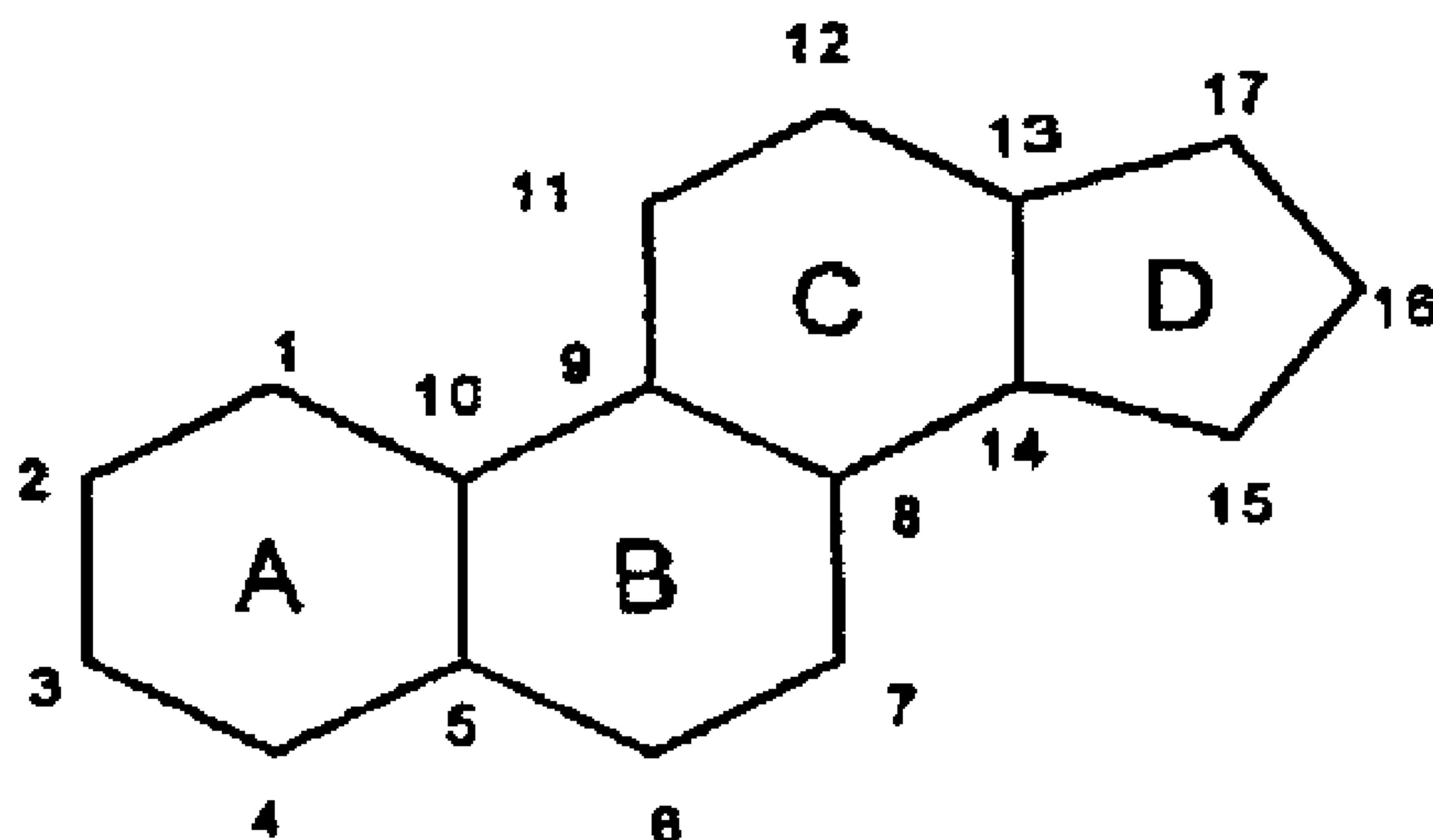




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(54) **Titre : PROCÉDE POUR LA RÉDUCTION ENANTIO-SELECTIVE DE L'OXYDATION, RESPECTIVEMENT, DES STÉROÏDES**
(54) **Title: PROCESS FOR THE ENANTIOSELECTIVE REDUCTION OF OXIDATION, RESPECTIVELY, OF STEROIDS**



(ABCD)

(57) **Abrégé/Abstract:**

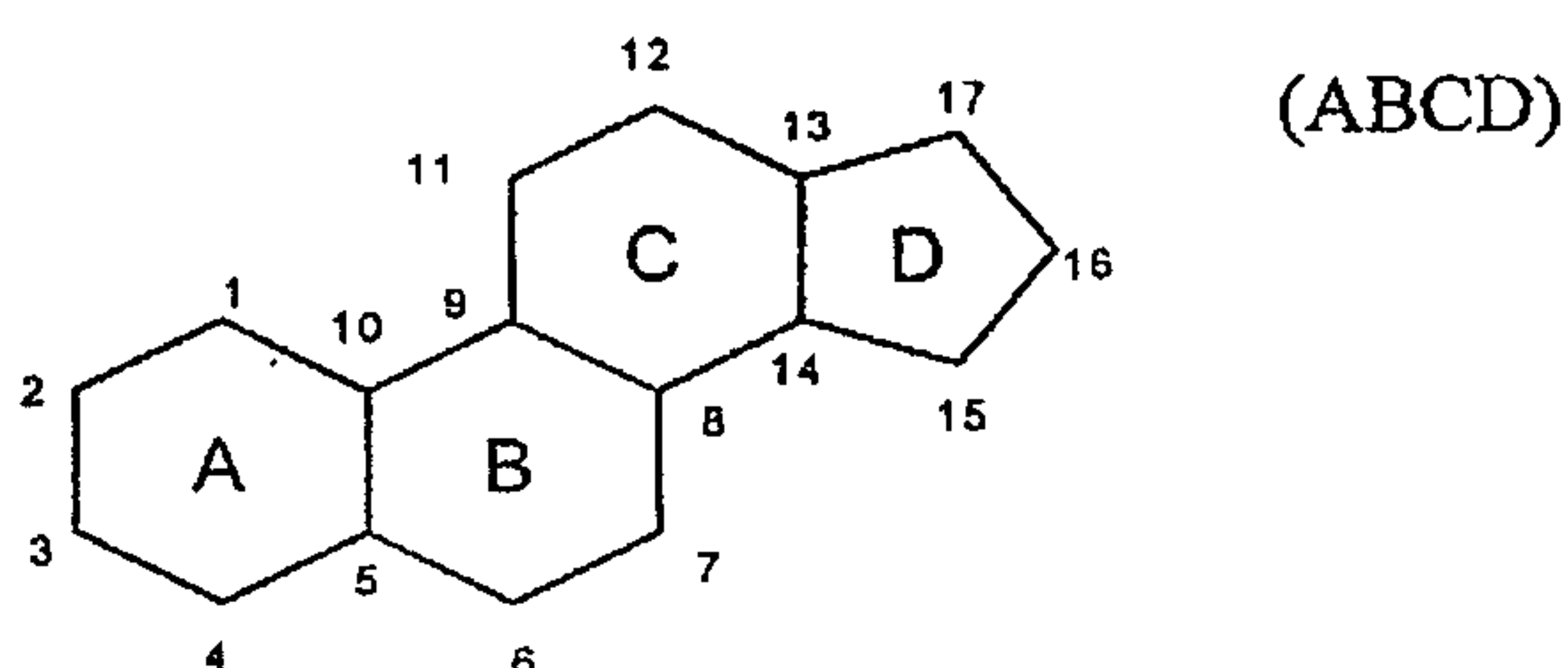
A process for the enantioselective enzymatic reduction of compounds which comprise a steroid structure (ABCD) including one or several heteroatoms, one or several double bonds and/or one aromatic component in the ring structure and have at least one oxo group at position 3, 7, 11, 12 or 17 in the steroid ring system or in the α -position at any carbon moiety on the steroid skeleton (=oxosteroid compound), (see formula above) wherein the oxosteroid compound is reduced with a hydroxysteroid dehydrogenase in the presence of a cofactor NADH or NADPH, is characterized in that a) the oxosteroid compound is provided in the reaction at a concentration of ≥ 50 g/l, b) the oxidized cofactor NAD or NADP formed by the hydroxysteroid dehydrogenase is regenerated continuously by oxidation of a secondary alcohol of general formula R_xR_yCHOH , wherein R_x , R_y independently represent hydrogen, a branched or unbranched C1-C8-alkyl and $C_{total} \geq 3$, or by oxidation of a C4-C6-cycloalkanol, and c) an additional oxidoreductase/alcohol dehydrogenase is used for the oxidation of the secondary alcohol of general formula R_xR_yCHOH or of the cycloalkanol, respectively.



Abstract:

Process for the Enantioselective Reduction of Steroids

A process for the enantioselective enzymatic reduction of compounds which comprise a steroid structure (ABCD) including one or several heteroatoms, one or several double bonds and/or one aromatic component in the ring structure and have at least one oxo group at position 3, 7, 11, 12 or 17 in the steroid ring system or in the α -position at any carbon moiety on the steroid skeleton (=oxosteroid compound),



wherein the oxosteroid compound is reduced with a hydroxysteroid dehydrogenase in the presence of a cofactor NADH or NADPH, is characterized in that

- a) the oxosteroid compound is provided in the reaction at a concentration of ≥ 50 g/l,
- b) the oxidized cofactor NAD or NADP formed by the hydroxysteroid dehydrogenase is regenerated continuously by oxidation of a secondary alcohol of general formula $R_X R_Y \text{CHOH}$, wherein R_X , R_Y independently represent hydrogen, a branched or unbranched C1-C8-alkyl and $C_{\text{total}} \geq 3$, or by oxidation of a C4-C6-cycloalkanol, and
- c) an additional oxidoreductase/alcohol dehydrogenase is used for the oxidation of the secondary alcohol of general formula $R_X R_Y \text{CHOH}$ or of the cycloalkanol, respectively.

Process for the Enantioselective Reduction and Oxidation, respectively, of Steroids

The present invention relates to a process for the enantioselective enzymatic reduction of compounds which comprise a steroid structure (ABCD) including one or several heteroatoms, one or several double bonds and/or one aromatic component in the ring structure and have at least one oxo group at position 3, 7, 11, 12 or 17 in the steroid ring system or in the α -position at any carbon moiety on the steroid skeleton (=oxosteroid compound), wherein the oxosteroid compound is reduced with a hydroxysteroid dehydrogenase in the presence of a cofactor NADH or NADPH.

Furthermore, the present invention relates to a process for the oxidation of compounds which comprise a steroid structure (ABCD) including one or several heteroatoms, one or several double bonds and/or one aromatic component in the ring structure and have at least one hydroxy group at position 3, 7, 11, 12 or 17 in the steroid ring system or in the α -position at any carbon moiety on the steroid skeleton (=hydroxysteroid compound), wherein the hydroxysteroid compound is oxidized with a hydroxysteroid dehydrogenase in the presence of a cofactor NAD or NADP.

Steroids are compounds which possess the ring system of cholesterol and differ in terms of the number of double bonds, the type, number and position of functional groups, the number of methyl groups, the alkyl side chain and the configuration of bonds. Steroid compounds are found both in the animal organism and in fungi and plants and exhibit manifold biological activities, for example, as male and female sex hormones, as hormones of the adrenal gland, as vitamins, as bile acids, as steroid sapogenines, as cardioactive substances and as toad venoms.

By oxosteroid compounds, steroids of the initially defined kind, i.e., those which have at least one keto function, are hereinafter understood, wherein said function can be included in the ring system or also in a side chain located at the steroid skeleton.

By hydroxysteroid compounds, steroids of the initially defined kind, i.e., those which have at least one hydroxy function, are hereinafter understood, wherein said function can be included in the ring system or also in a side chain located at the steroid skeleton.

Because of the manifold physiological effects of steroids it is obvious that steroid compounds and derivatives are also used, in large numbers, in medicine as therapeutically effective substances and medicaments.

For example, progestogen and estrogen derivatives are used worldwide as contraceptives; androgens (testosterone) are used as anabolics and antiandrogen is used, e.g., in the therapy of prostate carcinomas. Glucocorticoids (cortisone, cortisol, prednisolone and prednisone) and derivatives thereof are widely used in the therapeutic treatment of skin diseases, rheumatic diseases, allergic reactions, renal diseases, gastrointestinal diseases and many other disorders due to their antiphlogistic, antiallergic and immunosuppressive effects.

The global market of biologically active steroid compounds is enormous. In the production of various steroid derivatives having different effects, biotransformations play an important role. In particular, reactions catalyzed by hydroxylases and dehydrogenases are thereby of importance. In this connection, delta-1-dehydrogenation, 11 beta-reduction, 20 beta-reduction, 17 beta-reduction, stereoselective reductions at positions 3 and 7 and also oxidations of hydroxy groups, particularly at positions 3, 7, 12 and 17, play specific roles.

Industrially, bioreductions on steroids have so far been implemented exclusively with whole intact cells and at substrate concentrations of far below 10 g/l. First of all, this is due to the fact that the enzymes responsible for biotransformations have so far not been characterized and been expressible, respectively, and, secondly, that no satisfactory technological solution has been available which remedies, on the one hand, the problem of poor solubility of steroids in an aqueous medium and, on the other hand, the problem of regenerating the cofactors NADH and NADPH to a sufficient degree.

Oxidations on steroids have so far been performed chemically in an industrial manner.

Attempts at an enantioselective reduction of steroids with isolated enzymes were substantially described by G. Carrea from 1975 until 1988 (Eur. J. Biochemistry 44, 1974 p. 401-405; Biotechnology and Bioengineering, Vol 17, 1975, p. 1101-1108; Enzyme Microb. Technol. Vol 6, July, 1984, p. 307-311; Biotechnology and Bioengineering, Vol 26, 1984, p. 560-563; J. Org. Chem. 51, 1986, p. 2902-2906; J. Org. Chem. 58, 1993, p. 499-501; J. Org. Chem., 53, 1988, p. 88-92; Enzyme Microb. Technol., Vol 10, June, p. 333-339; Archives of Biochemistry and Biophysics, 159, 1973, p. 7-10).

In doing so, various hydroxysteroid dehydrogenases (HSHD) were used, whereby the regeneration of the cofactor NADH was achieved essentially by coupling with the enzymes lactate dehydrogenase, formate dehydrogenase or also alcohol dehydrogenase from yeast. The regeneration of NADP was effected by means of glucose dehydrogenase. In order to

overcome the solubility problems, experiments were also carried out in a two-phase system with ethyl acetate and butyl acetate as the organic phase. Also with isolated enzymes in the two-phase system, the operation was performed in ranges of concentration of usually far below 10 g/l, whereby the „total turn over numbers“ (TTN = mol of reduced oxosteroid compound / mol of cofactor used) that were achieved were likewise far below 1000, which is why said processes failed to provide a substantial economic advantage in comparison to whole-cell processes.

In addition, there are papers wherein the conversion of hydroxy groups from 7 alpha to 7 beta was accomplished by coupling oxidation and reduction. This was achieved by coupling 7 α HSDH and 7 β HSDH (Pedrini et al., Steroids 71 (2006), p. 189-198). Also in this process, the operation was performed in ranges of concentration of far below 10 g/l and the „total turn over numbers“ (TTN = mol of reduced oxosteroid compound / mol of cofactor used) achieved were below 100, which is why also these processes are economically not relevant.

The invention aims at avoiding said disadvantages and difficulties and has as its object to provide a process which enables the enantioselective reduction and oxidation, respectively, of oxosteroid compounds and hydroxysteroid compounds, respectively, with higher turnovers, in higher ranges of concentration and with higher TTN of the cofactors and hence in a more economical manner.

With a process of the initially mentioned kind, said object is achieved in that

- a) the oxosteroid compound is provided in the reaction at a concentration of ≥ 50 g/l,
- b) the oxidized cofactor NAD or NADP formed by the hydroxysteroid dehydrogenase is regenerated continuously by oxidation of a secondary alcohol of general formula $R_X R_Y \text{CHOH}$, wherein R_X , R_Y independently represent hydrogen, a branched or unbranched C1-C8-alkyl and $C_{\text{total}} \geq 3$, or by oxidation of a C4-C6-cycloalkanol, and
- c) an additional oxidoreductase/alcohol dehydrogenase is used for the oxidation of the secondary alcohol of general formula $R_X R_Y \text{CHOH}$ or of the cycloalkanol, respectively.

In a further process of the initially mentioned kind, said object is achieved in that

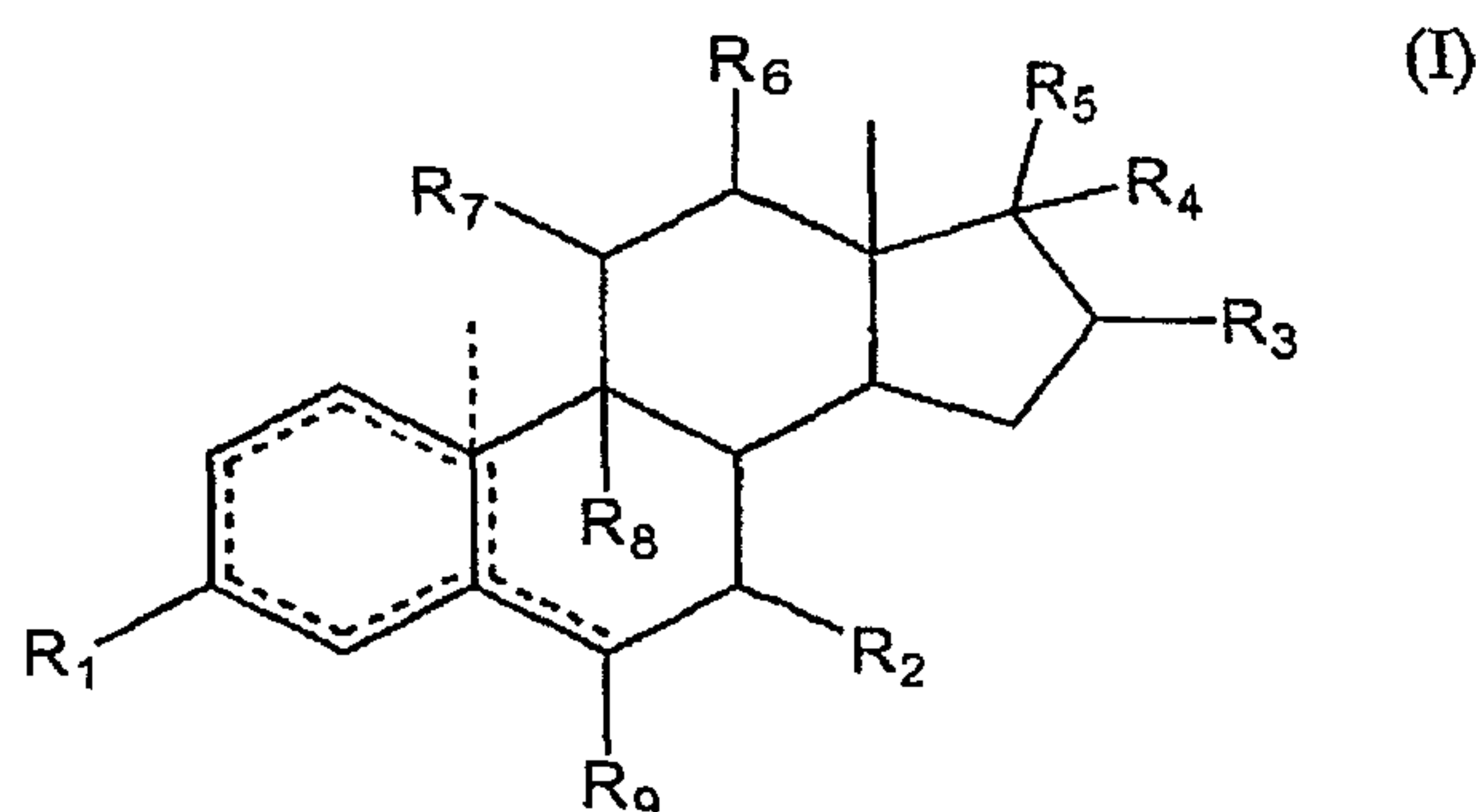
- a) the hydroxysteroid compound is provided in the reaction at a concentration of ≥ 50 g/l,
- b) the reduced cofactor NADH or NADPH formed by the hydroxysteroid dehydrogenase is regenerated continuously by reduction of a keto compound of general

formula $R_X R_Y CO$, wherein R_X , R_Y independently represent hydrogen, a branched or unbranched C1-C8-alkyl and $C_{total} \geq 3$, or by reduction of a C4-C6-cycloalkanone, and
 c) an additional oxidoreductase/alcohol dehydrogenase is used for the reduction of the keto compound of general formula $R_X R_Y CO$ or of the cycloalkanone, respectively.

The present invention constitutes a substantial improvement of the enantioselective enzymatic reduction and oxidation reactions at the steroid skeleton as compared to the prior art. The present invention enables the reduction and oxidation, respectively, of oxosteroid compounds to the corresponding hydroxysteroids with free enzymes in ranges of concentration which by far exceed those described in the prior art.

In the process according to the invention, NADH or NADPH is used as the cofactor. Under the term „NADP“, nicotinamide adenine dinucleotide phosphate is understood, by „NADPH“, reduced nicotinamide adenine dinucleotide phosphate is understood. The term „NAD“ denotes nicotinamide adenine dinucleotide, the term „NADH“ denotes reduced nicotinamide adenine dinucleotide.

According to a preferred embodiment, the process according to the invention is characterized in that a compound of general formula I



is used as the oxosteroid compound,
 wherein

R_1 represents hydrogen, a methyl group, a hydroxy group or an oxo group,
 R_2 represents hydrogen, a methyl group, a hydroxy group or an oxo group,
 R_3 represents hydrogen, a hydroxy group, an oxo group or a methyl group,
 R_4 represents hydrogen or a hydroxy group,

R_5 represents hydrogen, a moiety $-\text{COR}_{10}$, wherein R_{10} is a C1-C4-alkyl group that is unsubstituted or substituted with a hydroxy group or a substituted or unsubstituted C1-C4-carboxyalkyl group,

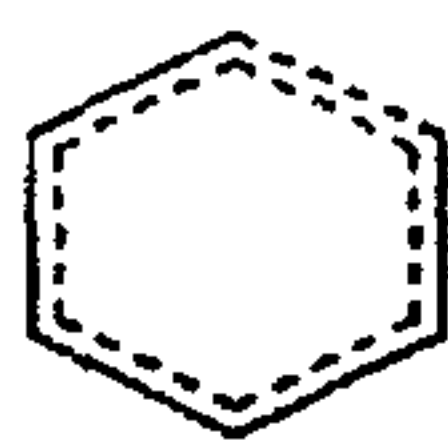
or R_4 and R_5 together represent an oxo group,

R_6 represents hydrogen, a methyl group, a hydroxy group or an oxo group,

R_7 represents hydrogen, a methyl group, a hydroxy group or an oxo group,

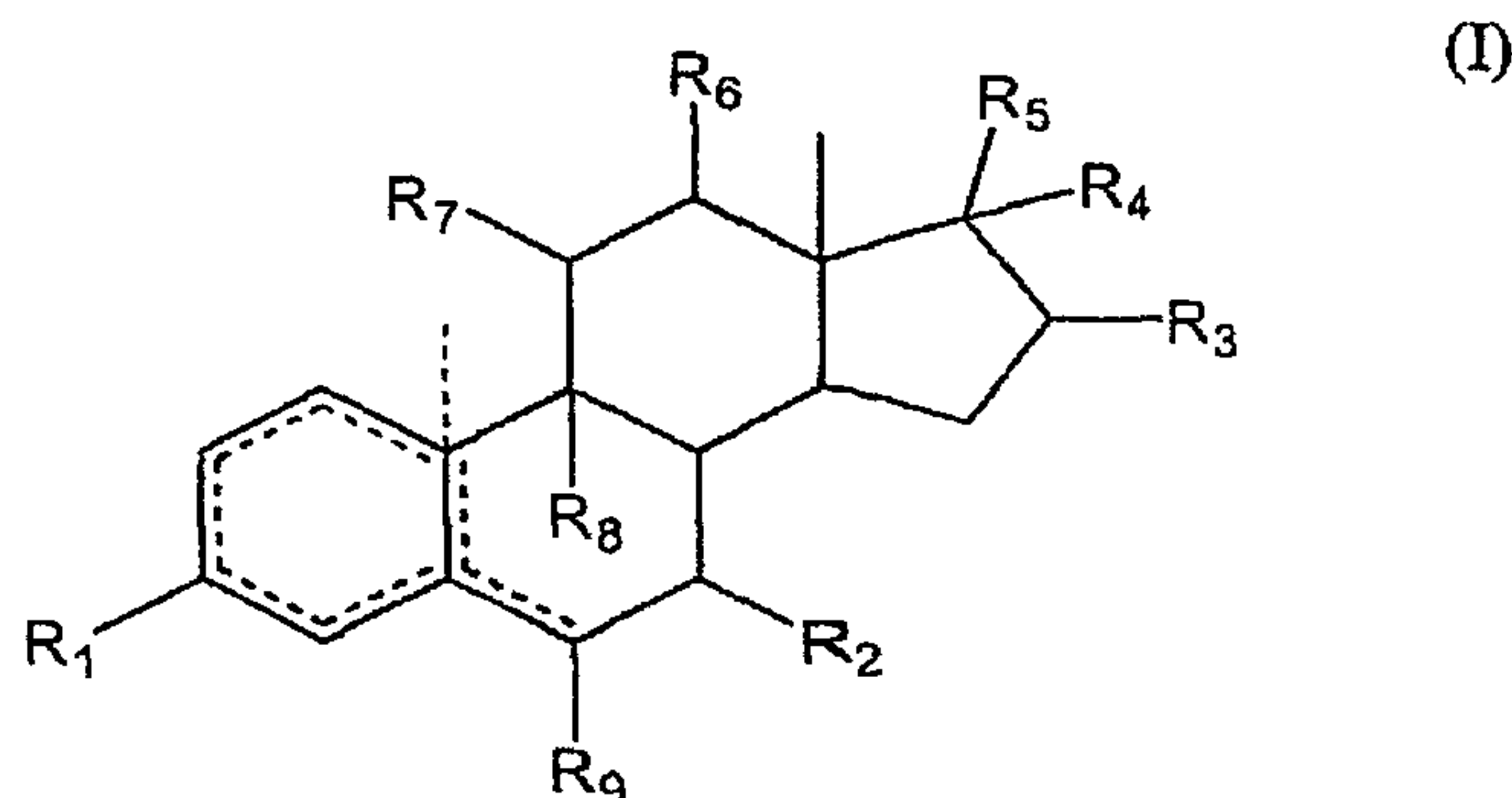
R_8 represents hydrogen, a methyl group or a halide, and

R_9 represents hydrogen, a methyl group, a hydroxy group, an oxo group or a halide, wherein at least one of R_1 , R_2 , R_4+R_5 , R_6 , R_8 or R_9 is an oxo group, or R_5 is a moiety $-\text{COR}_{10}$, respectively, and the structural element



represents a benzene ring or a C6-ring having 0, 1 or 2 C-C-double bonds.

According to a preferred embodiment, the process according to the invention is characterized in that a compound of general formula I



is used as the hydroxysteroid compound,

wherein

R_1 represents hydrogen, a methyl group, a hydroxy group or an oxo group,

R_2 represents hydrogen, a methyl group, an oxo group or a hydroxy group,

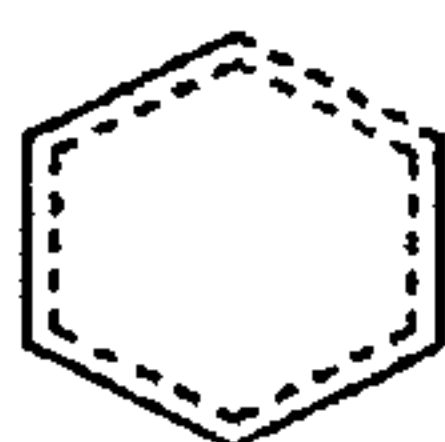
R_3 represents hydrogen, a hydroxy group, an oxo group or a methyl group,

R_4 represents hydrogen or a hydroxy group,

R_5 represents hydrogen, a moiety $-\text{COR}_{10}$, wherein R_{10} is a C1-C4-alkyl group that is unsubstituted or substituted with a hydroxy group or a substituted or unsubstituted C1-C4-carboxyalkyl group,

or R_4 and R_5 together represent an oxo group,

R_6 represents hydrogen, a methyl group, an oxo group or a hydroxy group,
 R_7 represents hydrogen, a methyl group, an oxo group or a hydroxy group,
 R_8 represents hydrogen, a methyl group or a halide, and
 R_9 represents hydrogen, a methyl group, a hydroxy group, an oxo group or a halide,
 wherein at least one of R_1 , R_2 , R_4 , R_6 , R_7 , R_8 or R_9 is a hydroxy group and the structural element



represents a benzene ring or a C6-ring having 0, 1 or 2 C-C-double bonds.

2-Propanol, 2-butanol, 2-pentanol, 4-methyl-2-pentanol, 2-hexanol, 2-heptanol, 5-methyl-2-hexanol or 2-octanol is preferably used as the secondary alcohol of general formula $R_X R_Y \text{CHOH}$, and cyclohexanol is used as the cycloalcohol.

Acetone, 2-butanone, 2-pentanone, 4-methyl-2-pentanone, 2-hexanone, 2-heptanone, 5-methyl-2-hexanone or 2-octanone is preferably used as the ketone of general formula $R_X R_Y \text{CO}$, and cyclohexanone is used as the cycloalkanone.

In the following, the ketone of general formula $R_X R_Y \text{CO}$ or the C4-C6-cycloalkanone, respectively, and the secondary alcohol of general formula $R_X R_Y \text{CHOH}$ or the C4-C6-cycloalkanol, respectively, are summarized under the general term cosubstrate.

The processes according to the invention are preferably carried out in an aqueous organic two-phase system. Thereby, the cosubstrate used for coenzyme regeneration is suitably not miscible with water and thus forms the organic phase of the aqueous organic two-phase system.

According to a further possible embodiment, an organic solvent not involved in the regeneration of the cofactor such as, for example, diethyl ether, tertiary butyl methyl ether, diisopropyl ether, dibutyl ether, ethyl acetate, butyl acetate, heptane, hexane or cyclohexane is additionally employed in the process.

Furthermore, it is preferred that the TTN of the process according to the invention is $\geq 10^3$.

In addition, preferably at least 50% of the employed oxosteroid compound or hydroxysteroid compound, respectively, is reduced to the corresponding hydroxysteroid compound or oxidized to the oxosteroid compound, respectively, within 2 to 96 h.

Preferred embodiments of the process according to the invention are characterized in that, for example, ketolithocholic acid (formula II), dexamethasone (formula III), 4-androstene-3,17-dione (formula IV), 1,4-androstadiene-3,17-dione (formula V), estrone (formula VI), pregnenolone (formula VII) and cortisone (formula VIII) are used as oxosteroid compounds.

Preferred embodiments of the process according to the invention are furthermore characterized in that, for example, various derivatives of bile acid such as cholic acid, chenodeoxycholic acid, 12-oxocholic acid, 3-hydroxy-12-oxocholic acid, ketolithocholic acid, lithocholic acid or also hydrocortisone are used as hydroxysteroid compounds.

By hydroxysteroid dehydrogenases are generally understood those enzymes which are capable of catalyzing the reduction of keto groups to hydroxy groups or the oxidation, respectively, of hydroxy groups to the corresponding keto groups on the steroid skeleton. The oxidation or reduction, respectively, can thereby occur on the ring system of the steroid itself (e.g., 7- α hydroxysteroid dehydrogenases) or also on carbon moieties of the skeletal structure of the steroid (e.g., 20- β hydroxysteroid dehydrogenases).

Suitable hydroxysteroid dehydrogenases for the reduction of oxosteroid compounds are, for example, 3- α hydroxysteroid dehydrogenase (HSDH), 3 β HSDH, 12 α HSDH, 20 β HSDH, 7 α HSDH, 7 β HSDH, 17 β HSDH and 11 β HSDH.

A suitable 3- α hydroxysteroid dehydrogenase is obtainable, for example, from *Pseudomonas testosteroni* (J. Biol. Chem. 276 (13), 9961-9970 (2001)) and can be used for the oxidation of 3- α -hydroxysteroids and for the reduction, respectively, of 3-ketosteroids such as, e.g., 3-keto-bile acids, progesterone, 4-androstene-3,17-dione, 5- α -androstane-3,17-dione etc.

Suitable enzymes having 3- β hydroxysteroid dehydrogenase activity are obtainable, for example, from *Clostridium innocuum* (Applied and Environmental Microbiology, June 1989, p. 1656-1659) or from *Pseudomonas testosteroni* and can be used for the oxidation of 3- β -hydroxysteroids and for the reduction, respectively, of 3-ketosteroids such as, e.g., 3-keto-bile acids, progesterone, 4-androstene-3,17-dione, 5- α -androstane-3,17-dione etc.

A 12 α HSDH is obtainable, for example, from Clostridia (Eur. J. Biochem. 196 (1991) 439-450) and can be used for the oxidation of 12 α -hydroxysteroids (e.g. cholic acid) and for the reduction, respectively, of 12-ketosteroids, such as, e.g., 12-keto-bile acids (12-ketochenodeoxycholic acid, dehydrocholic acid). Enzymes from Clostridia having 12- β

hydroxysteroid dehydrogenase activity are described as well (Biochim. Biophys. Acta 1988 Oct. 14; 962(3): 362-370).

Enzymes having 20- β hydroxysteroid dehydrogenase activity are obtainable, for example, from organisms of the group *Streptomyces* (The Journal of Biological Chemistry, 1977, Vol 252 No 1, Jan 10, 205-211) and can be used for the reduction of cortisone and cortisol derivatives (cortisone, cortisol, cortexolone, progesterone) to the corresponding 20- β -hydroxysteroids (e.g., 20- β -hydroxyprogesterone).

Corresponding enzymes having 20- α hydroxysteroid dehydrogenase activity are obtainable, for example, from Clostridia, in particular from *Clostridium scindens* (Journal of Bacteriology, June 1989, p. 2925-2932), and from *Tetrahymena pyriformis* (Biochem.J. (1994) 297, 195-200). Suitable 7- α hydroxysteroid dehydrogenases are obtainable, among other things, from organisms of the intestinal flora such as, e.g., from Clostridia (*Clostridium absonum*, *Clostridium sordellii*) (Journal of Bacteriology, Aug. 1994, p. 4865-4874), from *Escherichia coli* (Journal of Bacteriology Apr., 1991, p. 2173-2179), from *Bacteroides fragilis* (Current Microbiology, Vol 47 (2003) 475-484), *Brucella*, *Eubacterium* and can be used for the oxidation of 7- α -hydroxysteroids (chenolithocholic acid) and for the reduction, respectively, of 7-ketosteroids such as, e.g., 7-keto-bile acids (ketolithocholic acid).

Corresponding enzymes having 7- β hydroxysteroid dehydrogenase activity are likewise described to be obtainable from Clostridia, from microorganisms of the family of ruminococci (J. Biochemistry 102, 1987, p. 613-619) or peptostreptococci, respectively (Biochimica and Biophysica Acta 1004, 1989, p. 230-238), from *Eubacterium aerofaciens* (Applied and Environmental Microbiology, May 1982, p. 1057-1063) and from *Xanthomonas maltophilia* (Pedrini et al, Steroids 71 (2006) p. 189-198). By means of 7- β HSDH, ursodeoxycholic acid can, for example, be produced from ketolithocholic acid.

17- β hydroxysteroid dehydrogenases are known from fungi such as *Cylindrocarpon radicola* (J. Biochemistry 103, 1988, 1039-1044) and *Cochliobolus lunatus* (J. Steroid Biochem. Molec.Biol. Vol 59, 1996, No. 2, p. 205-214), from bacteria of the family of Streptomyces (Hoppe-Seyler's Z. Physiol. Chem, Vol. 356, 1975, 1843-1852), Pseudomonas (The Journal of Biological Chemistry, Vol. 252 No.11, June 10, 1977, p. 3775-3783) and Alcaligenes (The Journal of Biological Chemistry, Vol. 260, No. 25, Nov 5, 1985, p. 13648-13655).

17- β hydroxysteroid dehydrogenases can, for example, be used for the oxidation of 17- β -hydroxysteroids and for the reduction, respectively, of 17-ketosteroids such as, e.g., 4-androstene-3,17-dione, androsterone, estrone.

A corresponding enzyme having 17- α hydroxysteroid dehydrogenase activity is described to be obtainable from *Eubacterium* sp. (Journal of Lipid Research, Vol. 35, 1994, p. 922-929).

11- β hydroxysteroid dehydrogenases are known from higher mammals and can be used, for example, for oxidizing cortisol to cortisone.

However, any other oxidoreductase which catalyzes oxidations and reductions, respectively, on the steroid skeleton can also be used as the hydroxysteroid dehydrogenase.

Suitable secondary alcohol dehydrogenases for regenerating the NADH or NAD, respectively, when using, e.g., 17- β hydroxysteroid dehydrogenases from *Pseudomonas testosteroni*, 3- β hydroxysteroid dehydrogenases from *Clostridium innocuum* or 7- α hydroxysteroid dehydrogenase from *Bacteroides fragilis*, are, for example, those as described above and are isolated from yeasts of the genera *Candida* and *Pichia* such as, e.g.: Carbonyl reductase from *Candida parapsilosis* (CPCR) (US 5,523,223 and US 5,763,236; Enzyme Microb. Technol. 1993 Nov; 15(11):950-8), *Pichia capsulata* (DE 10327454.4), *Pichia farinosa* (A 1261/2005, Kl. C12N), *Pichia finlandica* (EP 1179595 A1), *Candida nemodendra* (A 1261/2005, Kl. C12N), *Pichia trehalophila* (A 1261/2005, Kl. C12N), *Rhodotorula mucilaginosa* (A 1261/2005, Kl. C12N), *Lodderomyces elongisporus* (A 1261/2005, Kl. C12N) *Pichia stipidis* (A 1261/2005, Kl. C12N)

Furthermore, the regeneration of NADH can also be effected with secondary alcohol dehydrogenases/oxidoreductase as described above and isolated from bacteria of the class of actinobacteria, e.g., from *Rhodococcus erythropolis* (US 5,523,223), *Norcardia fusca* (Biosci. Biotechnol. Biochem., 63 (10) (1999), pp. 1721-1729; Appl. Microbiol. Biotechnol. 2003 Sep; 62(4):380-6, Epub 2003 Apr 26), *Rhodococcus ruber* (J. Org. Chem. 2003 Jan 24; 68(2):402-6.) or *Microbacterium spec.* (A 1261/2005, Kl. C12N).

Suitable secondary alcohol dehydrogenases/oxidoreductases for regenerating the NADPH or NADP, respectively, when using, e.g., 12- α hydroxysteroid dehydrogenases from *Clostridium paraputrificum*, 17- α hydroxysteroid dehydrogenases from *Eubacterium* sp. or 7- α hydroxysteroid dehydrogenase from *Clostridium sordelli*, are, for example, those as

described above and isolated from organisms of the order of Lactobacillales (*Lactobacillus kefir* (US 5,200,335), *Lactobacillus brevis* (DE 19610984 A1; Acta Crystallogr. D. Biol. Crystallogr. 2000 Dec; 56 Pt 12:1696-8), *Lactobacillus minor* (DE 10119274), *Leuconostoc carnosum* (A 1261/2005, Kl. C12N) or are those as described from *Thermoanaerobium brockii*, *Thermoanaerobium ethanolicus* or *Clostridium beijerinckii*.

Both enzymes, hydroxysteroid dehydrogenase and oxidoreductase/alcohol dehydrogenase, are preferably used in a state of being recombinantly overexpressed in *Escherichia coli*. In the process according to the invention, both enzymes, hydroxysteroid dehydrogenase and alcohol dehydrogenase/oxidoreductase, can be used either in a completely purified state, in a partially purified state or in a state of being included in cells. The cells used can thereby be present in the native, in a permeabilized or in a lysed state.

Per kg of oxosteroid compound and hydroxysteroid compound, respectively, to be converted, 50 000 to 10 Mio U of hydroxysteroid dehydrogenase and 50 000 to 10 Mio U of alcohol dehydrogenase are used (no upper limit).

The enzyme unit 1 U thereby corresponds to the enzyme amount of hydroxysteroid dehydrogenase which is required for converting 1 μmol of oxosteroid compound per minute (min), or to the enzyme amount of alcohol dehydrogenase, respectively, which is required for oxidizing 1 μmol 2-alcohol per minute (min).

Analogically, per kg of oxosteroid compound or hydroxysteroid compound, respectively, to be converted, approx. 10 g to 500 g biological wet mass of *E. coli* containing the hydroxysteroid dehydrogenase and 10 g to 500 g biological wet mass of *E. coli* containing the alcohol dehydrogenase/oxidoreductases can be used (no upper limit).

In the described process, the regeneration of NAD(P)H is effected in an enzyme-coupled manner.

In the process according to the invention, the conversion of the oxosteroid compound or hydroxysteroid compound, respectively, preferably occurs in the two-phase system containing a 2-alcohol or a keto compound, respectively, for cofactor regeneration, a hydroxysteroid dehydrogenase, an alcohol dehydrogenase, water, cofactor and the oxosteroid compound or hydroxysteroid compound, respectively. Furthermore, additional organic solvents can also be included which are not involved in the cofactor regeneration, i.e., which do not contain any hydroxy groups oxidizable by the alcohol dehydrogenase used or any keto group, respectively, reducible by the same.

The portion of organic components not miscible with water in the two-phase system can range between 10% and 90%, preferably from 20% to 80%, based on the total volume of the reaction. The aqueous portion can range from 90% to 10%, preferably from 80% to 20%, based on the total volume of the reaction batch.

A buffer can be added to the water, for example, a potassium phosphate, tris/HCl, glycine or triethanolamine buffer having a pH value of from 5 to 10, preferably from 6 to 9. In addition, the buffer can comprise ions for the stabilization or activation of both enzymes, for instance, magnesium ions or zinc ions.

Additionally, further additives for stabilizing the enzymes hydroxysteroid dehydrogenase and alcohol dehydrogenase can also be used in the process according to the invention, such as, for example, glycerol, sorbitol, 1,4-DL-dithiothreitol (DTT) or dimethyl sulfoxide (DMSO).

The concentration of the cofactor NAD(P)H, based on the aqueous phase, ranges from 0.001 mM to 10 mM, in particular from 0.01 mM to 1.0 mM. Depending on the specific properties of the enzymes used, the temperature can range from 10°C to 70°C, preferably from 20°C to 35°C.

The TTN (total turn over number = mol of reduced oxosteroid compound / mol of cofactor used) achieved in the process according to the invention can thereby lie in the range of 10^2 to 10^5 , normally, a TTN $>10^3$ is preferred.

Usually, the oxosteroid compounds to be reduced and hydroxysteroid compounds, respectively, are poorly soluble in water. During the reaction, the substrate can be present in a completely or incompletely dissolved state. If the substrate is not completely dissolved in the reaction mixture, a portion of the substrate is provided in solid form and may thus form a third solid phase. During the conversion, the reaction mixture may also temporarily form an emulsion. In the process according to the invention, the oxosteroid compound to be reduced and the hydroxysteroid compounds to be oxidized, respectively, are used in amounts of ≥ 50 g/l, based on the total volume of the reaction batch. Preferably, between 50g/l and 400g/l of oxosteroid compound/hydroxysteroid compounds, particularly preferably between 50g/l and 200g/l, are used.

The preferred additional organic solvents not involved in the regeneration of the cofactor are, for example, ethyl acetate, butyl acetate, tertiary butyl methyl ether, diisopropyl ether,

heptane, hexane, toluene, dichloromethane or cyclohexane or mixtures thereof of different compositions.

The regeneration of NAD(P)H is achieved by oxidation of secondary alcohols of general formula R_XR_YCHOH or of C4-C6-cycloalkanols, respectively. In doing so, ketones of general formula $R_XR_YC=O$ or C4-C6-cycloalkanones, respectively, are formed as reaction products. Preferred secondary alcohols are aliphatic 2-alcohols such as, e.g., isopropanol, 2-butanol, 2-pentanol, 2-hexanol, 2-heptanol, 2-octanol, 4-methyl-2-pentanol, 5-methyl-2-hexanol, but also cyclic secondary alcohols such as cyclohexanol, cyclopentanol. In principle, the use of diols such as, e.g., 1,4-cyclohexanediol, is conceivable as well.

The regeneration of NAD(P) is achieved by reduction of keto compounds of general formula $R_XR_YC=O$ or of C4-C6-cycloalkanones, respectively. In doing so, secondary alcohols of general formula R_XR_YCHOH or C4-C6-cycloalkanols, respectively, are formed as reaction products. Preferred keto compounds are ketones such as, e.g., acetone, 2-butanone, 2-pentanone, 2-hexanone, 2-heptanone, 2-octanone, 4-methyl-2-pentanone, 5-methyl-2-hexanone but also cyclic ketones such as cyclohexanone, cyclopentanone. In principle, the use of diones such as, e.g., 1,4-cyclohexanedione, is conceivable as well.

The process according to the invention is carried out, for example, in a reaction vessel made of glass or metal. For this purpose, the components are transferred individually into the reaction vessel and stirred under an atmosphere of, e.g., nitrogen or air. Depending on the oxosteroid compound that is used, the reaction time ranges from 1 hour to 96 hours, in particular from 2 hours to 48 hours. In this period, the oxosteroid compound is reduced to the corresponding hydroxysteroid by at least 50%, or the hydroxysteroid is oxidized to the oxosteroid compound by at least 50%, respectively.

Below, the present invention is illustrated in further detail by way of examples.

Examples:

1. Reduction of androstene-3,17-dione to 17- β -hydroxy-4-androstene-3-one (testosterone)

A) Two-phase system with 4-methyl-2-pentanol for coenzyme regeneration

For the synthesis of 17 P-hydroxy-4-androstene-3-one (testosterone), 100 mg androstene-3,17-dione dissolved in 0.4 ml 4-methyl-2-pentanol are added to 0.5 ml of a buffer (100 mM triethanolamine, pH = 7, 1 mM MgCl₂, 10% glycerol) containing 0.1 mg NAD, 30 units of recombinant 17-(3-hydroxysteroid dehydrogenase from *Pseudomonas testosteroni* (1. Steroid Biochem. Mol. Biol. 44 (2), 133-139 (1993), Pubmed P19871) and 50 units of recombinant alcohol dehydrogenase from *Pichia capsulata* (DE-A 103 27 454). The mixture is incubated at room temperature for 24 h under constant mixing. The concentration of androstene-3,17-dione in the total reaction volume amounts to approx. 100g/l.

Upon completion of the reaction, the reaction mixture can, for example, be processed by extracting the reaction mixture with an organic solvent and subsequently removing the solvent via distillation.

After 24 h, approx. 94% of the androstene-3,17-dione used has been converted to 17-P-hydroxy-4-androstene-3-one (testosterone).

The conversion of androstene-3,17-dione to 17-13-hydroxy-4-androstene-3-one (testosterone) was monitored by gas chromatography. For this purpose, a gas chromatograph GC-17A of ShimadzuTM was used with a chiral separating column LipodexTM E, 12m (Machery-Nagel, Ditre, Germany), a flame ionization detector and helium as a carrier gas.

B) Two-phase system with butyl acetate and 2-propanol for coenzyme regeneration

For the synthesis of 17-P-hydroxy-4-androstene-3-one (testosterone), 100 mg androstene-3,17-dione dissolved in 0.3 ml butyl acetate and 0.1 ml 2-propanol are added to 0.5 ml of a buffer (100 mM triethanolamine, pH = 7, 1 mM MgCl₂, 10% glycerol) containing 0.1 mg NAD, 30 units of recombinant 17-P-hydroxysteroid dehydrogenase from *Pseudomonas testosteroni* (1. Steroid Biochem. Mol. Biol. 44 (2), 133-139 (1993), Pubmed P19871) and 50 units of recombinant alcohol dehydrogenase from *Pichia capsulata* (DE-A 103 27 454). The mixture is incubated at room temperature for 24 h under constant mixing. The concentration of androstene-3,17-dione in the total reaction volume amounts to approx. 100g/l.

Upon completion of the reaction, the reaction mixture can, for example, be processed by extracting the reaction mixture with an organic solvent and subsequently removing the solvent via distillation.

After 24 h, approx. 90-95% of the androstene-3,17-dione used has been converted to 17-n-hydroxy-4-androstene-3-one (testosterone).

2. Reduction of ketolithocholic acid to chenolithocholic acid

A) Coenzyme regeneration with ADH from *Thermoanaerobium brockii* / two-phase system

For the synthesis of 3a-7a-dihydroxy-5-13-cholanic acid (chenodeoxycholic acid), 100 mg 3a-hydroxy-7-oxo-5-p-cholanic acid (ketolithocholic acid) in 0.5 ml methylpentanol are added to 0.2 ml of a buffer (100 mM potassium phosphate buffer, pH = 8.5, 1 mM MgCl₂, 10% glycerol) containing 0.1 mg NADP, 10 units of recombinant 7-a-hydroxysteroid dehydrogenase from *Clostridium scindens* (Pubmed AAB61151) and 10 units of recombinant alcohol dehydrogenase from *Thermoanaerobium brockii*. The mixture is incubated at room temperature for 24 h under constant mixing. The concentration of ketolithocholic acid in the total reaction volume amounts to approx. 100g/l.

After 24 h, approx. 90-98% of the ketolithocholic acid used has been converted to chenodeoxycholic acid.

The conversion of ketolithocholic acid to chenodeoxycholic acid was monitored by HPLC. For this purpose, a separating column EC125/4 NucleodurTM 100-5 C18ec (Machery-Nagel, Diiren, Germany) was used isocratically with a mixture of MeOH/water (80:20).

13) Coenzyme regeneration with ADH from *Lactobacillus* (DE 10119274) / two-phase system

For the synthesis of 3a-7a-dihydroxy-5-13-cholanic acid (chenodeoxycholic acid), 100 mg 3a-hydroxy-7-oxo-5-13-cholanic acid (ketolithocholic acid) in 0.5 ml octanol are added, for example, to 0.3 ml of a buffer (100 mM triethanolamine buffer, pH = 7, 1 mM MgCl₂, 10% glycerol) containing 0.1 mg NADP, 10 units of recombinant 7-a-hydroxysteroid dehydrogenase from *Clostridium scindens* (Pubmed AAB61151) and 10 units of recombinant alcohol dehydrogenase from *Lactobacillus* (DE 10119274). The mixture is incubated at room temperature for 24 h under constant mixing. The concentration of ketolithocholic acid in the total reaction volume amounts to approx. 100g/l.

After 24 h, approx. 70-80% of the ketolithocholic acid used has been converted to chenodeoxycholic acid.

3. Reduction of 1,4-androstadiene-3,17-dione to 17-13-hydroxy-1,4-androstadiene-3-one

For the synthesis of 17-13-hydroxy-1,4-androstadiene-3-one, 100 mg 1,4-androstadiene-3,17-dione dissolved in 0.4 ml 4-methyl-2-pentanol are added to 0.5 ml of a buffer (100 mM triethanolamine, pH = 7, 1 mM MgCl₂, 10% glycerol) containing 0.1 mg NAD, 30 units of recombinant 17-13-hydroxysteroid dehydrogenase from *Pseudomonas testosteroni* (J. Steroid Biochem. Mol. Biol. 44 (2), 133-139 (1993), Pubmed P19871) and 5 units of recombinant alcohol dehydrogenase from *Pichia capsulate* (DE-A 103 27 454). The mixture is incubated at room temperature for 24 h under constant mixing. The concentration of 1,4-androstadiene-3,17-dione in the total reaction volume amounts to approx. 100g/l.

Upon completion of the reaction, the reaction mixture can, for example, be processed by separating the organic phase and subsequently removing the solvent via distillation.

After 24 h, approx. 90-98% of the androstene-3,17-dione used has been converted to 17-13-hydroxy-1,4-androstadiene-3-one.

The conversion of 1,4-androstadiene-3,17-dione to 17-13-hydroxy-1,4-androstadiene-3-one was monitored by gas chromatography. For this purpose, a gas chromatograph AutosystemTM XL of Perkin ElmerTM equipped with a mass spectrometer was used with an FS-capillary column Optima —5-MS (Machery-Nagel, Diiren, Germany) and helium as a carrier gas.

4. Oxidation of chenodeoxycholic acid to ketolithocholic acid

A) Coenzyme regeneration with ADH from *Thermoanaerobium brockii* / two-phase system

For the synthesis of 3a-hydroxy-7-oxo-5-13-cholanic acid (ketolithocholic acid), 100 mg 3a-7a-dihydroxy-5-3-cholanic-acid (chenodeoxycholic acid) in 0.5 ml methyl isobutyl ketone are added to 0.4 ml of a buffer (100 mM potassium phosphate buffer, pH = 8.5, 1 mM MgCl₂, 10% glycerol) containing 0.1 mg NADP, 10 mg biological wet mass E. coli containing the overexpressed 7-a-hydroxysteroid dehydrogenase from *Clostridium scindens* (Pubmed AAB61151) and 5 mg biological wet mass E. coli containing the overexpressed

alcohol dehydrogenase from *Thermoanaerobium Brockii*. The mixture is incubated at room temperature for 24 h under constant mixing. The concentration of ketolithocholic acid in the total reaction volume amounts to approx. 100g/l.

After 24 h, more than 80% of the chenodeoxycholic acid used has been converted to ketolithocholic acid.

The conversion of ketolithocholic acid to chenodeoxycholic acid was monitored by thin-layer chromatography.

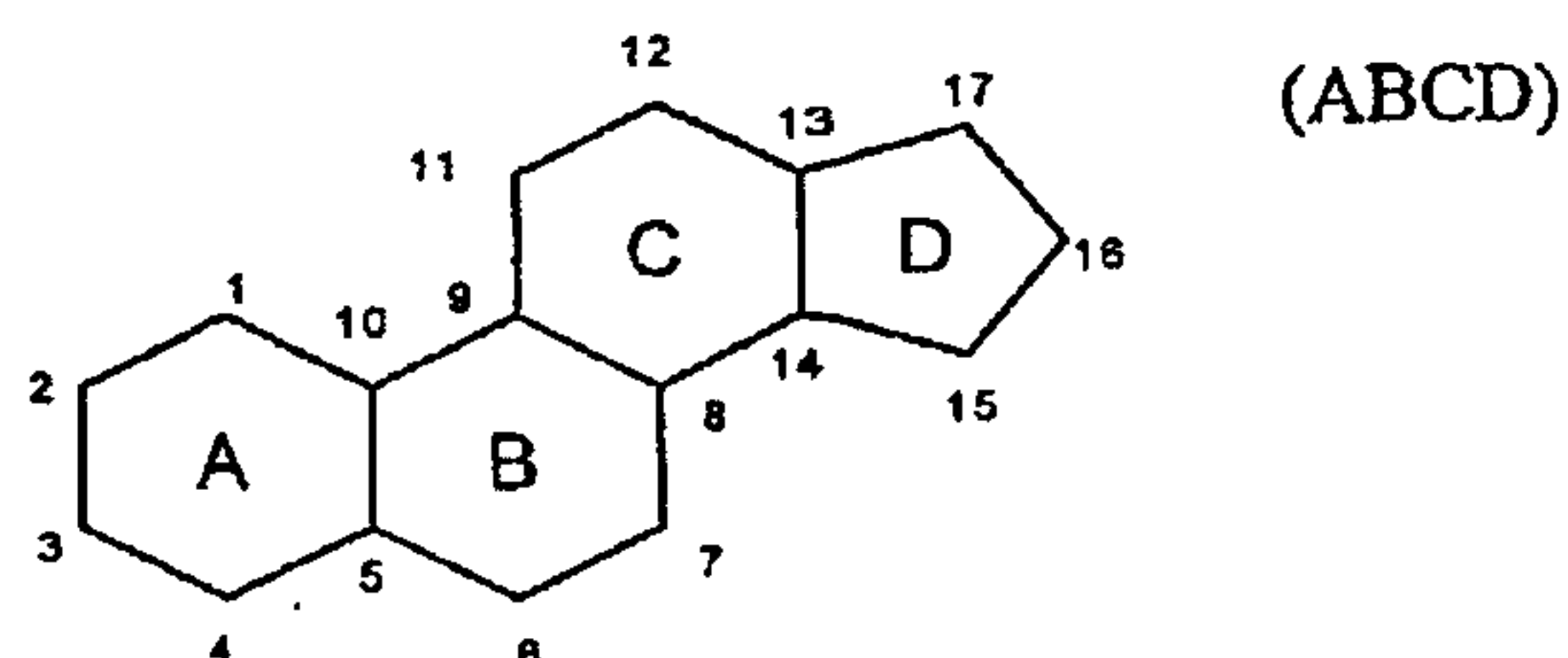
B) Coenzyme regeneration with ADH from Lactobacillus (DE 10119274) / two-phase system

For the synthesis of 3 α -hydroxy-7-oxo-5- β -cholan-3-ic acid (ketolithocholic acid), 100 mg 3 α -7 α -dihydroxy-5- β -cholan-3-ic acid (chenodeoxycholic acid) in 0.7 ml methyl isobutyl ketone are added to 0.15 ml of a buffer (100 mM potassium phosphate buffer, pH = 7.5, 1 mM MgCl₂, 10% glycerol) containing 0.1 mg NADP, 10 mg biological wet mass *E. coli* containing the overexpressed 7- α -hydroxysteroid dehydrogenase from *Clostridium scindens* (Pubmed AAB61151) and 5 mg biological wet mass *E. coli* containing the overexpressed alcohol dehydrogenase from Lactobacillus (DE 10119274). The mixture is incubated at room temperature for 24 h under constant mixing. The concentration of ketolithocholic acid in the total reaction volume amounts to approx. 100g/l.

After 24 h, more than 90% of the chenodeoxycholic acid used has been converted to ketolithocholic acid.

Claims:

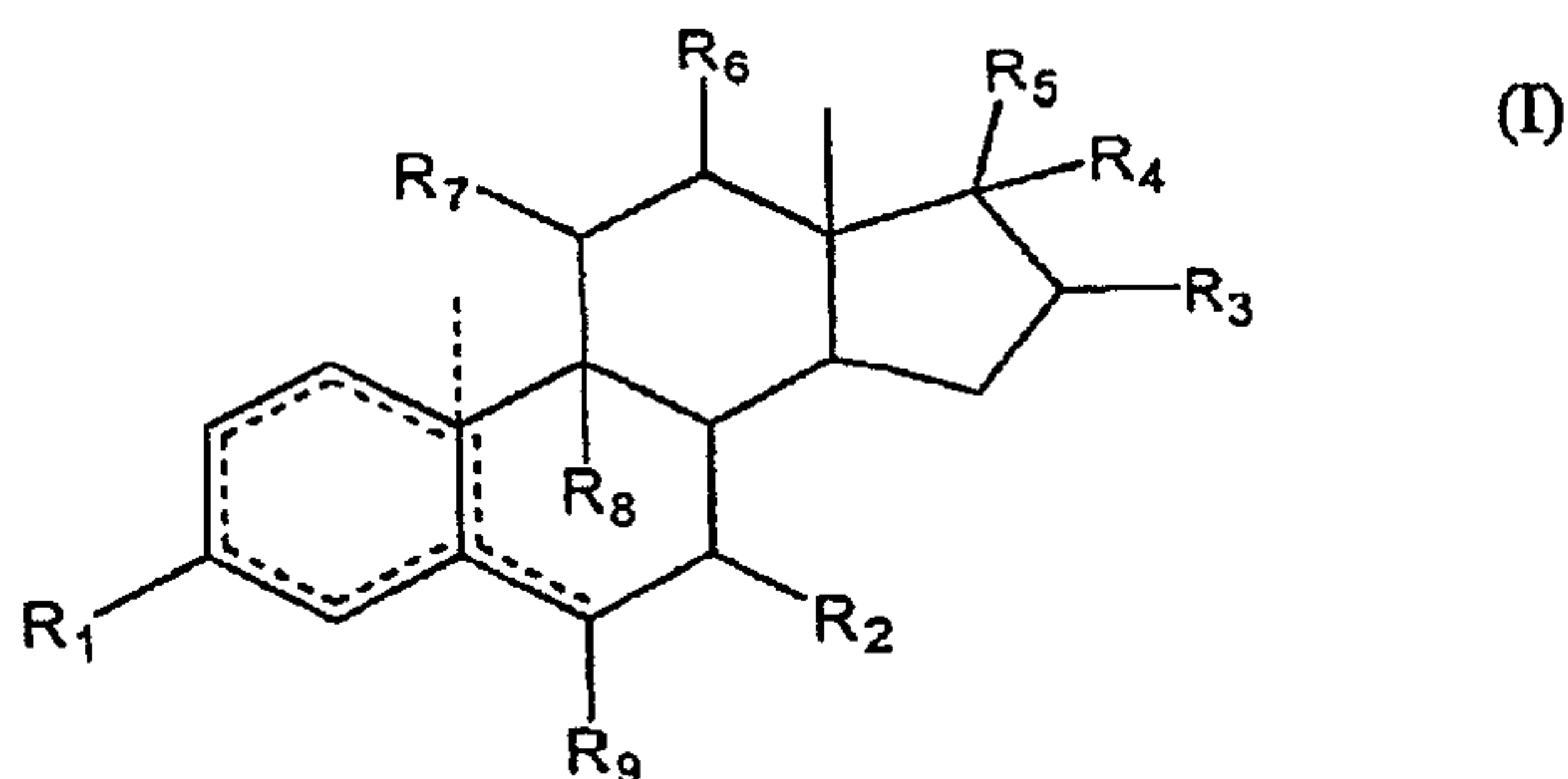
1. A process for the enantioselective enzymatic reduction of compounds which comprise a steroid structure (ABCD) including one or several heteroatoms, one or several double bonds and/or one aromatic component in the ring structure and have at least one oxo group at position 3, 7, 11, 12 or 17 in the steroid ring system or in the α -position at any carbon moiety on the steroid skeleton (=oxosteroid compound),



wherein the oxosteroid compound is reduced with a hydroxysteroid dehydrogenase in the presence of a cofactor NADH or NADPH, characterized in that

- a) the oxosteroid compound is provided in the reaction at a concentration of ≥ 50 g/l,
- b) the oxidized cofactor NAD or NADP formed by the hydroxysteroid dehydrogenase is regenerated continuously by oxidation of a secondary alcohol of general formula $R_X R_Y \text{CHOH}$, wherein R_X , R_Y independently represent hydrogen, a branched or unbranched C1-C8-alkyl and $C_{\text{total}} \geq 3$, or by oxidation of a C4-C6-cycloalkanol, and
- c) an oxidoreductase/alcohol dehydrogenase is used for the oxidation of the secondary alcohol of general formula $R_X R_Y \text{CHOH}$ or of the cycloalkanol, respectively.

2. A process according to claim 1, characterized in that a compound of general formula I



is used as the oxosteroid compound,

wherein

R₁ represents hydrogen, a methyl group, a hydroxy group or an oxo group,

R₂ represents hydrogen, a methyl group, a hydroxy group or an oxo group,

R₃ represents hydrogen, a hydroxy group, an oxo group or a methyl group,

R₄ represents hydrogen or a hydroxy group,

R₅ represents hydrogen, a moiety -COR₁₀, wherein R₁₀ is a C1-C4-alkyl group that is unsubstituted or substituted with a hydroxy group or a substituted or unsubstituted C1-C4-carboxyalkyl group,

or R₄ and R₅ together represent an oxo group,

R₆ represents hydrogen, a methyl group, a hydroxy group or an oxo group,

R₇ represents hydrogen, a methyl group, a hydroxy group or an oxo group,

R₈ represents hydrogen, a methyl group or a halide, and

R₉ represents hydrogen, a methyl group, a hydroxy group, an oxo group or a halide,

wherein at least one of R₁, R₂, R₄+R₅, R₆, R₈ or R₉ is an oxo group, or R₅ is a moiety -COR₁₀, respectively, and the structural element



represents a benzene ring or a C₆-ring having 0, 1 or 2 C-C-double bonds.

3. A process according to claim 1 or 2, characterized in that 2-propanol, 2-butanol, 2-pentanol, 4-methyl-2-pentanol, 2-hexanol, 2-heptanol, 5-methyl-2-hexanol or 2-octanol is used as the secondary alcohol of general formula R_xR_yCHOH, and cyclohexanol is used as the cycloalcohol.

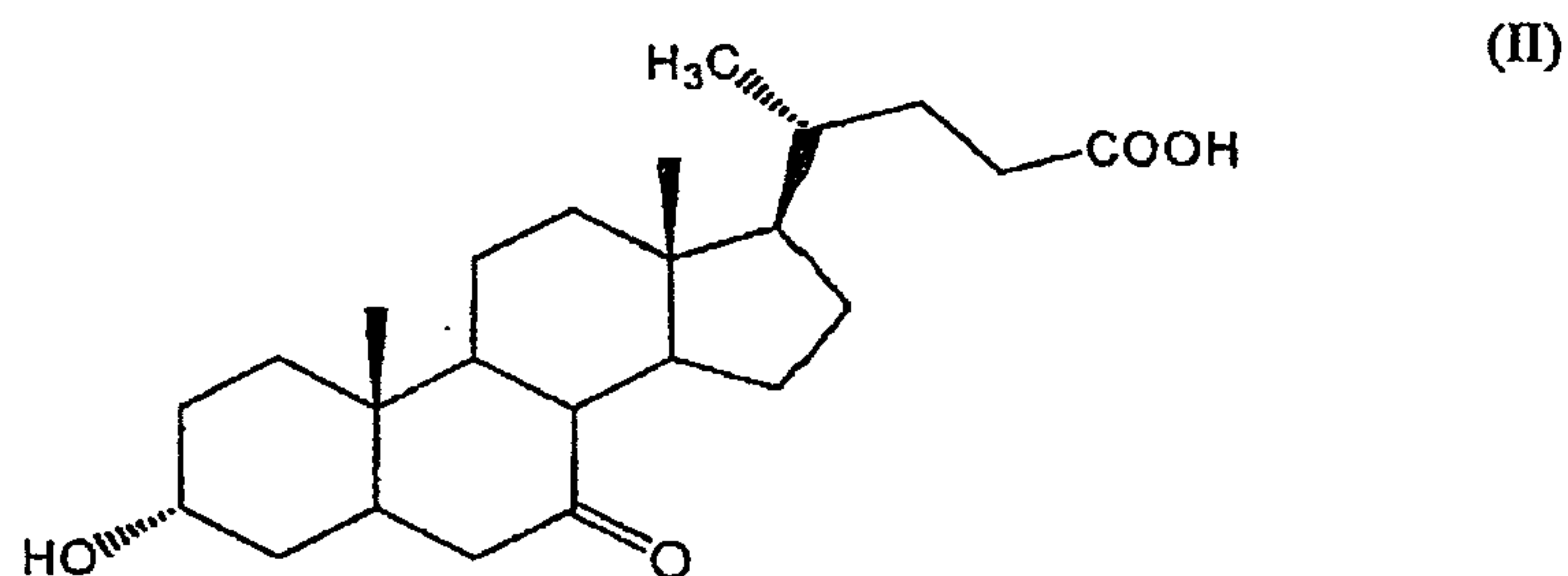
4. A process according to any of claims 1 to 3, characterized in that it is carried out in an aqueous organic two-phase system.

5. A process according to claim 4, characterized in that an organic solvent
diethyl ether, tertiary butyl methyl ether, diisopropyl ether, dibutyl ether, ethyl
acetate, butyl acetate, heptane, hexane or cyclohexane is employed.

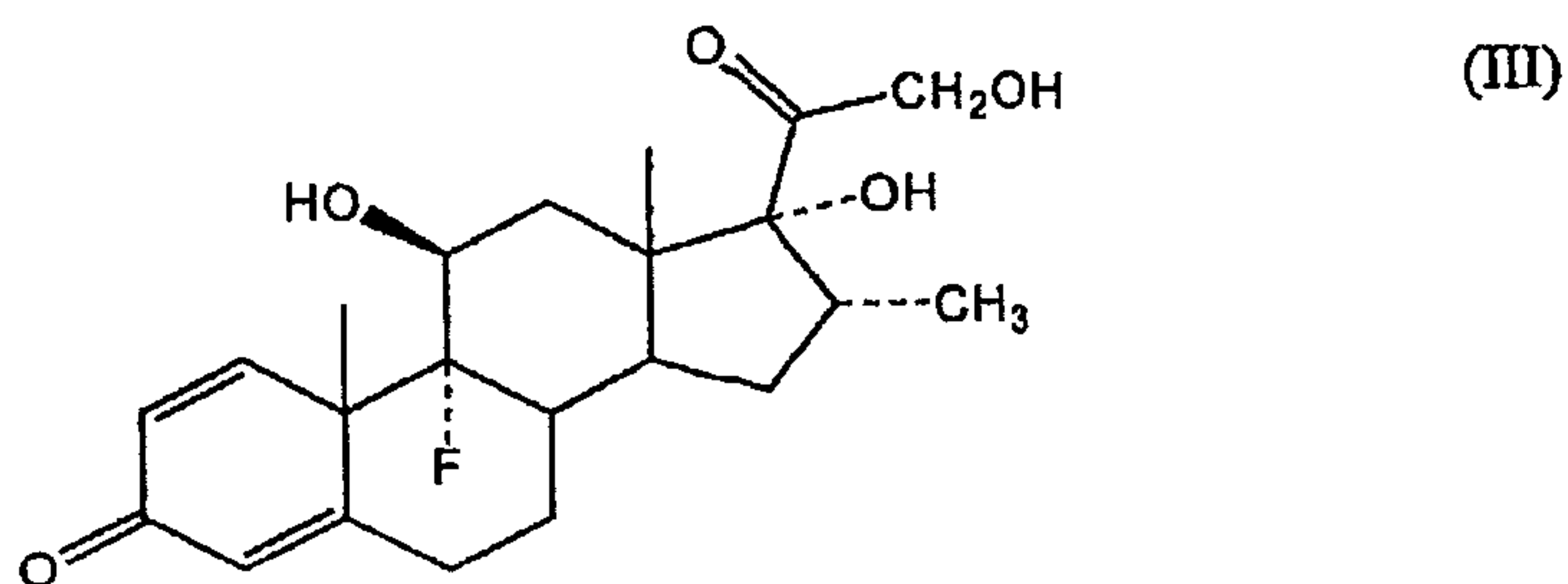
6. A process according to any of claims 1 to 5, characterized in that the TTN (total turn over number = mol of reduced oxosteroid compound / mol of cofactor used) is $\geq 10^3$.

7. A process according to any of claims 1 to 6, characterized in that at least 50% of the employed oxosteroid compound is reduced to the hydroxysteroid compound within 2 to 96 h.

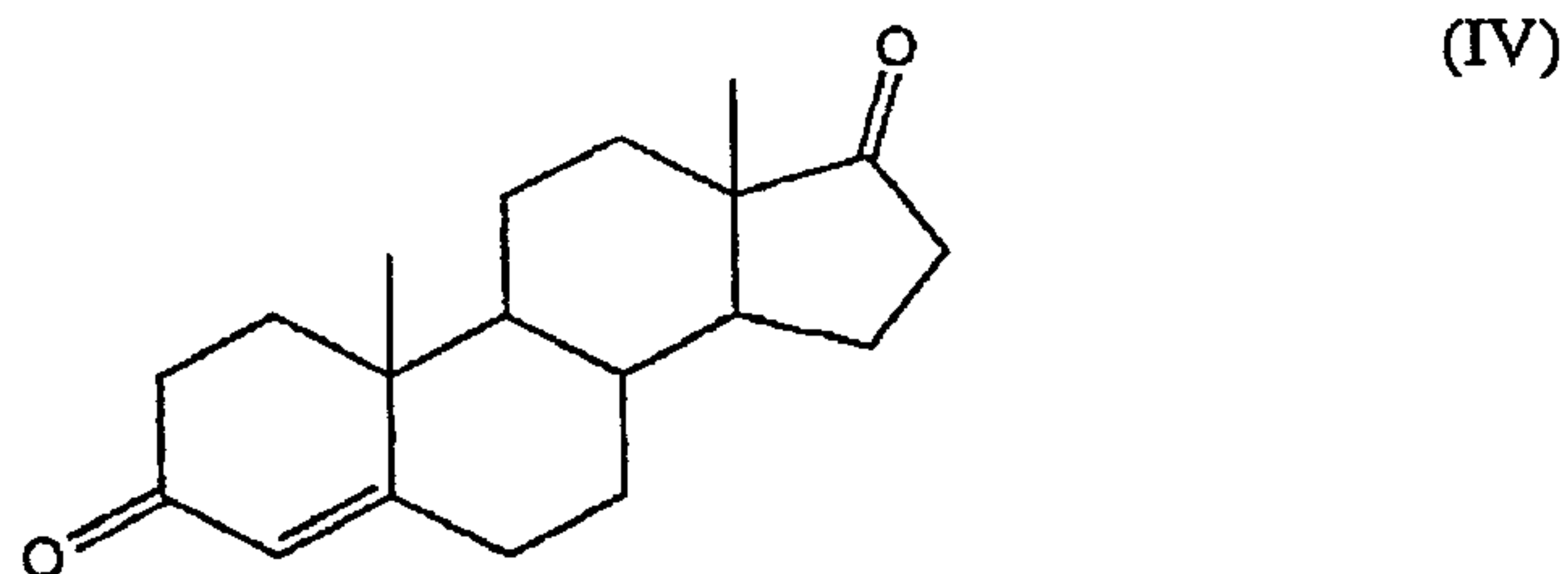
8. A process according to any of claims 1 to 7, characterized in that ketolithocholic acid (formula II) is used as the oxosteroid compound.



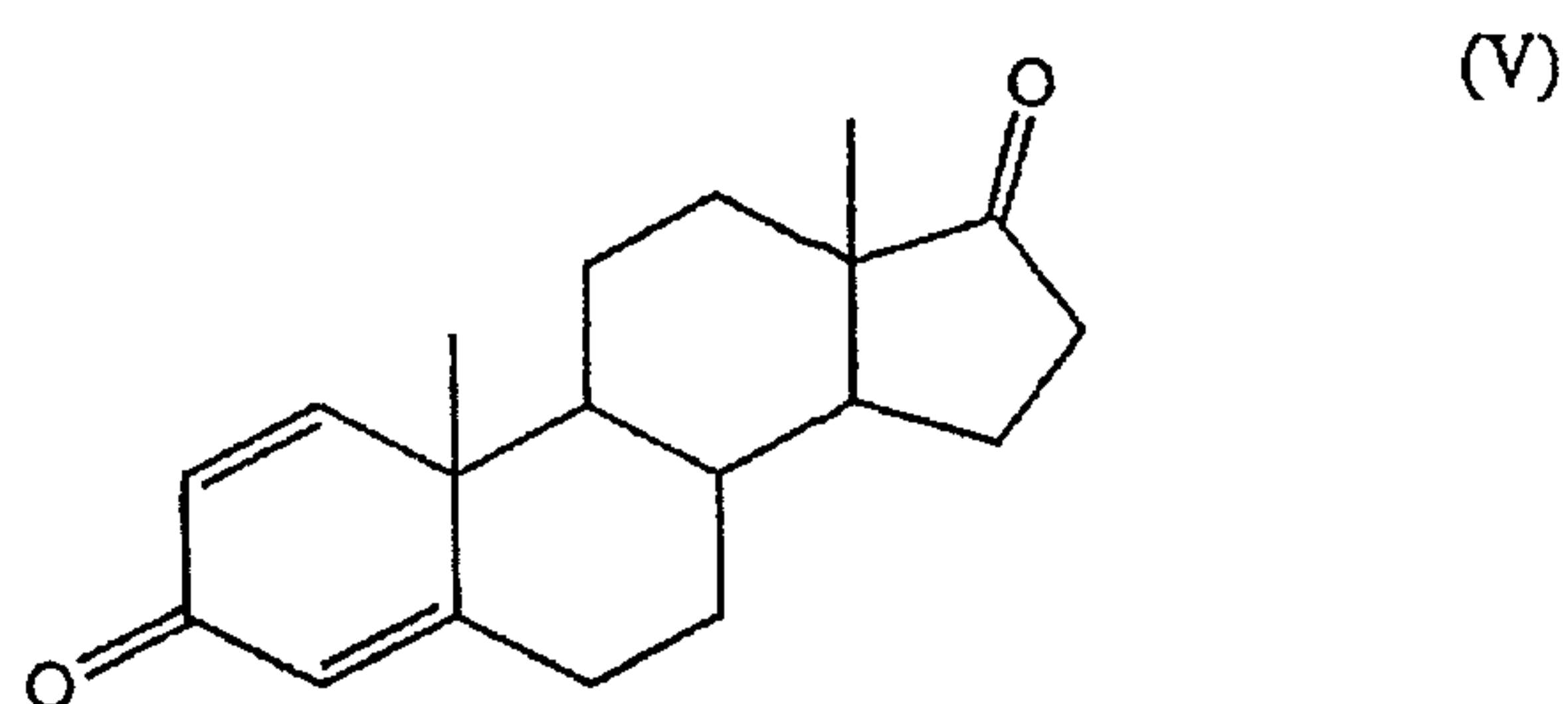
9. A process according to any of claims 1 to 7, characterized in that dexamethasone (formula III) is used as the oxosteroid compound.



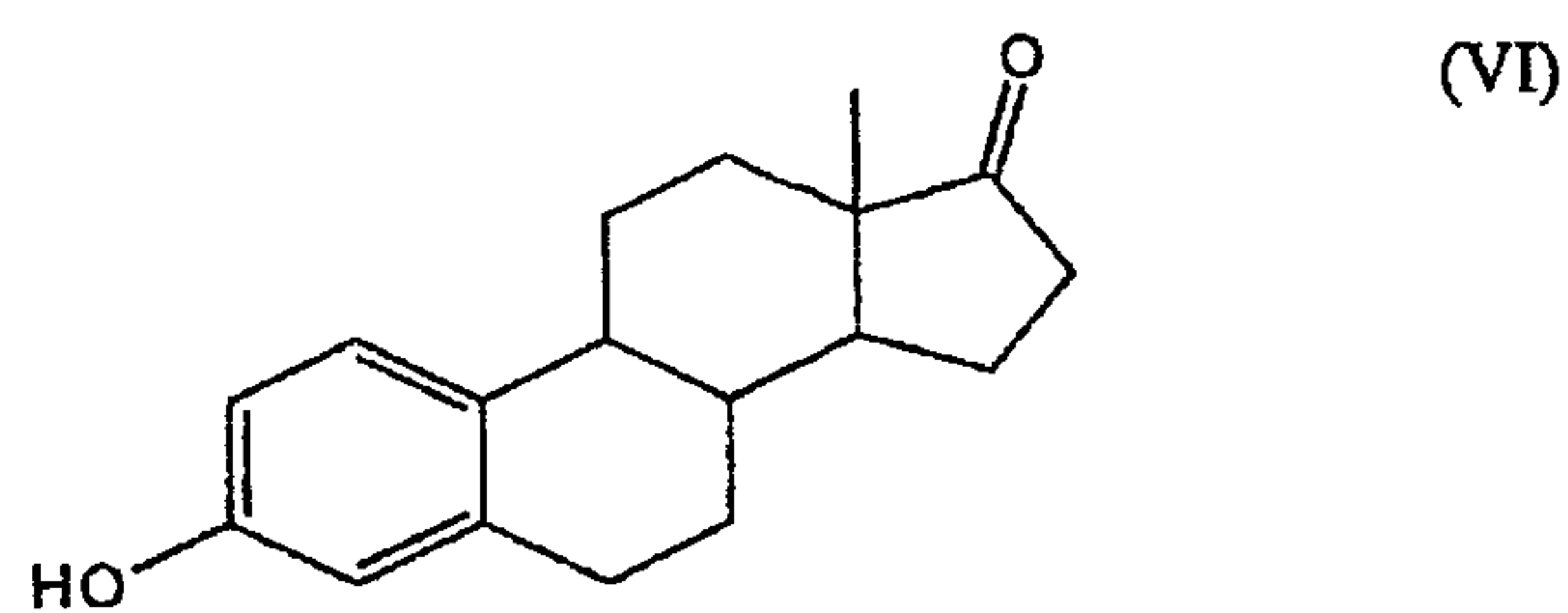
10. A process according to any of claims 1 to 7, characterized in that 4-androstene-3,17-dione (formula IV) is used as the oxosteroid compound.



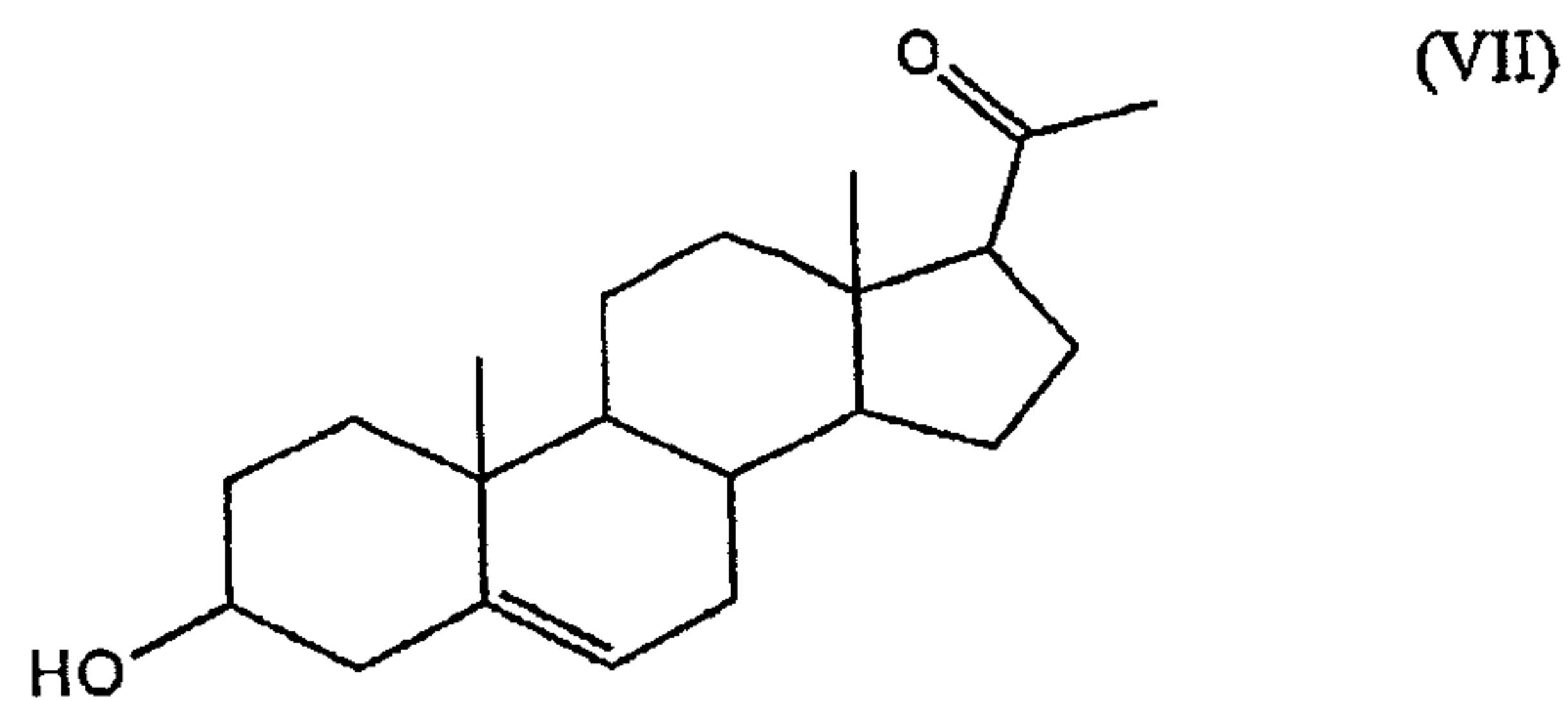
11. A process according to any of claims 1 to 7, characterized in that 1,4-androstadiene-3,17-dione (formula V) is used as the oxosteroid compound.



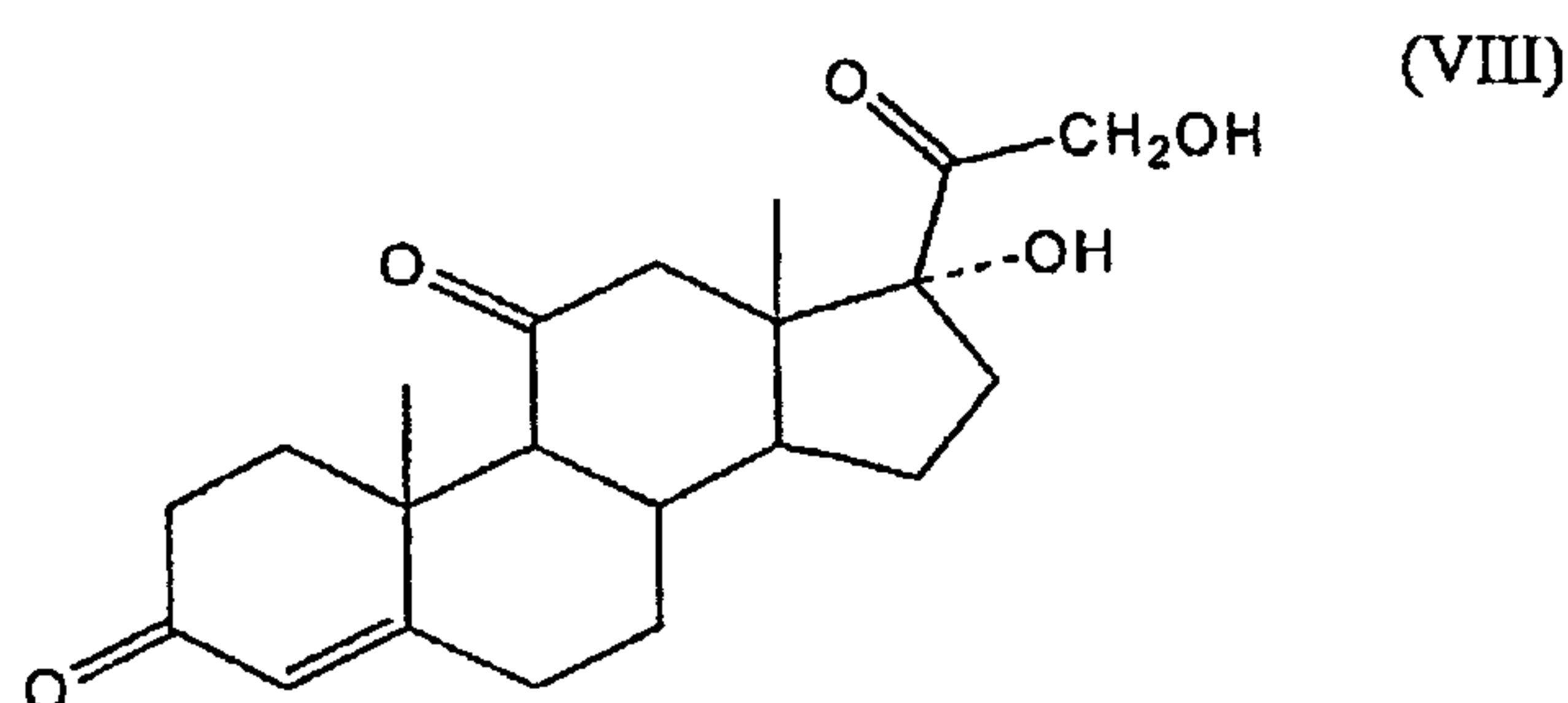
12. A process according to any of claims 1 to 7, characterized in that estrone (formula VI) is used as the oxosteroid compound.



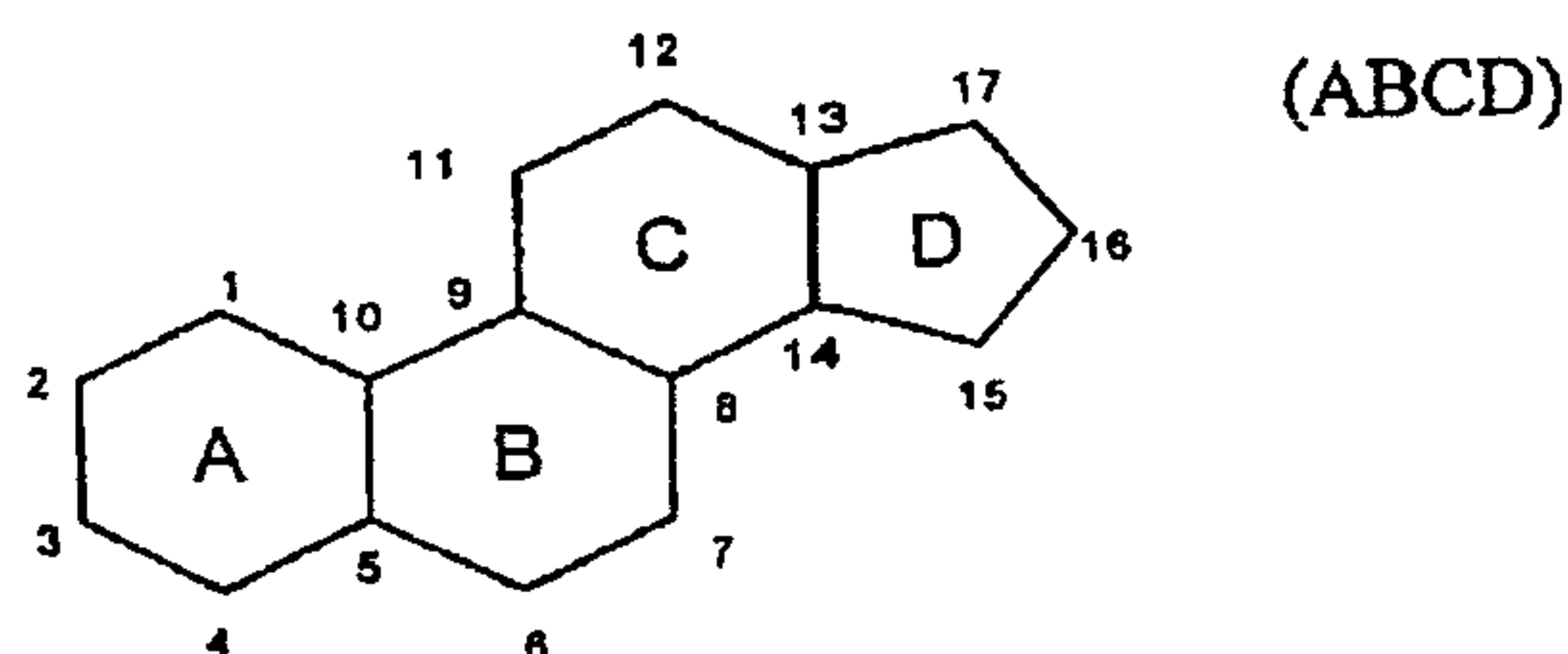
13. A process according to any of claims 1 to 7, characterized in that pregnenolone (formula VII) is used as the oxosteroid compound.



14. A process according to any of claims 1 to 7, characterized in that cortisone (formula VIII) is used as the oxosteroid compound.



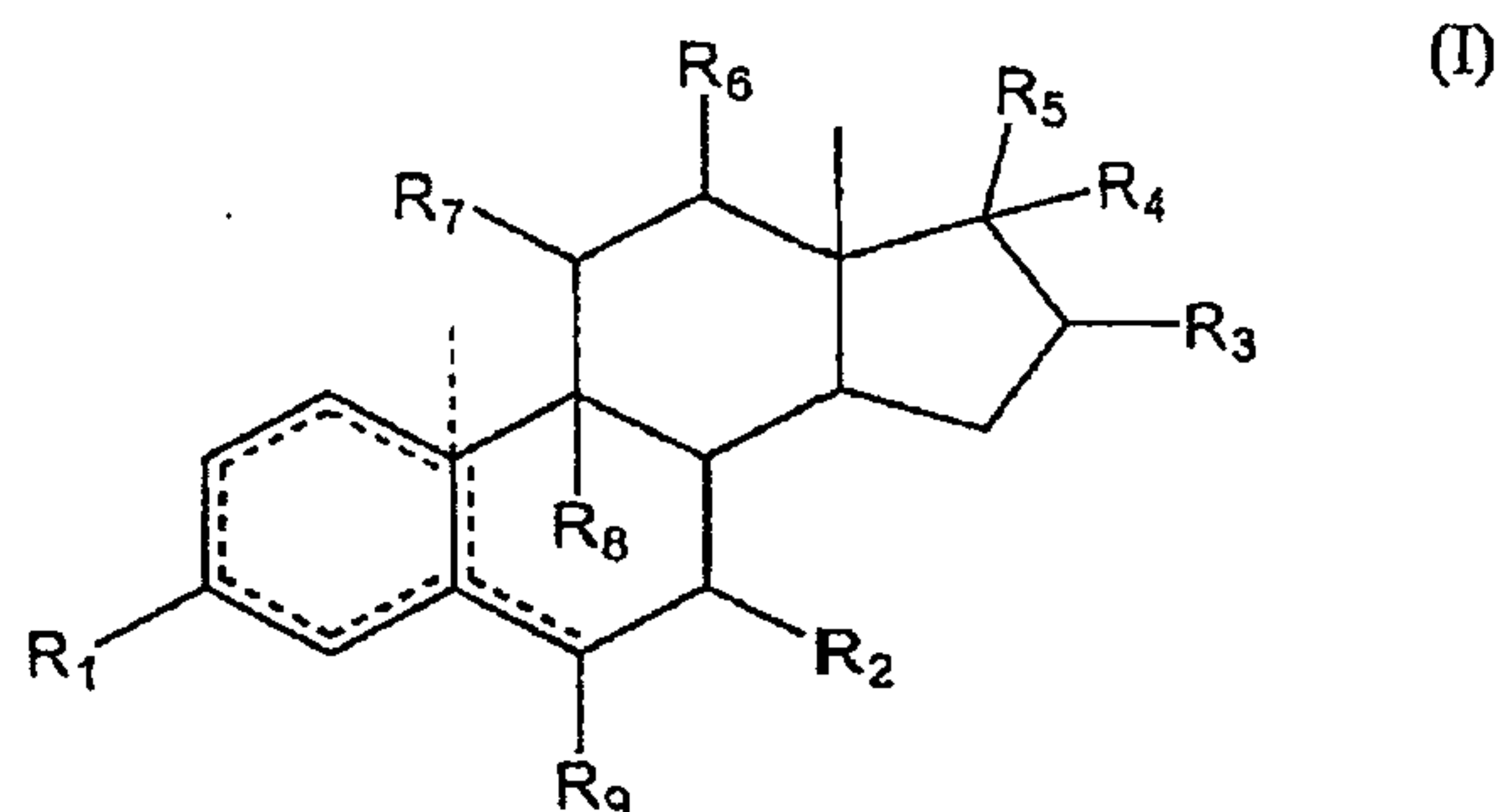
15. A process for the enzymatic oxidation of compounds which comprise a steroid structure (ABCD) including one or several heteroatoms, one or several double bonds and/or one aromatic component in the ring structure and have at least one hydroxy group at position 3, 7, 11, 12 or 17 in the steroid ring system or in the α -position at any carbon moiety on the steroid skeleton (=hydroxysteroid compound),



wherein the hydroxysteroid compound is oxidized with a hydroxysteroid dehydrogenase in the presence of a cofactor NAD or NADP, characterized in that

- the hydroxysteroid compound is provided in the reaction at a concentration of ≥ 50 g/l,
- the reduced cofactor NADH or NADPH formed by the hydroxysteroid dehydrogenase is regenerated continuously by reduction of a keto compound of general formula $R_X R_Y CO$, wherein R_X , R_Y independently represent hydrogen, a branched or unbranched C1-C8-alkyl and $C_{total} \geq 3$, or by reduction of a C4-C6-cycloalkanone, and
- an additional oxidoreductase/alcohol dehydrogenase is used for the reduction of the keto compound of general formula $R_X R_Y CO$ or of the cycloalkanone, respectively.

16. A process according to claim 15, characterized in that a compound of general formula I



is used as the hydroxysteroid compound,

wherein

R₁ represents hydrogen, a methyl group, a hydroxy group or an oxo group,

R₂ represents hydrogen, a methyl group, an oxo group or a hydroxy group,

R₃ represents hydrogen, a hydroxy group, an oxo group or a methyl group,

R₄ represents hydrogen or a hydroxy group,

R₅ represents hydrogen, a moiety -COR₁₀, wherein R₁₀ is a C1-C4-alkyl group that is unsubstituted or substituted with a hydroxy group or a substituted or unsubstituted C1-C4-carboxyalkyl group,

or R₄ and R₅ together represent an oxo group,

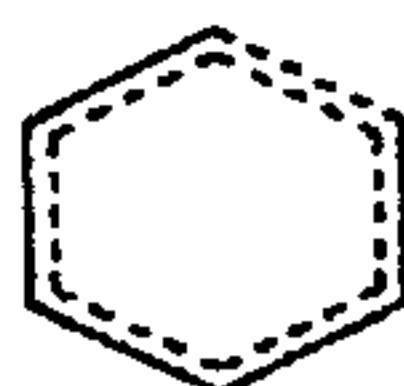
R₆ represents hydrogen, a methyl group, an oxo group or a hydroxy group,

R₇ represents hydrogen, a methyl group, an oxo group or a hydroxy group,

R₈ represents hydrogen, a methyl group or a halide, and

R₉ represents hydrogen, a methyl group, a hydroxy group, an oxo group or a halide,

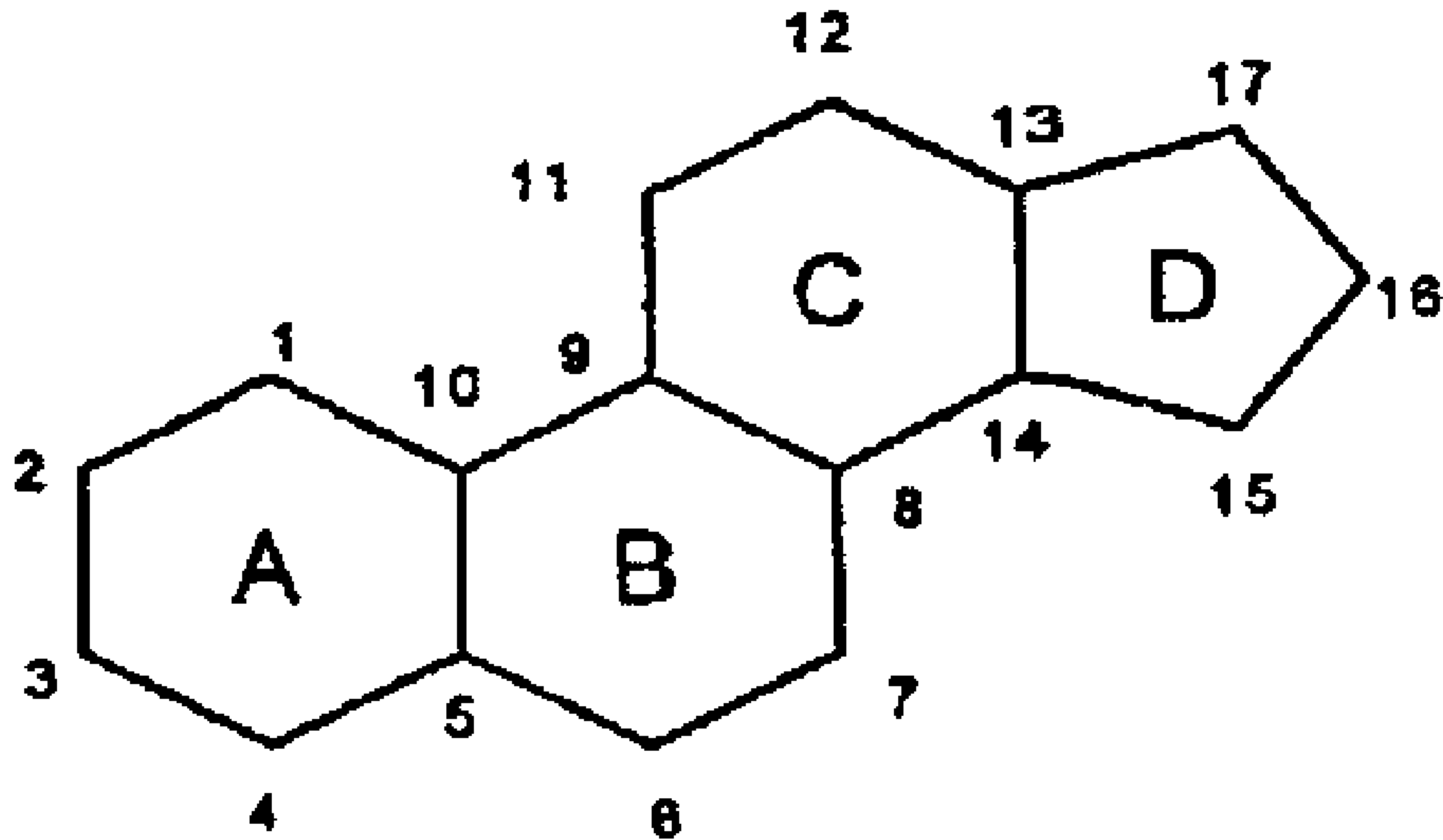
wherein at least one of R₁, R₂, R₄, R₆, R₇, R₈ or R₉ is a hydroxy group, and the structural element



represents a benzene ring or a C6-ring having 0, 1 or 2 C-C-double bonds.

17. A process according to claim 15 or 16, characterized in that acetone, 2-butanone, 2-pentanone, 4-methyl-2-pentanone, 2-hexanone, 2-heptanone, 5-methyl-2-hexanone or 2-octanone is used as the ketone of general formula R_xR_yCO, and cyclohexanone is used as the cycloalkanone.

18. A process according to any of claims 15 to 17, characterized in that it is carried out in an aqueous organic two-phase system.
19. A process according to claim 18, characterized in that an organic solvent
diethyl ether, tertiary butyl methyl ether, diisopropyl ether, dibutyl ether, ethyl
acetate, butyl acetate, heptane, hexane or cyclohexane is employed.
20. A process according to any of claims 15 to 19, characterized in that the TTN (total
turn over number = mol of oxidized hydroxysteroid compound / mol of cofactor used) is
 $\geq 10^3$.
21. A process according to any of claims 15 to 21, characterized in that at least 50% of
the employed hydroxysteroid compound is oxidized to the oxosteroid compound within 2 to
96 h.



(ABCD)