



(86) Date de dépôt PCT/PCT Filing Date: 2000/09/29
(87) Date publication PCT/PCT Publication Date: 2001/04/05
(45) Date de délivrance/Issue Date: 2007/02/06
(85) Entrée phase nationale/National Entry: 2002/03/25
(86) N° demande PCT/PCT Application No.: DK 2000/000546
(87) N° publication PCT/PCT Publication No.: 2001/022974
(30) Priorité/Priority: 1999/09/29 (DKPA 1999 01390)

(51) Cl.Int./Int.Cl. *A61K 33/06* (2006.01),
A61K 31/593 (2006.01), *A61K 31/616* (2006.01),
A61P 35/00 (2006.01)

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(54) Titre : PREVENTION DU CANCER COLORECTAL
(54) Title: PREVENTION OF COLORECTAL CANCER

(57) **Abrégé/Abstract:**

The present invention relates to a method for prevention of colorectal cancer or the initiation and/or progression of colorectal cancer in a human comprising administration to the human a combination dosage of a cyclooxygenase (COX) inhibitor, a vitamin D₃ including analogues and metabolites thereof and calcium. In a further embodiment the invention relates to the use of the combination dosage for the preparation of a medicament and to such pharmaceutical preparations. In a further aspect the invention relates method for reducing the effective dosage of ASA in a chemoprophylactic treatment of colorectal cancer in a human by co-administration with a non toxic dosage of a vitamin D₃ including analogues and metabolites thereof and Ca in the form of a combination dosage.



(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
5 April 2001 (05.04.2001)

PCT

(10) International Publication Number
WO 01/22974 A1

(51) International Patent Classification⁷: **A61K 33/06**,
31/593, 31/616, A61P 35/00

(21) International Application Number: PCT/DK00/00546

(22) International Filing Date:
29 September 2000 (29.09.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
PA 1999 01390 29 September 1999 (29.09.1999) DK

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(81) Designated States (*national*): AE, AG, AL, AM, AT, AT
(utility model), AU, AZ, BA, BB, BG, BR, BY, BZ, CA,
CH, CN, CR, CU, CZ, CZ (utility model), DE, DE (utility
model), DK, DK (utility model), DM, DZ, EE, EE (utility
model), ES, FI, FI (utility model), GB, GD, GE, GH, GM,
HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KR (utility
model), KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG,
MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD,
SE, SG, SI, SK, SK (utility model), SL, TJ, TM, TR, TT,
TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States (*regional*): ARIPO patent (GH, GM,
KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian
patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European
patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE,
IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG,
CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:

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

For two-letter codes and other abbreviations, refer to the "Guid-
ance Notes on Codes and Abbreviations" appearing at the begin-
ning of each regular issue of the PCT Gazette.

(54) Title: PREVENTION OF COLORECTAL CANCER

(57) Abstract: The present invention relates to a method for prevention of colorectal cancer or the initiation and/or progression of colorectal cancer in a human comprising administration to the human a combination dosage of a cyclooxygenase (COX) inhibitor, a vitamin D₃ including analogues and metabolites thereof and calcium. In a further embodiment the invention relates to the use of the combination dosage for the preparation of a medicament and to such pharmaceutical preparations. In a further aspect the invention relates method for reducing the effective dosage of ASA in a chemoprophylactic treatment of colorectal cancer in a human by co-administration with a non toxic dosage of a vitamin D₃ including analogues and metabolites thereof and Ca in the form of a combination dosage.

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PREVENTION OF COLORECTAL CANCER

The present invention relates to the chemoprophylaxis of colorectal cancer with combinations of a cyclooxygenase (COX) inhibitor, vitamin D₃ including analogues and metabolites thereof and/or calcium. In a further aspect the invention relates method for reducing the effective dosage of ASA in a chemoprophylactic treatment of colorectal cancer in a human by co-administration with a non toxic dosage of a vitamin D₃ including analogues and metabolites thereof and Ca in the form of a combination dosage. In a still further embodiment the invention relates to the use of a cyclooxygenase (COX) inhibitor, a vitamin D₃ and calcium together with a pharmaceutically acceptable carrier for the preparation of a medicament for preventing the initiation and/or progression of colorectal cancer in a human. The invention also relates to such a pharmaceutical medicament.

Colorectal cancer (CRC) is one of the leading cancer forms in the western world (1.3 million per year and over 600,000 annual deaths). In Denmark, the incidence is approximately 65 pr. 100.000 inhabitants and correlates to age. Concurrently with a fall in tobacco smoking in western industrial countries and an increased life expectancy, CRC is expected to become the most frequent solid cancer over the next decades.

The great majority of CRC cases are sporadic cancers, for which it is not possible to establish a genetic disposition. Effective CRC prevention in well-defined risk groups would have a significant effect on population health.

In the average population the lifetime risk of getting CRC is 6 per cent, and the risk of dying from the disease is 3 per cent (1,2,3). In first-degree relatives of patients with CRC, the risk is several times higher. In rare cases, the CRC disposing factors are hereditary non-polyposis colorectal cancer (HNPCC), where it is possible to establish the presence of mutations in mismatch repair genes, familial adenomatous polyposis (FAP, mutation in the APC gene), or inflammatory bowel diseases (ulcerative colitis and Crohn's disease), these factors accounting for 5 to 15 per cent in all.

There is no doubt that foods are the most important causal factor, including animal proteins and fats, which the western world is increasingly eating in excess amounts instead of cereals, fruits and vegetables. The incidence of CRC is increasing, but it is only half the

magnitude among vegetarians as among meat-eaters (4). The progress made during the last decades within surgical techniques, adjuvant treatment, etc, has not lowered mortality to any mentionable degree. CRC screening means tracing cancers at an early stage and removal of intestinal polyps, but so far, however, studies have not shown screening to reduce the incidence. The overall five-year survival in Denmark is approximately 30 per cent and depends on the stage at the time of diagnosis. Approximately 25 per cent of the patients have disseminated cancer at the time of diagnosis and are beyond a cure. Three fourths of CRC patients undergo surgery intended to cure; nevertheless, 50 per cent of these patients die within five years because of recurrence.

10

With the choices and results of treatment known today, only effective prophylaxis will be able to reduce decisively CRC morbidity and mortality (3,5).

In recent years, focus is very much on cancer prophylaxis, in acknowledgement of the fact that that surgery mostly does not suffice as the only modality and that most cytotoxic regimens are ineffective against solid tumours.

The term chemoprophylaxis covers the use of pharmacologically active, non-cytotoxic agents or naturally occurring nutrients that protect against the emergence and development of clones of mutated, malignant cells.

In 1994, to analyse existing data and to initiate new studies, the National Cancer Institute, USA, established a Chemopreventive Branch. The NCI-CB has concluded that CRC is an attractive target for cancer chemoprophylaxis, since it is a frequent cancer with a high mortality. However no acceptable treatment is available.

A well-defined multistage carcinogenesis has been mapped with well-defined precursors in the form of colorectal adenomas, and the groups at risk are also well defined.

Some studies have pointed to an inverted correlation between the individual intake of non-steroid anti-inflammatory drugs (NSAID), and on calcium and vitamin D₃ and the risk of developing CRC. The studies in question are animal experimental models of colorectal carcinogenesis, prospective studies of patients with FAP and epidemiological studies taking the form of retrospective case-control studies and prospective cohort and interventional studies (6-17). In conclusion, 21 of 23 epidemiological studies have shown that

regular use of NSAIDs reduces the risk of CRC by up to 50 per cent (18). However, data are not clear regarding dosage and duration of use. The most frequently studied drugs are acetylsalicylic acid, sulindac, piroxicam and indomethacin.

- 5 A few review articles and editorials have been published which find the results interesting, but existing data have not led to any recommendations proper nor proved any clinical significance (12). This is primarily because of the well-known undesirable effects of NSAIDs and the acknowledgement that the strongest evidence of the effect of these agents does not exist, ie, prospective, randomised double-blind trials in human populations.

10

The American Cancer Society has concluded that current data from epidemiological, clinical, pharmacological and toxicological studies show that acetylsalicylic acid protects against CRC development (13), and the FDA is currently assessing whether acetylsalicylic acid taken alone should be approved as chemoprophylaxis of CRC, or whether further phase III studies will be necessary.

Animal experimental data draw a promising picture of pharmacologically active drugs that, with different mechanisms of action, appear to be effective chemoprophylactic agents. However, individual epidemiological studies of calcium and vitamin D (or milk products) in relation to CRC are inconsistent. Out of 13 studies of calcium (nine case-control and four cohort studies), eight show a significant inverse correlation, three studies report insignificant correlation, whereas two fail to show any correlation.

Out of five epidemiological studies (three cohort studies and two case-control studies) of the impact of vitamin D on the CRC risk, two show a significant inverse correlation, whereas the rest have no significance.

The sequence of epithelium - adenoma - carcinoma is a process taking many years (5 to 10 years). CRC differs from many other cancers in as much as mutations in cell-cycle regulating genes and gene products are rarely seen. CRC is characterised by mutations in critical tumor suppressor genes (APC, DCC, p53, MCC) and oncogenes (K-ras) and upregulation in growth factors (especially the EGF-family) and enzymatic activity (especially cyclooxygenase).

In spite of CRC being a frequent cancer form, its incidence is only 65 per 100,000 inhabitants, and a clinical controlled study with the endpoints being invasive cancer and cancer-related deaths would require enrolment of tens of thousands individuals, and it would run for several decades and require astronomical financial resources. Healthy individuals do not feel impelled to participate in scientific studies including long-term medicine intake, and the results of any such studies would be subject to confounding.

Over 95 per cent of CRC develop from adenomas, which are accepted CRC precursors in scientific studies of humans and in animal experiments. Other biomarkers include genomic changes (mutations, etc), aberrant crypt foci (ACF), ornithine decarboxylase activity, cyclooxygenase activity and the prostaglandin level in mucosa, which are used as intermediary endpoints.

Colorectal carcinogenesis and Calcium:

In the western world, the daily average intake of calcium is substantially below the recommended dietary allowance (RDA) of 800 to 1200 mg/day, increasing to 1500 mg /day for the elderly. In western countries, each adult has an average daily intake of 750 to 850 mg of calcium (14).

Approximately 30 per cent of dietary calcium is absorbed from the intestinal canal, and vitamin D₃ stimulates the absorption. The absorption is both transcellular (at low dietary calcium content) and paracellular. The residual amount of calcium in the intestinal lumen binds free fatty acids and secondary bile acids by formation of insoluble soaps and reduces the local irritant effect of these acids in the colon.

25

Deoxycholic acid (DCA) in particular, which produces epitheliolysis in the epithelial surface of the colon, is considered to be the most carcinogenic and mitogenic of the secondary bile acids (15). Epitheliolysis induces potent proliferation in the crypts, probably as a result of exposure of the basal membrane. In the activated phase (S phase), the cells are more sensitive towards carcinogens like DCA, free fatty acids, etc (16). *In vivo* the harmful effect of 5mM of DCA can be prevented by increasing the Ca concentration in the intestinal lumen from 0 to 4 mM.

In several cases, the results of case-control studies and cohort studies have shown a significant correlation between a high dietary calcium level and a reduction in the risk of CRC

development. However, the results are not unambiguous, although large volumes of data from animal experimental studies all point in the same direction (14). One prospective study shows a significant reduction in the rate of polyp recurrence and a significant increase in cancer-related survival following CRC surgery at calcium supplements (calcium carbonate 2 g/day (17)).

The formation of insoluble calcium soaps is still considered to be the most important mechanism of the cancer preventive action of calcium, but in recent years focus has increasingly been directed at the central role of calcium in intracellular signal transduction, Calcium is a key factor in maintaining normal cell membrane function, and calcium flux over the cell membrane plays a central role in mediating intracellular signal transduction, which regulates multiple cellular functions. Furthermore, the expression of cellular surface cadherins, which is necessary to maintain intercellular contact, depends on the presence of calcium. Particularly on colon cancer cells the expression of cadherins correlates with the rate of differentiation and to the clinical outcome (19).

Reduction of calcium concentration in intercellular fluid lowers cell response to growth regulating factors and reduces the permeability of cell membranes. When the calcium concentration is reduced, the rates of proliferation and dedifferentiation increase.

Calcium contributes to regulating all cell division and cell differentiation phases, primarily through activation of various protein kinases (cAMP-dependent kinase, Ca-calmodulin-dependent protein kinases, protein kinase C) (20,21). Calcium suppresses ornithine-decarboxylase, a tumour-promoting enzyme (14) and reduces the number of K-ras mutations in colonic epithelium stimulated with the carcinogen 1,2 dimethylhydrazine (22). K-ras mutations are one of the early genomic changes in the carcinogenesis. K-ras mutations occur in approximately 85 per cent of adenocarcinomas and approximately 55 per cent of adenomas, but ras mutations exist even in up to 50 per cent of ACF.

Elevated calcium values produce increased differentiation of epithelial cells with concurrent growth suppression, but neoplastic colonic epithelial cells presumably lose their calcium response at one of the late stages of the epithelium - carcinoma sequence (16) .

Stimulation with carcinogens at the preneoplastic stage produces luminal proliferation of the colon crypt proliferating cells, an increased proliferation ratio and increased incidence

of ACF, which is also seen in individuals with an elevated risk of colon cancer (HNPCC and FAP patients). At calcium administration, cells in the crypts can be converted to a normal proliferation ratio and normal geographical distribution of non-dividing cells in the luminal two thirds of the crypts and proliferating cells at the bottom of the crypts (23).

5

Montoya R.G. et al, "Chemoprevention of gastrointestinal cancer." CANCER AND METASTASIS REVIEWS (1997) 16/3-4 (405-419) disclose several compounds used for the prevention of colon cancer. There is no mentioning of vit D₃ and the reference to Ca relates to the theory concerning formation of insoluble calcium soaps.

10

WO 96 41645 disclose use of COX₂ inhibitors for use in the treatment of inflammation.

Pence B C et al: Experimental chemoprevention of colon carcinogenesis by combined calcium and aspirin (Meeting Abstract), Proc. Annu Meet AM Assoc Cancer Res (1994).

15 Vol. 35, pp A3719. ISSN: 0197-016X describes that tumor burden was lowest in groups fed Ca or ASA during promotion only. Supplementation during progression was less effective.

Sokoloski, John A. et al: Introduction of the differentiation of HL-60 myelocytic leukemia
20 cells by nonsteroidal anti-inflammatory agents in combination with low levels of Vit D₃; Leuk. Res (1998), 22(2), 153-161, 1998. This article discloses that D₃ has an increasing effect on NSAID, however only derivatives with receptor-binding properties have this effect and not D₃ analogues without receptor-binding effect and with the Ca increasing effect has this increasing effect on the NSAID. The study is performed with leukemia cells.

25

Colorectal carcinogenesis and Vitamin D₃:

Vitamin D₃ (D₃) increases serum calcium by furthering the absorption of calcium and phosphate from the intestinal canal and mobilising calcium from bones. D₃ is present in food; it is formed by ultraviolet radiation of 7-dehydrocholesterol, a provitamin present in
30 human skin and in fatty tissues in many animals. D₃ metabolises by successive hydroxylation, first in the liver, into 25-hydroxycholecalciferol, and then in the kidneys to 1,25 dihydroxycholecalciferol (1,25DHC) or 24,25DHC, which are the hormonally active metabolites of D₃ (1,25DHC > 24,25DHC).

In addition to its anti-oxidative effect, 1,25DHC resembles steroid hormones in its chemical structure and mechanism of action, as 1,25DHC passes the cell membrane and binds to a specific cytoplasmatic receptor protein. This hormone-receptor complex is activated during translocation into the cell nucleus where it binds to DNA and initiates mRNA tran-
5 scription and protein synthesis. In the nuclear membrane receptors for 1,25DHC are situated (high affinity nuclear vitamin D receptors, VDR), which contribute to regulating the calcium flux over cell membranes (17).

1,25DHC modulates signal transduction, inhibits proliferation and DNA synthesis, modu-
10 lates c-myc, c-fos and c-jun oncogenic expression, induces differentiation and presumably apoptosis. VDRs have been identified both in normal colonic mucosa and in colorectal carcinomas (24). 1,25DHC increases intracellular calcium and stimulates various protein kinases. 1,25DHC stimulates transcription of the calbindin D gene in colonocytes, which is believed to increase transcellular calcium absorption.

15

A potent upregulation (300-400 per cent) of VDR takes place in neoplastic colonocytes; this can be interpreted as an adaptive response to tumoric cell growth, by which the cell increases its differentiation potential. This response disappears at more advanced stages of the disease (>T3), where it is assumed that the vitamin D defence mechanism be-
20 comes inactivated (25).

In vitro, 1,25DHC inhibits the growth of human colon cancer cell lines (LoVo) including CEA-producing cell lines. *In vivo* (mice), 1,25DHC can suppress growth of solid, human xenografts (17,26). A few cohort studies of human populations have shown a significant
25 reduction in the risk of developing colorectal cancer at intake of vitamin D₃ (or its active metabolite 1,25DHC), resulting in serum concentrations over 20 ng/ml (6,7).

RDA for vitamin D₃ is 10 µg/day increasing to 20 µg/day in elderly women without oestrogen substitution (6,7,27). The recommended dose of 1,25DHC is 0.01 µg/kg BW three
30 times a week. In osteoporosis studies, 0,75 µg/day has been shown to induce hypercalcaemia.

New synthetic D₃ analogue preparations have 100-200 times the antiproliferative effect and effect on differentiation and only 0.5 times the hypercalcaemic effect of 1,25DHC.

35

Two NCI-CB sponsored studies of 1,25DHC 0.5 µg or D3 400 IU and calcium carbonate 1500 mg have been initiated in 1994.

It is known from studies of bone mineral turnover that vitamin D and calcium are mutually dependent factors, and this has proved to be the case also in the regulation of cell division
5 and cell differentiation.

Colorectal carcinogenesis and cyclooxygenase inhibitors (acetylsalicylic acid (ASA) and other NSAIDs) and CRC:

- 10 The regulating effect of cyclooxygenase inhibitors (COX inhibitors) on the colonic epithelium has been investigated in connection with treatment of chronic inflammatory intestinal diseases and FAP.

A cancer preventive effect of COX inhibitors has not been identified in details at the level
15 of molecular biology, but it is considered to be related to the impact of these drugs on arachidonic acid metabolism and prostaglandin synthesis via blocking of cyclooxygenase enzymes (COX).

Two isomeric forms have been identified: COX₁ and COX₂:

20

COX₁ is the constitutive form. In the upper gastrointestinal tract it affects the protection of mucosa by inducing bicarbonate secretion and mucin production primarily through prostaglandin E (PGE), which is the quantitatively dominant product of the COX₁ turnover of arachidonic acid. COX₂ is an inducible form. It is particularly induced by inflammatory
25 stimuli, and it catalyses the formation of proinflammatory cytokines, including PGE₂ and PGF_α, which strengthen the mutagenic effect of carcinogens by proliferation induction, suppression of the immune system and stimulation of angiogenesis. PGE₂ exerts its inhibitory effect via negative feedback T-cell proliferation and lymphokine production.

- 30 Arachidonic acid (AA, 5,8,11,14-eicosatetraenoic acid) originates from the cell turnover of phospholipids (PL) located in the cell membrane. AA is primarily liberated from PL by hydrolysis of the ester binding that binds AA to PL. In most cell types this occurs by direct activation of the enzyme phospholipase A2. The phospholipase A2 activity constitutes the common factor regulating the rate of AA liberation, and thus the rate of production for all
35 eicosanoids (PG, prostacyclins, thromboxanes and leukotrienes).

AA metabolises via the COX pathway to eicosanoids, which stimulate cell division, as it is seen in inflammatory conditions, or via the lipoxygenase pathway to hydroperoxides (HPETE) and hydroxy compounds (HETE). The third pathway for arachidonic acid metabolism is via cytochrome P450 to HETE and EET (epoxyeicosatrienic acid). It has been shown that blocking of the lipoxygenase activity inhibits growth-factor-induced colonic tumor cell proliferation (28).

The COX inhibitor ASA (aspirin and others) and its metabolite salicylate block the formation of PG from AA by irreversible acetylation of COX (29), denying AA access to the active part of the enzyme. The COX activity can only be re-established by production of new COX molecules, and therefore cells without protein synthesis, such as platelets, are not capable of resuming COX activity. The main chemopreventive effect of ASA is deemed to be COX₂ inhibition (30), which results in metabolising of AA via a lipoxygenase pathway to 15-HETE (leukotriene with anti-inflammatory and antimitogenic effects).

Most other NSAIDs (piroxicam, sulindac and indomethacin) block COX in a reversible and dose-dependent manner, wherefore ASA is a more potent PG inhibitor. As it appears from the above, there are several mechanisms of action, and the PG cascade also depends on the calcium-regulated signal transduction system (21).

Several classic carcinogens are used as electron donors during the COX reaction, and they are activated by this reaction (high DNA affinity). Among them are polycyclic aromatic hydrocarbons, aflatoxins, halogenated pesticides, aromatic amines and phenol compounds. Thus, COX activates potential carcinogens into active DNA harmful metabolites.

In vitro studies show that most NSAIDs have an antiproliferative effect on human colon cancer cell lines (Ht-29, SW-80, DLD-1)(31).

30

In vitro studies also show that although the NSAID effect on the PG-synthesis is eliminated (for instance by the use of sulindac metabolites without COX inhibition), the growth of human colon cancer cell lines is inhibited nevertheless. This points to several mechanisms of action, including the ability significantly to induce apoptosis (28,32), and modulation of transmembrane calcium flux and intercellular junctions (33).

35

NSAID has been shown to inhibit several endonucleases; these are enzymes that cleave DNA molecules. Presumably they play a central part in the genomic instability that is one of the characteristics of colorectal multistep carcinogenesis (32). Other molecular biology
5 mechanisms are discussed in detail in (35).

An interesting point is that, contrary to other NSAIDs, ASA has been shown to inhibit proliferation and lumen formation in cocultivated normal colon epithelial cells (carcinoma cells in compartment 2). This is taken as an expression of inhibition of the growth promoting
10 signals of carcinoma cells. Other areas in which ASA differs from other NSAIDs are irreversible COX inhibition, lower plasma binding (approximately 50 per cent as compared with approximately 90 per cent).

Human colorectal neoplasms, both adenomas and adenocarcinomas, have been found to
15 produce large amounts of PG, especially of the E type (31,36), and precisely COX₂ activity has been found to be accentuated 2 to 50-fold in 85 to 90 per cent of colorectal carcinomas (35). Particularly APC loss of heterozygosity (LOH) is believed to stimulate COX₂ expression at an early stage of neoplastic development in both epithelial and stroma cells. However, it may precisely be the stromal COX₁ activity that stimulates the expression of
20 various angiogenetic factors (VEGF, bFGF and TGF_{β1}).

Other proneoplastic effects of COX are the change of TGF-beta from an anti-proliferative growth factor to an pro-proliferative growth factor and reduced intercellular and cellular-stromal contact/communication and thereby promoting angiogenesis and metastasis.
25 These properties of COX suggests that inhibitions of both isoforms may have important effects against CRC (38).

In itself, one of the three domains of the COX molecule (COX domain, EGF-domain and membrane binding motif) resembles the epidermal growth factor (ligand for the EGF re-
30 ceptor is also TGF_α). For this reason a possible inactivation of the entire domaine would be interesting in attempt to achieve optimum prophylaxis.

The COX inhibitor ASA (aspirin and others) and its metabolite salicylate block the formation of PG from AA by irreversible acetylation of COX (37), denying AA access to the ac-
35 tive part of the enzyme. The COX activity can only be re-established by production of new

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10

Several major epidemiological studies of COX inhibitors in the form of cohort studies, case-control studies and prospective interventional studies have shown a significant preventive effect (reduction of relative risk of 40 to 50 per cent) particularly of ASA on CRC after long-term (2 to 10 years) therapy in the doses used to prevent ischaemic heart disease (11-13,39-42). In a cohort of patients with ulcerative colitis, a relative risk reduction of 0.38 (0.2 to 0.7) has been found following only 3 months of sulphasalazine therapy.

Animal trials have been able to demonstrate a significant protective effect (50 to 60 per cent) of for instance indomethacin and piroxicam in rats exposed to the carcinogen dimethylnitrosamine or azoxymethane (methylazoxymethanol)(43-46).

The most frequent undesirable effects connected with long-term administration of NSAIDs are gastroduodenal ulceration and bleeding because of low PG and thromboxane A₂ levels in the gastrointestinal tract. PG stimulates mucin production and bicarbonate secretion, and thromboxane A₂ indicates platelet aggregation. These complications are primarily related to inhibition of the constitutive COX₁ enzyme.

Undesirable effects and complications primarily relate to the use of NSAIDs as analgesic or anti-inflammatory agents in significantly higher doses, but they are potential sequels after long-term use also in lower doses.

A review of 16 cohort studies and case-control studies showed that the risk of developing severe NSAID-induced gastrointestinal undesirable effects amounts to 2 to 4 per cent a year at analgesic and anti-inflammatory daily doses (14). In low-dose aspirin prophylaxis

of cardiovascular disease the relative risk-reduction in relation to stroke, acute myocardial infarction and/or cardiovascular death was found to be approx. 25% (47).

The Physicians' Health Study (325 mg of acetylsalicylic acid qod) found that, in addition to
5 a significant reduction of the risk of acute myocardial infarct, there were significantly more cases of melaena and epistaxis than in the placebo group, but of neither cerebral haemorrhage nor unspecific gastrointestinal bleeding (including haematemesis)(39).

There exist numerous data on the pharmacokinetics and toxicity of COX inhibitors, especially regarding ASA. The FDA has found that for instance acetylsalicylic acid is a safe
10 and efficient anti-inflammatory and analgesic agent and well suited for over-the-counter sales. No further toxicological studies are necessary to assess the usage of acetylsalicylic acid in chemoprevention (48-49).

15 The carcinogenesis in colorectal cancer involves a number of genetic changes and epigenetic factors such as increased expression of growth factors and suppression of growth inhibitors, which does not necessarily imply underlying mutations (but which for instance occurs at increased COX expression). Data from epidemiological studies and animal trials show that vitamin D₃ and calcium may be pharmacologically active when used as chemo-
20 prophylaxis of CRC. However, the effect is moderate. Some epidemiological studies in human populations indicate a reduction (40 to 50 per cent) of the relative cancer risk in populations using ASA continuously; there is not, however, consensus as to the dosage and duration of treatment. The risk-reduction in relation to CRC could be twice the risk-reduction of cardiovascular events.

25

BRIEF DESCRIPTION OF THE INVENTION

No prospective, randomised, double-blind studies exist. Studies of cancer chemoprophylaxis are extremely expensive, since they perforce have to include a very high number of
30 individuals and run for years, if the study endpoints are to be invasive cancer and cancer-related mortality. For these reasons there is an increasing tendency towards relying on epidemiological studies of intermediary endpoints (eg, polyps, ACF, etc), animal trials of genetically engineered or carcinogenically stimulated animal populations and biological models for examining different biomarkers (mutations, growth factors, etc).

35

According to the present invention, the CRC preventive effect of the following combination of preparations manifests itself by a significant reduction of the incidence and overall morbidity and mortality of colorectal cancer. To achieve this effect, however, it is believed to be important to take the preparation
5 consistently as prophylaxis over a long time (probably more than one year), exactly as for the prevention of ischaemic heart disease and osteoporosis.

By combining ASA with 1,25DHC (or an analogue preparation) or with calcium, an additive or synergistic effect is achieved, so that the amounts of
10 the individual drugs presumably are reduced and the toxicity thereby reduced to a negligible level. In a preferred embodiment ASA is combined with both 1,25DHC (or an analogue preparation) and calcium.

According to the present invention a surprising effect may be obtained by a
15 combination dosage comprising individual drugs which exert their effects on specific areas of the carcinogenesis: modification of signal transduction and expression of oncogenes, reduction of carcinogenic impact on the colonic epithelium and intracellular and intercellular signal transduction, COX inhibition and probably apoptosis.

20

In a further aspect of the invention, the incidence of undesirable gastrointestinal effects with COX inhibitors can be reduced or eliminated by prior eradication treatment of patients testing positive for *Helicobacter pylori*, who have been tested before initiation of chemoprophylaxis with a urease
25 breath-test.

According to an aspect of the present invention, there is provided a pharmaceutical medicament comprising a combination of acetylsalicylic acid (ASA), 1,25 dihydroxy-cholecalciferol (1,25 DHC) and calcium in a
30 combination dosage together with a pharmaceutically acceptable carrier.

13a

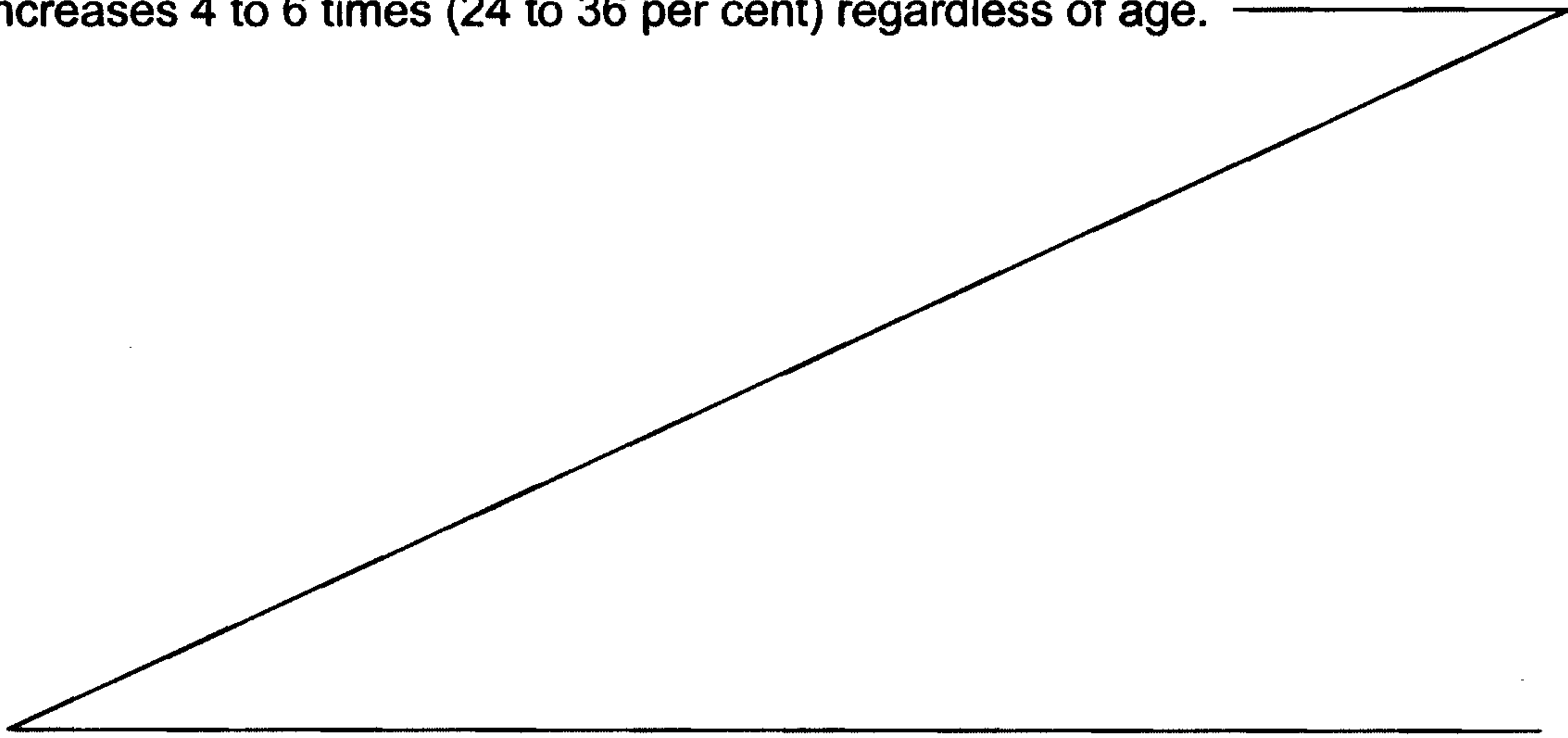
According to another aspect of the present invention, there is provided use of a combination dosage of acetylsalicylic acid (ASA), the vitamin D3 metabolite 1,25 dihydroxy-cholecalciferol (1,25 DHC) and calcium together with a
5 pharmaceutically acceptable carrier for the prevention of colorectal cancer in a human.

According to a further aspect of the present invention, there is provided use of a combination dosage of acetylsalicylic acid (ASA), the vitamin D3 metabolite
10 1,25 dihydroxy-cholecalciferol (1,25 DHC) and calcium together with a pharmaceutically acceptable carrier for the prevention of both the initiation of colorectal cancer and progression of colorectal cancer in a human.

In a preferred embodiment the human to be treated is a patient which due to
15 underlying disease or a genetic defect is in risk of developing colorectal cancer such as: HNPCC patients, polyp patients, patients with a history of CRC. In addition, individuals over 50 years, who are first-degree relatives of patients with colorectal cancer (risk of developing CRC 2 to 4 times increased (12 to 25 per cent).

20

For first-degree relatives of individuals with CRC diagnosed before the age of 50 years or for individuals with two first-degree relatives with CRC, the risk increases 4 to 6 times (24 to 36 per cent) regardless of age.



For carriers of HNPCC mutations, the risk of CRC is 75 per cent at the age of 65 years and the risk of metachronous cancer is 45 per cent ten years following resection of the primary tumour.

- 5 For patients with chronic inflammatory intestinal diseases (ulcerative colitis and Crohn's disease), the risk increases 4 to 25 times (lifetime risk 12 to 75 per cent in patients not treated with surgery after more than ten years of illness) depending on the dissemination and duration of the disease.
- 10 Accordingly, use and method according to the present invention is in a preferred embodiment suitable wherein the human is selected from the group being in risk of development of colorectal cancer due to being a first-degree relative to a patient with colorectal cancer, and/or carries the gene(s) for hereditary non-polyposis colorectal cancer (HNPCC), and/or having familial adenomatous polyposis, colorectal adenomas
- 15 and/or a inflammatory bowel disease such as ulcerative colitis or Crohns disease.

The preparations could be combined as follows:

- | | | |
|----|-----|--|
| | 500 | mg of Calcium (calcium carbonate 1250 mg) and/or |
| 20 | 0.5 | µg of 1,25DHC (or vitamin D ₃ 400 IU or D ₃ analogue, eg, 0.25 µg of calcitriol or 0,005 µg of calcitriol/kg BW) and |
| | 75 | mg of ASA or analogue reversible or irreversible COX ₂ inhibitor |

The main requirements for a preparation designed for chemoprophylaxis include: low price, high compliance and ultra-low toxicity; it is assumed that by adding 1,25DHC and calcium the amount of COX inhibitor (ASA) can be reduced, so that the ASA-related undesirable effects can be reduced to a negligible level without reducing its action. For acetylsalicylic acid, the undesirable effects following long-term use have made the FDA hesitate before approving acetylsalicylic acid as chemoprophylaxis of CRC.

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Preparations with specific action in the colon, such as for instance 5-ASA, may turn out to be appropriate, possibly in combination with mucosa-protective agents.

ASA and other NSAIDs are approved for over-the-counter sales for analgesic and anti-inflammatory use. Similarly, combination preparations containing D₃ and calcium (for in-

35

stance calcium carbonate 1250 mg = calcium 500 mg + D₃ 400 IU) are sold OTC for osteoporosis prophylaxis.

At first, *in vivo* studies of the effect of the above has been carried out in the form of animal experiments with the Institute for Toxicology of the Danish Veterinary and Food Administration (DVFA). The results of the studies is shown in Table 1.

DETAILED DESCRIPTION OF THE INVENTION

10 In one embodiment the invention relates to a method for reducing the effective dosage of ASA in a chemoprophylactic treatment of colorectal cancer in a human by co-administration with a non toxic dosage of a vitamin D₃ including analogues and metabolites thereof and/or Ca in the form of a combination dosage. This is due to the fact that it has surprisingly been shown that the addition of the vitamin D₃ and calcium may decrease the
15 necessary dosage of acetylsalicylic acid in order to decrease the formation of aberrant crypt foci in a model rat both with respect to size and numbers. Accordingly, the invention also relates to a method for prevention of the initiation and/or progression of colorectal cancer in a human comprising administration to the human a combination dosage of a cyclooxygenase (COX) inhibitor, a vitamin D₃ including analogues and metabolites thereof
20 and calcium.

By prevention and chemoprophylactic effect is meant prevention of colorectal cancer or the initiation and/or progression of colorectal cancer and or the effect of reducing the formation of conditions being pre-malignant of colorectal cancer.

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According to the present invention it is believed that the administration of the combination dosage should be administered regularly with an average daily dosage of the cyclooxygenase inhibitor corresponding to the inhibition of COX₁ and/or COX₂ provided by an dosage of ASA in the range of 50 mg and 500 mg, preferably in the range of 25 to 400
30 mg more preferred of in the range of 50 to 300 mg, still more preferred in the range of 75 to 150 mg such as in the range of 75 to 100 mg.

The combination dosage may further comprise a vitamin D₃ including analogues and metabolites thereof corresponding to the antiproliferative and/or cell differentiation effect
35 of the vitamin D₃ metabolite 1,25 dihydroxycholecalciferol in the range of 0,1 microgram

to 2 microgram. Any calcium in the combination dosage may preferable be in the range of 200 mg to 3500 mg such as calcium is in the range of from 250 mg to 3000 mg, such as in the range of from 300 mg to 2500 mg, preferable in the range of 400 to 2000 mg, more preferred in the range of from 500 to 1000 mg, such as 750 mg.

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As mentioned above, the treatment or prevention should be continued for a long period in order to give the best effect, however it is believed that a beneficial effect may be obtained after a treatment of at least 3 months. Accordingly the administration is preferably continued for at least 6 months, such as a least for 1 year, preferable for at least 2 years.

10 However, persons in high risk may be treated according with the present invention for the rest of lives.

In an important aspect, the administration of the combination dosage results in the prevention of the initiation or progression of colorectal cancer exceeding the effect of the administration of any of the individual ingredients in the same daily dosage and in the same period.

In another aspect, the method according to the invention is wherein the combination dosage has a preventive effect which is at least additive compared with the effect of the individual effective ingredients. In one embodiment, the preventive effect is at least synergistic compared with the effect of the individual effective ingredients.

An additive effect according to the invention may be calculated as an effect of the sum of prevention by each of the substances ASA, the Vitamin D₃, and the Ca, respectively or by an effect of the sum of prevention by the selection of two of the substances ASA, the Vitamin D₃, and the Ca and the preventive effect by the remaining substance.

By synergistic effect according to the present invention is preferable meant an effect which is higher than the additive effect as disclosed above. A suitable reference for the calculation is disclosed in Example 1 disclosing a study of the formation of aberrant crypt foci in induced rat colon.

The COX inhibitor may be any one acting on one or more of the mechanisms selected from reversible or irreversible acetylation of COX₁, reversible or irreversible acetylation of COX₂, inhibition of angiogenesis, inhibition of arachidonic acid metabolism, blocking of AA

metabolism, inhibits stimulation of proliferation stimulating from Epidermal Growth Factor, and stimulation of apoptosis. In a preferred embodiment, the COX inhibitor is primarily a COX₂ inhibitor. The preferred COX inhibitor is ASA. The preferred vitamin D₃ is 1,25 dihydroxycholecalciferol.

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The ASA is preferably acting by one or more of the following mechanisms: inhibiting of cell proliferation; inhibition of upregulation of pro-proliferative agents such as growth factors; modulation of signal transduction; and induction of apoptosis. Also inhibition of angiogenesis, inhibition of arachidonic acid metabolism may be the target for the ASA. In
10 a further aspect, the cyclooxygenase inhibitor is acting by decreasing the formation of potential carcinogens into DNA harmful metabolites.

The vitamin D₃ including analogues and metabolites thereof is preferable acting by one or more of the following mechanisms: inhibiting of cell proliferation; inhibition of DNA synthe-
15 sis; modulation of signal transduction; induction of differentiation; and induction of apoptosis.

In an important aspect of the invention, the vitamin D₃ is a synthetic analogue having an hypercalcaemic effect of 0.5 of the effect hypercalcaemic effect of 1,25 dihydroxychole-
20 calciferol. A number of synthetic Vit D analogues suitable according to the present invention are disclosed herein. In order to substitute one Vit D₃ analogue according to the present invention the, the relevant dosage may be correlated to the effect of 1,25 dihydroxycholecalciferol by reference to the antiproliferative effect which may be evaluated by methods well-known in the art, such as disclosed in ref 17 and 26 herein.

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The active mechanism of the calcium is according to the present invention preferable an effect on the expression of cellular surface cadherins and intra- and extracellular signal transmission.

30 An interesting embodiment, the invention relates to the finding that if the patient during or prior to the administration of the combination dosage receives treatment for Helicobacter Pylori, undesired effect of the dosage may be decreased.

One very important aspect of the the present invention is the finding that the method may
35 reduce the risk of developing colorectal cancer in the individual human receiving the

treatment by at least 10% or more compared to the effect of obtained by any of the individual ingredients in the same dosage and in the same period of administration. The reduction may be at least 20% or more, and in certain circumstances e.g. for high risk patients even 30% or more. The effect may be measured as disclosed in the example by
5 the number of aberrant crypt foci in AOM induced rats receiving administration of the combination dosage.

One preferred combination dosage according to the invention is the combination dosage comprising ASA, 1,25 DHC and Ca in that these ingredients are all well known drugs.
10

In a still further embodiment the invention relates to the use of a cyclooxygenase (COX) inhibitor, a vitamin D₃ and/or calcium together with a pharmaceutically acceptable carrier for the preparation of a medicament for preventing the initiation and/or progression of colorectal cancer in a human. In a preferred embodiment the medicament is in the form of a
15 combination dosage comprising the cyclooxygenase (COX) inhibitor, the vitamin D₃ and/or the calcium.

The use may be in accordance with any of the method described above and a still further embodiment, the invention relates any such pharmaceutical medicament. The pharmaceutical medicament may accordingly comprise a combination of a cyclooxygenase
20 (COX) inhibitor, a vitamin D₃ including analogues and metabolites thereof and/or calcium. In a further aspect, the pharmaceutical medicament is such a medicament as any of the combination dosages administered according to any of the methods described above.

25 Accordingly, a further aspect of the invention relates to the use of a cyclooxygenase (COX) inhibitor, a vitamin D₃ including analogues and metabolites thereof and calcium together with a pharmaceutically acceptable carrier for the preparation of a medicament for preventing the initiation and/or progression of colorectal cancer in a human.

30 In a still further aspect the present invention relates to a pharmaceutical medicament comprising a combination of a cyclooxygenase (COX) inhibitor, a vitamin D₃ including analogues and metabolites thereof and calcium in a combination dosage together with a pharmaceutically acceptable carrier.

The pharmaceutical medicament according to the invention is preferably a medicament wherein the combination dosage comprises vitamin D₃ including analogues and metabolites thereof corresponding to the antiproliferative and/or cell differentiation effect of the vitamin D₃ metabolite 1,25 dihydroxycholecalciferol in the range of 0,1 µg to 2 µg
5 such as in the range of 0,2µ to 1.5 µg, preferable in the range of from 0.3 to 1 µg, more preferred in the range of from 0.4 µg to 0,75 µg, such as 0.5 µg.

In a further embodiment, the pharmaceutical medicament is one wherein the combination dosage comprises calcium in the range of 200 mg to 3000 mg, such as in the range of
10 from 300 mg to 2500 mg, preferable in the range of 400 to 2000 mg, more preferred in the range of from 500 to 1000 mg, such as 750 mg.

In a preferred embodiment the pharmaceutical medicament according to the invention is wherein the combination dosage comprises ASA in the range of 50 mg and 500 mg,
15 preferably in the range of 25 to 400 mg more preferred of in the range of 50 to 300 mg, still more preferred in the range of 75 to 150 mg such as in the range of 75 to 100 mg, The most preferred medicament comprises 50 to 75 mg ASA, 500-1000 mg Ca, and 0.5 to 1µg 25 dihydroxycholecalciferol.

20 By a dosage according to the present invention is meant individual dosages for instance in a one packet or a physical entity of one or more of the ingredient. The combination dosage may also comprise different COX inhibitors as well as different Vit D₃ analogues and or metabolites.

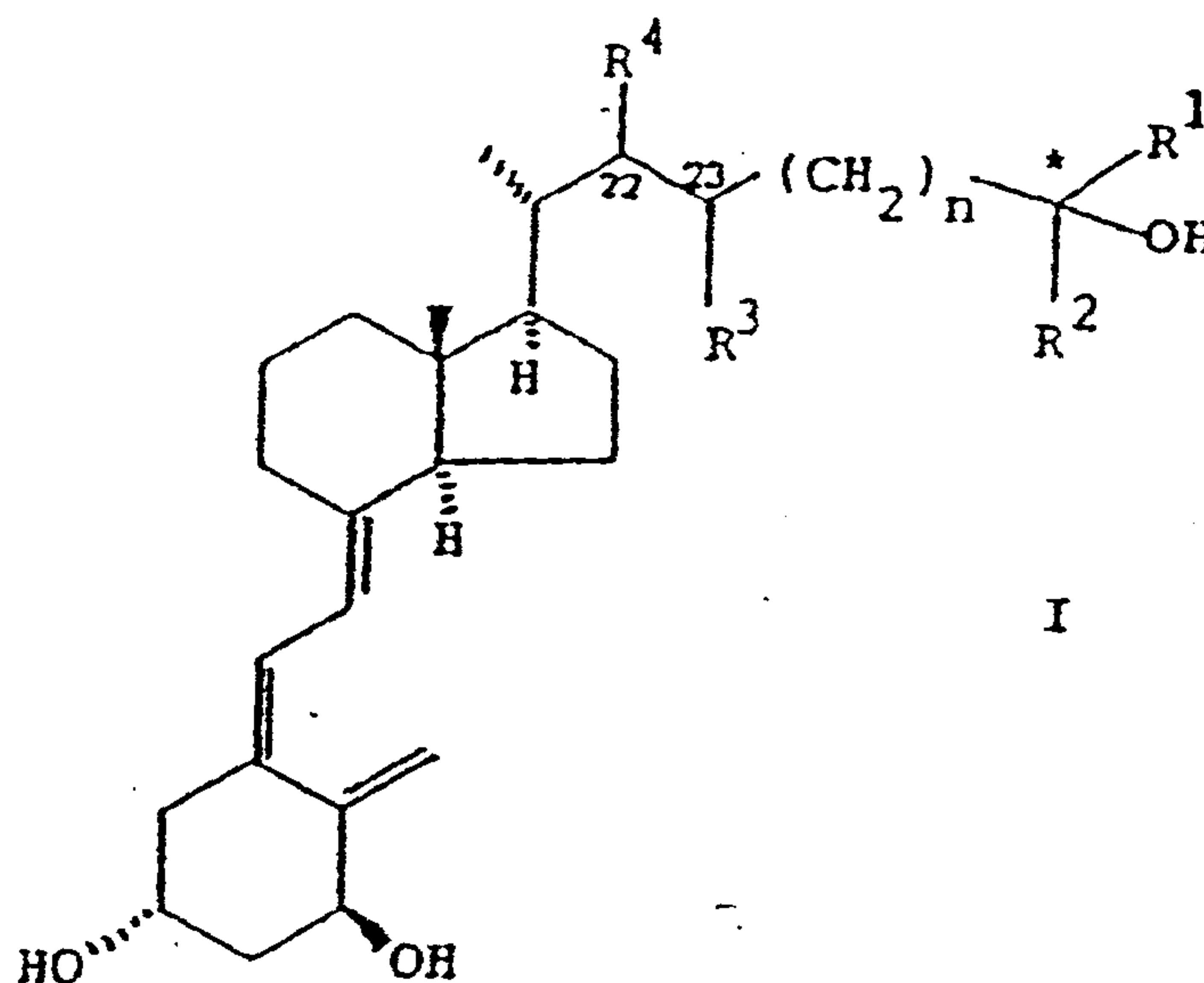
25 Accordingly, in a preferred embodiment, the pharmaceutical is one pharmaceutical comprising all three ingredients in order to secure the right individual dosage and patient compliance.

In a preferred embodiment the D₃ vitamin has a limited Ca inducing effect on the Ca level.
30 Accordingly, preferably the D₃ is an synthetic analogue having an hypercalcaemic effect of the most 0.5 of the hypercalcaemic effect of 1,25 dihydroxycholecalciferol calculated on a molar basis. This may be measured by methods known in the art. In the following relevant derivatives is disclosed.

In a still preferred embodiment, the vitamin D₃ analogues or derivatives is any one of the following disclosed in the references as mentioned.

5 WO 89/10351 mentions the following vitamin D analogues:

A compound of the formula I



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in which formula n is an integer from 1-7; and R¹ and R², which may be the same or different, stand for hydrogen, or straight or branched, saturated or unsaturated C₁-C₇-alkyl; with the provisos that when n = 1, R¹ and R² cannot simultaneously be hydrogen, nor can R¹ and R² simultaneously be an alkyl group independently chosen from methyl,

5 ethyl and normal-propyl, and when n=2, R¹ and R² cannot simultaneously be methyl; or C₃-C₈-cyclo-alkyl, or, taken together with the carbon (starred in formula I) bearing the hydroxyl group, R¹ and R² can form a saturated or unsaturated C₃-C₈ carbocyclic ring; and R³ and R⁴ represent either both hydrogen, or when taken together constitute a bond, such double bond (either in the Z or E configuration) connecting carbons numbered 22 and 23;

0 and derivatives of the compounds of formula I in which one or more hydroxy groups have been transformed into -O-acyl or -O-glycosyl or phosphate ester groups, such masked groups being hydrolyzable in vivo, or derivatives of the compounds of formula I in which the hydroxyl group at the starred carbon atom is lacking, these compounds being

converted to active compounds of formula I by enzymatic hydroxylation after administration.

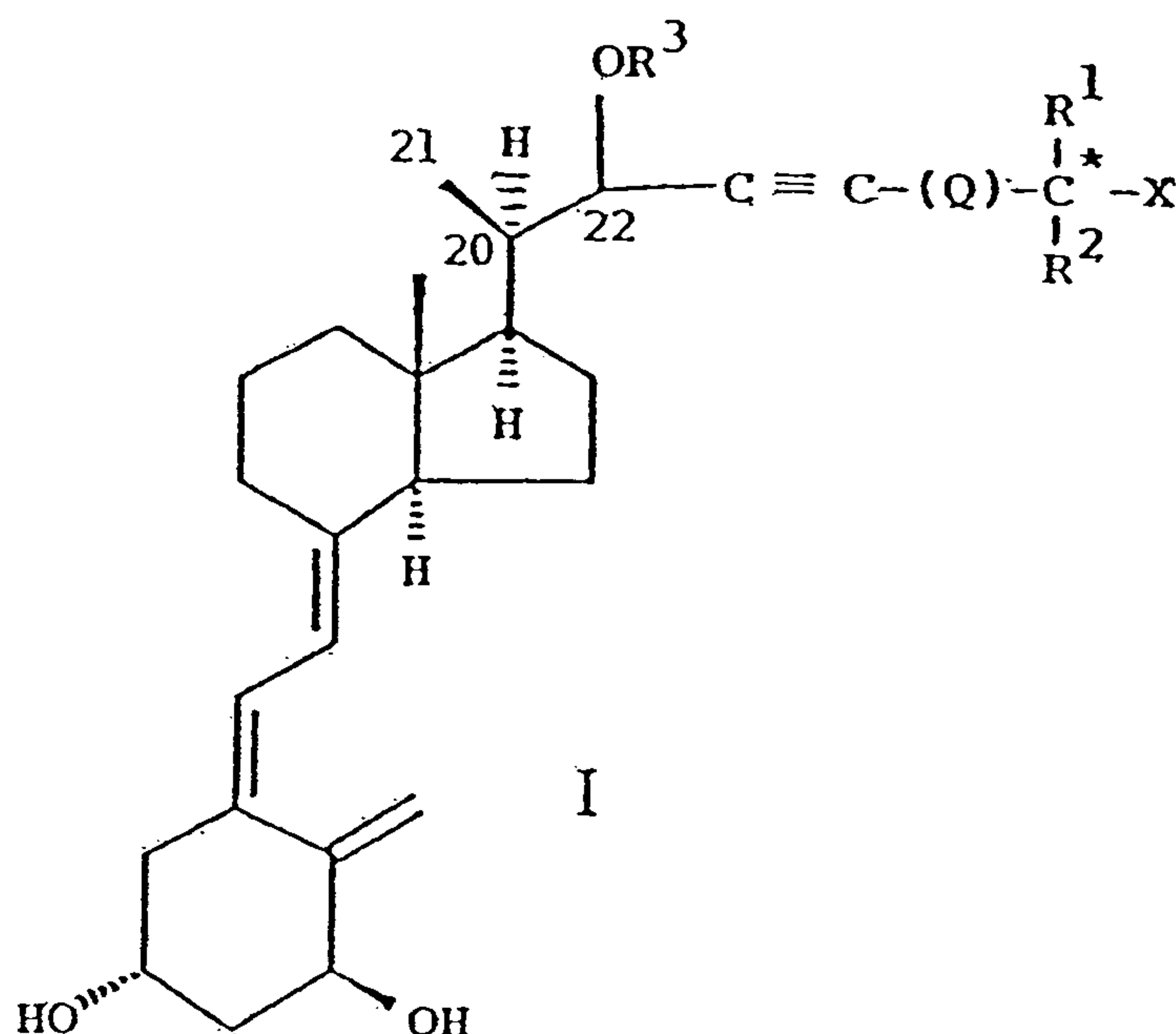
In particular a diastereoisomer of a compound mentioned above, in pure form; or a
5 mixture of diastereoisomers of a compound mentioned above.

Moreover, a compound according to the above mentioned specifications, selected from the group consisting of

- 0 1(S),3(R)-Dihydroxy-20(R)-(5-ethyl-5-hydroxy-1-heptyl)-9,10-secopregna-
5(Z),7(E),10(19)-triene;
- 1(S),3(R)-Dihydroxy-20(R)-(6-hydroxy-6-methyl-1-heptyl)-9,10-secopregna-
5(Z),7(E),10(19)-triene;
- 1(S),3(R)-Dihydroxy-20(R)-(6-hydroxy-6-methylhept-1(E)-en-1-yl-9,10)-secopregna-
5 5(Z),7(E),10(19)-triene;
- 1(S),3(R)-Dihydroxy-20(R)-(6-ethyl-6-hydroxy-1-octyl)-9,10-secopregna-
5(Z),7(E),10(19)-triene;
- 1(S),3(R)-Dihydroxy-20(R)-(7-hydroxy-7-methyl-1-octyl)-9,10-secopregna-
5(Z),7(E),10(19)-triene;
- 0 1(S),3(R)-Dihydroxy-20(R)-(6'-methyl-1'-heptyl)-9,10-secopregna-5(Z),7(E),10(19)-triene;

WO 93/19044 mentions the following vitamin D analogues:

A compound of the formula I



in which formula X is hydrogen or hydroxy; R^1 and R^2 , which may be the same or different, stand for hydrogen or a C_1 - C_6 hydrocarbyl radical; or R^1 and R^2 , taken together with the carbon atom (starred in formula I) bearing the group X, can form a C_3 - C_8 carbocyclic ring; R^3 stands for hydrogen or a C_1 - C_{10} hydrocarbyl radical or for YR^4 , in which Y stands for the radicals -CO-, -CO-O-, -CO-S-, -CS-, -CS-O-, -CS-S-, S-SO- OR -SO₂-, and R^4 stands for hydrogen or a C_1 - C_{10} hydrocarbyl radical; Q is a single bond or a C_1 - C_8 hydrocarbylene diradical; R^1 , R^2 , R^3 , and/or Q may be optionally substituted with one or more deuterium or fluorine atoms.

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In particular a diastereoisomer of a compound mentioned above, in pure form; or a mixture of diastereoisomers of a compound mentioned above.

Moreover, a compound according to the above mentioned specifications, selected from the group consisting of

1(S),3(R)-Dihydroxy-20(R)-(1,5-dihydroxy-5-ethyl-2-heptyn-1-yl)-9,10-seco-pregna-5(Z),7(E),10(19)-triene;

1(S),3(R)-Dihydroxy-20(R)-(5-ethyl-5-hydroxy-1-methoxy-2-heptyn-1-yl)-9,10-seco-pregna-5(Z),7(E),10(19)-triene;

20 1(S),3(R)-Dihydroxy-20(R)-(1-ethoxy-5-ethyl-5-hydroxy-2-heptyn-1-yl)-9,10-seco-pregna-5(Z),7(E),10(19)-triene;

1(S),3(R)-Dihydroxy-20(R)-(1-methoxy-4-hydroxy-4-ethyl-2-hexyn-1-yl)-9,10-seco-pregna-5(Z),7(E),10(19)-triene;

1(S),3(R)-Dihydroxy-20(R)-(1-ethoxy-4-hydroxy-4-ethyl-2-hexyn-1-yl)-9,10-seco-pregna-5(Z),7(E),10(19)-triene;

5 isomer A.

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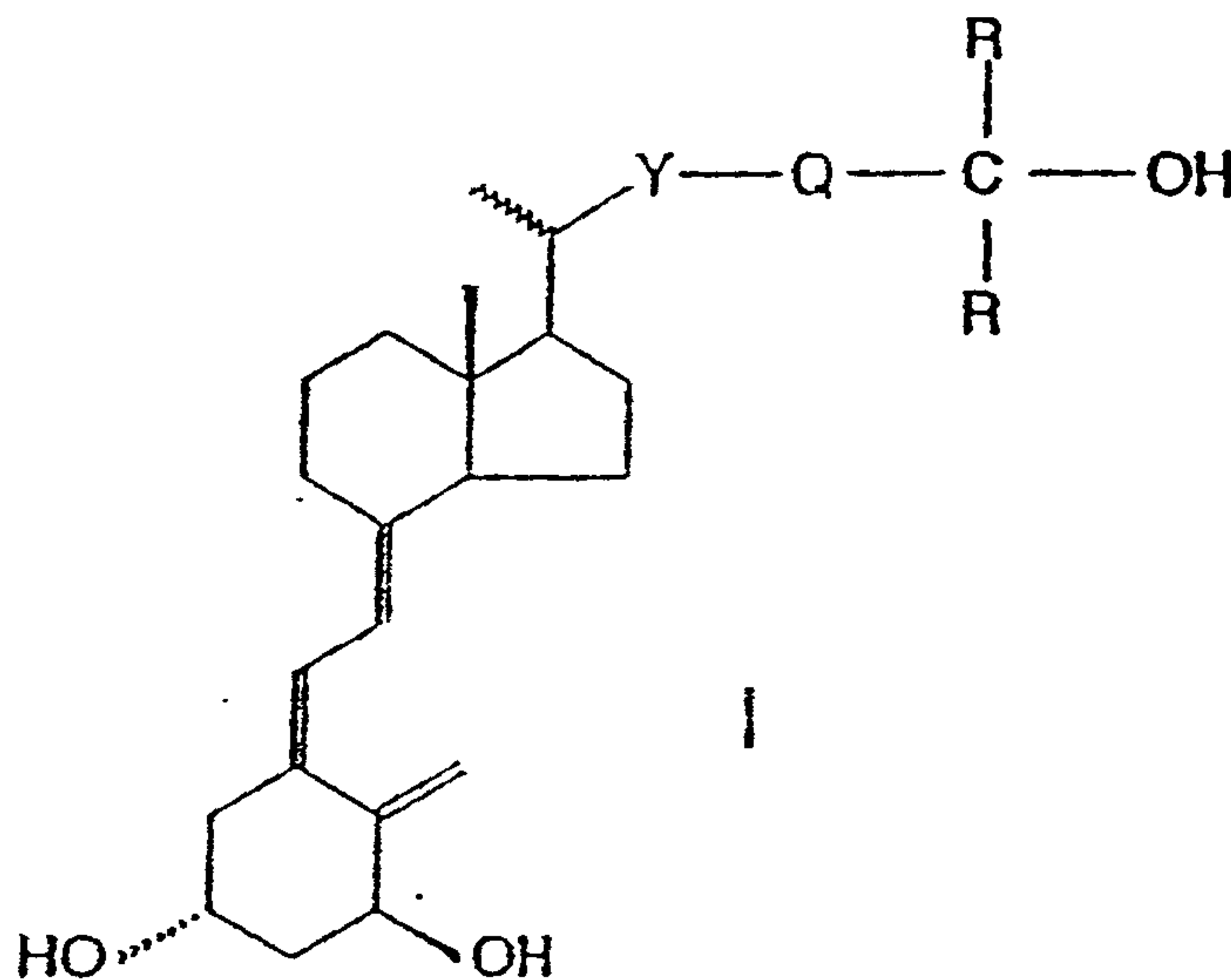
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WO 94/14766 mentions the following vitamin D analogues:

A compound of the formula I



in which formula Y is sulfur, S(O), or S(O)₂; R stands for C₁-C₃ alkyl; or R - C - R can form a C₃-C₈ carbocyclic ring; Q is a C₁-C₈ hydrocarbylene diradical; and prodrugs of I in which one or more of the hydroxy groups are masked as groups which can be reconverted to hydroxy groups in vivo.

A compound of the formula I shown above in which Y is sulfur and Q is C₂-C₇ alkylene.

Moreover a stereoisomer of a compound according to the above mentioned specifications, in pure form; or a mixture of such stereoisomers.

Furthermore, a stereoisomer of a compound according to the above mentioned specifications having a saturated side chain with the R-configuration at C-20.

5 Furthermore, a compound according to the formula I which is

a) 1(S), 3(R) - Dihydroxy-20(R) - (4-ethyl-4-hydroxy-1-hex-
ylthio) - 9,10-seco-pregna-5(Z), 7(E), 10(19) - triene,

10 b) 1(S), 3(R) - Dihydroxy-20(R) - [5-methyl-5-hydroxy-1-hex-
ylthio] - 9,10-seco-pregna-5(Z), 7(E), 10(19) - triene,

c) 1(S), 3(R) - Dihydroxy-20(R) - [3- (1-methyl-1-hydroxyeth-
yl)benzylthio] - 9,10-seco-pregna-5(Z), 7(E), 10(19) -
15 - triene,

or

d) 1(S), 3(R) - Dihydroxy-20(R) - (3-methyl-3-hydroxy-1-but-
ylthio) - 9,10-seco-pregna-5(Z), 7(E), 10(19) - triene.

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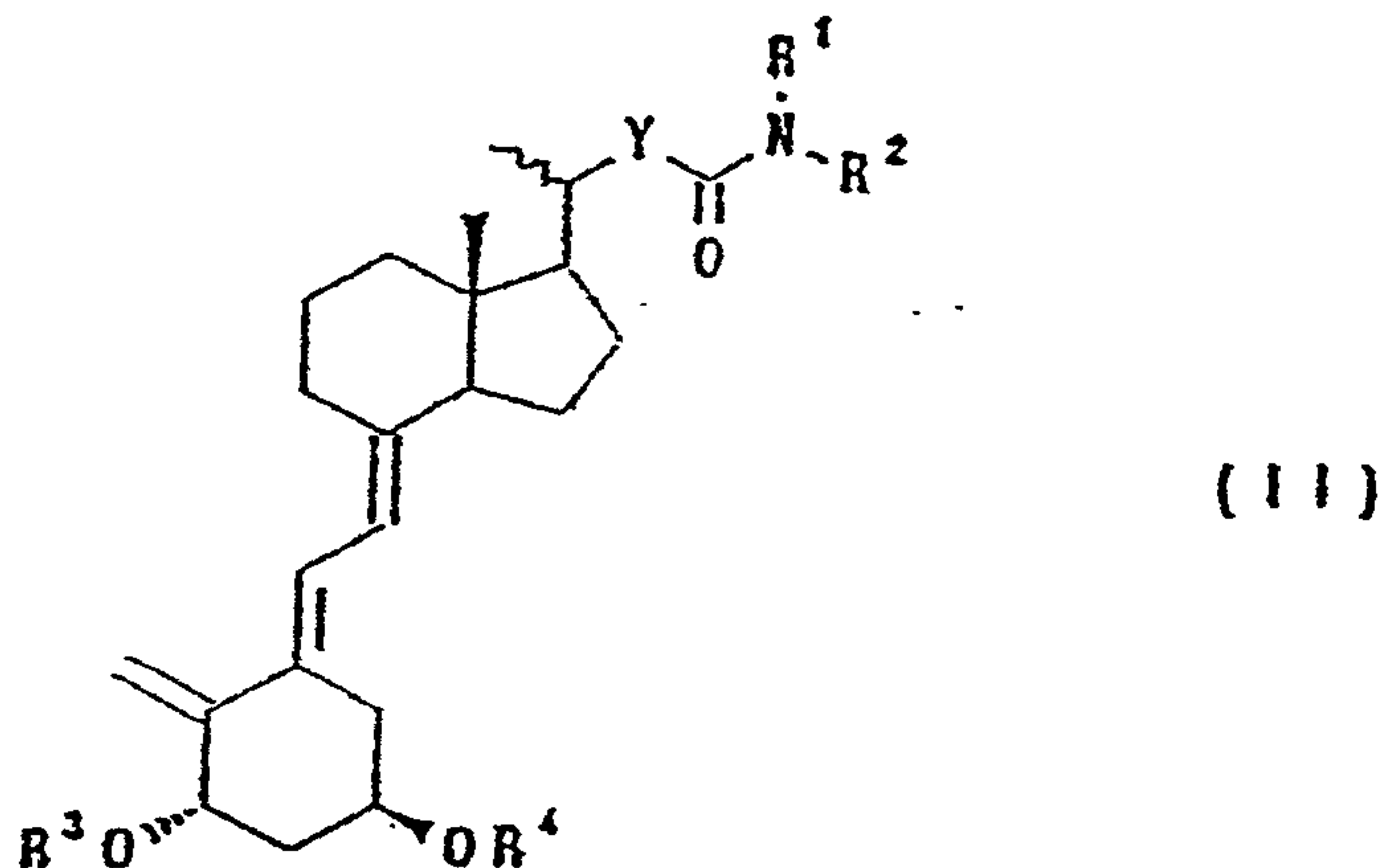
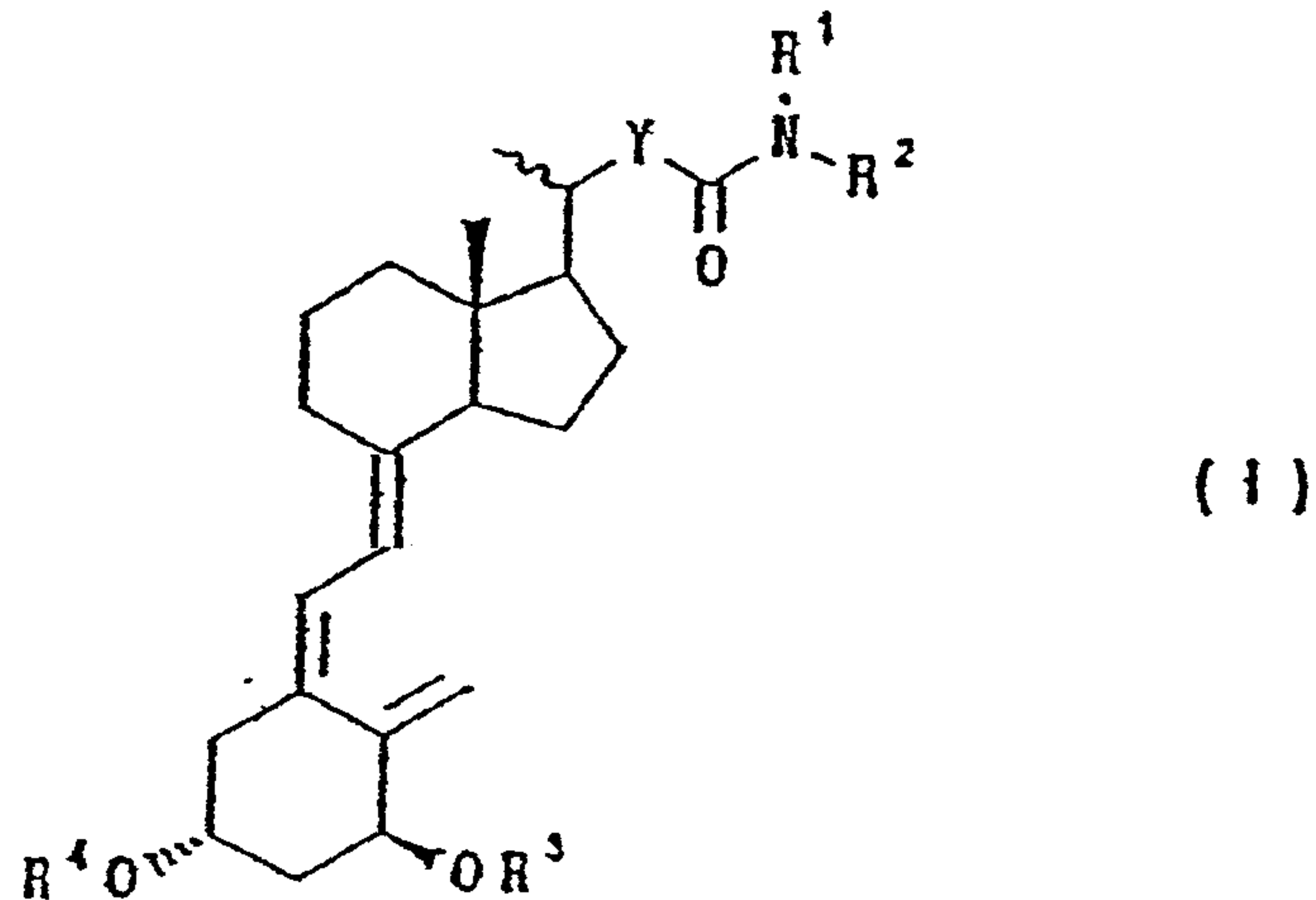
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WO 93/0909 mentions the following vitamin D analogues:

Compounds of general formulae (I) and (II)



(wherein Y represents an alkylene or alkenylene group containing up to 4 carbon atoms; R^1 and R^2 , which may be the same or different, each represents a hydrogen atom or a lower alkyl or cycloalkyl group or together with the nitrogen atom to which they are attached form a heterocyclic group; and R^3 and R^4 , which may be the same or different, each represents a hydrogen atom or an O-protecting group).

Moreover compounds according to the above mentioned compounds wherein Y represents a group of formula



(wherein R^A is $-\text{CH}=\text{CH}-$, R^B is $-\text{CH}_2-$, m is 0, 1 or 2 and n is 0 or an integer such that $2m+n = 1, 2, 3$ or 4.

10 Furthermore, compounds according to the above mentioned compounds wherein Y is a C2-4 alkylene group.

Compounds according to the above mentioned compounds wherein at least one of R^1 and R^2 is other than hydrogen.

15 Compounds according to the above mentioned compounds wherein R^1 and R^2 are selected from hydrogen atoms, methyl and cyclopropyl groups, or R^1R^2N- represents a piperidino group.

20 Compounds according to the above mentioned compounds wherein R^3 and R^4 represents etherifying silyl groups.

Compounds according to the above mentioned compounds wherein R^3 and R^4 are selected from hydrogen atoms and metabolically labile etherifying or esterifying groups.

25 Furthermore, the following compounds

$1\alpha, 3\beta$ -dihydroxy-9,10-seco-25-azacholesta-5(Z),7,10(19)-trien-24-one;

30 $1\alpha, 3\beta$ -dihydroxy-23,23-bishomo-24-aza-9,10-secocholesta-5(Z),7,10(19)-trien-24-one;

$1\alpha, 3\beta$ -dihydroxy-27-nor-9,10-secocholesta-5(Z),7,10(19),22,24-pentaene-26-carboxylic acid, 26-dimethyl amide;

35 N,N-pentamethylene- $1\alpha, 3\beta$ -dihydroxy-9,10-secocholanamide-5(Z),7,10(19)-triene;

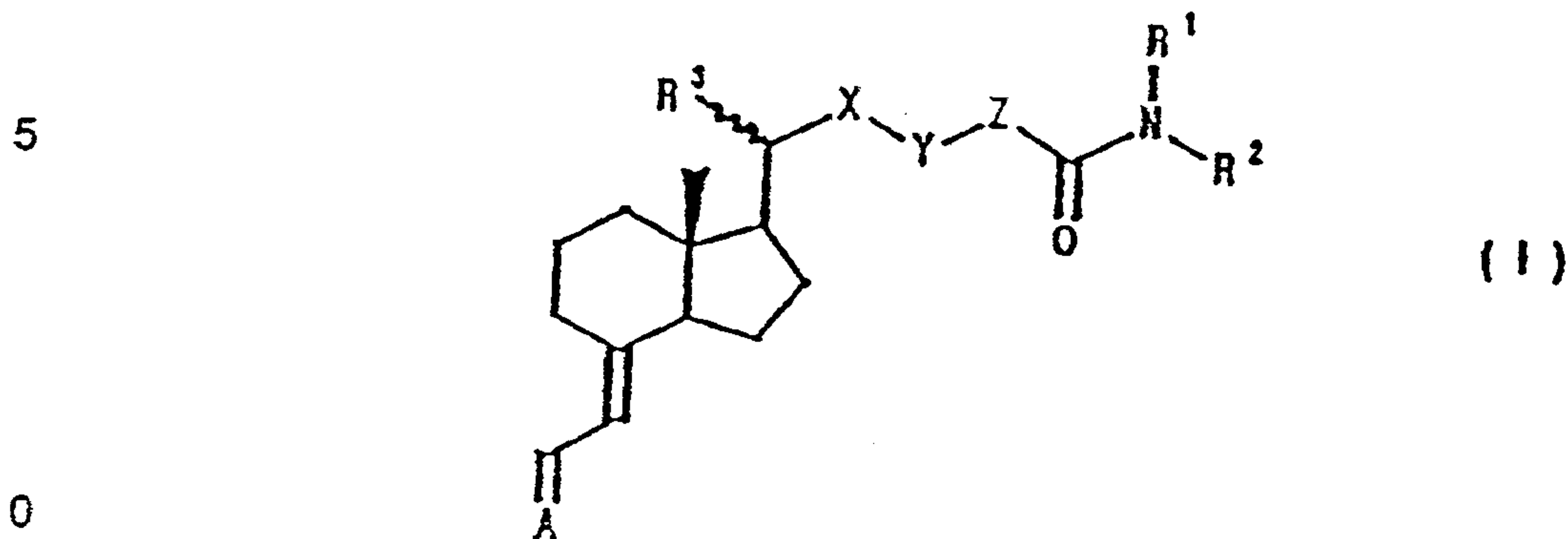
N-cyclopropyl-1 α ,3 β -dihydroxy-9,10-secocholanamide-5(Z),7,10(19)-triene;

1 α ,3 β -dihydroxy-9,10-secocholanamide-5(Z),7,10(19)-triene;

5 N,N-pentamethylene-1 α ,3 β -dihydroxy-9,10-seco-20-epi-cholanamide-5(Z),7,10(19)-triene;
and corresponding 5(E)-isomers thereof.

0 WO 94/26707 mentions the following vitamin D analogues:

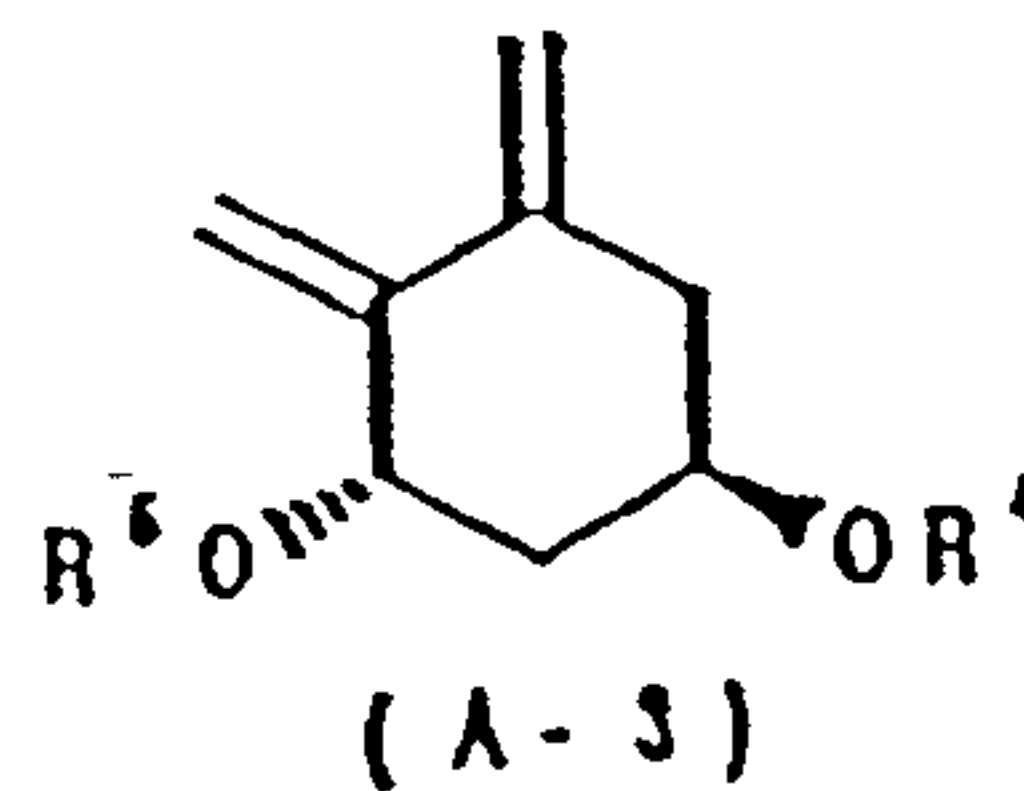
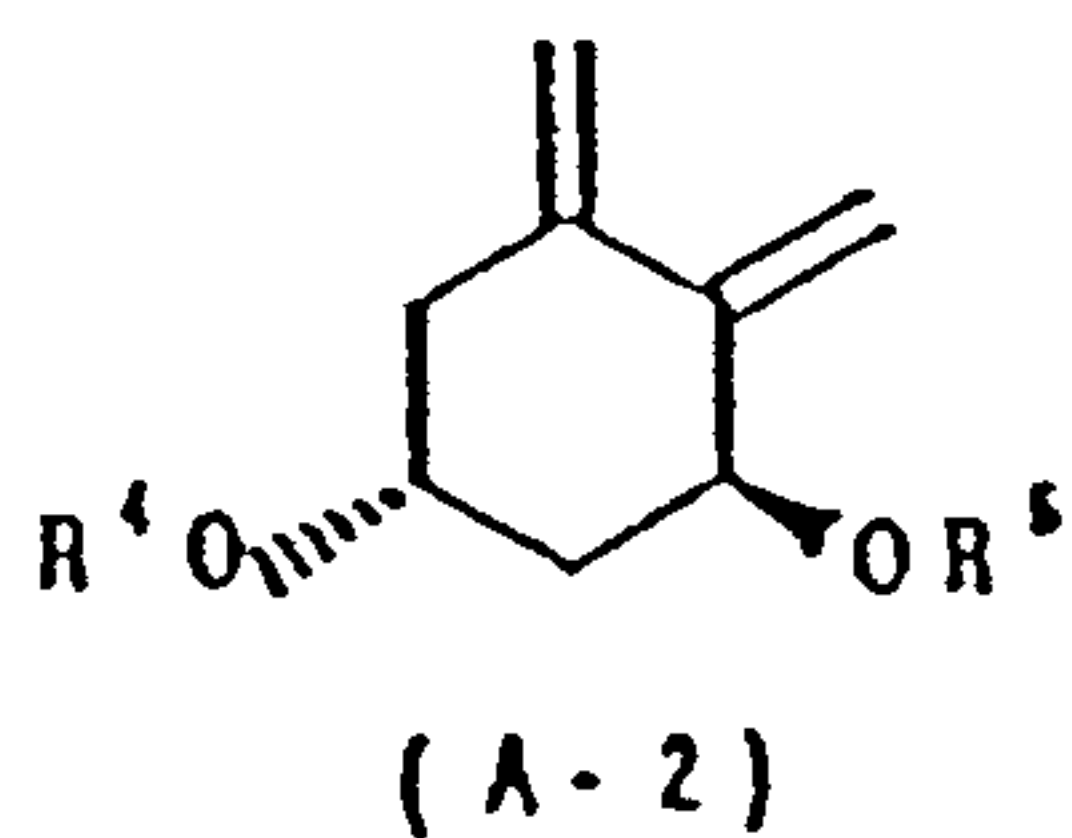
Compounds of general formula (I)



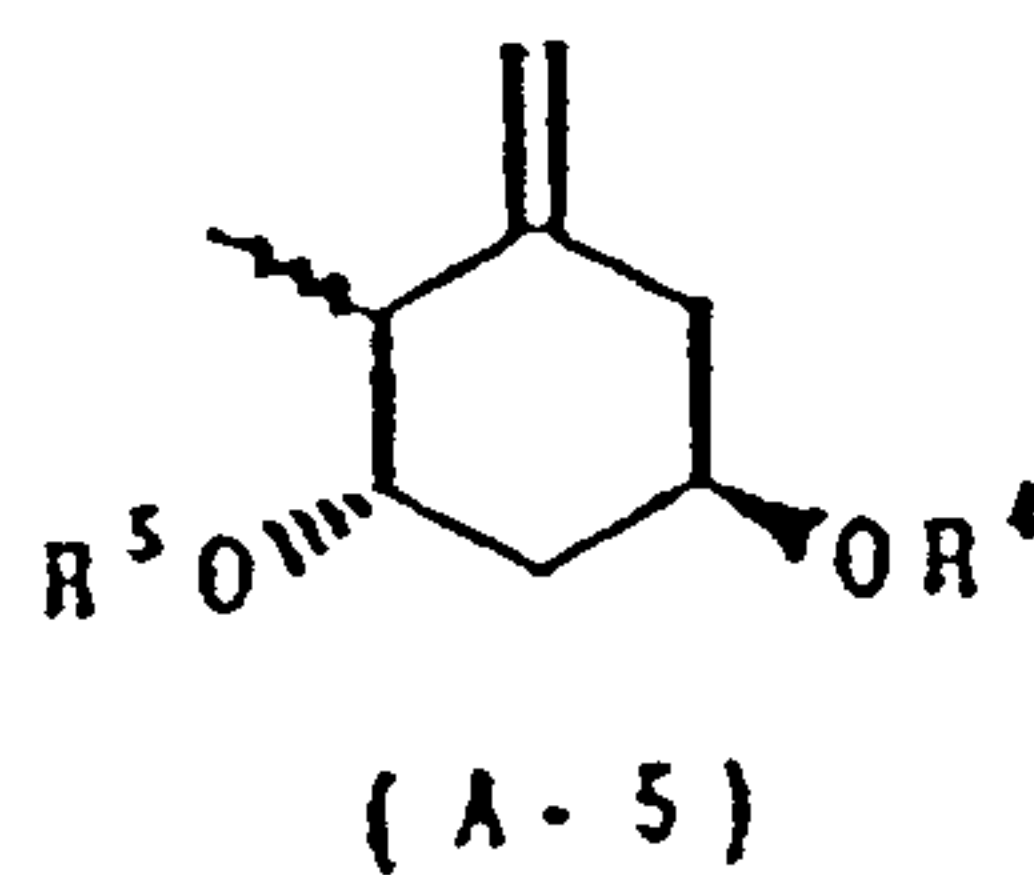
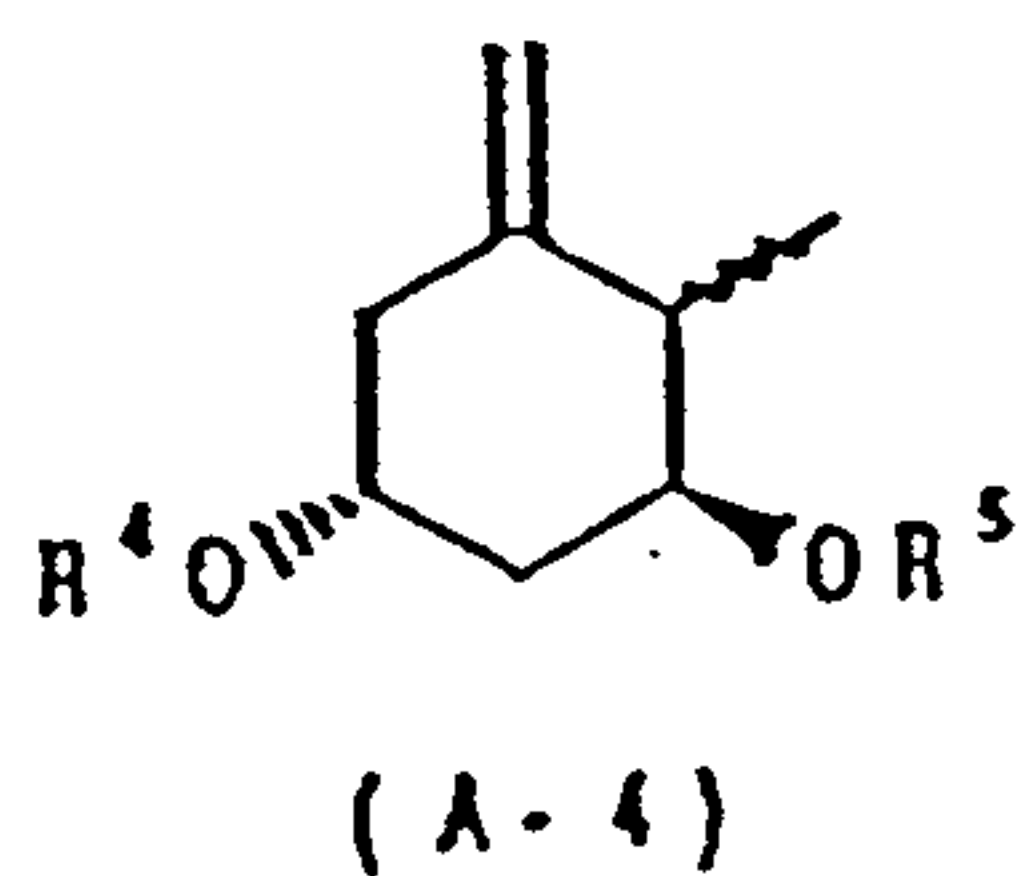
where R¹ and R², which may be the same or different, each represent a hydrogen atom or an aliphatic, cycloaliphatic, araliphatic or aryl group or together with the nitrogen atom to which they are attached form a heterocyclic group; R³ represents a methyl group having α - or β -configuration; X represents a valence bond or a C₁₋₂ alkylene group; Y represents -O-, -S-, -CH₂- or -NR- where R is a hydrogen atom or an organic group; Z represents a valence bond or a C₁₋₃ alkylene group; and A= represents a cyclohexylidene moiety characteristic of the A-ring of a 1 α -hydroxylated vitamin D or analogue thereof, with the proviso that when -X-Y-Z- together represent an alkylene group containing up to 4 carbon atoms A= does not carry an exocyclic methylene group at the 10-position.

Compounds of the general formula (I) as shown above wherein A= represents one of the groups

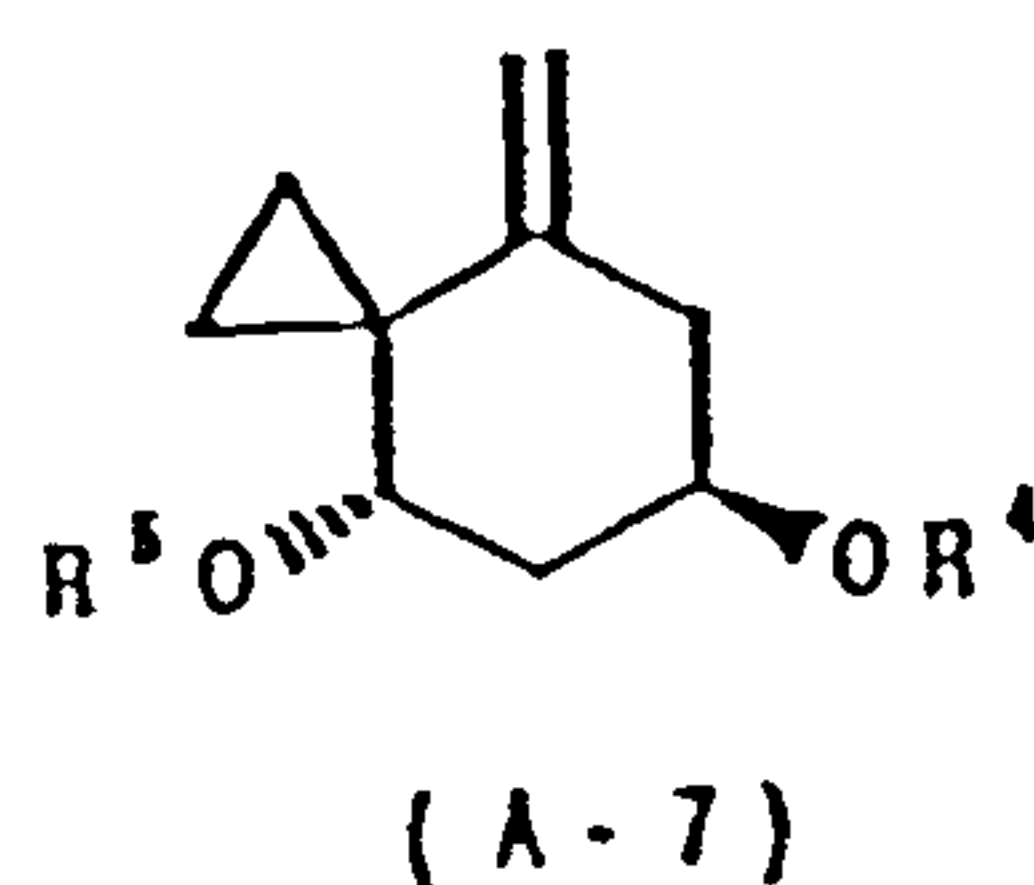
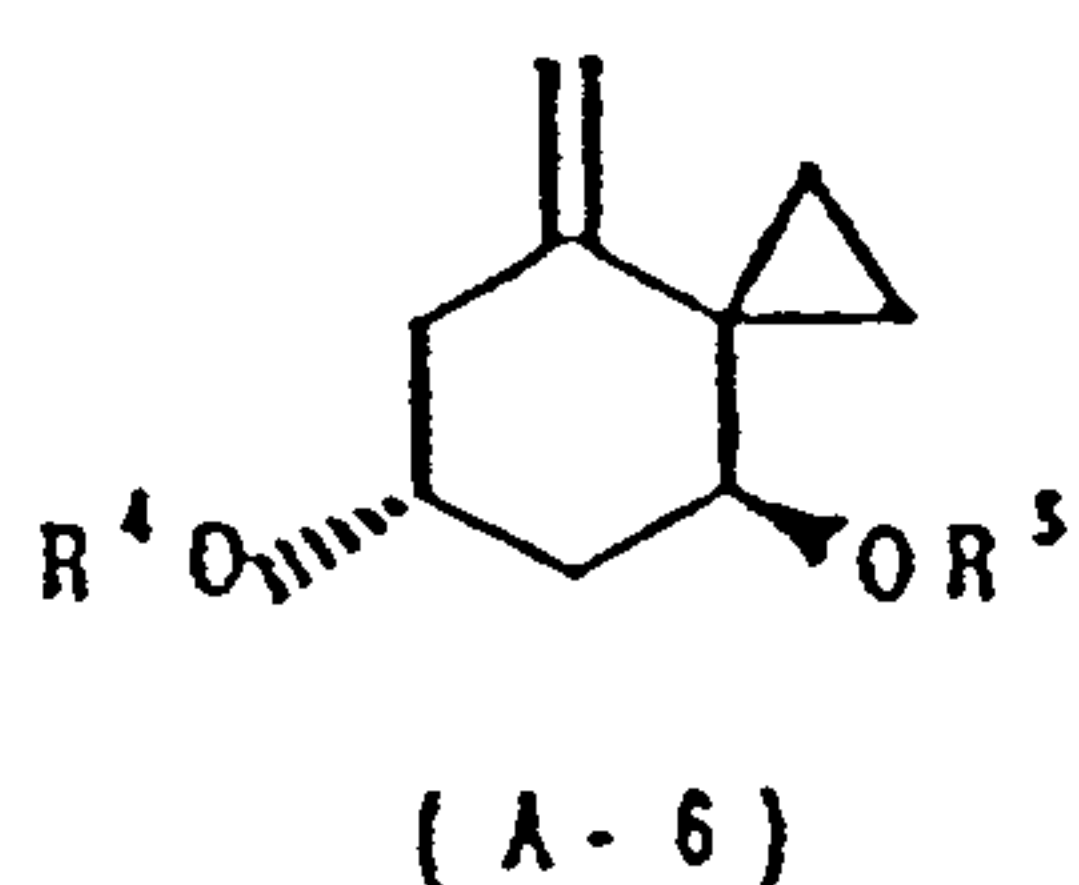
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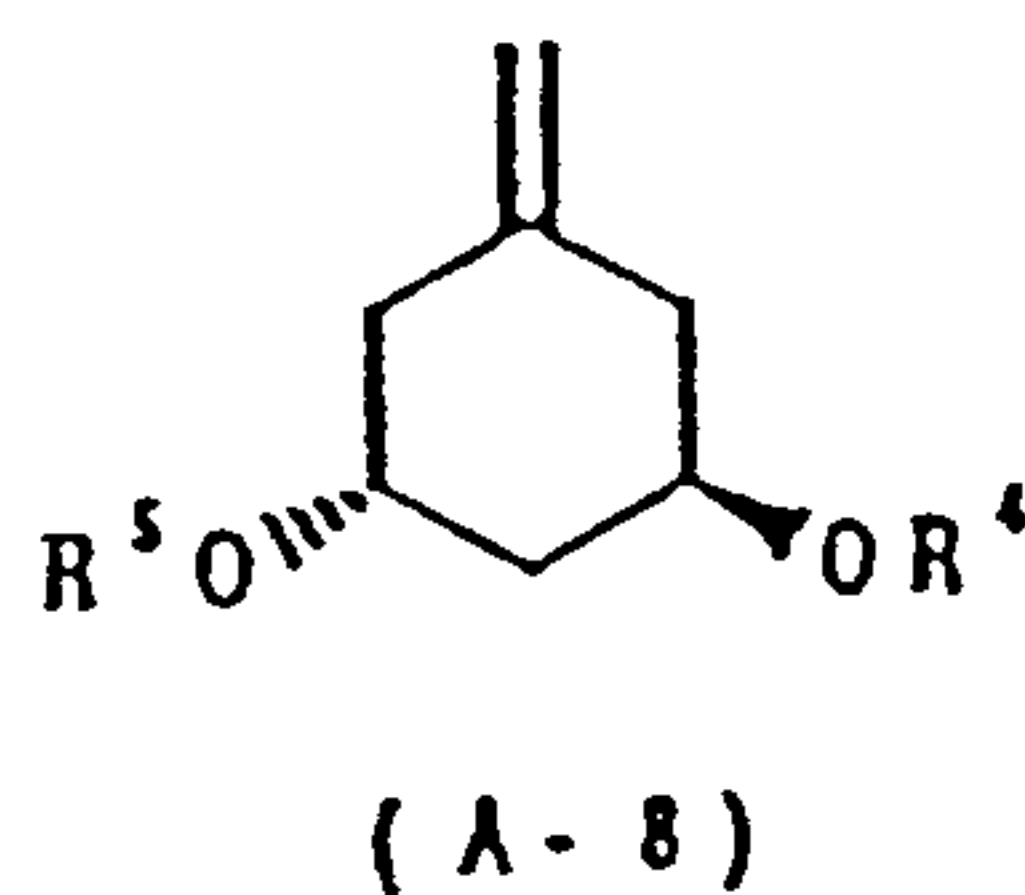


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and



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(where R^4 and R^5 , which may be the same or different, each represent a hydrogen atom or an O-protecting group).

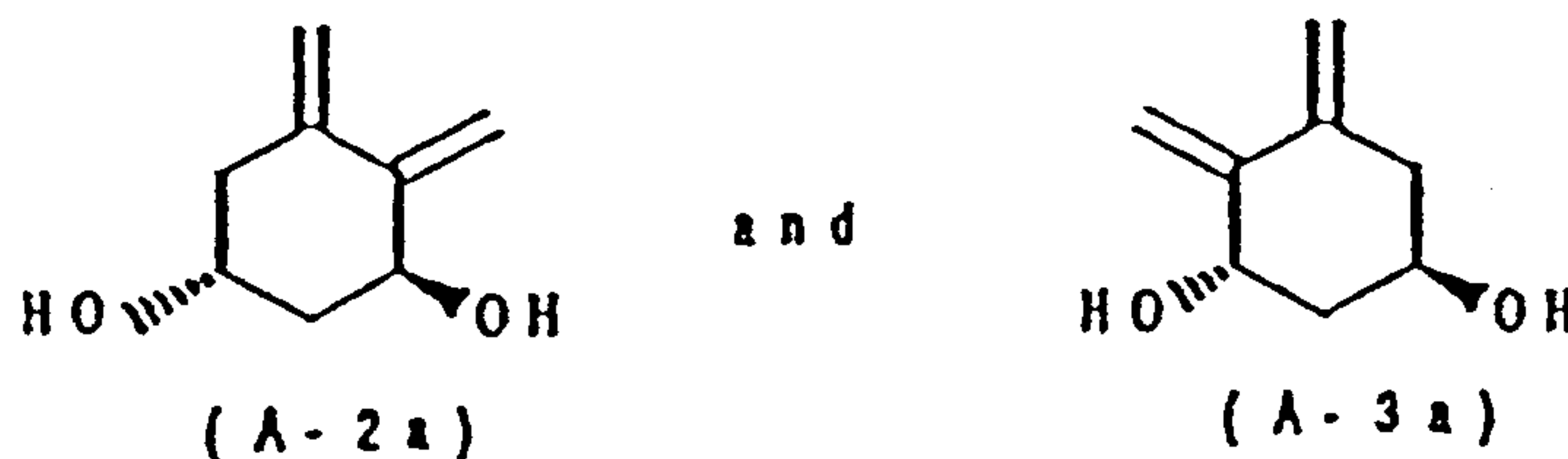
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Moreover compounds of the general formula (I) and the specification mentioned above wherein R^4 and R^5 represent etherifying silyl groups.

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Moreover compounds of the general formula (I) and the specification mentioned above wherein R^4 and R^5 are selected from hydrogen atoms and metabolically labile etherifying or esterifying groups.

Furthermore compounds of general formula (I) according to the above specification wherein A= represents one of the groups



15 Compound of the above general formula (I) wherein R^1 and R^2 are selected from hydrogen atoms, C_{1-6} alkyl groups, C_{3-8} cycloalkyl groups, C_{6-12} aryl C_{1-4} alkyl and optionally substituted C_{6-12} carbocyclic aryl groups.

Compounds of the above-specified general formula (I) wherein R^1 and R^2 are selected from hydrogen atoms, C_{1-6} alkyl groups and C_{3-8} cycloalkyl groups.

20 Compounds of the above-specified general formula (I) wherein R^1 and R^2 are selected from hydrogen atom, methyl, ethyl, and cyclopropyl groups.

Furthermore, the following compounds

25 $1\alpha, 3\beta$ -dihydroxy-20-epi-22-oxa-9,10-secochola-5(Z),7,10(19)-trienic acid, piperidine amide;

30 $1\alpha, 3\beta$ -dihydroxy-20-epi-23-homo-22-oxa-9,10-secochola-5(Z),7,10(19)-trienic acid, dimethylamine, cyclopropylamine and piperidine amides;

$1\alpha, 3\beta$ -dihydroxy-20-epi-22-oxa-9,10-secochola-5(Z),7,10(19)-trienic acid, morpholine amide;

35 $1\alpha, 3\beta$ -dihydroxy-20-epi-23-bis-homo-22-oxa-9,10-secochola-5(Z),7,10(19)-trienic acid, piperidine amide;

1 α ,3 β -dihydroxy-20-epi-23-homo-23-oxa-9,10-secochola-5(Z),7,10(19)-trienic acid, piperidine amide;

5 1 α ,3 β -dihydroxy-23-homo-23-oxa-9,10-secochola-5(Z),7,10(19)-trienic acid, diethylamine, cyclopropylamine and piperidine amides and 20-epi analogues thereof;

10 1 α ,3 β -dihydroxy-23-homo-23-oxa-9,10-secochola-5(Z),7-dienic acid, diethylamine, cyclopropylamine and piperidine amides and 29-epi analogues thereof;

15 1 α ,3 β -dihydroxy-23-homo-23-oxa-10-spirocyclopropyl-9,10-secochola-5(Z),7-dienic acid, diethylamine, cyclopropylamine and piperidine amides and 20-epi analogues thereof;

1 α ,3 β -dihydroxy-9,10-secochola-5(Z),7-dienic acid, piperidine amide;

20 1 α ,3 β -dihydroxy-10-spirocyclopropyl-9,10-secochola-5(Z),7-dienic acid, piperidine amide;

25 1 α ,3 β -dihydroxy-23-homo-9,10-secochola-5(Z),7-dienic acid, piperidine amide;

1 α ,3 β -dihydroxy-23-homo-10-spirocyclopropyl-9,10-secochola-5(Z),7-dienic acid, piperidine amide;

30 1 α ,3 β -dihydroxy-23-homo-19-nor-9,10-secochola-5,7-dienic acid, piperidine amide;

1 α ,3 β -dihydroxy-20-epi-23-homo-9,10-secochola-5(Z),7-dienic acid, piperidine amide;

35 1 α ,3 β -dihydroxy-20-epi-23-homo-9,10-seco-23-thiachola-5(Z),7,10(19)-trienic acid, piperidine amide;

23-aza-1 α ,3 β -dihydroxy-20-epi-23-bis-homo-9,10-
secochola-5(Z),7,10(19)-trienic acid, piperidine amide;

5 23-aza-1 α ,3 β -dihydroxy-20-epi-23-homo-9,10-secochola-
5(Z),7,10(19)-trienic acid, piperidine amide;

1 α ,3 β -dihydroxy-20-epi-19-nor-9,10-secochola-5,7-dienic
acid, piperidine amide;

10 1 α ,3 β -dihydroxy-23-homo-19-nor-23-oxa-9,10-secochola-
5,7-dienic acid, piperidine amide;

23-aza-1 α ,3 β -dihydroxy-9,10-secochola-5(Z),7,10(19)-
trienic acid, piperidine amide;

15 22-aza-1 α ,3 β -dihydroxy-20-epi-23-homo-9,10-secochola-
5(Z),7,10(19)-trienic acid, piperidine amide;

20 23-aza-1 α ,3 β -dihydroxy-9,10-secochola-5(Z),7,10(19)-
trienic acid, diethyl amide and the 20-epi analogue
thereof;

1 α ,3 β -dihydroxy-9,10-secochola-5(Z),7,10(19)-trienic
acid, N-methyl-N-phenyl amide and the 20-epi analogue
thereof; and

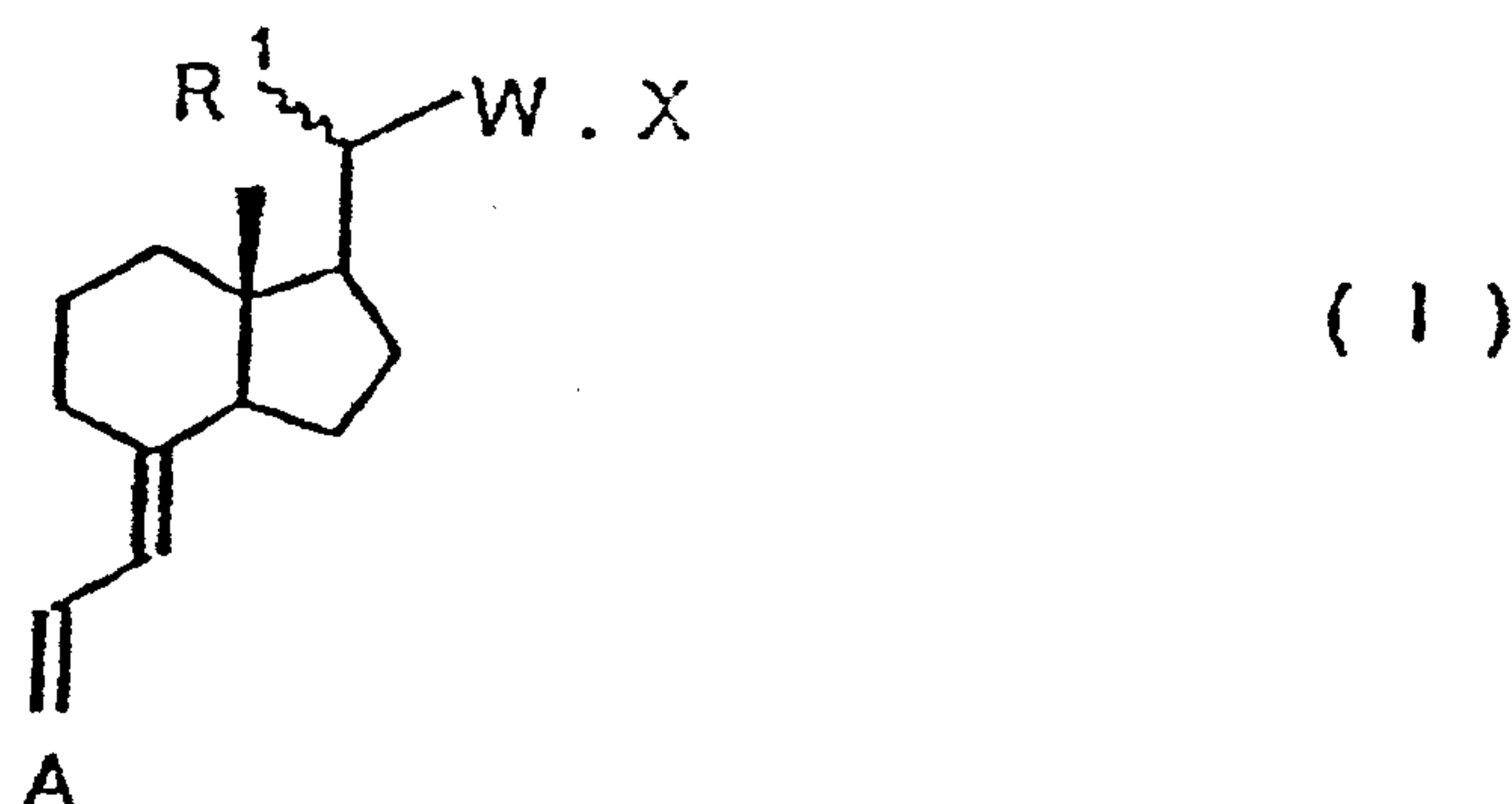
25 1 α -3 β -dihydroxy-9,10-secochola-5(Z),7-dienic acid, N-
methyl-N-phenyl amide and the 20-epi analogue thereof.

30

35

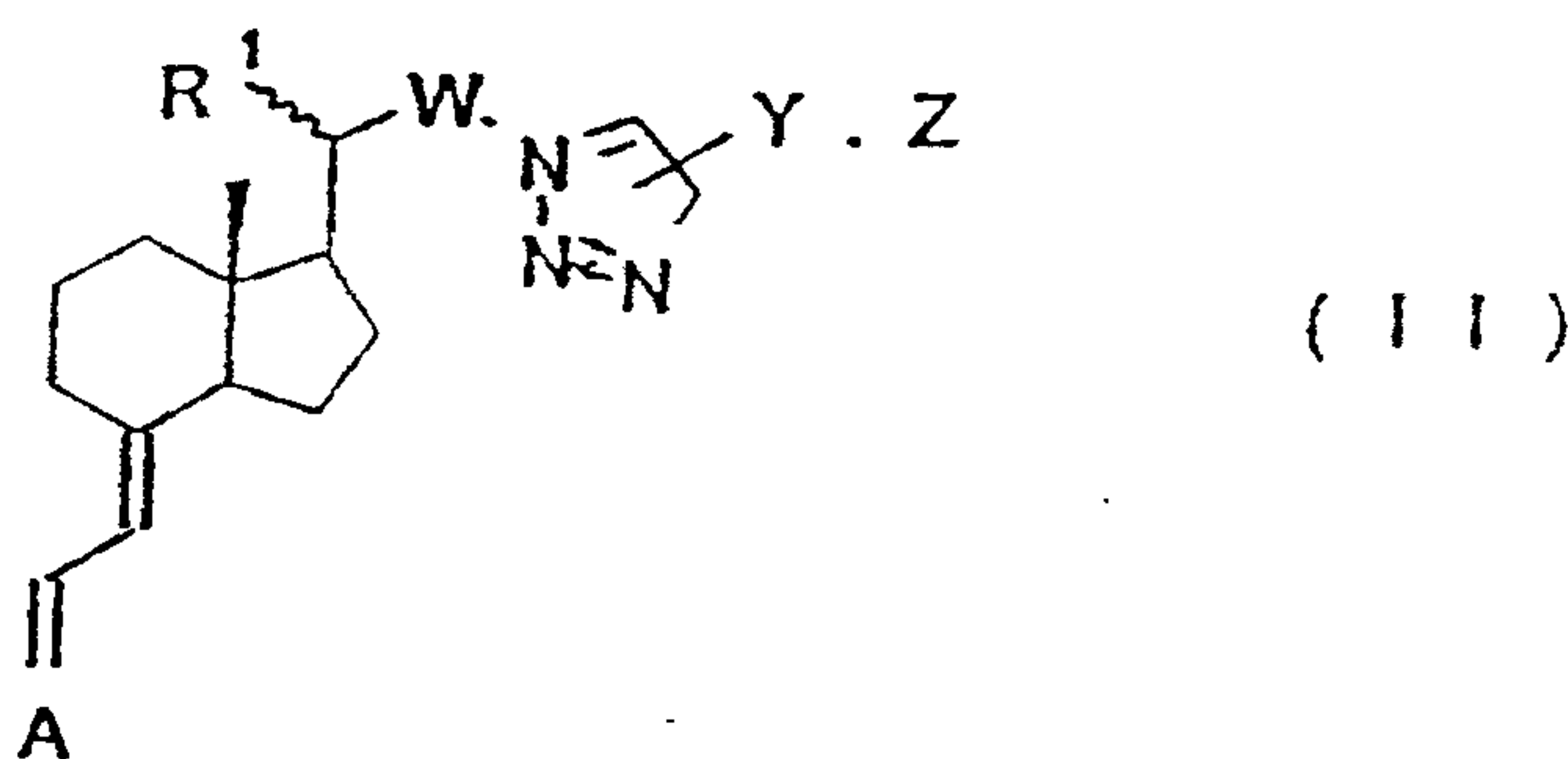
WO 95/03273 mentions the following vitamin D analogues:

Compounds of general formula (I):



where R^1 represents a methyl group having α - and β -configuration; W represents a valence bond or a C_{1-5} alkylene group; X represents azide or an optionally substituted triazole group; and A= represents a cyclohexylidene moiety characteristic of the A-ring of a 1α -hydroxylated vitamin D or analogue thereof.

Moreover compounds of the general formula (I) as shown above having the general formula (II)



wherein R^1 , W and A are as defined above; Y represents a valence bond or a lower alkylene group attached to the 4- or 5-position of the triazole ring; and Z represents either (i) a group $-CO-NR^2R^3$ in which R^2 and R^3 may be the same or different and are selected from hydrogen atoms, aliphatic, cycloaliphatic, araliphatic and aryl groups, or R^2 and R^3 together with the nitrogen atom to which they are attached form a heterocyclic group; or (ii) a group $-(R^4)(R^5)-OH$ in which R^4 and R^5 may be the same or different and are

selected from hydrogen atoms, aliphatic, cycloaliphatic, araliphatic and aryl groups, or R² and R³ together with the carbon atom to which they are attached form a C₃₋₈ carbocyclic ring.

5 Furthermore the compounds:

- 20α-(3-azidopropyl)-1α,3β-dihydroxy-9,10-secopregna-5(Z),7,10(19)-triene;
 20α-azido-1α,3β-dihydroxy-9,10-secopregna-5(Z),7,10(19)-triene;
 1α,3β-dihydroxy-20α-[4-(2-hydroxyprop-2-yl)-1,2,3-triazol-1-yl]-9,10-secopregna-
 10 5(Z),7,10(19)-triene;
 1α,3β-dihydroxy-20α-[4-(3-hydroxypent-3-yl)-1,2,3-triazol-1-yl]-9,10-secopregna-
 5(Z),7,10(19)-triene;
 1α,3β-dihydroxy-20α-[4-(2-hydroxyprop-2-yl)-1,2,3-triazol-1-ylmethyl]-9,10-secopregna-
 5(Z),7,10(19)-triene;
 15 1α,3β-dihydroxy-20β-[4-(3-hydroxypent-3-yl)-1,2,3-triazol-1-ylmethyl]-9,10-secopregna-
 5(Z),7,10(19)-triene;
 1α,3β-dihydroxy-20α-[4-(3-hydroxypent-3-yl)-1,2,3-triazol-1-ylmethyl]-9,10-secopregna-
 5(Z),7,10(19)-triene;
 1α,3β-dihydroxy-20α-[4-(N,N-pentamethylenecarbamoyl)-1,2,3-triazol-1-ylmethyl]-9,10-
 20 secopregna-5(Z),7,10(19)-triene;
 1α,3β-dihydroxy-20α-[4-(N,N-diethylcarbamoyl)-1,2,3-triazol-1-ylmethyl]-9,10-
 secopregna-5(Z),7,10(19)-triene;
 1α,3β-dihydroxy-20α-[4-(N-cyclopropylcarbamoyl)-1,2,3-triazol-1-ylmethyl]-9,10-
 secopregna-5(Z),7,10(19)-triene;
 25 1α,3β-dihydroxy-20α-[4-(N,N-3-oxapentamethylencarbamoyl)-1,2,3-triazol-1-ylmethyl]-
 9,10-secopregna-5(Z),7,10(19)-triene;
 1α,3β-dihydroxy-20α-[4-(N,N-diisopropylcarbamoyl)-1,2,3-triazol-1-ylmethyl]-9,10-
 secopregna-5(Z),7,10(19)-triene;
 1α,3β-dihydroxy-20β-[4-(N,N-pentamethylencarbamoyl)-1,2,3-triazol-1-ylmethyl]-9,10-
 30 secopregna-5(Z),7,10(19)-triene;
 1α,3β-dihydroxy-20β-[4-(N,N-diethylcarbamoyl)-1,2,3-triazol-1-ylmethyl]-9,10-secopregna-
 5(Z),7,10(19)-triene;
 1α,3β-dihydroxy-20β-[4-(N-cyclopropylcarbamoyl)-1,2,3-triazol-1-ylmethyl]-9,10-
 secopregna-5(Z),7,10(19)-triene;

1 α ,3 β -dihydroxy-20 β -[4-(N,N-3-oxapentamethylenecarbamoyl)-1,2,3-triazol-1-ylmethyl]-
9,10-secopregna-5(Z),7,10(19)-triene;

1 α ,3 β -dihydroxy-20 β -[4-(N,N-diisopropylcarbamoyl)-1,2,3-triazol-1-ylmethyl]-9,10-
secopregna-5(Z),7,10(19)-triene;

5 1 α ,3 β -dihydroxy-20 α -{2-[4-(N,N-pentamethylenecarbamoyl)-1,2,3-triazol-1-yl]ethyl}-9,10-
secopregna-5(Z),7,10(19)-triene;

1 α ,3 β -dihydroxy-20 α -{2-[4-(N,N-diethylcarbamoyl)-1,2,3-triazol-1-yl]ethyl}-9,10-
secopregna-5(Z),7,10(19)-triene;

1 α ,3 β -dihydroxy-20 α -{2-[4-(N-cyclopropylcarbamoyl)-1,2,3-triazol-1-yl]ethyl}-9,10-
10 secopregna-5(Z),7,10(19)-triene;

- 1 α ,3 β -dihydroxy-20 α -{2-[4-(N,N-3-oxapentamethylene-carbamoyl)-1,2,3-triazol-1-yl]ethyl}-9,10-secopregna-5(Z),7,10(19)-triene;
- 5 1 α ,3 β -dihydroxy-20 α -{2-[4-(N,N-diisopropylcarbamoyl)-1,2,3-triazol-1-yl]ethyl}-9,10-secopregna-5(Z),7,10(19)-triene;
- 1 α ,3 β -dihydroxy-20 α -[4-(1-hydroxycyclohex-1-yl)-1,2,3-triazol-1-ylmethyl]-9,10-secopregna-5(Z),7,10(19)-triene;
- 10 1 α ,3 β -dihydroxy-20 α -{2-[4-(3-hydroxypent-3-yl)-1,2,3-triazol-1-yl]ethyl}-9,10-secopregna-5(Z),7,10(19)-triene;
- 1 α ,3 β -dihydroxy-20 β -{2-[4-(3-hydroxypent-3-yl)-1,2,3-triazol-1-yl]ethyl}-9,10-secopregna-5(Z),7,10(19)-triene;
- 15 1 α ,3 β -dihydroxy-20 α -{3-[4-(3-hydroxypent-3-yl)-1,2,3-triazol-1-yl]propyl}-9,10-secopregna-5(Z),7,10(19)-triene;
- 1 α ,3 β -dihydroxy-20 α -{3-[4-(3-methyl-3-hydroxybutyl)-1,2,3-triazol-1-yl]propyl}-9,10-secopregna-5(Z),7,10(19)-triene;
- 20 1 α ,3 β -dihydroxy-20 α -{3-[4-(2-methyl-2-hydroxypentyl)-1,2,3-triazol-1-yl]propyl}-9,10-secopregna-5(Z),7,10(19)-triene;
- 1 α ,3 β -dihydroxy-20 α -{3-[4-(4-ethyl-4-hydroxyhexyl)-1,2,3-triazol-1-yl]propyl}-9,10-secopregna-5(Z),7,10(19)-triene;
- 25 1 α ,3 β -dihydroxy-20 α -{3-[4-(2-hydroxybut-2-yl)-1,2,3-triazol-1-yl]propyl}-9,10-secopregna-5(Z),7,10(19)-triene;
- 30 1 α ,3 β -dihydroxy-20 α -{3-[4-(4-methyl-2-hydroxypent-2-yl)-1,2,3-triazol-1-yl]propyl}-9,10-secopregna-5(Z),7,10(19)-triene;
- 1 α ,3 β -dihydroxy-20 α -{3-[4-(2,4-dimethyl-3-hydroxypent-3-yl)-1,2,3-triazol-1-yl]propyl}-9,10-secopregna-5(Z),7,10(19)-triene;
- 35 1 α ,3 β -dihydroxy-20 α -[4-(2-ethyl-2-hydroxybutyl)-1,2,3-

triazol-1-ylmethyl]-9,10-secopregna-5(Z),7,10(19)-triene;

1 α ,3 β -dihydroxy-20 α -[5-(2-ethyl-2-hydroxybutyl)-1,2,3-triazol-1-ylmethyl]-9,10-secopregna-5(Z),7,10(19)-triene;

1 α ,3 β -dihydroxy-20 α -[4-(3-hydroxypent-3-yl)-1,2,3-triazol-1-ylmethyl]-9,10-secopregna-5(E),7-diene;

1 α ,3 β -dihydroxy-20 α -[4-(3-hydroxypent-3-yl)-1,2,3-triazol-1-ylmethyl]-9,10-secopregna-5(Z),7-diene;

1 α ,3 β -dihydroxy-20 α -[4-(3-hydroxypent-3-yl)-1,2,3-triazol-1-ylmethyl]-10-spirocyclopropyl-9,10-secopregna-5(E),7-diene;

1 α ,3 β -dihydroxy-20 α -[4-(3-hydroxypent-3-yl)-1,2,3-triazol-1-ylmethyl]-10-spirocyclopropyl-9,10-secopregna-5(Z),7-diene;

1 α ,3 β -dihydroxy-20 β -[4-(3-hydroxypent-3-yl)-1,2,3-triazol-1-ylmethyl]-19-nor-9,10-secopregna-5,7-diene;

1 α ,3 β -dihydroxy-20 α -[4-(3-methyl-3-hydroxybutyl)-1,2,3-triazol-1-ylmethyl]-9,10-secopregna-5(Z),7,10(19)-triene;

1 α ,3 β -dihydroxy-20 α -[4-(2-methyl-2-hydroxypentyl)-1,2,3-triazol-1-ylmethyl]-9,10-secopregna-5(Z),7,10(19)-triene;

1 α ,3 β -dihydroxy-20 α -[4-(4-ethyl-4-hydroxyhexyl)-1,2,3-triazol-1-ylmethyl]-9,10-secopregna-5(Z),7,10(19)-triene;

1 α ,3 β -dihydroxy-20 α -[4-(2-hydroxybut-2-yl)-1,2,3-triazol-1-ylmethyl]-9,10-secopregna-5(Z),7,10(19)-triene;

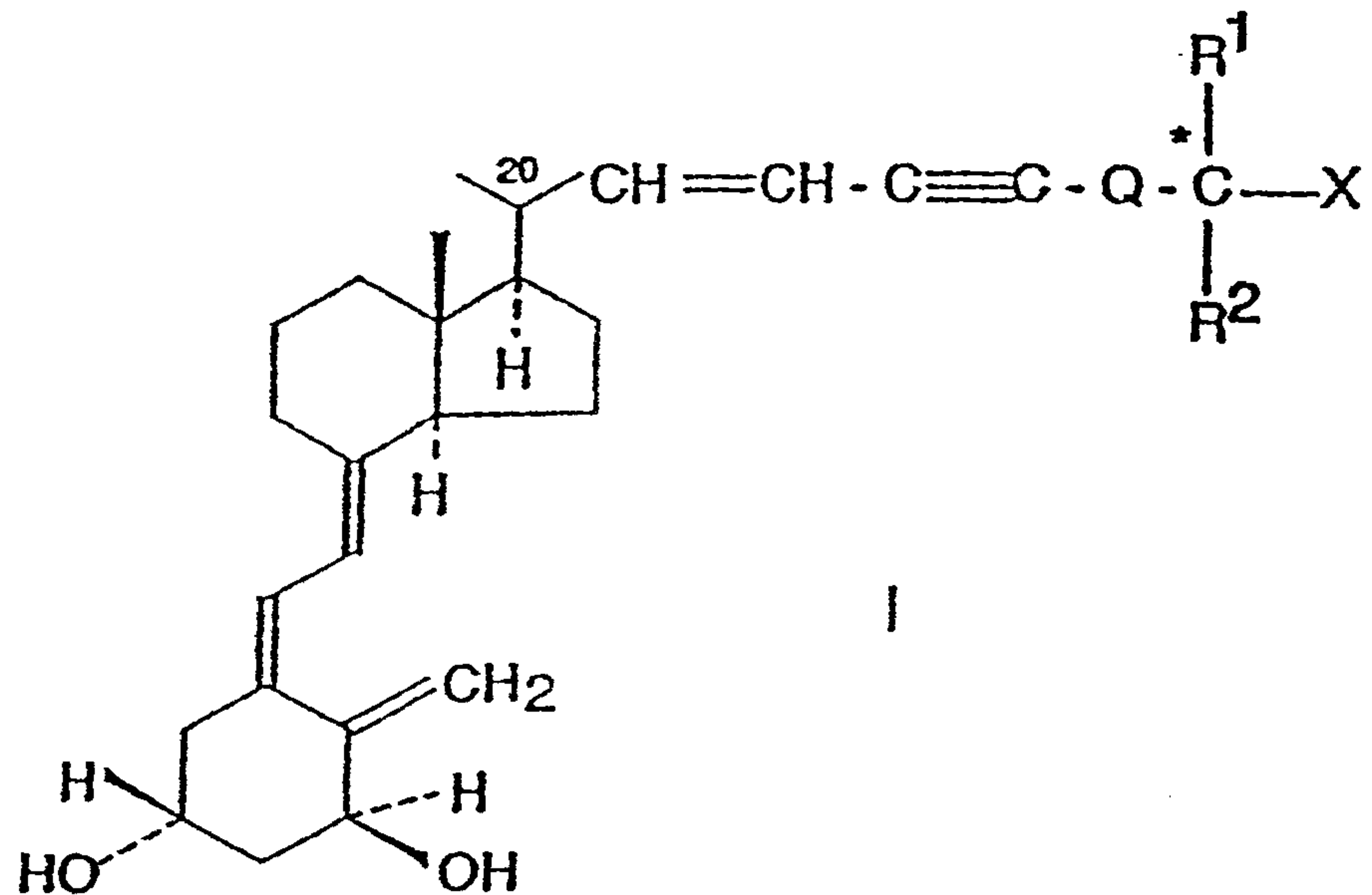
1 α ,3 β -dihydroxy-20 α -[4-(4-methyl-2-hydroxypent-2-yl)-1,2,3-triazol-1-ylmethyl]-9,10-secopregna-5(Z),7,10(19)-triene;

1 α ,3 β -dihydroxy-20 α -[4-(2,4-dimethyl-3-hydroxypent-3-yl)-1,2,3-triazol-1-ylmethyl]-9,10-secopregna-5(Z),7,10(19)-triene; and

1 α ,3 β -dihydroxy-20 α -[4-(2-hydroxypheneth-2-yl)-1,2,3-triazol-1-ylmethyl]-9,10-secopregna-5(Z),7,10(19)-triene.

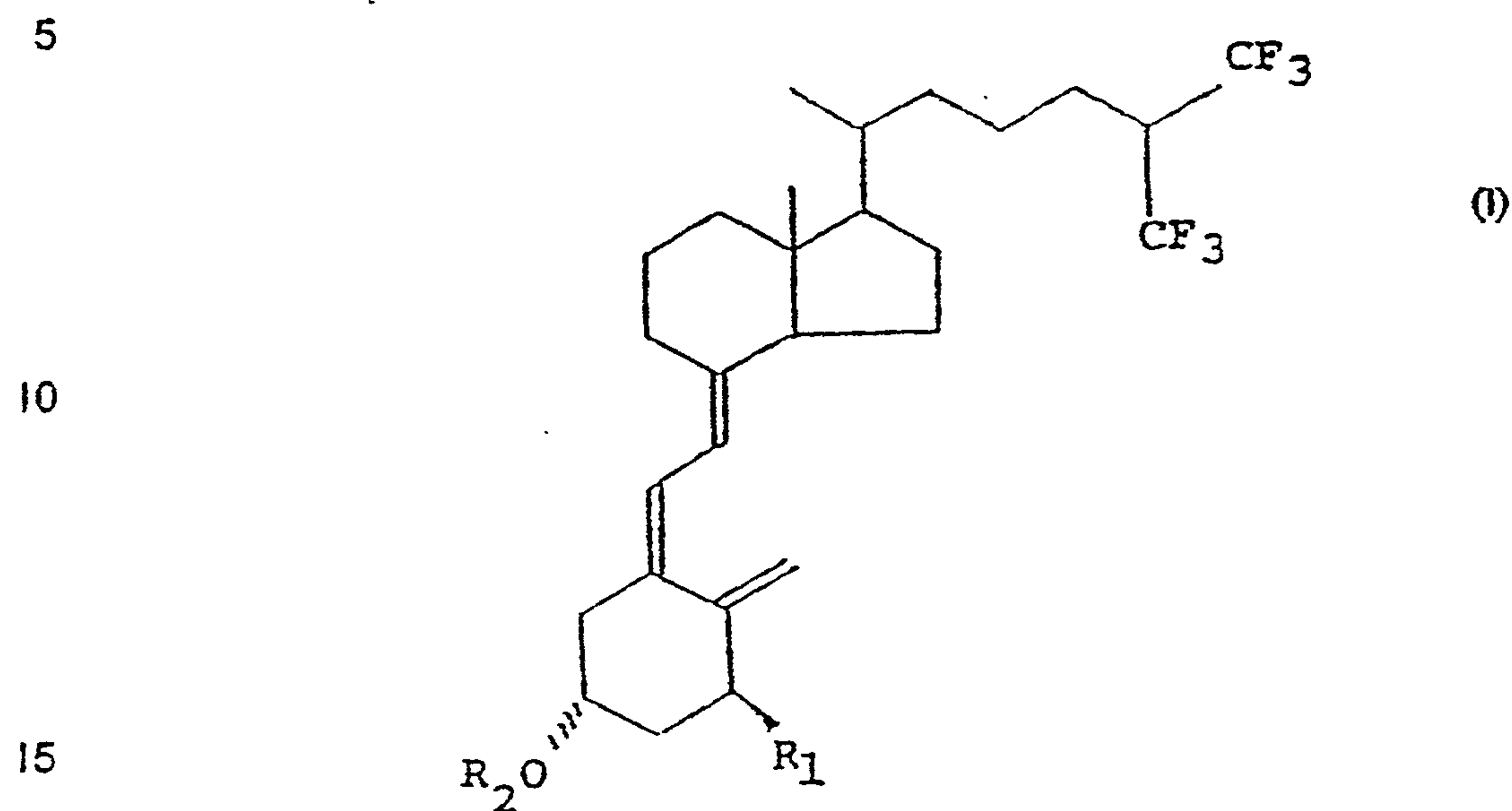
WO 95/02577 mentions the following vitamin D analogues:

A compound of the formula I



in which formula X is hydrogen or hydroxy; R^1 and R^2 , which may be the same or different, stand for hydrogen or C_1 - C_4 hydrocarbyl; or R^1 and R^2 , taken together with the carbon atom bearing the group X, can form a C_3 - C_8 carbocyclic ring; Q is a single bond or a C_1 - C_4 hydrocarbylene diradical, the expression hydrocarbyl radical (hydrocarbylene diradical) indicating the residue after removal of 1 (2) hydrogen atom(s) from a straight, branched or cyclic saturated or unsaturated hydrocarbon; R^1 , R^2 and/or Q may be optionally substituted with one or more fluorine atoms; and prodrugs of I in which one or more of the hydroxy groups are masked as groups which can be reconverted to hydroxy groups in vivo.

A compound of the formula (I)



wherein R₁ is a hydrogen atom, a hydroxyl group or a protected hydroxyl group and R₂ is a hydrogen atom or a protecting group.

such as

26,26,26,27,27,27-Hexafluoro-1 α -hydroxyvitamin D₃.

26,26,26,27,27,27-Hexafluorovitamin D₃.

The COX inhibitors according to the invention in addition to the ASA include other NSAIDS known in the art.

5 EXAMPLE 1

The influence of $1\alpha,25(\text{OH})_2$ -vitamin D_3 , calcium and acetylsalicylic acid on AOM-induced aberrant crypt foci and colorectal tumors in rat colon

Materials and Methods

10 Animals

128 male F344/Mol-rats, SPF (F344/Ntac@Mol) three to four weeks old were purchased from M & B (Li. Skensved, Denmark).

Diets

- 15 All groups of rats are offered a powdered purified diet. The amounts of $1\alpha,25(\text{OH})_2 \text{D}_3$, calcium and acetylsalicylic acid in the diet for each group are presented in table 1.

Chemical

Azoxymethane (AOM) was obtained from Sigma Chemical (St. Louis, MO).

- 20 $1\alpha,25(\text{OH})_2 \text{D}_3$ was provided by Leo Pharmaceutical Products, Ballerup, Denmark.
Acetylsalicylic acid (ASA) was obtained from Nycomed Danmark A/S.

Housing

- The animals were kept in disposable plastic cages with an inserted steel grid floor, two
15 animals per cage, in flexible film isolaters (IsotecTM 12134, Olac, Oxford, UK) during the dosing period, and one week after the termination of the dosing with AOM. For the remaining period of the study, the animals were kept in stainless steel wire cages with two animals per cage. During the study, the temperature was maintained at $21 \pm 1^\circ \text{C}$, a relative humidity at $55 \pm 5\%$, air was changed 10 times/hr, and fluorescent light was on
0 from 2100 to 0900.

Experimental design

The animals were randomly assigned to eight experimental groups of 16 animals and fed their respective diets for 19 days (table 1). Then all groups were dosed AOM, 15 mg/kg body weight subcutaneously twice one week apart. The animals were maintained on their respective diets for a 16-week observation period. Body weight, food and water consumption were measured weekly. At termination of the study, ten animals from each of the groups 1, 6, 7, and 8 were placed separately in metabolism cages for 24 hours. After rinsing with 10 ml water the urine was collected for determination of volume, pH, calcium and creatinine. Eighteen weeks after the first AOM injection the animals were sacrificed and serum was collected for future analysis of acetylsalicylic acid. The abdomen and thoracic cavity of all animals were examined to reveal macroscopic changes. The weight of kidneys and adrenals were recorded. These organs, the stomach, small intestine, and thyroid gland with parathyroids were preserved in 4% buffered formaldehyde, pending future requirement for histopathological examination. The large intestine was cut longitudinally, rinsed in 0.9% NaCl, and divided into two pieces of equal length, pinned on a cork slab, and fixed in cold 4% neutral buffered formaldehyde according to (1).

Table 1:

20 Scheme of treatment

Group	Animal no.	ASA ppm	1α,25(OH)₂D₃ μg/ kg *	Ca Ppm
1	1-16	0	0	5000
2	17-32	0	0	2500
3	33-48	0	0	7500
4	49-64	0	0.02	7500
5	65-80	300	0	7500
6	81-96	300	0.02	7500
7	97-112	300	0.02	5000
8	113-128	300	0.02	2500

*) The content of vitamin D₂ in SYN 8 is unchanged 1000 IU/ kg.

Urine analysis

Analysis for creatinine and calcium were analysed using a Combas Mira S analyser using the relevant kit for each parameter (Roche Diagnostic Systems).

5 Assessment of ACF

The ACF were visualised by Giemsa staining, recorded using a stereomicroscope at x40 magnification, and grouped into small (1-3 crypts), medium (4-6 crypts), large (7-9 crypts), extra-large (≥ 10 crypts), and ACF >7 (> 7 crypts). The ACF were distinguished from normal crypts according to (1).

10

Assessment of tumors

Colonic tissue deviations suspected to be tumors at gross examination or under stereomicroscope were embedded in paraffin, sectioned 4-6 μm , and stained with hematoxylin and eosin for histopathological examination.

15

Statistical analysis

All data are presented as mean $\pm$ SE. One way analysis of variance with repeated measures on one factor was used to analyse body weight and food and water consumption. The analyses were followed by a Least Significant Difference test if significant. Data on organ weights, urine chemistry, and ACF was analysed by an one-way analysis of variance followed by a Least Squares Means. The homogeneity of variance among groups was evaluated by judgement of standard residual plots (General Linear Model procedure). Data on tumor bearing animals was analysed by Fishers exact test. A $p < 0.05$ was considered significant. All statistical analysis was carried out using SAS release 6.12.

25

Results and discussion

Urine chemistry

30 The urinary volume, pH, concentration of calcium and creatinine of group 1, 6, 7, and 8 were examined (see Table 2. The concentration of calcium was statistically significant

decreased in group 6 and 8. A non-significant decrease was observed in group 7 as well. The effect is likely to be caused by a $1\alpha,25(\text{OH})_2 \text{D}_3$ induced renal retention of calcium. The pH of group 7 was statistically significant higher than the control value. As no significant changes were observed in groups 6 and 8 this is probably a fortuitousness.

- 5 The results further demonstrate that no side effect of the combination treatment according to the invention is observed.

Table 2. Urine chemistry ^a

Group ^b	N	volume ml	pH	calcium mmol/l	creatinine $\mu\text{mol/l}$
1	10	11.3 ± 1	7.2 ± 1.1	6.57 ± 1.9	7062 ± 560
6	10	12.1 ± 2	6.9 ± 1.1	$4.49 \pm 1.6^*$	7412 ± 858
7	10	11.8 ± 1	$8.6 \pm$	5.80 ± 2.0	7164 ± 429
8	10	12.0 ± 2	7.8 ± 1.1	$2.8 \pm 1.7^*$	6370 ± 512

a: Mean $\pm$ SD.

- 10 b: Group 1, control diet (5000 ppm Ca); group 6, $0.02 \mu\text{g } 1\alpha,25(\text{OH})_2 \text{D}_3$, 300 ppm acetyl salicylic acid, and 7500 ppm Ca in the diet; group 7, $0.02 \mu\text{g } 1\alpha,25(\text{OH})_2 \text{D}_3$, 300 ppm acetyl salicylic acid, and 5000 ppm Ca in the diet; group 8, $0.02 \mu\text{g } 1\alpha,25(\text{OH})_2 \text{D}_3$, 300 ppm acetyl salicylic acid, and 2500 ppm Ca in the diet.

- * mark statistically significance to control group when a Least Square Means test was
15 performed ($p < 0.05$).

Organ weight

- The relative organ weight was calculated per 100g body weight. The terminal body weight, the absolute weight of kidneys and adrenals, and the relative weight of adrenals
20 were not affected by treatment. The relative kidney weight of group 5 and 8 was increased when compared to the control group. The changes are minor and not believed to be related to treatment.

ACF and Tumors

- 25 ACF as intermediary bio-markers and not tumors/cancers were end-points for this study. Nevertheless a number of animals developed tumors during the trial. According to the

adenoma – carcinoma sequence tumors represent late-stage carcinogenesis and are therefore included in the results. Large ACF, x-large ACF and tumors represent lesions with significant correlation to subsequent invasive cancer and cancer related death. The group of animals with lesions containing more than seven crypts (ACF>7) is considered
5 as high-risk animals with regard to cancer development.

The total number and distribution of ACF and tumors are presented in Table 3 and appendix 3.

Animals fed on a low calcium diet of 2500 ppm (mimicking an older ,western-world,
10 human population) were most susceptible to ACF development. Increasing calcium levels in the diet significantly reduced the number of ACF. In animals fed 7500 ppm calcium and 1,25(OH)₂-D₃ a statistically significant decrease in the total number of ACF was observed when compared to group 1 and 2 (35.1 vs. 61.7, and 35.1 vs. 88.3, respectively). In
15 animals fed 7500 ppm calcium, 1,25 (OH)₂D₃ and ASA a non-significant reduction in total ACF was observed when compared to group 1 (44.6 vs. 61.7).

Table 3

Mean number of aberrant crypt foci (ACF) or tumors in AOM-induced rats fed diets containing 1 α ,25(OH)₂ D₃, acetyl salicylic acid, and various doses of Calcium^{a-b}

20

Grou p ^c	N	Total	Small	Medium	Large	Extra Large	Tumors	ACF>7 crypts
1	15	61.7 $\pm$ 8.4	39.8 $\pm$ 5.0	18.9 $\pm$ 3.4	2.3 $\pm$ 0.7	0.7 $\pm$ 0.3	0.2 $\pm$ 0.1	3.2 $\pm$ 0.8
2	16	88.3 $\pm$ 9.4	55.1 $\pm$ 6.3	27.6 $\pm$ 3.8	4.5 $\pm$ 0.9	1.1 $\pm$ 0.4	0.6 $\pm$ 0.2	6.1 $\pm$ 1.3
3	16	46.8 $\pm$ 8.7	35.4 $\pm$ 6.5	9.9 $\pm$ 2.3	1.0 $\pm$ 0.3	0.4 $\pm$ 0.2	0.9 $\pm$ 0.3	2.4 $\pm$ 0.6
4	16	35.1 $\pm$ 5.0	26.1 $\pm$ 3.5	7.5 $\pm$ 1.5	1.1 $\pm$ 0.3	0.4 $\pm$ 0.1	1.1 $\pm$ 0.3	2.6 $\pm$ 0.5
5	16	45.4 $\pm$ 5.7	33.0 $\pm$ 5.1	10.9 $\pm$ 1.7	1.2 $\pm$ 0.4	0.3 $\pm$ 0.1	0.5 $\pm$ 0.2	1.9 $\pm$ 0.6
6	16	44.6 $\pm$ 6.7	32.9 $\pm$ 5.0	10.4 $\pm$ 2.1	0.6 $\pm$ 0.3	0.6 $\pm$ 0.3	0.5 $\pm$ 0.2	1.8 $\pm$ 0.6
7	16	73.6 $\pm$ 8.8	52.2 $\pm$ 6.0	19.3 $\pm$ 2.9	1.8 $\pm$ 0.3	0.3 $\pm$ 0.1	0.8 $\pm$ 0.3	2.9 $\pm$ 0.7
8	16	90.3 $\pm$	63.7 $\pm$ 7.0	23.3 $\pm$ 3.4	3.1 $\pm$ 0.9	0.3 $\pm$ 0.1	0.1 $\pm$ 0.1	3.4 $\pm$ 0.9

a: Mean $\pm$ SE

b: Small foci: 1-3 crypts; Medium foci: 4-6 crypts; Large foci: 7-9 crypts; Extra large foci: $\geq$ 10 crypts; ACF>7: >7 crypts

c: Group 1: Normal Calcium control group (5000 ppm Ca); Group 2: Low Calcium control group (2500 ppm Ca); Group 3: Supplemental Calcium group (7500 ppm Ca); Group 4: 0.02 μg $1\alpha,25(\text{OH})_2\text{D}_3$ and 7500 ppm Ca; Group 5: 300 ppm acetyl salicylic acid and 7500 ppm Ca; Group 6: 0.02 μg $1\alpha,25(\text{OH})_2\text{D}_3$, 300 ppm acetyl salicylic acid, and 7500 ppm Ca; Group 7: 0.02 μg $1\alpha,25(\text{OH})_2\text{D}_3$, 300 ppm acetyl salicylic acid, and 5000 ppm Ca; Group 8: 0.02 μg $1\alpha,25(\text{OH})_2\text{D}_3$, 300 ppm acetyl salicylic acid, and 2500 ppm Ca

When comparing the high-risk group (ACF>7) on the low calcium diet (2500 ppm) to the high-risk group treated with the three active components (group 6), a highly significant reduction in the number of colonic lesions (ACF>7) (6.1 vs. 1.8, $p=0.0001$) is seen.

Table A

General Linear Models Procedure

Least Squares Means

GROUP: ACF>7

LSMEAN	Number
1	3.20000000
2	6.12500000
3	2.37500000
4	2.56250000
5	1.93750000
6	1.75000000
7	2.93750000
8	3.43750000

Probability of LSMEAN(i) = LSMEAN(j)

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46

	i/j	1	2	3	4	5	6	7	8
	1	.	0.0094	0.4579	0.5661	0.2567	0.1931	0.8131	0.8306
	2	0.0094	.	0.0008	0.0014	0.0002	0.0001	0.0041	0.0151
	3	0.4579	0.0008	.	0.8637	0.6888	0.5674	0.6067	0.3316
5	4	0.5661	0.0014	0.8637	.	0.5674	0.4574	0.7314	0.4236
	5	0.2567	0.0002	0.6888	0.5674	.	0.8637	0.3607	0.1713
	6	0.1931	0.0001	0.5674	0.4574	0.8637	.	0.2781	0.1242
	7	0.8131	0.0041	0.6067	0.7314	0.3607	0.2781	.	0.6472
	8	0.8306	0.0151	0.3316	0.4236	0.1713	0.1242	0.6472	.

10

Separate addition of Calcium, $1\alpha,25(\text{OH})_2\text{D}_3$ or ASA, or combinations of two, also gave significant reductions in ACF indicating a protective effect. The most pronounced effect was obtained with the combination of all 3 ingredients, suggesting an additive/synergistic effect.

15

When regarding low calcium diets, the increase in total number of ACF in group 8 was primarily due to an increase in small ACF. Whereas in group 2, in addition to a non statistically significant increase in small ACF ($p=0.061$), statistically significant increases in medium, large sized ACF and $\text{ACF}>7$ were observed. This demonstrates that addition of $1\alpha,25(\text{OH})_2\text{D}_3$ and ASA to the fodder protects against progression of small ACF in animals given sub-optimal calcium (2500 ppm). When increasing calcium to a higher level (5000 ppm), an obvious trend is seen (1.8 vs. 3.2, $p=0.19$) supporting the anticancer effect of $1\alpha,25(\text{OH})_2\text{D}_3$ and ASA is recognised.

20

In conclusion, treatment of animals given a low calcium diet with the combination of $1\alpha,25(\text{OH})_2\text{D}_3$, Ca, and ASA results in a statistically significant reduction in the development of pre-neoplastic colonic lesions.

25

Tumors

Tumor bearing rats were observed in all groups (Table 4). The presented results should be taken with caution as further histological examination is needed for final evaluation. At autopsy, some of the lesions appeared very prominent and tumor-like, reddish, elevated
5 and one to fifteen millimetre in diameter. Whereas others lesions were less elevated and had the same colour as the mucosa. Some animals had more than one tumor often 2 to 3. Other lesions were only observed when the intestine was investigated under stereomicroscope for counting of ACF. Tumors were also observed in the small intestine of one animal of group 1, 5, 7, and 8. In group 2, two animals carried tumors in their small
10 intestine (not histologically examined). The highest number of tumor bearing animals and total number of tumors were seen in group 3 and 4. Group 8 receiving the lowest level of calcium in combination with ASA and $1\alpha,25(\text{OH})_2 \text{D}_3$ had the lowest tumor burden, followed by the group 1. When comparing the numbers of tumor bearing animals of group 2 and 8 (both receiving calcium at the lowest level), the results indicate a protective effect
15 of the ASA and $1\alpha,25(\text{OH})_2 \text{D}_3$ combination. A similar but less pronounced tendency is seen when comparing group 3 and 6 (both receiving the highest level of calcium).

Tumor data indicate that the combination of $1\alpha,25(\text{OH})_2 \text{D}_3$ and ASA given to animals fed either the high or low level of calcium (group 6 and 8) tended to reduce the number of
20 tumor bearing animals and the total number of tumors when compared to the relevant control group receiving the same level of calcium. Although the reduction is non-statistically significant, this indicates protection against progression of ACF to adenomas. Especially at the low calcium level, where the highest total number of ACF was recorded but the lowest number of tumor bearing animals.

Table 4. Colo-rectal tumor in AOM-induced rats fed diets containing $1\alpha,25(\text{OH})_2\text{D}_3$, acetyl salicylic acid, and various doses of Calcium - **preliminary data**^a

Group	N	No. of tumor bearing rats	No. of rats with adenoma	No. of rats wi adenocarcino
1	15	2	1	2
2	16	6	4	4 ^b
3	16	9	8	3
4	16	9	7	4
5	16	6	4	3
6	16	6	5	3
7	16	8	6	4 ^b
8	16	1	0	1 ^b

- a: Group 1, control diet (5000 ppm Ca); group 2, 2500 ppm Ca in the diet; group 3, 7500 ppm Ca in the diet; group 4, 0.02 μg $1\alpha,25(\text{OH})_2\text{D}_3$ in the diet; group 5, 300 ppm acetyl salicylic acid and 7500 ppm Ca in the diet; group 6, 0.02 μg $1\alpha,25(\text{OH})_2\text{D}_3$, 300 ppm acetyl salicylic acid, and 7500 ppm Ca in the diet; group 7, 0.02 μg $1\alpha,25(\text{OH})_2\text{D}_3$, 300 ppm acetyl salicylic acid, and 5000 ppm Ca in the diet; group 8, 0.02 μg $1\alpha,25(\text{OH})_2\text{D}_3$, 300 ppm acetyl salicylic acid, and 2500 ppm Ca in the diet.
- 10 b: including mucinous adenocarcinoma and signet-ring carcinoma.

Example 2

The *in vivo* study of the effect on prevention of CRC in AOM (Azoxymethane) induced rats

- 5 The rats are induced s.c 1 x week in 2 weeks with an AOM solution.

The effect of the specific treatment is evaluated due to the number of aberrant crypt foci (ACF) of the colon/rectum in the AOM-induced rats. The aberrant crypt score is evaluated by stereomicroscopia (40 X) upon staining with Giemsa-solution (6 ml conc. Giemsa in 50
10 ml PBS, pH 7.1). The crypt size is categorised as small, medium, large and X-large. The number of animals is 80 with 16 in each group. The medicaments are administered with the food. The section is 18 weeks from the first dosage. The Ca in the food is 5000/10000 ppm, the 1,24 DHC is 2.5 microg/kg. Data for weight, food and water are registeres and analyzed

15

Treatment:

- | | |
|-------------|---|
| Group 1. | Control animals |
| Group 2. | 200 ppm aspirin (acetyl salicylic acid) |
| 20 Group 3. | Ca and 1,25 DHC |
| Group 4. | 200 ppm aspirin, Ca and 1,25 DHC |
| Group 5. | 100 ppm aspirin, Ca and 1,25 DHC |

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Table 1

	Small	Medium	Large	X-Large
Control Group 1	25	3	0	0
	27	29	4	0
	34	15	1	0
	46	32	4	0
	16	21	1	0
	31	25	2	1
	55	42	7	1
	30	16	2	0
	99	61	4	0
	69	59	11	1
	53	32	1	0
	58	42	9	2
	40	26	9	2
	68	54	8	0
	67	49	3	0
	60	36	4	0
Mean	48.63	33.88	4.38	0.44

Small	Large	Total
28	0	28
56	4	60
49	1	50
78	4	82
37	1	38
56	3	59
97	8	105
46	2	48
160	4	164
128	12	140
85	1	86
100	11	111
66	11	77
122	8	130
116	3	119
96	4	100
82.50	4.81	87.31

Asp 200 Group 2	92	74	13	2
	87	45	8	1
	20	8	2	0
	51	34	5	3
	31	10	2	0
	82	51	5	0
	73	35	3	0
	44	31	3	0
	55	22	3	0
	47	49	5	1
	40	21	1	0
	22	9	2	0
	46	26	6	1

166	15	181
132	9	141
28	2	30
85	8	93
41	2	43
133	5	138
108	3	111
75	3	78
77	3	80
96	6	102
61	1	62
31	2	33
72	7	79

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	73	47	5	0
	44	18	4	0
	63	50	1	0
Mean	54.38	33.13	4.25	0.50
F-Test	0.9237	0.660965	0.6512	0.4327
T-test	62	0.452385	26	93
(mean)	0.2313		0.4566	0.4149
	06		34	29

120	5	125
62	4	66
113	1	114
87.50	4.75	92.25
0.8267	0.7820	0.8051
73	59	88
0.3569	0.4816	0.3665
96	08	36

D3 + Ca Group 3	41	37	4	1
	31	43	1	0
	53	37	4	0
	35	7	2	0
	89	57	2	0
	88	52	5	1
	25	12	1	0
	26	13	0	0
	65	29	1	0
	53	30	3	0
	42	36	2	0
	52	49	3	0
	32	26	1	0
	36	19	2	0
	25	6	0	0
	30	17	0	0
Mean	45.19	29.38	1.94	0.13
F-Test	0.8483	0.921093	0.0035	0.0057
(Var)	89	0.220913	71	84
T-test	0.3238		0.0081	0.0672
(mean)	2		45	87

78	5	83
74	1	75
90	4	94
42	2	44
146	2	148
140	6	146
37	1	38
39	0	39
94	1	95
83	3	86
78	2	80
101	3	104
58	1	59
55	2	57
31	0	31
47	0	47
74.56	2.06	76.63
0.7807	0.0036	0.7155
25	53	55
0.2681	0.0093	0.2132
6	82	05

	Small	Medium	Large	X-Large
Asp 200	101	62	6	0
	71	58	8	0

Small	Large	Total
163	6	169
129	8	137

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D3 + Ca Group 4	31	21	0	0
	79	60	2	0
	64	26	3	0
	52	42	2	0
	56	23	3	0
	41	25	1	0
	88	44	3	0
	64	25	1	0
	61	29	1	0
	40	11	1	0
	37	46	8	1
Mean	60.38	36.31	3.00	0.08
F-Test	0.9106	0.957009	0.4073	0.0017
(Var)	34	0.349196	29	74
T-test	0.0742		0.1169	0.0413
(mean)	18		57	86

52	0	52
139	2	141
90	3	93
94	2	96
79	3	82
66	1	67
132	3	135
89	1	90
90	1	91
51	1	52
83	9	92
96.69	3.08	99.77
0.7985	0.2610	0.7580
13	02	68
0.1477	0.0900	0.1900
47	54	09

Asp 100 D3 + Ca Group 5	21	11	1	0
	57	20	5	0
	62	72	16	2
	67	52	1	1
	34	48	2	0
	31	44	18	4
	53	39	5	0
	36	16	0	0
	16	7	2	0
	44	19	4	0
	41	29	3	0
	33	39	10	2
	69	20	0	0
	34	16	3	0
	18	20	1	0
	26	10	0	0
Mean	40.13	28.88	4.44	0.56
F-Test	0.3723	0.703108	0.0687	0.0845

32	1	33
77	5	82
134	18	152
119	2	121
82	2	84
75	22	97
92	5	97
52	0	52
23	2	25
63	4	67
70	3	73
72	12	84
89	0	89
50	3	53
38	1	39
36	0	36
69.00	5.00	74.00
0.4728	0.0550	0.6031

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(Var)	2	0.211847	88	34
T-test	0.1132		0.4848	0.3584
(mean)	69		12	15

62	55	21
0.1357	0.4614	0.1573
54	5	56

"Small" in the third column is the sum of small and medium of the first column

"Large" in the third column is the sum of large and x-large the of second column

- 5 The risk of malignant development (APC and ras-mutations) is correlated to large and x-large crypts.

The aspirin dosage general considered as being preventive is 400-500 ppm in AOM-induced F344 rats.

10

The results shows that it is not possible to reduce the aspirin dosage to 200 ppm as this dosage is without effect (Group 2). However, the addition of Ca and 1,25 DHC results a significant reduction in large ACF (Group 4). The results of Group 3 and 4 are peculiar as it seems as though the presence of aspirin is an adverse effect. It should be noted that the

- 15 rats suffered from hypercalcaemia as shown by kidney stones.

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CLAIMS:

1. A pharmaceutical medicament comprising a combination of acetylsalicylic acid (ASA), 1,25 dihydroxy-cholecalciferol (1,25 DHC) and calcium in a combination dosage together with a pharmaceutically acceptable carrier.
5
2. A pharmaceutical medicament according to claim 1 wherein the combination dosage comprises 1,25 DHC in the range of 0.1 μ g to 2 μ g.
3. A pharmaceutical medicament according to claim 1 or 2 wherein the combination dosage comprises calcium in the range of 200 mg to 3000 mg.
- 10 4. A pharmaceutical medicament according to any one of claims 1 to 3 wherein the combination dosage comprises ASA in the range of 50 mg to 500 mg.
5. A pharmaceutical medicament according to any one of claims 1 to 4 wherein the combination dosage comprises 50 to 75 mg ASA, 500 to 1000 mg
15 calcium, and 0.5 μ g to 1 μ g 1,25 DHC.
6. Use of a combination dosage of acetylsalicylic acid (ASA), the vitamin D₃ metabolite 1,25 dihydroxy-cholecalciferol (1,25 DHC) and calcium together with a pharmaceutically acceptable carrier for the prevention of colorectal cancer in a human.
- 20 7. Use of a combination dosage of acetylsalicylic acid (ASA), the vitamin D₃ metabolite 1,25 dihydroxy-cholecalciferol (1,25 DHC) and calcium together with a pharmaceutically acceptable carrier for the prevention of both the initiation of colorectal cancer and progression of colorectal cancer in a human.
8. Use according to claim 6 or 7 wherein the acetylsalicylic acid (ASA) is
25 in the range of 50 mg to 500 mg.

9. Use according to any one of claims 6 to 8 wherein calcium is in the range of 200 mg to 3000 mg.

5 10 Use according to any one of claims 6 to 9 wherein 1,25 DHC is present in an amount of 0.1 μ g to 2 μ g.

11. Use according to any one of claims 6 to 10 wherein the combination is in the form of a combination dosage comprising ASA, 1,25 DHC and calcium.