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(54) Title: PROCESS FOR THE PREPARATION OF C–4 DEACETYLTAXANES

(57) Abstract

A process useful for the preparation of intermediates in synthesis or semi–synthesis of paclitaxel analogs wherein a starting taxane such as 10–deacetylbaccatin III is deacetylated at the C–4 position using a microorganism or an enzyme derived therefrom to provide 4–deacetyltaxanes, such as 4,10–dideacetylbaccatin III.

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PROCESS FOR THE PREPARATION OF C-4 DEACETYLTAXANES

Brief Description of the Invention

The present invention is directed to a process using microorganisms or enzymes derived therefrom for deacetylation of taxanes at C-4 to give 4-deacetyltaxanes, useful intermediates for the synthesis of new anti-cancer agents.

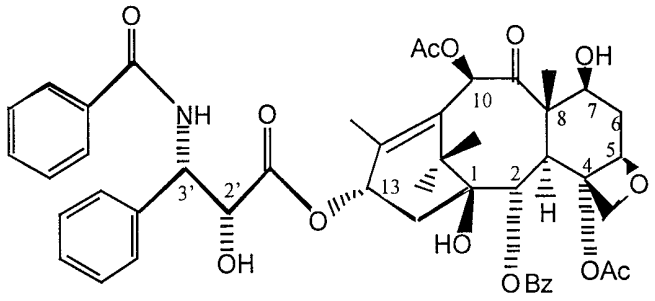
Background of the Invention

Taxanes are diterpene compounds which find utility in the pharmaceutical field. For example, taxanes containing aryl heterocyclic or cycloalkyl groups on the C-13 sidechain find utility as anti-cancer agents. Taxanes include pacltitaxel, cephalomannine, taxol c, 10-deacetylpaclitaxel, 10-deacetylcephalomannine, 7-ß-xylosylpaclitaxel, baccatin-III, 10-deacetylbaccatin III, 7-ß-xylosyl-10-deacetyl cephalomannine, 7-ß-xylosyl-10-deacetylbaccatin III, 7-ß-xylosylbaccatin III, and 10-deacetyl-taxol c.

Paclitaxel (Taxol), a diterpene taxane compound, is a natural product extracted from the bark of the Pacific yew tree, *Taxus Brevifolia*. It has been shown to have excellent antitumor activity in *in vivo* animal models, and recent studies have elucidated its unique mode of action, which involves abnormal polymerization of tubulin and disruption of mitosis during the cell cycle. Taxol has recently been approved for the treatment of refractory advanced ovarian cancer, breast cancer, non-small cell lung cancer, and most recently, AIDS-related Kaposi's Sarcoma. The results of paclitaxel clinical studies are replete in scientific periodicals and have been reviewed by numerous authors, such as Rowinsky and Donehower in "The Clinical Pharmacology and Use of Antimicrotubule Agents in Cancer Chemotherapeutics", *Phamac. Ther.*, 52, pp. 35-84 (1991); Spencer and

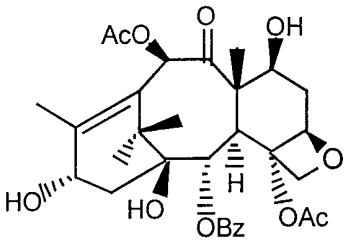
Faulds, Paclitaxel, A Review of its Pharmacodynamic and Pharmacokinetic Properties and Therapeutic Potential in the Treatment of Cancer, *Drugs*, 48 (5), pp. 794-847 (1994); K.C. Nicolau et al., Chemistry and Biology of Taxol, *Angew. Chem., Int. Ed. Eng.*, 33, pp. 15-44 (1994); F. A. Holmes, A. P. Kudelka, J. J. Kavanaugh, M. H. Huber, J. A. Ajani, and V. Valero, "Taxane Anticancer Agents - Basic Science and Current Status", edited by Gunda I. Georg, Thomas C. Chen, Iwao Ojima, and Dolotrai M. Vyas, pp. 31-57 American Chemical Society, Wash., D.C. (1995); Susan G. Arbuck and Barbara Blaylock, "Taxol(Science and Applications", edited by Matthew Suffness, pp. 379-416, CRC Press, Boca Raton, FL (1995) and the references cited therein.

The structure of Taxol® is shown below along with the conventional numbering system for molecules belonging to the Taxane class; such numbering system is also employed in this application.

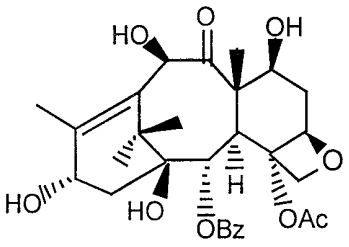


With reference to the numbering of the taxane, reference to a particular carbon on the taxane structure shall be indicated throughout this application by a "C-number", which signifies the carbon on the taxane according to the above numbering system. For example, "C-13" refers to the carbon at position 13 on the taxane ring as shown above, having a sidechain coupled thereto.

The central backbone structural unit of paclitaxel is baccatin III, a diterpenoid having the chemical structure:



It is also very similar in structure to 10-deacetylbaccatin III ("10-DAB III"), which has the chemical structure:



but which lacks an acetate ester at the 10-position alcohol.

Chemical modification of the paclitaxel structure at C-4 and other positions has been explored by many groups to determine structure/activity relationships and to try to obtain compounds with superior efficacy to taxol to develop as second generation drugs. See U.S. Pat. No. 5,773,461; Gunda I. George, Syed M. Ali, Thomas C. Boge, Apurba Datta, and Lise Falborg, "Selective C-2 and C-4 Deacylation of Taxol: The First Synthesis of a C-4 Substituted Taxol Analogue", *Tetrahedron Let.*, 35:48, pp. 8931-8934 (1994); Shu-Hui Chen, John F. Kadow, Vittorio Farina, Craig R. Fairchild and Kathy A. Johnston, "First Synthesis of Novel Paclitaxel (Taxol) Analogs Modified at the C-4 Position", *J. Org. Chem.* 59, pp. 6156-6158 (1994); S. Py, and F. Khuong-Huu, "A Novel Rearrangement of The Taxane Skeleton", *Bull. Soc. Chim. Fr.*, 130, pp. 189-191 (1993).

Replacement of the C-4-acetyl group of paclitaxel with other substituents has lead to compounds with improved potency in activity assays (S. Chen et al., Biorganic and Medicinal Chemistry Letters, 5:2741-2748 (1995)). An enzyme capable of specifically removing the C-4-acetyl group from taxanes will be useful in the synthesis of C-4-modified paclitaxel analogs

to provide starting material to allow incorporation of other groups at this position, for example C-4 butyrate esters, C-4 cyclobutyl esters, C-4 propyl esters, C-4 cyclopropyl esters and C-4 methyl and ethyl carbonates.

Description of the Drawings

Figure 1 illlustrates the effect of methanol concentration on the C-4 deacetylation by strain SC16249;

Figure 2 illlustrates the effect of methanol concentration on the C-4 deacetylation by strain SC16250.

Description of the Invention

It is an object of the present invention to provide a new, useful and efficient protocol for the preparation of 4-deacetyltaxanes.

Another object of the present invention is the provision of a process using microorganisms or enzymes derived therefrom for deacetylation of taxanes at the C-4 position to provide 4-deacetyltaxanes.

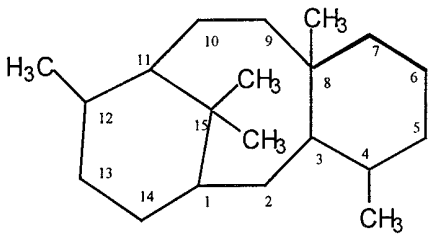
An additional object of the present invention is the provision of a simple, efficient, and cost effective protocol for the provision of second-generation taxol analogues having various substituents at the C-4 position.

Accordingly, the present invention encompasses a novel method by which 10-deacetyl baccatin III can be efficiently converted to 4,10-dideacetylbacatin III using a microorganism or one or more enzymes derived from the microorganism. The resulting 4,10-dideacetylbacatin III compound may then be utilized as part of novel processes for the synthesis and semi-synthesis of paclitaxel analogs.

The present disclosure is broadly directed to a process for the efficient deacetylation of 10-DAB III at the C-4 position. The deacetylation at C-4 transpires as a result of a biotransformation caused by a microorganism or an enzyme derived from said microorganism. More specifically, the present invention is directed to the use of an organism that transforms 10-DAB III into 4,10-dideacetylbaccatin III.

The terms "enzymatic process" or "enzymatic method", as used herein, denote a process or method of the present invention employing an enzyme or microorganism. Use of "an enzyme or microorganism" in the present method includes use of a single, as well as two or more, enzymes or microorganisms.

The term "taxane", as used herein, denotes compounds having a taxane moiety as described following. The term "taxane moiety", as used herein, denotes moieties containing the core structure (with numbering of ring system positions used herein shown):



which core structure may be substituted and which may contain ethylenic unsaturation in the ring system thereof. Such moieties having an oxetane ring fused at the 4- and 5- positions, such as is found in paclitaxel, are preferred.

The enzyme or microorganism employed in the present invention may be any enzyme or microorganism capable of catalyzing the enzymatic hydrolysis described herein. The enzymatic or microbial materials, regardless of origin or purity may be employed in the free state or immobilized on a support such as by physical adsorption or entrapment.

Exemplary microorganisms, which have been identified through a screening process, include *Rhodococcus sp.* ATCC 202192 (SC 16249) and *Rhodococcus sp.* ATCC 202191 (SC 16250). The term "ATCC" as used herein refers to the accession number of the American Type Culture Collection, 10801 University Blvd., Manassas, VA, the depository for the organism referred to. The above microorganisms ATCC 202192 and ATCC 202191 were deposited on January 14, 1999. The term "SC" denotes the

designation given to the microorganism as part of the Squibb culture collection.

It should be understood that mutants of the biologically pure microorganisms ATCC 202192 (SC 16249), and ATCC 202191 (SC 16250) are also contemplated by the present invention for use in the biotransformation described herein, such as those modified by the use of chemical, physical (for example, x-rays) or biological means (for example, molecular biology techniques).

Rhodococcus sp. ATCC 202192 (SC 16249) and *Rhodococcus sp.* ATCC 202191 (SC 16250) may be cultivated on a medium of 0.5% toasted nutrisoy, 2% glucose, 0.5% yeast extract 0.5% K_2HPO_4, 0.5% NaCl, adjusted to pH 7 with HCl. The organisms were isolated from soil (from a sample from Parsippany, N.J.), and are gram positive, non-motile rods with an aerobic requirement

Preferred enzymes include those derived from microorganisms, particularly those microorganisms described above. Enzymes may be isolated, for example, by extraction and purification methods, such as ion exchange chromatography, followed by hydrophobic interaction chromatography and gel filtration. The present invention further provides the enzymes capable of the present hydrolysis which may be isolated from *Rhodococcus sp.* ATCC 202192 (SC 16249) and *Rhodococcus sp.* ATCC 202191 (SC 16250), for example by the above techniques.

Where microorganisms are employed, the cells may be used in the form of intact wet cells or dried cells such as lyophilized, spray-dried or heat-dried cells, or in the form of treated cell material such as ruptured cells or cell extracts. The use of genetically engineered organisms is also contemplated. The host cell may be any cell, e.g. *Escherichia coli*, modified to contain a gene or genes for expressing one or more enzymes capable of catalysis as described herein.

Where one or more microorganisms are employed, the enzymatic deacetylation process of the present invention may be carried out subsequent

to the fermentation of the microorganism (two-stage fermentation and hydrolysis), or concurrently therewith, that is, in the latter case, by *in situ* fermentation and hydrolysis (single-stage fermentation and hydrolysis).

Growth of the microorganisms may be achieved by one of ordinary skill in the art by the use of an appropriate medium. Appropriate media for growing microorganisms include those which provide nutrients necessary for the growth of the microbial cells. A typical medium for growth includes necessary carbon sources, nitrogen sources, and elements (e.g. in trace amounts). Inducers may also be added. The term "inducer", as used herein, includes any compound enhancing formation of the desired enzymatic activity within the microbial cell.

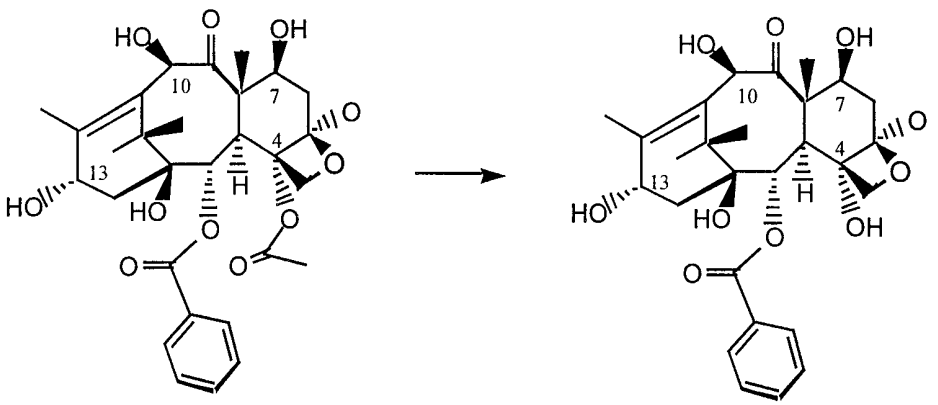
Carbon sources may include sugars such as maltose, lactose, glucose, fructose, glycerol, sorbitol, sucrose, starch, mannitol, propylene glycol, and the like; organic acids such as sodium acetate, sodium citrate, and the like; and alcohols such as ethanol, propanol and the like.

Nitrogen sources may include N-Z amine A, corn steep liquor, soy bean meal, beef extracts, yeast extracts, molasses, baker's yeast, tryptone, nutrisoy, peptone, yeastamin, amino acids such as sodium glutamate and the like, sodium nitrate, ammonium sulfate and the like.

Trace elements may include magnesium, manganese, calcium, cobalt, nickel, iron, sodium and potassium salts.

Phosphates may also be added in trace or, preferably, greater than trace amounts.

The general biotransformation process described herein using the aforementioned microorganism can be illustrated according to the following reaction scheme:



The specific examples that follow illustrate the synthesis of representative compounds of the instant invention and are not to be construed as limiting the invention in sphere or scope. The methods may be adapted to variations in order to produce intermediates and compounds embraced by this invention but not specifically disclosed. Further, variations of the methods to produce the same compounds in somewhat different fashion will also be evident to one skilled in the art.

Example 1

Deacetylation of 10-deacetylbaccatin III

Medium: 0.5% toasted nutrisoy, 2% glucose, 0.5% yeast extract, 0.5% K_2HPO_4. 0.5% NaCl, adjusted to pH 7 with HCl (R.V. Smith and J. P. Rosazza, Arch. Biochem. Biophys., 161, 551-558 (1974)).

Strain SC16249 (ATCC 202192) isolated from a soil sample collected in Parsippany, N.J. was maintained on a plate containing the above medium plus 1.5% agar. 10 ml of the medium in a 50 ml flask was inoculated with a loopful of the culture. After 24 h incubation at 28 °C, 200 rpm, the culture was centrifuged at 11951xg for 10 min. The cell pellet was resuspended in a 50 ml flask with 10 ml 50 mM potassium phosphate buffer, pH 7.
2 mg 10 deacetylbaccatin III dissolved in 0.2 ml methanol was added and the flask was shaken at 28 °C, 200 rpm for 16 h. A sample of 0.7 ml was diluted

with 0.7 ml methanol and analyzed by the HPLC method below. The molar yield of 4,10 dideacetylbaccatin III was 86%. HPLC retention time was the same as a chemically prepared standard. LC/MS analysis showed $(M+CH_3COO^-)^-$ = 561 indicating a molecular weight of 502.

HPLC method

column: Hewlett Packard Hypersil 5 (ODS C 18 200x4.6 mm)
mobile phase: 45% methanol; 55% water
flow rate: 1 ml/min
detection: 235 nm
temperature: 40 °C

TABLE 1

Compound	ret. time min.
10-deacetylbaccatin III	10.221
4,10-dideacetylbaccatin III	5.131

Example 2

Deacetylation of 10-deacetylbaccatin III

Strains SC16249 (ATCC 202192) and SC 16250 (ATCC 202191) (both isolated from a soil sample collected in Parsippany, NJ) were grown in 500 ml flasks containing 100 ml of the medium given in Example 1. The flasks were inoculated with a loopful of the culture and shaken for 64 h at 28° C, 200 rpm. Cells were collected by centrifugation, washed with 50 mM potassium phosphate buffer, pH 7, and centrifuged again. The cell pellets were resuspended at a concentration of 10% w/v in 50 mM potassium phosphate buffer, pH 7. Samples of cell suspension containing 2 mg 10-

deacetylbaccatin III and 2%, 5%, 10% or 20% methanol in a total volume of 10 ml were shaken in 50 ml flasks at 28° C, 200 rpm. Samples of 0.5 ml were diluted with 0.5 ml methanol and analyzed by the HPLC method given in Example 1. The molar yield of 4,10-dideacetylbaccatin III was 96% after 2h for strain SC16249 (ATCC 202192) with 10% methanol. The molar yield of 4,10-dideacetylbaccatin III was 89% after 18h for strain SC16250 (ATCC 202191) with 5% methanol.

Claims

We Claim:

1. A method for the preparation of 4-deacetyltaxanes, comprising the steps of contacting a taxane with a microorganism or an enzyme derived therefrom which is capable of deacetylating said taxane at the 4-position thereon to obtain 4-deacetyltaxane.

2. The method according to claim 1, wherein said taxane is 10-deacetylbaccatin III, paclitaxel, cephalomannine, taxol c, 10-deacetylpaclitaxel, 10-deacetylcephalomannine, 7-β-xylosylpaclitaxel, baccatin-III, 7-β-xylosyl-10-deacetylcephalomannine, 7-β-xylosyl-10-deacetylbaccatin III, and 10-deacetyltaxol c.

3. The method according to claim 1, wherein said 4-deacetyltaxane is 4,10-dideacetylbaccatin III, 4-deacetylpaclitaxel, 4-deacetylcephalomannine, 4-deacetyltaxol c, 4,10-dideacetylpaclitaxel, 4,10-dideacetylcephalomannine, 4-deacetyl-7-β-xylosylpaclitaxel, 4-deacetylbaccatin III, 4-deacetyl-7-β-xylosyl-10-deacetylcephalomannine, 4-deacetyl-7-β-xylosyl-10-deacetylbaccatin III, 4-deacetyl-7-β-xylosylbaccatin III, and 4,10-dideacetyltaxol c.

4. The method according to claim 1, wherein said taxane is obtained by plant cell culture of, and/or extraction from plant tissue, wherein said plant is a member of the T*axus* genus.

5. The method according to claim 1, wherein said microorganism is within the genus *Rhodococcus*.

6. The method according to claim 5, wherein said microorganism is *Rhodococcus sp.* ATCC 202192 (SC 16249) or *Rhodococcus sp.* ATCC 202191 (SC 16250).

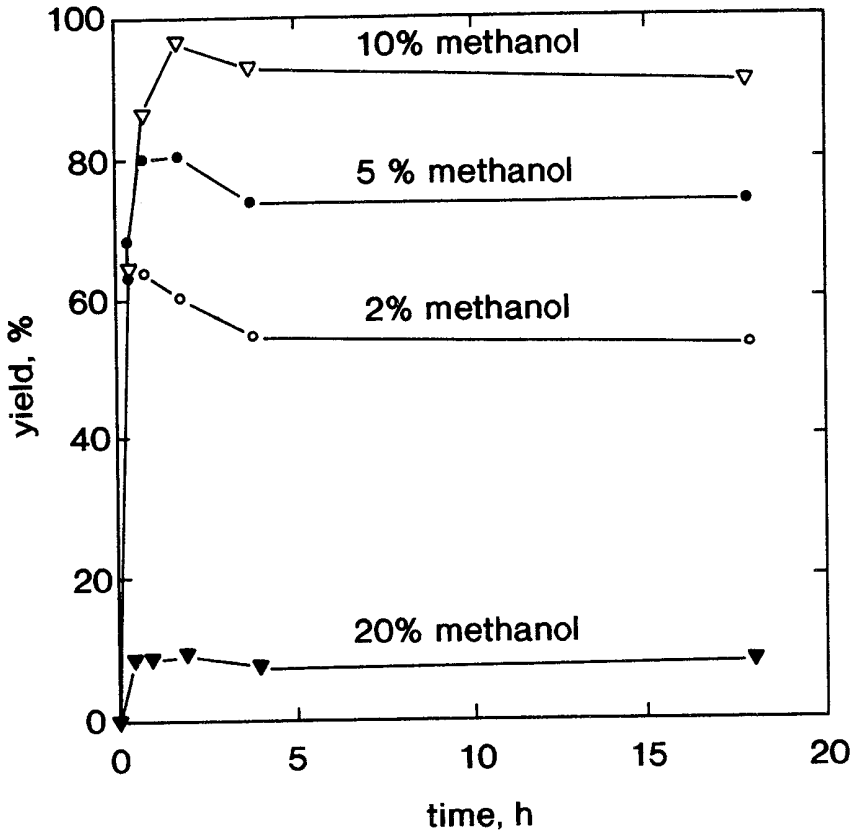
7. The method according to claim 1, wherein said enzyme is derived from a microorganism which is within the genus *Rhodococcus*.

8. The method according to claim 7, wherein said enzyme is derived from the microorganism *Rhodococcus sp.* ATCC 202192 (SC 16249) or *Rhodococcus sp.* ATCC 202191 (SC 16250).

9. An enzyme isolated from a microorganism which is capable of catalyzing the C-4 deacetylation of a taxane.

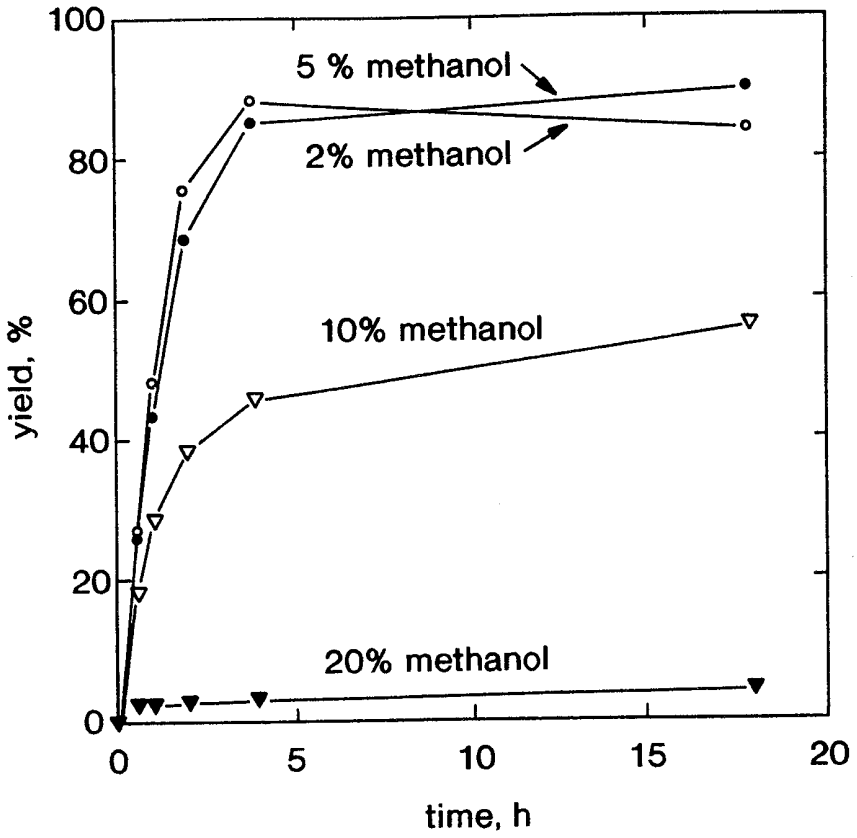
10. The enzyme as recited in claim 9, wherein said microorganism is within the genus *Rhodococcus*.

11. The enzyme as recited in claim 9, wherein said microorganism is *Rhodococcus sp.* ATCC 202192 (SC 16249) or *Rhodococcus sp.* ATCC 202191 (SC 16250).



strain SC16249
10% w/v
50 mM KPi pH 7
0.2 mg/ml 10-DAB

FIG. 1



strain SC16250
10% w/v
50 mM KPi pH 7
0.2 mg/ml 10-DAB

FIG. 2

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/17547

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C07D 305/14; C12N 9/00; C12P 17/02
US CL :549/510, 511; 435/123, 183

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 549/510, 511; 435/123, 183

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

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Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

none

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5,516,676 A (HANSON et al) 14 May 1996, see the entire document.	9-11
A	US 5,547,866 A (DURZAN et al) 20 August 1996,see the entire document.	1-11

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