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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification [4] : C12P 21/02	A1	(11) International Publication Number: WO 87/ 06268
		(43) International Publication Date: 22 October 1987 (22.10.87)

(21) International Application Number: PCT/AU87/00092

(22) International Filing Date: 8 April 1987 (08.04.87)

(31) Priority Application Number: PH 5408

(32) Priority Date: 10 April 1986 (10.04.86)

(33) Priority Country: AU

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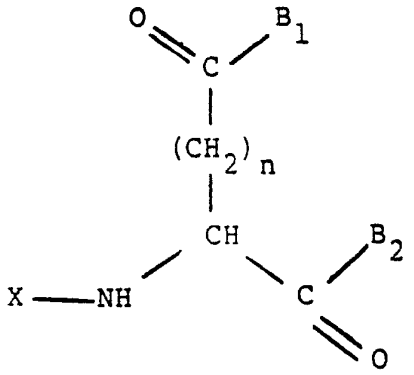
(81) Designated States: AT (European patent), AU, BE (European patent), CH (European patent), DE (European patent), FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent), US.

Published
With international search report.

(54) Title: ENZYMATIC SYNTHESIS



(I)

(57) Abstract

A process for the linking of either aspartate or glutamate radicals to the N-terminal of an amino acid or derivative thereof or to the N-terminal residue of a peptide. The process is applicable for all amino acids with the exception of proline and hydroxy-proline and involves reacting an aspartate or glutamate radical of formula (I), in which n is 1 or 2, B_1 is any group capable of forming an ester linkage, B_2 is any group capable of forming an ester linkage and cleavable by a thiol proteinase and X is an aliphatic or aromatic hydrophobic group, with an amino acid derivative or peptide in the presence of a thiol proteinase.

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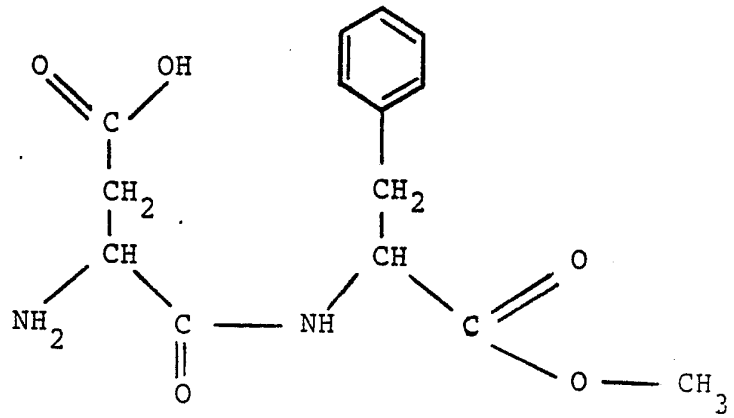
ENZYMATIC SYNTHESIS

The present invention relates to a method of linking either an aspartate or glutamate radical to the N-terminal of an amino acid or derivative thereof. This method is applicable for amino acids, with the exception of proline or hydroxy-proline, and is functional regardless of whether the amino acid is in isolation or present as the N-terminal residue of a peptide. The method is therefore useful in peptide synthesis.

This method is particularly applicable to the production of the artificial sweetening agent known under the generic name of aspartame. Aspartame was first discovered in 1966 and is a dipeptide of the following structure:



Aspartic acid Phenylalanine Methyl ester

and has a sweetening power of 100 to 200 times that of sugar.

It is well known in the art to link amino acids enzymatically. This is witnessed by the number of patent applications relating to such processes which include US 4,086,136, US 4,116,768, US 4,289,721 and US 4,521,514 and Australian Patent No. 558330. Each of these processes involves the linking of an amino acid derivative with a free hydroxyl group to an amino acid derivative or peptide, the reaction being carried out in the presence of an enzyme. All these prior art processes involve a condensation reaction to bring about the joining of the amino acids. A general example of this type of reaction is shown below.

$$A_1\text{-OH} + \text{H-}A_2 \xrightarrow{\text{Enzyme}} A_1 - A_2 + H_2O$$

where A_1 and A_2 are amino acids or peptides.

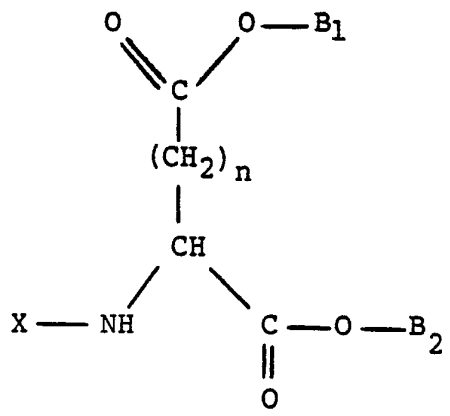
During the course of investigations into the use of the thiol proteinases in enzymic peptide synthesis the present inventor discovered that thiol proteinases were able to catalyse the reaction between Z-L-aspartic acid dibenzyl ester and L-phenylalanine methyl ester to form a derivate of aspartame, Z-L-aspartyl(β-benzyl ester)-L-phenylalanine methyl ester (Z is an abbreviation used in the art that refers to benzyoxycarbonyl). Further work has shown that this method is applicable to linking either aspartate or glutamate to the N-terminal of an amino acid derivative or peptide.

The reaction takes advantage of the esterase activity of the thiol proteinase giving rise to a much faster and more efficient reaction than results from the use of prior art processes involving condensation reactions. The use of an aspartate or glutamate derivative with a free carboxyl group is a different and less efficient method with a reaction time of days as compared to minutes. Further it should be noted that in the present process the

use of the esterase activity does not generate a free carboxyl group, rather, in the enzyme-C-component complex the ester of the C-component is cleaved by a nucleophilic attack by the amino group of the incoming N-component during formation of the peptide bond.

The present invention consists in a method for the addition of an aspartate or glutamate radical to the N-terminal of an amino acid, wherein said amino acid exists either singly, as a derivative having a C-terminal protective group, or as the N-terminal residue of a peptide, said method comprising the following steps:

reacting, in the presence of a thiol proteinase, a compound of a general formula:



wherein: n is either 1 or 2,

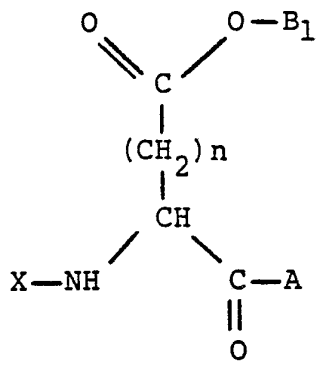
B_1 is any group capable of forming an ester linkage,

B_2 is any group capable of forming an ester linkage and cleavable by the thiol proteinase,

X is an aliphatic or aromatic hydrophobic group,

with an amino acid or derivative thereof or a peptide, the amino acid derivative having a C-terminal protective

group, to obtain a compound of the following general formula:



wherein A is a residue of said amino acid or derivative thereof or said peptide, and optionally removing the X and B_1 groups from the molecule.

In a preferred embodiment of the invention the thiol proteinase is either papain or chymopapain, n is 1, X is benzyloxycarbonyl (known in the art as Z), B_1 and B_2 are both benzyl groups, A is a methyl ester of phenylalanine and a hydrogenation process is used to remove the Z and B_1 benzyl group from the reaction product to produce aspartame.

Thiol proteinases have been well characterized in the literature (e.g. Advances in Enzymology Vol. 53 pp 239-306), and include the enzymes papain, chymopapain, ficin, bromelain, cathepsins B and C and Streptococcal proteinase. Apart from Z there are a number of other aliphatic or aromatic hydrophobic groups which could be used in this invention. These include t-BOC, B.poc, and Fmoc. The use and characteristics of these compounds has been described in the literature by Schechter I and Berger A (Biochem. Biphys. Res. Comm. Vol. 27 pp 157-162) and by Fruton J.S. (Advances in Enzymology Vol. 53 pp 239-306).

Examples of groups which can be substituted for benzyl at B_1 and/or B_2 are ethyl or methyl groups. However the rate of reaction in the case of production of aspartame is greater when B_1 and B_2 are both benzyl.

When the method of the present invention is employed to link an aspartate or glutamate radical to a single amino acid derivative, as is the case of the production of aspartame, it is preferable that C-terminal of the amino acid be protected. The protective groups for the carboxyl group of this amine component include alkoxy groups, substituted or unsubstituted benzyloxy groups, or amino groups. As will be appreciated by the person skilled in the art when the method of the present invention is employed to link an aspartate or glutamate radical to a peptide it is not necessary to protect the C-terminal of peptide, although this may optionally be done, as the carboxyl group of the amino acid residue of the peptide to which the aspartate or glutamate radical is to be linked is already indirectly protected by the other amino acid residue(s) making up the peptide.

The invention will now be described by means of example.

Example 1

(i) Formation of Z-L-aspartyl (β-benzyl ester)-L-phenylalanine methyl ester

a) 1.0 M Z-L-aspartic acid dibenzyl ester in dimethyl formamide.

b) 278mM L-phenylalanine methyl ester in 55% ethanol containing 11mM EDTA and 28mM mercapto-ethanol plus 5.9uM activated papain, pH8.5.

Reaction 1 part "a" was added to 9 parts "b" at room temperature and the pH maintained at 8.5. Synthesis was monitored by high pressure liquid chromatography (HPLC). The reaction proceeded with approximately 70 to 80% efficiency in terms of the amount of "a" used with a

reaction time of approximately 2-3 hours.

The synthesis product Z-L-aspartyl (β-benzyl ester)-L-phenylalanine methyl ester precipitated during the reaction and was harvested by filtration.

All other reaction products are recoverable and the hydrolysis product Z-L-aspartic acid β-benzyl ester can be recycled to reform Z-L-aspartic acid dibenzyl ester. Experiments indicate that the papain is stable under the reaction conditions although it may need to be reactivated periodically.

(ii) Conversion to L-aspartyl-L-phenylalanine methyl ester

This conversion was carried out by a hydrogenation reaction involving a palladium catalyst and hydrogen gas.

The entire reaction is summarized in Figure 1.

This method of production of aspartame has a number of advantages over prior art process in that the use of Z-L-aspartic acid dibenzyl ester has advantages over other aspartic acid derivatives. It is a relatively cheap reagent to prepare as both carboxyl groups have the same substitution. The side chain remains protected after the coupling of the L-phenylalanine methyl ester enabling the product to precipitate. In this regard it should be noted that some prior art processes require the use of addition compounds to cause precipitation e.g. U.S. 4,521,024. Finally both the Z and benzyl groups can be removed from the synthesis product in a single catalytic hydrogenation to yield aspartame

Example 2

Formation of Z-L-glutamyl (γ-benzyl ester)-L-phenylalanine methyl ester

a) 1.0 M Z-L glutamic acid dibenzyl ester in dimethylformamide.

b) 222 mM L-phenylalanine methyl ester in 44% dimethylformamide containing 11 mM EDTA and 28 mM mercaptoethanol plus 2.4 uM activated papain, pH 8.5.

Reaction

One part of "a" was added to nine parts "b" at room temperature and the pH maintained at 8.5. Synthesis was monitored by HPLC. The reaction proceeded with approximately 90% efficiency in terms of the amount of "a" used.

The synthesis product Z-L-glutamyl (γ - benzyl ester)-L-phenylalanine methyl ester precipitated during the reaction and was harvested by filtration.

Example 3

Formation of Z-L-aspartyl (β-benzyl)-L-alanine ethyl ester.

a) 800 mM Z-L-aspartic acid dibenzyl ester in dimethylformamide.

b) 222 mM L-alanine ethyl ester in 55% dimethylformamide containing 11 mM EDTA and 28 mM mercaptoethanol plus 2.4 uM activated papain, pH 8.5.

Reaction

One part "a" was added to nine parts "b" at room temperature and the pH maintained at 8.5. Synthesis was monitored by HPLC. The reaction proceed with approximately 40% efficiency in terms of the amount of "a" incorporated into Z-L-aspartyl (β-benzyl)-L-alanine ethyl ester.

The synthesis product precipitated during the reaction and was harvested by filtration.

Example 4

Formation of Z-L-aspartyl (β-benzyl ester)-L- serine amide

a) 1.0 M Z-L-aspartic acid dibenzyl ester in dimethylformamide.

b) 667 mM L-serine amide in 55% dimethylformamide containing 11mM EDTA and 28 mM mercaptoethanol plus 5.9 uM activated papain, pH 8.5.

Reaction

One part "a" was added to nine parts "b" at room

temperature and the pH maintained at 8.5. The reaction was monitored by HPLC. The reaction proceeded with approximately 40-50% efficiency in terms of the amount of "a" incorporated into Z-L-aspartyl (β-benzyl)-L- serine amide.

Example 5

Formation of t-BOC-L-aspartyl (β-benzyl ester)-L-alanyl-L-isoleucyl-L-phenylalanine methyl ester

a) 1.0 M t-butyloxycarboxyl-L-aspartic acid dibenzyl ester in dimethyl formamide.

b) 111mM L-alanyl-L-isoleucyl-L-phenylalanine methyl ester in 55% dimethylformamide containing 28 mM EDTA plus 4.3 uM activated papain, pH 8.5.

Reaction

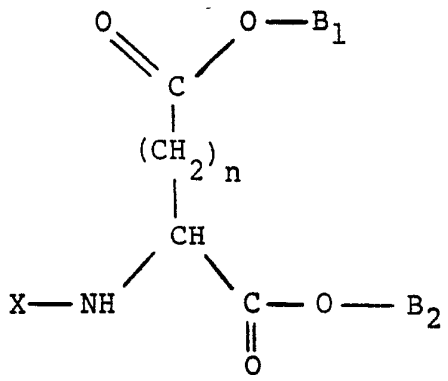
One part "a" was added to nine parts "b" at room temperature and the pH maintained at 8.5. The reaction was monitored by HPLC. The reaction proceeded with approximately 30-40% efficiency in terms of the amount of "a" used.

The synthesis product t-BOC-L-aspartyl (β-benzyl)-L-alanyl-L-isoleucyl-L-phenylalanine methyl ester precipitated during the reaction and was harvested by filtration.

CLAIMS

1. A method for the addition of an aspartate or glutamate radical to the N-terminal of an amino acid, wherein said amino acid exists either singly, as a derivative having a C-terminal protective group, or as the N-terminal residue of a peptide, said method comprising the following steps:

reacting, in the presence of a thiol proteinase, a compound of a general formula:



wherein: n is either 1 or 2,

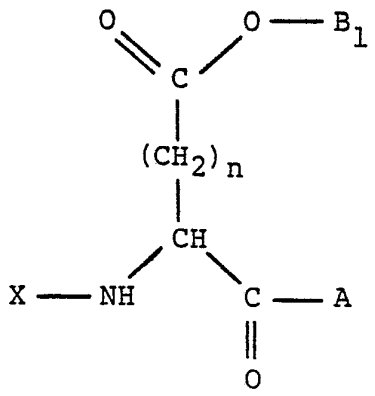
B_1 is any group capable of forming an ester linkage,

B_2 is any group capable of forming an ester linkage and cleavable by the thiol proteinase,

X is an aliphatic or aromatic hydrophobic group,

with an amino acid or derivative thereof or a peptide, the amino acid derivative having a C-terminal protective

group, to obtain a compound of the following general formula:



wherein A is a residue of said amino acid or derivative thereof or said peptide, and optionally removing the X and B_1 groups from the molecule.

2. A method as claimed in claim 1 in which B_1 and B_2 are the same or different and are selected from the group comprising methyl, ethyl and benzyl, and wherein the amino acid derivative has a C-terminal protective group.

3. A method as claimed in claim 1 or 2 in which the thiol proteinase is either papain or chymopapain, and in which X is either benzyloxycarboxyl (Z) or tertiary butyloxy carboxyl (t-BOC).

4. A method as claimed in claim 3 in which B_1 and B_2 are both benzyl; the thiol protease is papain; and X is Z and in which the X and B_1 groups are subsequently removed by hydrogenation.

5. A method of producing L-aspartyl-L- phenylalanine methyl ester (aspartame) comprising the steps of:

a) reacting Z-L-aspartic acid dibenzyl ester with

L-phenylalanine methyl ester in the presence of papain;

b) recovering formed Z-L-aspartyl (β-benzyl ester)-L-phenylalanine methyl ester; and

c) subjecting the recovered product of (b) to a hydrogenation reaction to remove the benzyl and Z groups.

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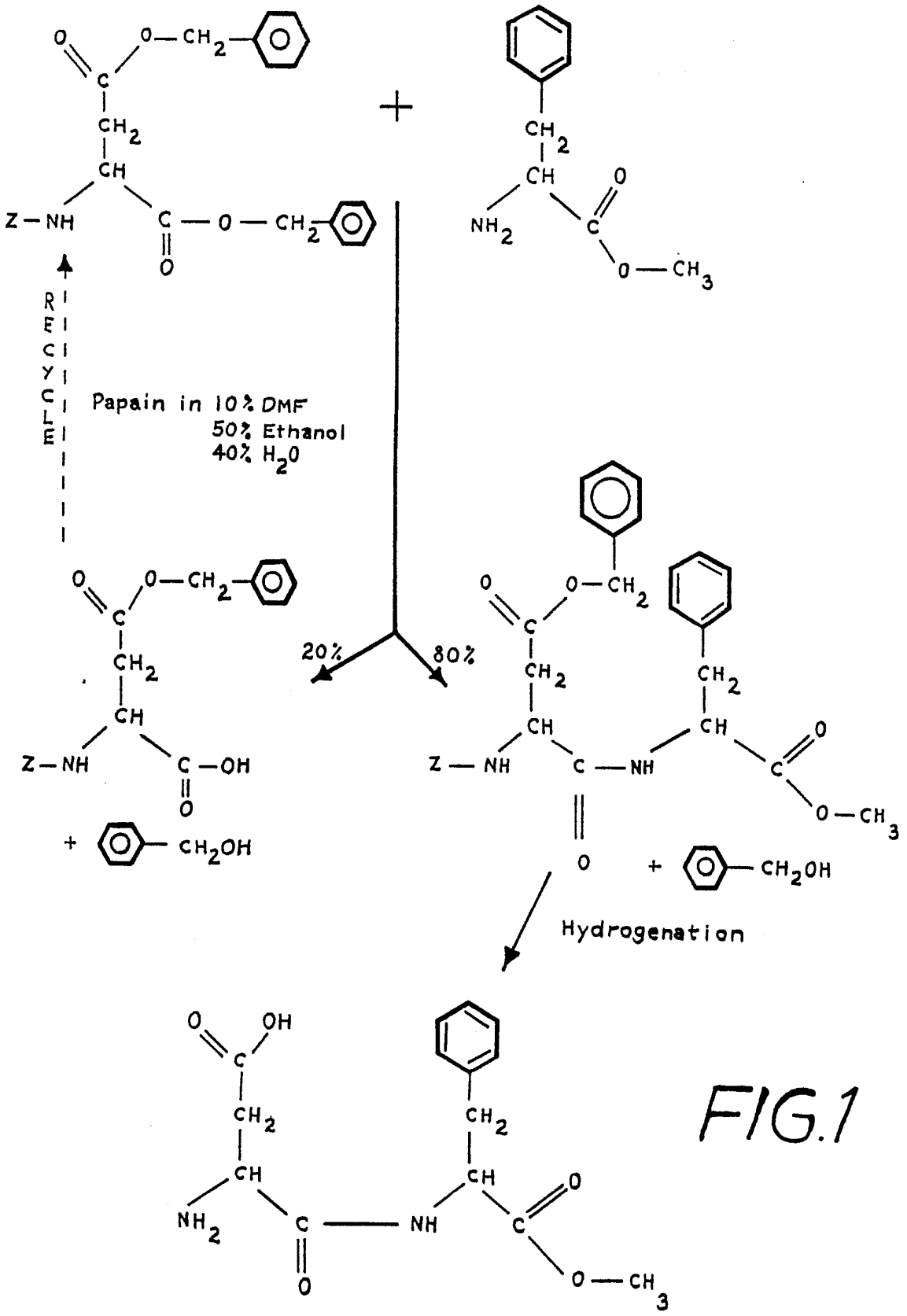

FIG.1

INTERNATIONAL SEARCH REPORT

International Application No PCT/AU 87/00092

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

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

Int. Cl.[4] C12P 21/02

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC	C12P 21/00, 21/02

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

AU : IPC as above

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X	US,A, 4339534 (JOHANSEN et al) 13 July 1982 (13.07.82) See column 5 lines 27-44	(1-5)
A	US,A, 4116768 (ISOWA et al) 26 September 1978 (26.09.78)	
A	US,A, 4086136 (ISOWA et al) 25 April 1978 (25.04.78)	
A	GB,A, 2092161 (G.D. SEARLE AND CO.) 11 August 1982 (11.08.82)	
A	GB,A, 2066266 (SOCIETE DES PRODUITS NESTLE SA) 8 July 1981 (08.07.81)	
A	DE,A, 2224644 (TATE & LYLE LTD) 1 February 1973 (01.02.73)	

* Special categories of cited documents: [10]

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"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
9 July 1987 (09.07.87)	(21.07.87) 21 JULY 1987
International Searching Authority	**Signature of Authorized Officer**
Australian Patent Office	G.J. McNEICE

Form PCT/ISA/210 (second sheet) (January 1985)

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON
INTERNATIONAL APPLICATION NO. PCT/AU 87/00092

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report	Patent Family Members		
US 4339534	AU 59815/80	BR 8008024	CA 1160973
	CA 1177429	DK 5202/80	EP 17485
	FI 801035	IL 59776	NO 803672
	NZ 193375	WO 8002157	ZA 8001929
	US 4645740	AU 74508/81	CA 1175372
	DK 3197/80	DK 1300/82	DK 3204/83
	EP 45187	ES 504462	ES 514463
	NO 820958	WO 8200301	ZA 8105066
US 4116768	CA 1059051	CH 623807	DE 2647189
	FR 2328695	GB 1523546	IT 1077079
	JP 52128290	NL 7611569	US 4119493
	JP 52072885	JP 52128291	
GB 2092161	DE 3203292	FR 2499098	GB 2092161
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END OF ANNEX