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

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
9 October 2003 (09.10.2003)

PCT

(10) International Publication Number
WO 03/082794 A1

(51) International Patent Classification[7]: C07C 65/40, A61K 31/192

(21) International Application Number: PCT/IN03/00073

(22) International Filing Date: 24 March 2003 (24.03.2003)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
10/107,806 28 March 2002 (28.03.2002) US

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

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

Declaration under Rule 4.17:

— *as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii)) for the following designations AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NI, NO, NZ, OM, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, UZ, VC, VN, YU, ZA, ZM, ZW, ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, RO, SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, 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 "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 03/082794 A1

(54) Title: A COMPOUND AS CHOLINESTERASE INHIBITOR AND ITS ISOLATION FROM FUNGUS *SPOROTRICHUM* SPECIES

(57) Abstract: The present invention provides a novel bioactive compound 12- (2'-CARBOXY- 5'-METHOXYPHENYL)-2,12-DIHYDROXY-DODECA-4-ONE "Sporotricolone", mainly as acetylcholinesterase (AchE) inhibitor, along with a process for the isolation of said compound from fungus *Sporotrichum* species.

A COMPOUND AS CHOLINESTERASE INHIBITOR AND ITS ISOLATION FROM FUNGUS *SPOROTRICHUM* SPECIES

TECHNICAL FIELD

The present invention relates to a compound 12- (2'-CARBOXY- 5'-METHOXYPHENYL)-2,12-DIHYDROXY-DODECA-4-ONE "Sporotricolone", mainly as acetylcholinesterase (AchE) inhibitor. The present invention also relates to a process for the isolation of said compound from fungus *Sporotrichum* species.

BACKGROUND ART

Enzyme inhibitors are important class of molecules that are used as drugs and pesticides. The enzyme acetylcholinesterase (AchE) is involved in the synaptic transmission of the nerve impulse and its inhibition leads to accumulation of the neurotransmitter, acetylcholine leading to overexcitation of the postsynaptic neuron. This property of the inhibitor has been exploited to develop newer insecticides against a wide range of insect pests as well as drugs effective against worms, and, recently a new class of neuroactive drugs against dementia (Alzheimer's).

Although earlier authors have isolated metabolites such asasteric acis, questin and questinol from *Sororticum* sp., no AchE inhibitor activity has been reported (Slater, GP, Haskins, RH and Hogge, L.R. Can J Mirobiol 17 (1971), 1576-79). The fungi, *Aspergillus terreus* (Ling, KH, Liou,HH, Yang, CM and Yang CK, Appl. Env. Microbiol, 37 (1979) 355-57) and *Penicillium* sp. (Omura, Skuno,F, Otoguro,K, Shiomi,K. Mauma,R and Iwai,Y. J. Antibiot. 48(1995) 745-46) have been reported to produce an AchE inhibitor named Arusigacin. However, the AchE inhibitor of the present invention is isolated from Sporotrichum having distinct chemical structure and properties and therefore a novel inhibitor molecule.

After screening of various microorganisms, a fungal culture is selected which shows inhibition against a serine esterase/ protease/cholinesterase enzyme. This imperfect deuteromycetes, *Sporotrichum* species and was first isolated in 1966. The taxonomic features of *Sporotrichum* species (deuteromyces) are broad hyphae and septate in nature; has hyalline conidiophores with little differentiation from vegetatative hyphae and solitary conidia with broad attachment to the hyphae.

This culture has previously been a subject of research investigation at the Central Food Technological Research Institute (CFTRI) India, for its ability to grow on lignocellulosic

wastes for the production of enzymes and organic acids (Sreekantaiah, KR, PhD thesis (1976) University of Mysore; Manonmani, HK, PhD thesis (1986) University of Mysore). This culture has now been used in the present invention to produce a fermented extract containing a serine esterase / protease/ cholinesterase inhibitor.

The conditions of fermentation have been described earlier in Indian Patent Application No. 303/DEL/2000 Sattur, AP, Shivanandappa, T, Divakar, S and Karanth, NG.

OBJECTS OF THE INVENTION

The main object of the present invention is to provide bioactive compound 12- (2'-CARBOXY-5'-METHOXYPHENYL)-2,12-DIHYDROXY-DODECA-4-ONE "Sporotricolone" having inhibitory activity against acetylcholinesterase (AchE).

Another object of the present invention is to provide a compound having inhibitory activity against seiren esterase and protease.

Yet another object of the present invention is to provide a compound having insecticidal properties.

Still another object of the present invention is to provide a compound having enhanced cholinergic activity.

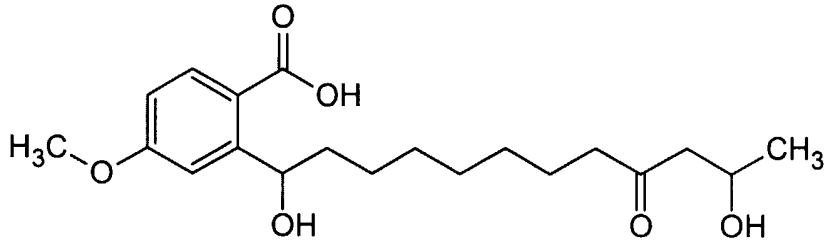
Yet another object of the present invention is to provide a process for the isolation of the above said compound.

SUMMARY OF THE INVENTION

The present invention provides a compound 12- (2'-CARBOXY- 5'-METHOXYPHENYL)-2,12-DIHYDROXY-DODECA-4-ONE "Sporotricolone" mainly as acetylcholinesterase (AchE) inhibitor. The present invention also provides a process for the isolation of said compound from fungus *Sporotrichum* species.

DETAILED DESCRIPTION OF THE INVENTION

Accordingly, the present invention provides a bioactive compound 12- (2'-CARBOXY-5'-METHOXYPHENYL)-2,12-DIHYDROXY-DODECA-4-ONE "Sporotricolone" of formula (I) having acetylcholinesterase (AchE) enzyme inhibition activity obtained from fungus *Sporotrichum* species.



FORMULA (I)

An embodiment of the present invention wherein the compound 12- (2'-CARBOXY- 5'-METHOXYPHENYL)-2,12-DIHYDROXY-DODECA-4-ONE "Sporotricolone" of formula (I) having the following characteristic properties:

Solubility: Highly soluble in ethyl acetate, methanol and acetone.

UV (ethyl acetate) λ_{max}: 265 nm, 312 nm.

^{1}HNMR spectrum (DMSO, δ_{TMS} = 0.00 ppm);

δ 1.03 (3H, d, J=6.3Hz, -CH-C$\underline{H_3}$)

δ 1.2-2.7 (14H, m, 7x – C$\underline{H_2}$)

δ 3.6-3.8 (m, Ar-O-C$\underline{H_3}$ and Ar-C$\underline{H}$-OH)

δ 7.20 (1H, d, J=2.5Hz, $C_{6'}$-$\underline{H}$)

δ 7.30 (2H, d, J = 7.1 Hz, $C_{3'}$ –$\underline{H}$ and $C_{4'}$-$\underline{H}$)

Mass spectrum (EI, 70 eV, 25^0 C, 200-ul amp):

m/e: 336 (M+), 279(366-87), 167(274-112), 57 (CH_2COCH_3), 43, 29.

Another embodiment of the present invention, wherein the purity of the compound is established by TLC and RP HPLC.

Yet another embodiment of the present invention, wherein said compound is named as 12-(2'-CARBOXY- 5'-METHOXYPHENYL)-2,12-DIHYDROXY-DODECA-4-ONE "Sporotricolone".

Still another embodiment of the present invention, wherein said compound is an inhibitor of the enzyme acetylcholinesterase from the rat brain as well as erythrocytes with a IC_{50} value of 20 x 10^{-6} M.

Yet another embodiment of the present invention, wherein said compound also acts as an inhibitor of serine esterase of the rat liver serum.

Still another embodiment of the present invention, wherein said compound having insecticidal properties.

Yet another embodiment of the present invention, wherein said compound effective against mosquito larvae at an optimum concentration of 70 μg/ml water (70 ppm) when exposed for 24 hrs.

Still another embodiment of the present invention, wherein the insecticidal activity of the compound against mosquito larvae is selected from *culex quinquifasciatus.*

Yet another embodiment of the present invention, wherein said compound as acetylcholineesterase inhibitor having potential application as a drug for Alzheimer's disease or dermentia.

The present invention also provides a process for the isolation of 12- (2'-CARBOXY- 5'-METHOXYPHENYL)-2,12-DIHYDROXY-DODECA-4-ONE Sporotricolone from the fungus Sporotrichum species, said process comprising the steps of:

(a) extracting the fermented solid with an organic solvent;

(b) filtering the extract of step (a) through a cloth or Whatman filter paper to obtain a clear solution;

(c) evaporating the solution of step (b) under reduced pressure to obtain a crude extract;

(d) purifying the crude extract of step (c) by column chromatography over silica gel and eluting with mixture of organic solvents of increasing polarity;

(e) pooling active eluted fraction of step (d) and further subjected to column chromatography over silica gel by eluting with mixture of organic solvents with increasing polarity;

(f) repooling the active eluted fractions of step (e);

(g) evaporating the pooled fractions of step (f) to get a residue; and

(h) dissolving the residue in step (g) in ethyl acetate to yield the pure compound "Sporotricolone".

Yet another embodiment of the present invention, a process wherein in step (a) the organic solvent is selected from a group consisting of ethyl acetate, acetone or methanol and preferably ethyl acetate.

Still another embodiment of the present invention, a process wherein in step (d) the mixture of organic solvents is selected from the combination of hexane: diethyl ether and chloroform: methanol mixtures.

Further embodiment of the present invention, a process wherein in step (e) the mixture of organic solvent used is chloroform: ethyl acetate mixture.

Yet another embodiment of the present invention, wherein the said compound is separated and purified by column chromatography on silica gel and RP HPLC.

Still another embodiment of the present invention, wherein said compound having an UV absorption at 265 and 312 nm.

The present invention is further explained in the form of following embodiments

In the present invention a process for the isolation of an acetylcholinesterase inhibitor, which comprises the extraction of the fermented broth culture with solvents such as ethyl acetate. The crude extract is further extracted with 10-20 ml of methanol and subjected to column chromatography using silica gel and eluted with various combinations of solvents

such as hexane: diethyl ether (85:15, 50:50) followed by chloroform: methanol (95:5, 50:50, 10:90). Fractions are evaporated under nitrogen, dissolved in ethyl acetate and assayed for acetylcholinesterase (AchE) inhibition. The active fractions are pooled and further subjected to purification on silica gel column chromatography and eluted with chloroform: ethyl acetate (90:10, 50:50, 0:100). The active fractions pooled and the solvent evaporated and dissolved in 2 ml ethyl acetate. The purity, as checked by TLC, showed a single spot and HPLC on reverse phase column (C18) with chloroform and methanol as mobile phase. The yield is about 10 mg. The purified inhibitor showed inhibitor potency against rat brain AchE with an IC_{50} of 15-20 x 10^{-6} M.

A general process for the production of the novel Acetylcholinesterase inhibitor is given in the flow sheet:

Solid State Fermentation

↓

Solvent extraction

↓

Crude extract

↓

Silica gel column chromatography I

(hexane, diethylether, chloroform & methanol)

↓

enriched fraction

↓

silica gel column chromatography II

(Chloroform: ethyl acetate)

↓

purified inhibitor

12- (2'-CARBOXY- 5'-METHOXYPHENYL)-2,12-DIHYDROXY-DODECA-4-ONE "Sporotricolone"

The structure of the isolated inhibitor is determined by UV, ^{1}H NMR and mass spectrometry.

The following examples are given by way of illustration of the present invention and should not be construed to limit the scope of the invention.

EXAMPLE – 1

The fermentation culture is extracted with 100 ml of ethyl acetate, filtered through a cotton filter and concentrated in vacuo to obtain 1 ml of the crude extract. This is used as a source of the enzyme inhibitor and 20 µl of the extract gave 60-90 % inhibition of rat brain acetylcholinesterase enzyme.

EXAMPLE -2

The crude extract is further extracted with 10-20 ml of methanol and subjected to column chromatography using silica gel and eluted with various combinations of solvents such as hexane: diethyl ether (85:15, 50:50) followed by chloroform: methanol (95:5, 50:50, 10:90). Fractions are evaporated under nitrogen, dissolved in ethyl acetate and assayed for acetyl cholinesterase (AchE) inhibitor. The active fractions (# 11-19) are pooled and further subjected to purification on silica gel column chromatography and eluted with chloroform: ethyl acetate (90:10, 50:50, 0:100). The active fractions pooled and the solvent evaporated and dissolved in 2 ml ethyl acetate. The purity, as checked by TLC, showed a single spot. RP HPLC also ascertained the purity on a C18 column with chloroform and methanol as the mobile phase wherein it is a single peak. The yield is about 10 mg.

EXAMPLE – 3

The purified inhibitor showed inhibitor potency against rat brain AchE with an IC50 of 15-20 x 10^{-6} M. as assayed according to Ellman et al., (Biochem. Pharmacol. 7(1961), 88-95) and is given as follows: The enzyme inhibition is carried out by pre- incubating the enzyme (rat brain acetylcholinesterase) with 2-20ul of the culture extract or the column fraction at room temperature (25 ^{0} C) for 15 minutes followed by the addition of the substrate, acetyl thiocholine iodide (0.5mM), in 3ml phosphate buffer (0.1.M, pH.7.4) containing 0.25mM dithiobisnitrobenzoic acid. Absorbance change at 412 nm is monitored every 30 seconds for 2 min in an UV-VIS Spectrophotometer. Inhibition is calculated relative to the solvent control. IC_{50} is determined by regression analysis.

EXAMPLE – 4

Inhibition of serine esterase: The isolated compound (inhibitor) was demonstrated to be an inhibitor of serine esterase as follows:

Inhibition of esterase activity was assayed *in vitro* with the substrate, *p*-nitrophenyl acetate in tris buffer (.05 M, pH 7.4) and the rat liver extract as the source of the enzyme. The

inhibitor, at various concentrations, was pre-incubated with the enzyme in buffer at room temperature for 30 minutes and the residual activity was measured. The product of enzymatic hydrolysis (*p*-nitrophenol) was monitored spectrophotometrically by measuring the absorbance change per minute at 405 nm, according to the method of Sidhu and Blair (J. Biol. Chem., 1975, 250: 7891). Percentage inhibition was calculated with respect to the control (without inhibitor) and IC_{50} (the concentration required to inhibit 50% activity) was calculated by regression analysis. IC_{50} for the microbial inhibitor was found to be 0.067 µmole/ml which indicates that it is a potent inhibitor of serine esterase.

EXAMPLE – 5

Insecticidal properties: The insecticidal property of the inhibitor isolated from Sporotrichum sps was investigated as follows:

***a. Toxicity to mosquito larvae*:**

Late III instar larvae of the mosquito, *Culex quinquefaciatus* (from laboratory culture) were released in four beakers containing tap water (ten larvae in each beaker). The treatment group (2 replicates) was added the inhibitor compound at 100 ppm in 100 µl acetone whereas the control group received acetone only. After 24 hrs, 75 % mortality (7/10 and 8/10) was observed in treated group whereas no mortality was seen in the control group (0/10, 0/10). This experiment demonstrates the insecticidal activity of the compound.

***b. Acetyl cholinesterase of the house fly brain*:**

Insecticidal potential of the compound was further investigated by the inhibition of the target enzyme, acetylcholinesterase(AchE) from the housefly brain *in vitro*. Ten house fly heads were homogenized in 3 ml buffer(tris .05M, pH 7.4) which served as the source of the enzyme. AchE inhibition was assayed with acetyl thiocholine as the substrate with various concentrations of the inhibitor as described earlier for the rat brain enzyme. Percentage inhibition was calculated with respect to the control (without inhibitor) and IC_{50} determined by regression analysis. The compound was found to be a potent inhibitor of the housefly brain AchE with an IC_{50} value of 0.98 µmole/ml.

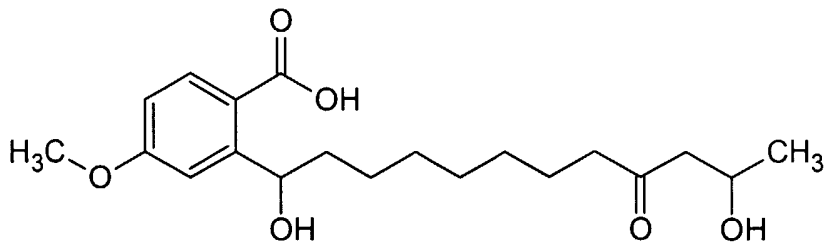
From the above experiment, it is very clear that the compound of the invention is effective in killing or inhibiting the growth of insects, and evidence that the compound functions as an acetylcholinesterase inhibitor. It is well known to those of skill in the art that acetylcholinesterase inhibitors are effective as insecticides.

MAIN ADVANTAGES OF THE PRESENT INVENTION

1. The present invention provides an AchE /serine esterase/protease inhibitor from a microbial source.
2. The present invention provides a simple extraction and chromatographic procedure to purify the AchE inhibitor from the crude mixture.
3. In the present invention the isolated inhibitor is a novel bioactive molecule

Claims

1. A compound having the formula:



2. Use of the compound of formula 1 for killing or inhibiting the growth of an insect comprising administering to the insect an effective amount of the compound.
3. Use as claimed in calim2 wherein said insect is a mosquito.
4. Use as claimed in claim 3 wherein said mosquito is C*ulex quinquifasciatus.*
5. The compound according to claim 1 is as an inhibitor of serine esterase.
6. Use of the compound of formula 1 for application as a drug for Alzheimer's disease or dermentia
7. A process for the isolation of the compound according to claim 1, from the fungus Sporotrichum species, said process comprising the steps of:
   (a) extracting fermented solid with an organic solvent;
   (b) filtering the extract of step (a) through a cloth or Whatman filter paper to obtain a clear solution;
   (c) evaporating the solution of step (b) under reduced pressure to obtain a crude extract;
   (d) purifying the crude extract of step (c) by column chromatography over silica gel and eluting with mixture of organic solvents of increasing polarity;
   (e) pooling active eluted fractions of step (d) and further subjected to column chromatography over silica gel by eluting with a mixture of organic solvents with increasing polarity;
   (f) repooling the active eluted fractions of step (e);
   (g) evaporating the pooled fractions of step (f) to yield the pure compound of formula I and
   (h) dissolving the residue in step (g) in ethyl acetate to yield the pure compound of claim 1.

8. The process according to claim 7, wherein in step (a) the organic solvent is selected from the group consisting of ethyl acetate, acetone and methanol.
9. The process according to claim 7, wherein in step (d) the mixture of organic solvents is selected from the combination of hexane: diethyl ether and chloroform: methanol mixtures.
10. The process according to claim 7, wherein in step (e) the mixture of organic solvent used is chloroform: ethyl acetate mixture.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/IN 03/00073

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C07C65/40 A61K31/192

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)
IPC 7 C07C A61K A61P

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

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
CHEM ABS Data, WPI Data, EPO-Internal, MEDLINE, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	OMURA, SATOSHI ET AL: "Arisugacin, a novel land selective inhibitor of acetylcholinesterase from Penicillium sp. FO-4259" JOURNAL OF ANTIBIOTICS , vol. 48, no. 7, 1995, pages 745-746, XP009013727 cited in the application the whole document	1

☐ Further documents are listed in the continuation of box C.

☐ Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
15 July 2003	06/08/2003

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Goetz, G

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IN 03/00073

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claim 6 is directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.