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(54) **Titre : AGENTS DE CIBLAGE OSSEUX A BASE D'OXYSTEROL OSTEOGENE A SELECTIVITE OSSEUSE**
(54) **Title: BONE-SELECTIVE OSTEOGENIC OXYSTEROL-BONE TARGETING AGENTS**

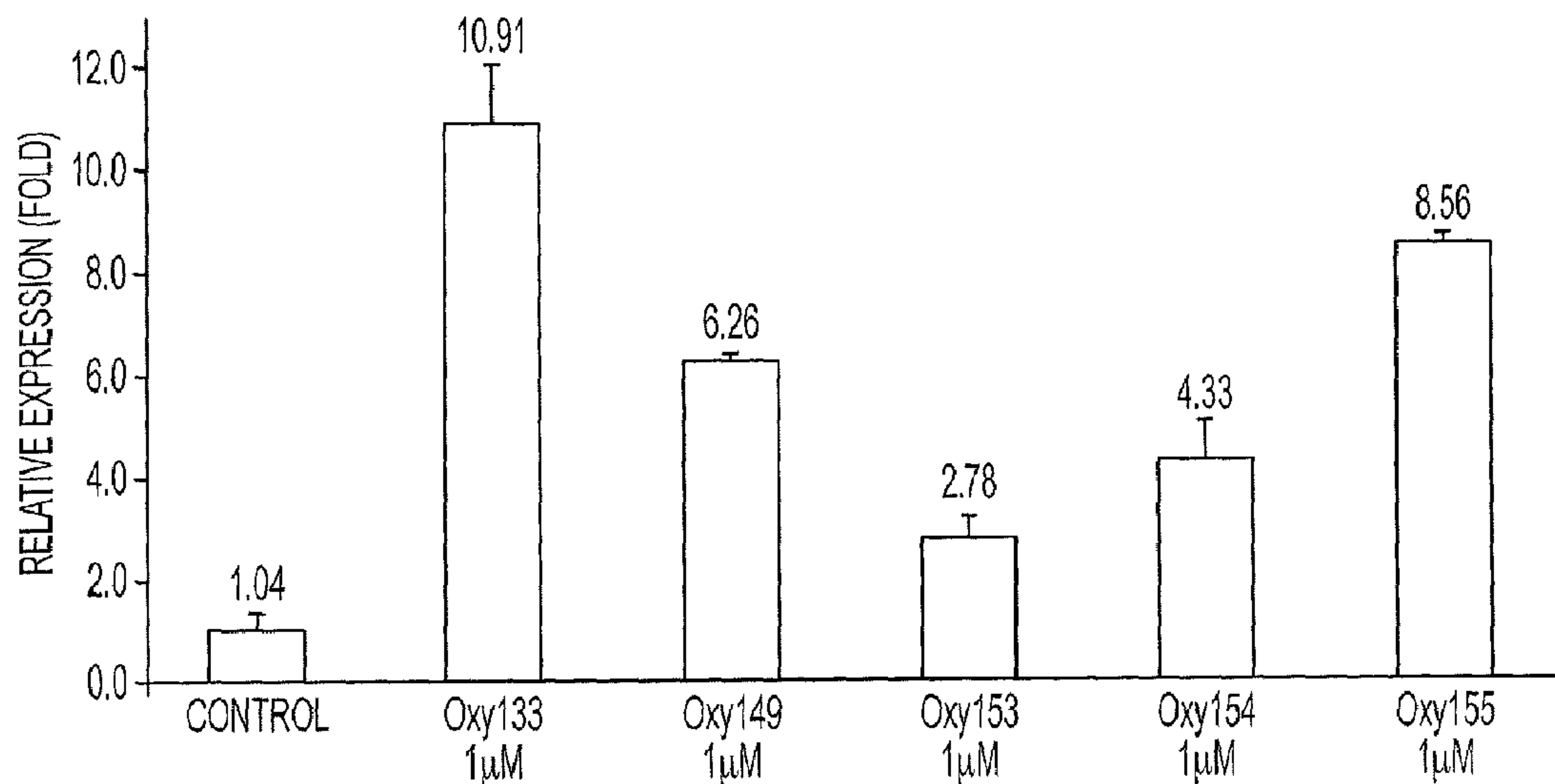


FIG. 7

(57) **Abrégé/Abstract:**

Compounds and compositions for the treatment of bone disorders are presented. Oxysterol compounds and compositions for the treatment of bone disorders are presented. The compounds are conjugates or derivatives of (3S,5S,6S,8R,9S,10R,13S,14S,17S)-17-[(2S)-2-hydroxyoctan-2-yl]-10,13-dimethyl-2,3,4,5,6,7,8,9,11,12,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthrene-3,6-diol with a BTA-linker, such as from a tetracycline fragment, attached to 3-position, 6-position, or 20-position. These osteogenic oxysterol compounds stimulate hedgehog signaling pathway. The various linker units are susceptible to certain esterases.

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(54) Title: BONE-SELECTIVE OSTEOGENIC OXYSTEROL-BONE TARGETING AGENTS

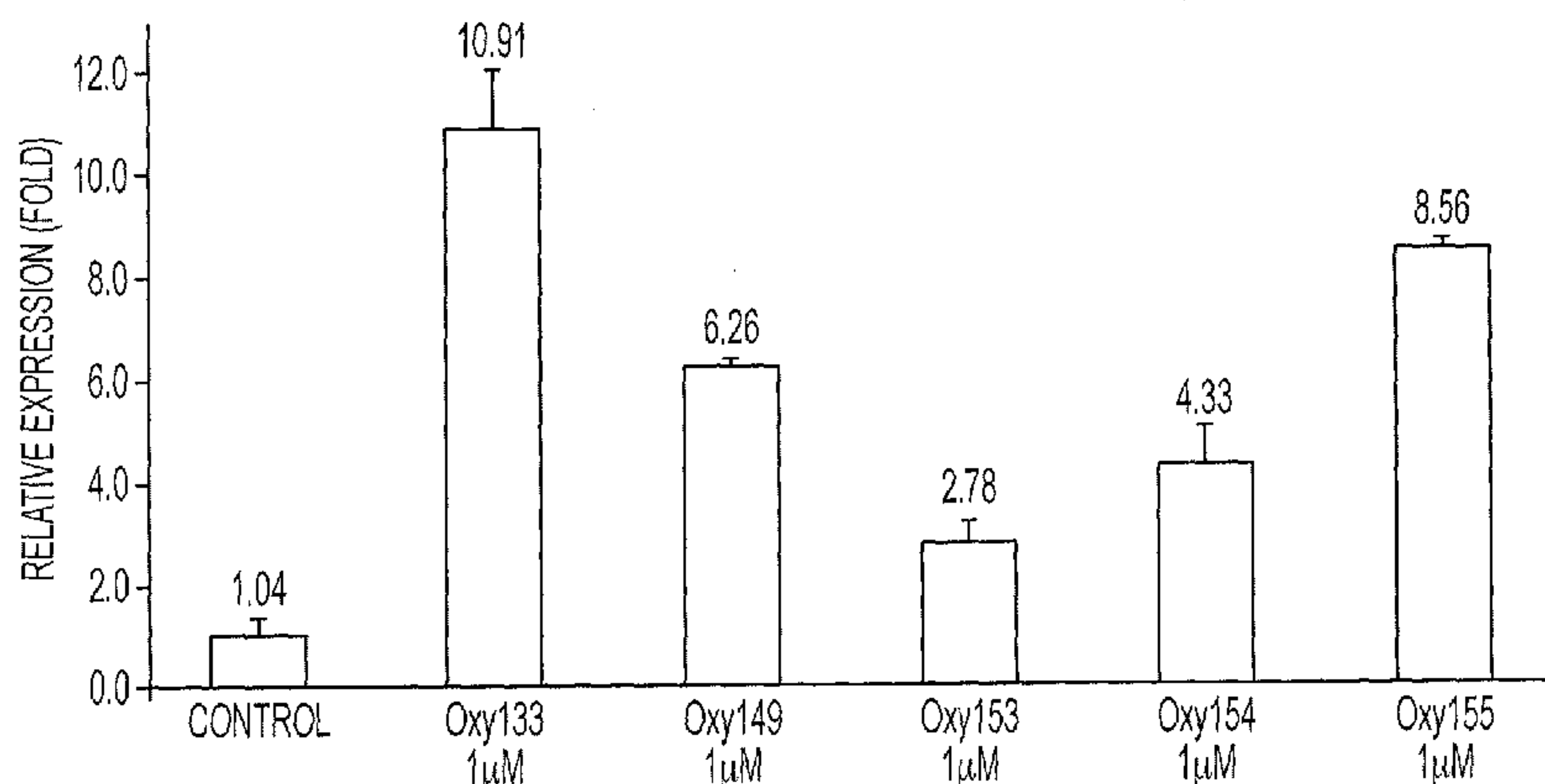


FIG. 7

(57) Abstract: Compounds and compositions for the treatment of bone disorders are presented. Oxysterol compounds and compositions for the treatment of bone disorders are presented. The compounds are conjugates or derivatives of (3S,5S,6S,8R,9S,10R,13S,14S,17S)-17-[(2S)-2-hydroxyoctan-2-yl]-10,13-dimethyl-2,3,4,5,6,7,8,9,11,12,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthrene-3,6-diol with a BTA-linker, such as from a tetracycline fragment, attached to 3-position, 6-position, or 20-position. These osteogenic oxysterol compounds stimulate hedgehog signaling pathway. The various linker units are susceptible to certain esterases.

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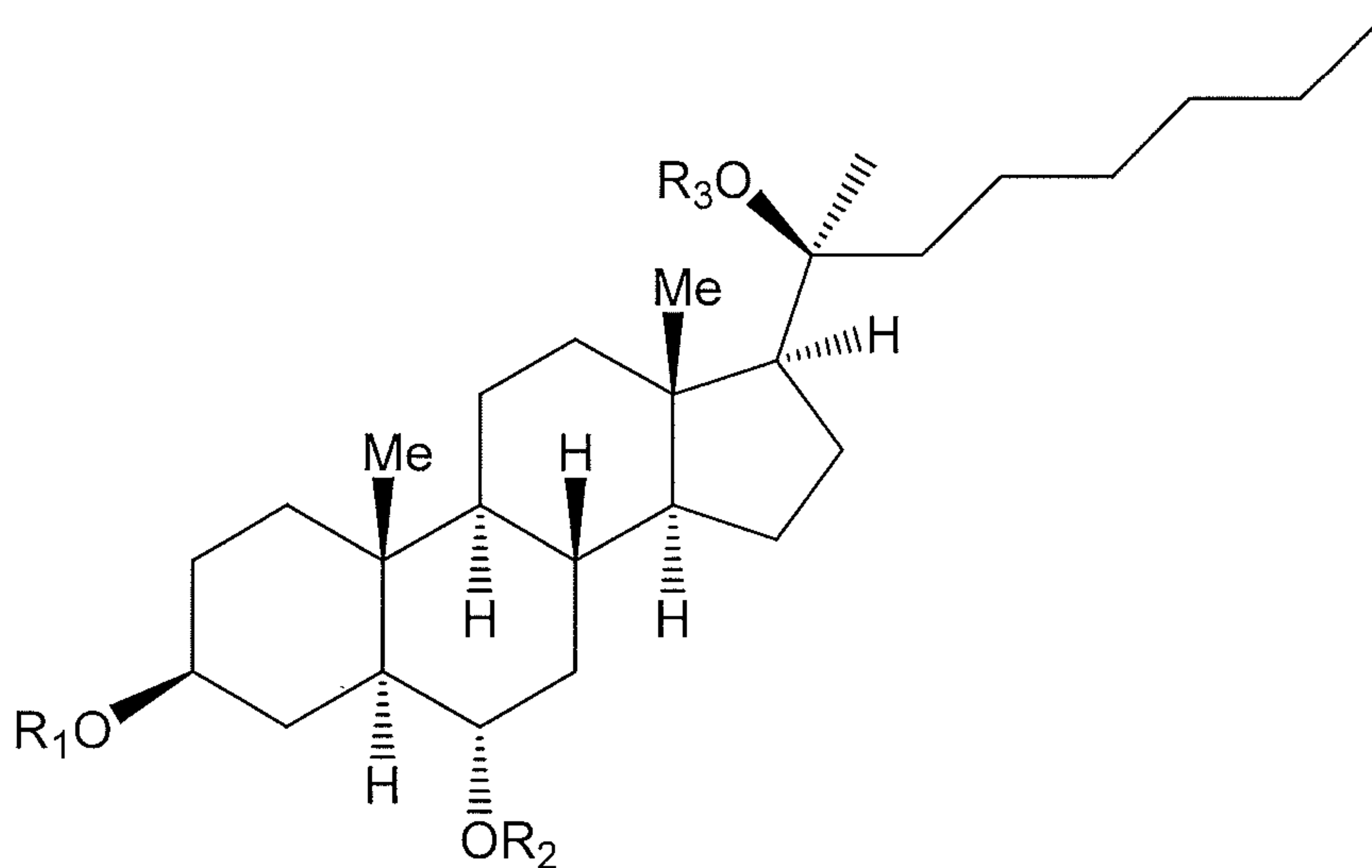
Bone-Selective Osteogenic Oxysterol-Bone Targeting Agents

Background of the Invention

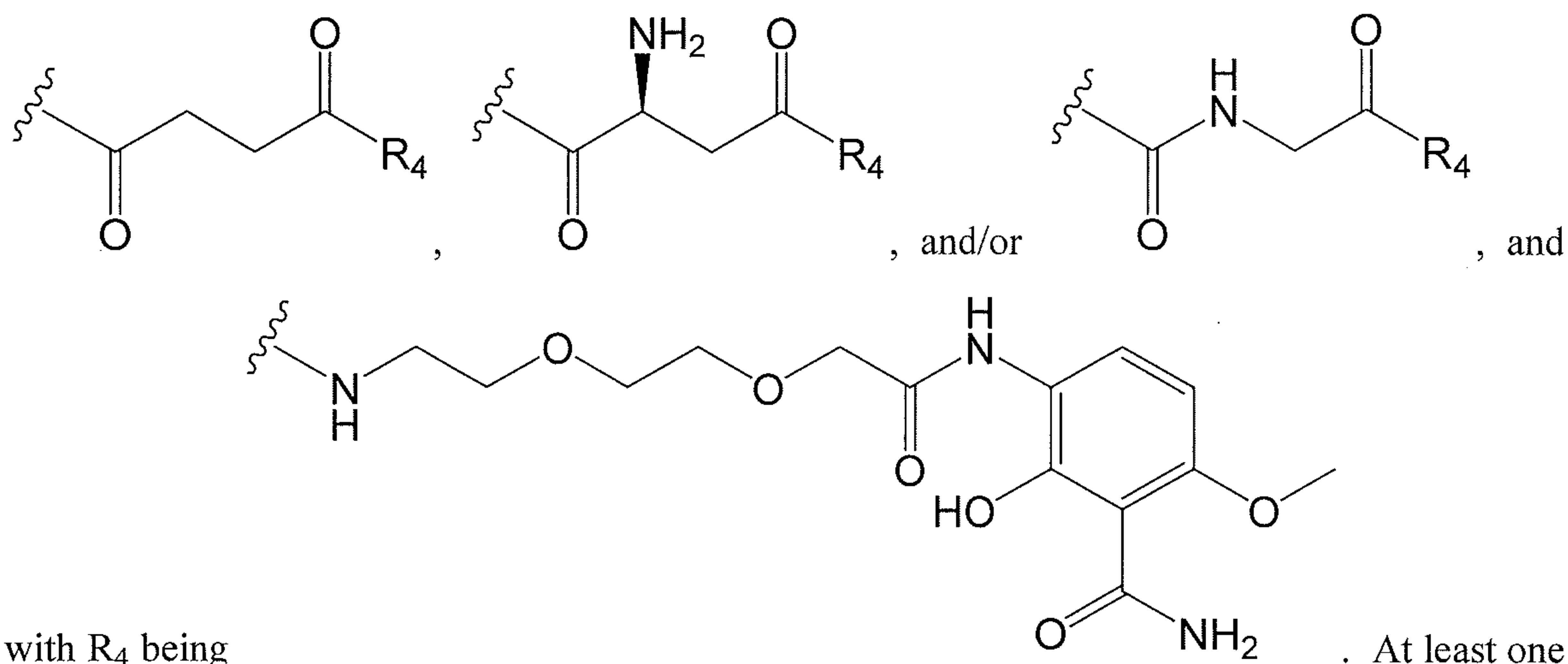
[0001] The present invention is relevant to the field of treatment of bone disorders.

Summary of the Invention

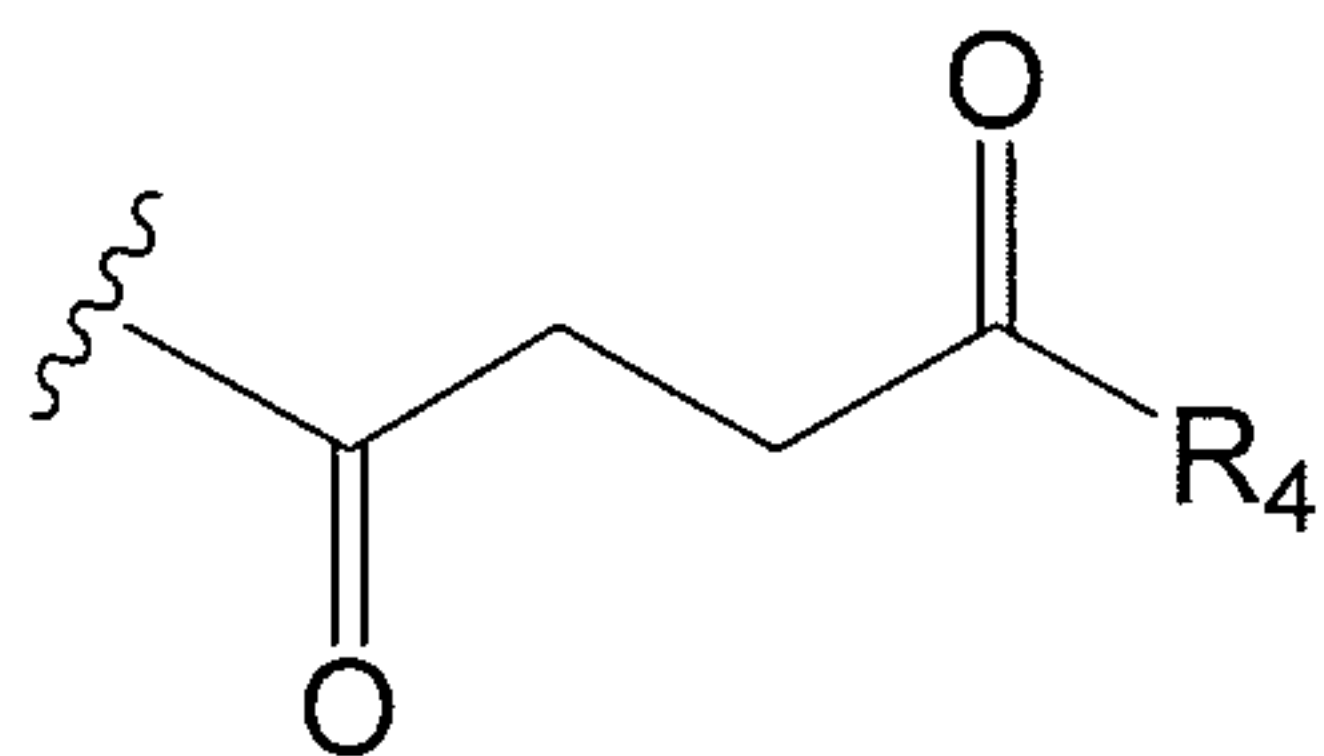
[0002] An embodiment of the present invention is a composition comprising an oxysterol-bone targeting agent compound, such as set forth herein, for example, an Oxy133-tetracycline derivative compound. An oxysterol-bone targeting agent compound can include a compound of the formula



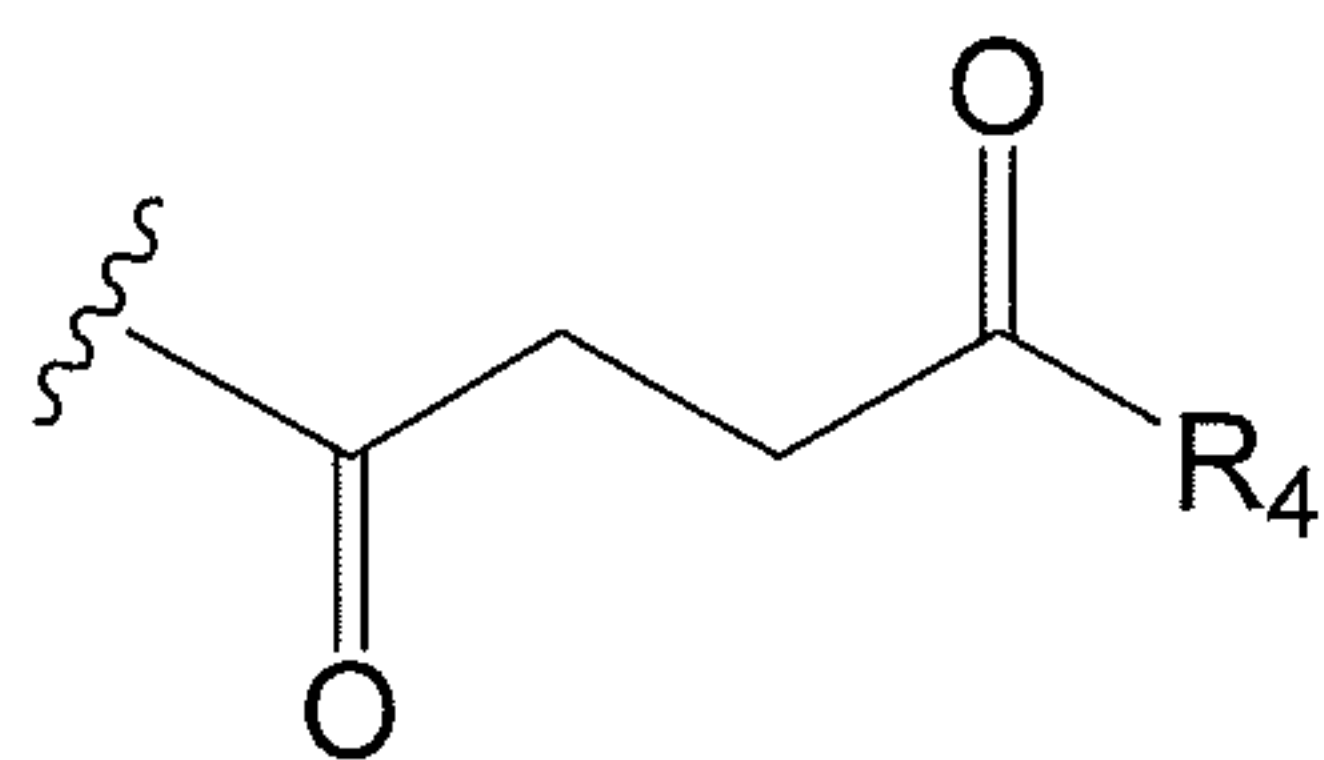
with R_1 , R_2 , and R_3 being independently hydrogen,



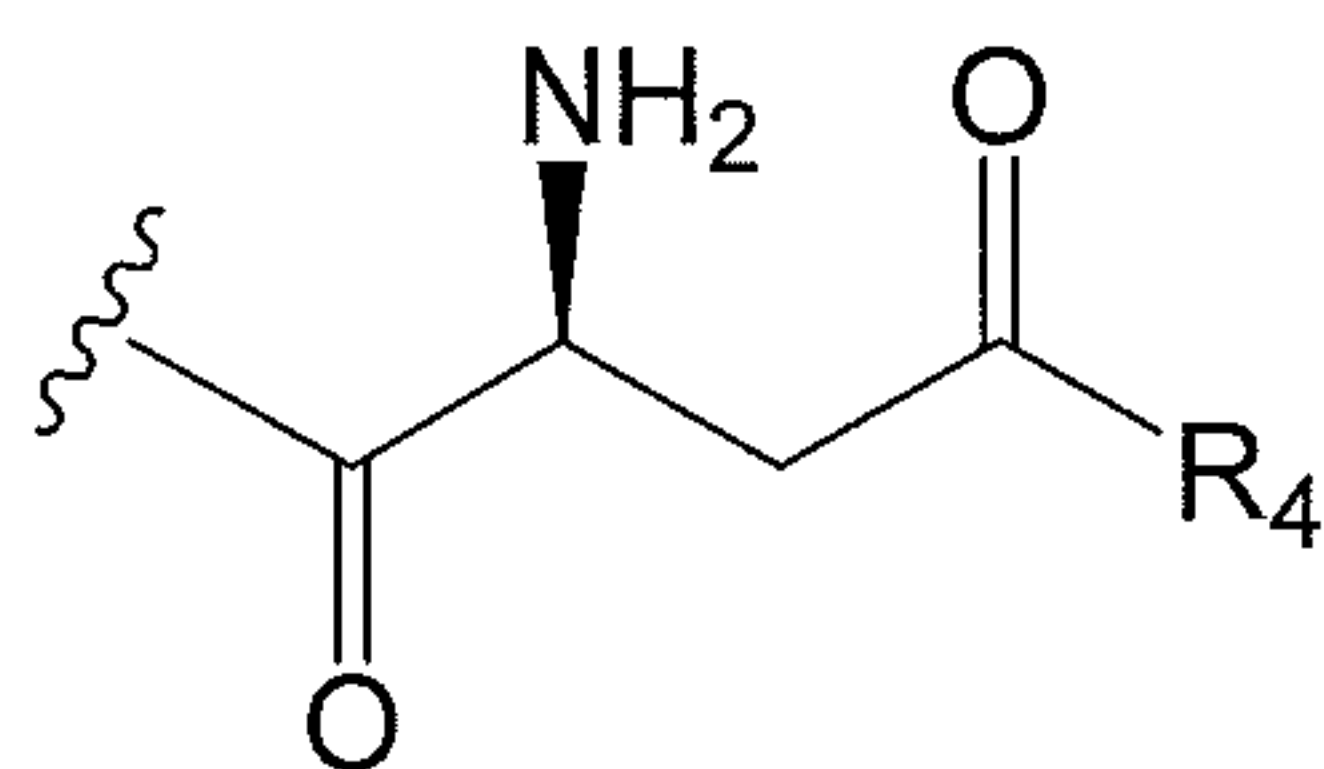
of R_1 , R_2 , and R_3 is not hydrogen, and when R_1 and R_3 are hydrogen, then R_2 is not



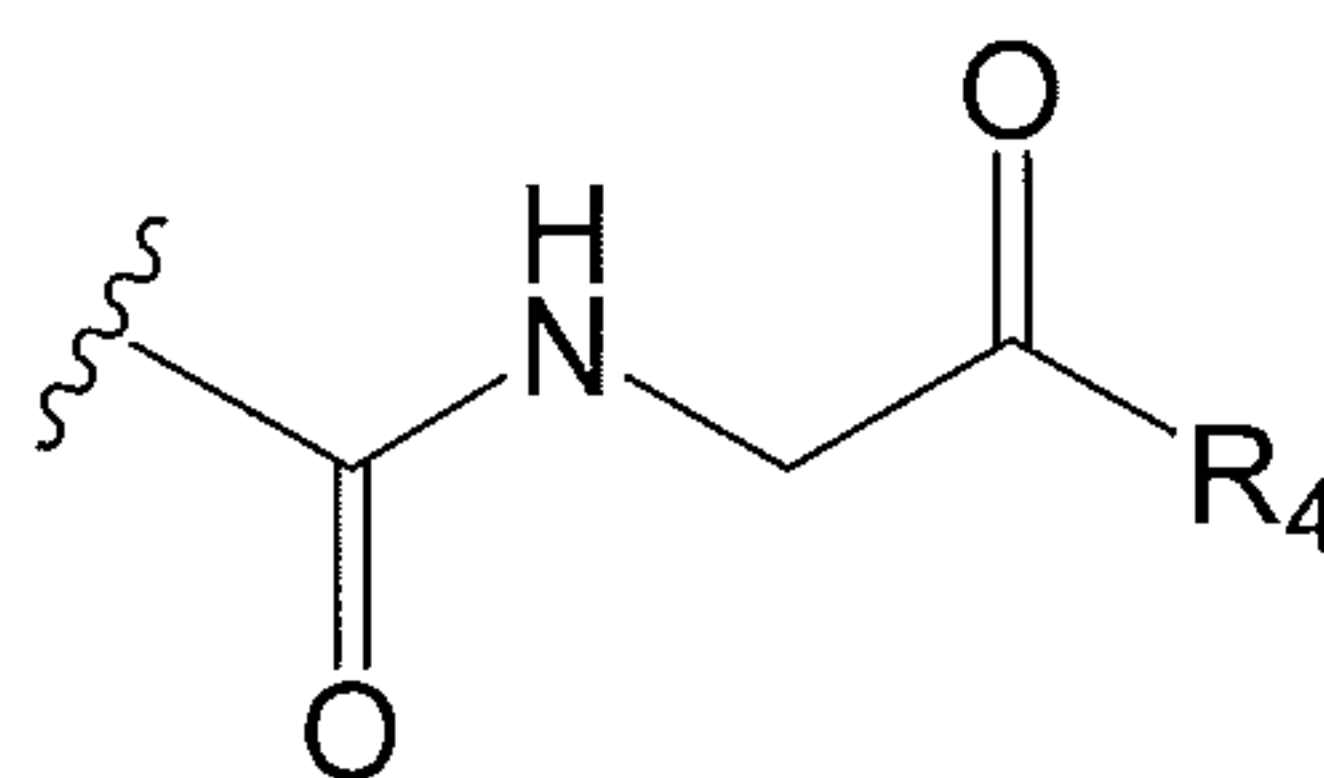
. For example, R_1 , R_2 , and/or R_3 can be hydrogen. For example, R_1 , R_2 , and/or R_3 can be not hydrogen. R_3 can be hydrogen. R_2 can be hydrogen and R_3 can be hydrogen. R_1 can be hydrogen and R_3 can be hydrogen. R_3 can be hydrogen and R_1 can be not hydrogen and R_2 can be not hydrogen. R_3 can be hydrogen and R_1 and R_2 can each be



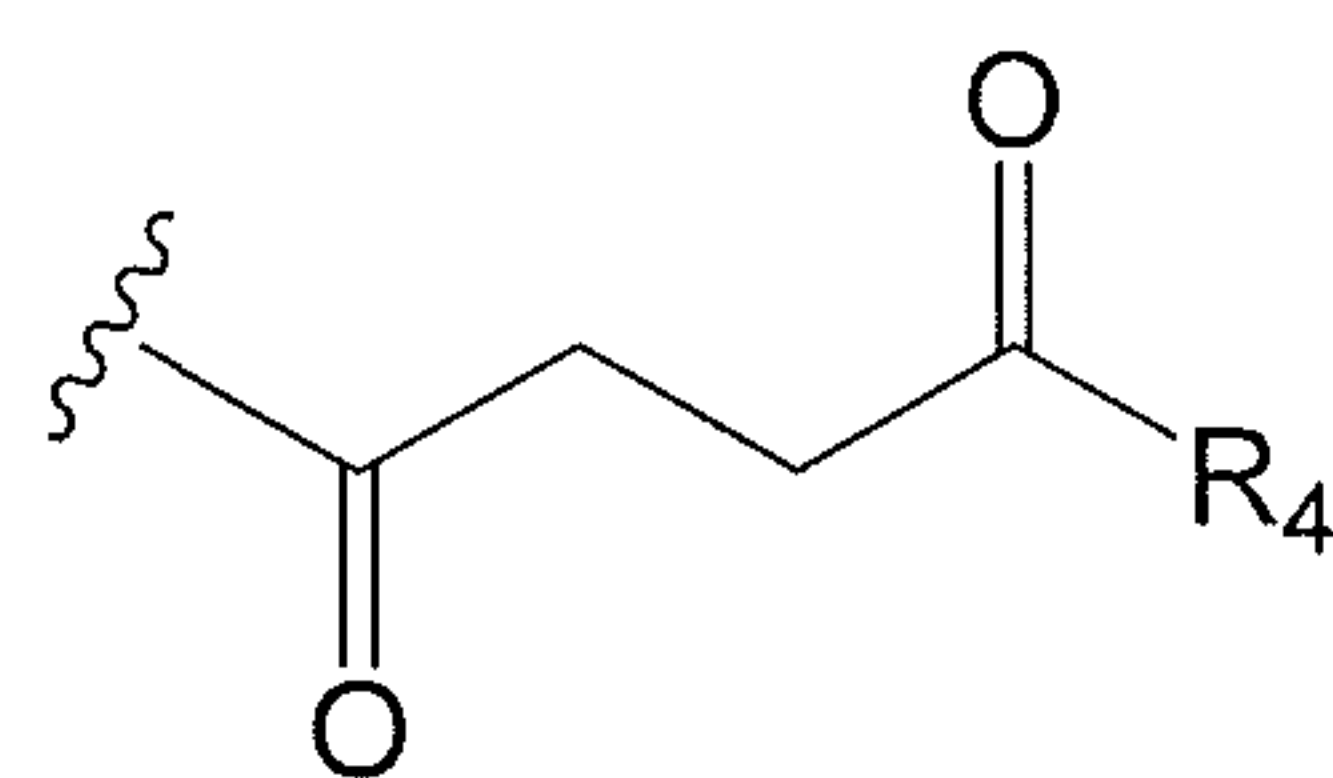
. R_3 can be hydrogen and R_1 and R_2 can each be



. R_3 can be hydrogen and R_1 and R_2 can each be

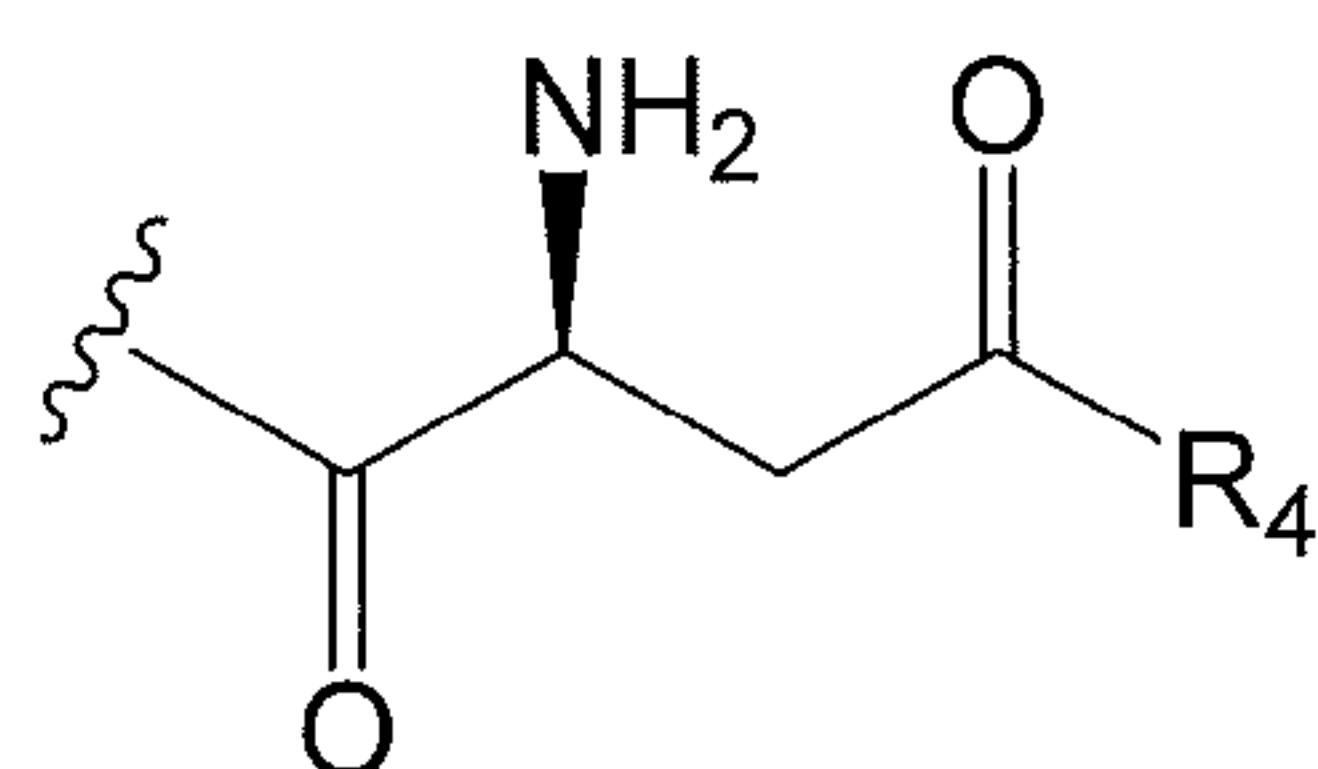


. R_1 , R_2 , and R_3 can each be

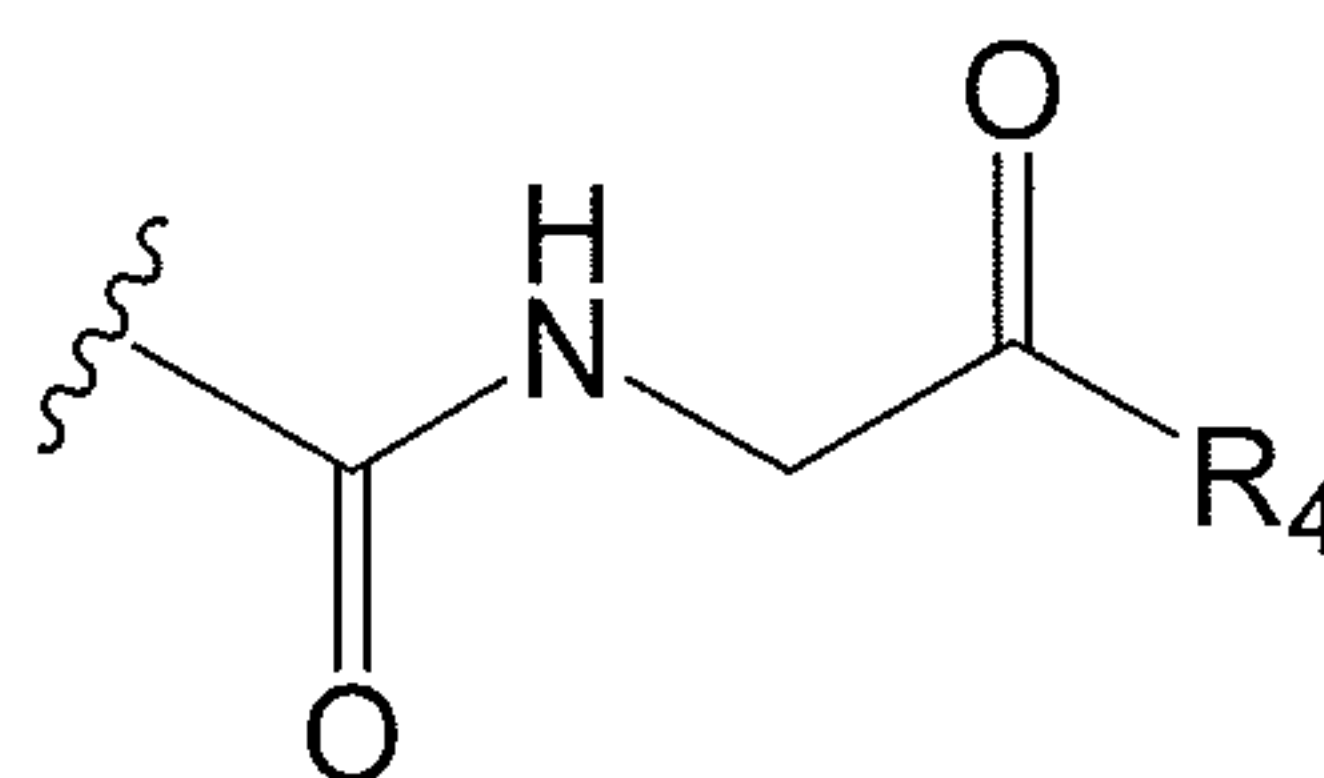


. R_1 , R_2 , and

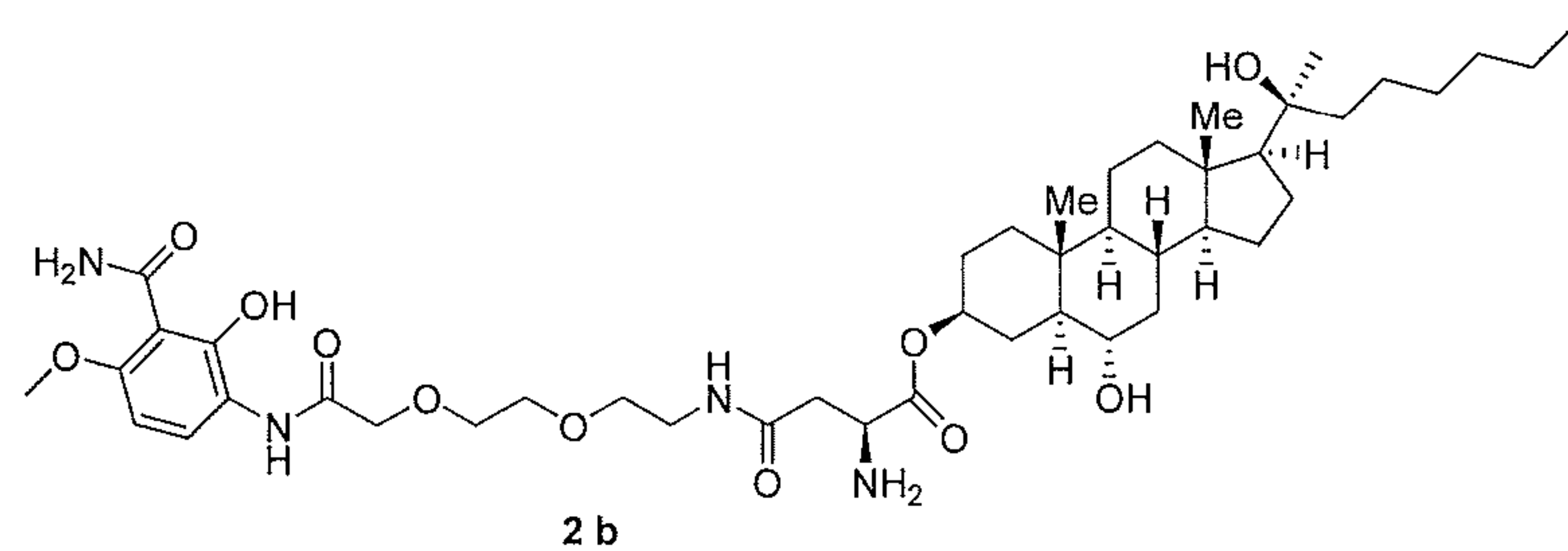
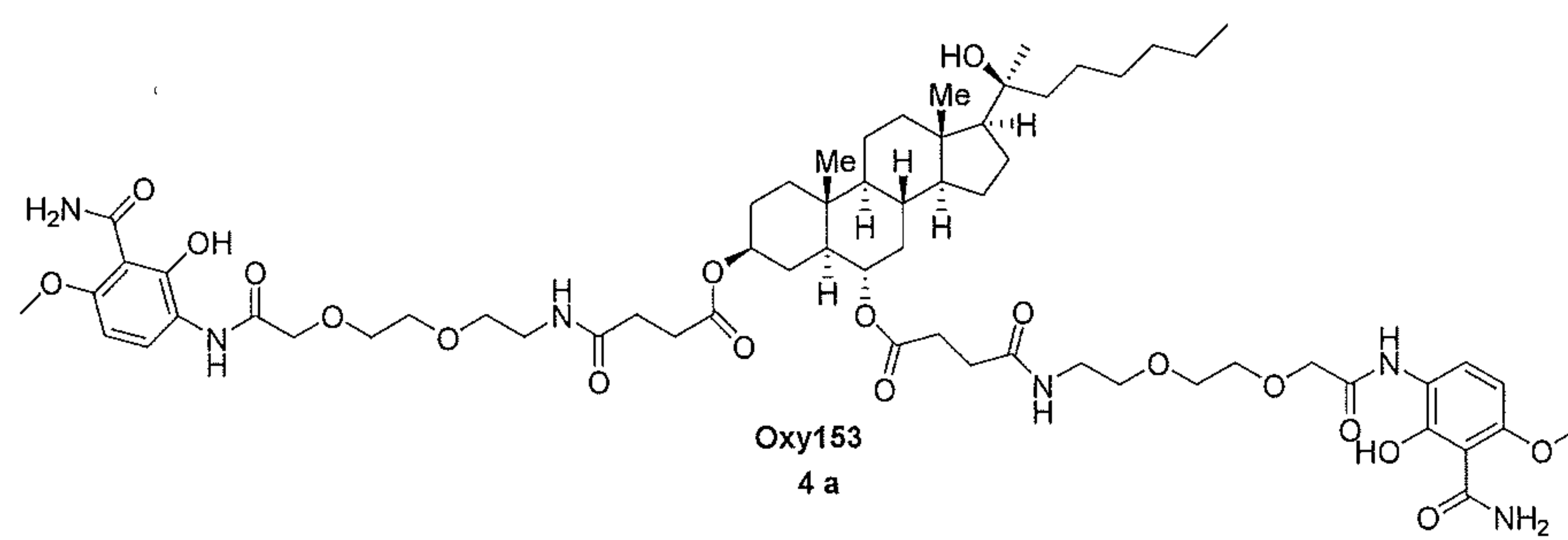
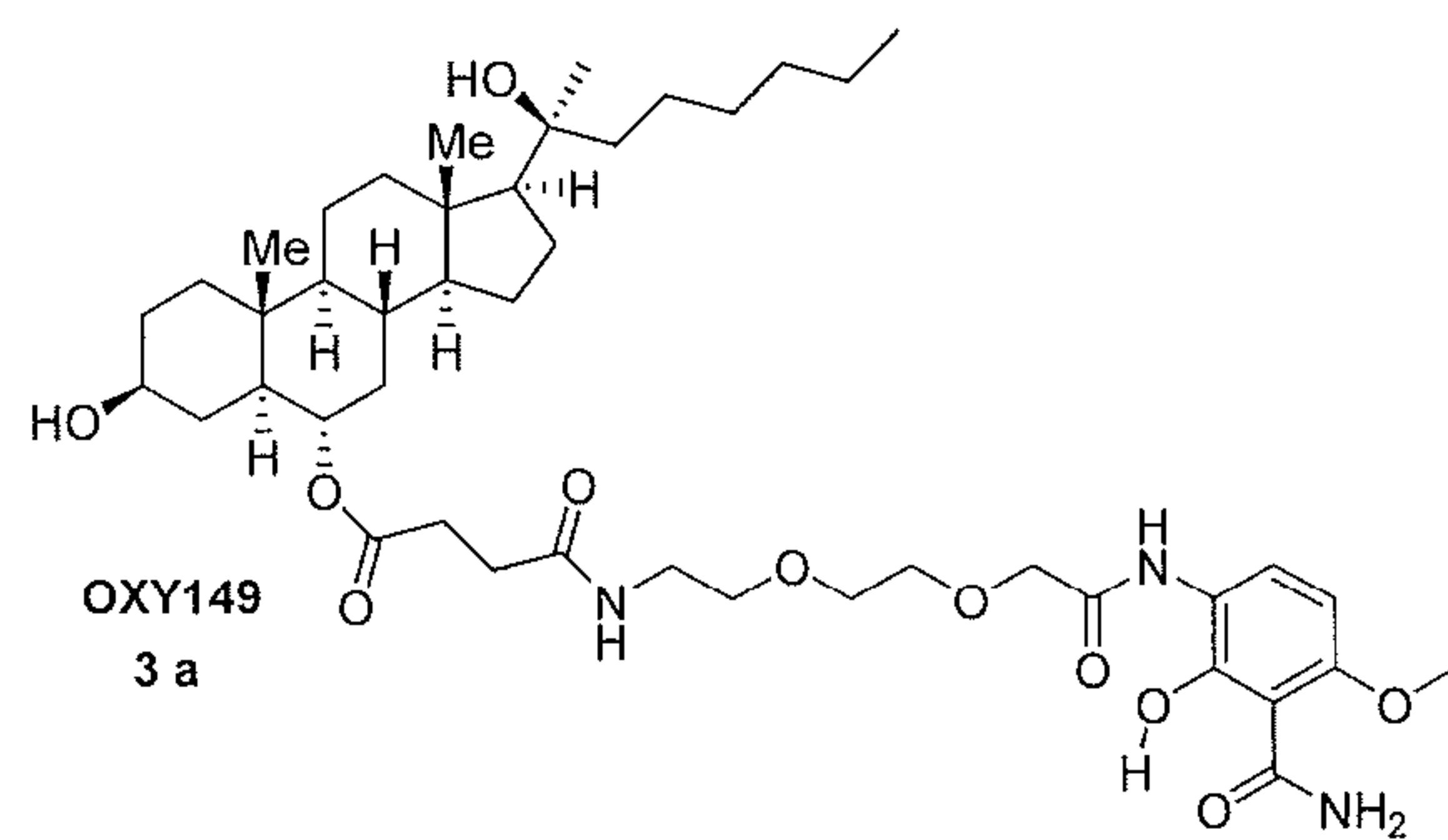
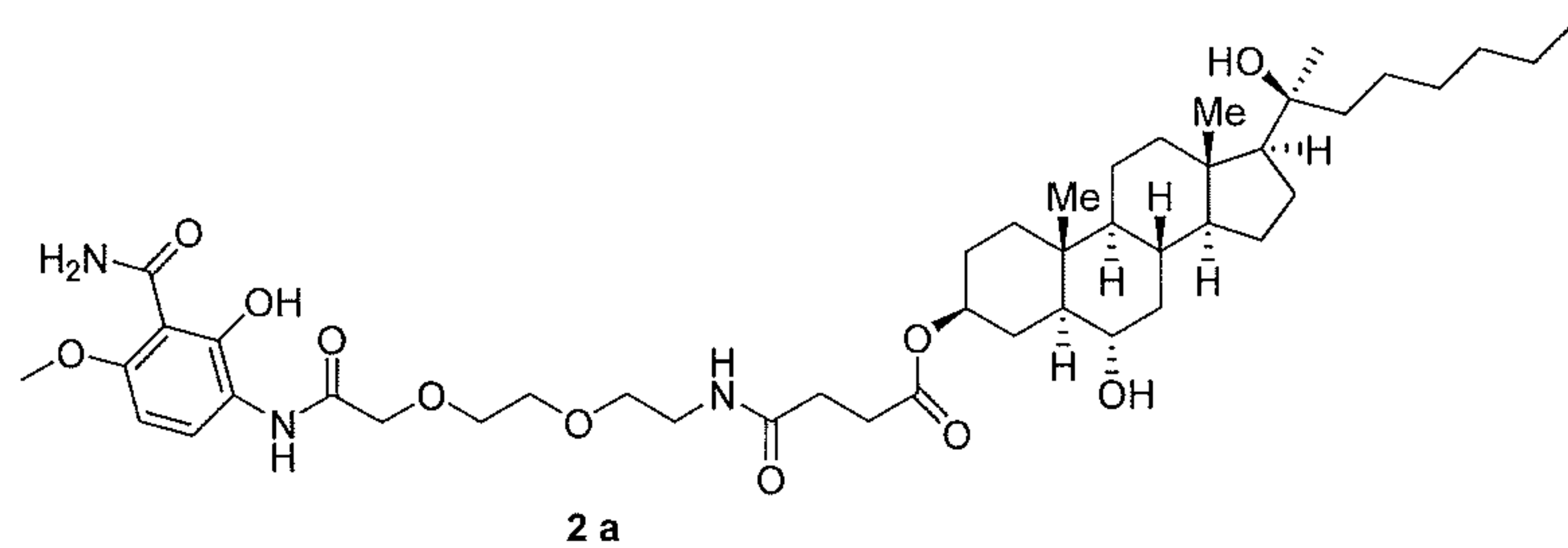
R_3 can each be

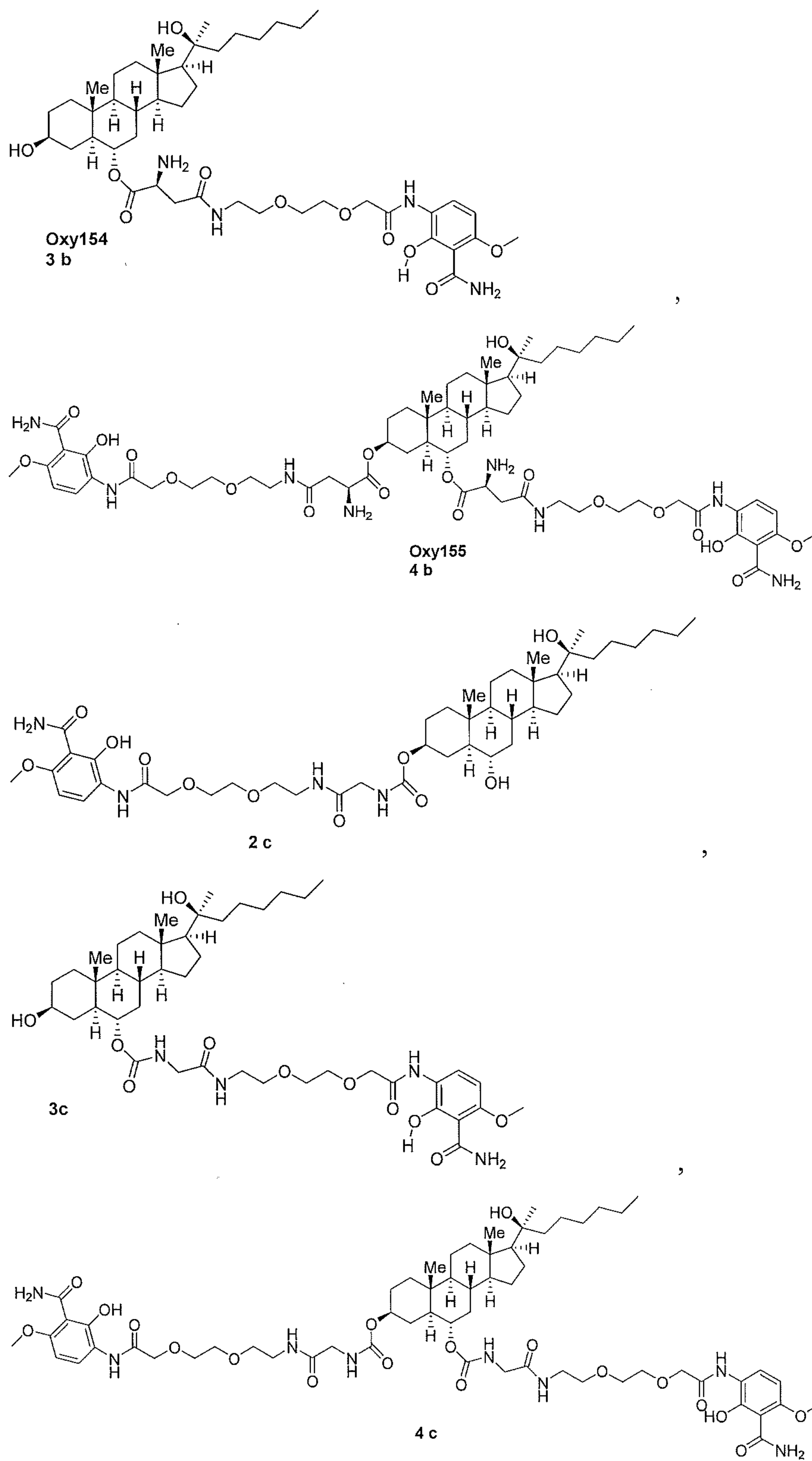


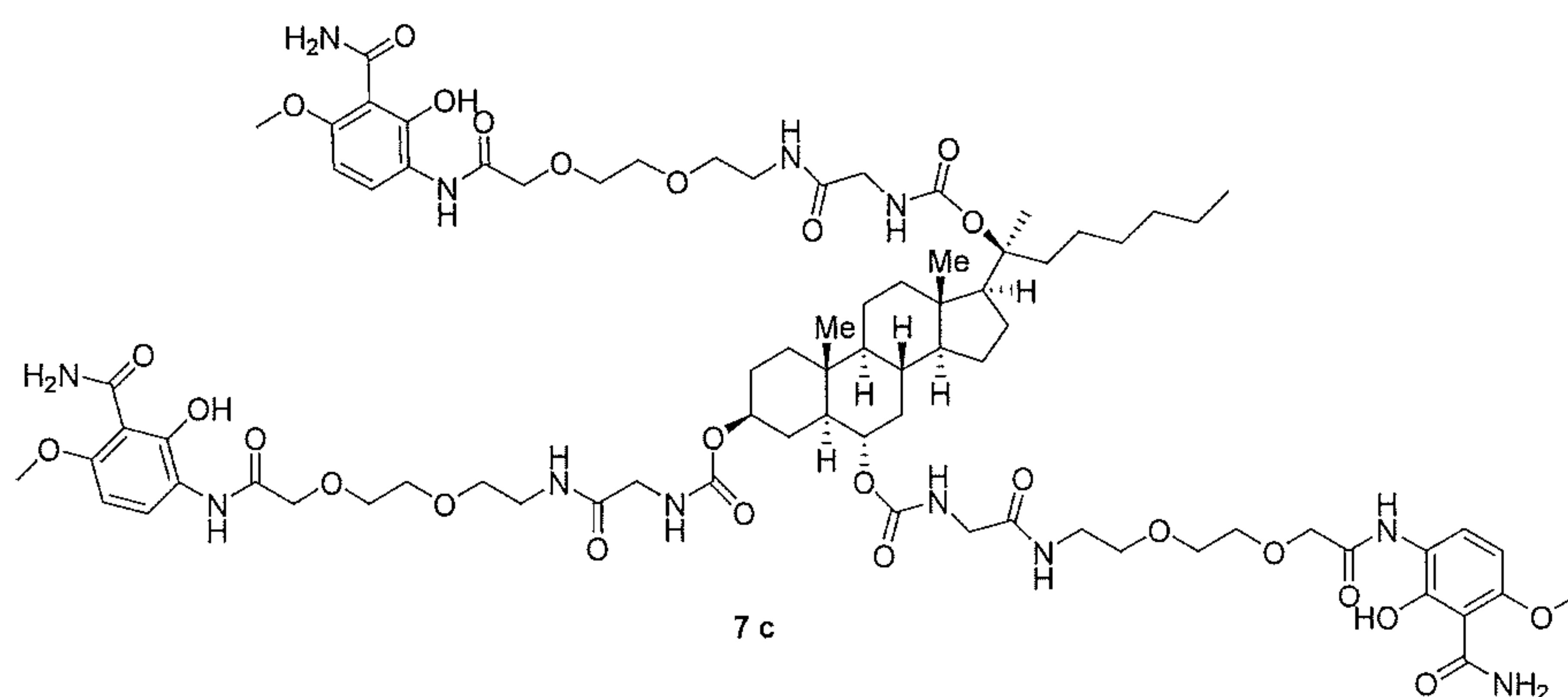
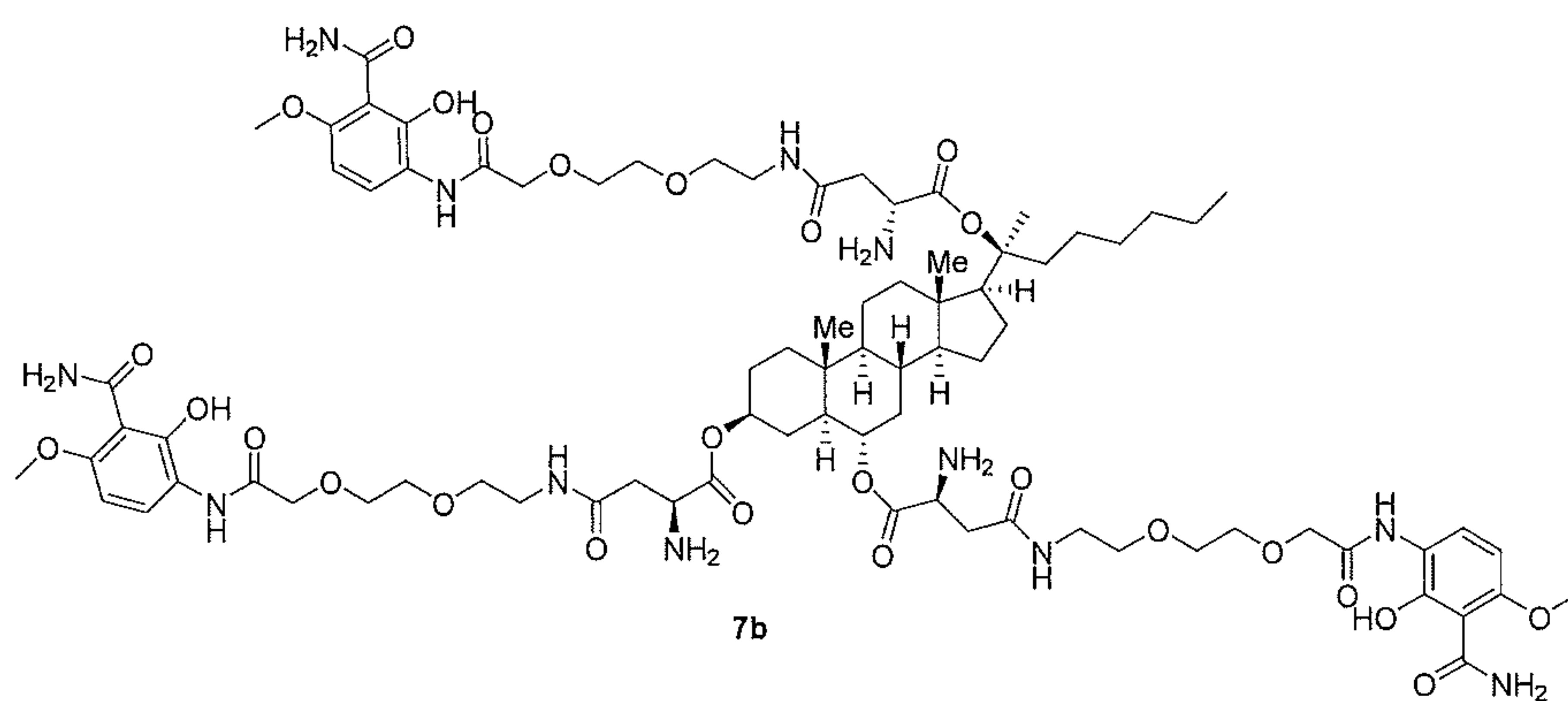
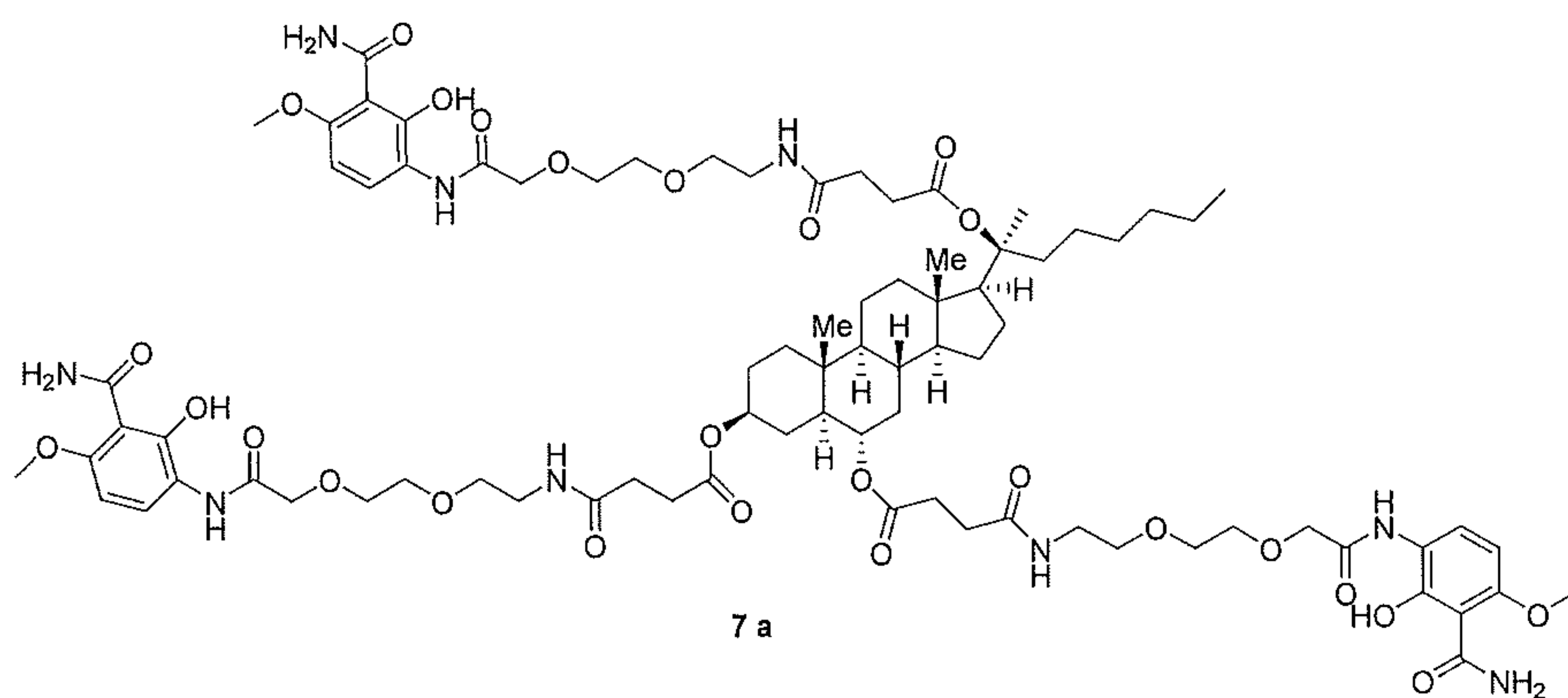
. R_1 , R_2 , and R_3 can each be



. For example, the compound can be







[0003] The composition can include a pharmaceutically acceptable carrier or diluent and can be a pharmaceutical formulation. A method of the present invention includes the administration and/or delivery, locally and/or systemically, of an oxysterol-bone targeting agent compound into a subject, which can be a person or an animal, for the treatment of a bone disorder including, but not limited to, a bone fracture, osteoporosis, and/or osteopenia. A method of the present invention includes *in vitro* treatment of osteoblast progenitor cells with an oxysterol-bone targeting agent compound, and their (the osteoblast progenitor cells) subsequent local and/or

systemic administration and/or delivery into a subject, which can be a person or an animal, for the treatment of a bone disorder including, but not limited to, a bone fracture, osteoporosis, and/or osteopenia. A method of the present invention includes making and/or administering, such as locally and/or systemically, to a cell, an oxysterol-bone targeting agent compound, for example, so that a Hedgehog signaling pathway in the cell is stimulated. The cell can be part of a tissue or an organ, and the compound can be administered *in vivo* (locally or systemically). A method according to the present invention includes treating a subject, which can be a human or an animal, for example, that would benefit from therapeutic activation of a Hedgehog signaling pathway in a tissue or an organ, by treating a cell of the tissue or organ by administering an oxysterol-bone targeting agent to the cell, so that the Hedgehog signaling pathway of the tissue or organ is stimulated.

Brief Description of the Drawings

[0004] Figure 1 shows the structure of the Oxy133 compound, with the sites of BTA-linker attachments.

[0005] Figure 2 shows the structure of tetracycline and the BTA linker 1 formed from a PEG (polyethylene glycol) linker and tetracycline fragment.

[0006] Figure 3 illustrates the relative susceptibility of various linker units to cleavage by esterases.

[0007] Figure 4 shows the alkaline phosphatase (ALP) enzymatic activity of *in vitro* bone marrow stromal cells, M2-10B4, in contact with oxysterols.

[0008] Figure 5 shows the expression of ALP gene by M2-10B4 cells *in vitro* 4 days after initial contact with oxysterols.

[0009] Figure 6 shows the expression of bone sialoprotein (BSP) gene of M2-10B4 cells *in vitro* 4 days after initial contact with oxysterols.

[0010] Figure 7 shows the expression of osterix (OSX) gene of M2-10B4 cells *in vitro* 4 days after initial contact with oxysterols.

[0011] Figure 8 shows the expression of Patched1 (Ptch) gene of M2-10B4 cells *in vitro* 4 days after initial contact with oxysterols.

[0012] Figure 9 shows the expression of Hedgehog (Hh) interacting protein (HIP) gene of M2-10B4 cells *in vitro* 4 days after initial contact with oxysterols.

[0013] Figure 10 shows the concentration of analytes without and with contact with hydroxyapatite (HAP).

Detailed Description

[0014] Embodiments of the invention are discussed in detail below. In describing embodiments, specific terminology is employed for the sake of clarity. However, the invention is not intended to be limited to the specific terminology so selected. A person skilled in the relevant art will recognize that other equivalent parts can be employed and other methods developed without parting from the spirit and scope of the invention.

[0015] This application claims the benefit of U.S. Provisional Application number 61/818,825, filed May 2, 2013, which is hereby incorporated by reference in its entirety.

[0016] Osteoporosis is a common metabolic bone disease affecting more than 10 million Americans, nearly 50% of the elderly female and more than 10% of the elderly male population (T.D. Rachner et al., *Lancet* **2011**, 377, 1276-1287; B.C. Silva et al., *Annu. Rev. Med.* **2011**, 62, 307-322; G.P. Lyritis et al., *Ann. N. Y. Acad. Sci.* **2010**, 1205, 277-283; S. Khosla et al., *J. Clin. Endocrinol. Metab.* **2012**, 97, 2272-2282; T.J. Aspray et al., *Maturitas* **2012**, 71, 76-78; D.M. Black et al., *N. Engl. J. Med.* **2012**, 366, 2051-2053). Osteopenia (reduced bone mass), a major risk factor for developing osteoporosis, is even more common, affecting 34 million Americans (Silva; Lyritis; Khosla; Aspray). Bone fractures are a widespread complication of osteoporosis and osteopenia resulting in significant socio-economic cost, such as hospitalization and disability, and very often they are the cause of deterioration and death of otherwise healthy and functioning elderly individuals (Lyritis). Age-related osteoporotic bone loss and its resulting complications cause significant morbidity and mortality in the aging population (Rachner; Silva; Lyritis; Khosla; Aspray; Black). Among two possible therapeutic strategies for osteoporosis, prevention of bone loss/resorption or stimulation of bone growth, anti-resorptive therapy with bisphosphonate drugs is more established (Khosla; M. Sharpe, et al., *Drugs*, **2001**, 61, 999-1039). Nearly all current therapies for osteoporosis as well as the majority of potential new treatments under clinical investigation aim to reduce the level of bone resorption in osteoporotic patients (Khosla; Aspray; Black; L. Brewer et al., *Eur. J. Clin. Pharmacol.* **2011**, 67, 321-331). Therapies on the market or in clinical trials that target mechanisms of bone resorption include bisphosphonates (e.g., Alendronate), Denosumab (Prolia), Zoledronic Acid (Reclast), Odanacatib, and Saracatinib (Brewer). Anti-resorptive drug therapy has been most effective in

treating early and mild cases of the disease, unlike advanced osteoporosis where a massive loss of bone mineral density has already occurred (Khosla; Brewer).

[0017] Alternatively, bone anabolic agents can provide additional treatment options, particularly with advanced disease, and significantly improve osteoporosis management, in spite of a paucity of FDA approved drugs in this area (E. Canalis, *J. Clin. Endocrinol. Metab.* **2010**, 95, 1496-1504). Currently, the only FDA approved bone anabolic agent available for treatment of severely osteoporotic patients is teriparatide (Forteo), a recombinant form of parathyroid hormone (PTH), which has to be administered intermittently, by daily injection (F.Vescini et al., *Clin. Cases Miner. Bone Metab.* **2012**, 9, 31-36). Forteo can produce significant bone formation and reduce fracture risk, but its use is severely restricted due to safety concerns (Canalis; Vescini; R.Dimitriou et al., *BMC Medicine* **2011**, 9, 1-10). Due to adverse side effects, such as an increased risk of osteosarcoma, drug labeling for Forteo is highly restricted with respect to patient population and duration of use (less than 24 months). Other anabolic agents under clinical investigation include calcilytic drugs that stimulate endogenous intermittent PTH secretion, and inhibitors of antagonists of Wnt signaling (Rachner; Dimitriou).

[0018] In patients with mild osteoporosis, bisphosphonate drugs (e.g., alendronic acid, Fosamax) can produce significant benefits such as improved bone density and reduced fracture risk (Khosla; Sharpe). However, bisphosphonate drugs, including alendronic acid, display low oral bioavailability, 0.6-0.7% on average, even when ingested under fasting conditions. Drug intake together with meals and beverages (other than water) further reduces the bioavailability, and intake under fasting conditions entails serious upper GI tract irritation in a majority of patients (Aspray). Hence, repeated, often daily, oral dosing under fasting conditions is necessary to maximize delivery of the bisphosphonate drugs to what is pharmacologically achievable while more than 99% of the dose cannot be absorbed and is ejected from the body unused. The fraction of bisphosphonate drug that can be absorbed, can rapidly partition in the human body, with about 50% of the drug binding to bone surface and the rest being excreted unchanged via the kidneys. The physicochemical basis of low oral absorption is thought to be associated with the negatively charged phosphonate moieties that are unavoidably part of all bisphosphonate drugs. To overcome this drawback, strategies have been investigated, including prodrug approaches with fatty acid and bile acid conjugation that aim to mask the phosphonate charge effect (P.Vachal et al., *J. Med. Chem.* **2006**, 49, 3060-3063; O.Bortolini et al., *Euro. J. Med. Chem.* **2012**, 52, 221-229).

[0019] Biologics are commonly employed to promote bone growth in medical applications including fracture healing and surgical management of spinal disorders (E.E.Johnson et al., *Clin. Orthop. Relat. Res.* **2000**, 371, 61–74; G.R.Mundy, *Annu. Rev. Med.* **2002**, 53, 337–54; G.A. Rodan et al., *Science* **2000**, 289, 1508–14; S.T.Yoon, *Clin. Orthop. Relat. Res.* **2002**, 395, 33–43). Spine fusion is often performed by orthopedic surgeons and neurosurgeons alike to address degenerative disc disease and arthritis affecting the lumbar and cervical spine. Historically, autogenous bone graft, commonly taken from the iliac crest of the patient, has been used to augment fusion between vertebral levels. However, the associated donor site morbidity, increased operating time, and increased blood loss associated with harvesting autogenous bone graft (E.D.Arrington et al., *Clin. Orthop. Relat. Res.* **1996**, 329, 300–9.; A.R.Vaccaro et al., *Spine J.* **2002**, 2, 206–15; J.A.Rihn et al., *Spine* **2010**, 35, 1629–39) has provided incentive to find a safe and effective alternative.

[0020] Recombinant human bone morphogenetic protein-2 (rhBMP-2) is commonly used to promote spine fusion in humans. Its use was approved in 2002 by the US Food and Drug Administration (FDA) for single-level anterior lumbar interbody fusion (M.Mitka, *JAMA* **2011**, 306, 1311–2.). The use of rhBMP-2 has increased significantly since this time and indications for its use have expanded to include posterior lumbar spinal fusion as well cervical spine fusion. Despite the efficacy of rhBMP-2, recent reports have called into question its safety when employed during spine fusion surgery. Reported complications have included seroma formation, soft tissue swelling, vertebral osteolysis, ectopic bone formation, retrograde ejaculation, and carcinogenicity (K.-U.Lewandrowski, *Spine J.* **2007**, 7, 609–14; D.A.Wong, *Spine J.* **2008**, 8, 1011–8; J.D.Smucker et al., *Spine*, **2006**, 31, 2813–9; E.J.Carragee, *Spine J.*, **2011**, 11, 471–91). Moreover, airway edema has been observed with its use in the cervical spine, prompting the FDA to issue a Public Health Notification warning for its use in cervical spine operations.

[0021] In an embodiment of the invention, novel molecules are synthesized that are combinations of an osteogenic oxysterol, Oxy133, with a bone targeting agent (BTA). When administered systemically these molecules may selectively home to bone tissue and enhance bone formation. These molecules can be used as bone anabolic agents for the treatment of osteoporosis. Bone targeted osteogenic oxysterols are not expected to have significant toxic or immunogenic effects when administered systemically.

[0022] The cellular differentiation of multipotent mesenchymal stem cells (MSCs) into bone forming osteoblasts can constitute a driver of anabolic bone growth. Certain naturally occurring

oxysterols can induce osteogenic while preventing adipogenic differentiation of MSCs *in vitro*, and can stimulate localized bone formation in a rat calvarial defect model *in vivo* (T.L.Aghaloo et al., *J. Orthop. Res.* **2007**, *25*, 1488-1497). The synthesis and characterization of novel semi-synthetic oxysterols with greater osteogenic activity than the naturally occurring oxysterols when used *in vitro* or in a rat spine fusion model *in vivo* has been reported (J.S.Johnson et al., *J. Cell. Biochem.* **2011**, *112*, 1673-1684; S.R.Montgomery et al., *J. Bone Miner. Res.* **2014**, accepted for publication). In an embodiment of the present invention, novel osteogenic oxysterols as bone anabolic agents in the context of systemic dosing (iv (intravenous), ip (intraparenteral), or oral), as required for the treatment of osteoporosis, are set forth.

[0023] Oxysterols, products of cholesterol oxidation, are formed *in vivo*, and have been implicated in various biologic processes including cellular differentiation and cholesterol metabolism (G.J.Schroepfer, *Physiol. Rev.* **2000**, *80*, 362-554; S.Gill et al., *Prog. Lipid Res.* **2008**, *47*, 391-404; B.Sottero et al., *Curr. Top. Med. Chem.* **2009**, *16*, 685-705). Naturally occurring oxysterols, which are found in human and animal circulation and in various tissues, can have bone-forming, osteogenic properties (J.R.Dwyer et al., *J. Biol. Chem.* **2007**, *282*, 8959-8968; W.K.Kim et al., *J. Bone Miner. Res.* **2010**, *25*, 782-795; H.T.Kha et al., *J. Bone Miner. Res.* **2004**, *19*, 830-840; J.A.Richardson et al., *J. Cell. Biochem.* **2007**, *100*, 1131-1145; C.M.Amantea et al., *J. Cell. Biochem.* **2008**, *105*, 424-436). The administration of these oxysterols to pluripotent mesenchymal osteoprogenitor cells, including bone marrow stromal cells (mesenchymal stem cells, MSC) and embryonic fibroblasts, can cause robust osteogenic differentiation and formation of an abundant mineralized bone matrix *in vitro* (Kim; Kha; Richardson). Without being bound by theory, these effects may be mediated in part through activation of the Hedgehog (Hh) signaling pathway independent of the classical Hh proteins (Dwyer). A family of more potent oxysterols can possess osteogenic and anti-adipogenic activity superior to the naturally occurring oxysterols from which they are derived (J.S.Johnson; Montgomery). Such molecules can display potent osteogenic activity *in vitro* and stimulate robust bone formation and spine fusion *in vivo*. They are not expected to elicit significant immunogenic responses (J.S.Johnson; Montgomery).

[0024] Bone health in adult life depends on a coordinated balance of anabolic and catabolic cellular activities of bone-forming osteoblasts and bone-resorbing osteoclasts, respectively. Multipotent mesenchymal stem cells (aka marrow stromal cells, MSCs) form the precursor population for a variety of cell types, including osteoblasts and adipocytes. Formation of new

bone is driven by osteoblastic differentiation of MSCs, a process that can be disrupted by a number of factors. Aging, disease and lifestyle factors such as tobacco and alcohol abuse tend to push MSC populations toward adipogenesis at the expense of osteoblast differentiation, resulting in osteopenic disorders that often lead to full-fledged osteoporosis and impaired fracture repair (A.Sloan et al., *The Surgeon*, **2010**, 8, 111-116; D.A.Chakkalakal, *Alcohol Clin. Exp. Res.* **2005**, 12, 2077-2090). The mechanisms behind lineage-specific differentiation of MSC can be important. Factors can stimulate osteoblast formation while inhibiting adipogenesis.

[0025] Naturally-occurring oxysterols can act as drug-like molecules with an effect on MSCs and other multipotent mesenchymal cells (Aghaloo; Dwyer; Kim; Kha; Richardson; Amantea). Oxysterols that occur in human circulation and various tissues can be short-lived intermediates in metabolic transformations of cholesterol to form steroid hormones and bile acids (Schroepfer). Beyond their role as passive metabolites, however, natural oxysterols can function as signaling molecules, capable of modulating a range of physiological phenomena, among them homeostasis of lipids as well as control over cellular states such as differentiation, inflammation and apoptosis (Gill). That is, oxysterols can play a role as regulators of tissue specific signaling. Early research on oxysterols considered their pathological contributions and assumed that all oxysterols have similar properties, regardless of their distinct chemical composition (Sottero). Oxysterol chemotypes can have more individualized characteristics that depend on the cellular context and the exact chemical composition of the oxysterol (S.Nachtergaele et al., *Nat. Chem. Biol.* **2012**, 8, 211-220). Some oxysterols can promote oxidative stress (Sottero). However, osteogenic oxysterols can inhibit the adverse effects of oxidative stress on osteogenic differentiation of progenitor cells (D.Shouhed, *J. Cell. Biochem.* **2005**, 95, 1276-1283). Some oxysterols are thought to be endogenous ligands of LXR receptors. However, the osteogenic activity of oxysterols may not be a consequence of LXR activation, but can be mediated through the activation of Hh signaling (Dwyer). The oxysterol-induced activation of Hh signaling can occur independent of Hh proteins and result in the activation of non-canonical Wnt and Notch signaling (Kha; Amantea). Baseline PKA/cAMP, PKC, MAPK, and PI3-Kinase signaling can be involved in mediating various aspects of the cellular responses to these oxysterols (Kha; Richardson). In spite of reported cytotoxicity of some oxysterols (Schroepfer), no toxic effects were found with osteogenic oxysterols *in vitro* when dosed at 1-20 μ M with osteoprogenitor cells or, *in vivo*, during local administration in the rat spine fusion

model (40 mg), or, in mice, dosed *ip* at 50 mg/kg 3 times per week for a total of 8 weeks as determined by the absence of behavioral changes.

[0026] Naturally-occurring oxysterols, 20(*S*)-hydroxycholesterol, 22(*S*)-hydroxycholesterol and 22(*R*)-hydroxycholesterol can be used as potential osteogenic agents (Kha; Richardson; Amantea). A series of potent osteogenic oxysterol analogues was identified, which are efficacious both *in vitro* and *in vivo*. Members of this family of semi-synthetic oxysterols induce robust bone formation and spine fusion in rats when applied locally between transverse processes via a collagen sponge (J.S.Johnson). The compound OXY133 is an analog in the series with enhanced osteogenic activity (Figure 1) (Montgomery). Oxy133 can induce osteogenesis in cultured human primary mesenchymal stem cells and induce spine fusion in rats in an accelerated manner compared to other analogues. Oxy133 can induce robust osteogenesis in non-rodent models of bone disease such as rabbit spine fusion and rabbit calvarial defect models. Oxy133 is a drug development candidate for local administration with potential application in spine fusion and repair of non-union fractures. However, when contemplating systemic administration of oxysterols like Oxy133 as a potential anabolic factor to stimulate bone formation in osteoporosis, one has to consider their short half-lives (< 5 min) in human liver microsomes (HLM), and tissue distribution that does not necessarily favor deposition in bone tissue. Furthermore, due to the possible mechanism of osteogenesis, a transient activation of the Hh-pathway in MSCs, increasing selectivity for bone tissue while minimizing the exposure to other tissues may be prudent. This can be accomplished by linking a bone targeting agent (BTA) to the oxysterol molecule that selectively delivers it to bone.

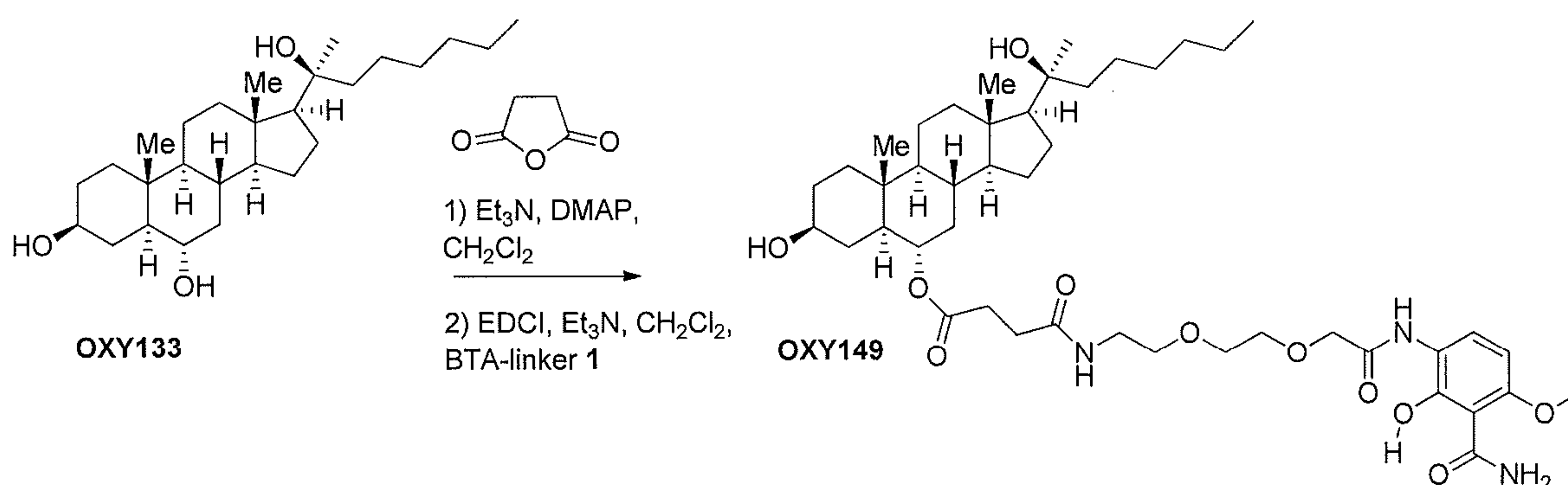
[0027] Bone specific drug delivery is not only applicable to drugs marginally acceptable for bone disease (e.g., estradiol and diclofenac) to increase efficacy, minimize side effects and allow for appropriate dosing (S.Zhang et al., *Chem. Soc. Rev.* **2007**, 36, 507-31). In an embodiment of the present invention, the concept of bone specific drug delivery is applied to osteogenic molecules not previously tested for systemic bone disease, to render them as effective treatments for osteoporosis. Oxysterol-based agonists of Hh signaling with osteogenic properties can fall into this category. Bone specific drug delivery agents can be attached to their drug molecules via hydrolysable linker bonds (M.W. Orme et al., *Bioorg. Med. Chem. Lett.* **1994**, 4, 1375-1380; Zhang).

[0028] The compound Oxy133 (Figure 1) can act as a potent osteogenic oxysterol, which induces osteogenic differentiation of osteoprogenitor cells *in vitro* and robust bone formation *in*

vivo in rat and rabbit spine fusion models. An osteogenic oxysterol, Oxy149, is a combination of Oxy133 and a bone targeting agent (BTA) that is a derivative of tetracycline. As shown in Figure 1, we have identified three sites for the attachment of BTA to Oxy133, i.e., at the hydroxyl groups of carbon 3, carbon 6, and carbon 20 through either succinic acid or aspartic acid linkers. Linker attachments of the tetracycline BTA fragments include, but are not limited to, succinate-based linkers and polyethylene glycol (PEG)-based linker units, such as those resulting in BTA-linker 1, depicted in Figure 2. For example, an Oxy133-BTA analog can have BTA attached at the hydroxyl group of carbon 6. Also set forth herein are other analogues where BTA can be linked to carbon 3 or carbon 20, or to more than one carbon resulting in a total of 2 or 3 BTA units on Oxy133.

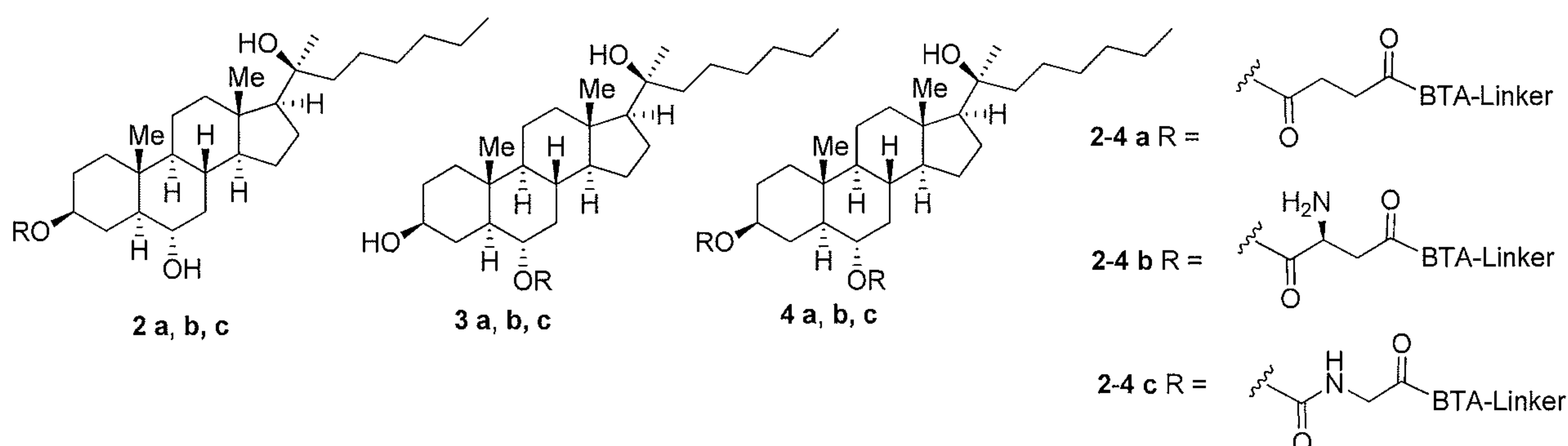
[0029] Bone specific drug delivery by bone specific drug delivery systems, for example, for orthopedic medicine, is not only applicable to drugs marginally acceptable for bone disease (e.g.: estradiol and diclofenac) to increase efficacy, minimize side effects and allow for appropriate dosing. This concept can be extended to osteogenic molecules not previously tested for systemic bone disease, potentially making them into effective treatments for osteoporosis. Oxysterol-based agonists of Hedgehog (Hh) signaling with osteogenic properties mostly fall into this category. Among known chemical entities with high bone affinity ranging from oligopeptides to various bisphosphonates, the antibiotic tetracycline is a relatively nontoxic, orally available and “human-experienced” bone targeting agent or moiety. However, its powerful antibiotic activity, chemical complexity, and lack of stability limit the clinical potential of unmodified tetracycline as a bone targeting agent (BTA). Therefore, tetracycline fragments are considered as BTA-units. The latter are devoid of antimicrobial activity and undue chemical complexity, while retaining most (80%) of the bone affinity compared to tetracycline in a hydroxyapatite binding assay. The linker attachments of tetracycline BTA fragments can include succinate-based linkers and polyethylene glycol (PEG)-based linker units, for example, for BTA-linker 1, depicted in Figure 2. BTA agents can be attached to drug molecules via hydrolysable linker bonds. Non-hydrolysable bonds may be used in cases where the drug molecule after conjugation to the BTA unit retains pharmacological activity. Ester groups can be used, as they populate a favorable stability range relative to more labile thioesters and more stable amides (L.Gil et al., *Bioorg. Med. Chem.* **1999**, 7, 901-919). The *in vivo* stability of ester groups can be further fine-tuned by substitutions placed adjacent to the ester group (T.C.Bruice et al., *Bioorganic Mechanisms*, Vol. 1, W. A. Benjamin, New York, **1966**, 1-258). Hence,

Oxy133-BTA ester conjugates can be suitable for systemic dosing (oral, ip, or iv) that entails selective deposition in bone tissue followed by enzymatic linker hydrolysis and release of the osteogenic agent, Oxy133, at controlled rates into the target tissue. Such attachment of BTA-linker 1 to the 6-position of Oxy133 to form the conjugate Oxy149 (3a) can be achieved by a straightforward coupling to succinic anhydride via ester linkage, as depicted below.



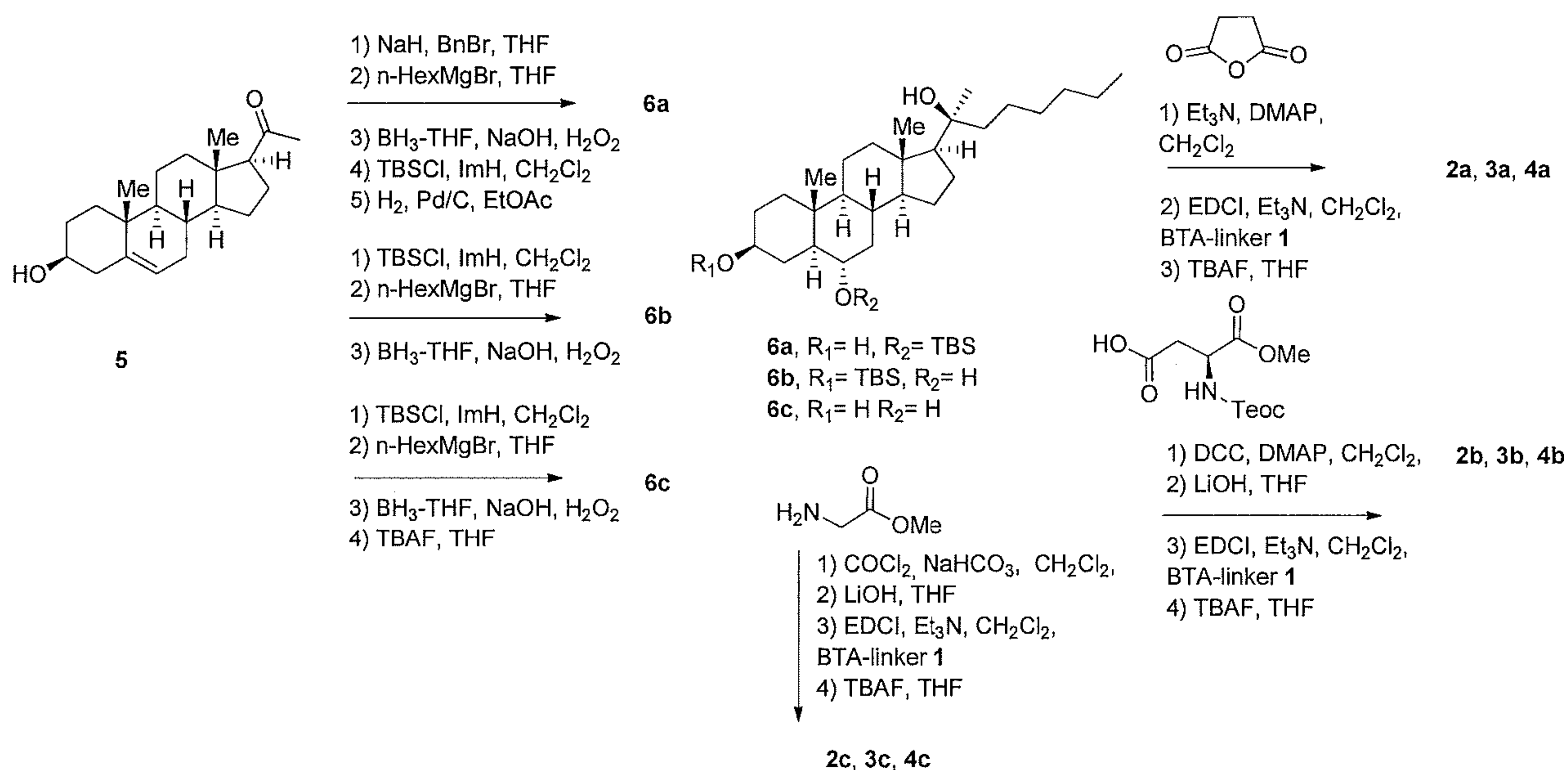
(In Oxy133 the 6-hydroxyl group is more reactive toward succinic anhydride than the 3-hydroxyl.) The resulting conjugate, OXY149, retains most of the osteogenic activity of the parent Oxy133 in C3H10T1/2 cells determined by the level of induction of osteogenic differentiation marker, alkaline phosphatase activity after 4 days of treatment (Control: 2 ± 1 ; Oxy133(1 μM): 390 ± 10 ; Oxy149(1 μM): 100 ± 12 ; Oxy149(5 μM): 500 ± 18 ; Oxy149(10 μM): 480 ± 12 ; Oxy149(20 μM): 470 ± 25). BTA-linker 1 can be attached via the 3- and/or 6-positions of OXY133 using tunable linker attachments.

[0030] Analogues 2, 3, and 4, in which Oxy133 is conjugated to the BTA via the 3- and/or 6-positions with ester linker units derived from succinic (a series) or aspartic acid (b series) and a carbamate linker unit (c series), that is, oxysterol-BTA conjugates with a tunable linker attachment, are depicted below.



[0031] The carbamate linker should be more stable toward esterase hydrolysis compared to ester linkers. The succinic acid-based linker should be more stable toward esterase hydrolysis compared to the aspartic acid-based linker, which may undergo enzymatic hydrolysis of the amino ester bond more readily. A difference in the rate of ester hydrolysis can be used to fine tune the release of Oxy133 in the target bone tissue. For example, Oxy 133 conjugated to BTA using a urethane unit should be more stable than when the conjugate is formed using an aspartate. This relative susceptibility of various linker units to cleavage by esterases is illustrated in Figure 3.

[0032] The synthesis of the Oxy133-BTA conjugated analogues **2**, **3**, and **4** starting from pregnenolone (**5**) is shown below. The latter can be transformed by known methods to differentially protected Oxy133 derivatives, **6 a-c**, by protection of the 3-hydroxyl, addition of the side chain, hydroboration-oxidation of the 5-alkene, and then, depending on the analogue desired, selective protection or deprotection of the hydroxyl groups. The coupling partners for these compounds can be prepared.

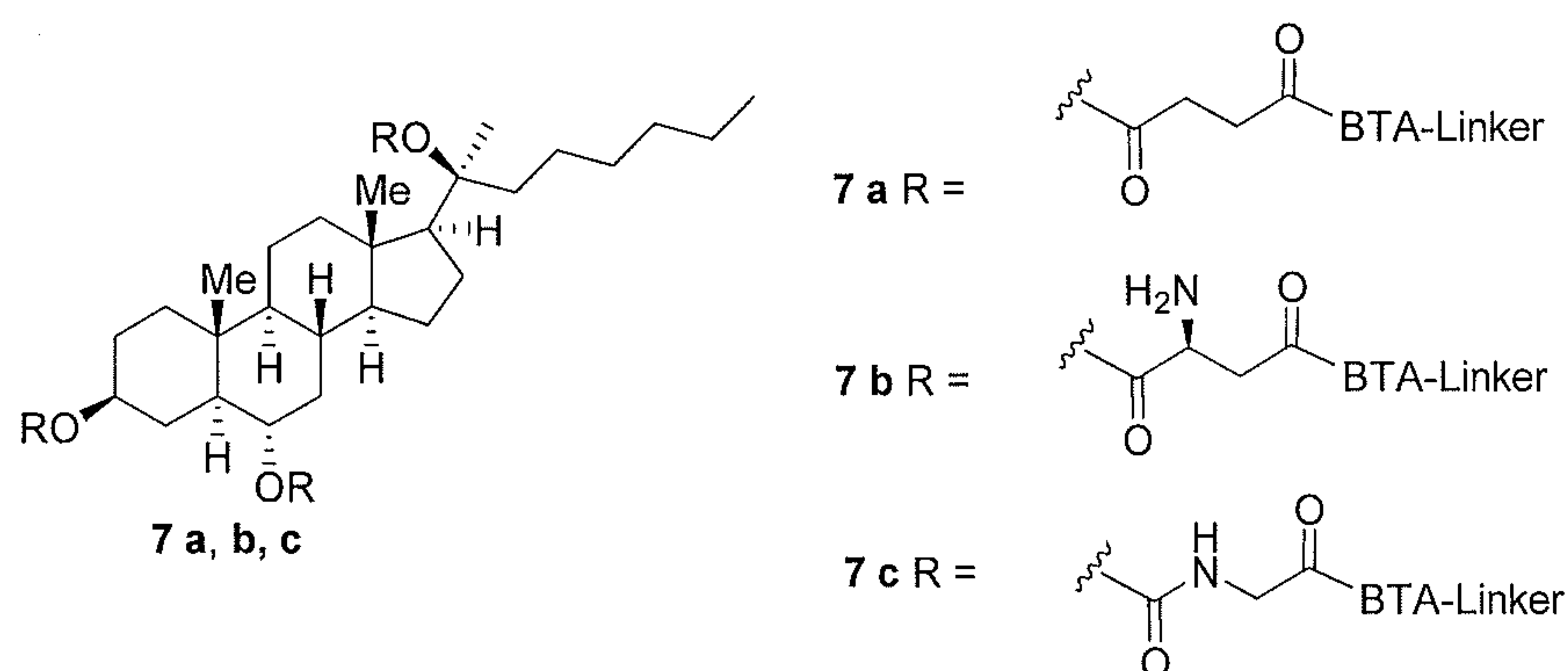


[0033] First, differentially protected Oxy133 derivatives **6 a-c** are acylated at the 3 and/or 6-hydroxyl with succinic anhydride or protected aspartic acid and the resulting carboxylic acids are then coupled to BTA-linker **1** to afford ester conjugates, as depicted in Figure 6. After the ester coupling reactions, the *tert*-butyldimethylsilyl ethers (TBS) and, in the case of **2-4b**, the 2-trimethylsilylethyl carbamate (Teoc) unit can be cleaved with tetra-*n*-butylammonium fluoride

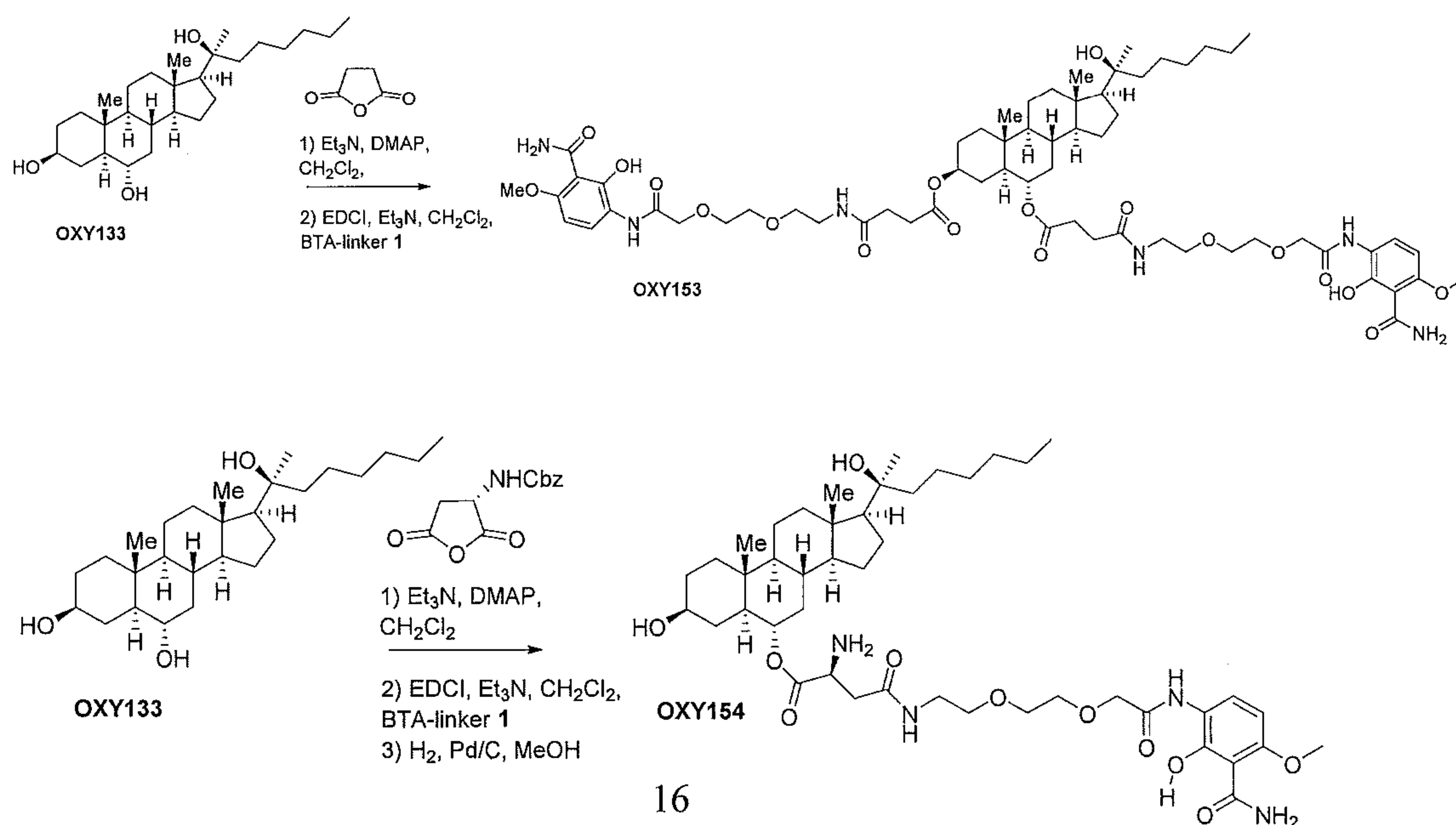
(TBAF) to afford the final products incorporating the succinic acid linker (2-4 a) and the aspartic acid linker (2-4 b). Compound 3a corresponds to Oxy149, obtained in the preliminary study shown in Figure 3. The carbamate linked analogues, 2-4 c, are prepared by converting glycine methylester into the isocyanate to carbamylate Oxy133 derivatives 6 a-c after which the synthesis proceeds in an analogous fashion.

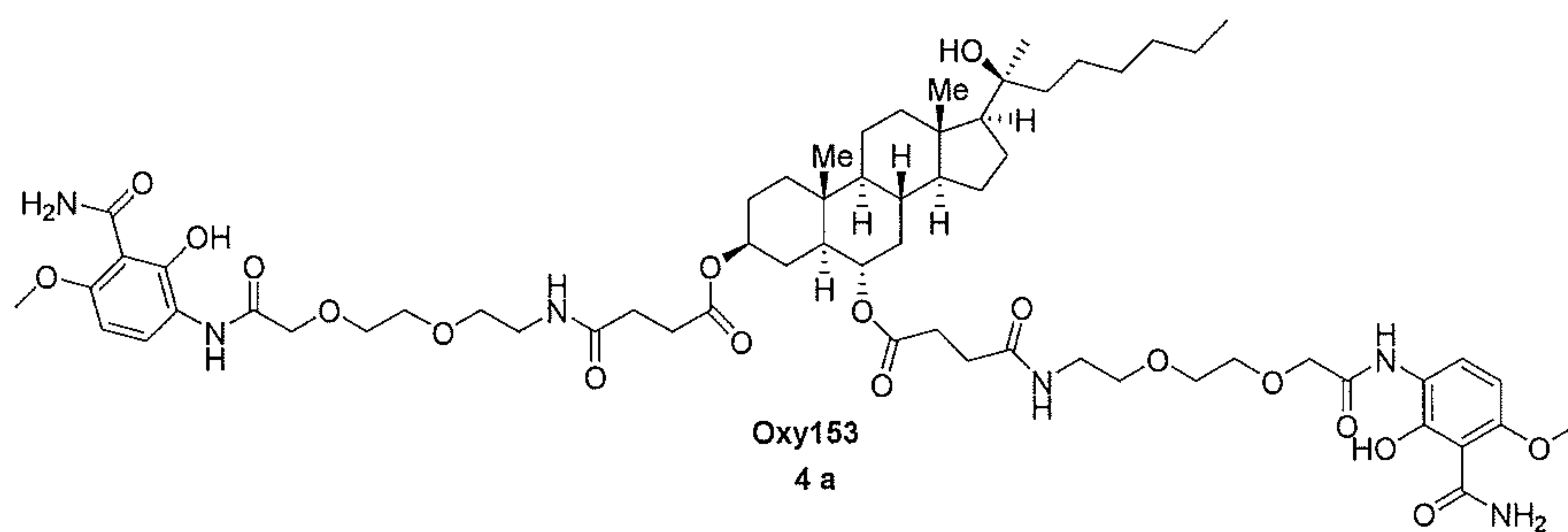
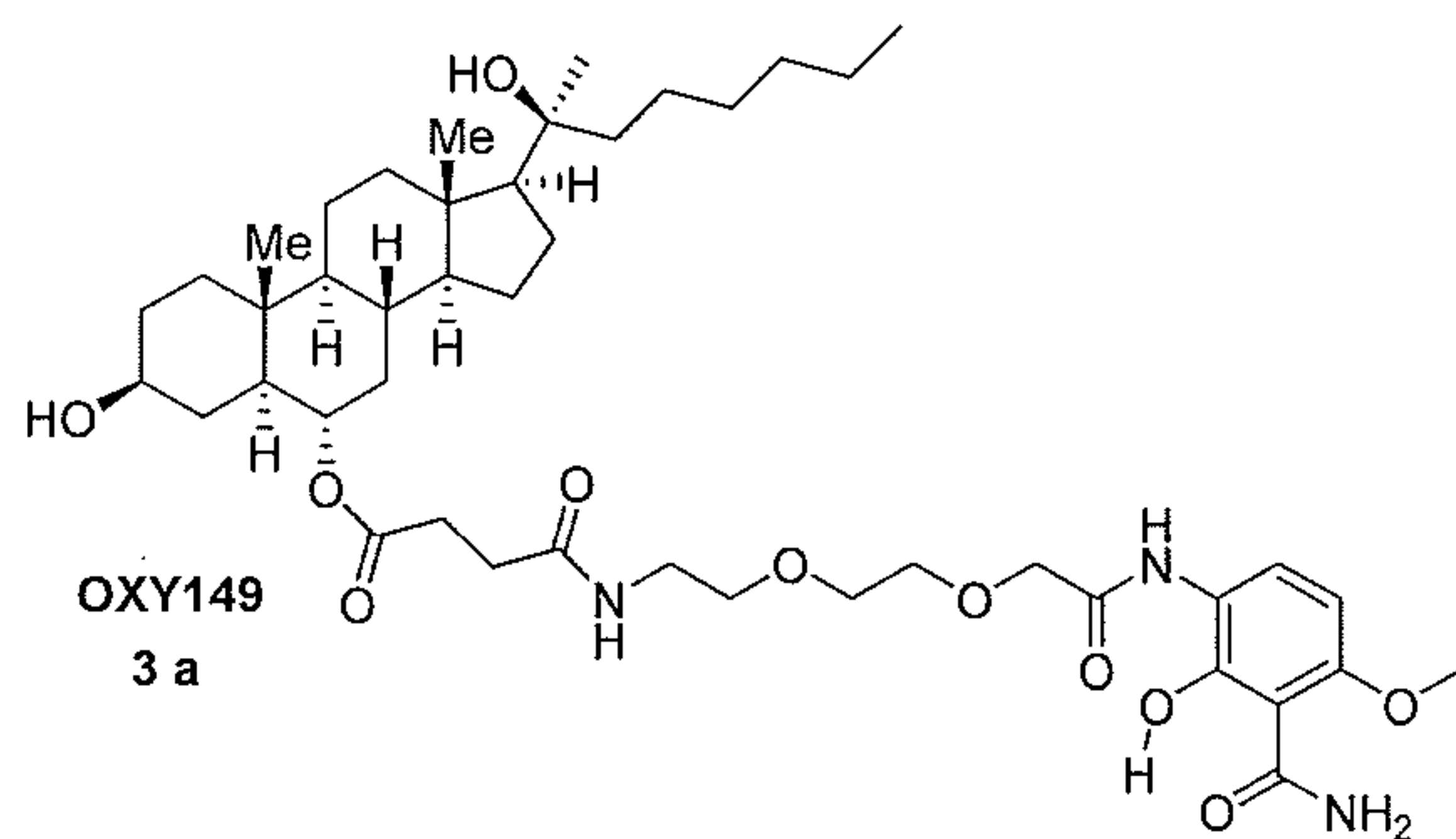
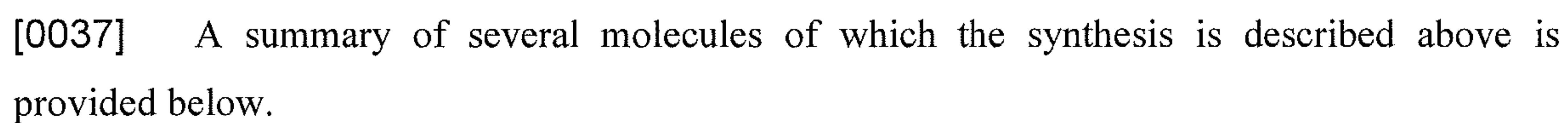
[0034] A derivative of Oxy133-BTA may 1) have greater osteogenic activity in a C3H10T1/2 cell based assay screens than Oxy149, perhaps based on a more favorable cell-cleavable property, and 2) show optimized hydroxyapatite binding capacity.

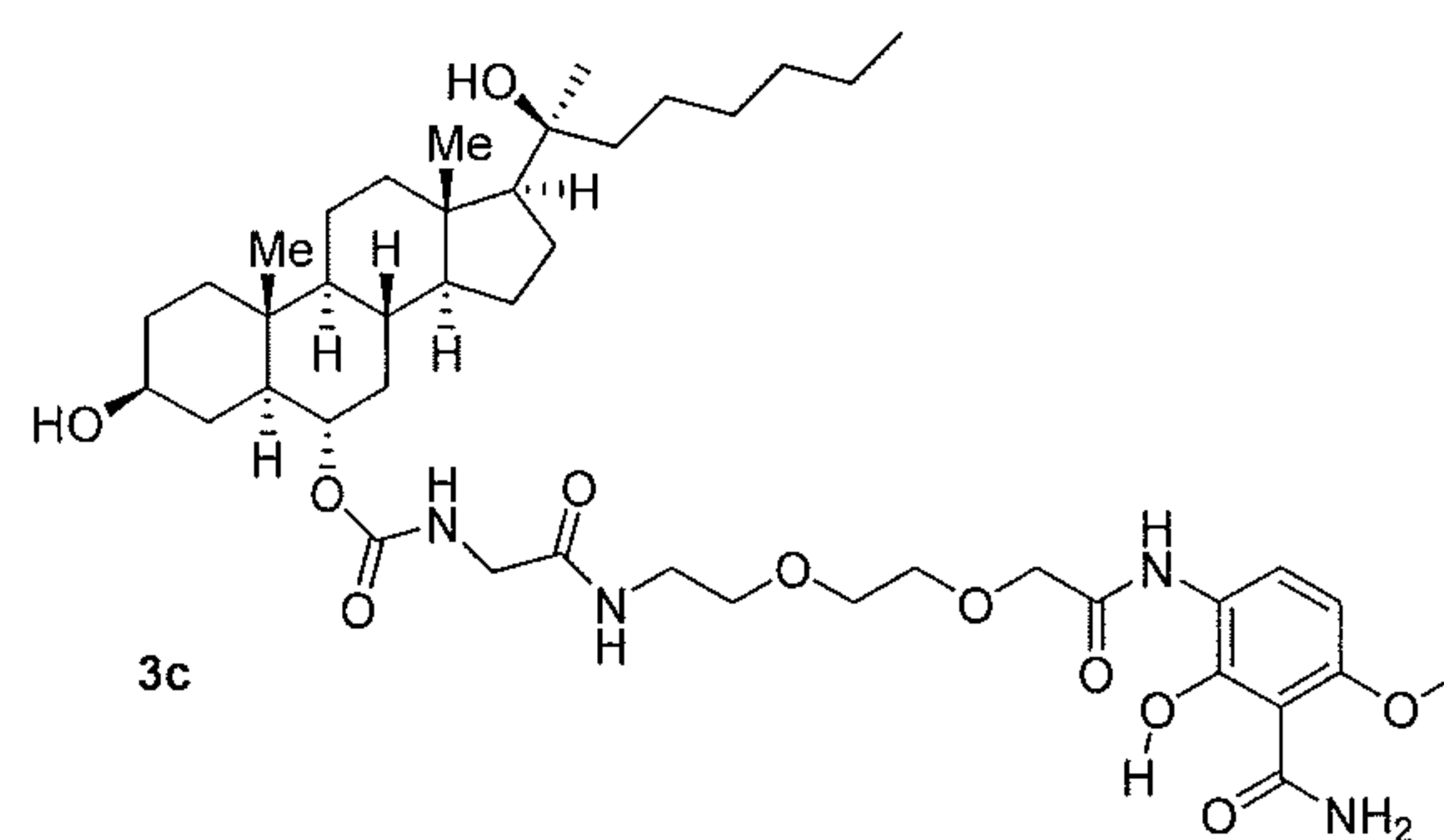
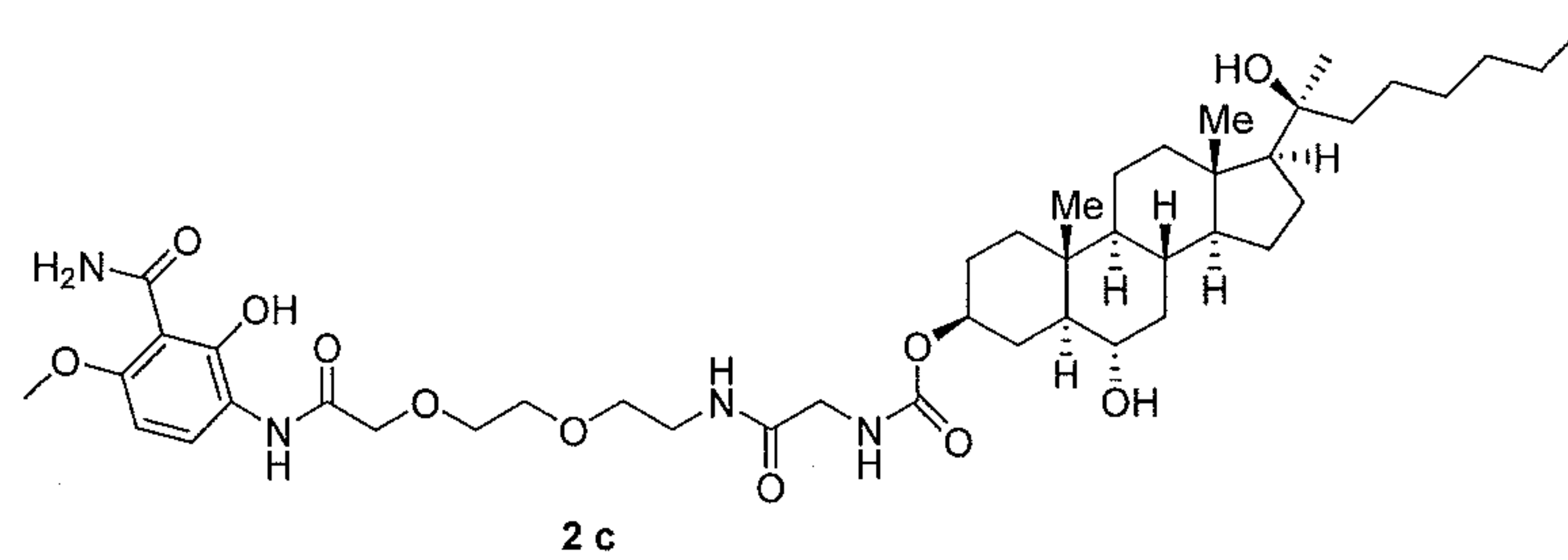
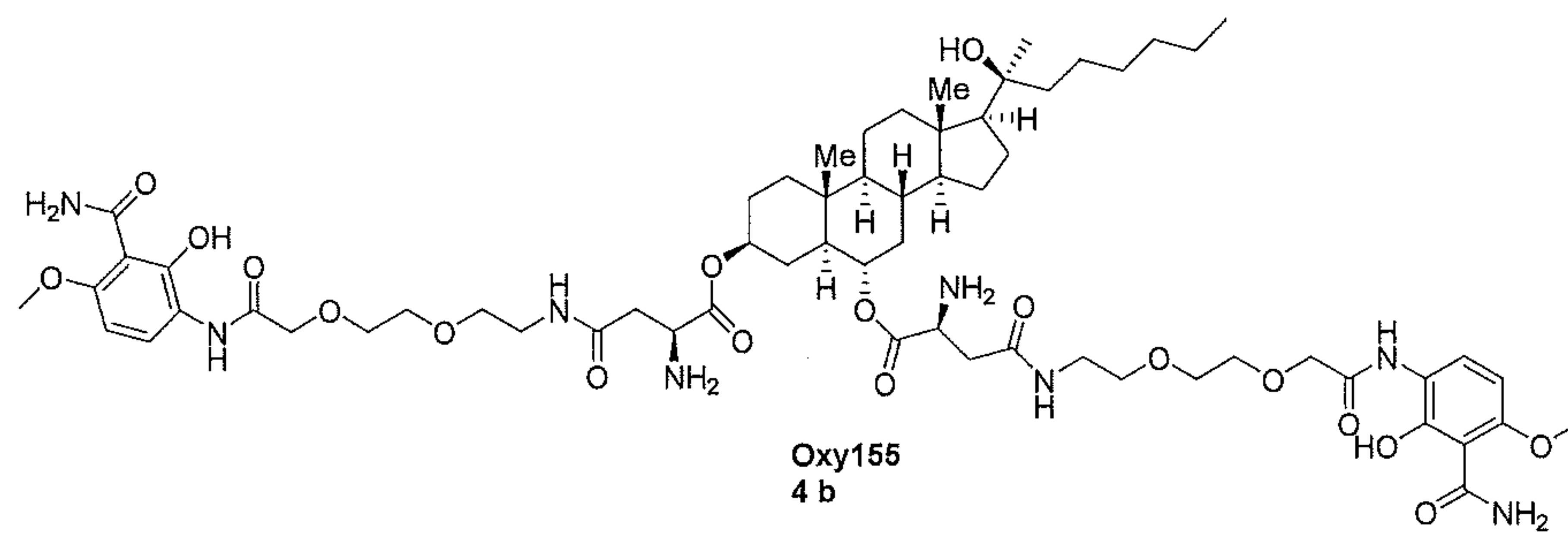
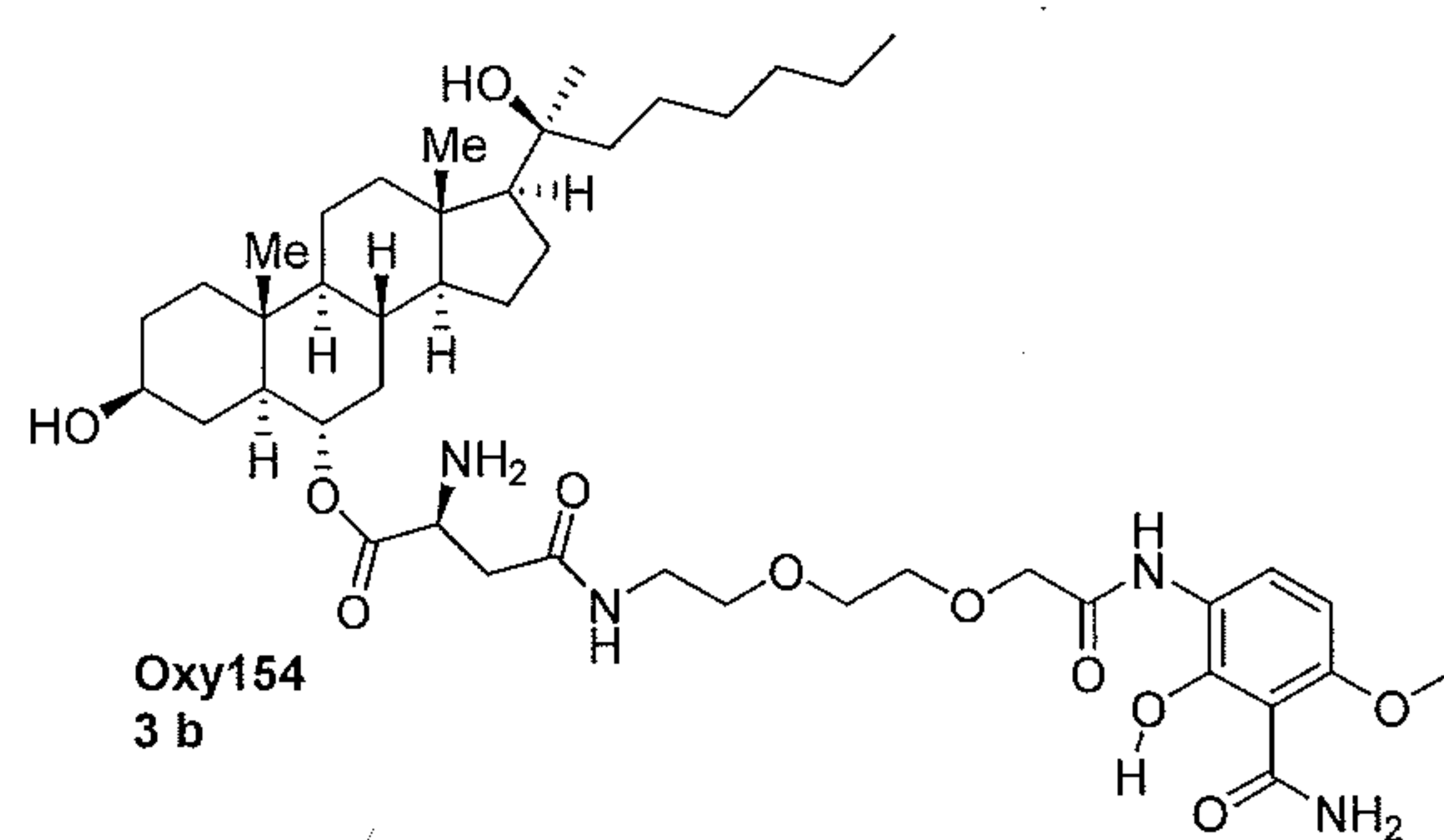
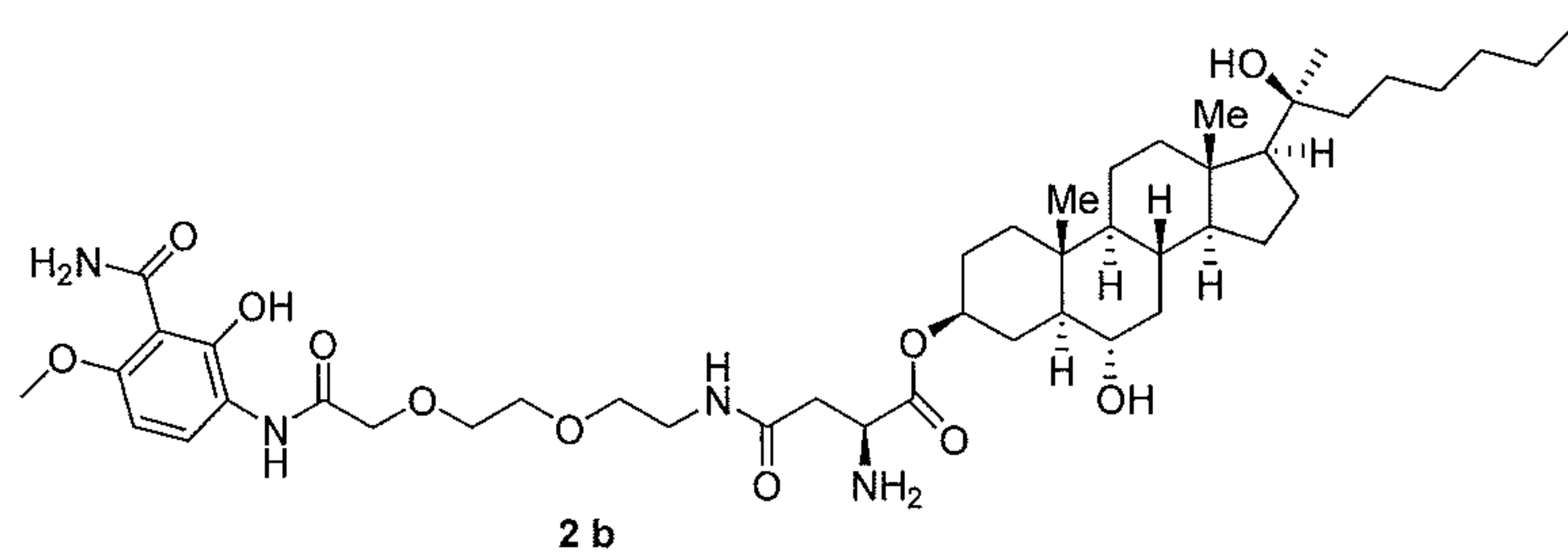
[0035] Using exhaustive acylation conditions, the tertiary alcohol at C-20 can be acylated, to afford peracylated oxysterol-BTA conjugate analogs as depicted below.

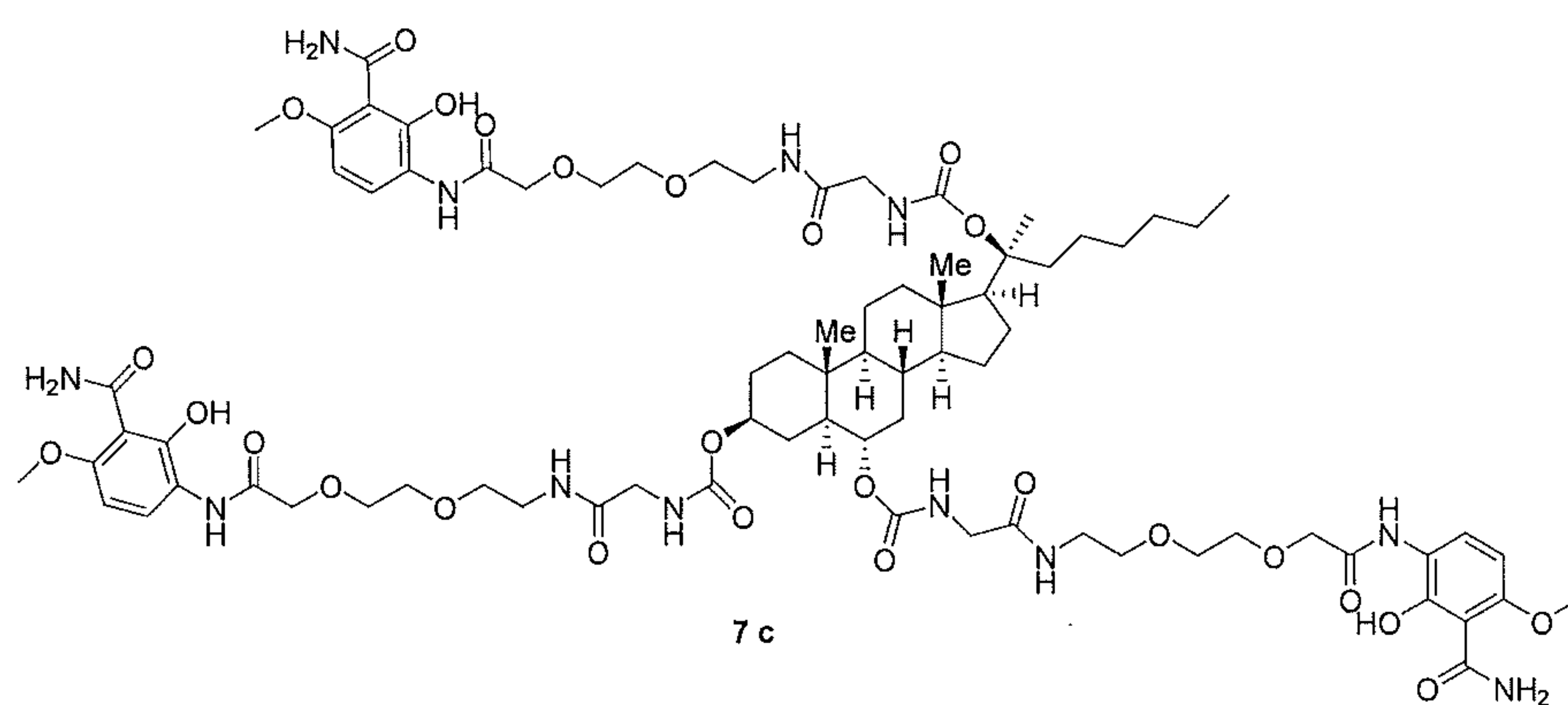
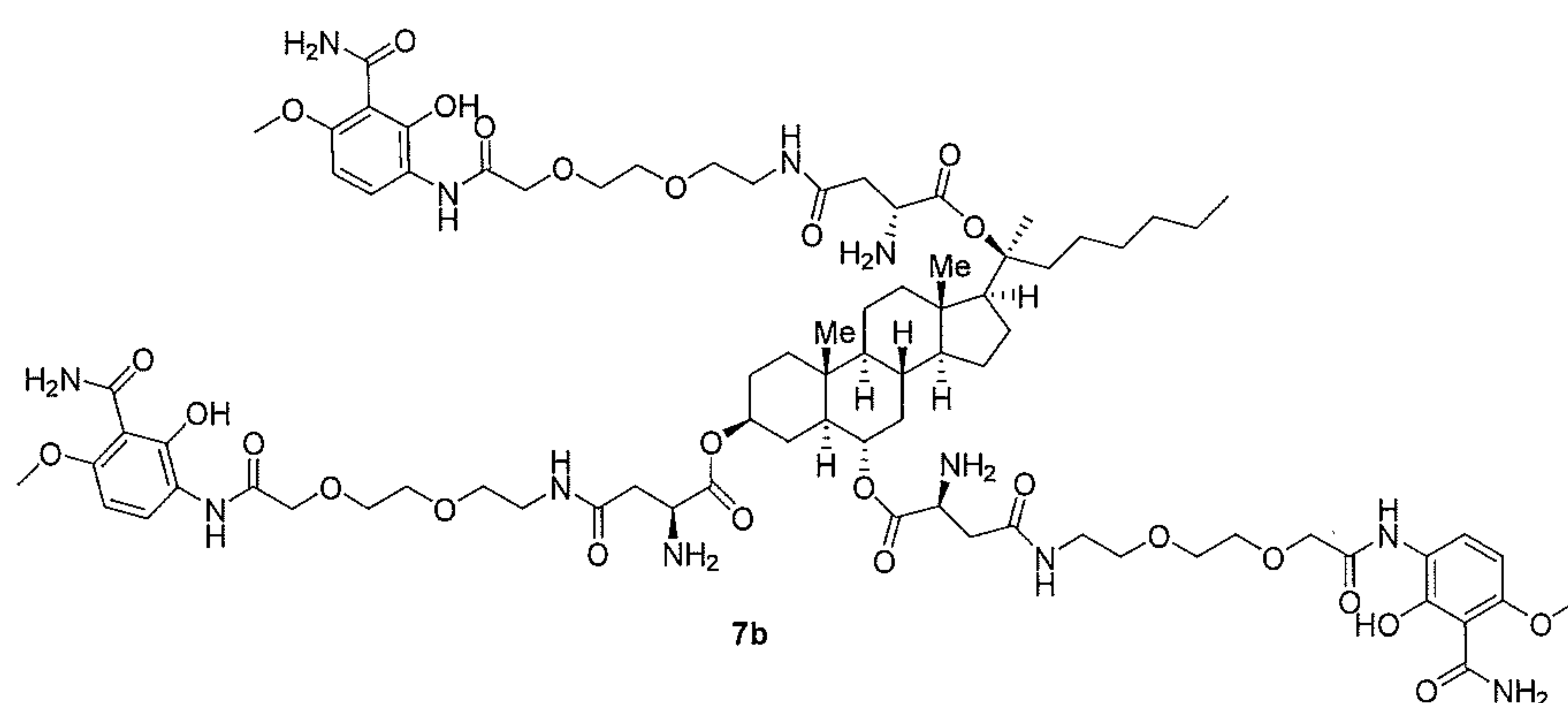
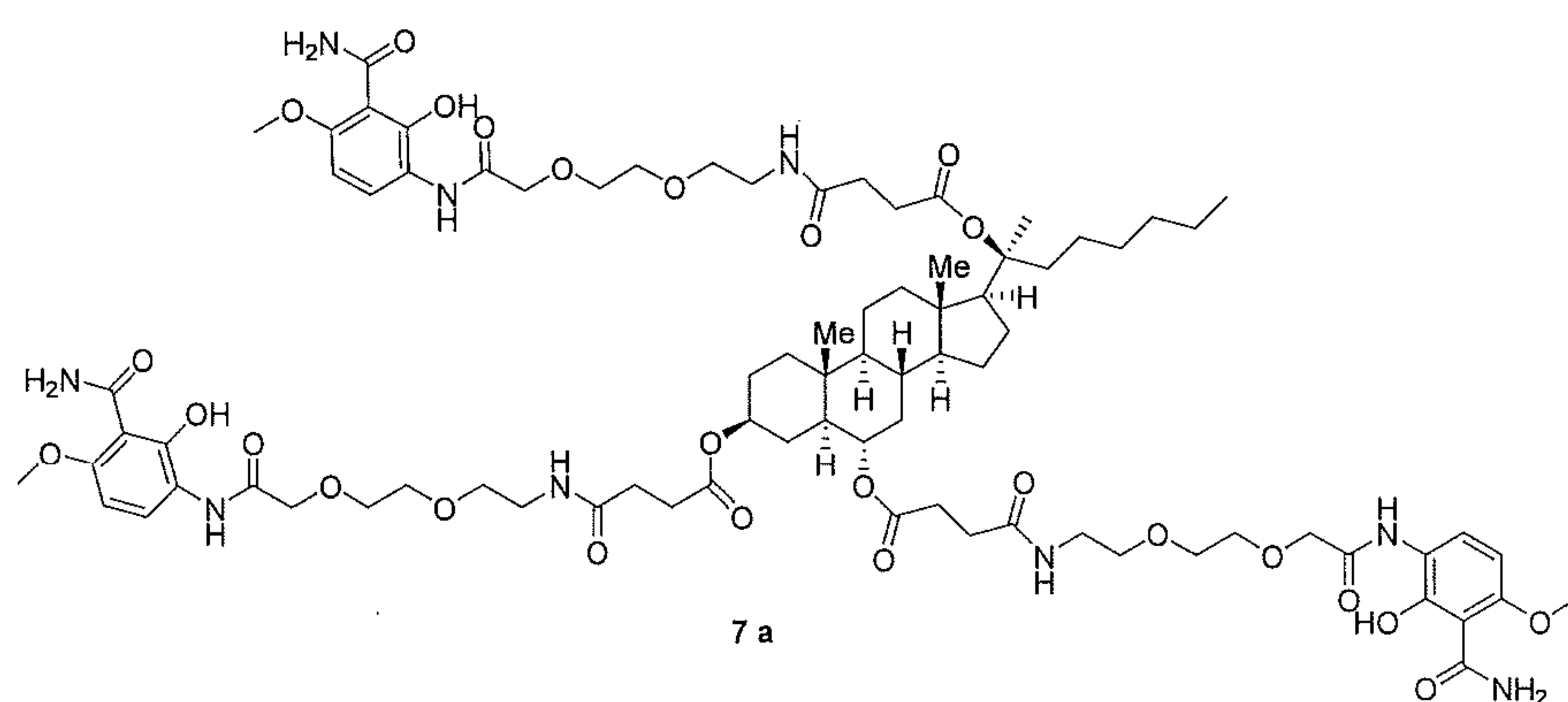
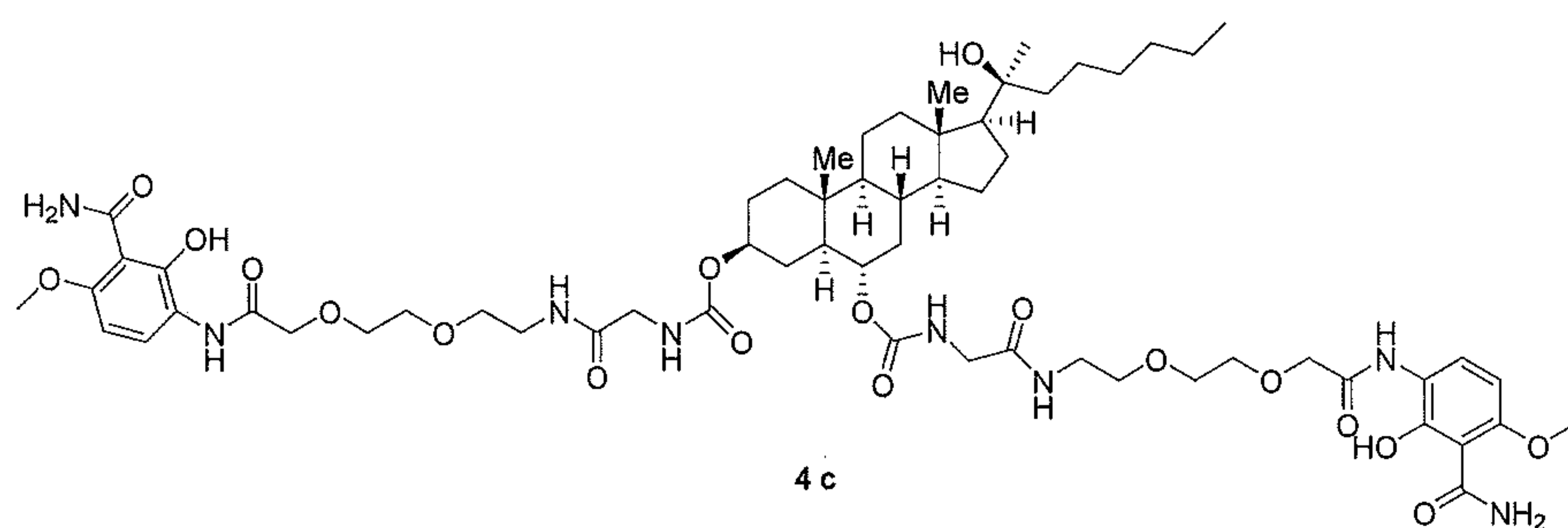


[0036] Synthetic routes to obtain compounds 3b (Oxy 154), 4a (Oxy 153), and 4b (Oxy 155) (shown in Figure 4), starting from the Oxy 133 compound, are shown below.





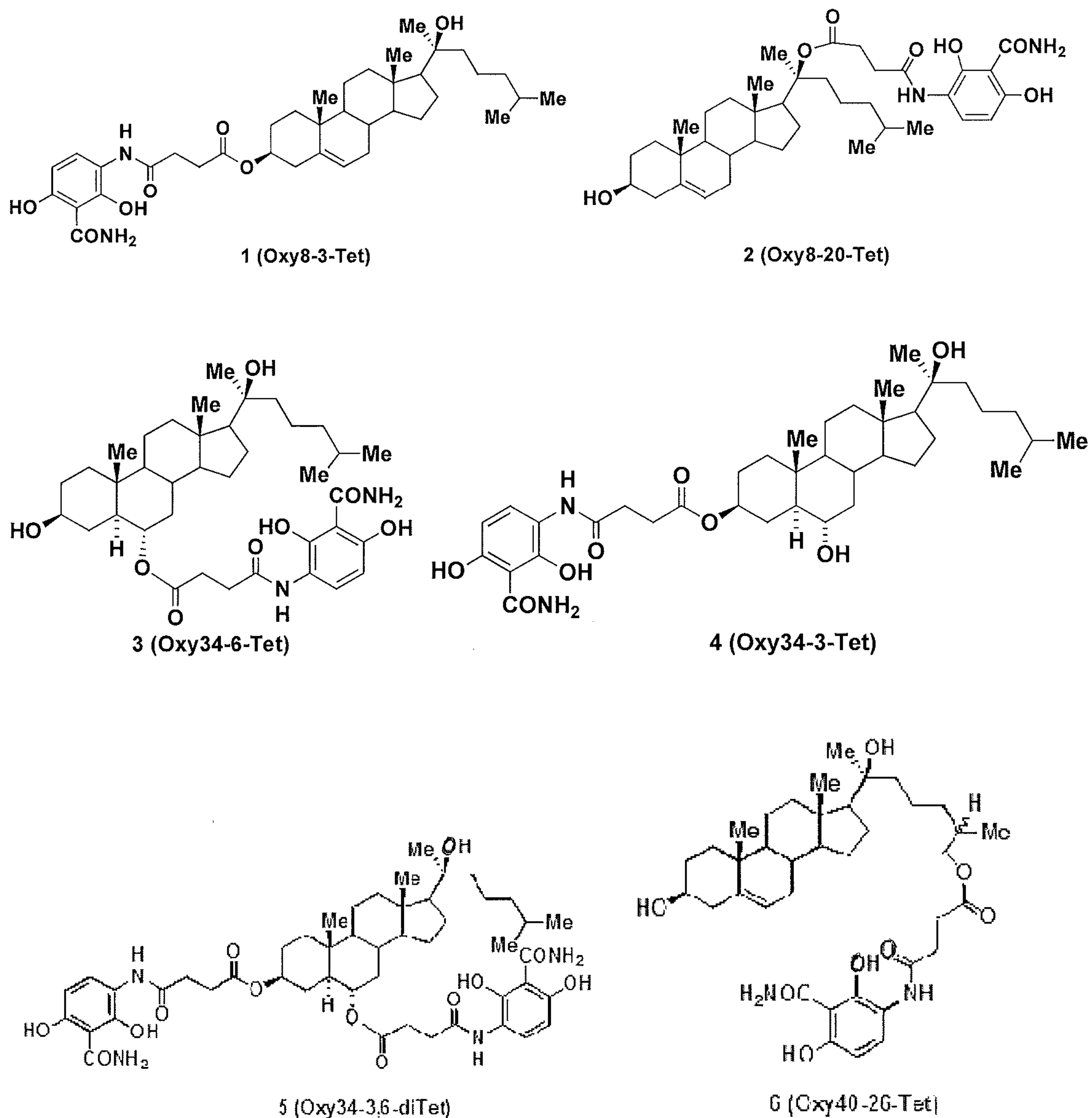


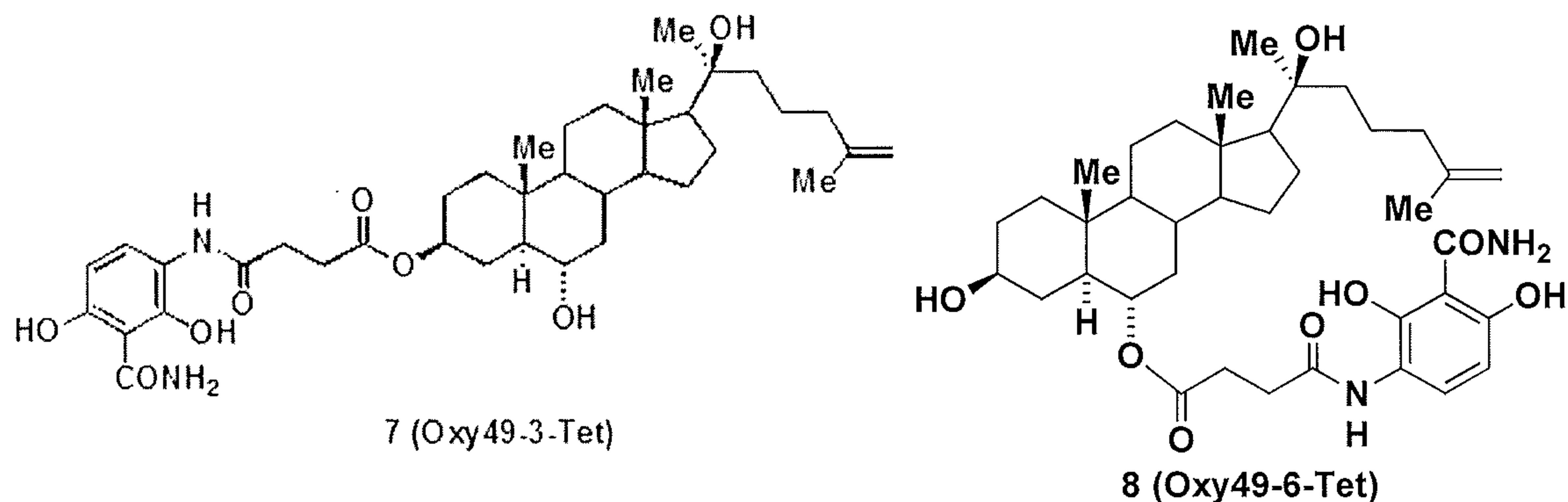


[0038] An embodiment of the invention relates to hybrid molecules comprising Oxy133 or other osteogenic oxysterols described previously by the some of the present inventors, wherein

the oxysterols are linked to other versions of tetracycline-derived bone targeting moieties described by some of the present inventors. Some such moieties are described, *e.g.*, in US Pat. 7,196,220 and US Pat. 7,196,220.

[0039] An osteogenic oxysterol molecule can be linked (conjugated) to such a tetracycline derivative and used as described herein. Representative such oxysterols include Oxy8, Oxy34, Oxy40, and Oxy49, or other suitable oxysterols previously described by the present inventors or by others. Some such hybrid molecules include the following.





[0040] These oxysterols and oxysterol-bone targeting agent (BTA) conjugates can be a part of a pharmaceutical composition that can be used as a therapeutic agent for the treatment of osteoporosis.

[0041] Oxysterols form a family of oxygenated derivatives of cholesterol that are present in the circulation, and in human and animal tissues. Oxysterols have been found to be present in atherosclerotic lesions and play a role in various physiologic processes, such as cellular differentiation, inflammation, apoptosis, and steroid production. Some naturally occurring oxysterols can have osteogenic properties (Kha). A naturally occurring oxysterol, 20(S)-hydroxycholesterol ("20S") (W.-K.Kim, *J. Bone Miner. Res.* **2007**, 22, 1711–9), is both osteogenic and anti-adipogenic when applied to multipotent mesenchymal cells capable of differentiating into osteoblasts and adipocytes. Structural modifications of 20S can be made to synthesize more potent analogues of 20S including Oxy34 and Oxy49, which can induce the osteogenic and inhibit the adipogenic differentiation of bone marrow stromal cells (MSC) through activation of Hedgehog (Hh) signaling (J.S.Johnson). Oxy34 and Oxy49 can stimulate spine fusion *in vivo* in a rat model of posterolateral spine fusion (J.S.Johnson). Oxysterols can make more feasible clinical options for physicians treating, for example, long bone fractures, spine disorders, and osteoporosis.

[0042] The compounds of embodiments of the present invention are useful as pharmaceutical compositions prepared with a therapeutically effective amount of a compound of the invention, as defined herein, and a pharmaceutically acceptable carrier or diluent.

[0043] The compounds of the invention can be formulated as pharmaceutical compositions and administered to a subject in need of treatment, for example a mammal, such as a human patient, in a variety of forms adapted to the chosen route of administration, for example, orally,

nasally, intraperitoneally, or parenterally, by intravenous, intramuscular, topical or subcutaneous routes, or by injection into tissue.

[0044] Thus, compounds of the invention may be systemically administered, e.g., orally, in combination with a pharmaceutically acceptable vehicle such as an inert diluent or an assimilable edible carrier, or by inhalation or insufflation. They may be enclosed in hard or soft shell gelatin capsules, may be compressed into tablets, or may be incorporated directly with the food of the patient's diet. For oral therapeutic administration, the compounds may be combined with one or more excipients and used in the form of ingestible tablets, buccal tablets, troches, capsules, elixirs, suspensions, syrups, wafers, and the like. The compounds may be combined with an inert powdered carrier and inhaled by the subject or insufflated. Such compositions and preparations should contain at least 0.1% of a compound of an embodiment of the present invention. The percentage of the compositions and preparations may, of course, be varied and may conveniently be between about 2% to about 60% of the weight of a given unit dosage form. The amount of compound in such therapeutically useful compositions is such that an effective dosage level will be obtained.

[0045] The tablets, troches, pills, capsules, and the like may also contain the following: binders such as gum tragacanth, acacia, corn starch or gelatin; excipients such as dicalcium phosphate; a disintegrating agent such as corn starch, potato starch, alginic acid and the like; a lubricant such as magnesium stearate; and a sweetening agent such as sucrose, fructose, lactose or aspartame or a flavoring agent such as peppermint, oil of wintergreen, or cherry flavoring may be added. When the unit dosage form is a capsule, it may contain, in addition to materials of the above type, a liquid carrier, such as a vegetable oil or a polyethylene glycol. Various other materials may be present as coatings or to otherwise modify the physical form of the solid unit dosage form. For instance, tablets, pills, or capsules may be coated with gelatin, wax, shellac or sugar and the like. A syrup or elixir may contain the active compound, sucrose or fructose as a sweetening agent, methyl and propylparabens as preservatives, a dye and flavoring such as cherry or orange flavor. Of course, any material used in preparing any unit dosage form should be pharmaceutically acceptable and substantially non-toxic in the amounts employed. In addition, the compounds may be incorporated into sustained-release preparations and devices. For example, the compounds may be incorporated into time release capsules, time release tablets, time release pills, and time release polymers or nanoparticles.

[0046] The compounds may also be administered intravenously or intraperitoneally or subcutaneously by infusion or injection. Solutions of the compounds can be prepared in water, optionally mixed with a nontoxic surfactant. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, triacetin, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations can contain a preservative to prevent the growth of microorganisms.

[0047] The pharmaceutical dosage forms suitable for injection or infusion can include sterile aqueous solutions or dispersions or sterile powders comprising the compounds which are adapted for the extemporaneous preparation of sterile injectable or infusible solutions or dispersions, optionally encapsulated in liposomes. In all cases, the ultimate dosage form should be sterile, fluid and stable under the conditions of manufacture and storage. The liquid carrier or vehicle can be a solvent or liquid dispersion medium comprising, for example, water, ethanol, a polyol (for example, glycerol, propylene glycol, liquid polyethylene glycols, and the like), vegetable oils, nontoxic glyceryl esters, and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the formation of liposomes, by the maintenance of the required particle size in the case of dispersions or by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars, buffers or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

[0048] Sterile injectable solutions are prepared by incorporating the compounds in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filter sterilization. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze drying techniques, which yield a powder of the active ingredient plus any additional desired ingredient present in the previously sterile-filtered solutions.

[0049] For topical administration, the compounds may be applied in pure form. However, it may be desirable to administer them to the skin as compositions or formulations, in combination with a dermatologically acceptable carrier, which may be a solid or a liquid.

[0050] Useful solid carriers include finely divided solids such as talc, clay, microcrystalline cellulose, silica, alumina and the like. Other solid carriers include nontoxic polymeric

nanoparticles or microparticles. Useful liquid carriers include water, alcohols or glycols or water/alcohol/glycol blends, in which the compounds can be dissolved or dispersed at effective levels, optionally with the aid of non-toxic surfactants. Adjuvants such as fragrances and additional antimicrobial agents can be added to optimize the properties for a given use. The resultant liquid compositions can be applied from absorbent pads, used to impregnate bandages and other dressings, or sprayed onto the affected area using pump-type or aerosol sprayers.

[0051] Thickeners such as synthetic polymers, fatty acids, fatty acid salts and esters, fatty alcohols, modified celluloses or modified mineral materials can also be employed with liquid carriers to form spreadable pastes, gels, ointments, soaps, and the like, for application directly to the skin of the user.

[0052] Examples of useful dermatological compositions which can be used to deliver the compounds to the skin are known to the art; for example, see Jacquet et al. (U.S. Pat. No. 4,608,392), Geria (U.S. Pat. No. 4,992,478), Smith et al. (U.S. Pat. No. 4,559,157) and Wortzman (U.S. Pat. No. 4,820,508), all of which are hereby incorporated by reference.

[0053] Useful dosages of the compounds of formula I can be determined by comparing their *in vitro* activity, and *in vivo* activity in animal models. Methods for the extrapolation of effective dosages in mice, and other animals, to humans are known to the art; for example, see U.S. Pat. No. 4,938,949, which is hereby incorporated by reference.

[0054] For example, the concentration of the compounds in a liquid composition, such as a lotion, can be from about 0.1-25% by weight, or from about 0.5-10% by weight. The concentration in a semi-solid or solid composition such as a gel or a powder can be about 0.1-5% by weight, or about 0.5-2.5% by weight.

[0055] The amount of the compounds required for use in treatment will vary not only with the particular salt selected but also with the route of administration, the nature of the condition being treated and the age and condition of the patient and will be ultimately at the discretion of the attendant physician or clinician.

[0056] Effective dosages and routes of administration of agents of the invention are conventional. The exact amount (effective dose) of the agent will vary from subject to subject, depending on, for example, the species, age, weight and general or clinical condition of the subject, the severity or mechanism of any disorder being treated, the particular agent or vehicle used, the method and scheduling of administration, and the like. A therapeutically effective dose can be determined empirically, by conventional procedures known to those of skill in the

art. See, e.g., *The Pharmacological Basis of Therapeutics*, Goodman and Gilman, eds., Macmillan Publishing Co., New York. For example, an effective dose can be estimated initially either in cell culture assays or in suitable animal models. The animal model may also be used to determine the appropriate concentration ranges and routes of administration. Such information can then be used to determine useful doses and routes for administration in humans. A therapeutic dose can also be selected by analogy to dosages for comparable therapeutic agents.

[0057] The particular mode of administration and the dosage regimen will be selected by the attending clinician, taking into account the particulars of the case (e.g., the subject, the disease, the disease state involved, and whether the treatment is prophylactic). Treatment may involve daily or multi-daily doses of compound(s) over a period of a few days to months, or even years.

[0058] In general, however, a suitable dose will be in the range of from about 0.001 to about 100 mg/kg, e.g., from about 0.01 to about 100 mg/kg of body weight per day, such as above about 0.1 mg per kilogram, or in a range of from about 1 to about 10 mg per kilogram body weight of the recipient per day. For example, a suitable dose may be about 1 mg/kg, 10 mg/kg, or 50 mg/kg of body weight per day.

[0059] The compounds are conveniently administered in unit dosage form; for example, containing 0.05 to 10000 mg, 0.5 to 10000 mg, 5 to 1000 mg, or about 100 mg of active ingredient per unit dosage form.

[0060] The compounds can be administered to achieve peak plasma concentrations of, for example, from about 0.5 to about 75 μ M, from about 1 to 50 μ M, from about 2 to about 30 μ M, or from about 5 to about 25 μ M. Exemplary desirable plasma concentrations include at least or no more than 0.25, 0.5, 1, 5, 10, 25, 50, 75, 100, or 200 μ M. For example, plasma levels may be from about 1 to 100 micromolar or from about 10 to about 25 micromolar. This may be achieved, for example, by the intravenous injection of a 0.05 to 5% solution of the compounds, optionally in saline, or orally administered as a bolus containing about 1-100 mg of the compounds. Desirable blood levels may be maintained by continuous infusion to provide about 0.00005 - 5 mg per kg body weight per hour, for example at least or no more than 0.00005, 0.0005, 0.005, 0.05, 0.5, or 5 mg/kg/hr. Alternatively, such levels can be obtained by intermittent infusions containing about 0.0002 - 20 mg per kg body weight, for example, at least or no more than 0.0002, 0.002, 0.02, 0.2, 2, 20, or 50 mg of the compounds per kg of body weight.

[0061] The compounds may conveniently be presented in a single dose or as divided doses administered at appropriate intervals, for example, as one dose per day or as two, three, four or more sub-doses per day. The sub-dose itself may be further divided, e.g., into a number of discrete loosely spaced administrations; such as multiple inhalations from an insufflator.

[0062] An aspect of the invention is a bioactive or pharmaceutical composition comprising a compound set forth herein or a pharmaceutically acceptable salt or solvate thereof and a pharmaceutically acceptable carrier. The terms “bioactive” composition or “pharmaceutical” composition are used interchangeably herein. Both terms refer to compositions that can be administered to a subject, used to coat or be present in a medical device that is introduced into a subject, or the like. These bioactive or pharmaceutical compositions are sometimes referred to herein as “pharmaceutical compositions or bioactive compositions of the invention.” Sometimes the phrase “administration of a compound” is used herein in the context of administration of this compound to a subject (*e.g.*, contacting the subject with the compound). It is to be understood that the compound for such a use can generally be in the form of a pharmaceutical composition or bioactive composition comprising the compound.

[0063] Another aspect of the invention is a method for inducing (stimulating, enhancing) a Hedgehog (Hh) pathway mediated response, in a cell or tissue, *e.g.*, in a subject, comprising contacting the cell or tissue with an effective amount (*e.g.*, a therapeutically effective amount) of a compound, wherein the Hedgehog (Hh) pathway mediated response is the stimulation of osteoblastic differentiation, osteomorphogenesis, and/or osteoproliferation. The Hh mediated response can be useful in regenerative medicine.

[0064] Another aspect of the invention is a method for treating a subject having a bone disorder, osteopenia, osteoporosis, or a bone fracture, comprising administering to the subject an effective amount of a bioactive composition or pharmaceutical composition comprising a compound. The subject can be administered the bioactive composition or pharmaceutical composition at a therapeutically effective dose in an effective dosage form at a selected interval to, *e.g.*, increase bone mass, ameliorate symptoms of osteoporosis, or reduce, eliminate, prevent or treat other conditions which would benefit from an increase in osteomorphogenesis and/or osteoproliferation.. The subject can be administered the bioactive composition or pharmaceutical composition at a therapeutically effective dose in an effective dosage form at a selected interval to ameliorate the symptoms of osteoporosis. In one embodiment, the subject is treated to induce

bone formation by harvesting mammalian mesenchymal stem cells (*e.g.*, from the subject or from a suitable mammal, or from a tissue or cell bank), treating the mammalian mesenchymal cells with a compound to induce osteoblastic differentiation of the cells, and administering the differentiated cells to the subject.

[0065] In any of the methods of the invention, the compound can be administered to a cell, tissue or organ by local administration. For example, the compound can be applied locally with a cream or the like, or it can be injected or otherwise introduced directly into a cell, tissue or organ, or it can be introduced with a suitable medical device (*e.g.*, an implant). Alternatively, the compound can be administered systemically, *e.g.*, orally, intravenously (though IV), or via injection such as intraperitoneal (ip) injection or subcutaneous (subcu) injection.

[0066] Another aspect of the invention is a kit for carrying out one or more of the methods described herein. The kit can comprise an effective amount (*e.g.*, a therapeutically effective amount) of a compound, optionally in a container.

[0067] Another aspect of the invention is an implant for use in the body of a subject (*e.g.*, an animal such as a human) comprising a substrate having a surface. The surface or insides of the implant comprises a bioactive composition or pharmaceutical composition comprising the compound in an amount sufficient to induce bone formation in the surrounding bone tissue.

[0068] Optionally, a bioactive composition, method, kit or medical device of the invention can comprise one or more other suitable therapeutic agents, such as, *e.g.*, parathyroid hormone, sodium fluoride, insulin-like growth factor I (ILGF-I), insulin-like growth factor II (ILGF-II), transforming growth factor beta (TGF- β), a cytochrome P450 inhibitor, an osteogenic prostanoid, BMP 2, BMP 4, BMP 7, BMP 14, and/or an anti-resorptive agent such as, *e.g.*, bisphosphonate.

[0069] In addition to the compounds set forth herein, other embodiments of the invention encompass any and all individual stereoisomers at any of the stereocenters shown in the Formulas, including diastereomers, racemates, enantiomers, and other isomers of the compounds. In embodiments of the invention, all polymorphs and solvates of the compound, such as hydrates and those formed with organic solvents, are included. A "solvate" is a complex or aggregate formed by one or more molecules of a solute, *e.g.*, a compound or a pharmaceutically-acceptable salt thereof, and one or more molecules of a solvent. Such solvates can be crystalline solids having a substantially fixed molar ratio of solute and solvent. Suitable solvents will be known by those of ordinary skill in the art, *e.g.*, water, ethanol or

dimethylsulfoxide. Such isomers, polymorphs, and solvates may be prepared by methods known in the art, such as by regiospecific and/or enantioselective synthesis and resolution.

[0070] The ability to prepare salts depends on the acidity or basicity of a compound. Suitable salts of the compound include, but are not limited to, acid addition salts, such as those made with hydrochloric, hydrobromic, hydroiodic, perchloric, sulfuric, nitric, phosphoric, acetic, propionic, glycolic, lactic pyruvic, malonic, succinic, maleic, fumaric, malic, tartaric, citric, benzoic, carbonic cinnamic, mandelic, methanesulfonic, ethanesulfonic, hydroxyethanesulfonic, benzenesulfonic, p-toluene sulfonic, cyclohexanesulfamic, salicylic, p-aminosalicylic, 2-phenoxybenzoic, and 2-acetoxybenzoic acid; salts made with saccharin; alkali metal salts, such as sodium and potassium salts; alkaline earth metal salts, such as calcium and magnesium salts; and salts formed with organic or inorganic ligands, such as quaternary ammonium salts.

[0071] Additional suitable salts include, but are not limited to, acetate, benzenesulfonate, benzoate, bicarbonate, bisulfate, bitartrate, borate, bromide, calcium edetate, camsylate, carbonate, chloride, clavulanate, citrate, dihydrochloride, edetate, edisylate, estolate, esylate, fumarate, gluceptate, gluconate, glutamate, glycolylarsanilate, hexylresorcinate, hydrabamine, hydrobromide, hydrochloride, hydroxynaphthoate, iodide, isothionate, lactate, lactobionate, laurate, malate, maleate, mandelate, mesylate, methylbromide, methylnitrate, methylsulfate, mucate, napsylate, nitrate, N-methylglucamine ammonium salt, oleate, pamoate (embonate), palmitate, pantothenate, phosphate/diphosphate, polygalacturonate, salicylate, stearate, sulfate, subacetate, succinate, tannate, tartrate, teoclate, tosylate, triethiodide, and valerate salts of the compounds.

[0072] It is to be understood that references to compounds herein include pharmaceutically acceptable salts or solvates thereof.

[0073] In any of the methods, compositions or kits of the invention, particularly for use in treating a subject, a composition of the invention may optionally be in combination with one or more other suitable therapeutic agents. Any therapeutic agent that is suitable for treatment of a particular condition can be used. Suitable such agents or drugs will be evident to one skilled in the art. For example, for the treatment of bone disorders, a conventional therapeutic drug can be used in combination with a composition of the invention. Some such agents include, *e.g.*, parathyroid hormone, sodium fluoride, insulin-like growth factor I (ILGF-I), insulin-like growth factor II (ILGF-II), transforming growth factor beta (TGF- β), a cytochrome P450 inhibitor, an

osteogenic prostanoid, BMP 2, BMP 4, BMP 7, BMP 14, and/or bisphosphonates or other inhibitors of bone resorption.

[0074] A composition or compound of the invention can be formulated as a pharmaceutical composition, which comprises a composition of the invention and pharmaceutically acceptable carrier. By a "pharmaceutically acceptable carrier" is meant a material that is not biologically or otherwise undesirable, *i.e.*, the material may be administered to a subject without causing any undesirable biological effects or interacting in a deleterious manner with any of the other components of the pharmaceutical composition in which it is contained. The carrier is naturally selected to minimize any degradation of the active ingredient and to minimize any adverse side effects in the subject, as would be well known to one of skill in the art. For a discussion of pharmaceutically acceptable carriers and other components of pharmaceutical compositions, see, *e.g.*, Remington's Pharmaceutical Sciences, 18th ed., Mack Publishing Company, 1990. Some suitable pharmaceutical carriers will be evident to a skilled worker and include, *e.g.*, water (including sterile and/or deionized water), suitable buffers (such as PBS), physiological saline, cell culture medium (such as DMEM), artificial cerebral spinal fluid, dimethylsulfoxide (DMSO), or the like.

[0075] One of skill in the art will appreciate that a particular formulation of the invention will depend, at least in part, upon the particular agent or combination of agents that is employed and the chosen route of administration. Accordingly, there is a wide variation of suitable formulations of compositions of the present invention. Some representative formulations are discussed below. Others will be evident to a skilled worker. A compound can be administered locally or directly to a cell, tissue or organ in need of treatment, or it can be administered systemically.

[0076] Formulations or compositions suitable for oral administration can consist of liquid solutions, such as an effective amount of compound dissolved in diluents, such as water, saline, or fruit juice; capsules, sachets or tablets, each containing a predetermined amount of the active ingredient, as solid, granules or freeze-dried cells; solutions or suspensions in an aqueous liquid; and oil-in-water emulsions or water-in-oil emulsions. Tablet forms can include one or more of lactose, mannitol, corn starch, potato starch, microcrystalline cellulose, acacia, gelatin, colloidal silicon dioxide, croscarmellose sodium, talc, magnesium stearate, stearic acid, and other excipients, colorants, diluents, buffering agents, moistening agents, preservatives, flavoring agents, and pharmacologically compatible carriers. Suitable formulations for oral delivery can

also be incorporated into synthetic and natural polymeric microspheres, or other means to protect the agents of the present invention from degradation within the gastrointestinal tract.

[0077] Formulations suitable for parenteral administration (e.g., intravenous) include aqueous and non-aqueous, isotonic sterile injection solutions, which can contain anti-oxidants, buffers, bacteriostats, and solutes that render the formulation isotonic with the blood of the intended recipient, and aqueous and non-aqueous sterile suspensions that can include suspending agents, solubilizers, thickening agents, stabilizers, and preservatives. The formulations can be presented in unit-dose or multi-dose sealed containers, such as ampules and vials, and can be stored in a freeze-dried (i.e., lyophilized) condition requiring only the addition of the sterile liquid carrier, for example, water, for injections, immediately prior to use. Extemporaneous injection solutions and suspensions can be prepared from sterile powders, granules, and tablets of the kind previously described.

[0078] A compound, alone or in combination with other therapeutic agents, can be made into aerosol formulations to be administered via inhalation. These aerosol formulations can be placed into pressurized acceptable propellants, such as dichlorodifluoromethane, propane, nitrogen, and the like.

[0079] Suitable formulations for topical administration include lozenges comprising the active ingredient in a flavor, usually sucrose and acacia or tragacanth; pastilles comprising the active ingredient in an inert base, such as gelatin and glycerin, or sucrose and acacia; mouthwashes comprising the active ingredient in a suitable liquid carrier; or creams, emulsions, suspensions, solutions, gels, creams, pastes, foams, lubricants, sprays, suppositories, or the like.

[0080] Other suitable formulations include, e.g., hydrogels and polymers suitable for timed release of a compound, or nanoparticles for small dose delivery of a compound. Such formulations are well-known to those of skill in the art.

[0081] A person skilled in the art will appreciate that a suitable or appropriate formulation can be selected, adapted or developed based upon the particular application at hand. In addition, the pharmaceutical compositions of the present invention may be prepared for administration by a variety of different routes, whether systemic, local or both. Such examples include, but are not limited to, administrations performed intraarticularly, intracranially, intradermally, intrahepatically, intramuscularly, intraocularly, intraperitoneally, intrathecally, intravenously, subcutaneously, transdermally, or directly into a bone region atherosclerotic site, such as by

direct injection, introduction with a catheter or other medical device, topical application, direct application, and/or by implanting a device into an artery or other appropriate tissue site.

[0082] A compound may be formulated to be contained within, or adapted to release by a surgical or medical device or implant. In certain aspects, an implant may be coated or otherwise treated with a compound. For example, hydrogels, or other polymers, such as bio-compatible and/or biodegradable polymers, may be used to coat an implant with the compositions of the present invention (*i.e.*, the composition may be adapted for use with a medical device by using a hydrogel or other polymer). Polymers and copolymers for coating medical devices with an agent are well-known in the art. Examples of medical devices and implants include, but are not limited to, sutures and prostheses such as prosthetic joints, and can be in the shape, *e.g.*, of a pin, screw, plate or prosthetic joint.

[0083] An "effective amount" of a compound, as used herein, refers to an amount that can bring about at least a detectable effect. A "therapeutically effective amount," as used herein, refers to an amount that can bring about at least a detectable therapeutic response in a subject being treated (*e.g.*, the amelioration of one or more symptoms) over a reasonable period of time.

[0084] In embodiments of the invention, a compound can stimulate or inhibit a therapeutic response, as measured by any of a variety of conventional assays, by about 1%, 5%, 10%, 20%, 30%, 40%, 50% 150%, 200%, or more of that in an untreated control sample. Intermediate values in these ranges are also included.

[0085] Dosages for a compound can be in unit dosage form, such as a tablet or capsule. The term "unit dosage form," as used herein, refers to physically discrete units suitable as unitary dosages for animal (*e.g.*, human) subjects, each unit containing a predetermined quantity of an agent of the invention, alone or in combination with other therapeutic agents, calculated in an amount sufficient to produce the desired effect in association with a pharmaceutically acceptable diluent, carrier, or vehicle.

[0086] One skilled in the art can routinely determine the appropriate dose, schedule, and method of administration for the exact formulation of the composition being used, in order to achieve the desired effective amount or effective concentration of the agent in the individual patient. One skilled in the art also can readily determine and use an appropriate indicator of the "effective concentration" of the compounds by a direct or indirect analysis of appropriate patient samples (*e.g.*, blood and/or tissues), in addition to analyzing the appropriate clinical symptoms of the disease, disorder, or condition.

[0087] The exact dose of a compound or composition thereof administered to an animal, such as a human, in the context of the present invention will vary from subject to subject, depending on the species, age, weight, and general condition of the subject, the severity or mechanism of any disorder being treated, the particular agent or vehicle used, its mode of administration, other medications the patient is taking, and other factors normally considered by an attending physician, when determining an individual regimen and dose level appropriate for a particular patient, and the like. The dose used to achieve a desired concentration *in vivo* will be determined by the potency of the form of the compound, the pharmacodynamics associated with the compound in the host, with or without additional agents, the severity of the disease state of infected individuals, as well as, in the case of systemic administration, the body weight and age of the individual. The size of the dose may also be determined by the existence of any adverse side effects that may accompany the particular agent, or composition thereof, employed. It is generally desirable, whenever possible, to keep adverse side effects to a minimum.

[0088] For example, a dose can be administered in the range of from about 5 ng (nanograms) to about 1000 mg (milligrams), or from about 100 ng to about 600 mg, or from about 1 mg to about 500 mg, or from about 20 mg to about 400 mg. For example, the dose can be selected to achieve a dose to body weight ratio of from about 0.0001 mg/kg to about 1500 mg/kg, or from about 1 mg/kg to about 1000 mg/kg, or from about 5 mg/kg to about 150 mg/kg, or from about 20 mg/kg to about 100 mg/kg. For example, a dosage unit can be in the range of from about 1 ng to about 5000 mg, or from about 5 ng to about 1000 mg, or from about 100 ng to about 600 mg, or from about 1 mg to about 500 mg, or from about 20 mg to about 400 mg, or from about 40 mg to about 200 mg of a compound or a composition comprising a compound. In one embodiment of the invention, amounts of a compound as above (*e.g.*, a few grams) are administered locally, such as in a spine fusion procedure as part of a scaffold.

[0089] A dose can be administered once per day, twice per day, four times per day, or more than four times per day as required to elicit a desired therapeutic effect. For example, a dose administration regimen can be selected to achieve a blood serum concentration of a compound of the present invention in the range of from about 0.01 to about 1000 nM, or from about 0.1 to about 750 nM, or from about 1 to about 500 nM, or from about 20 to about 500 nM, or from about 100 to about 500 nM, or from about 200 to about 400 nM. For example, a dose administration regime can be selected to achieve an average blood serum concentration with a

half maximum dose of a compound of the present invention in the range of from about 1 µg/L (microgram per liter) to about 2000 µg/L, or from about 2 µg/L to about 1000 µg/L, or from about 5 µg/L to about 500 µg/L, or from about 10 µg/L to about 400 µg/L, or from about 20 µg/L to about 200 µg/L, or from about 40 µg/L to about 100 µg/L.

[0090] Certain embodiments of the invention may also include treatment with an additional agent which acts independently or synergistically with a compound to improve the therapeutic results. When given in combined therapy, the agent other than the compound can be given at the same time as the compound, or the dosing can be staggered as desired. The two (or more) drugs also can be combined in a composition. Doses of each can be less when used in combination than when either is used alone. Suitable doses can be determined by a skilled worker, using standard dosage parameters.

[0091] As used herein, the singular forms "a," "an" and "the" include plural referents unless the context clearly dictates otherwise.

[0092] A "subject," as used herein, includes any animal that exhibits a symptom of a condition that can be treated with a compound. Suitable subjects (patients) include laboratory animals (such as mouse, rat, rabbit, or guinea pig), farm animals, and domestic animals or pets (such as a cat, dog, or horse). Non-human primates and humans, including human patients, are included. Typical subjects include animals that exhibit aberrant amounts (lower amounts than a "normal" or "healthy" subject) of one or more physiological activities that are stimulated by Hedgehog signaling. The aberrant activities may be regulated by any of a variety of mechanisms, including activation of a Hedgehog activity. The aberrant activities can result in a pathological condition.

[0093] One embodiment of the invention is a kit useful for any of the methods disclosed herein, either *in vitro* or *in vivo*. Such a kit comprises a compound or a bioactive or pharmaceutical composition thereof, and can comprise one or more other oxysterols, *e.g.*, which result in an increase in a Hh pathway-mediated activity, or other suitable therapeutic agents. Optionally, the kits comprise instructions for performing the method. Optional elements of a kit of the invention include suitable buffers, pharmaceutically acceptable carriers, or the like, containers, or packaging materials. The reagents of the kit may be in containers in which the reagents are stable, *e.g.*, in lyophilized form or stabilized liquids. The reagents may also be in single use form, *e.g.*, in single dosage form. A skilled worker will recognize components of kits suitable for carrying out any of the methods of the invention.

[0094] A variety of conditions can be treated with a compound, used alone or in combination with other therapeutic agents.

[0095] A compound can result in an increase in Hedgehog pathway activity.

[0096] One effect of a compound can be to target pluripotent cells to induce their lineage specific differentiation into various cell types, *e.g.*, osteoblasts. For example, mesenchymal stem cells treated with a compound can show induced expression of markers of osteoblast differentiation. Without wishing to be bound by any particular mechanism, it is suggested that this lineage specific differentiation is due to the induction of Hedgehog signaling in these cells. However, methods of treatment discussed herein are included in the present invention, regardless of the mechanism by which the compound functions. A compound can be useful for treating conditions which would benefit from stimulation of bone formation, osteoblastic differentiation, osteomorphogenesis and/or osteoproliferation. Among these conditions or treatments are, *e.g.*, osteoinductive therapy for stimulation of localized bone formation in spine fusion or osteoporosis, bone fracture repair or healing, dental procedures for which increased bone formation in the jaw is of clinical benefit, repair of craniofacial bone defects induced by trauma or congenital defects such as cleft palate/lip, and a number of other musculoskeletal disorders in which native bone growth is inadequate, which will be evident to skilled workers. Treatment can be administered to treat open fractures and fractures at high risk of non-union, and in subjects with spinal disorders, including subjects in need of spine fusion (*e.g.*, anterior lumbar interbody fusion, posterior lumbar spinal fusion, and cervical spine fusion) or subjects having degenerative disc disease or arthritis affecting the lumbar and cervical spine. Furthermore, a compound can be used to treat osteoporosis, particularly in the aging and post-menopausal population, resulting from increased bone resorption by osteoclasts in parallel with decreased bone formation by osteoblasts.

[0097] More particularly, the following types of bone-related treatments can be carried out:

[0098] 1. A compound can be used as an osteogenic agent delivered locally in the body in order to stimulate localized bone formation, using a scaffold that is composed of a compatible molecule such as but not limited to collagen I, which absorbs the compound and then is placed inside the body. For example, the scaffold containing the compound and which can be placed in between transverse processes or in the intervertebral disc where the fusion of two or more vertebrae is indicated, for example in spine fusion, pseudoarthrosis, and non-union fusions. In other embodiments, the scaffold containing the compound is placed in a fractured bone in order

to simulate bone formation and healing of the fracture; is placed in a bone defect such as calvarial or maxillofacial bone defects where bone regeneration by the compound is indicated; or is placed in the jaw bone in order to stimulate bone formation as a means of regenerating bone prior to dental procedures such as dental implants.

[0099] 2. A compound can be used as an osteogenic agent *in vitro*. For example, it can be administered to osteoprogenitor cells, for example mesenchymal stem cells, in order to stimulate their osteogenic differentiation prior to the application of such cells in orthopedic and other procedures as indicated in 1) above in order to stimulate localized bone formation.

[00100] 3. A compound can be used *in vitro* in order to stimulate the Hedgehog signaling pathway in osteoprogenitor cells, thereby leading to the osteogenic differentiation of the cells *in vitro* or *in vivo*.

[00101] In the foregoing and in the following examples, all temperatures are set forth in uncorrected degrees Celsius; and, unless otherwise indicated, all parts and percentages are by weight.

[00102] The osteogenic oxysterols described above are useful for direct, localized administration to target cells, tissues, or organs of interest.

Examples

[00103] Oxy149 (3a), Oxy153 (4a), Oxy154 (3b), and Oxy155 (4b) are BTA-conjugated analogs of compound Oxy133, which induce osteogenic differentiation of osteoprogenitor bone marrow stromal cells, M2-10B4. Osteogenic differentiation was assessed by the induction of alkaline phosphatase (ALP) enzymatic activity, as well as induced expression of osteogenic differentiation marker genes ALP, bone sialoprotein (BSP), and osterix (OSX).

[00104] The ALP activity of the bone marrow stromal cells, M2-10B4, in contact with each of these oxysterols at a concentration of 1 μ M *in vitro* is shown in Fig. 4. The gene expression of ALP in M2-10B4 cells *in vitro* 4 days after initial contact with each oxysterol at a concentration of 1 μ M in comparison with a control in which no oxysterol was contacted with the cells (control had a relative expression of 1.01) is shown in Fig. 5.

[00105] A summary of the EC50 of these oxysterols based on activation of ALP in M2-10B4 cells is presented in Table 1.

Oxysterol	EC50
Oxy149	0.40 μ M
Oxy153	1.40 μ M
Oxy 154	0.63 μ M
Oxy155	0.33 μ M

Table 1

[00106] The gene expression of BSP in M2-10B4 cells *in vitro* 4 days after initial contact with each oxysterol at a concentration of 1 μ M is shown in Fig. 6. The gene expression of OSX in M2-10B4 cells *in vitro* 4 days after initial contact with each oxysterol at a concentration of 1 μ M is shown in Fig. 7.

[00107] Oxy149, Oxy153, Oxy154, and Oxy155 activate Hedgehog (Hh) signaling in M2-10B4 cells as determined by the induced expression of Hh target genes Patched1 (Ptch) and Hh interacting protein (HIP). In addition, these oxysterols induce osteogenic differentiation by activating Hh signaling as assessed by the inhibitory effect of Hh pathway inhibitor cyclopamine (Cyp) on oxysterol-induced osteogenic differentiation. The expression of Ptch gene in M2-10B4 cells *in vitro* 4 days after initial contact with each oxysterol at a concentration of 1 μ M is shown in Fig. 8. The expression of HIP gene in M2-10B4 cells *in vitro* 4 days after initial contact with each oxysterol at a concentration of 1 μ M is shown in Fig. 9.

[00108] The Hh pathway inhibitor cyclopamine can inhibit oxysterol-induced alkaline phosphatase activity. The effect of contacting cyclopamine (Cyc; 5 μ M) with M2-10B4 bone marrow stromal cells *in vitro*, with and without contact with each oxysterol is shown in Table 2.

Treatment	ALP Activity (units/mg protein \pm SD)
Control	1 \pm 1
Oxy133 (1 μ M)	714 \pm 5
Oxy133 + Cyc	31 \pm 7
Oxy149 (1 μ M)	358 \pm 51
Oxy149 + Cyc	26 \pm 3
Oxy153 (5 μ M)	154 \pm 36
Oxy153 + Cyc	19 \pm 7

Oxy154 (1 μ M)	390 \pm 68
Oxy154 + Cyc	18 \pm 2
Oxy155 (1 μ M)	582 \pm 8
Oxy155 + Cyc	39 \pm 2
Cyc	1 \pm 1

Table 2

[00109] The BTA-conjugated analogues of Oxy133, that is, Oxy149, Oxy153, Oxy154, and Oxy155, can bind hydroxyapatite (HAP) bone mineral *in vitro*. An *in vitro* HAP binding assay was conducted as follows. A 1 mM solution of each analyte was made in 100% dimethylsulfoxide (DMSO). A further dilution was then made to form a 20 μ M solution of each analyte in 50 mM Tris-HCl Buffer at pH 7.5 with 20% DMSO. Estradiol was used as a negative control and showed no binding to HAP at the concentration of 20 μ M. The HAP concentration used was 50 mg/mL.

[00110] For each analyte, samples were prepared in triplicate. The control samples (1 mL of 20 μ M analyte) were transferred into a microcentrifuge tube. To a second set of samples (1 mL of 20 μ M analyte) in a microcentrifuge tube was added 50 mg of HAP. The samples were vortexed, gently mixed with inversion for 10 minutes at room temperature, and then centrifuged at 4,400 rpm for 2 minutes to sediment the HAP contained in the samples. The supernatant was transferred to another set of microcentrifuge tubes.

[00111] An electronic spectral scan (ultraviolet-visible) from 220-520 nm was obtained for each analyte using a Bio-Rad Smart-Spec 3000. The blank was 50 mM Tris-HCl Buffer, pH 7.5, 20% DMSO. The wavelength of maximum absorbance (λ_{\max}) was determined, and the extinction coefficient (ϵ) was calculated using the Beer-Lambert Law.

[00112] The absorbance of the samples incubated with HAP was measured at λ_{\max} and the molar concentration of the analyte was then determined using the Beer-Lambert Law and the previously calculated ϵ . The concentration of the analytes 17 β -estradiol, Oxy149, Oxy153, and Oxy155 without and with contact with HAP is shown in Fig. 10.

[00113] The fraction of analyte absorbed to HAP for each sample was subsequently calculated using the following formula for % binding,

$$(H_0 - H) / H_0 * 100 = \% \text{ binding}$$

with H_0 being the mean concentration of control samples and H being the mean calculated concentration of samples treated with HAP. The percent binding of 17 β -estradiol, Oxy149, Oxy153, and Oxy155 to HAP is summarized in Table 3.

Compound	% Binding to Hydroxyapatite
17 β -estradiol	-1.95%
Oxy149	67.44%
Oxy153	95.78%
Oxy155	76.68%

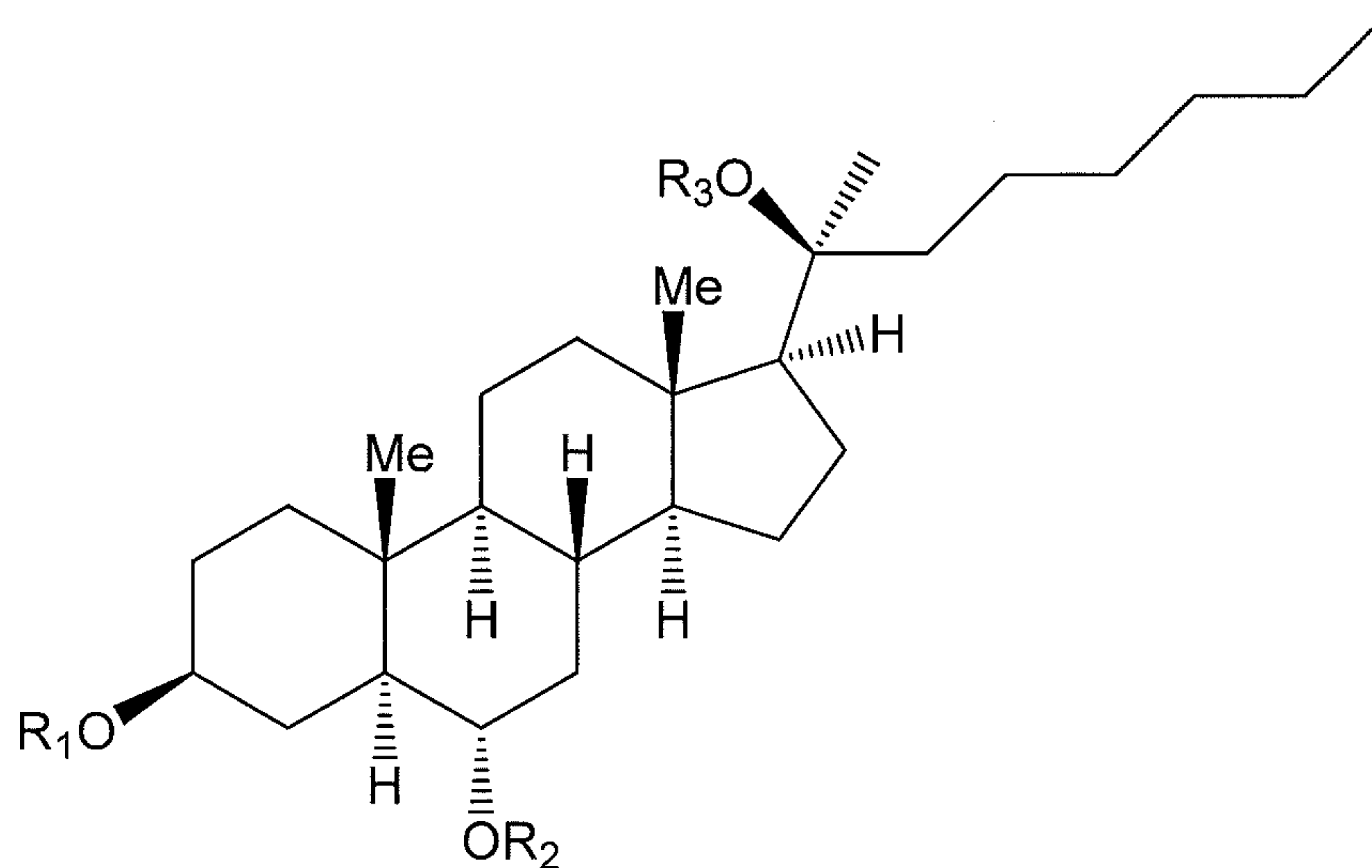
Table 3

[00114] All documents, references, and information, including, but not limited to, journal articles, patent applications, and patents, that are mentioned, cited, or referred to in this application are hereby incorporated by reference in their entirety as if each had been individually incorporated. Such documents include, but are not limited to, U.S. Provisional Application for Patent bearing Serial number 61/643,746 (filed May 7, 2012), International Application bearing Serial number PCT/US2013/032693 (filed March 15, 2013), U.S. Provisional Application for Patent bearing Serial number 61/643,776 (filed May 7, 2012), and International Application bearing Serial number PCT/US2013/032650 (filed March 15, 2013). Such documents also include, but are not limited to, Patent Cooperation Treaty (PCT) international applications published as WO/2008/115469, WO/2008/082520, WO/2007/098281, WO/2007/028101, WO/2006/110490, WO/2005/020928, and WO/2004/019884.

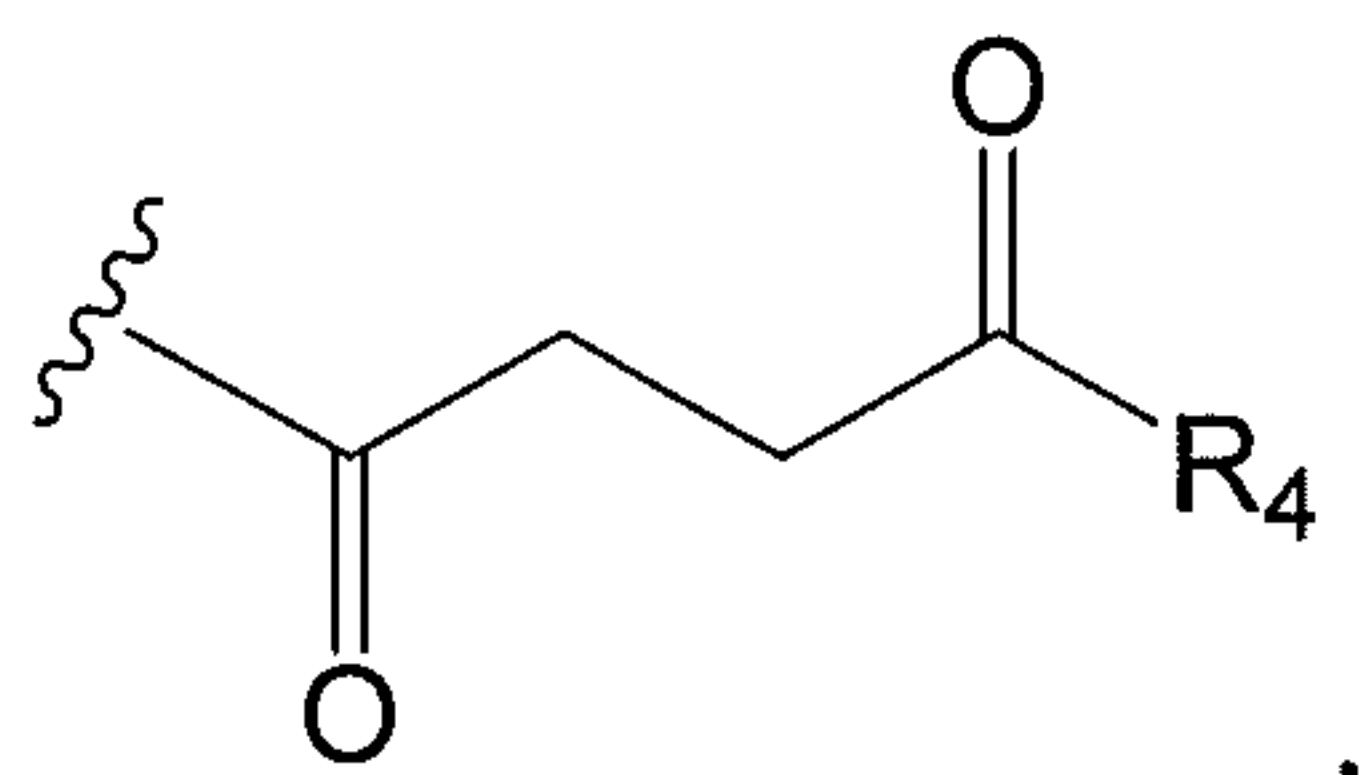
[00115] The embodiments illustrated and discussed in this specification are intended only to teach those skilled in the art the best way known to the inventors to make and use the invention. Nothing in this specification should be considered as limiting the scope of the present invention. All examples presented are representative and non-limiting. The above-described embodiments of the invention may be modified or varied, without departing from the invention, as appreciated by those skilled in the art in light of the above teachings. It is therefore to be understood that, within the scope of the claims and their equivalents, the invention may be practiced otherwise than as specifically described.

Claims:

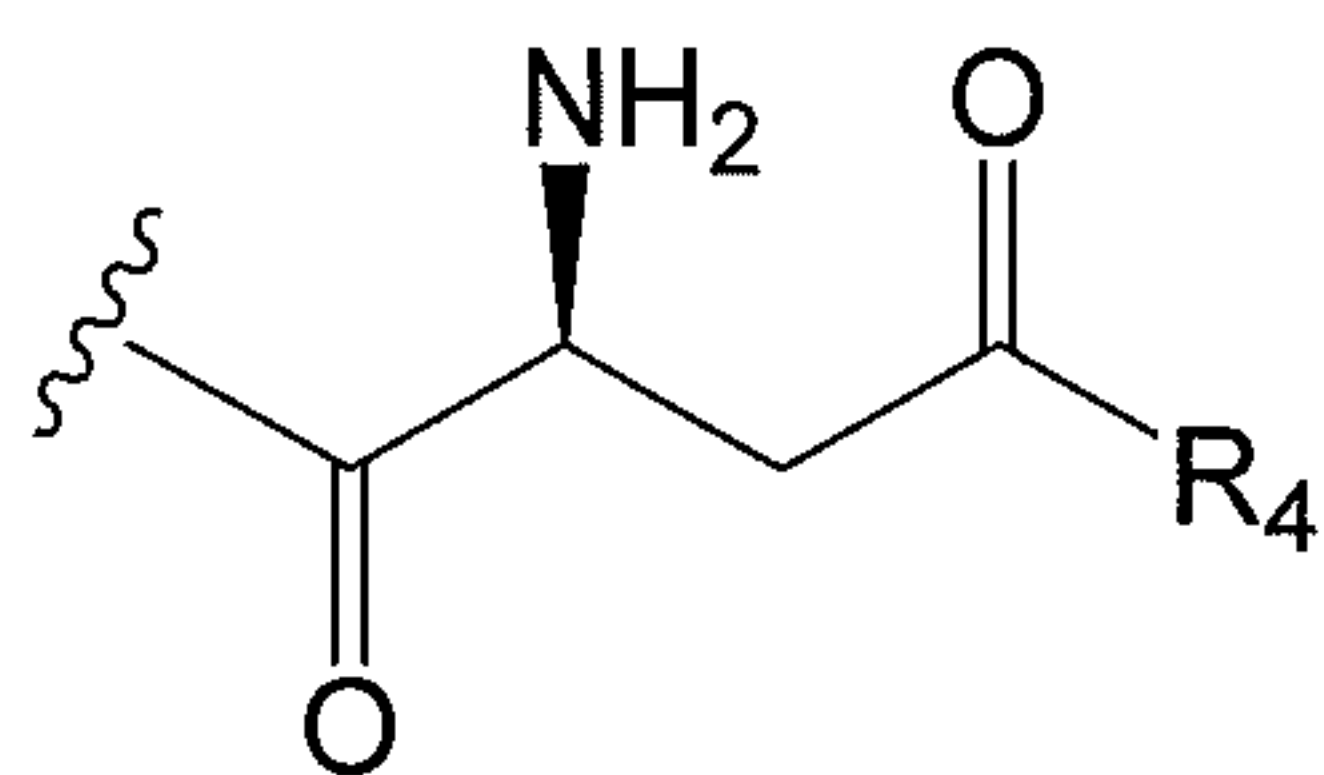
Claim 1. A compound of the formula



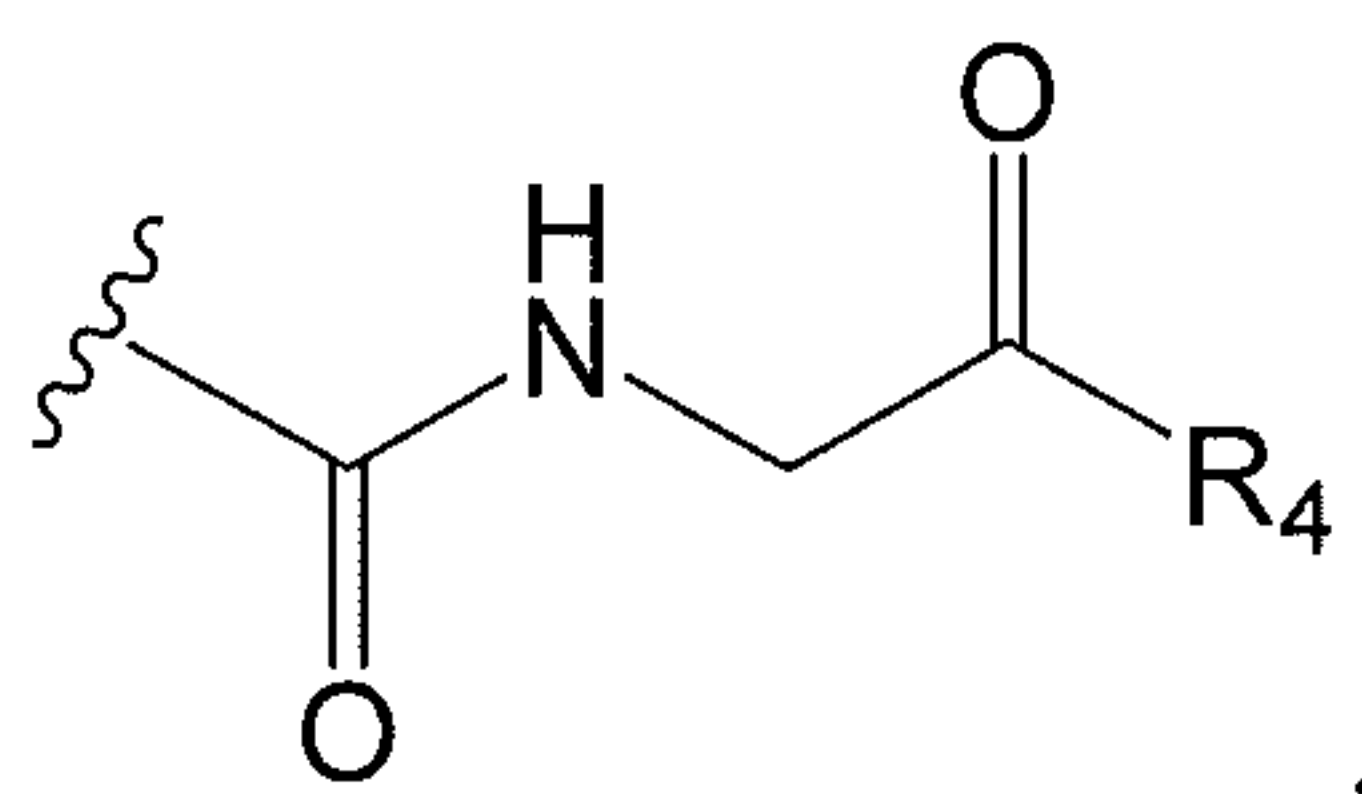
wherein R_1 , R_2 , and R_3 are independently selected from the group consisting of hydrogen,



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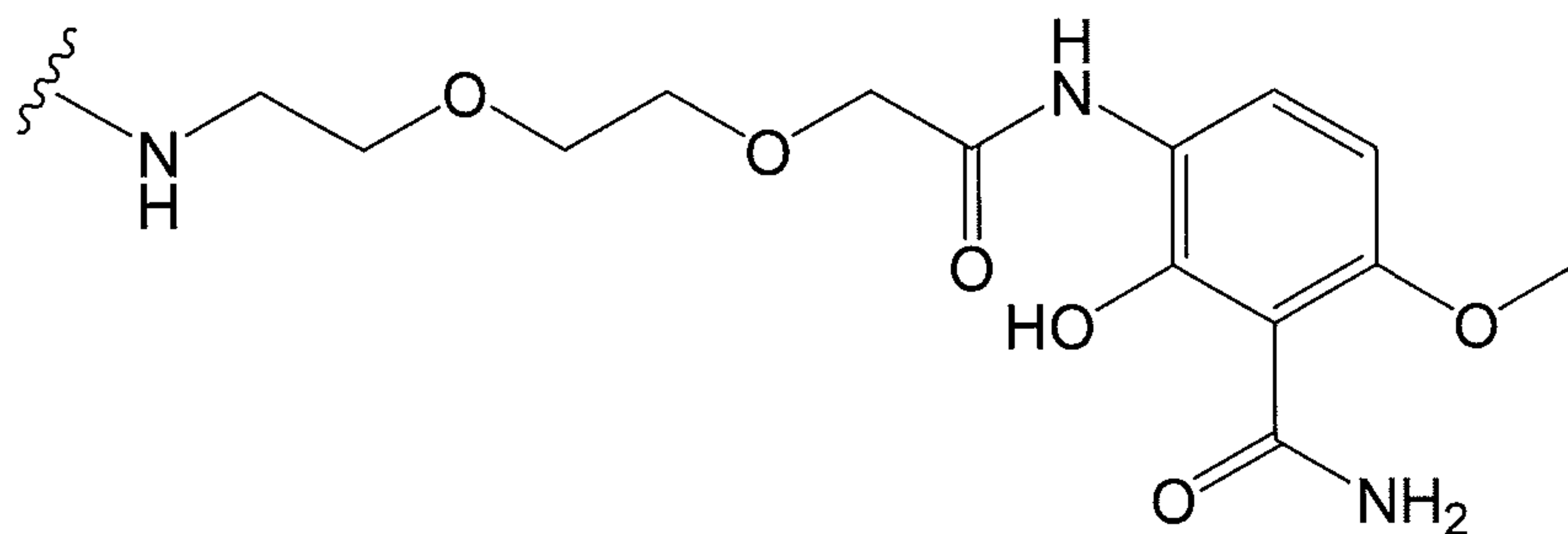


, and

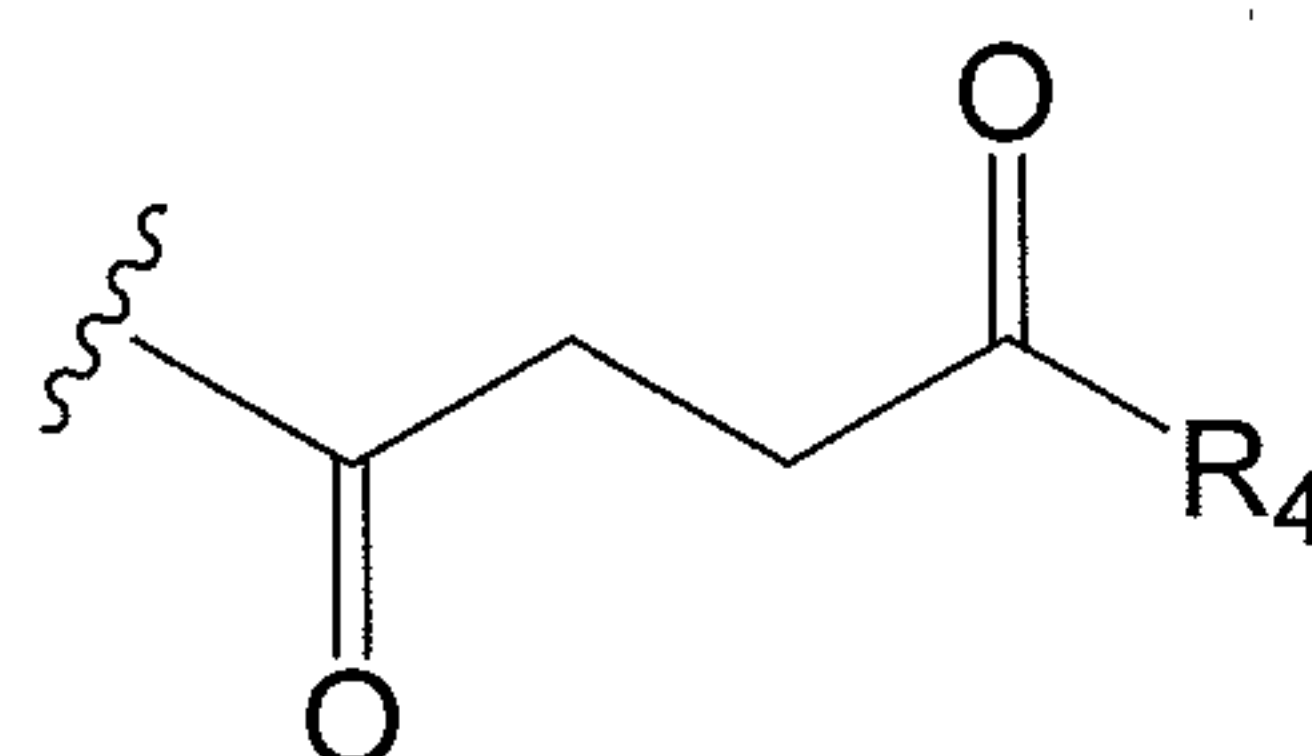


,

wherein R_4 is



wherein at least one of R₁, R₂, and R₃ is not hydrogen, and



wherein when R₁ and R₃ are hydrogen, then R₂ is not

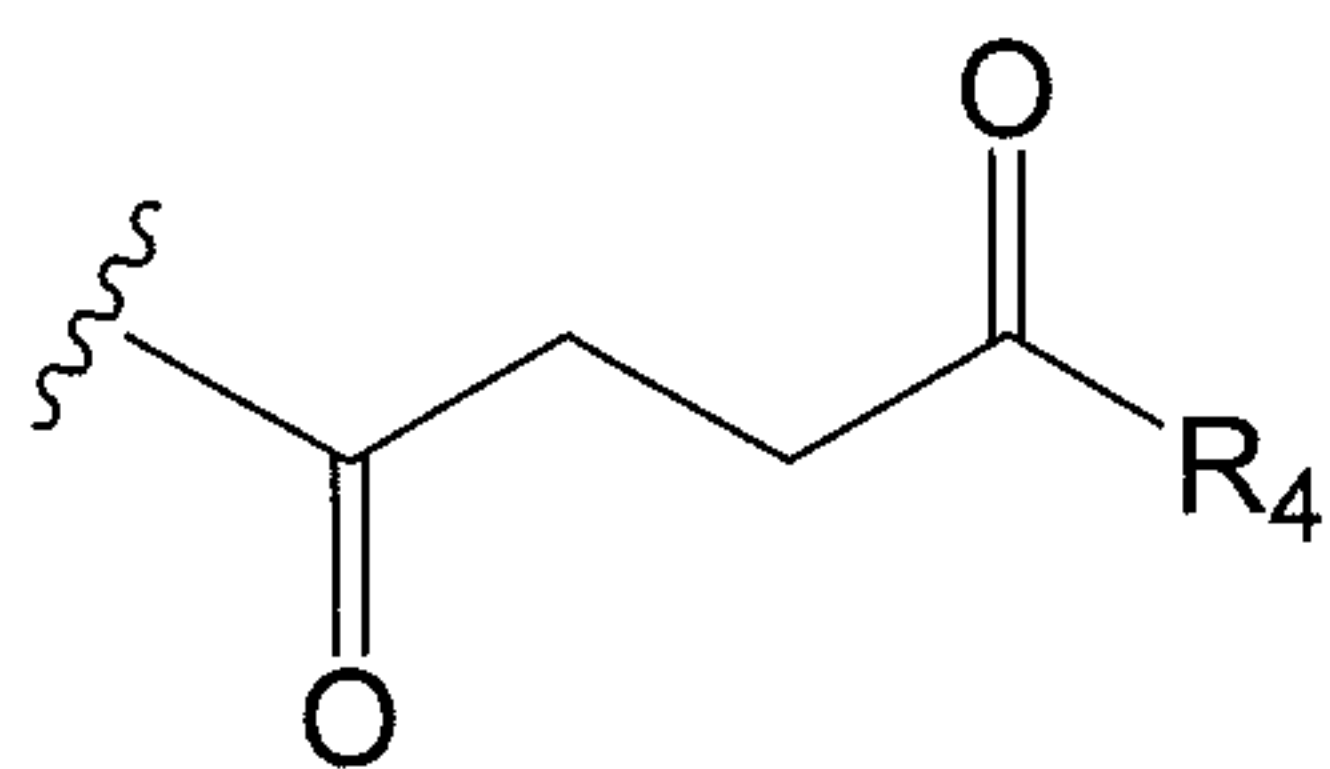
Claim 2. The compound of claim 1, wherein R₃ is hydrogen.

Claim 3. The compound of claim 1, wherein R₂ is hydrogen and R₃ is hydrogen.

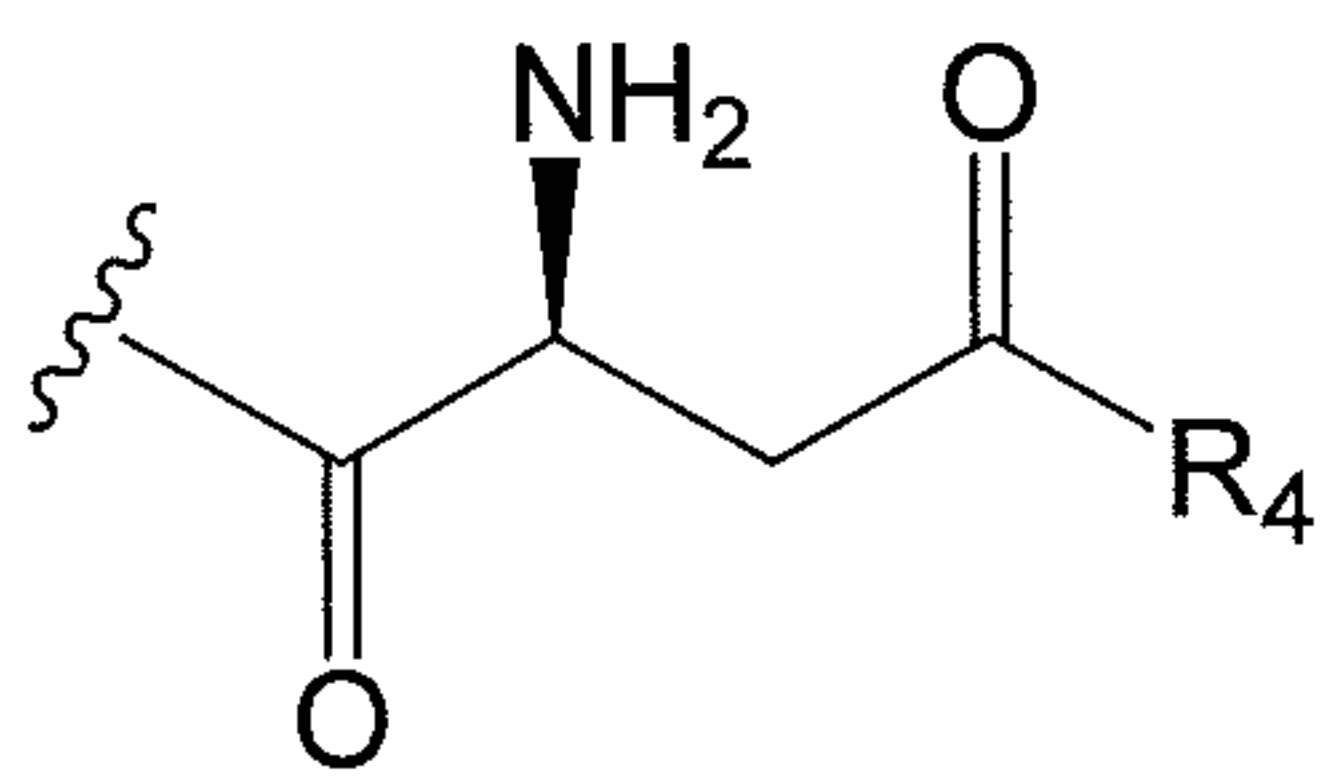
Claim 4. The compound of claim 1, wherein R₁ is hydrogen and R₃ is hydrogen.

Claim 5. The compound of claim 1, wherein R₃ is hydrogen and R₁ is not hydrogen and R₂ is not hydrogen.

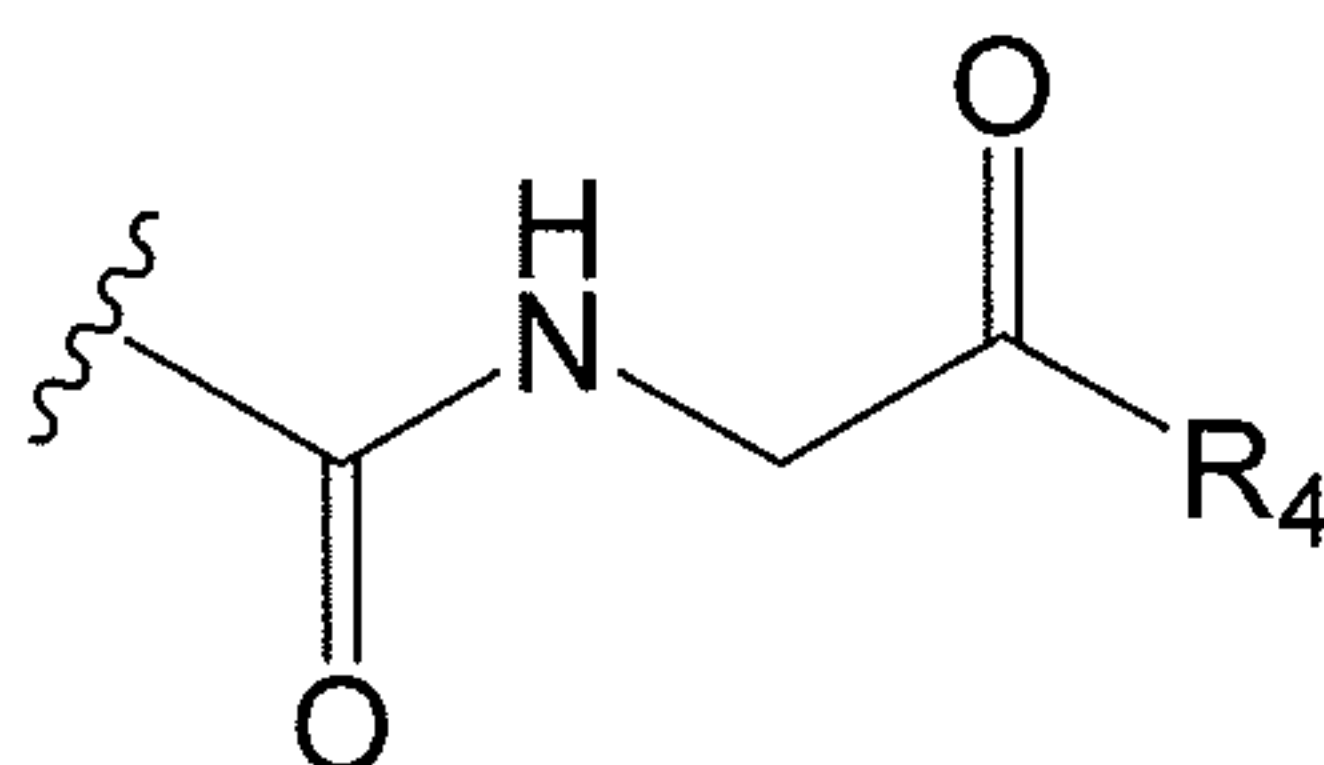
Claim 6. The compound of claim 1, wherein R₃ is hydrogen and R₁ and R₂ are each



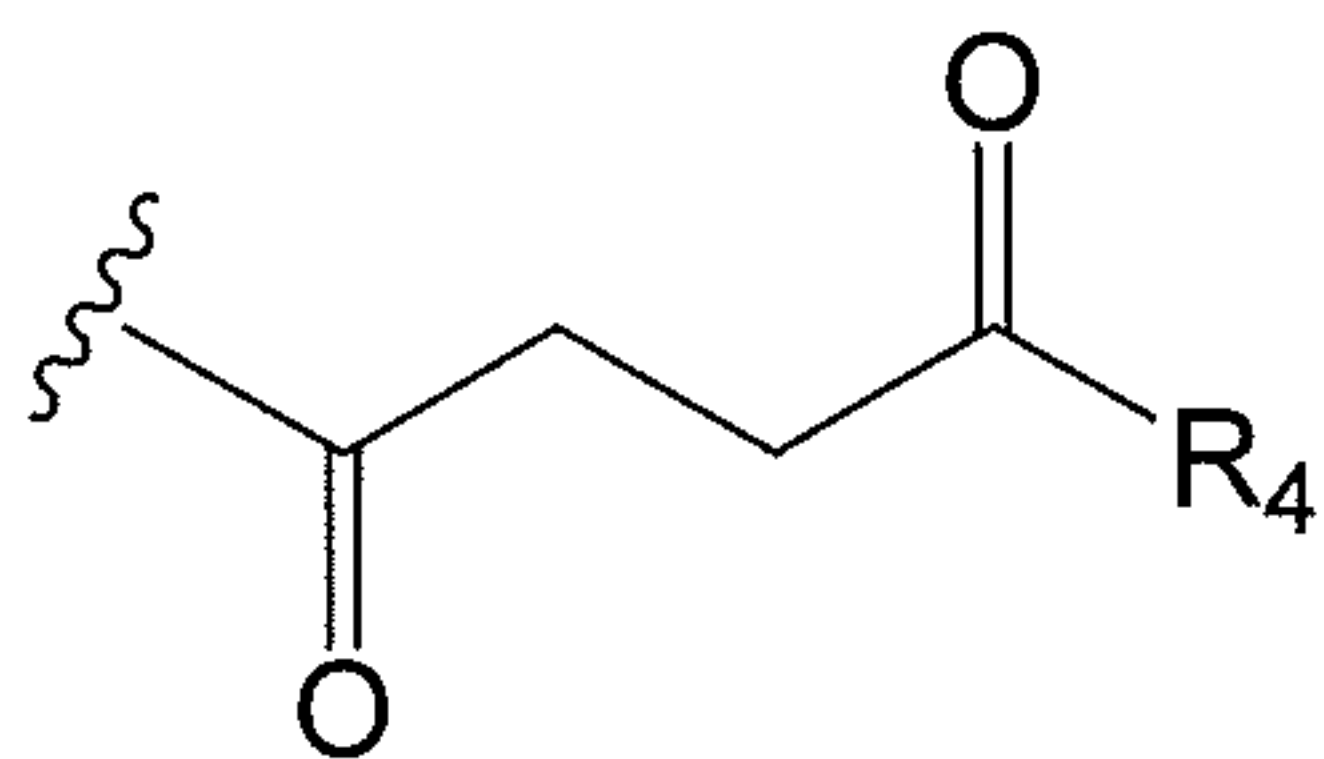
Claim 7. The compound of claim 1, wherein R₃ is hydrogen and R₁ and R₂ are each



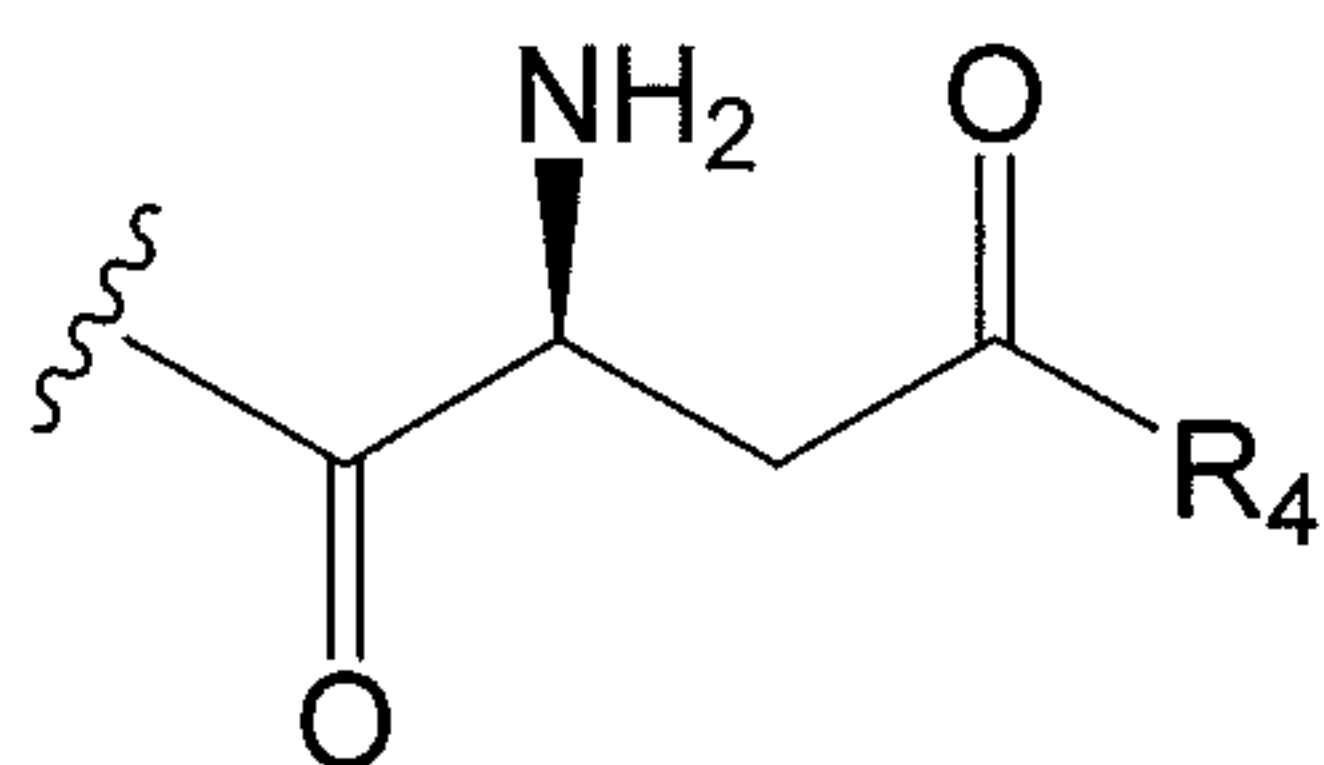
Claim 8. The compound of claim 1, wherein R_3 is hydrogen and R_1 and R_2 are each



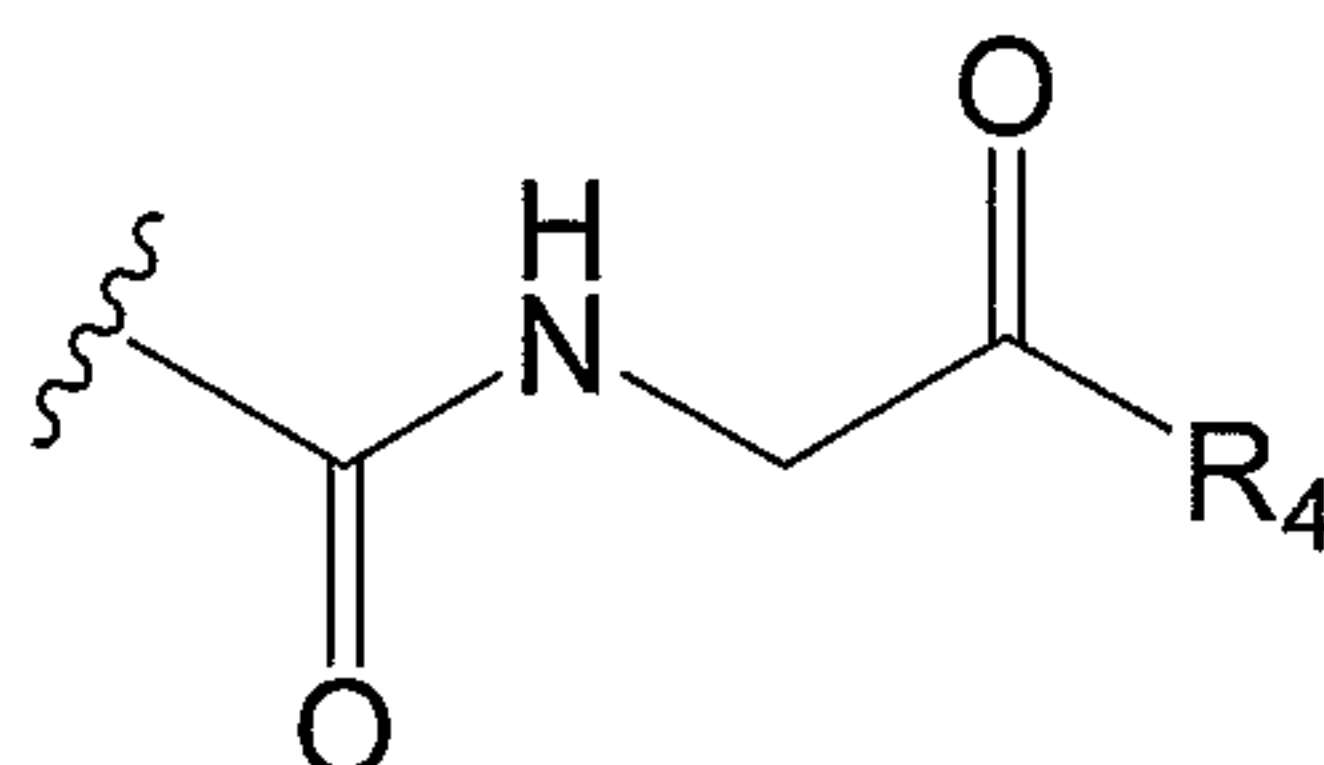
Claim 9. The compound of claim 1, wherein R_1 , R_2 , and R_3 are each



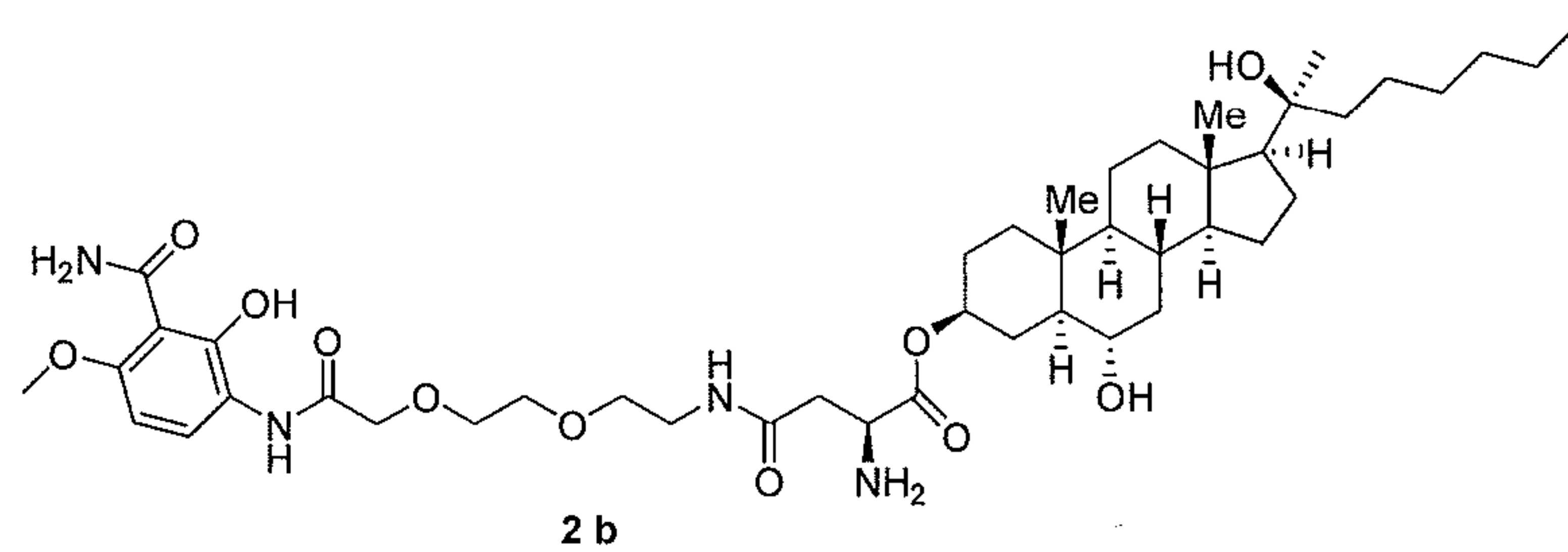
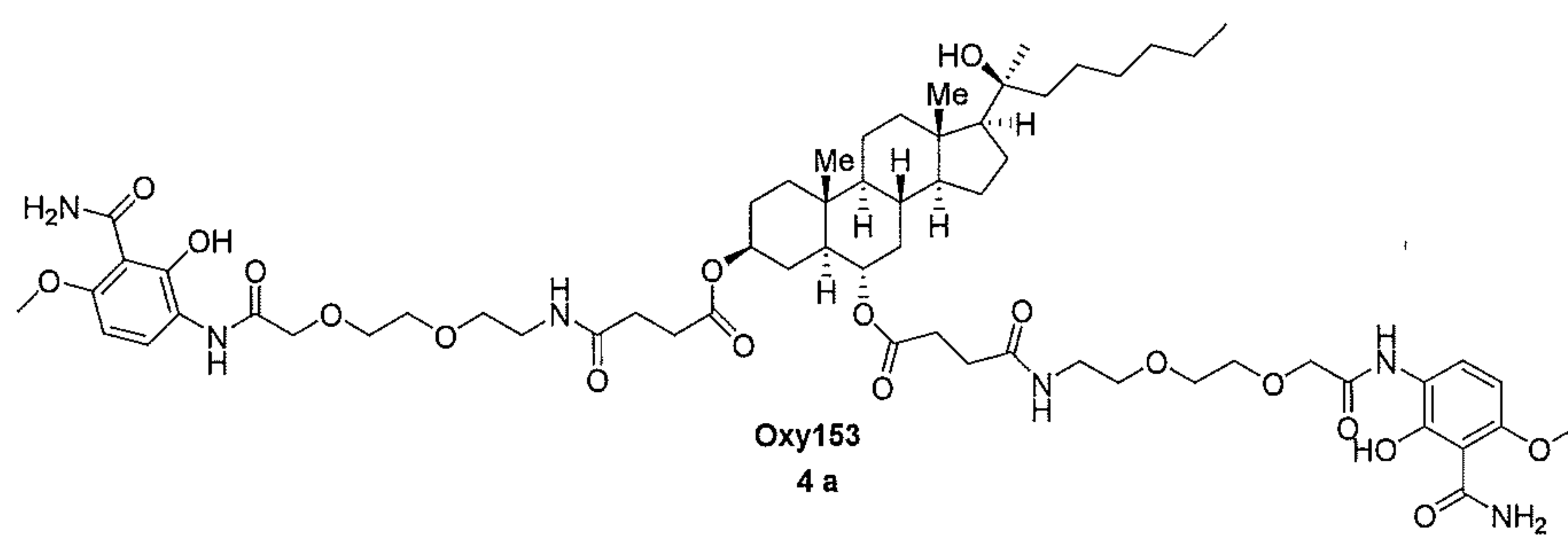
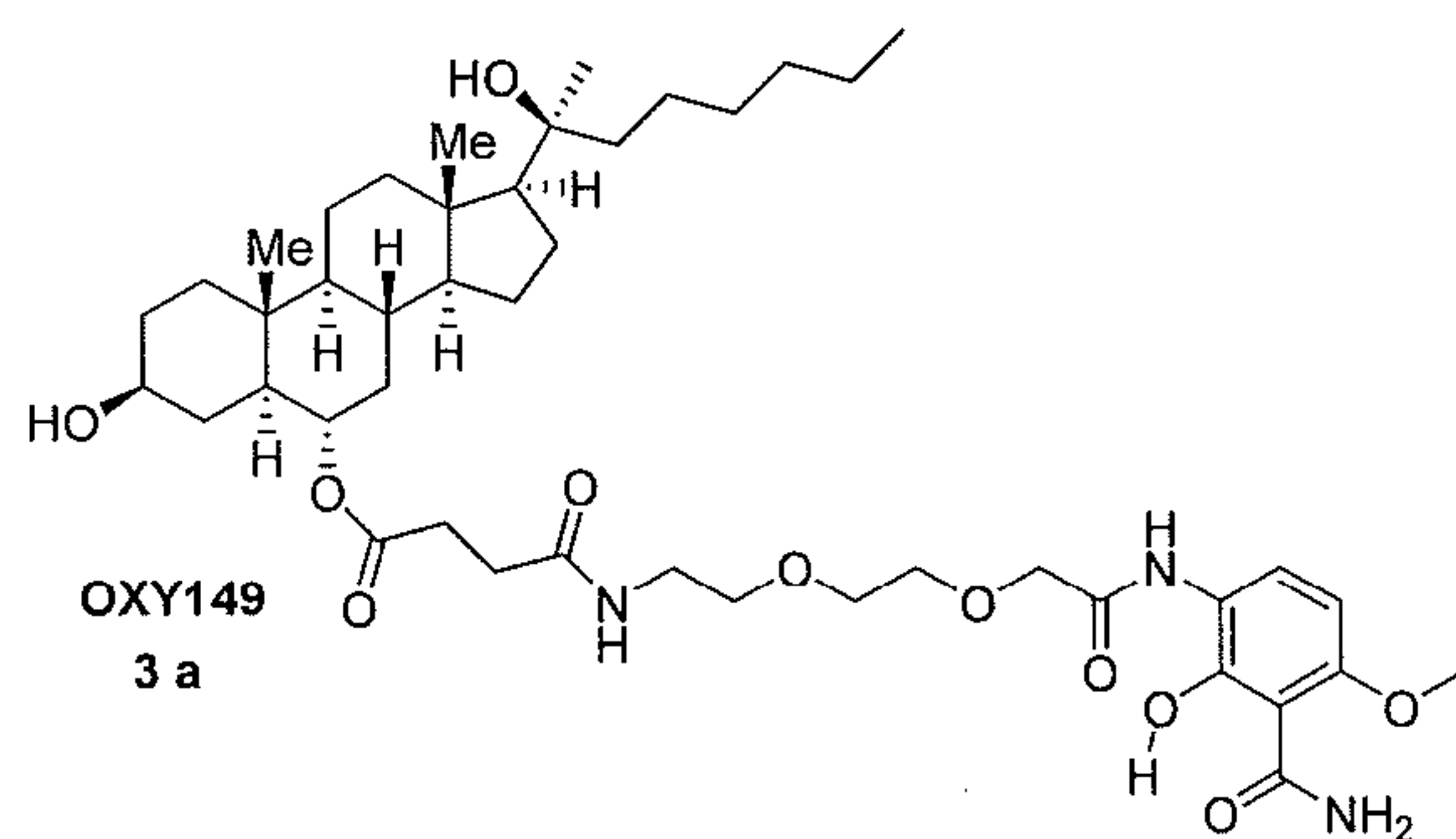
Claim 10. The compound of claim 1, wherein R_1 , R_2 , and R_3 are each

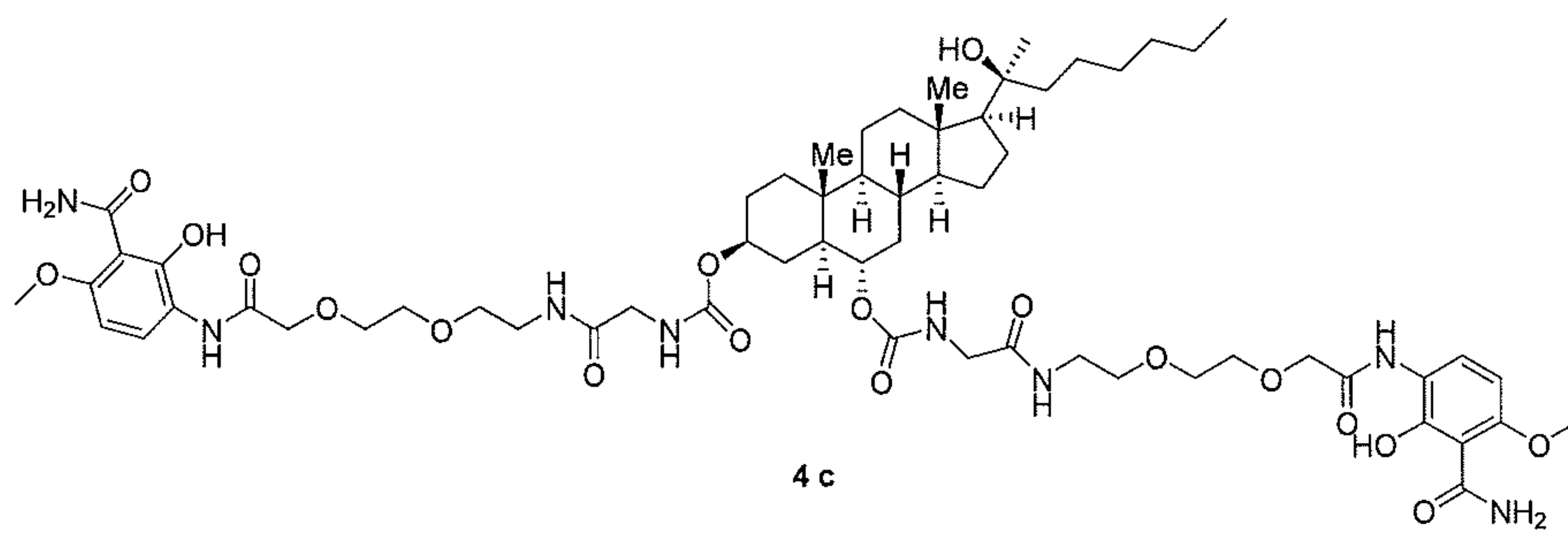
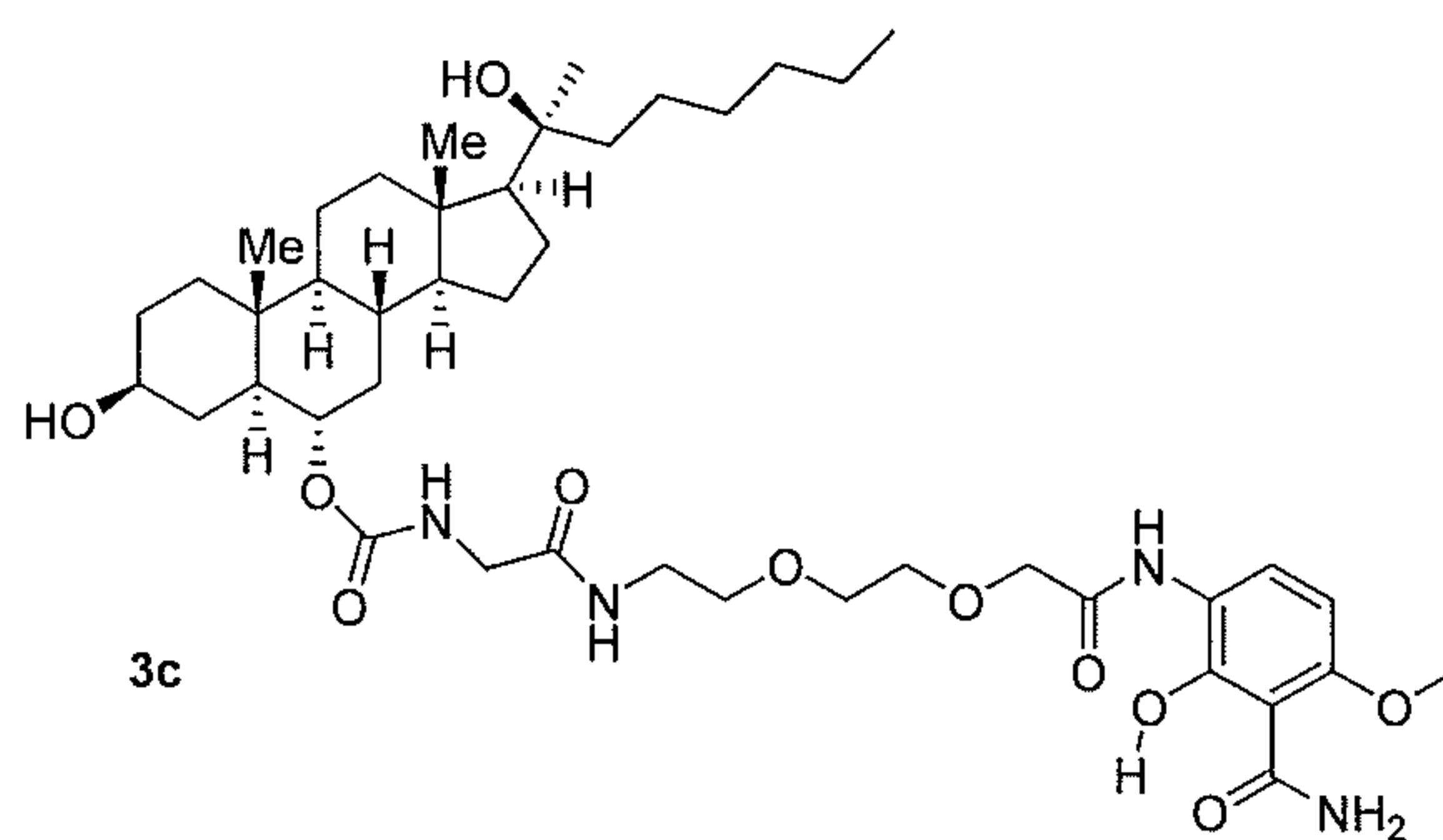
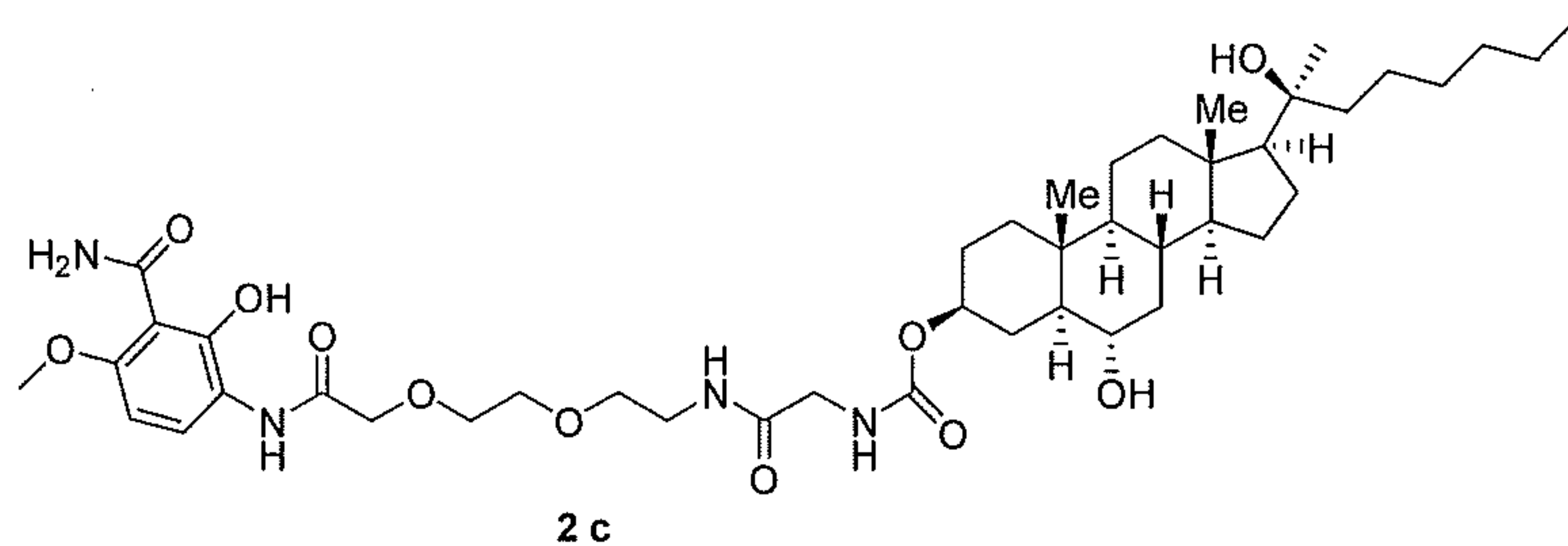
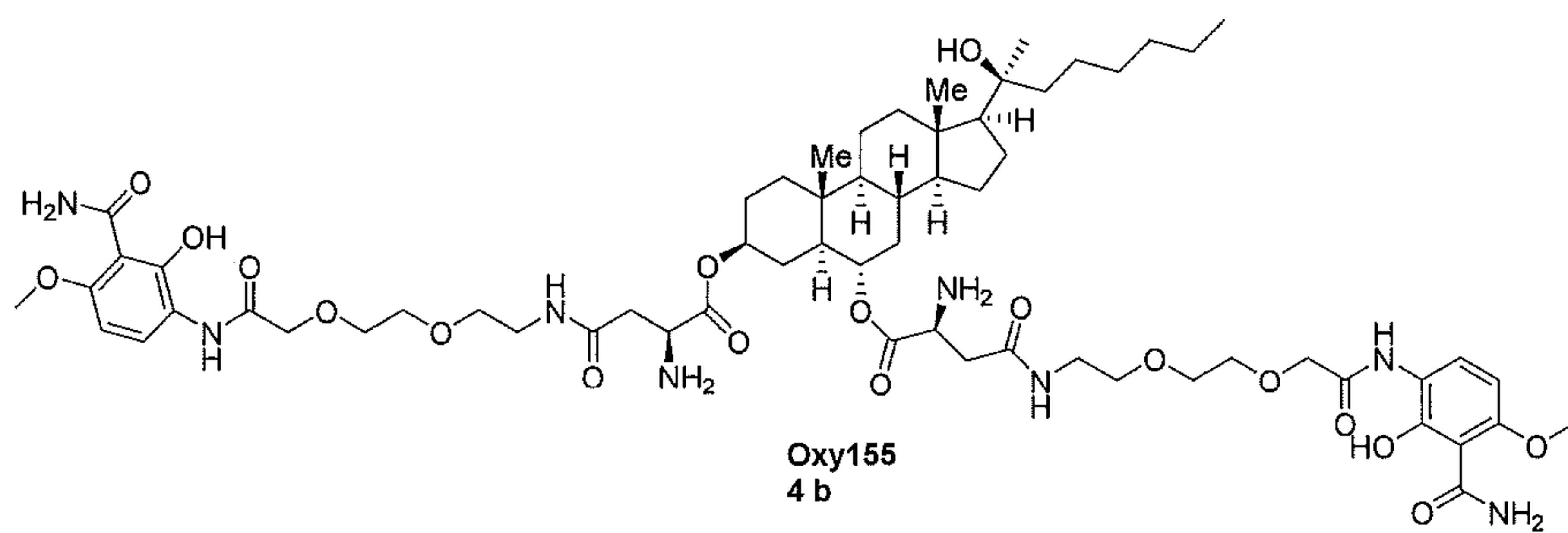
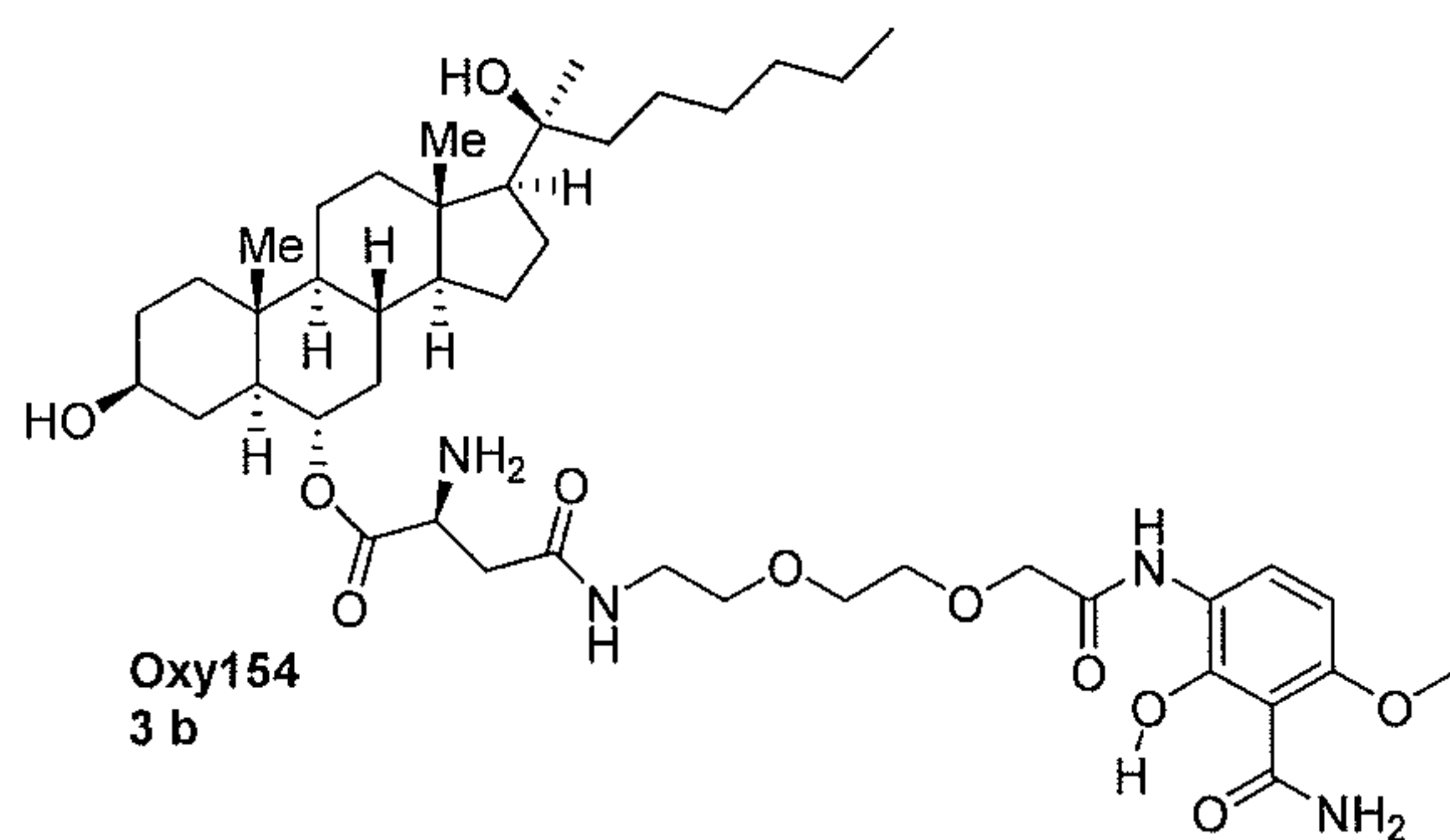


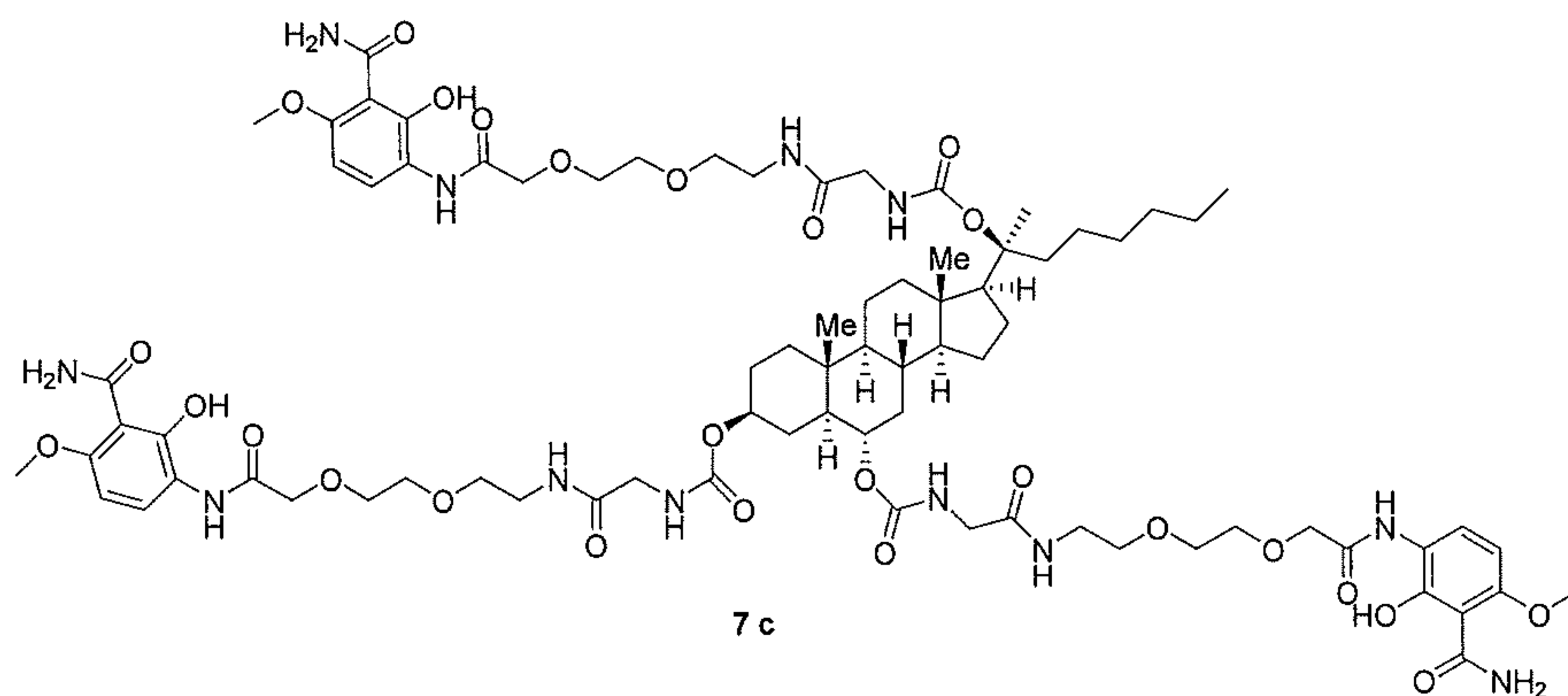
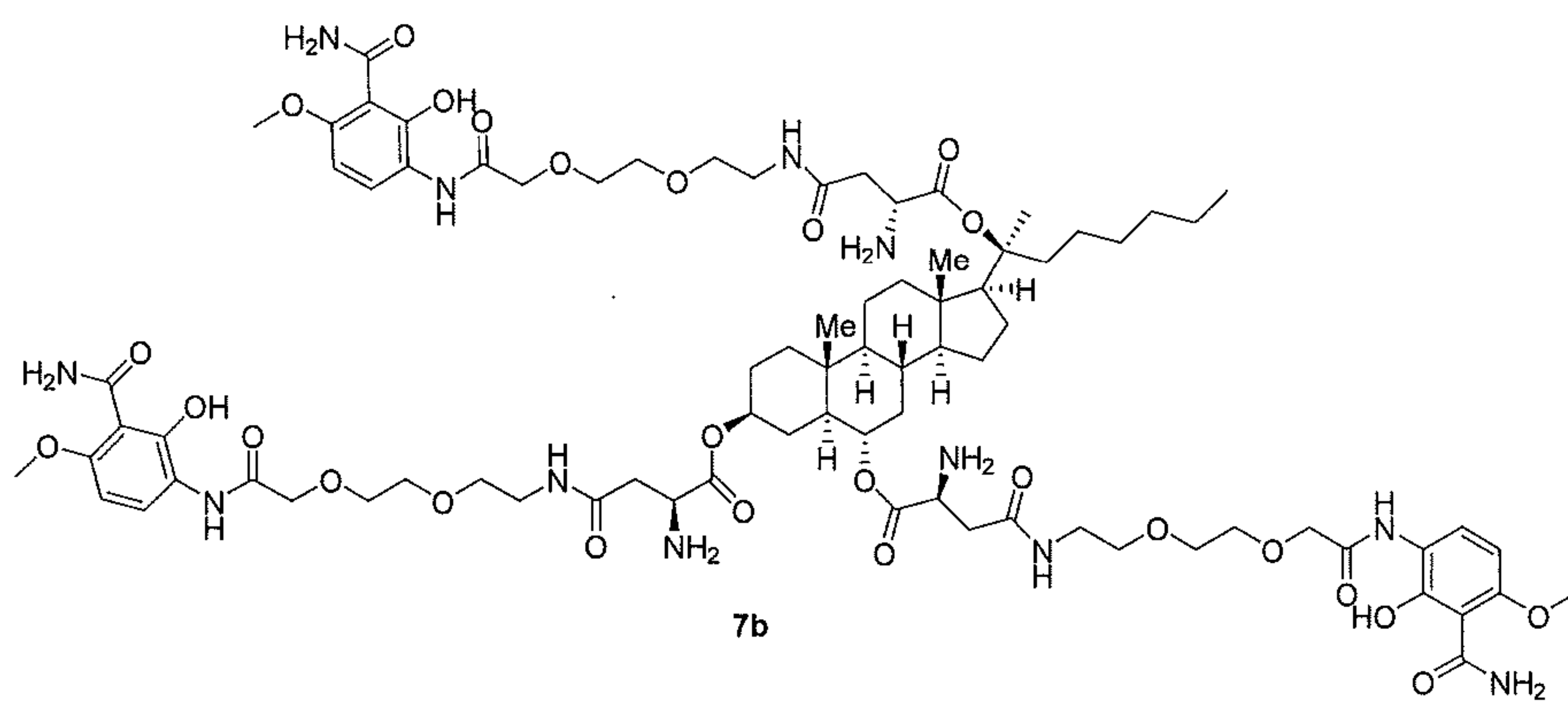
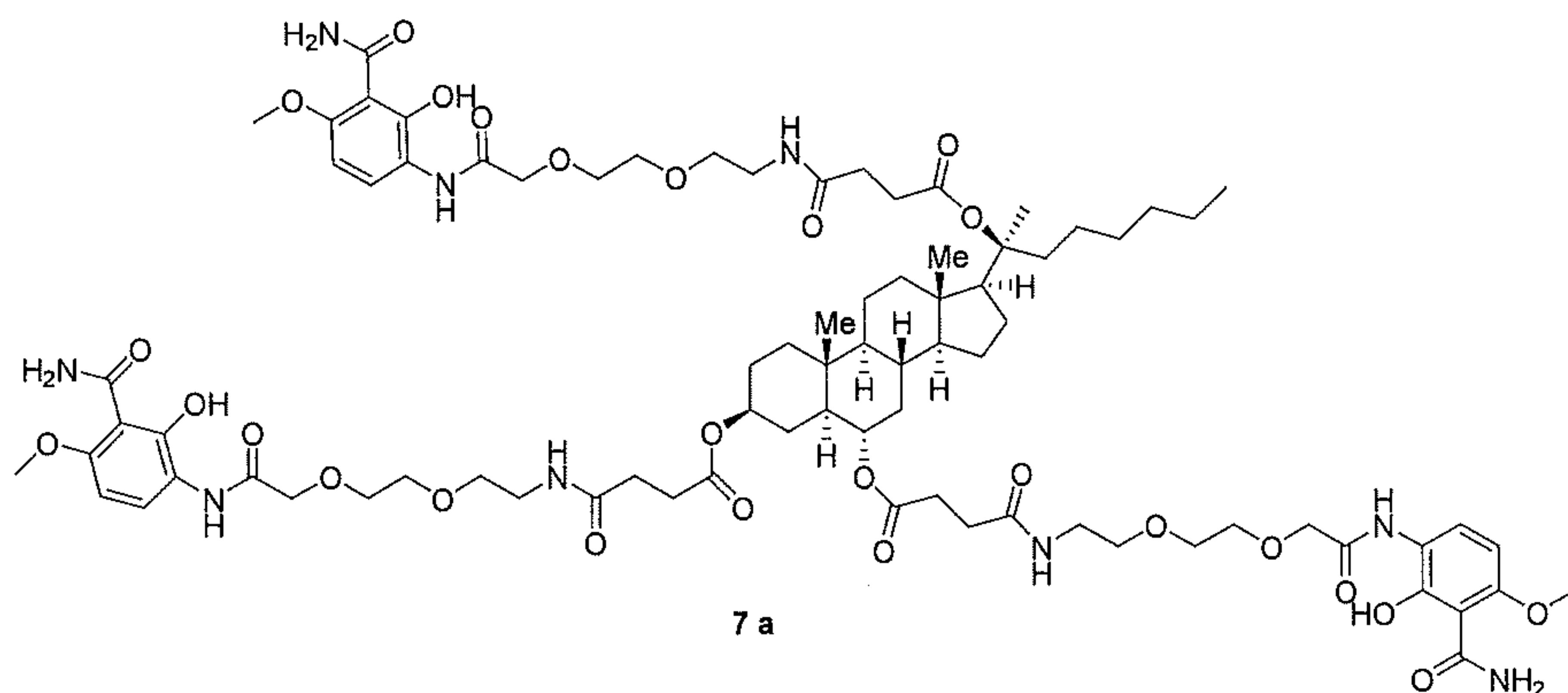
Claim 11. The compound of claim 1, wherein R_1 , R_2 , and R_3 are each



Claim 12. The compound of claim 1, selected from the group consisting of







Claim 13. A pharmaceutical composition comprising a compound of claim 1 and a pharmaceutically acceptable carrier or diluent.

Claim 14. A method for treating a subject (a human or an animal) suffering from a bone disorder, comprising administering to the subject an effective amount of the compound of claim 1.

Claim 15. The method of claim 14, wherein the compound is administered to effect localized delivery to the subject.

Claim 16. The method of claim 14, wherein the compound is administered to effect systemic delivery to the subject.

Claim 17. The method of claim 14, wherein the bone disorder is selected from the group consisting of a bone fracture, osteoporosis, and osteopenia

Claim 18. A method for treating a subject (a human or an animal) suffering from a bone disorder, comprising contacting an osteoblast progenitor cell with an effective amount of the compound of claim 1.

Claim 19. The method of claim 18, wherein the osteoblast progenitor cell is contacted with the compound *in vitro*.

Claim 20. A method for treating a subject (a human or an animal) suffering from a bone disorder, comprising administering the contacted osteoblast progenitor cell of claim 18 to the subject.

Claim 21. The method of claim 20, wherein the contacted osteoblast progenitor cell is administered locally to the subject.

Claim 22. The method of claim 20, wherein the contacted osteoblast progenitor cell is administered systemically to the subject.

Claim 23. A method for treating a cell, comprising administering an effective amount of the compound of claim 1 to the cell, so that a Hedgehog signaling pathway in the cell is stimulated.

Claim 24. The method of claim 23, wherein the cell is part of a tissue or an organ.

Claim 25. The method of claim 23, wherein the compound is administered *in vivo* (locally and/or systemically).

Claim 26. A method for treating a subject (a human or an animal) that would benefit from therapeutic activation of a Hedgehog signaling pathway in a tissue or an organ, comprising treating a cell of the tissue or organ as set forth in claim 23, so that the Hedgehog signaling pathway of the tissue or organ is stimulated.

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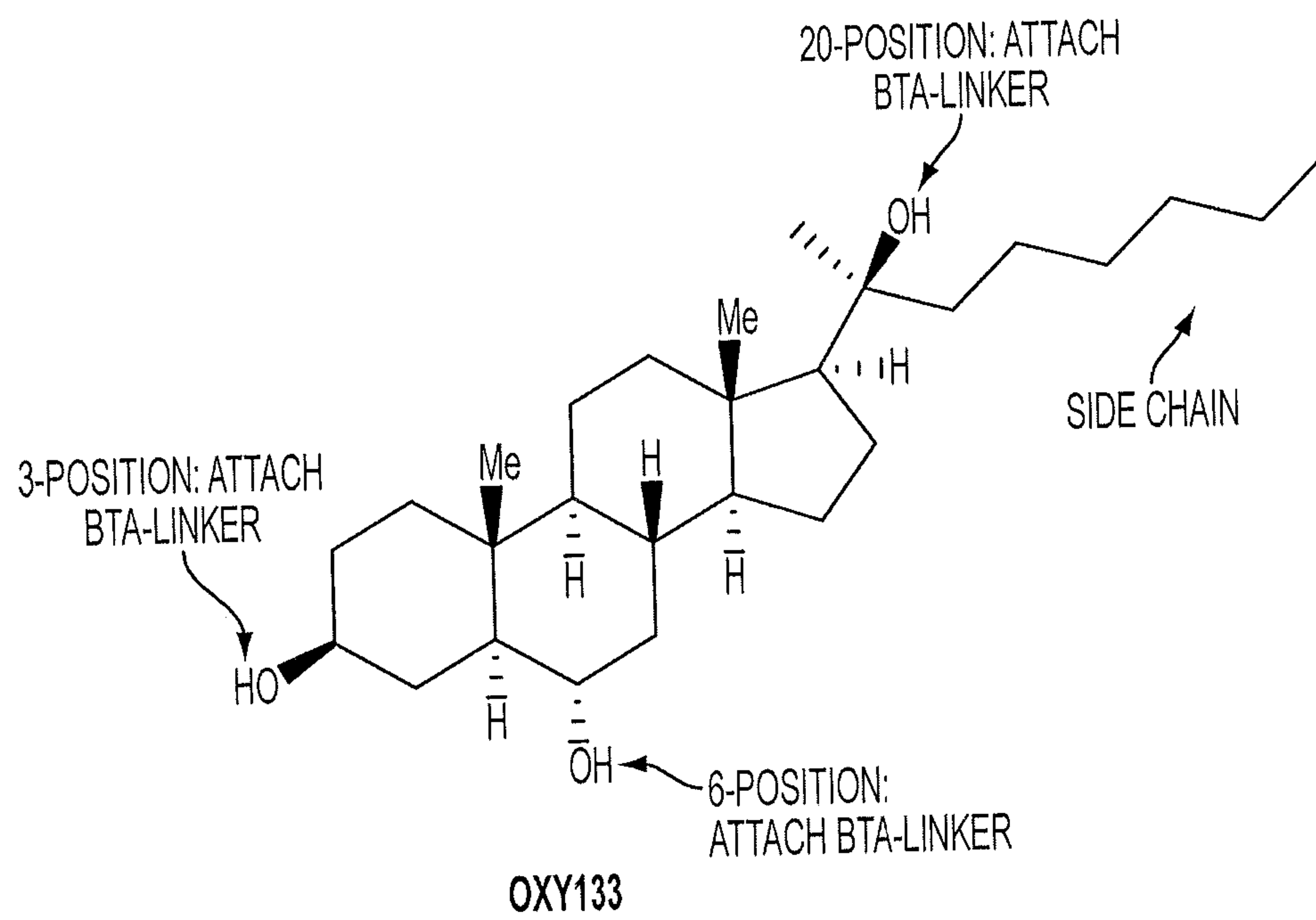


FIG. 1

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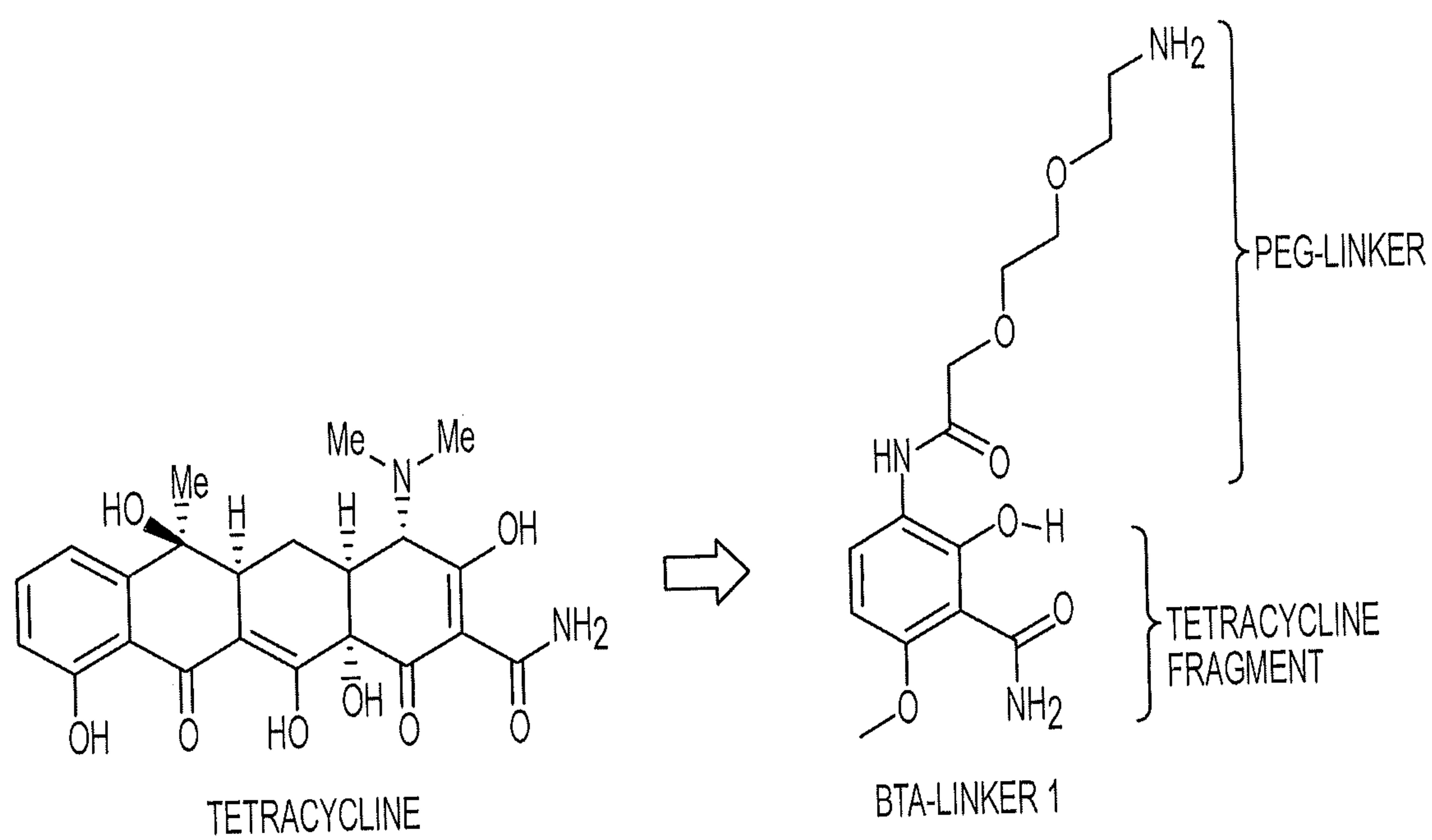
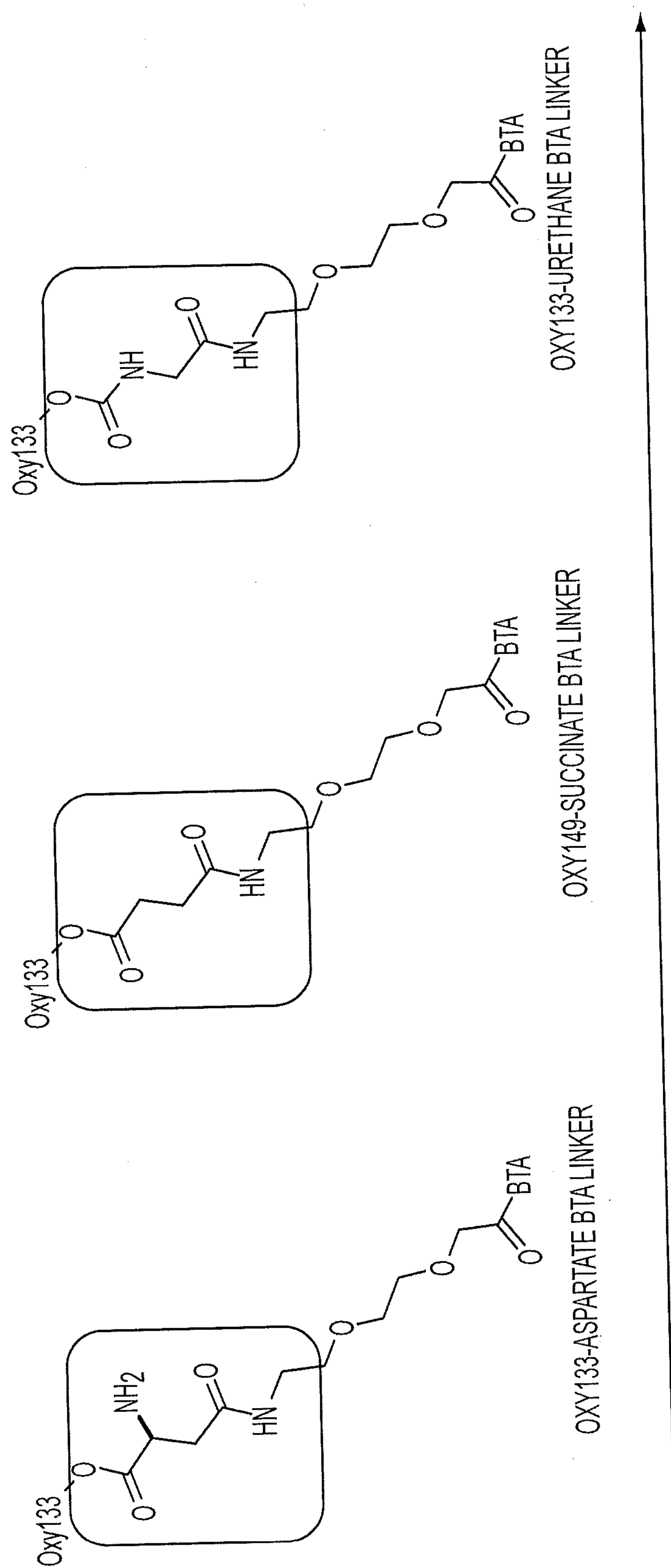


FIG. 2



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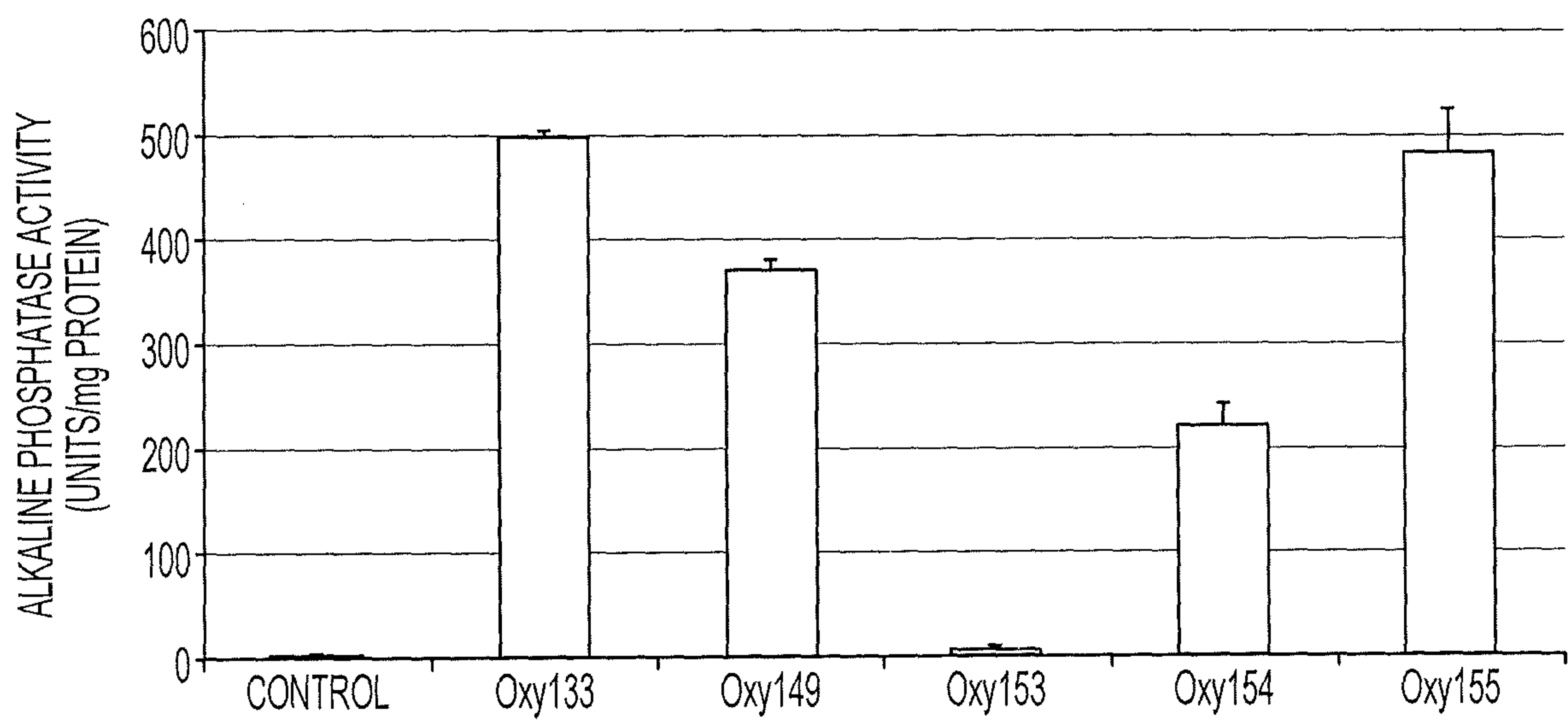


FIG. 4

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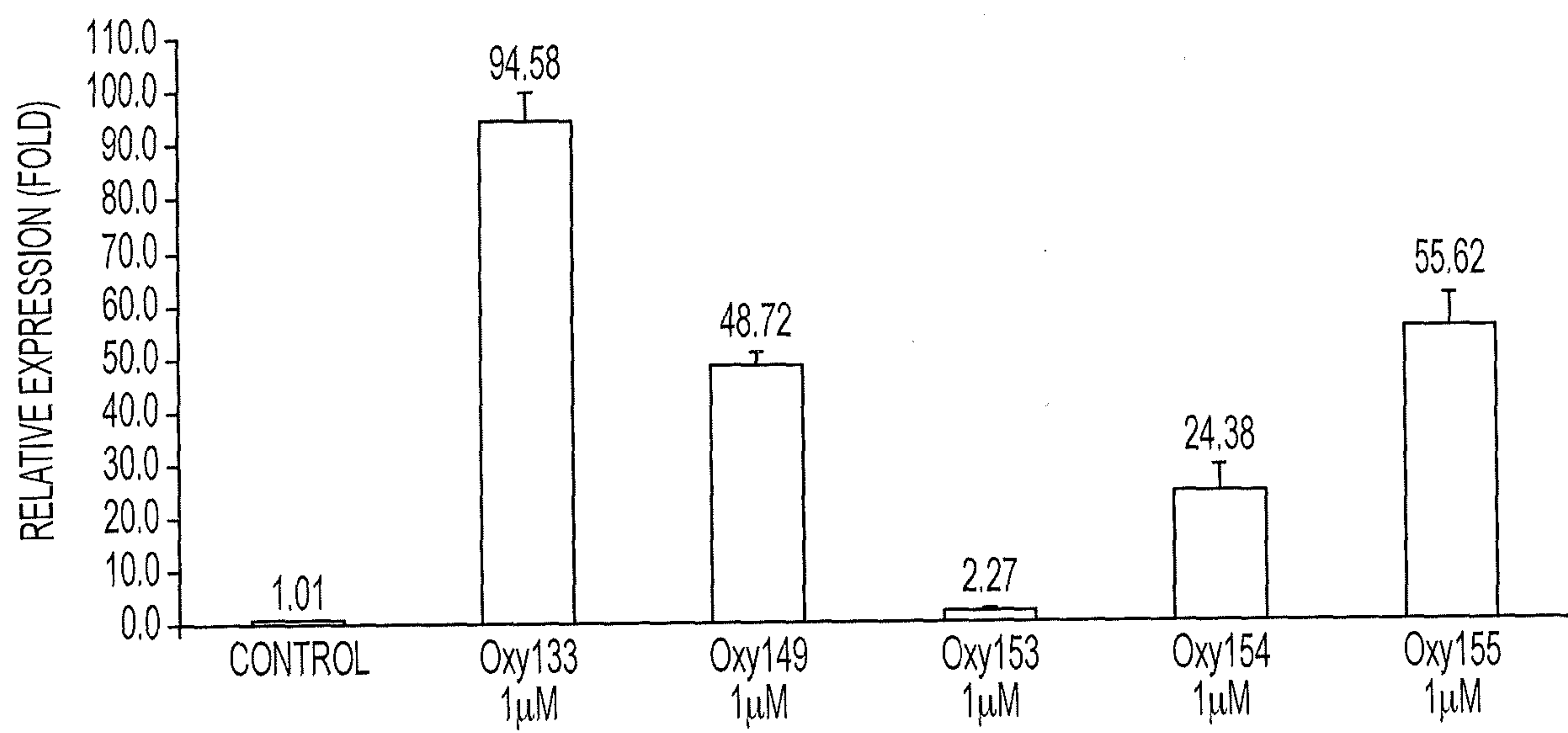


FIG. 5

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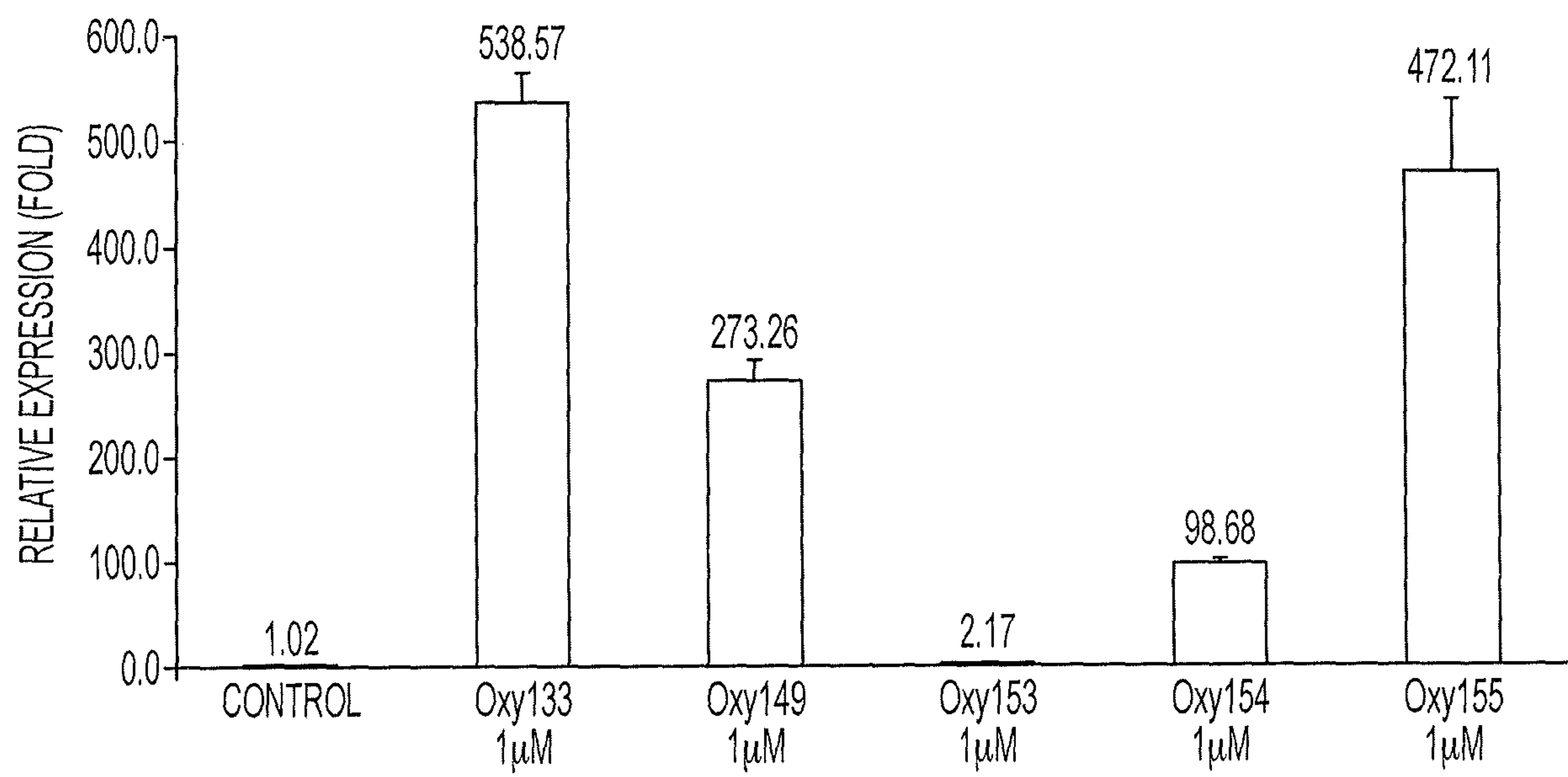


FIG. 6

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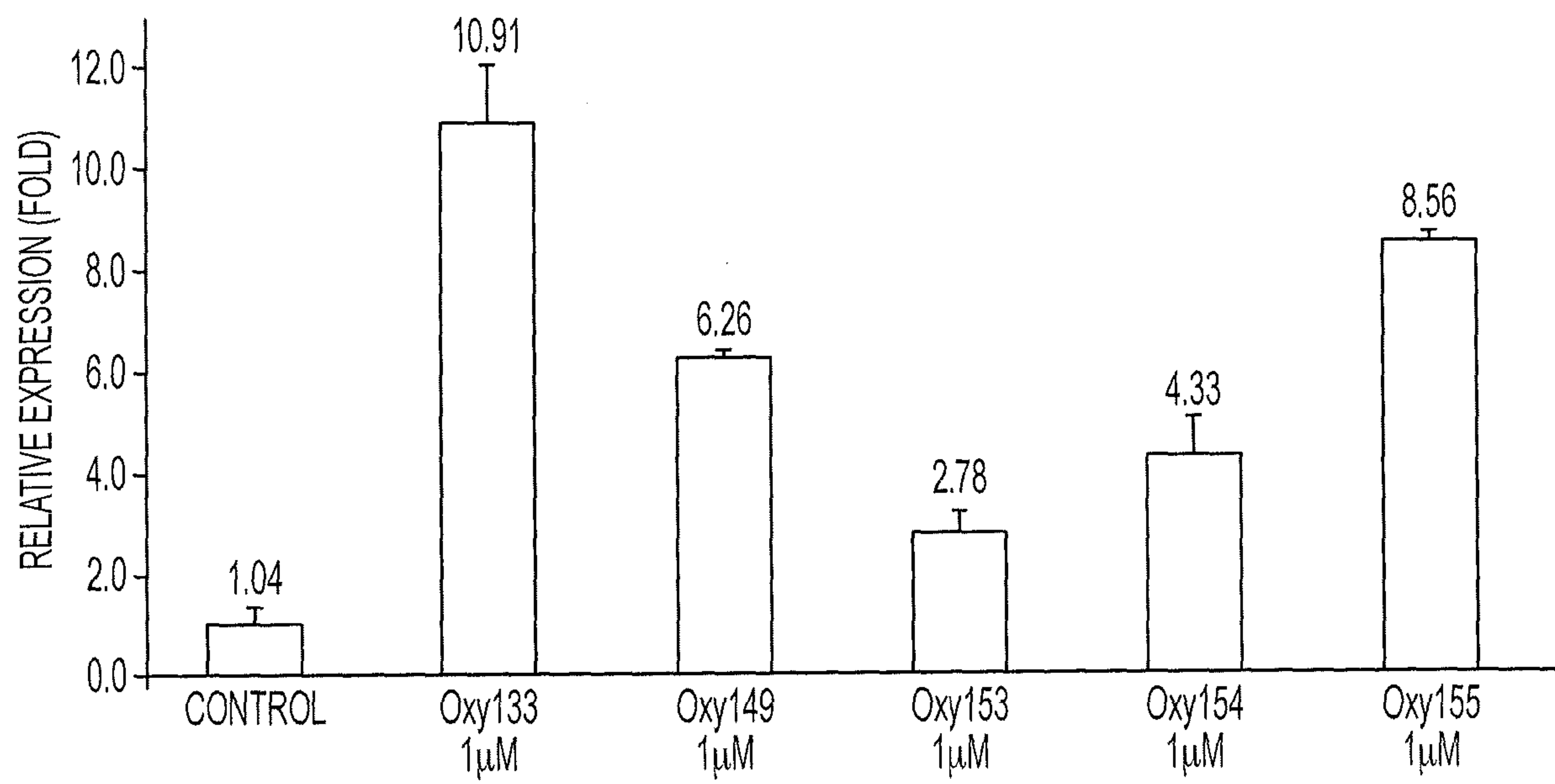


FIG. 7

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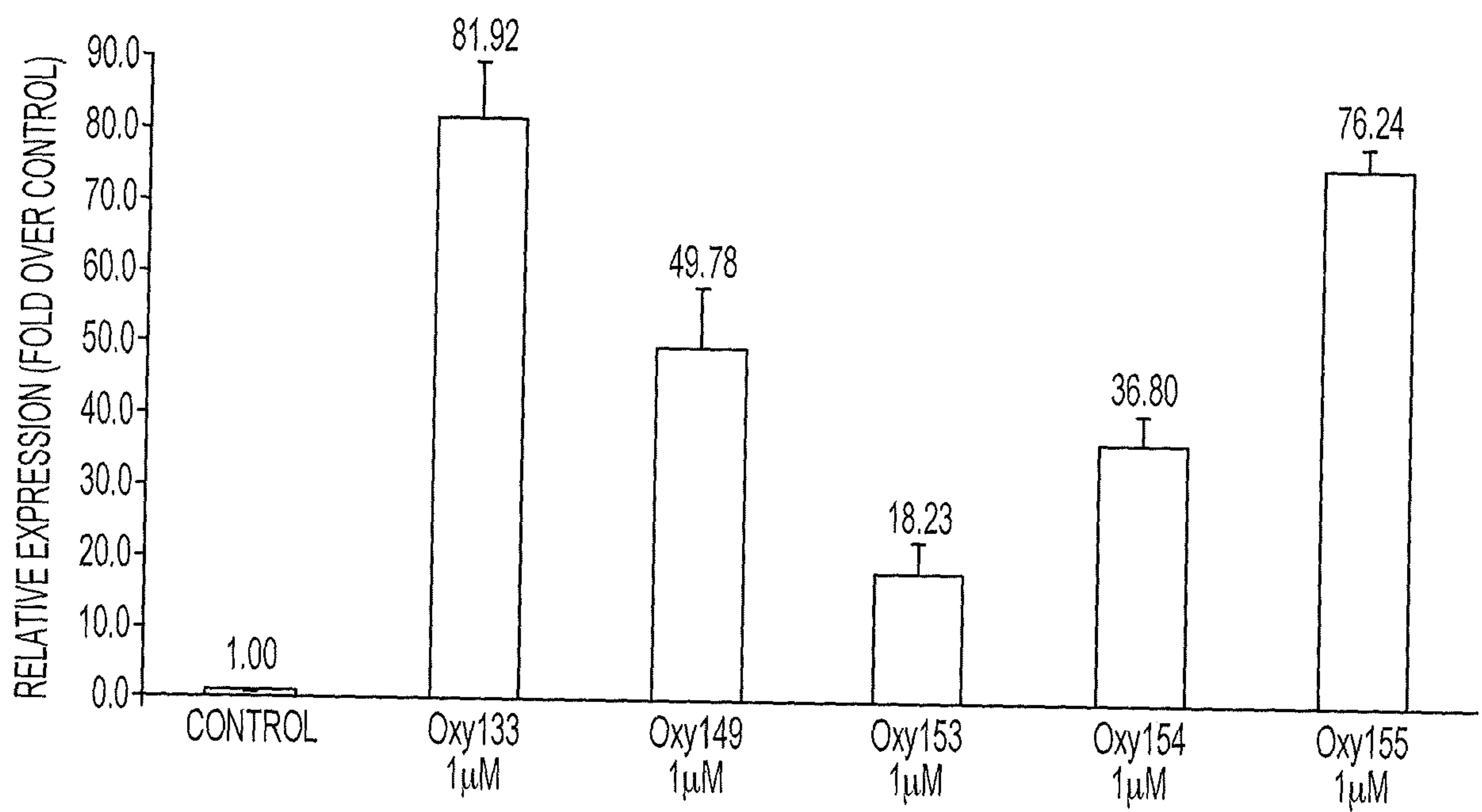


FIG. 8

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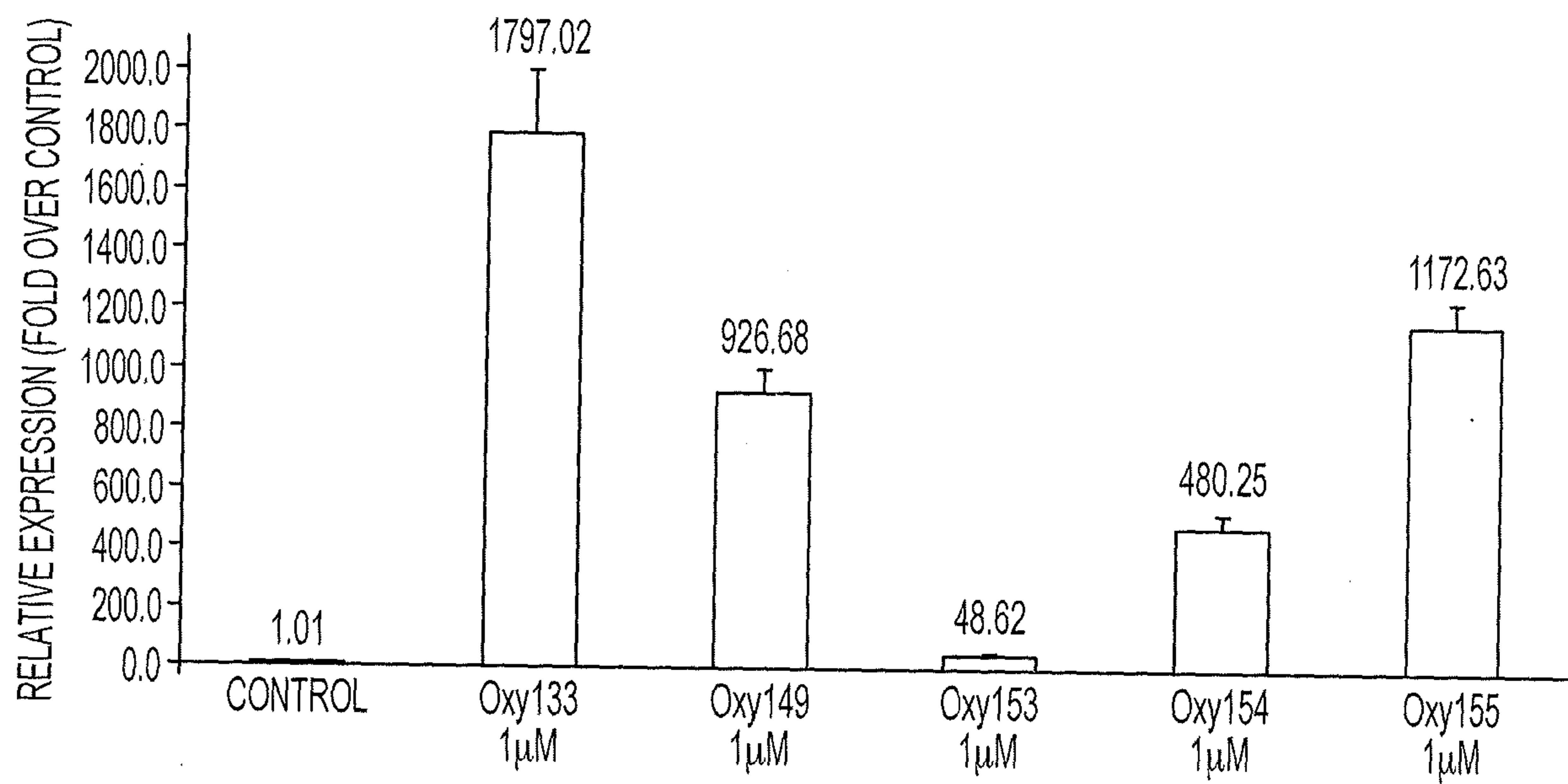


FIG. 9

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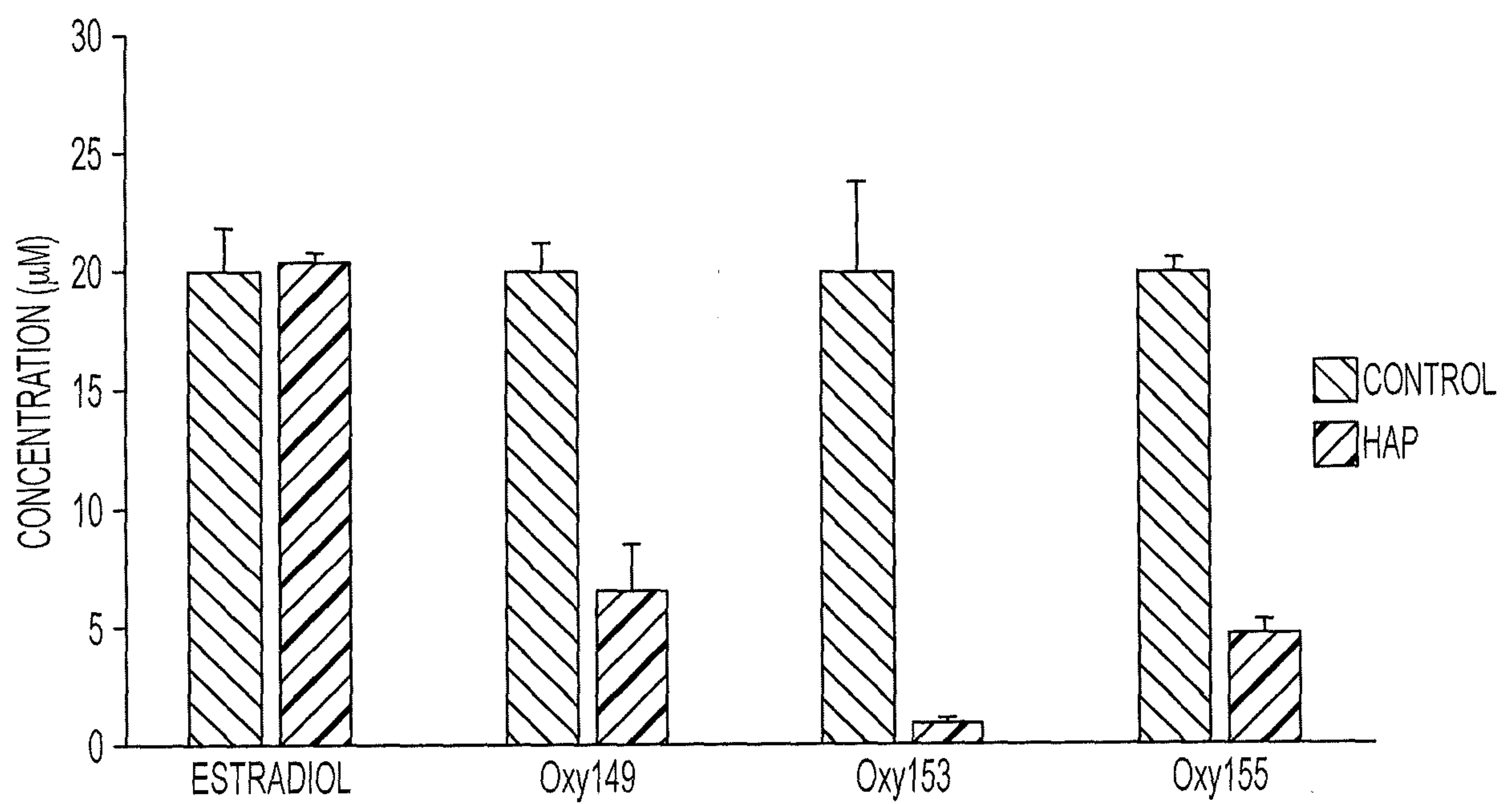


FIG. 10

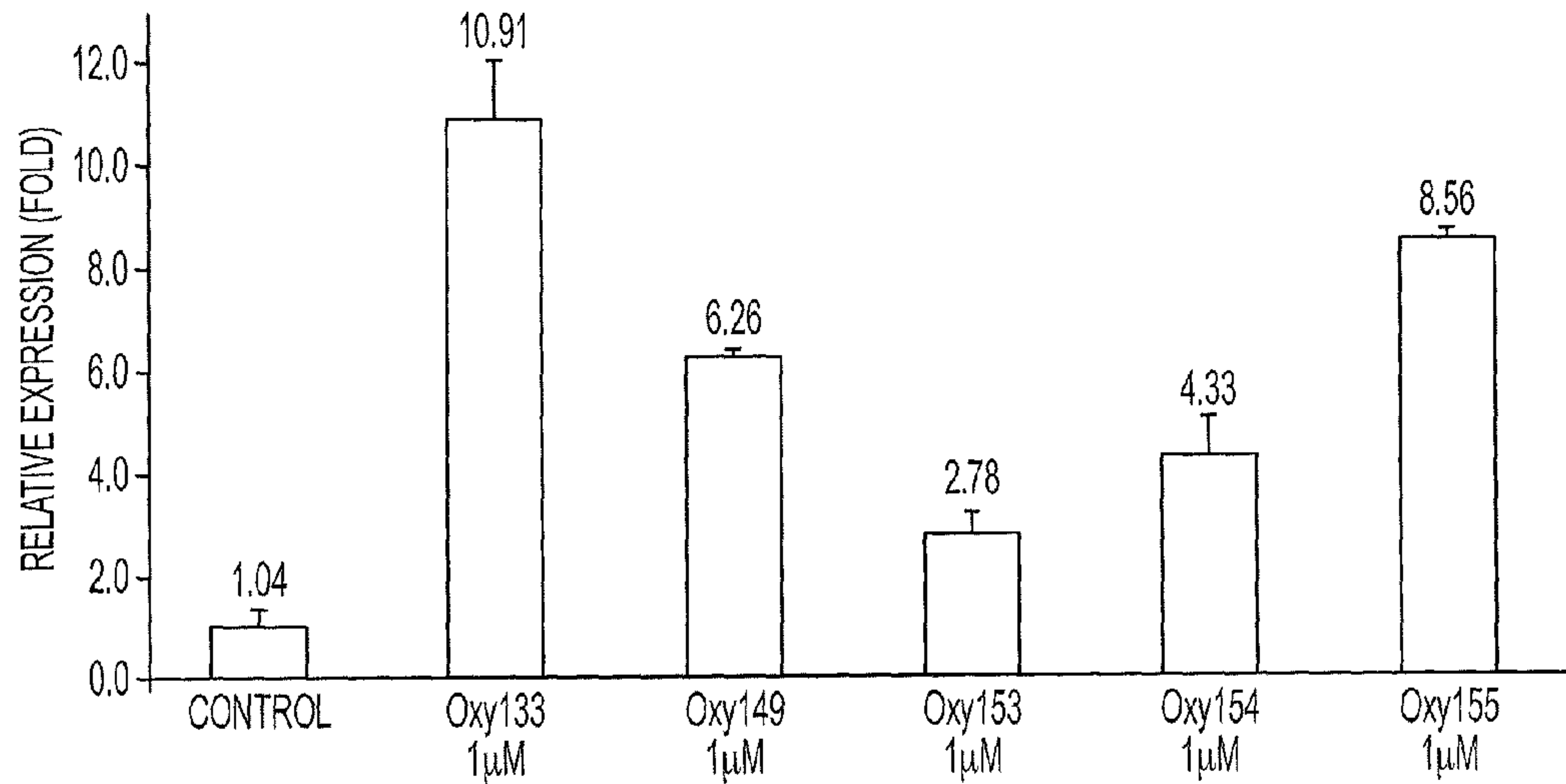


FIG. 7