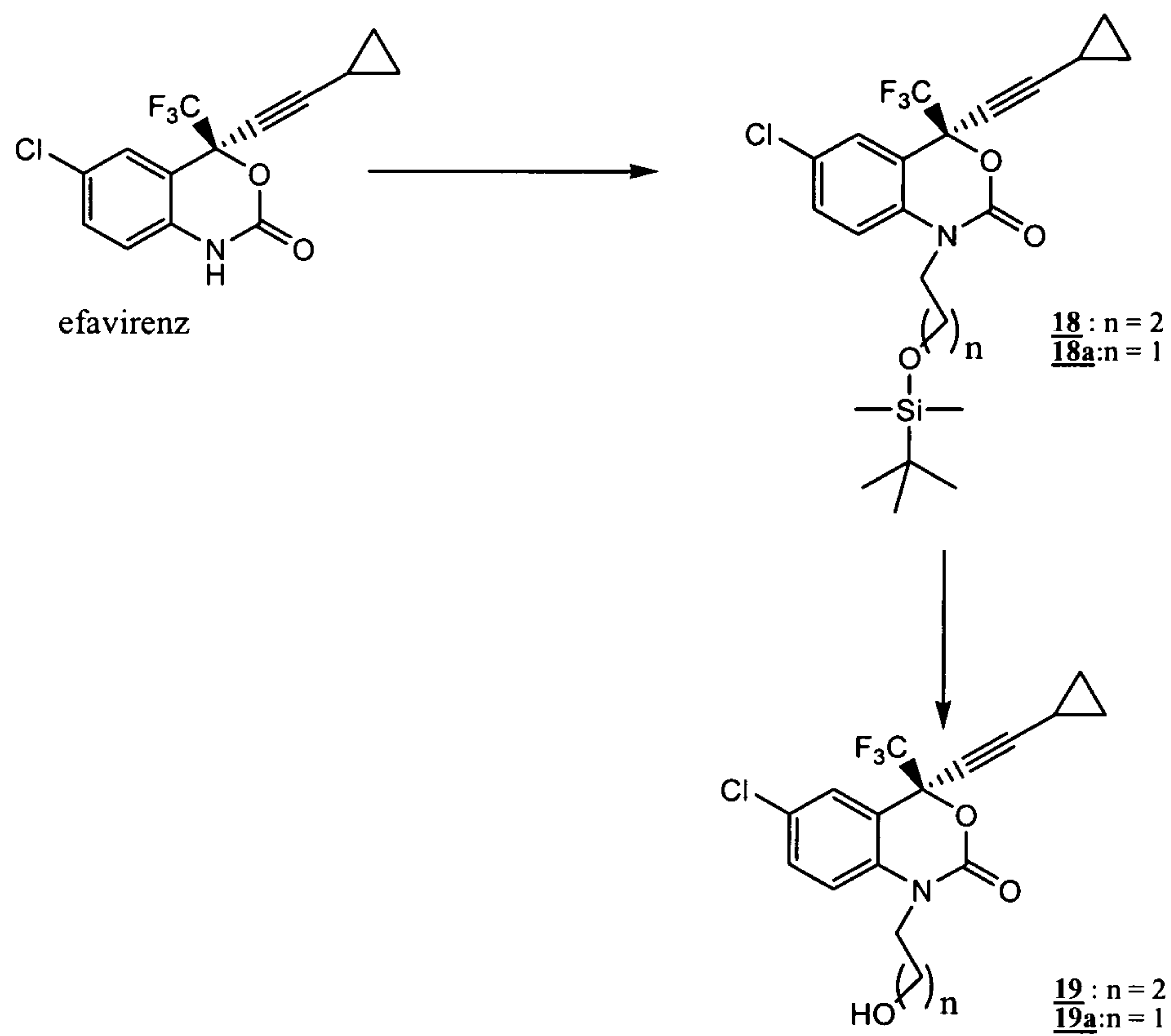


Figure 9

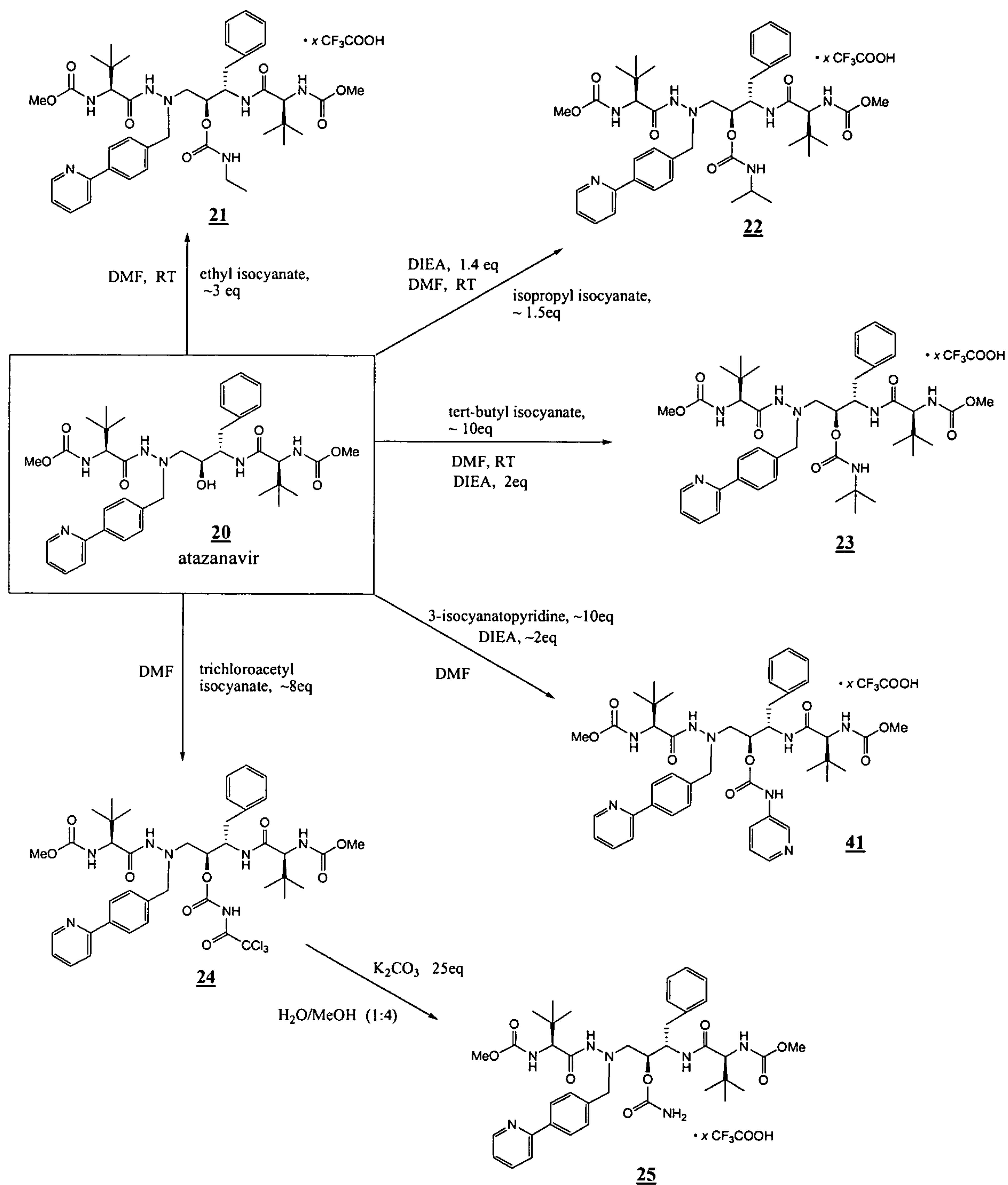


Figure 10

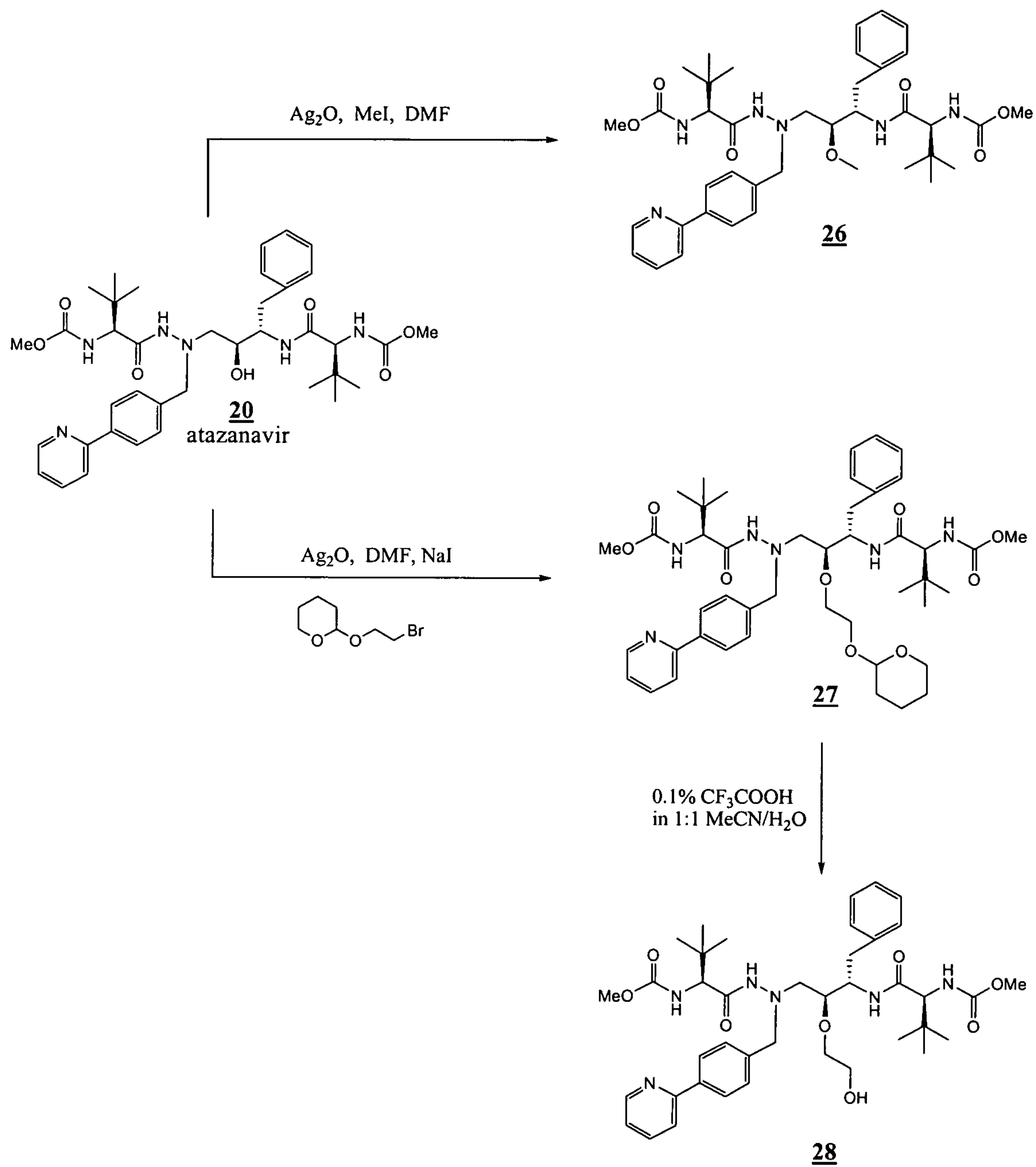


Figure 11

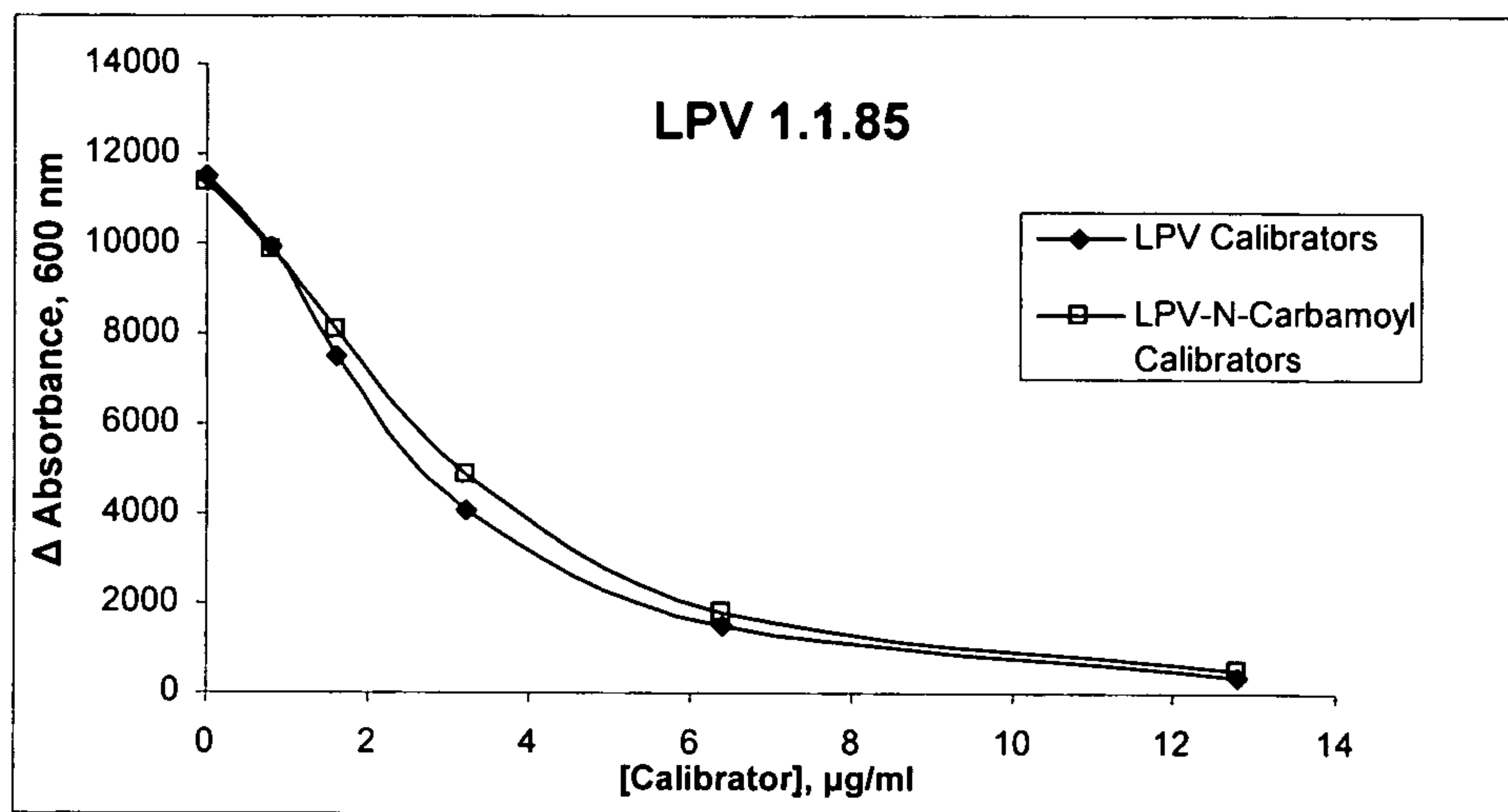


Figure 12a

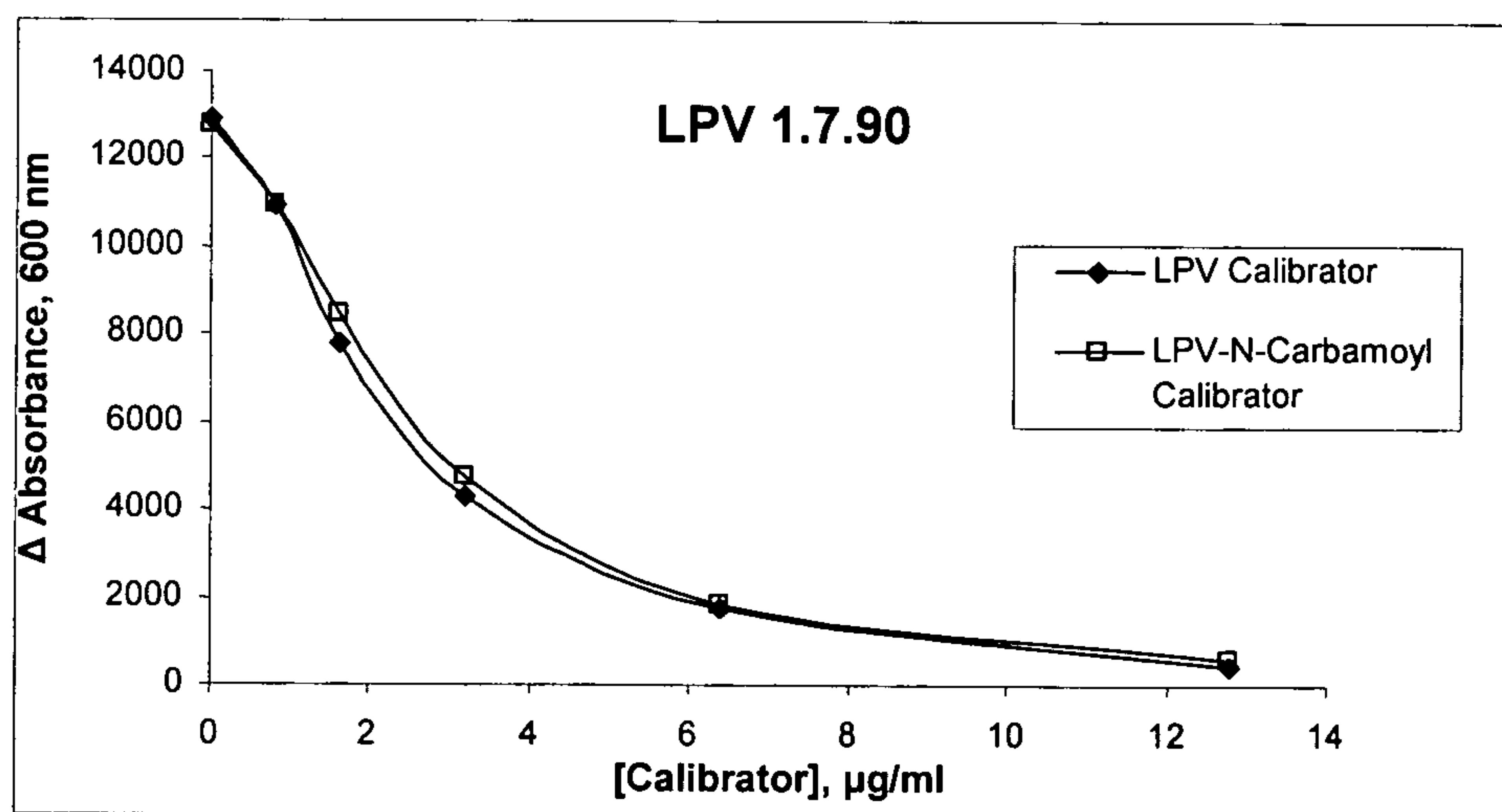


Figure 12b

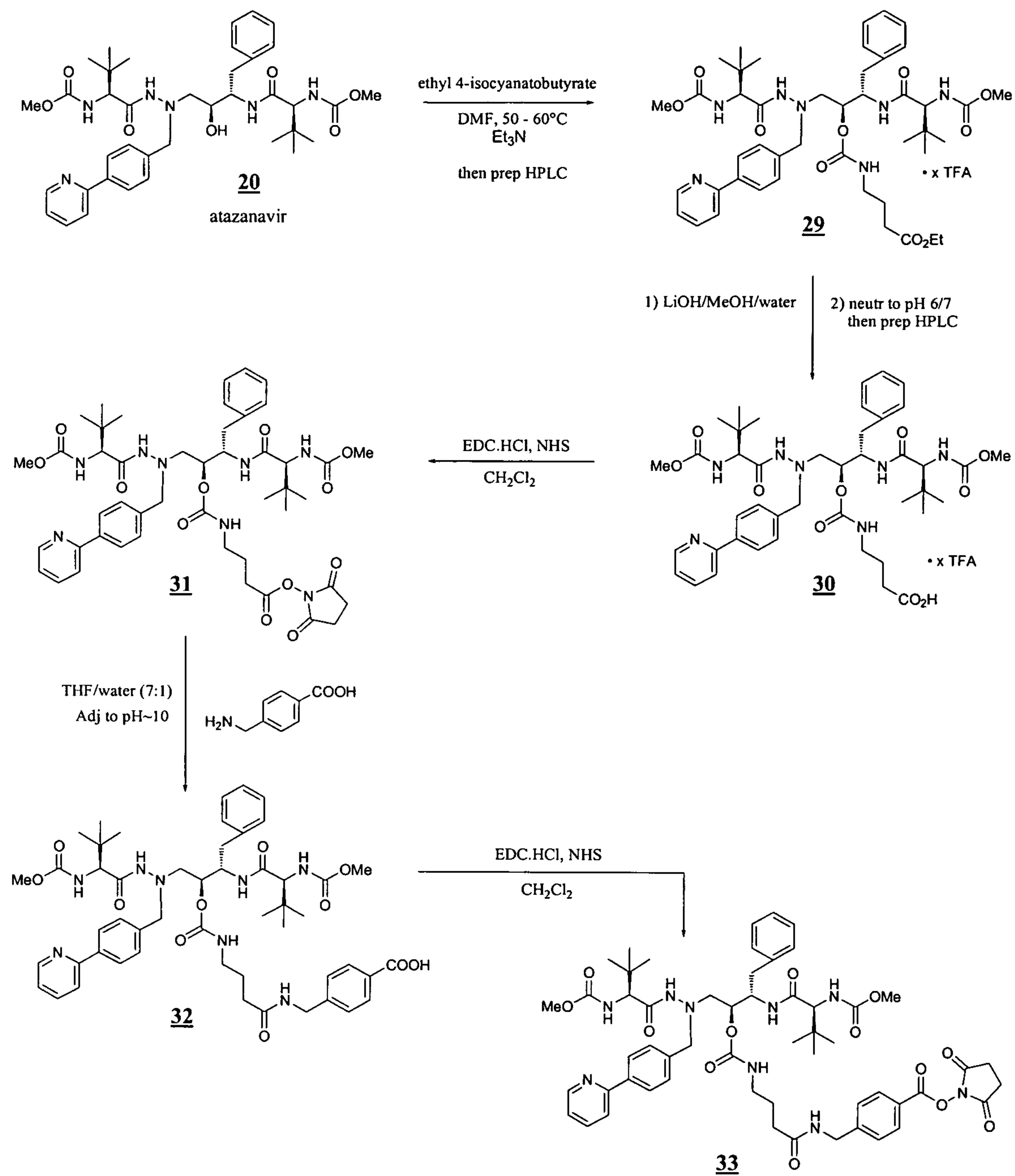


Figure 13

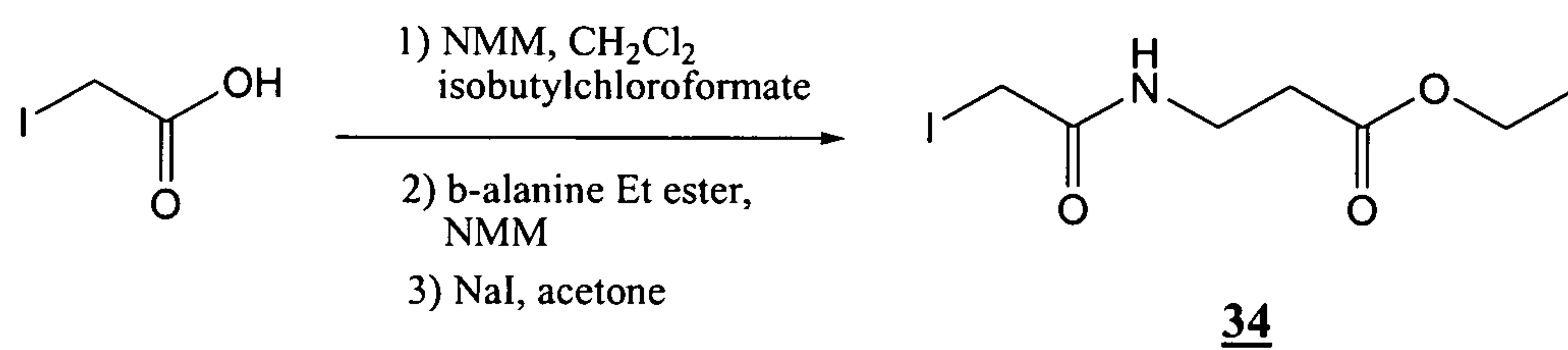


Figure 14

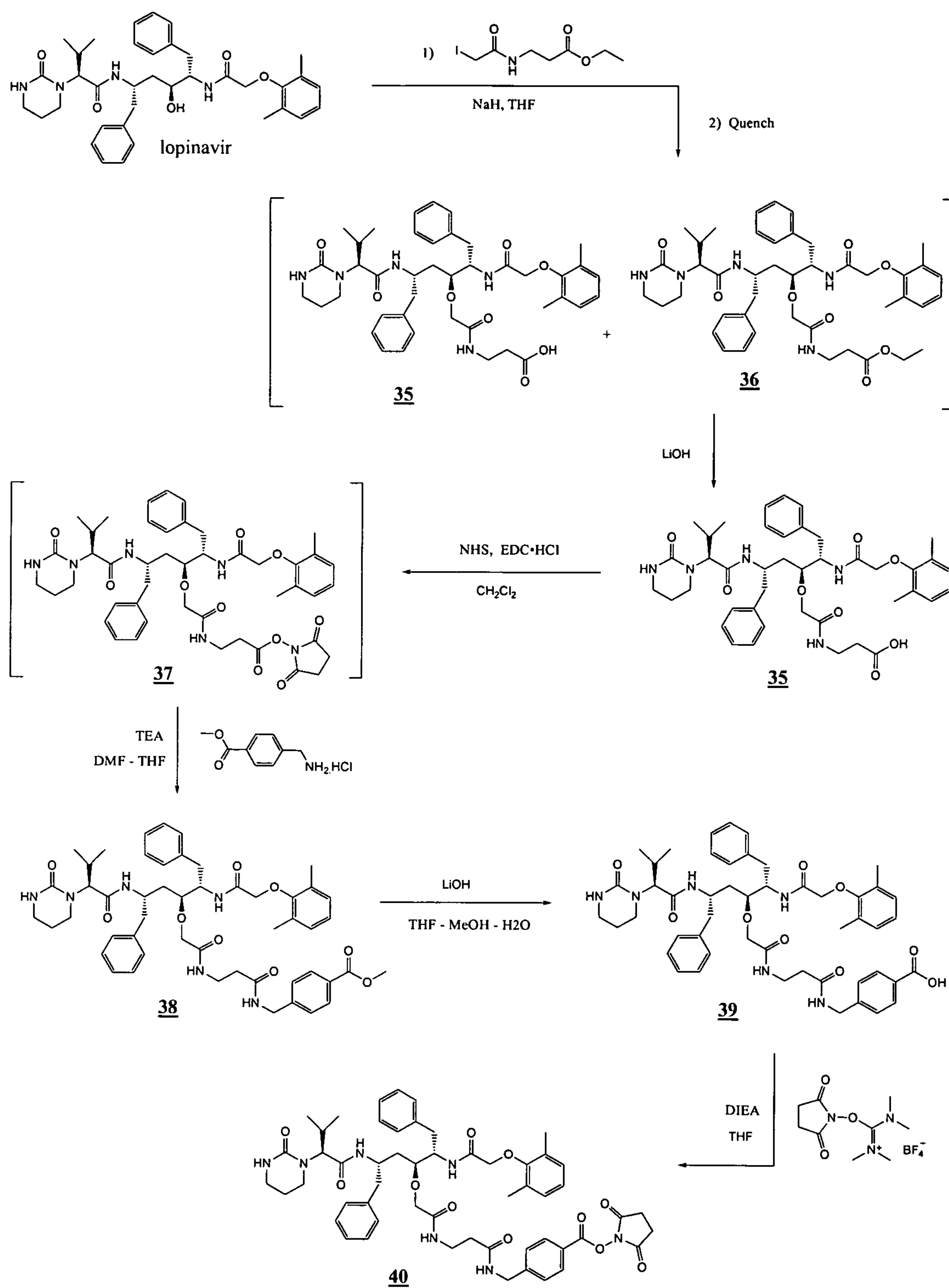


Figure 15

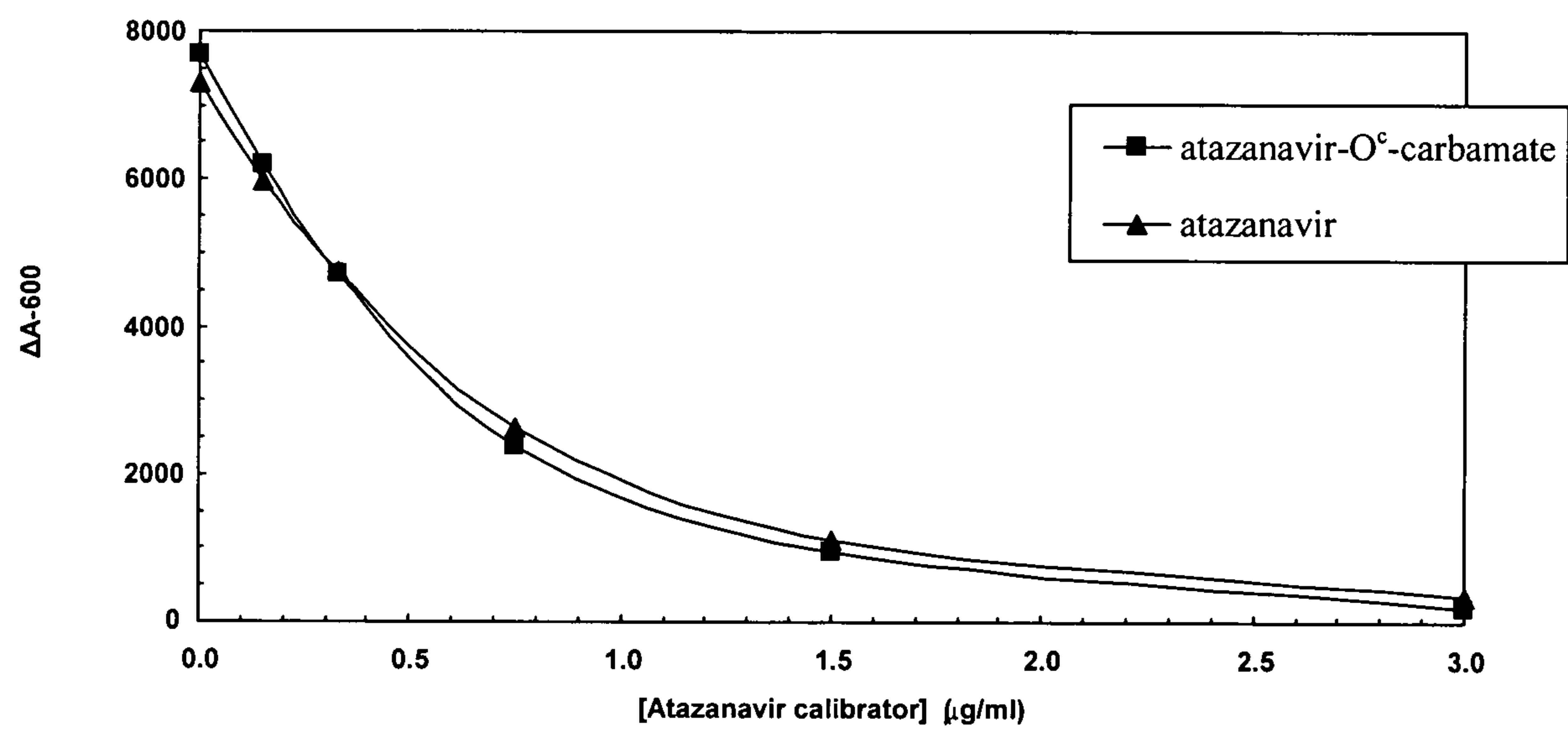


Figure 16

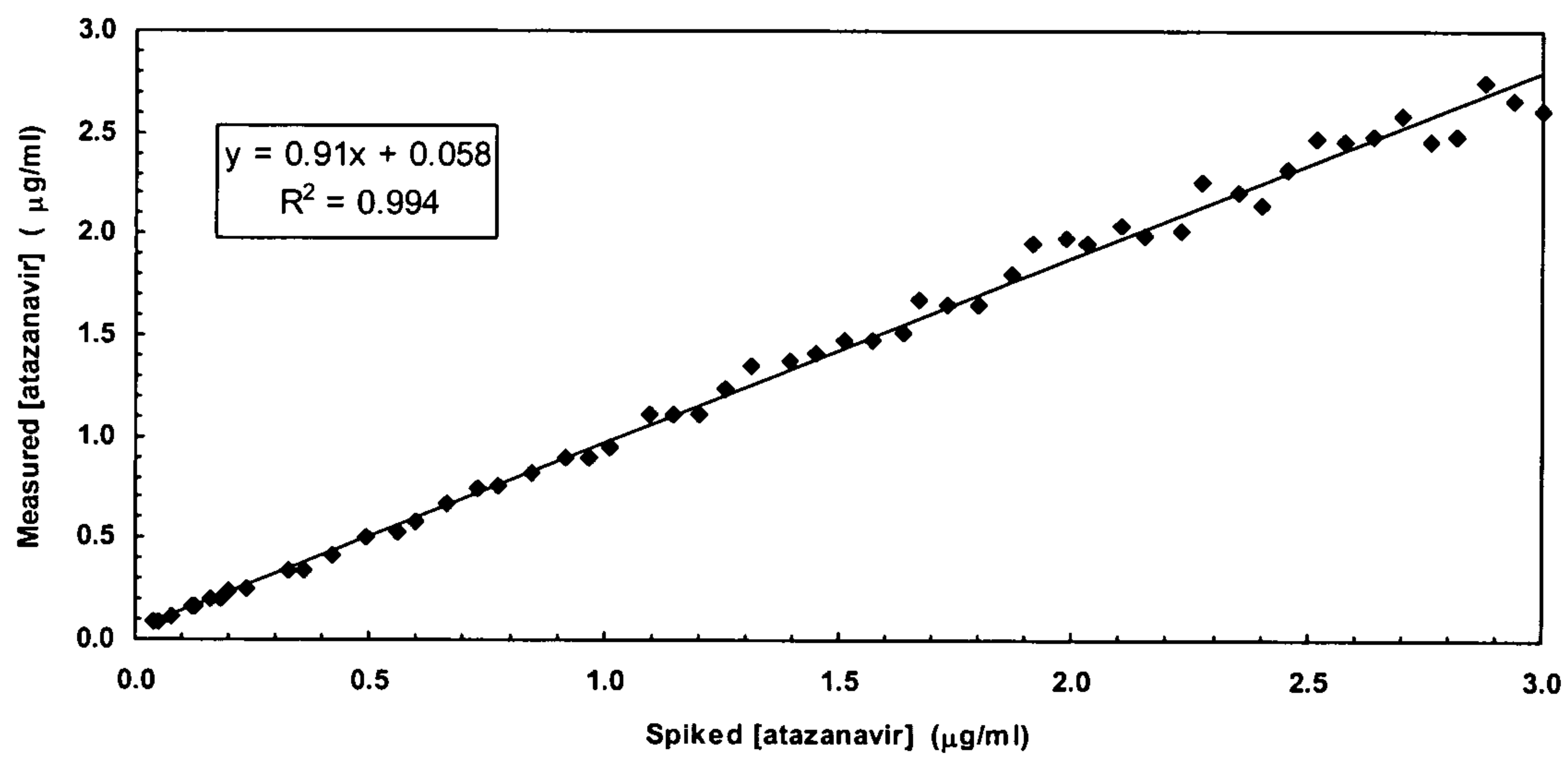


Figure 17

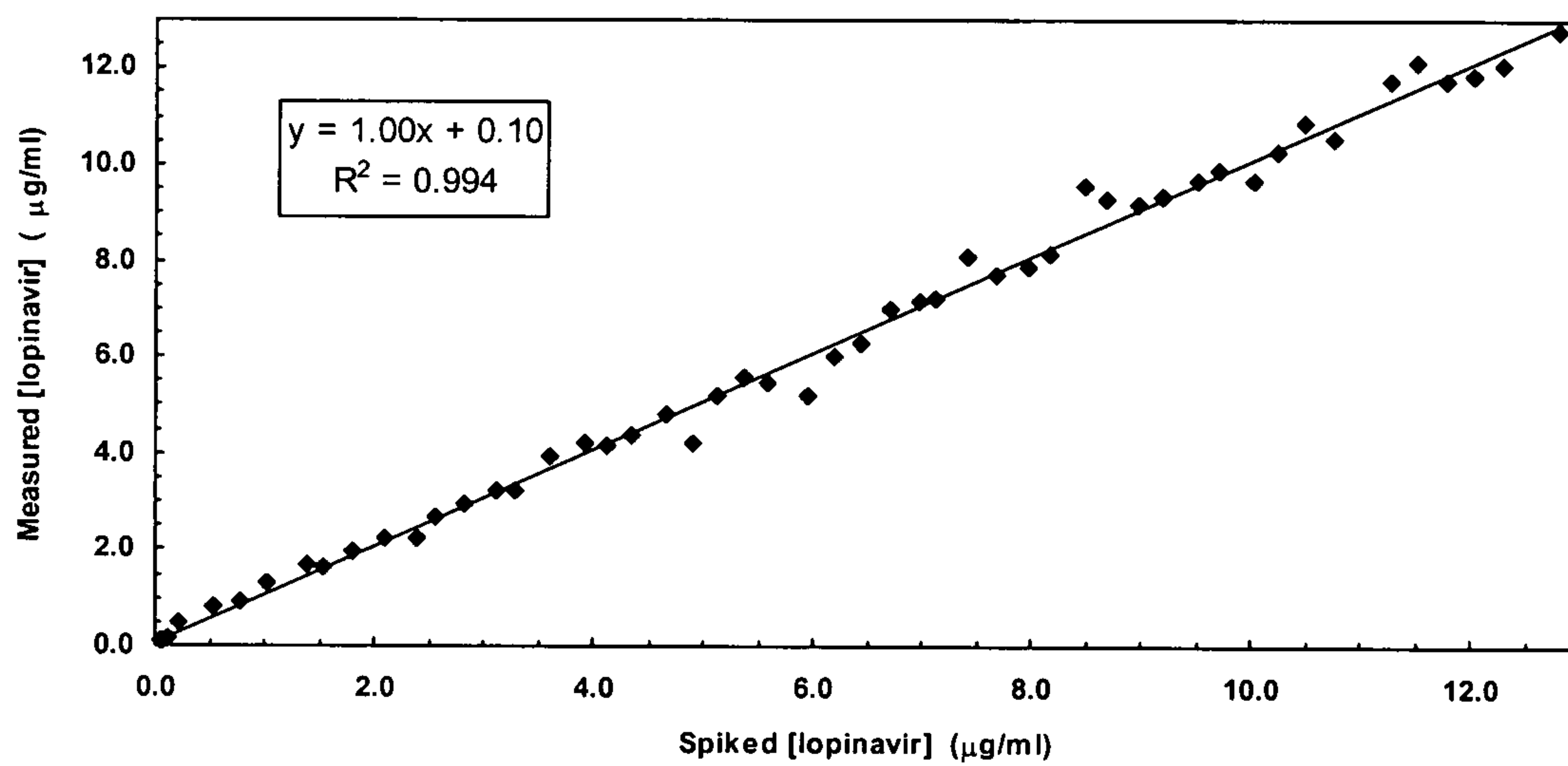


Figure 18

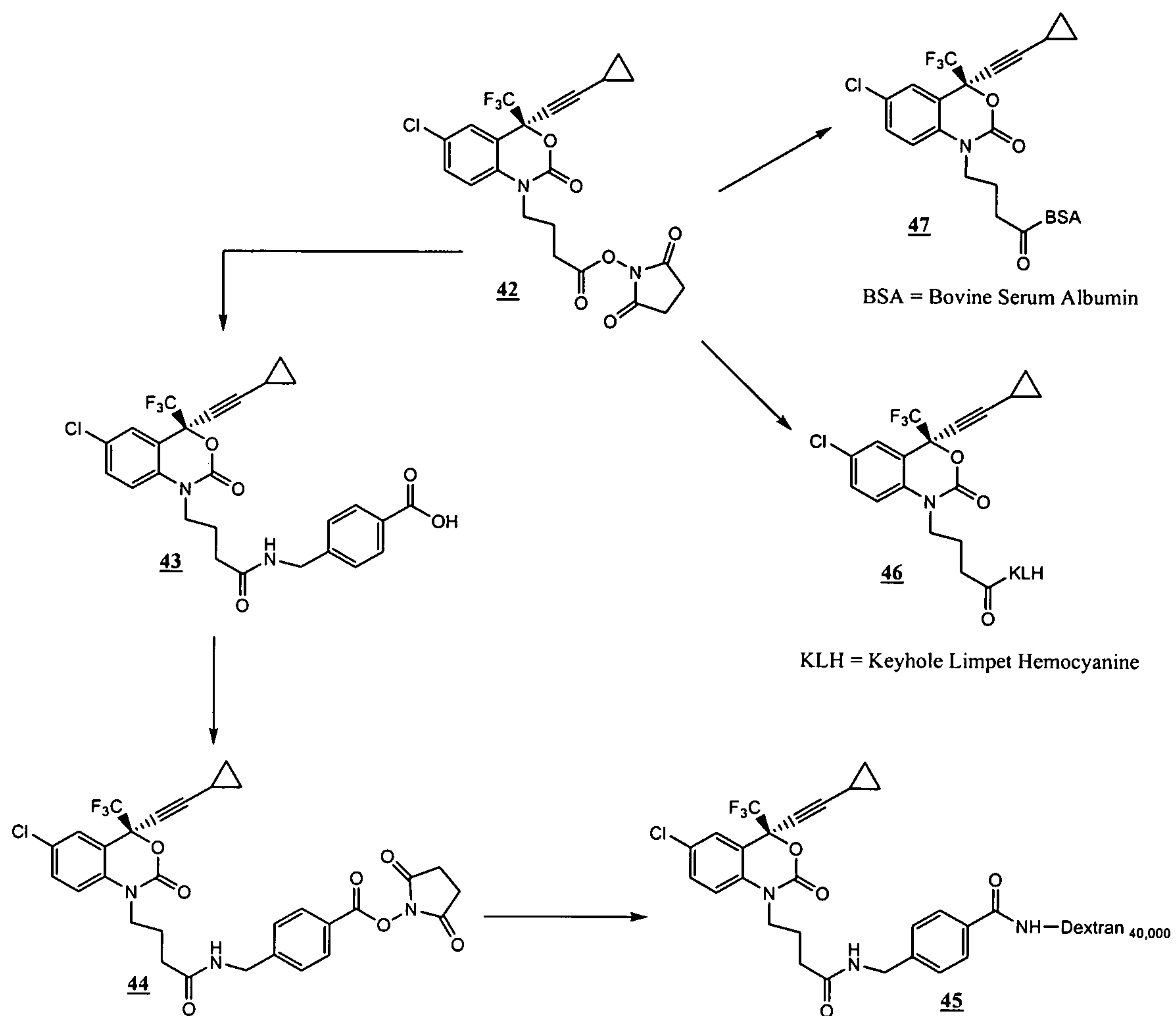


Figure 19