CORRECTED VERSION*

EP 18993.

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 3 : C07C 103/52	A1	(11) International Publication Number: WO 80/00562
		(43) International Publication Date: 3 April 1980 (03.04.80)

(21) International Application Number: PCT/US79/00691

(22) International Filing Date: 6 September 1979 (06.09.79)

(31) Priority Application Number: 942,002

(32) Priority Date: 13 September 1978 (13.09.78)

(33) Priority Country: US

(71) Applicant: ABBOTT LABORATORIES [US/US]; 14th Street and Sheridan Road, North Chicago, IL 60064 (US).

(72) Inventor: ALI, Akhtar; 13 Montebello Drive, Vernon Hills, IL 60061 (US).

(74) Agent: MCDONNELL, John, J.; 14th Street and Sheridan Road, North Chicago, IL 60064 (US).

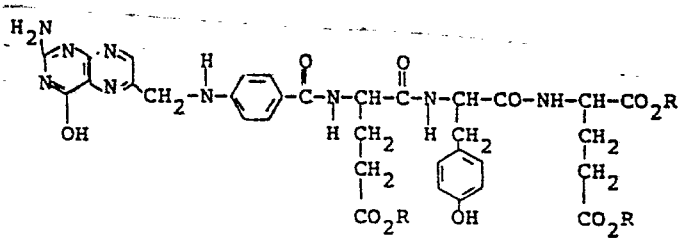
(81) Designated States: DE, FR (European patent), GB, JP.

Published
With international search report

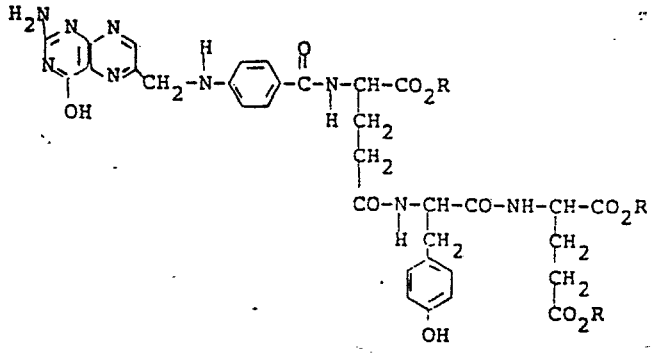
(54) Title: FOLIC ACID DERIVATIVES FOR USE IN RADIOIMMUNOASSAY

(57) Abstract

The present invention encompasses compounds A and B, mixtures of A and B and acid addition salts thereof wherein R is hydrogen or loweralkyl having 1-3 carbon atoms, and the phenol ring is labeled with ^{125}I or ^{131}I. The radiolabeled compounds are useful in radiochemical assaying for folic acid.



A



B

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria
AU	Australia
BR	Brazil
CF	Central African Republic
CG	Congo
CH	Switzerland
CM	Cameroon
DE	Germany, Federal Republic of
DK	Denmark
FR	France
GA	Gabon
GB	United Kingdom
HU	Hungary
JP	Japan
KP	Democratic People's Republic of Korea
LI	Liechtenstein
LU	Luxembourg
MC	Monaco
MG	Madagascar
MW	Malawi
NL	Netherlands
NO	Norway
RO	Romania
SE	Sweden
SN	Senegal
SU	Soviet Union
TD	Chad
TG	Togo
US	United States of America

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 3 : C07C 103/52	A1	(11) International Publication Number: WO 80/00562 (43) International Publication Date: 3 April 1980 (03.04.80)

(21) International Application Number: PCT/US79/00691

(22) International Filing Date: 6 September 1979 (06.09.79)

(31) Priority Application Number: 942,654

(32) Priority Date: 13 September 1978 (13.09.78)

(33) Priority Country: US

(71) Applicant: ABBOTT LABORATORIES [US/US]; 14th Street and Sheridan Road, North Chicago, IL 60064 (US).

(72) Inventor: ALI, Akhtar; 13 Montebello Drive, Vernon Hills, IL 60061 (US).

(74) Agent: MCDONNELL, John, J.; 14th Street and Sheridan Road, North Chicago, IL 60064 (US).

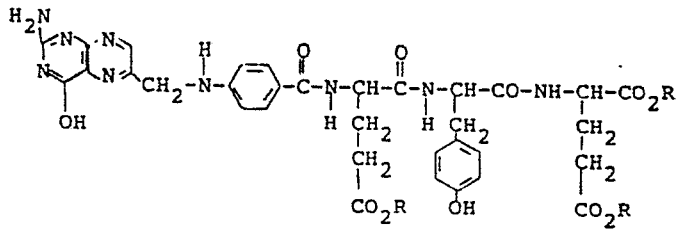
(81) Designated States: DE, FR (European patent), GB, JP.

Published
With international search report

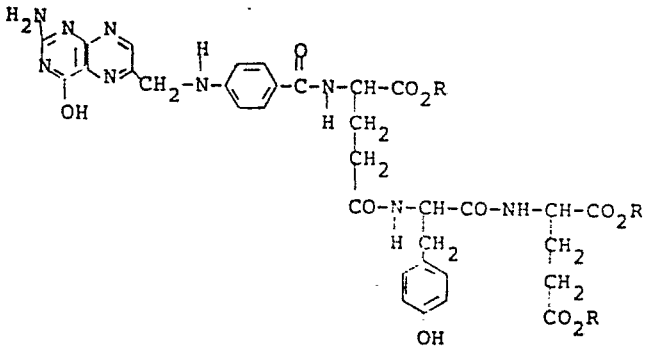
(54) Title: FOLIC ACID DERIVATIVES FOR USE IN RADIOIMMUNOASSAY

(57) Abstract

The present invention encompasses compounds A and B, mixtures of A and B and acid addition salts thereof wherein R is hydrogen or loweralkyl having 1-3 carbon atoms, and the phenol ring is labeled with ^{125}I or ^{131}I. The radiolabeled compounds are useful in radiochemical assaying for folic acid.



A



B

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT

AT	Austria	**LU**	Luxembourg
BR	Brazil	**MC**	Monaco
CF	Central African Republic	**MG**	Madagascar
CG	Congo	**MW**	Malawi
CH	Switzerland	**NL**	Netherlands
CM	Cameroon	**RO**	Romania
DE	Germany, Federal Republic of	**SE**	Sweden
DK	Denmark	**SN**	Senegal
FR	France	**SU**	Soviet Union
GA	Gabon	**TD**	Chad
GB	United Kingdom	**TG**	Togo
JP	Japan	**US**	United States of America

Description

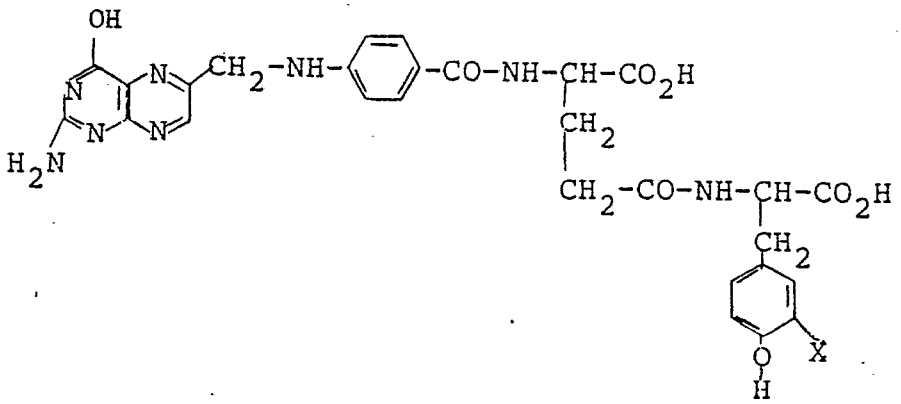
Folic Acid Derivatives For Use In Radioimmunoassay

Technical Field

The present invention relates to folic acid derivatives useful in radioassaying folic acid and its metabolites in biological fluids such as blood serum. There are many methods known for the radioassay of folic acid and its derivatives in biological fluid. Competitive binding of labeled folic acid (compounds of the present invention) and test sample folic acid for the folate binding protein(s) present in milk (Clinical Chem. 19, 1101, 1973) is a suitable method for determining folic acid.

Background Art

U. S. Patent 3,989,812 describes compounds of the formula:



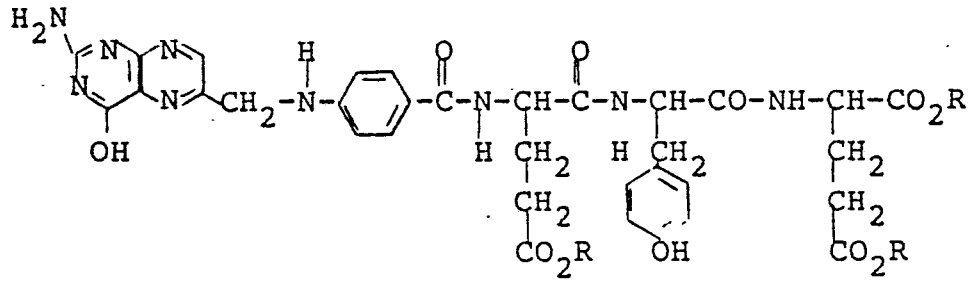
wherein X is ^{125}I or ^{131}I substituted in the phenol ring having utility for determining folic acid in serum.

Belgium Patent 840,196 describes tyramine derivatives of folic acid labeled ^{125}I or ^{131}I.

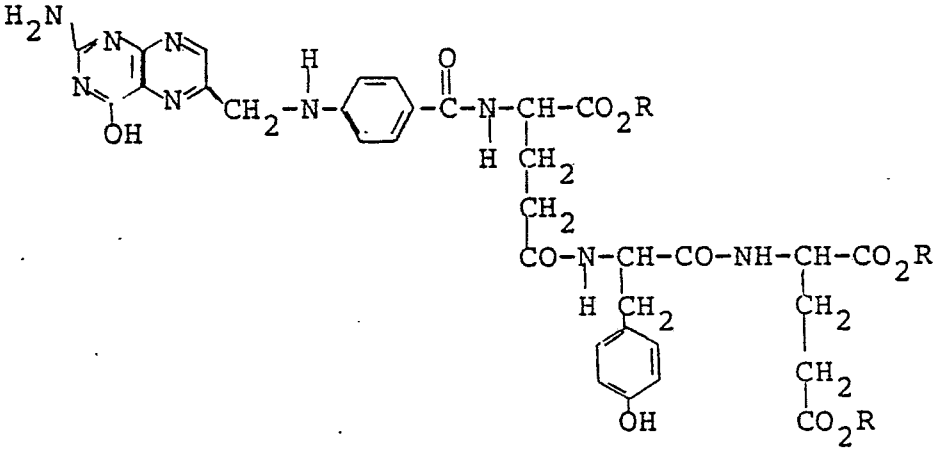
The compounds of the present invention differ in that the labeled tryosine moiety is not in the terminal position and that the terminal position is occupied by glutamic acid. Labeled compounds of the present invention more closely resemble folic acid in that both have glutamic acid in the terminal position.

Disclosure of Invention

The present invention encompasses compounds of the formula A and B:



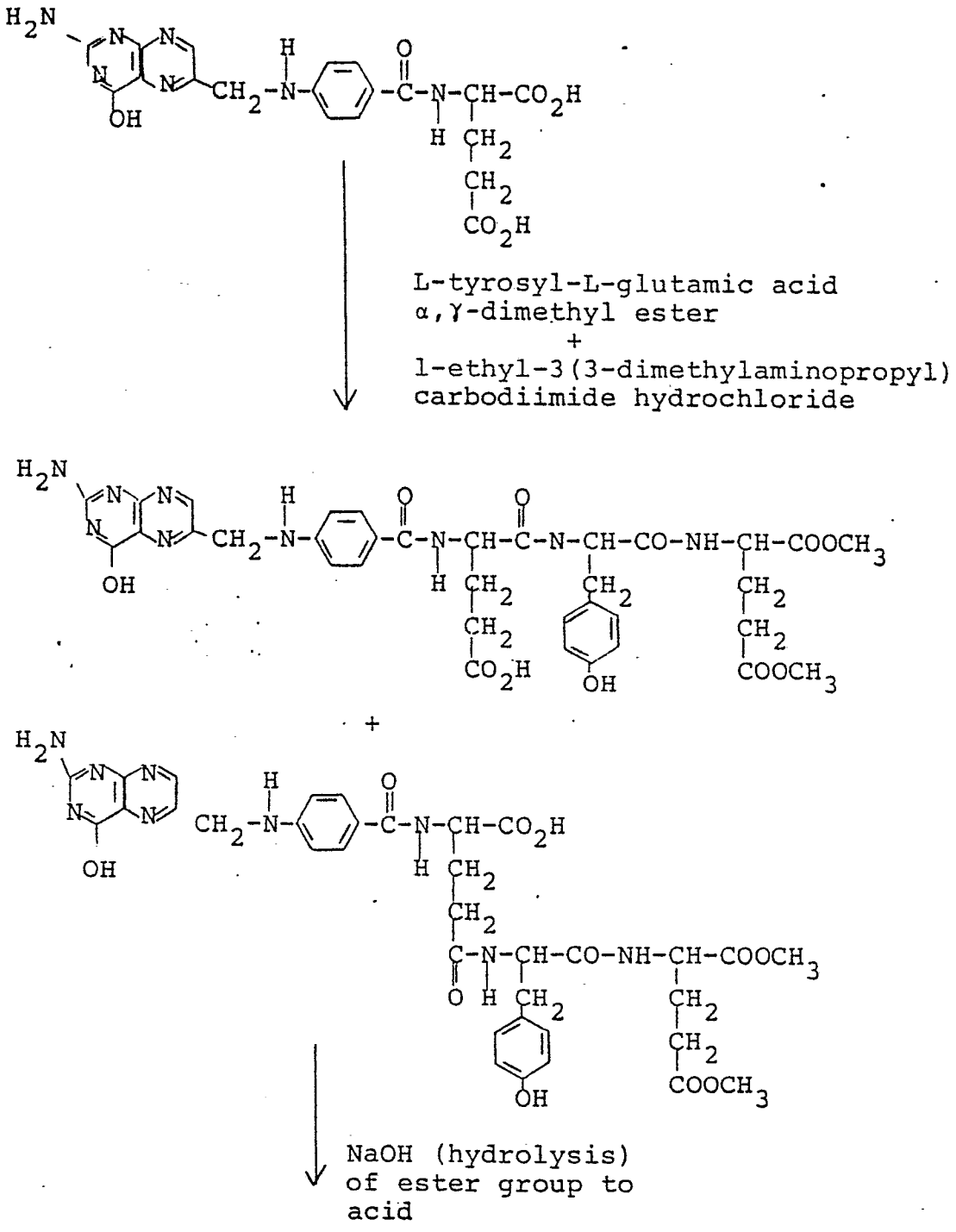
A



B

mixtures of A and B and acid addition salts thereof wherein R is hydrogen or loweralkyl having 1-3 carbon atoms and the phenol moiety is labeled with ^{125}I or ^{131}I to provide radiolabeled folic acid derivatives useful for assaying for folic acid in biological fluids.

Compounds of the present invention are prepared according to the following scheme:



These compounds are separated by conventional chromatography techniques. The residual acid groups may be esterified by reaction with diazomethane or acid-catalyzed esterified with alcohols. Alternately, one of the carboxylic acid groups of the glutamic acid portion of the starting folic acid can be esterified providing a

single condensation product. Esters are converted to acids by conventional base catalyzed hydrolysis.

Radioactive iodine, ^{125}I or ^{131}I, is conveniently introduced by the phenol ring of tyrosine by the chloramine-T method of Greenwood, et al, Biochem. J., 89, 114 (1963).

Compounds of the present invention are most conveniently used as the acid addition salt of organic and mineral acids such as hydrochloric, hydrobromic, hydroiodic, sulfuric, nitric, benzoic, acetic, trichloroacetic, toluene-sulfonic acids and the like.

Best Mode For Carrying Out The Invention

Folic acid (485 mg, 1.1 mmole) is suspended in water (8.0 ml) and stirred magnetically, while a solution of L-tyrosyl-L-glutamic acid α,γ-dimethyl ester (396 mg, 1.17 mmole) in pyridine (8 ml) is added. To this reaction mixture is added 1-ethyl 3(3-dimethylaminopropyl)-carbodiimide hydrochloride (2.87 mg, 1.5 mmole). The mixture is stirred at 4°C overnight. 20 ml of 0.5% sodium bicarbonate solution is added to the reaction mixture and filtered. The filtrate is acidified with 1N hydrochloride acid to pH 3.0. The resulting precipitate is filtered and washed with about 20 ml of cold water.

This precipitate of the folic acid-tyrosylglutamic acid dimethyl ester is suspended by stirring in 10 ml of 0.2N sodium hydroxide, while nitrogen gas is bubbled through the reaction mixture. After 15-20 minutes of stirring at room temperature, the clear solution is acidified with 1N hydrochloric acid to pH 3.0. This precipitated material is filtered, washed with cold water and dried in a vacuum desiccator to provide a mixture of Compound A and B where R is hydrogen. These compounds are separated by conventional techniques.

Iodination was performed by chloramine-T procedure, Biochem. J., 89, 114 (1963).

100µl of 0.5M sodium phosphate pH 7.5 is added to a glass test tube, followed by 10.0 mCi of Na I^{125}. To this solution is added 25µl (8.3µg) of folic acid-tyrosylglutamic acid in sodium bicarbonate solution and 50µl of 0.4% chloramine-T solution in a 0.05M sodium phosphate buffer pH 7.5, respectively. After 60 seconds, mixing at room temperature, 50µl of 0.8% sodium metabisulfite is added to the reaction mixture.

Incorporation of ^{125}I into folic acid derivatives is in excess of 85%. The purification of the iodinated reaction mixture is achieved by ion exchange followed by cellulose column chromatography.

Industrial Applicability

Example 1

Binding Activity Of Folate Derivatives

The ^{125}I folate compounds are tested for their ability to bind to specific folate binders.

Partially purified bovine milk and goat's milk folate binders (J. Dairy Res., 36, 435 (1969)) are diluted serially with 0.05M borate buffer containing 0.1% dithiothreitol and 0.05% gelatin pH 9.0. ^{125}I folate (0.01 - 0.02µCi) is added to 0.5 ml of each respective binder dilution (final reaction volume is 0.6 ml). The mixture is vortexed and allowed to incubate 45 minutes at room temperature. At the end of the incubation period, 1 ml of a 0.7% dextran-coated charcoal suspension is added to each tube, vortexed and allowed to stand 5 minutes at room temperature. The tubes are centrifuged at 1000g for 15 minutes. The supernatants are decanted into clean tubes and counted in a gamma scintillation counter.

Results

The ^{125}I folate derivatives typically show a maximum binding ability of between 75-90%.

Example 2

Determination Of Folate Concentration in Biological Fluids Preparation Of Samples

Serum is obtained, free of hemolysis and stored at 4°C for 24 hours. For longer storage periods samples are stored at -20°C.

Samples for red cell folate determinations are prepared by diluting one part of whole blood with 19 parts of a 0.2% ascorbic acid solution. Samples are allowed to stand at room temperature for 1-1/2 hours to allow for hydrolysis of folate polyglutamates. Samples are then stored at -20°C if not used immediately.

Example 3

Folate Assay

Pteroylglutamic acid (folic acid) is diluted serially with 0.05M borate buffer containing 0.50% gelatin and 0.1% dithiothreitol; pH 9.0. The concentrations of folic acid used are 0, 2.5, 5, 10, 15 and 20 ng/ml.

Borate buffer (0.4 ml) is added to a series of polypropylene tubes. To this solution is added 0.05 ml of appropriate standard dilution or sample (serum or hemolysate).

The contents of the tubes are mixed and heated in a boiling water bath for 15 minutes. The tubes are loosely covered during the extraction.

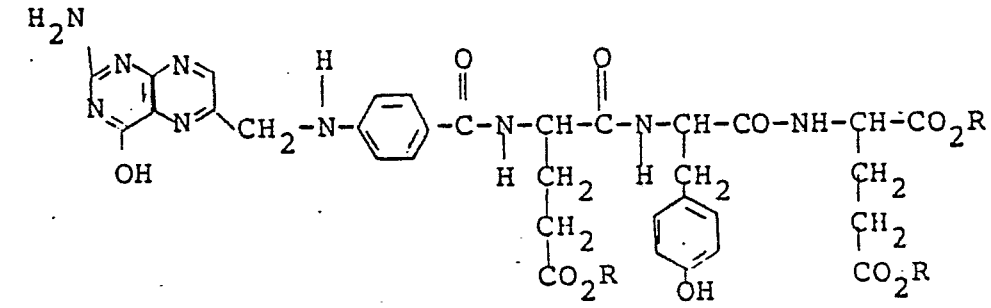
The tubes are cooled to room temperature and 0.10 ml of ^{125}I folate derivative in borate buffer (0.01-0.02μCi) is added to each tube followed by 0.01 ml of an appropriate dilution of folate milk binder in borate buffer to give about 50% binding. The tubes are vortexed and allowed to stand 5 minutes at room temperature. The tubes are then centrifuged at 1000xg for 15 minutes. The supernatants are decanted into clean tubes and counted in a suitable scintillation counter.

Results

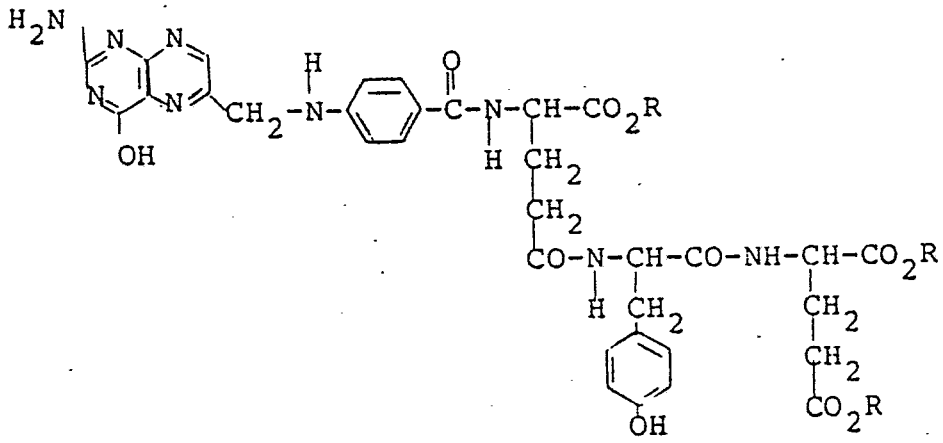
The folate in the patient samples are determined by comparison with a logit-log plot of the standard curve. Red cell folates are corrected for dilution and hematocrit (Clin. Biochem., 6, 274 (1973)).

Claims

1. A compound of the formula A or B:



A



B

mixtures of A and B, and acid addition salts thereof wherein R is hydrogen or loweralkyl having 1-3 carbon atoms and wherein the phenol ring is labeled with ^{125}I or ^{131}I.

INTERNATIONAL SEARCH REPORT

International Application No PCT/US79/00691

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int. Cl. C07C 103/52
US Cl. 260/112.5R

WO 80/00562

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System	Classification Symbols
US	260/112.5R

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [5]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
A	US, A, 3,989,812 Published 02 November 1976, Barrett	

* Special categories of cited documents: [15]

"A" document defining the general state of the art

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

"L" document cited for special reason other than those referred to in the other categories

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

"P" document published prior to the international filing date but on or after the priority date claimed

"T" later document published on or 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

IV. CERTIFICATION

Date of the Actual Completion of the International Search [2]	Date of Mailing of this International Search Report [2]
20 December 1979	15 JAN 1980
International Searching Authority [1]	Signature of Authorized Officer [20]
ISA/US	Paul M. Coughlan Jr.
	Paul M. Coughlan Jr.

Form PCT/ISA/210 (second sheet) (October 1977)