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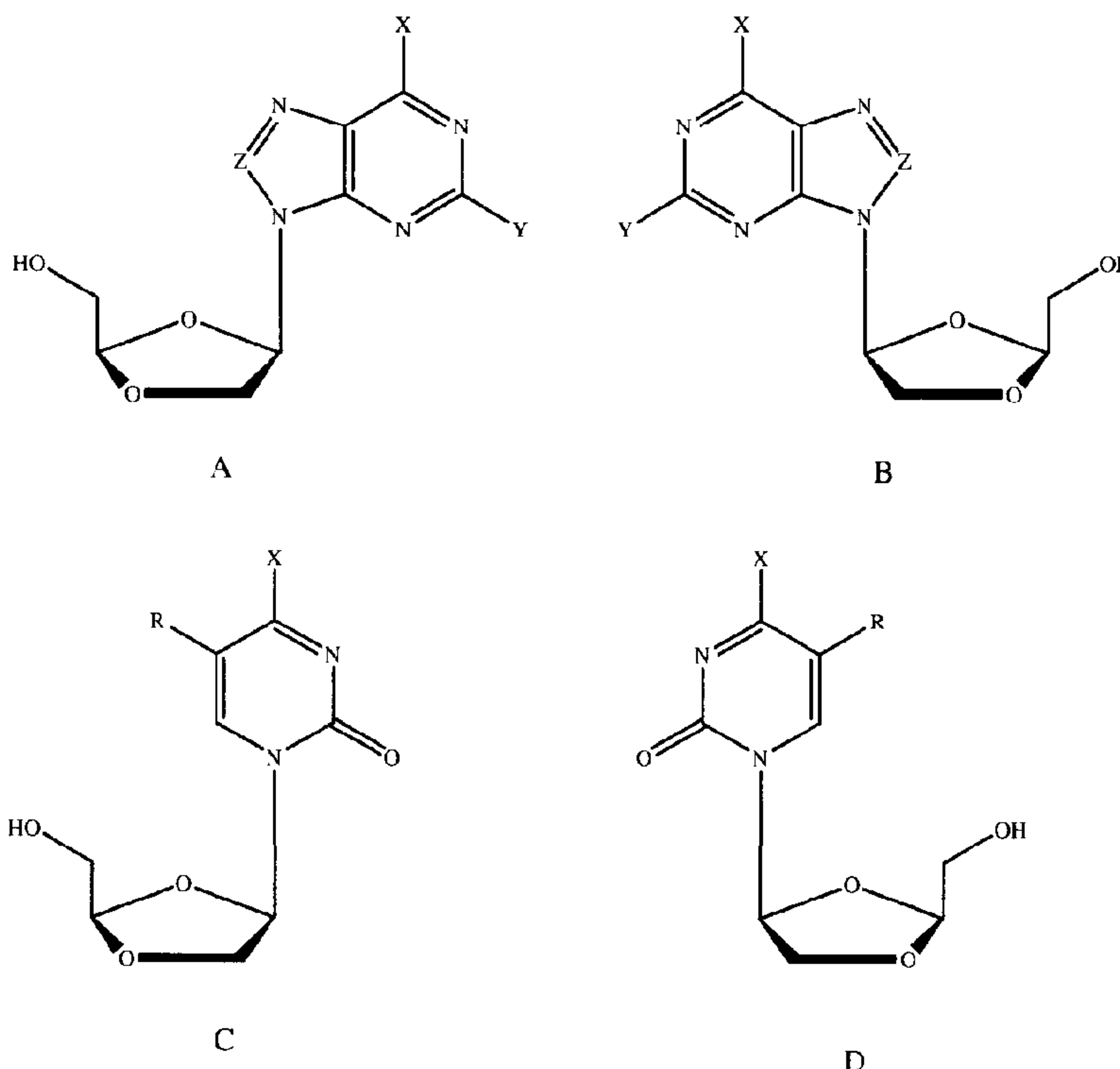
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(54) Titre : METHODES DE FABRICATION DE NUCLEOSIDES DE 1,3-DIOXOLANE

(54) Title: METHODS TO MANUFACTURE 1,3-DIOXOLANE NUCLEOSIDES



(57) Abrégé/Abstract:

This application provides a process for preparing enantiomerically pure β -D-dioxolane nucleosides. In particular, a new synthesis of (-)-DAPD, suitable for large scale development, is described. In one embodiment the invention provides a process for preparing a substantially pure β -D or β -L-1,3-dioxolane nucleosides comprising a) preparing or obtaining an esterified 2,2-dialkoxy ethanol; b) cyclizing the esterified 2,2-dialkoxy ethanol with glycolic acid to obtain a 1,3-dioxolane lactone; c) resolving the 1,3-dioxolane lactone to obtain a substantially pure D- or L-lactone; d) selectively reducing and activating the D- or L-chiral lactone to obtain a substantially pure D- or L-1,3-dioxolane; e) coupling the D- or L-1,3-dioxolane to an activated and/or protected purine or pyrimidine base; and f) optionally purifying the nucleoside to obtain a substantially pure protected β -D or β -L-1,3-dioxolane nucleoside.



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(54) Title: METHODS TO MANUFACTURE 1,3-DIOXOLANE NUCLEOSIDES

(57) Abstract: This application provides a process for preparing enantiomerically pure β -D-dioxolane nucleosides. In particular, a new synthesis of (-)-DAPD, suitable for large scale development, is described. In one embodiment the invention provides a process for preparing a substantially pure β -D or β -L-1,3-dioxolane nucleosides comprising a) preparing or obtaining an esterified 2,2-dialkoxy ethanol; b) cyclizing the esterified 2,2-dialkoxy ethanol with glycolic acid to obtain a 1,3-dioxolane lactone; c) resolving the 1,3-dioxolane lactone to obtain a substantially pure D- or L-lactone; d) selectively reducing and activating the D- or L-chiral lactone to obtain a substantially pure D- or L-1,3-dioxolane; e) coupling the D- or L-1,3-dioxolane to an activated and/or protected purine or pyrimidine base; and f) optionally purifying the nucleoside to obtain a substantially pure protected β -D or β -L-1,3-dioxolane nucleoside.



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METHODS TO MANUFACTURE 1,3-DIOXOLANE NUCLEOSIDES

REFERENCE TO PRIOR APPLICATIONS

This application claims priority to U.S. Provisional application 60/541,545, entitled "Methods To Manufacture 1,3-Dioxolane Nucleosides" filed February 3, 2004.

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FIELD OF INVENTION

This application provides a process for preparing enantiomerically pure β -D-dioxolane nucleosides. In particular, a new synthesis of (-)-DAPD, suitable for large scale development, is described.

BACKGROUND OF THE INVENTION

10

AIDS, Acquired Immune Deficiency Syndrome, is a catastrophic disease that has reached global proportions. Currently an estimated 40 million people worldwide are living with AIDS, with approximately 5 million new infections every year. The yearly death toll is still over 3 million people worldwide. Another virus that causes a serious human health problem is the hepatitis B virus (HBV). HBV is second only to tobacco as

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a cause of human cancer. Some estimates put the number of people worldwide that have been infected with HBV as high as two billion people, up to a third of the world's population, with approximately 400 million chronically infected.

20

A number of 2',3'-dideoxynucleosides have been found to be potent antiviral agents against HIV and/or hepatitis B virus. After cellular phosphorylation to the 5'-triphosphate by cellular kinase, these synthetic nucleosides are incorporated into a growing strand of viral DNA, causing chain termination due to the absence of the 3'-hydroxyl group. They can also inhibit the viral enzyme reverse transcriptase.

25

There has also been interest in the synthesis of nucleoside derivatives in which the 3'-carbon of the nucleoside has been replaced with a heteroatom. Both 3TC and its 5-fluorocytosine analog (FTC) exhibit activity against HIV and HBV (Furman, et al., *Antimic. Ag. Chemo.*, 1992, 2686-2692; and Cheng, et al., *J. Biol. Chem.*, 1992, 267(20),13938-13942).

The discovery that a racemic oxathiolane nucleoside BCH-189 possessed a potent activity against replication of HIV prompted Chu et al. to synthesize the chiral products

(+)- and (-)-BCH-189 (Belleau, et al., 5th International Conference on AIDS, Montreal, Canada, June 4-9, 1989, #T.C.O. 1; Chu, et al. *Tetr. Lett.*, 1991, 32, 3791). The latter compound, lamivudine, otherwise known as 3TC or epivir, is currently used clinically in the treatment of both HIV infection and HBV infection. The (-) enantiomer of 5-fluorocytosine oxathiolane analogue (FTC), is particularly active against HIV (Choi, W. et al., *J. Am. Chem. Soc.*, 1991, 113, 9377; Schinazi, R. F., et al., *Antimic. Ag. Chemo.* 1992, 2423; U.S. Patent Nos. 5,204,4665; 5,210,085; 5,914,331; and 5,639,814).

The above-described 1,3-oxathiolane nucleosides are manufactured by condensation of silylated purine or pyrimidine base with a 1,3-oxathiolane intermediate. U.S. Patent No. 5,204,466 discloses a method to condense a 1,3-oxathiolane with a silylated pyrimidine using tin chloride as a Lewis acid, which provides virtually complete β -stereoselectivity. A number of U.S. patents describe processes for the preparation of 1,3-oxathiolane nucleosides via condensation of a 1,3-oxathiolane-2-carboxylic acid ester with a protected silylated base in the presence of a silicon-based Lewis acid, followed by reduction of the ester to the corresponding hydroxymethyl group to afford the final product (see U.S. Patent Nos. 5,663,320; 5,693,787; 5,696,254; 5,744,596; 5,756,706 and 5,864,164). In addition, these patents contain generic disclosures for the synthesis of 1,3-dioxolane nucleosides in a similar fashion using the corresponding 1,3-dioxolane intermediate.

U.S. Patent No. 5,272,151 discloses a process using a 2-O-protected-5-O-acylated-1,3-oxathiolane for the preparation of nucleosides by condensation with a silylated purine or pyrimidine base in the presence of a titanium catalyst. U.S. Patent No. 6,215,004 discloses a process for producing 1,3-oxathiolane nucleosides that includes condensing 2-O-protected-methyl-5-chloro-1,3-oxathiolane with a silylated 5-fluorocytosine without a Lewis acid catalyst. In these cases, the 1,3-oxathiolane ring is prepared in one of the following ways: (i) reaction of an aldehyde derived from a glyoxylate or glycolic acid with mercaptoacetic acid in toluene in the presence of p-toluenesulfonic acid to give 5-oxo-1,3-oxathiolane-2-carboxylic acid; (ii) cyclization of anhydrous glyoxylates with 2-mercaptoacetaldehyde diethylacetal at reflux in toluene to give 5-ethoxy-1,3-oxathiolane lactone; (iii) condensation of glyoxylic acid ester with mercaptoacetaldehyde (dimeric form) to give 5-hydroxy-1,3-oxathiolane-2-carboxylic ester or (iv) coupling of an acyloxyacetaldehyde with 2,5-dihydroxy-1,4-dithiane, the dimeric form of 2-mercaptoacetaldehyde to form a 2-(acyloxy)methyl-5-hydroxy-1,3-

oxathiolane. The lactone, 5-oxo compound, has to be reduced to the corresponding lactol during the process. The 2-carboxylic acid or its ester also has to be reduced to the corresponding 2-hydroxymethyl derivatives with borane-methylsulfide complex.

The key intermediate, aldehyde, can be prepared using several methods: (i) lead
5 tetraacetate oxidation of 1,4-di-O-benzoyl meso-erythritol, 1,6-di-O-benzoyl D-mannitol
or 1,5-di-O-benzoyl-D-arabitol; (ii) preparation of monoacylated ethylene glycol
followed by oxidation to aldehyde; (iii) acylation of ethylene chlorohydrin followed by
dimethylsulfoxide oxidation; (v) lead tetraacetate oxidation; (vi) ozonolysis of allyl or 3-
methyl-2-buten-1-ol acylate; (vii) and more recently, by acylation of 2-butene-1,4-diol
10 followed by ozonolysis. Also, U.S. Patent No. 6,215,004 discloses a process to prepare
acyloxyacetaldehyde diethylacetal by acylation of 2,2-diethoxyethanol.

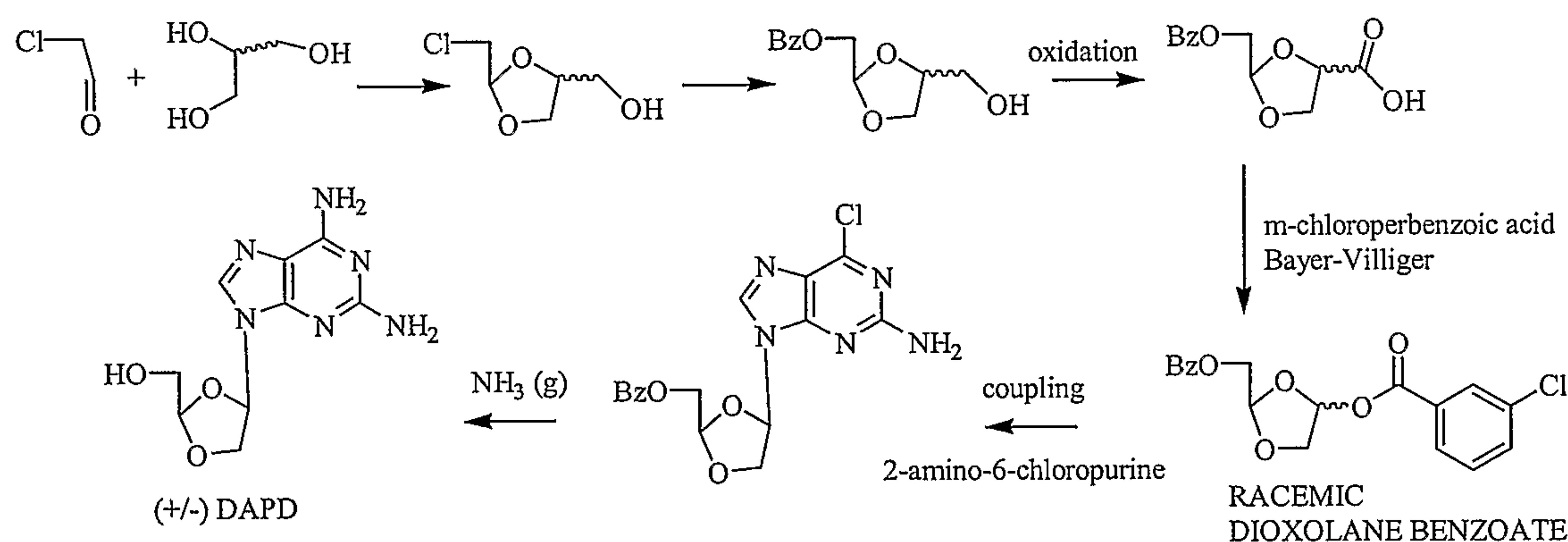
Norbeck, D. W., et al. (*Tet. Lett.*, 1989, 30, 6263) reported the synthesis of ($\pm$)-1-
(2 β ,4 β)-2-(hydroxymethyl)-4-dioxolanyl-thymine, that results in a racemic mixture of
diastereomers about the C4' atom. The X-ray crystallographic analysis of the product
15 revealed that the dioxolane ring adopts the $_3T_4$ conformation commonly observed in
ribonucleosides, with the O3' atom in the endo position, which is quite distinct from the
distorted $_3E$ conformations observed in AZT, AZDU, ddA, ddC, and 3'-deoxy-3'-
fluorothymidine, all of which exhibit potent in vitro activity against HIV.

The antiviral activity of dioxolane nucleosides prompted Chu *et al.* to synthesize
20 a series of analogs in a search for potent antiviral and/or anticancer agents. For example,
9-(β -D hydroxymethyl-1,3-dioxolanyl) aminopurine (β -D-DAPD), and its metabolite 9-
(β -D hydroxymethyl-1,3-dioxolanyl)-guanine (β -D-DXG) have been reported to have
potent and selective activity against human immunodeficiency virus (HIV) and hepatitis
B virus (HBV) (Rajagopalan et al., *Antiviral Chem. Chemother.*, 1996, 7(2), 65-70).
25 (-)-DAPD is a potent and selective inhibitor of HIV *in vitro* and *in vivo* and HBV
replication *in vitro* (Furman, et al. *Drugs of the Future* 2000, 25 (5), 454-461).
Similarly, 1-(β -L hydroxymethyl-1,3-dioxolanyl)-thymine (Dioxolane-T) (Norbeck et
al., *Tet. Lett.*, 1989, 30, 6263-66) possess anti-HIV and anti-HBV activity. 1-(β -L
hydroxymethyl 3-dioxolanyl)-cytidine (β -L-OddC) was discovered to have potent anti-
30 tumor activity towards human prostate as well as renal carcinoma (Kadhim et al., *Can.*
Cancer Res., 57(21), 4803-10, 1997). (-)-(2'S,4'R)-1'-[2'-(hydroxy-methyl)-1',3'-
dioxolan-4'-yl]-5-iodouracil) L-IOddU is currently in pre-clinical or clinical studies to
assess its value as an antiviral or anticancer agent (see Kim, *et al.*, *J. Med. Chem.* 1993,

36, 519-528 and references therein; Corbett, & Rublein, *Curr. Opin. Investig. Drugs* 2001, 2, 348-353; Gu, et al., *Antimicrob. Agents Chemother.* 1999, 43, 2376-2382; Mewshaw, et al., *J. Acquir. Immune Defic. Syndr.* 2002, 29, 11-20).

US 5,041,449 and US 5,270,315 to Belleau et al. disclose a generic group of racemic 2-substituted-4-substituted-1,3-dioxolanes. Table 1 of the reference shows data for two racemic 1,3-dioxolane nucleosides – a racemic trans (α) 1,3-dioxolane nucleoside with a cytosine base (Compound XII) and a racemic cis (β) 1,3-dioxolane nucleoside with an adenine base (Compound XIV) (see also EP 0 337 713 to IAF BioChem International).

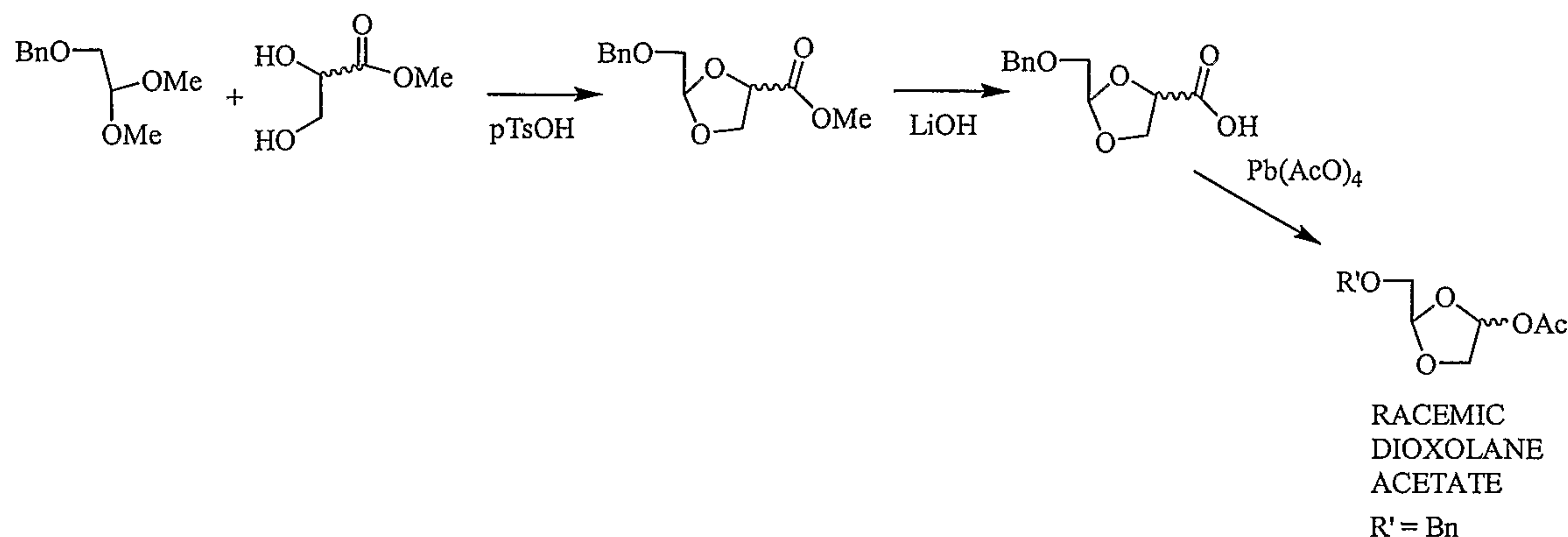
In June 1989, Belleau, et al., reported a method of synthesis of cytidine nucleosides that contain oxygen or sulfur in the 3'-position (Belleau, B., et al. Fifth International Conference on AIDS, Montreal; International Development Research Centre: Ottawa, Ontario, 1989; T.C.O.1.). The dioxolane ring was prepared by the condensation of $\text{RCO}_2\text{CH}_2\text{CHO}$ with glycerin. The synthesis resulted in a racemic mixture of diastereoisomers about the C4' carbon of the nucleoside. Racemic DAPD was synthesized as depicted in Scheme 1.



Scheme 1

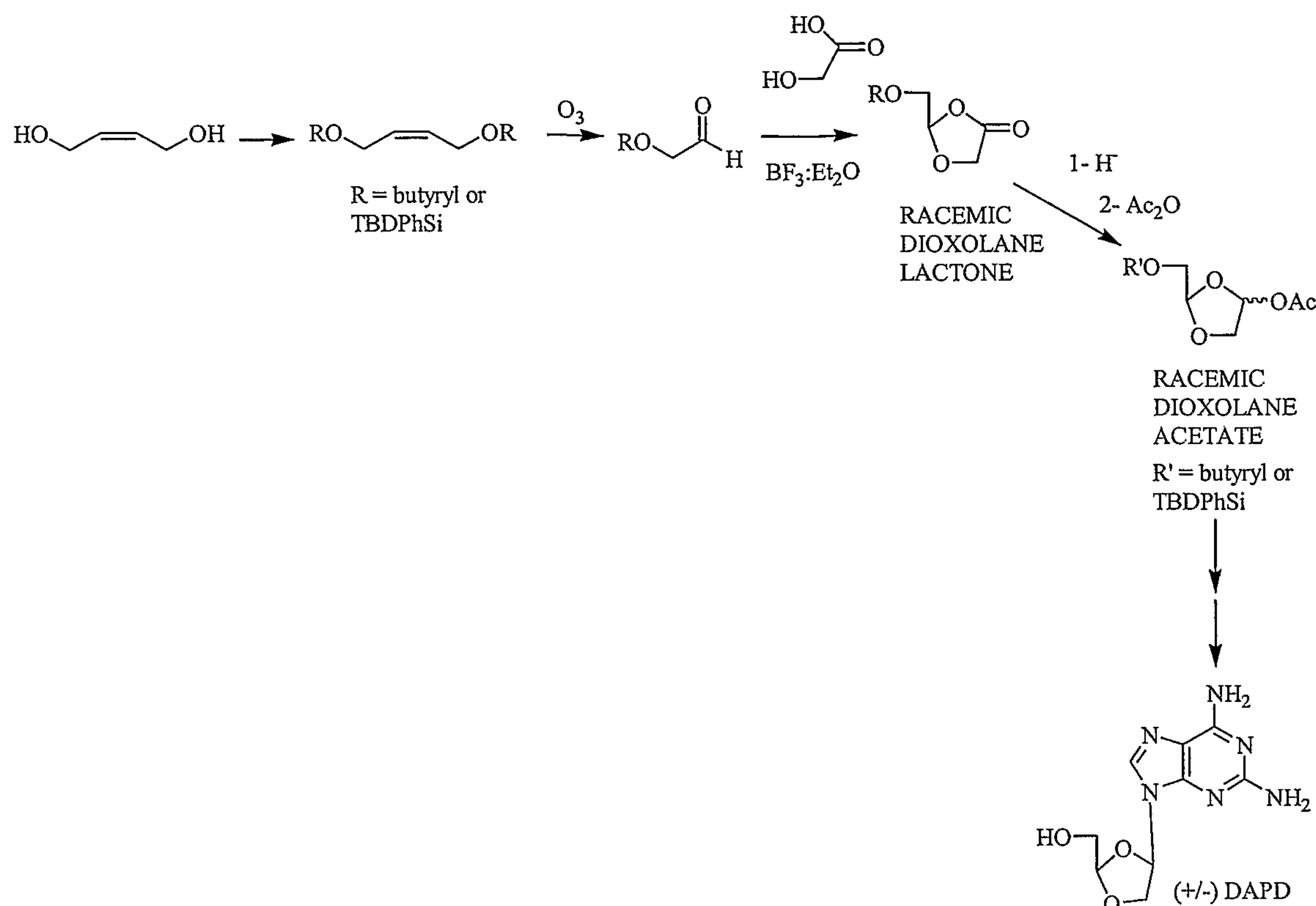
Belleau et al. reacted glycerol and chloroacetaldehyde to generate a dioxolane intermediate. After chlorine displacement with a benzoic acid salt, oxidation of the primary alcohol to a carboxylic acid and Baeyer-Villiger rearrangement with m-chloroperbenzoic acid, the corresponding racemic dioxolane benzoate was obtained. This compound was then coupled with 2-amino-6-chloropurine and the resulting nucleoside intermediate was reacted with ammonia under pressure to afford racemic DAPD. ($\pm$)-Dioxolane-T was also synthesized in similar fashion by Choi et al. (Choi, et al., *J. Am. Chem. Soc.* 1991, 113, 9377-9378 and U. S. Patent No. 5,852,027).

As discussed above, in late 1989, Norbeck et al. published an article which described the synthesis of racemic cis-1,3-dioxolane thymidine which also had anti-HIV activity *in vitro* (Norbeck, et al. *Tet. Let.* 1989, 30 (46), 6263-6266). The product was synthesized in five steps from benzyloxyaldehyde dimethylacetal and (±)-methyl glycerate to produce a 79% yield of the 1:1 diastereomeric mixture. As with the Belleau synthesis, the Norbeck synthesis results in a racemic mixture of diastereoisomers about the C4' carbon of the nucleoside. See Scheme 2.



Scheme 2

The same racemic dioxolane acetate intermediate was synthesized by Liotta et. al. starting from cis-2-buten-1,4-diol (Choi, et al. *J. Am. Chem. Soc.* 1991, 113 (24), 9377-0379 and Wilson, et al. *Bioorg. Med. Chem. Let.* 1993, 3 (2), 169-174). See Scheme 3.



Scheme 3

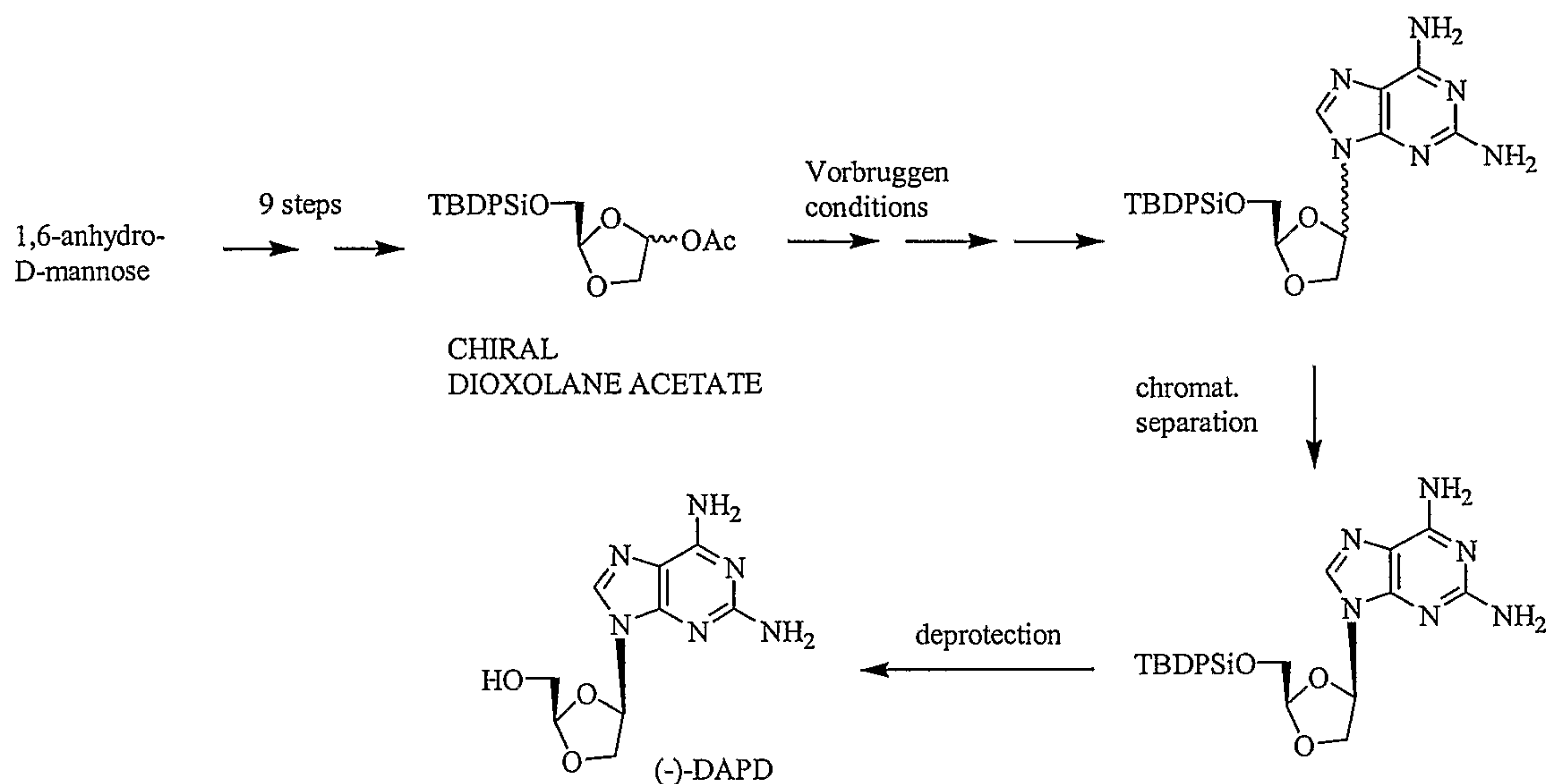
The drawback of these procedures, shown in Scheme 3, is that they involve the synthesis of an unstable aldehyde and difficult oxidative step(s).

US 5,179,104 to Chu and Schinazi, discloses a method to obtain enantiomerically pure β -D-1,3-dioxolane nucleosides via a stereospecific synthesis (see also related U.S. Patent Nos. 5,925,643; 5,767,122; 5,444,063; 5,684,010; 5,834,474; and 5,830,898).

EP 0 515 156 to BioChem Pharma discloses a method to obtain the enantiomers of 1,3-dioxolane nucleosides using a stereoselective synthesis that includes condensing a 1,3-dioxolane intermediate covalently bound to a chiral auxiliary with a silyl Lewis acid (see also related U.S. Patent Nos. 5,753,706 and 5,744,596).

Chu, et al., published a stereospecific synthesis of β -D-1,3-dioxolane nucleosides from 1,6-anhydromannose (Chu, et al. *Tet. Let.*, 1991, 32, 3791-3794). At about the same time, Thomas and Surber published an article which described that (i) a thorough search of the literature on chiral chromatography failed to reveal any examples of nucleoside separations and that (ii) their paper appears to be the first separation of the enantiomers of a nucleoside by chiral high performance liquid chromatography. The nucleoside resolved was not a 1,3-dioxolane nucleoside, and four out of the five chiral columns attempted did not work (Thomas, et al., *J Chromat.*, 1991, 586, 265-270).

Kim et al. (Kim, et al. *J. Med. Chem.* 1993, 36 (1), 30-37) subsequently published a paper which discloses an asymmetric synthesis of β -D and α -D enantiomers of 1,3-dioxolane pyrimidine nucleosides from 1,6-anhydro-D-mannose. The synthesis of (-)-DAPD was described as a thirteen step process from 1,6-anhydro-D-mannose including a



Scheme 4

After coupling of the acetate under Vorbruggen conditions and several purification and deprotection steps, (-)-DAPD was obtained in modest yield. This process is time consuming, difficult and involves complicated oxidation steps.

In 1992, Belleau et al. (Belleau, et al. *Tet. Let.* 1992, 33, 6949-6952) published a synthesis of enantiomerically pure 2',3'-dideoxy-3-oxacytidine stereoisomers via an eight step process using L-ascorbic acid as a chiral auxiliary. L-ascorbic acid was used to produce a set of diastereomers which could be separated. The use of lead tetraacetate makes this process unsuitable for scale-up. In 1992, Kim, et al., also published an article disclosing how to obtain (-)-L- β -dioxolane-C and (+)-L- β -dioxolane-T from 1,6-anhydro-L- β -gulopyranose (Kim, et al., *Tet. Let.* 1992, 32(46), 5899-6902).

Liotta and Shinazi, in US 5,276,151, found that racemic 2-O-protected-5-O-acylated-1,3-dioxolanes could be coupled with purine or pyrimidine bases in the presence of a titanium-containing Lewis acid to predominately generate the racemic β -isomers (see also WO 92/14729).

Jin et al. (Jin, et al. *Tet. Asym.* 1993, 4 (2), 211-214), discloses that Lewis acid catalysts play a crucial role in the preparation 1,3-dioxolane nucleosides. TiCl_4 and

SnCl₄ promote the formation of dioxolane nucleosides with racemization in the coupling of enantiomerically pure 2'-deoxy-3'-oxaribosides with silylated N-acetylcytosine. The use of the Lewis acids trimethylsilyltriflate, trimethylsilyl iodide, and TiCl₂(Oi-Pr)₂ furnishes enantiomerically pure cytosine dioxolane nucleosides in low
5 diastereoselectivity.

An asymmetric synthesis of dioxolane nucleosides was reported by Evans, *et al.* (*Tet. Asym.* 1993, 4, 2319-2322). Reaction of D-mannitol with BnOCH₂CH-(OCH₃)₂ in the presence of SnCl₂ in 1,2-dimethoxyethane followed by RuCl₃/NaOCl oxidation gave cis- and trans-dioxolane-4-carboxylic acid, which was then converted to D- and L-
10 dioxolane nucleosides by decarboxylation, coupling and deprotection reactions. An alternative route to these carboxylic acids by reaction of BnOCH₂CH(OCH₃)₂ with L-ascorbic acid was also reported in the paper. The chiral carboxylic acid can also be prepared by reacting commercially available 2,2-dimethyl-1,3-dioxolane-4-(S)-carboxylic acid with a protected derivative of hydroxy-acetaldehyde such as
15 benzoyloxyacetaldehyde, under acidic conditions (see U.S. Patent Nos. 5,922,867 and 6,358,963).

Siddiqui, et al., discloses that cis-2,6-diaminopurine dioxolane can be selectively deaminated using adenosine deaminase (Siddiqui, et al., *Bioorg. Med. Chem. Let.*, 1993, 3 (8), 1543-1546).

20 While the synthesis of dioxolane nucleosides is possible using processes described in the literature, the chemistry is not applicable to the synthesis of (-)-DAPD on large scale. See Chu, et al. *Tet. Let.* 1991, 32, 3791-3794; Siddiqui, et al. *Bioorg. Med. Chem. Let.* 1993, 3, 1543-1546; Kim, et al. *Tet. Let.* 1992, 46, 6899-6902).

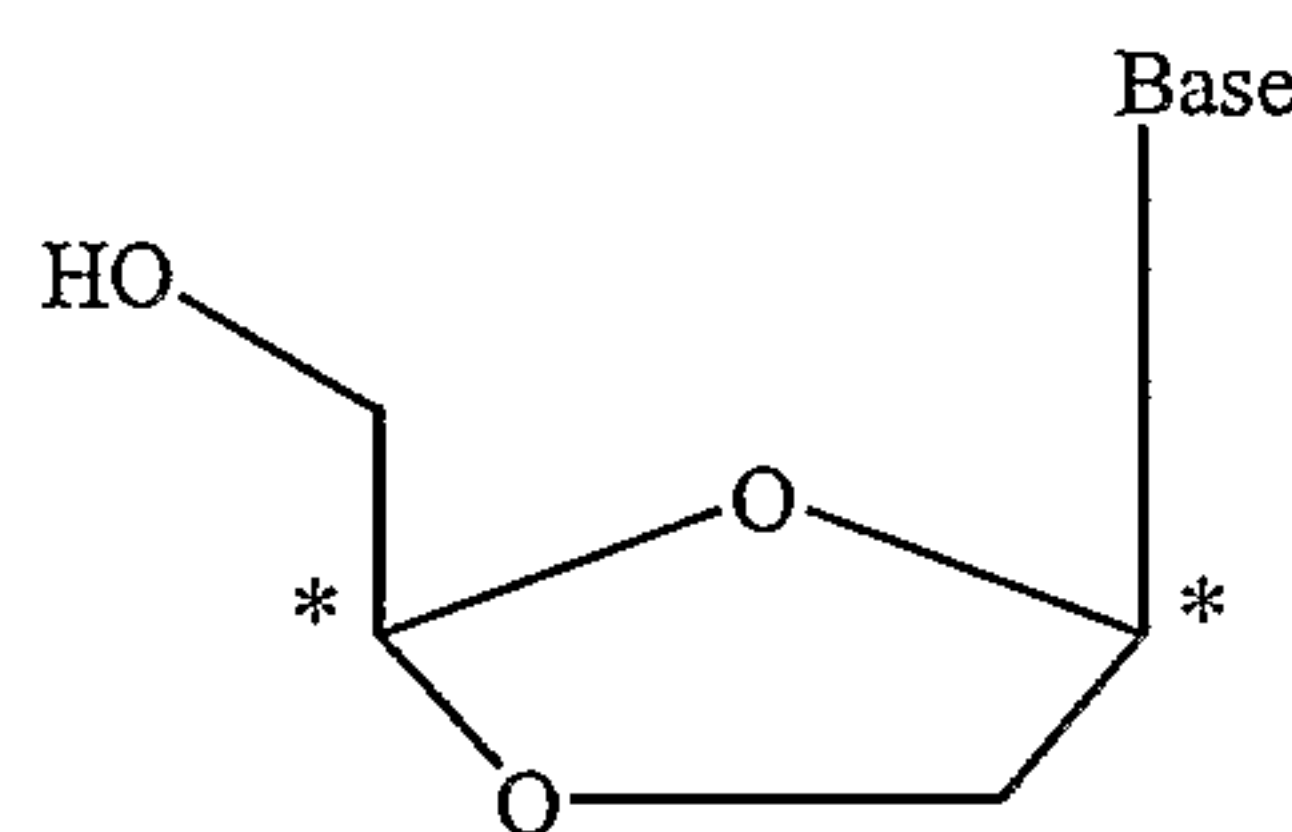
US 5,763,606 to Mansour et al. describes processes for producing predominately
25 pure 1,3-oxathiolane and 1,3-dioxolane nucleosides via coupling of a silylated purine or pyrimidine base with a bicyclic intermediate (see also WO 94/29301).

US 6,215,004 to Painter et al. discloses process for preparing 2-[R¹C(O)OCH₂]-1,3-dioxolanyl-5-one by reacting glycolic acid with an acetal of the formula (R¹O)₂CHR; a hemiacetal of the formula (R²O)(HO)CHR; or a mixture thereof, wherein R is -(CH₂-O-C(O)R¹), and R¹ and R² are independently alkyl, aryl, heteroaryl, heterocyclic, alkaryl,
30 alkylheteroaryl, or alkylheterocyclic, or aralkyl, in the presence of a Lewis acid, such as boron trifluoride diethyl etherate (see also WO 00/09494).

WO 00/47759 and WO 01/58894 both to BioChem Pharma disclose processes of separating β and α anomers from an anomeric mixture of dioxolane analogs with a COOR moiety at the C4' position prior to coupling with a purine or pyrimidine base. The process for resolving the dioxolane analogues to obtain dioxolanes having a predominant β -L-configuration, involves the use of enzymes, namely hydrolases.

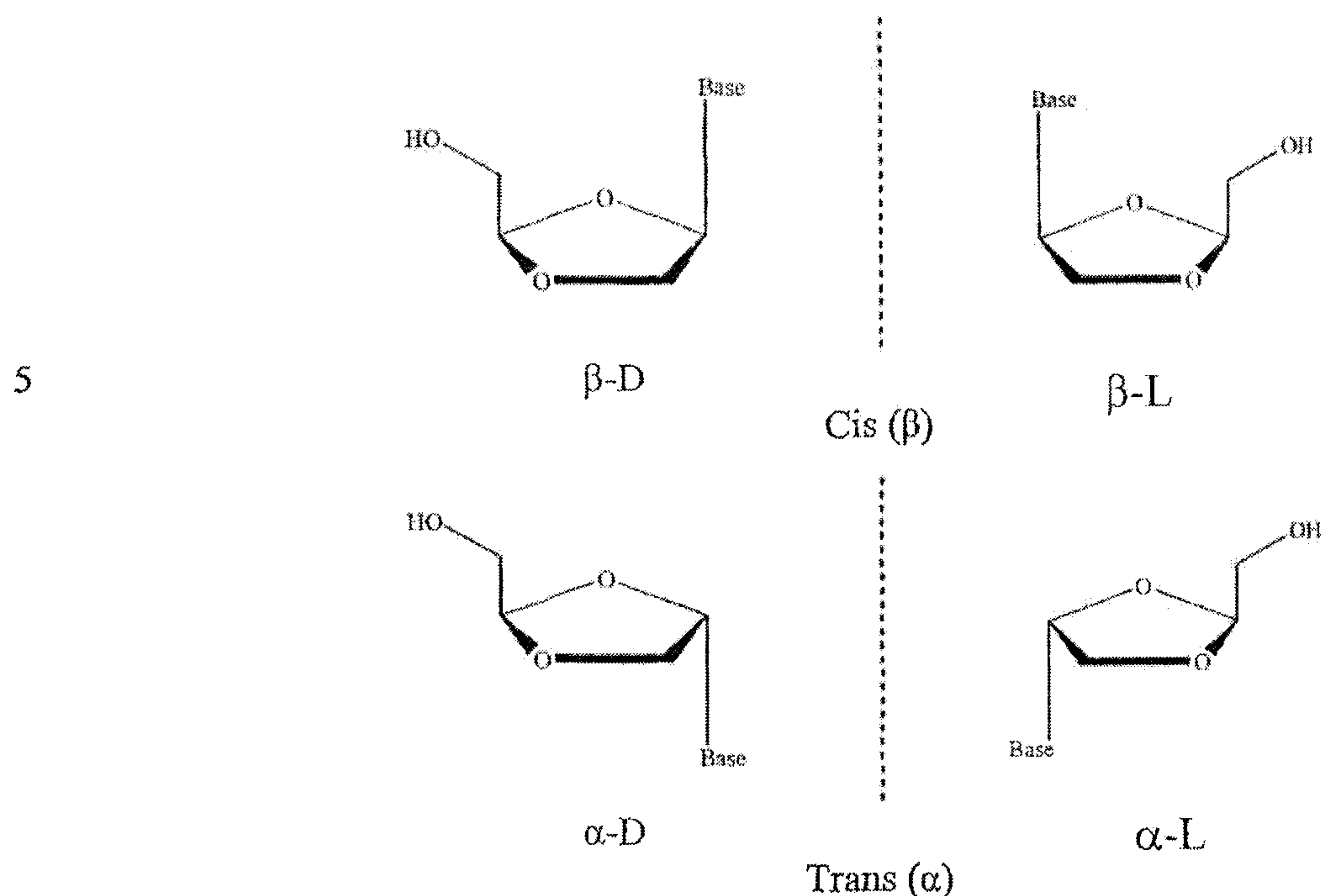
WO 03/062229 to Shire BioChem Inc. discloses a single reaction vessel process for producing a dioxolane nucleoside analogue by adding a Lewis acid, a silylating agent and a non-silylated purine or pyrimidine base to a dioxolane. The publication also describes a process for producing a dioxolane compound by reacting a dioxolane compound in a solvent in the presence of DIB and I2, using a suitable source of energy.

The stereochemistry of 3'-oxa-substituted 2',3'-dideoxynucleoside analogues ("dioxolane nucleoside analogues") can play an important role in their biological activity. The C1' position of the ribose in the nucleoside is a chiral center because the carbon is attached to four different moieties. Likewise, there is an optically active center at C4' of the nucleoside.



As shown below, the substituents on the chiral carbons (the specified purine or pyrimidine base and CH₂OH) of 1,3-dioxolane nucleosides can be either *cis* (on the same side) or *trans* (on opposite sides) with respect to the dioxolane ring system. For the purpose of consistency, the same stereochemical designation is used when the methoxy moiety or the base moiety is replaced with another substituent group. Both the *cis* and *trans* racemates consist of a pair of optical isomers. Hence, each compound has four individual optical isomers. The four optical isomers are represented by the following configurations (when orienting the dioxolane moiety in a horizontal plane such that the —O-CH₂- moiety is in front): (1) *cis*, with both groups "up", which is a β -*cis* configuration (referred to as β -D); (2) *cis*, with both groups "down", which is the opposite β -*cis* configuration (referred to as β -L); (3) *trans* with the C4' substituent "up" and the C1' substituent "down"; and (4) *trans* with the C4' substituent "down" and the C1' substituent "up". The two *cis* enantiomers together are referred to as a racemic mixture of β -enantiomers, and the two *trans* enantiomers are referred to as a racemic mixture of

α -enantiomers. In general, it is difficult to separate or otherwise obtain the individual enantiomers of the *cis*-configuration. The four possible stereoisomers of *cis*-1,3-dioxolane nucleosides are illustrated below:



10 Since stereoisomers of dioxolane nucleosides usually have different biological activities and toxicity, obtaining the pure therapeutically active isomer becomes crucial. Frequently, one stereoisomer is considerably more active than the other.

15 Chu *et al.* developed methods for the *asymmetric* synthesis of dioxolane nucleosides from D-mannose and L-gulonic lactone for D- and L-dioxolane nucleosides, respectively (U.S. Patent Nos. 5,767,122, 5,792,773). However, these processes involved many steps and most of the intermediates need to be purified by silica gel column chromatography (see Kim *et al. J. Med. Chem.* 1993, 36, 519-528).

20 To prepare a sufficiently large quantity of dioxolane nucleoside drug substance for clinic trials, a chiral 2-acyloxymethyl-5-oxo-1,3-dioxolane has been used as the key intermediate. This was prepared by cyclization of ROCH_2CHO or its acetal with glycolic acid in the presence of BF_3 , followed by column separation on chiral resin or by enzymatic resolution, which are expensive and difficult techniques.

Thus, there remains a need for cost-effective and stereoselective processes to produce biologically active isomers of dioxolane nucleosides.

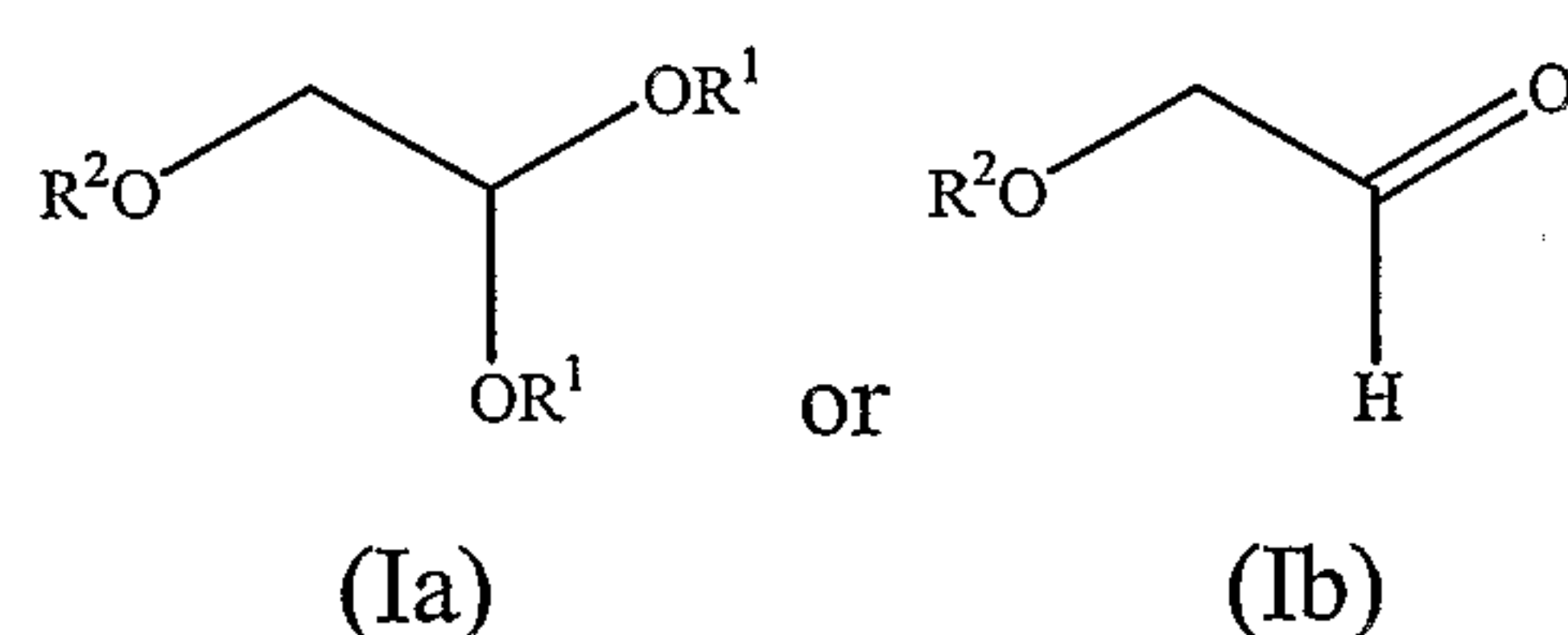
25 It is an object of the present invention to provide novel and cost-effective processes for the synthesis of enantiomerically pure dioxolane nucleosides.

SUMMARY OF THE INVENTION

The present invention includes an efficient synthetic route to 1,3-dioxolane nucleosides from inexpensive precursors, with the option of introducing functionality as needed. The processes allow the stereoselective preparation of the biologically active isomer of these compounds.

In one embodiment of the present invention, a process for preparing a substantially pure β -D- or β -L-1,3-dioxolane nucleoside, such as β -D-DAPD, is provided, comprising:

- a) preparing or obtaining an esterified 2,2-dialkoxy ethanol of the formula (Ia) or (Ib);



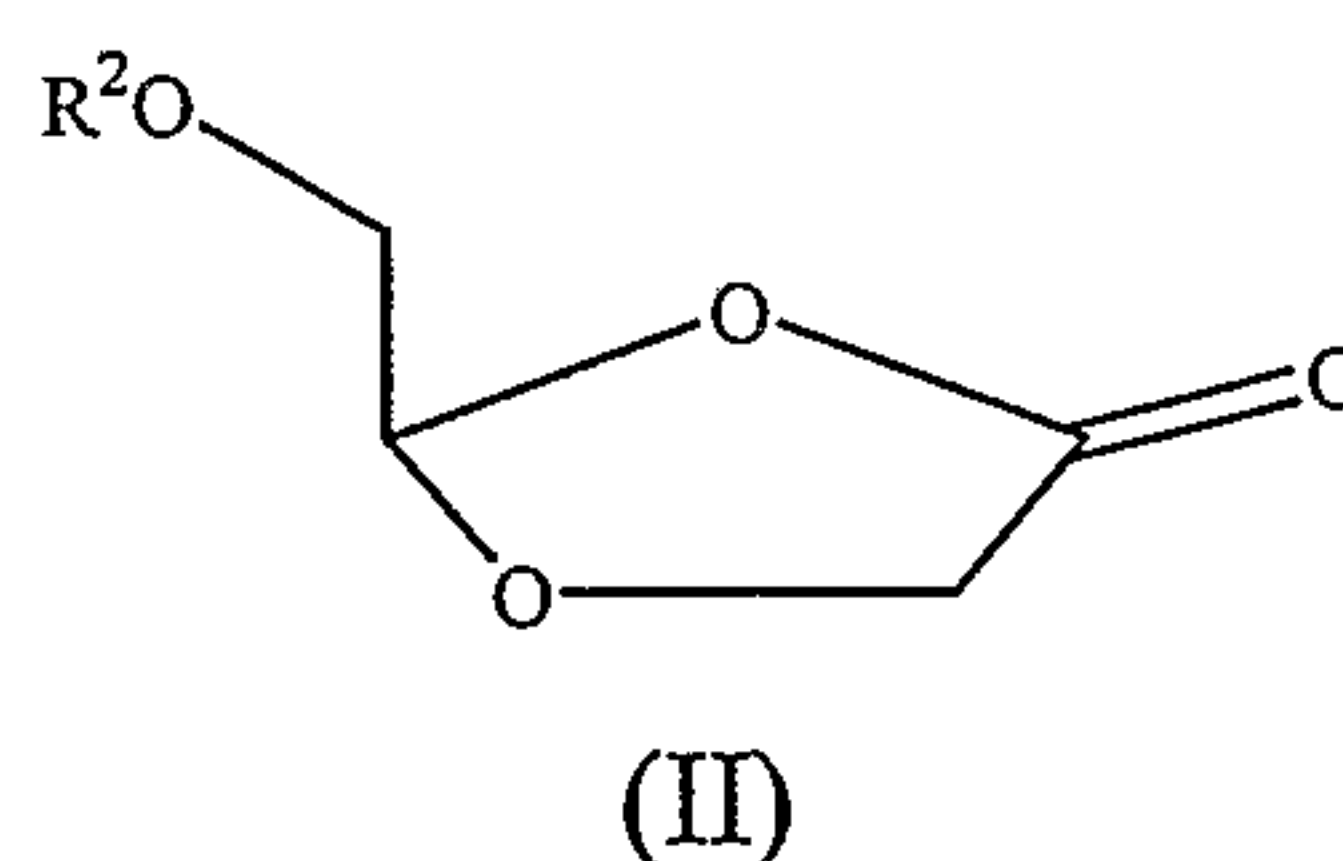
wherein:

each R^1 is independently alkyl, aryl, heteroaryl, heterocyclic, alkaryl, alkylheteroaryl, or alkylheterocyclic, or aralkyl; and

R^2 is any suitable removable group such that (1) it is easily removable at the end of the synthesis, (2) it has a low molecular weight to avoid carrying large mass during the process, (3) it is commercially available and inexpensive, (4) the corresponding ester is stable under the reductive acetylation conditions, (5) the subsequent lactone is resolvable, and (6) after coupling, the corresponding anomers are easily separated, preferentially by crystallization (e.g. *iso*-butyryl or *p*-methoxy benzoyl);

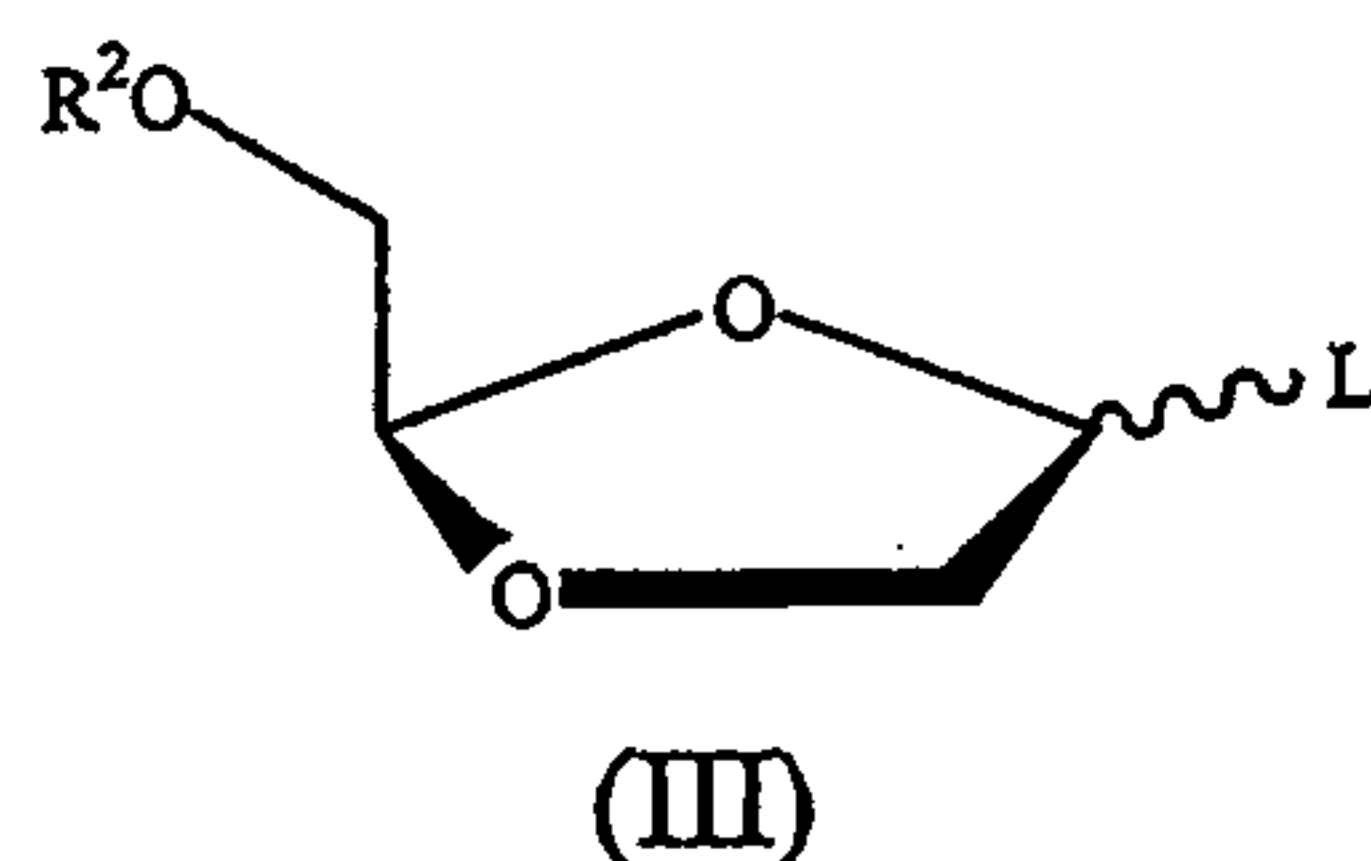
and then,

- b) cyclizing the esterified 2,2-dialkoxy ethanol of the formula (Ia) or (Ib) with glycolic acid, preferably in the presence of a Lewis acid, such as $BF_3 \cdot Et_2O$, to obtain a 1,3-dioxolane lactone of the formula (II):



and then,

- c) resolving the 1,3-dioxolane lactone of the formula (II) to obtain a substantially pure D- or L-lactone; and then,
- d) selectively reducing with a reducing agent, such as $\text{LiAlH}(\text{OtBu})_3$ and activating the substantially pure D- or L-chiral lactone to obtain a substantially pure D- or L-1,3-dioxolane of the formula (III):

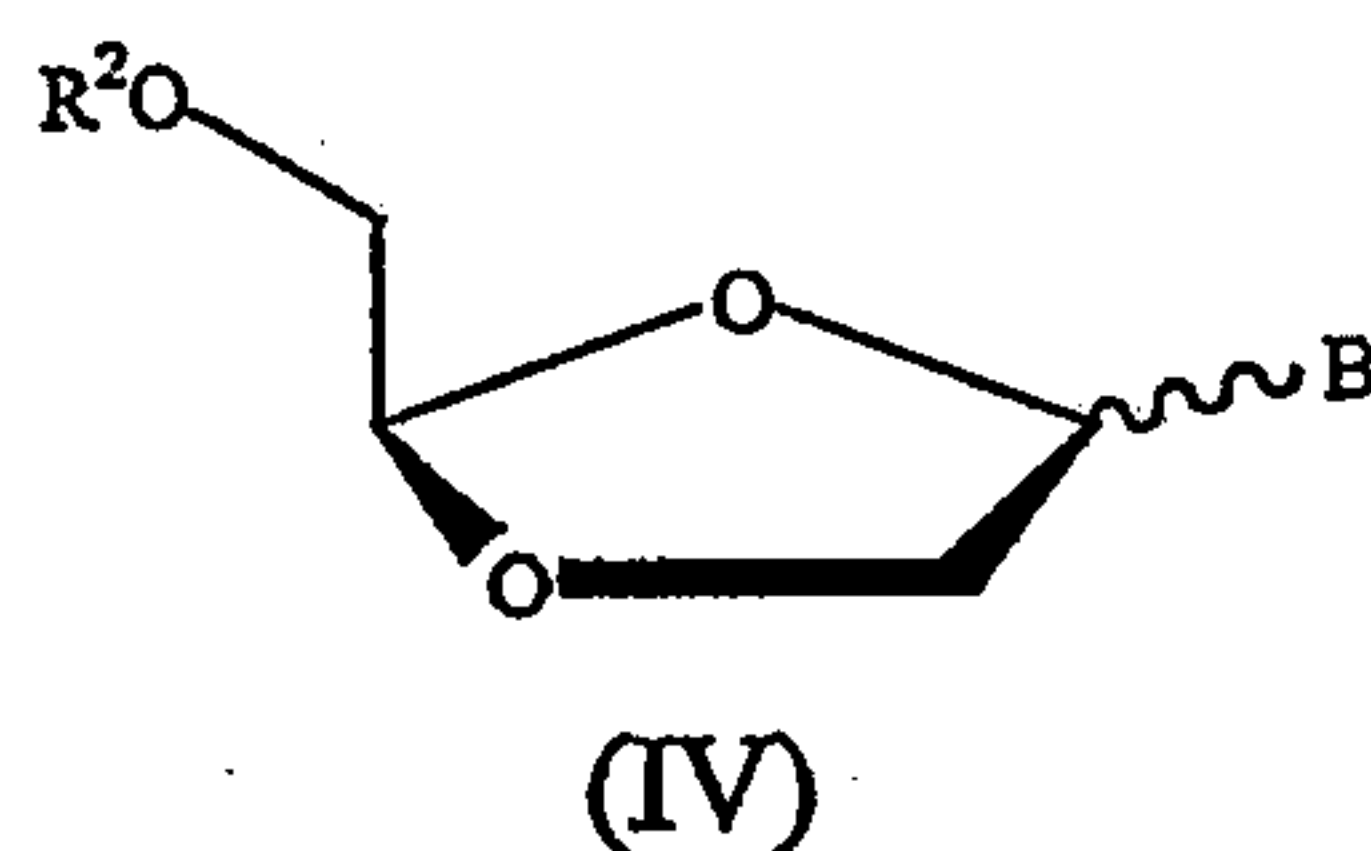


wherein:

L is a suitable leaving group, such as an O-acyl, such as OAc, halogen (F, Br, Cl or I), OMesylates (OMs) and OToluates (OTs), or the like;

and then,

- e) coupling the substantially pure D- or L-1,3-dioxolane of the formula (III) to an activated and/or protected purine or pyrimidine base or its derivative, such as activated 2,6-dichloropurine, to obtain $\alpha:\beta$ mixture of substantially pure protected D- or L-1,3-dioxolane nucleosides of the formula (IV):



wherein:

B is a purine or pyrimidine base or its derivative;

and then,

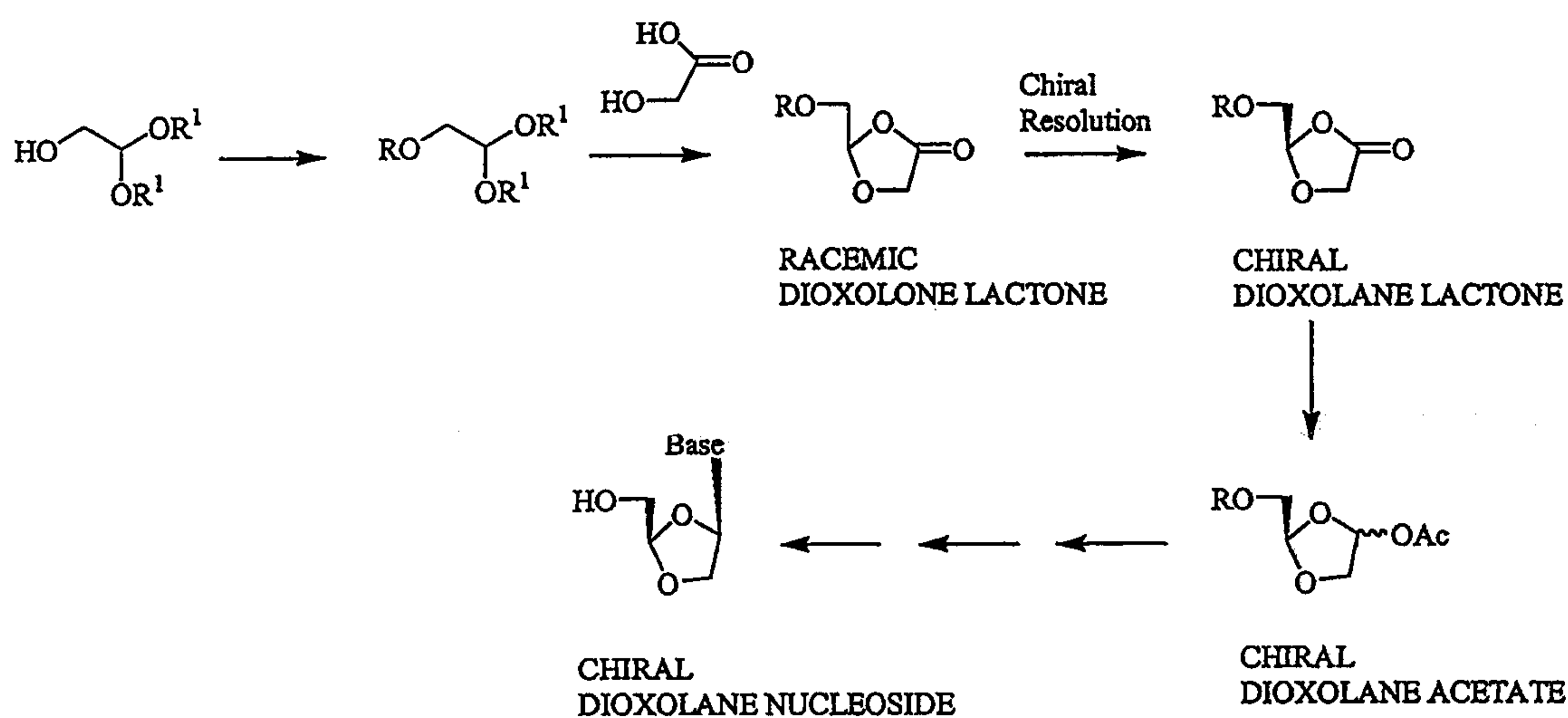
- f) optionally purifying the $\alpha:\beta$ mixture of substantially pure protected D- or L-1,3-dioxolane nucleosides of the formula (IV) to obtain a substantially pure protected β -D or β -L-1,3-dioxolane nucleoside; and then
- g) optionally deprotecting the substantially pure protected β -D- or β -L-1,3-dioxolane nucleoside, if necessary, to obtain a substantially pure β -D or β -L-1,3-dioxolane nucleoside.

In one embodiment of the present invention, the esterified 2,2-dialkoxy ethanol of the formula (Ia) is hydrolyzed to the corresponding aldehyde of formula (Ib). In a particular embodiment the esterified 2,2-dialkoxy ethanol of the formula (Ia) is

hydrolyzed to the corresponding aldehyde of formula (Ib) when R^2 is *p*-methoxy benzoyl.

In another embodiment of the present invention, the esterified 2,2-dialkoxy ethanol of the formula (Ia) is not hydrolyzed to the corresponding aldehyde of formula (Ib). In a particular embodiment the esterified 2,2-dialkoxy ethanol of the formula (Ia) is not hydrolyzed to the corresponding aldehyde of formula (Ib) when R^2 is *iso*-butyryl.

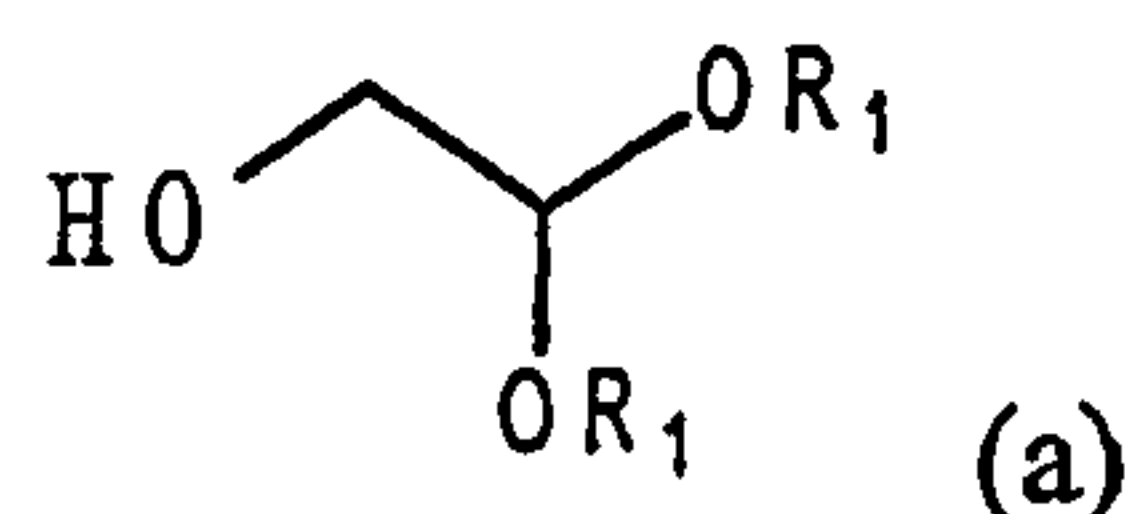
In one embodiment of the present invention, the resolution of the 1,3-dioxolane lactone of the formula (II) to obtain a substantially pure D- or L-lactone is accomplished using chiral chromatography. In an alternate embodiment of the present invention, the resolution of the 1,3-dioxolane lactone of the formula (II) to obtain a substantially pure D- or L-lactone is accomplished using enzymatic resolution.



Scheme 5

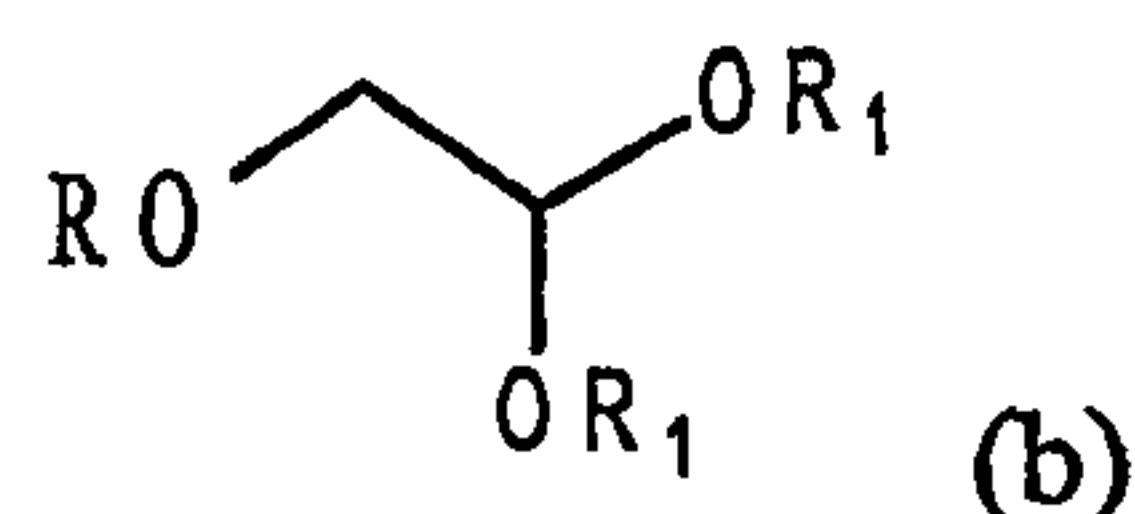
In another embodiment of the present invention, there is provided a process for preparing substantially pure β -D-DAPD, comprising:

- a) providing a compound of formula (a):



wherein each R^1 is independently alkyl, aryl, heteroaryl, heterocyclic, alkaryl, alkylheteroaryl, or alkylheterocyclic, or aralkyl;

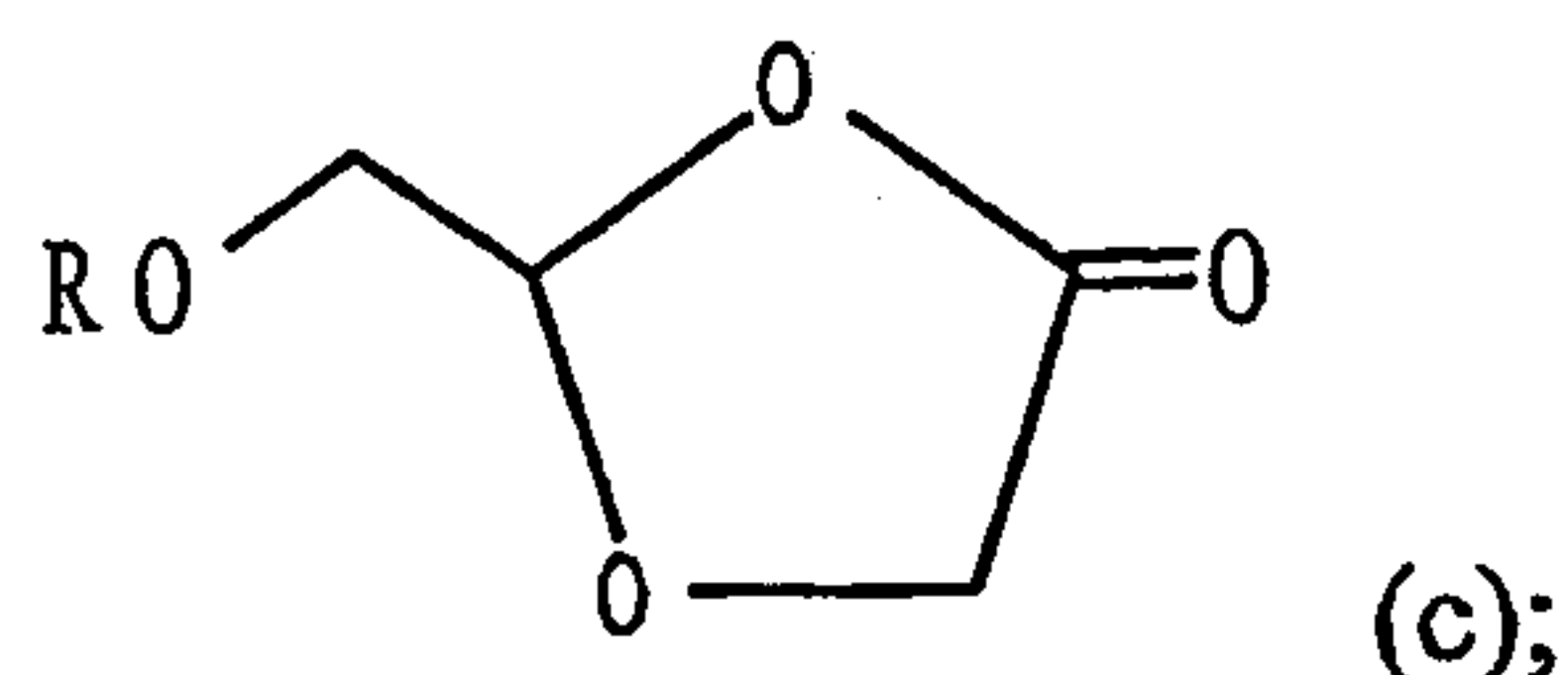
- b) converting the compound of formula (a) to a compound of formula (b):



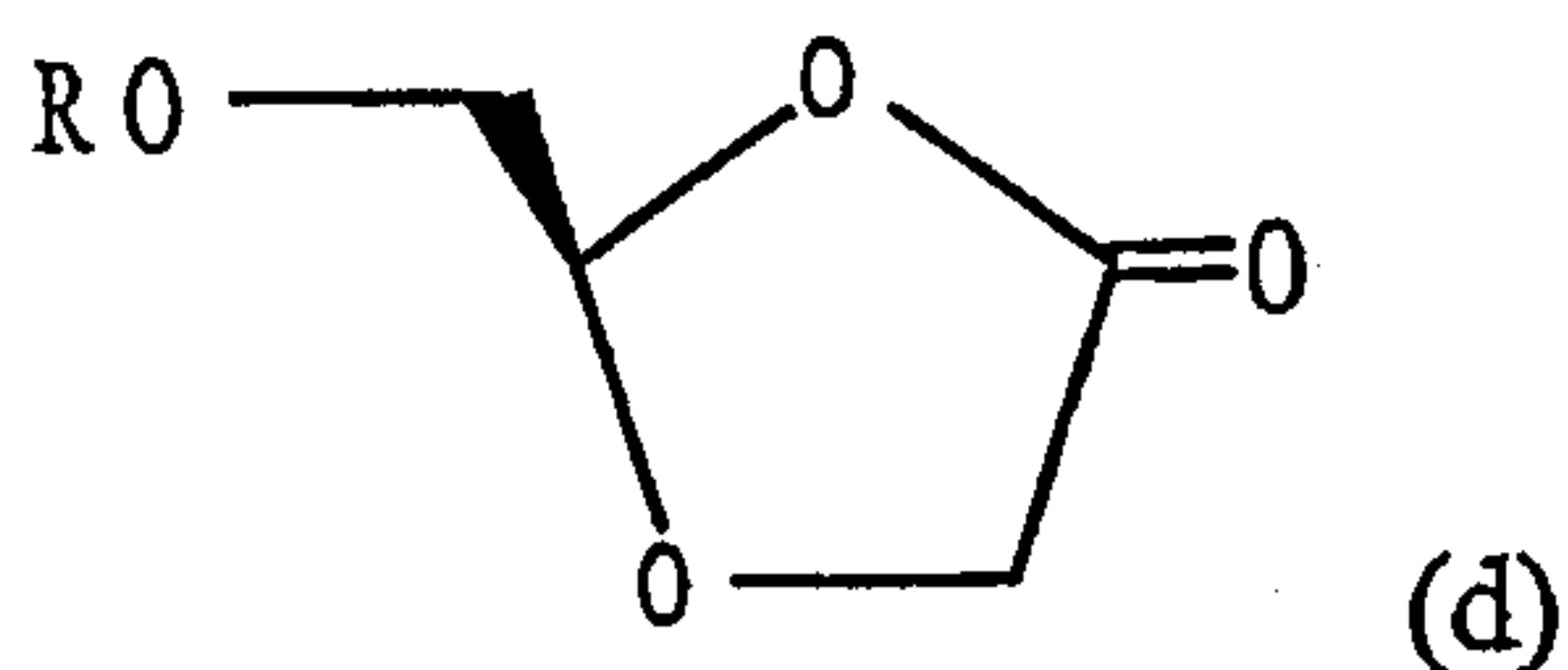
13a

wherein R is independently H, halogen, OH, OR', OCH₃, SH, SR', SCH₃, NH₂, NHR', NR'₂, lower alkyl of C₁-C₄, CH₃, CH=CH₂, N₃C=CH₂, CO₂H, CO₂R', CONH₂, CONHR', CH₂OH, CH₂CH₂OH, CF₃, CH₂CH₂F, CH=CHCO₂H, CH=CHCO₂R', CH=CHCl, CH=CHBr, or CH=CHI;

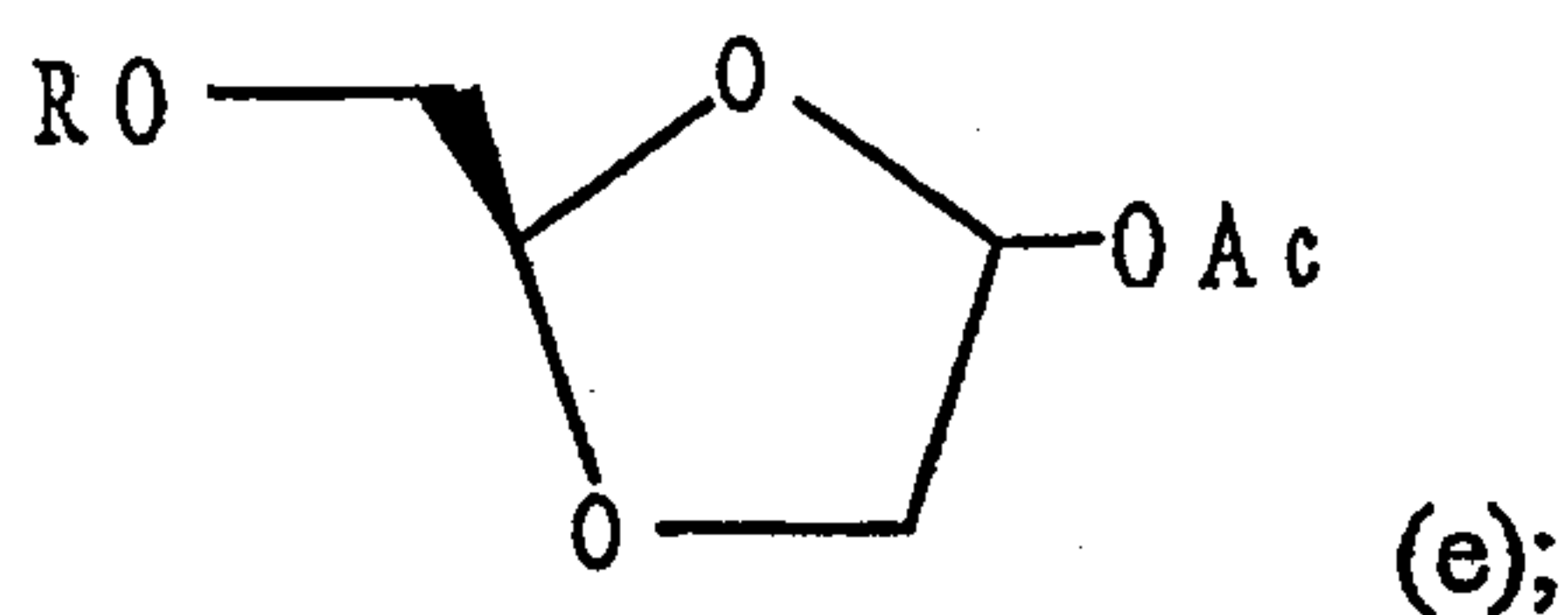
- c) cyclizing the compound of formula (b) to a racemic 1,3-dioxolane lactone of formula (c):



- d) resolving the compound of formula (c) to a chiral dioxolane lactone of formula (d);

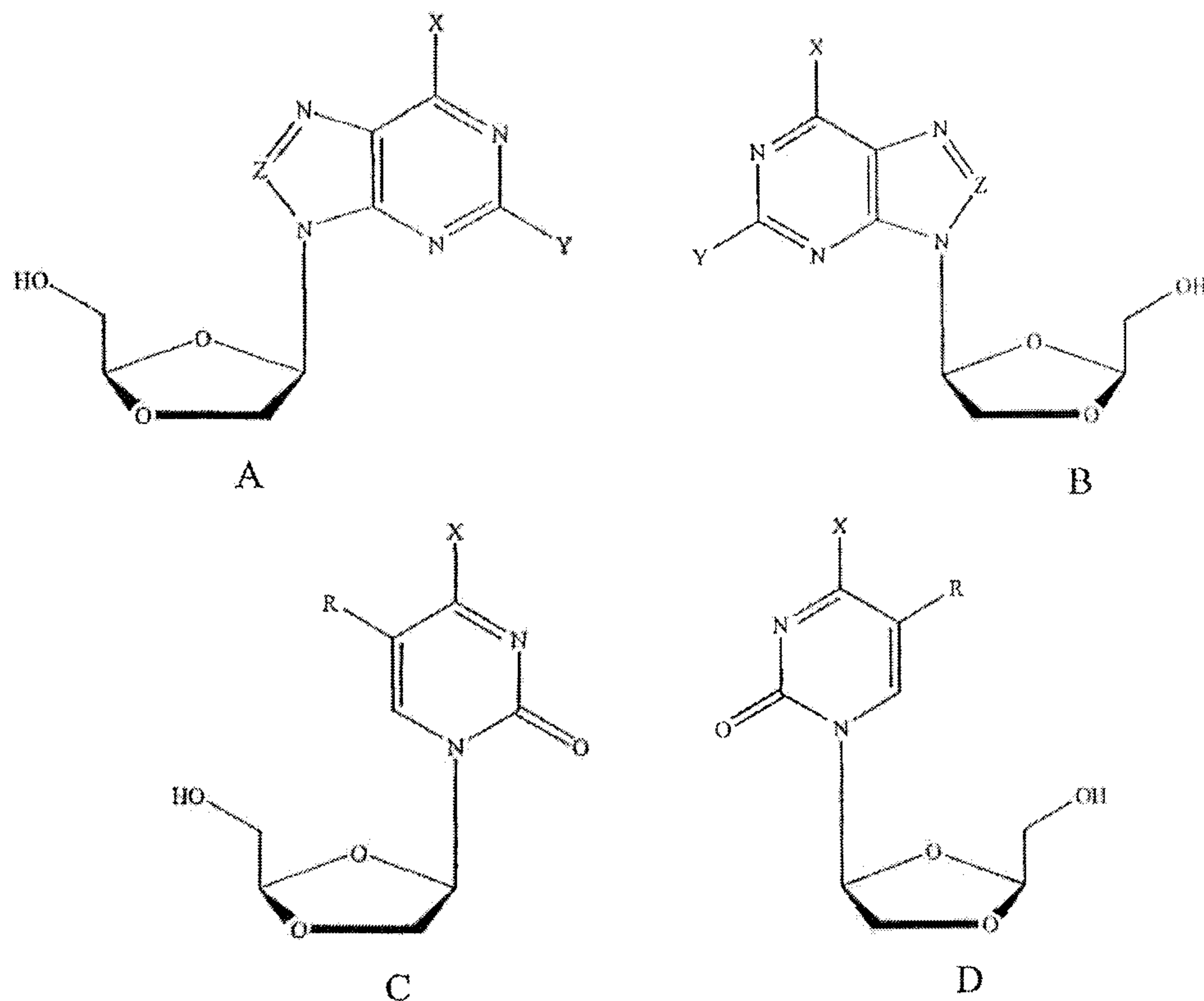


- e) acetylating the chiral dioxolane lactone of formula (d) to a chiral dioxolane acetate of formula (e); and



- f) coupling the compound of formula (e) to an activated and/or protected adenine base; and
- g) optionally purifying and deprotecting the compound of step (f) to obtain β -D-2,6-diaminopurine dioxolane.

Therefore, the processes of the present invention may be used to prepare compounds of formula A to D:



and pharmaceutically acceptable salts or esters thereof, wherein:

R is independently H, halogen (F, Cl, Br, I), OH, OR', OCH₃, SH, SR', SCH₃, NH₂, NHR', NR'₂, lower alkyl of C₁-C₄, CH₃CH=CH₂, N₃C=CH₂, CO₂H, CO₂R', CONH₂, CONHR', CH₂OH, CH₂CH₂OH, CF₃, CH₂CH₂F, CH=CHCO₂H, CH=CHCO₂R', CH=CHCl, CH=CHBr, or CH=CHI;

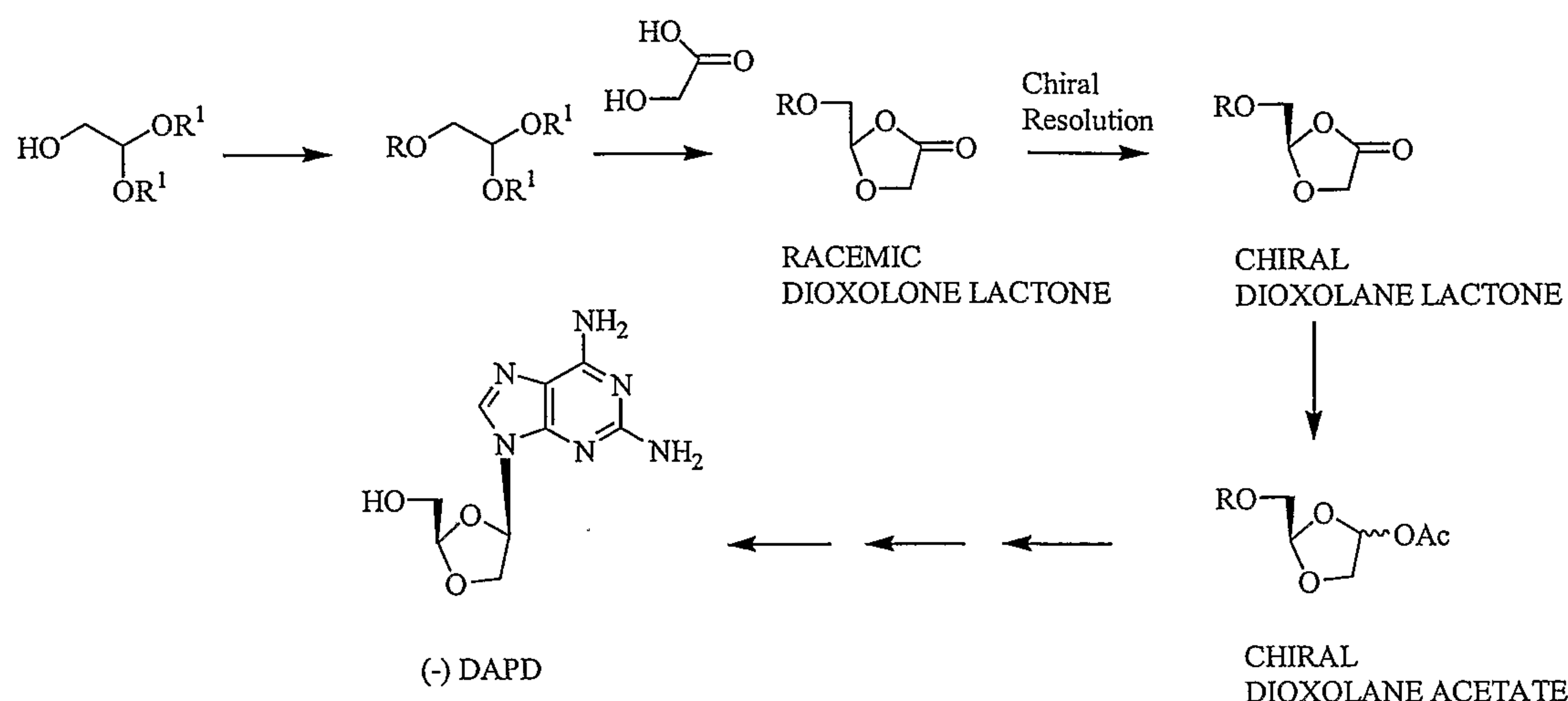
each R' is independently a lower alkyl of C₁-C₄;

Z is either one of CH or C-X; and

each X and Y are independently H, halogen (F, Cl, Br, I), OH, OR', OCH₃, SH, SR', SCH₃, NH₂, NHR', NR'₂, or CH₃.

In a particular embodiment of the present invention, a process for preparing substantially pure β-D-DAPD, is provided. See Scheme 6.

15



Scheme 6

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 are typical chromatograms for the compounds of the present invention.

Figure 1A is a typical chromatogram for the butyrl ester lactone (compound 1). **Figure 1B** is a typical chromatogram for the butyrl ester acetate (compound 3). **Figure 1C** is a typical chromatogram for the *p*-methoxybenzoyl lactone (compound 11). **Figure 1D** is a typical chromatogram for the *p*-methoxybenzoyl lactone (compound 11) using the optimized SFC method. **Figure 1E** is a typical chromatogram for the benzoyl lactone (compound 12). **Figure 1F** is a typical chromatogram for the benzoyl lactone (compound 12) using the optimized SFC method. **Figures 1G -1J** are typical chromatogram for the iso-butyrl ester lactone (compound 13). **Figure 1K** is a typical chromatogram for the *t*-butyl-diphenyl-silyl ether lactone (compound 4). **Figure 1L** is a typical chromatogram for the *t*-butyl-diphenyl-silyl ether lactone (compound 4) using the optimized method. **Figure 1M** is a typical chromatogram for DAPD. **Figure 1N** is a typical chromatogram for DAPD using the optimized SFC method.

Figure 2 is a graphical representation of a theoretical phase diagram for the *t*-butyl-diphenyl-silyl ether lactone (compound 4).

Figure 3 are graphical representations of the selectivity of certain microbial enzymes for a particular enantiomer of the compounds of the present invention. **Figures 3A** and **3B** depict the results from the screen of the butyrl ester lactone (compound 1) against various microbial enzymes. **Figure 3C** depicts the results from the screen of the *p*-methoxybenzoyl lactone (compound 11) against various microbial enzymes. **Figure**

3D depicts the results from the screen of the benzoyl lactone (compound 12) against various microbial enzymes. **Figure 3E** depict the results from the screen of the *iso*-butyryl ester lactone (compound 13) against various microbial enzymes.

Figure 4 is a graphical representation of the microbial resolution of the *iso*-butyryl ester lactone (compound 13) against the microbial enzymes CMC 103669 and CMC 103661 using various concentrations, buffers, pH ranges and temperatures.

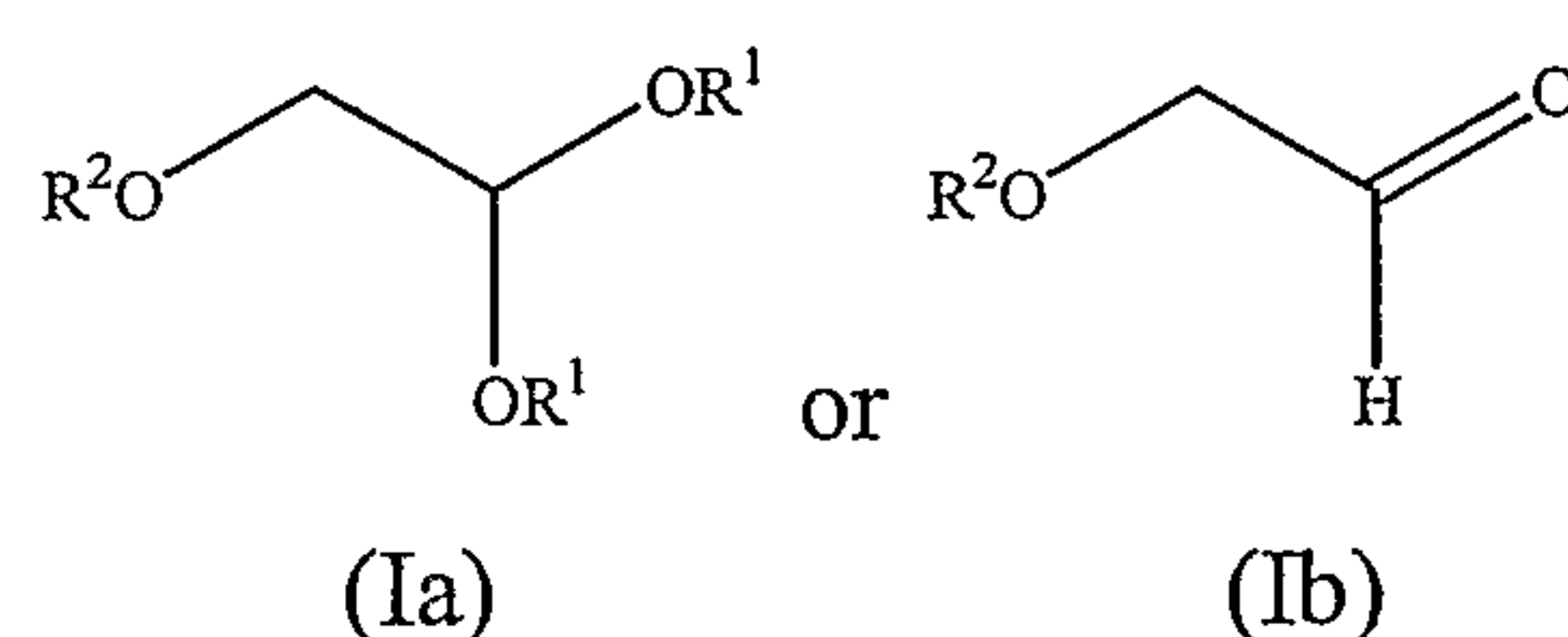
Figure 5 is a 400 MHz ^1H NMR spectrum for the resolved *iso*-butyryl ester lactone (compound 13).

DETAILED DESCRIPTION OF THE INVENTION

The present invention includes an efficient synthetic routes to 1,3-dioxolane nucleosides from inexpensive precursors, with the option of introducing functionality as needed. These processes allow the stereoselective preparation of the biologically active isomer of these compounds.

In one embodiment of the present invention, a process for preparing a substantially pure β -D- or β -L-1,3-dioxolane nucleoside, such as β -D-DAPD, is provided, comprising:

- a) preparing or obtaining an esterified 2,2-dialkoxy ethanol of the formula (Ia) or (Ib);



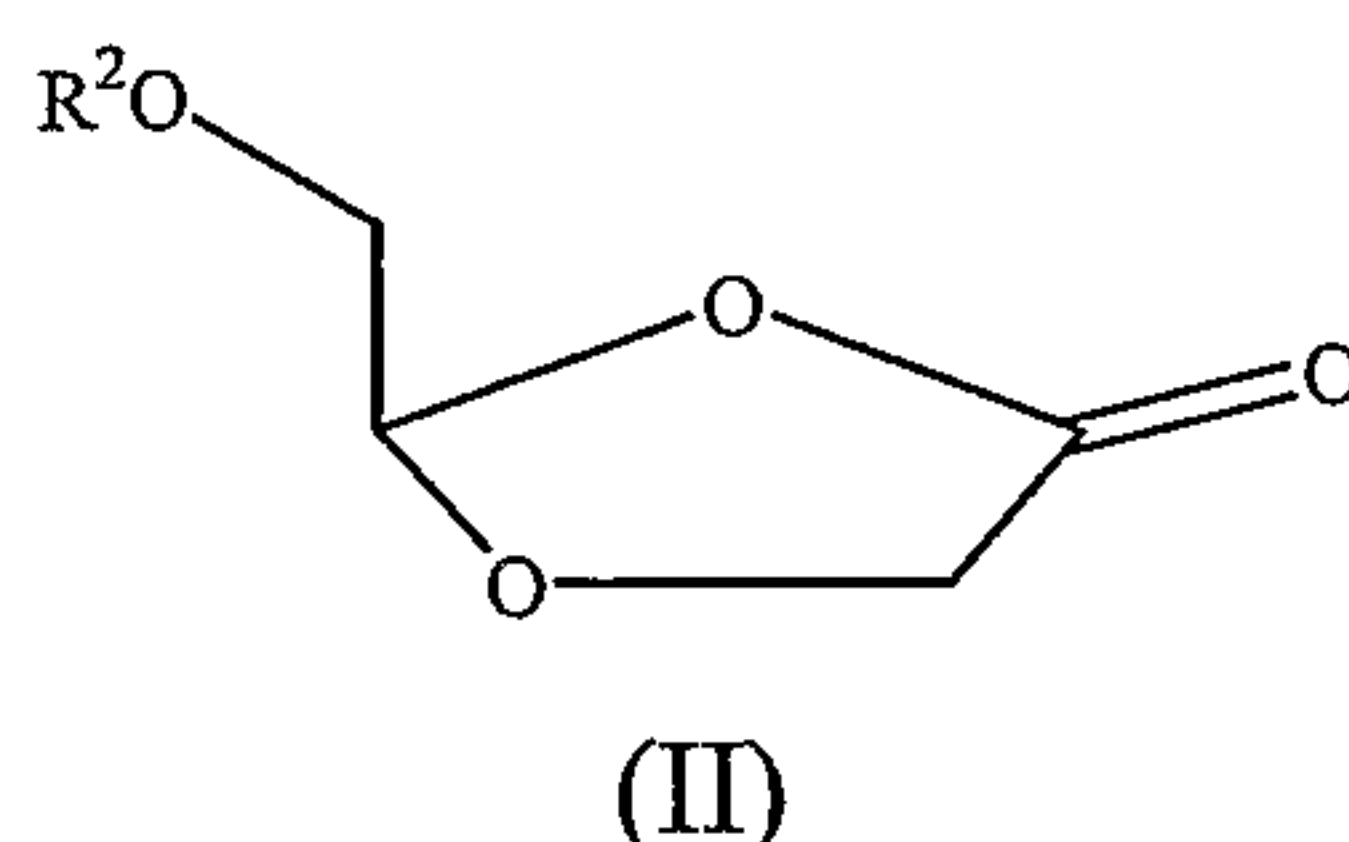
wherein:

each R^1 is independently alkyl, aryl, heteroaryl, heterocyclic, alkaryl, alkylheteroaryl, or alkylheterocyclic, or aralkyl; and

R^2 is any suitable removable group such that (1) it is easily removable at the end of the synthesis, (2) it has a low molecular weight to avoid carrying large mass during the process, (3) it is commercially available and inexpensive, (4) the corresponding ester is stable under the reductive acetylation conditions, (5) the subsequent lactone is resolvable, and (6) after coupling, the corresponding anomers are easily separated, preferentially by crystallization (e.g. *iso*-butyryl or *p*-methoxy benzoyl);

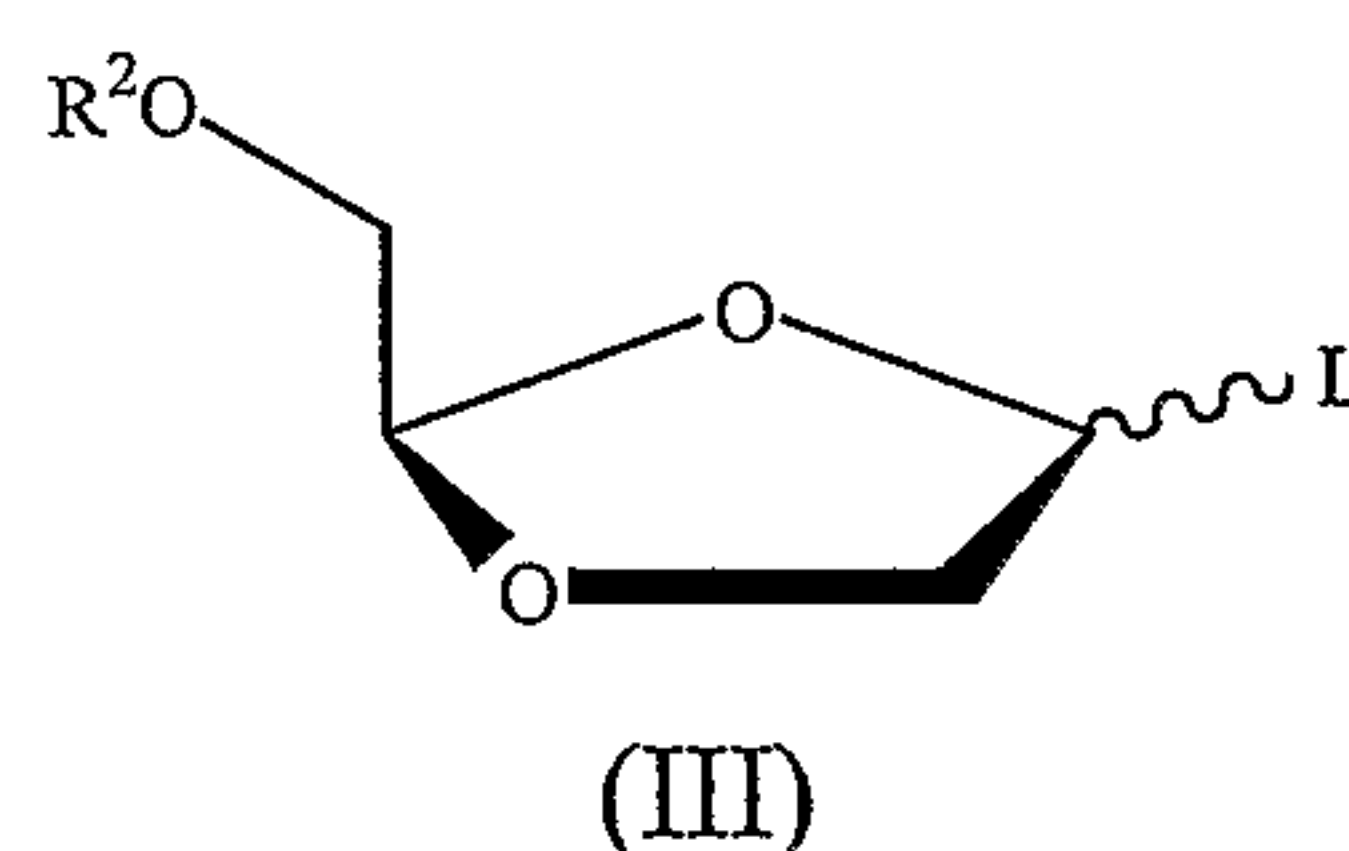
and then,

- b) cyclizing the esterified 2,2-dialkoxy ethanol of the formula (Ia) or (Ib) with glycolic acid, preferably in the presence of a Lewis acid, such as $\text{BF}_3 \cdot \text{Et}_2\text{O}$, to obtain a 1,3-dioxolane lactone of the formula (II):



and then,

- c) resolving the 1,3-dioxolane lactone of the formula (II) to obtain a substantially pure D- or L-lactone; and then,
- d) selectively reducing with a reducing agent, such as $\text{LiAlH}(\text{OtBu})_3$ and activating the substantially pure D- or L-chiral lactone to obtain a substantially pure D- or L-1,3-dioxolane of the formula (III):

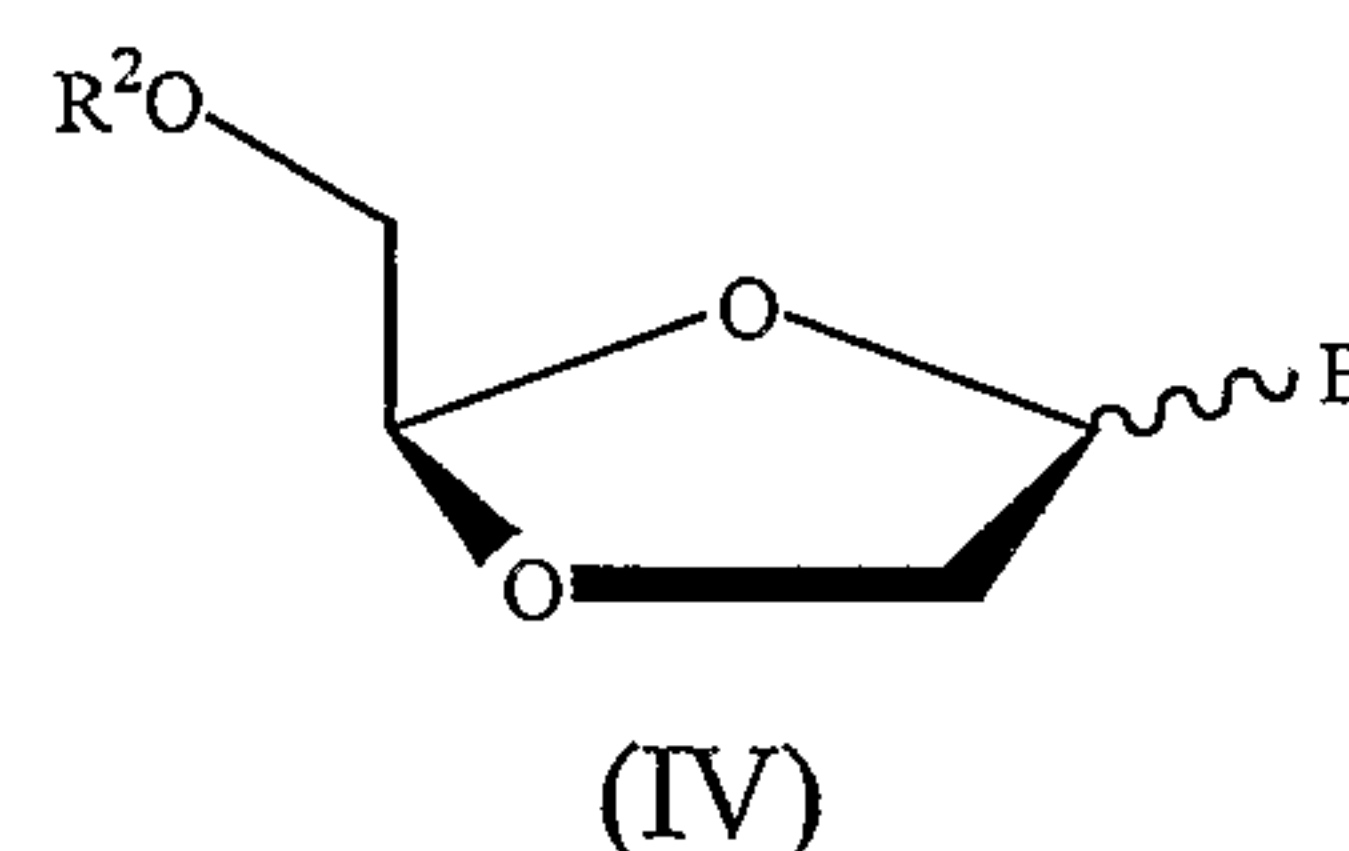


wherein:

L is a suitable leaving group, such as an O-acyl, such as OAc, halogen (F, Br, Cl or I), OMs, OTs, or the like;

and then,

- e) coupling the substantially pure D- or L-1,3-dioxolane of the formula (III) to an activated and/or protected purine or pyrimidine base or its derivative, such as activated 2,6-dichloropurine, to obtain $\alpha:\beta$ mixture of substantially pure protected D- or L-1,3-dioxolane nucleosides of the formula (IV):



wherein:

B is a purine or pyrimidine base or its derivative;

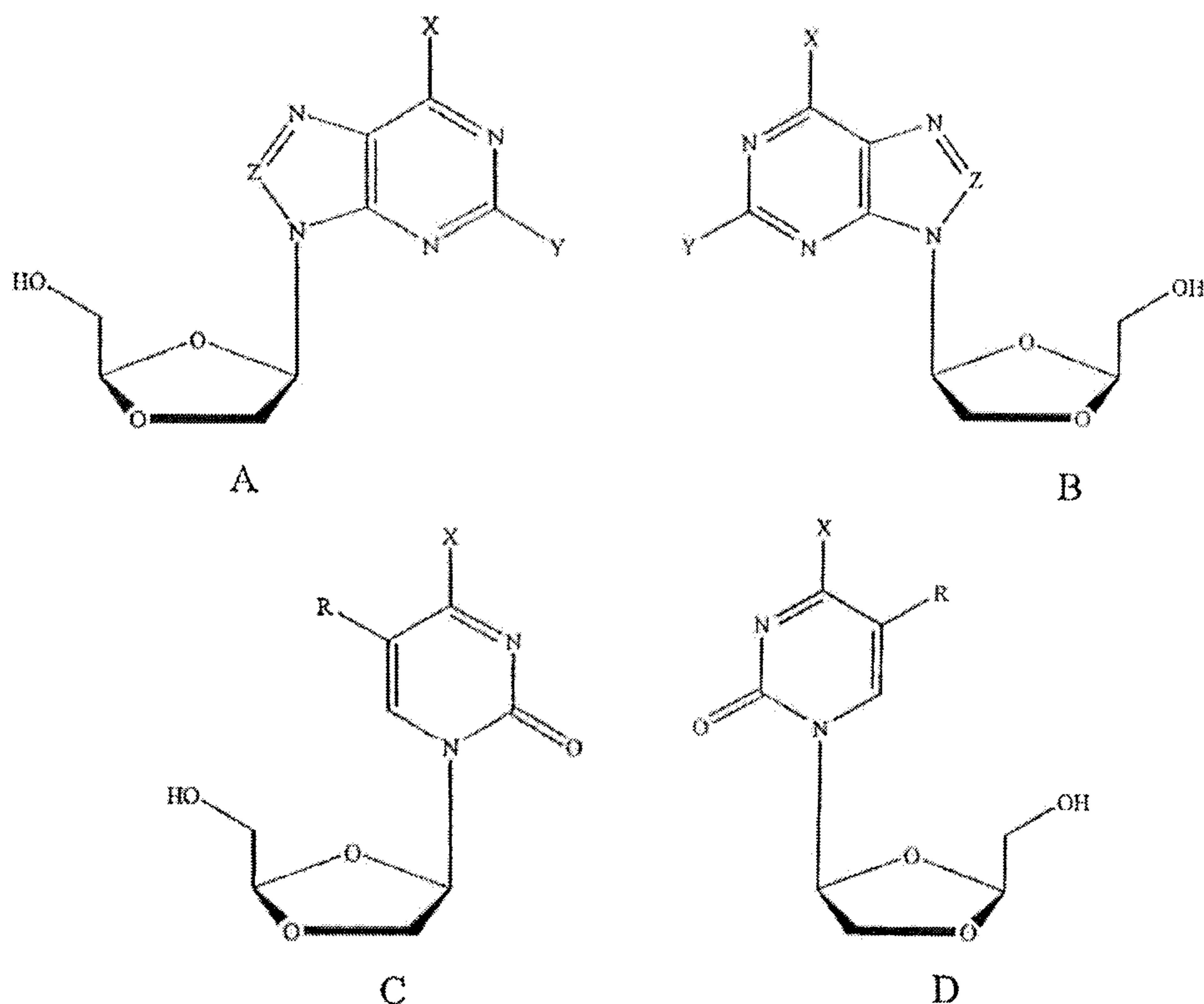
and then,

- f) purifying the $\alpha:\beta$ mixture of substantially pure protected D- or L-1,3-dioxolane nucleosides of the formula (IV) to obtain a substantially pure protected β -D- or β -L-1,3-dioxolane nucleoside; and then
- g) deprotecting the substantially pure protected β -D- or β -L-1,3-dioxolane nucleoside, if necessary, to obtain a substantially pure β -D- or β -L-1,3-dioxolane nucleoside.

In one embodiment of the present invention, the esterified 2,2-dialkoxy ethanol of the formula (Ia) is hydrolyzed to the corresponding aldehyde of formula (Ib). In a particular embodiment the esterified 2,2-dialkoxy ethanol of the formula (Ia) is hydrolyzed to the corresponding aldehyde of formula (Ib) when R^2 is *p*-methoxy benzoyl.

In one embodiment of the present invention, the resolution of the 1,3-dioxolane lactone of the formula (II) to obtain a substantially pure D- or L-lactone is accomplished using chiral chromatography. In an alternate embodiment of the present invention, the resolution of the 1,3-dioxolane lactone of the formula (II) to obtain a substantially pure D- or L-lactone is accomplished using enzymatic resolution.

Therefore, the processes of the present invention may be used to prepare compounds of formula A to D:



and pharmaceutically acceptable salts or esters thereof, wherein:

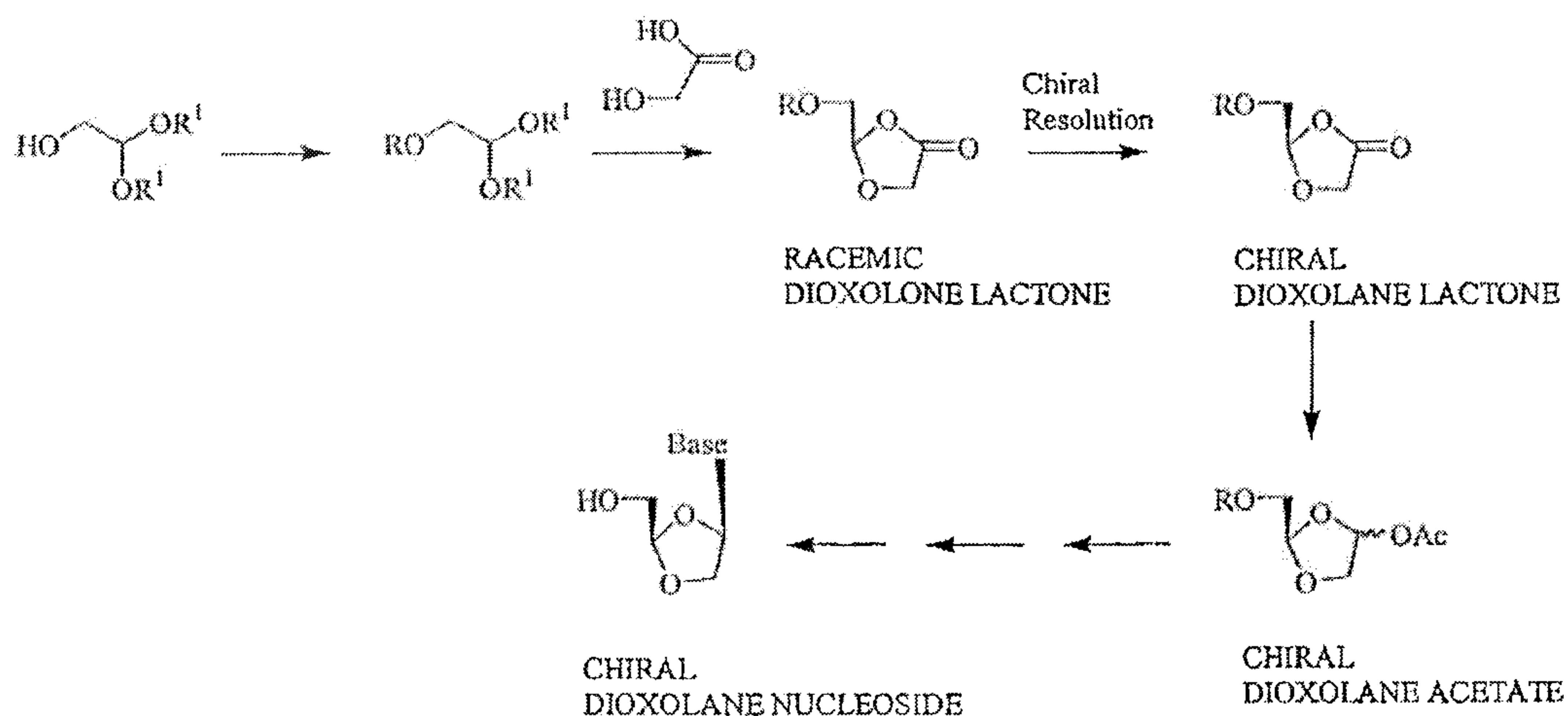
R is independently H, halogen (F, Cl, Br, I), OH, OR', OCH₃, SH, SR', SCH₃, NH₂, NHR', NR'₂, lower alkyl of C₁-C₄, CH₃CH=CH₂, N₃C=CH₂, CO₂H, CO₂R', CONH₂, CONHR', CH₂OH, CH₂CH₂OH, CF₃, CH₂CH₂F, CH=CHCO₂H, CH=CHCO₂R', CH=CHCl, CH=CHBr, or CH=CHI;

each R' is independently a lower alkyl of C₁-C₄;

Z is either one of CH or C-X; and

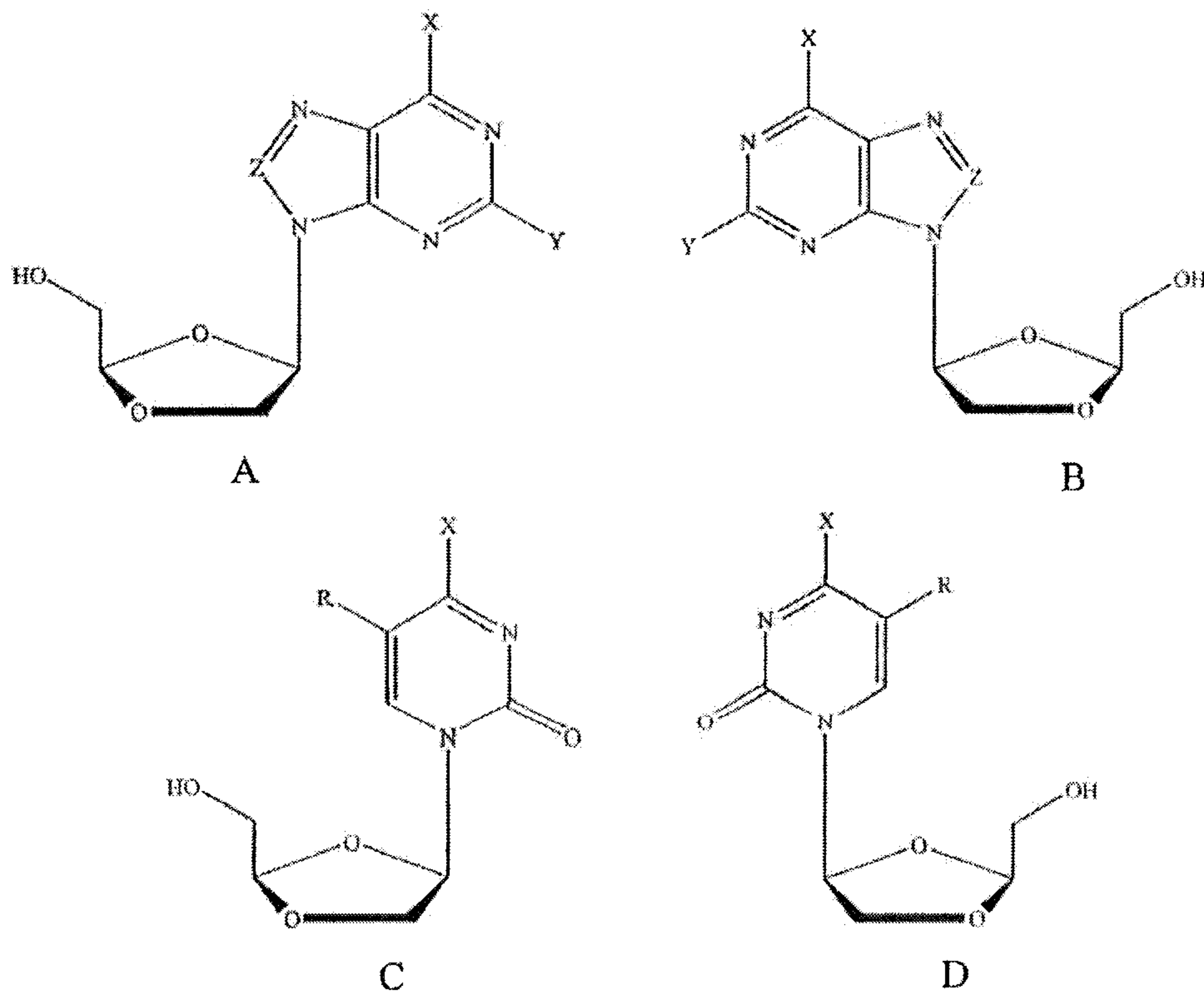
each X and Y are independently H, halogen (F, Cl, Br, I), OH, OR', OCH₃, SH, SR', SCH₃, NH₂, NHR', NR'₂, or CH₃.

20



Scheme 5

Therefore, the processes of the present invention may be used to prepare compounds of formula **A** to **D**:



and pharmaceutically acceptable salts or esters thereof, wherein:

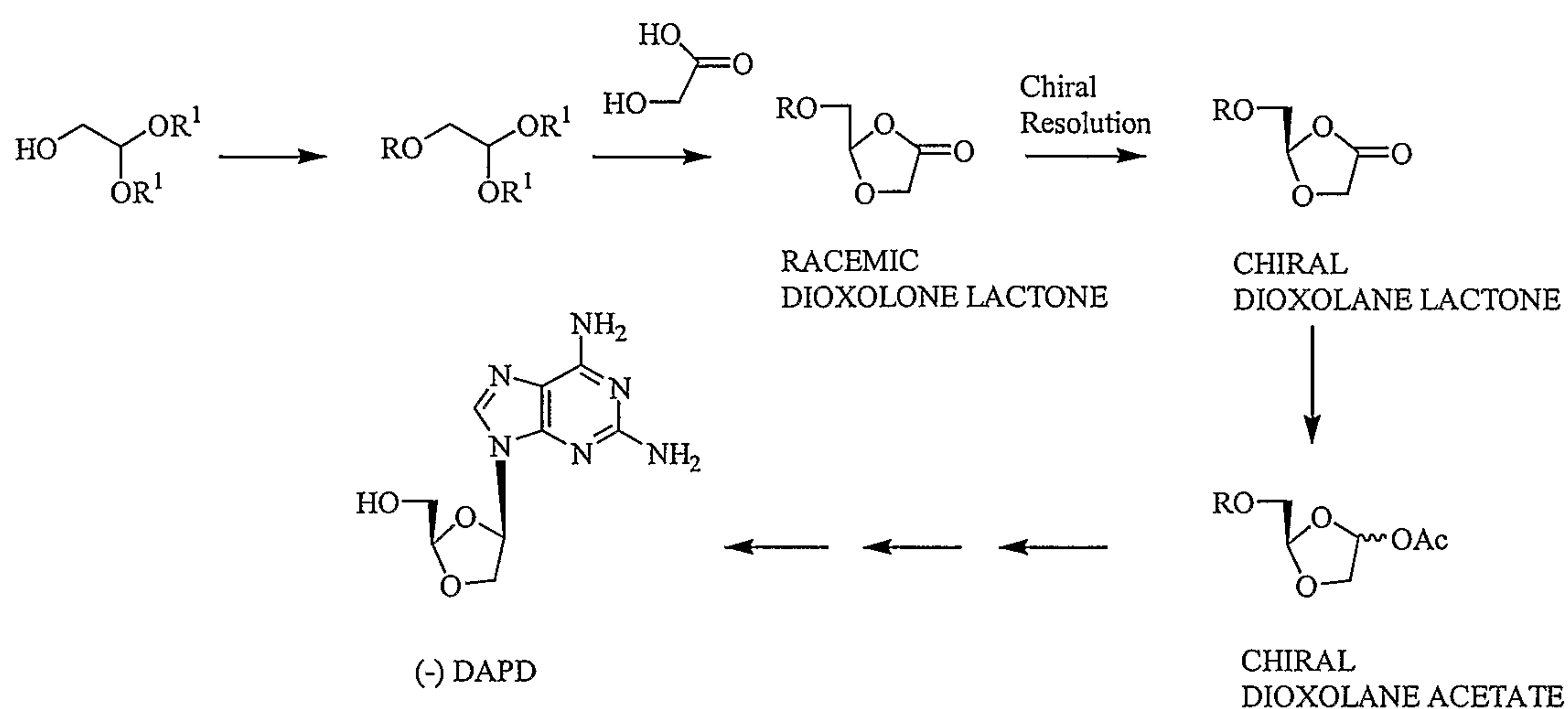
10 R is independently H, halogen (F, Cl, Br, I), OH, OR', OCH₃, SH, SR', SCH₃, NH₂, NHR', NR'₂, lower alkyl of C₁-C₄, CH₃CH=CH₂, N₃C=CH₂, CO₂H, CO₂R', CONH₂, CONHR', CH₂OH, CH₂CH₂OH, CF₃, CH₂CH₂F, CH=CHCO₂H, CH=CHCO₂R', CH=CHCl, CH=CHBr, or CH=CHI;

each R' is independently a lower alkyl of C₁-C₄;

Z is either one of CH or C-X; and

each X and Y are independently H, halogen (F, Cl, Br, I), OH, OR', OCH₃, SH, SR', SCH₃, NH₂, NHR', NR'₂, or CH₃.

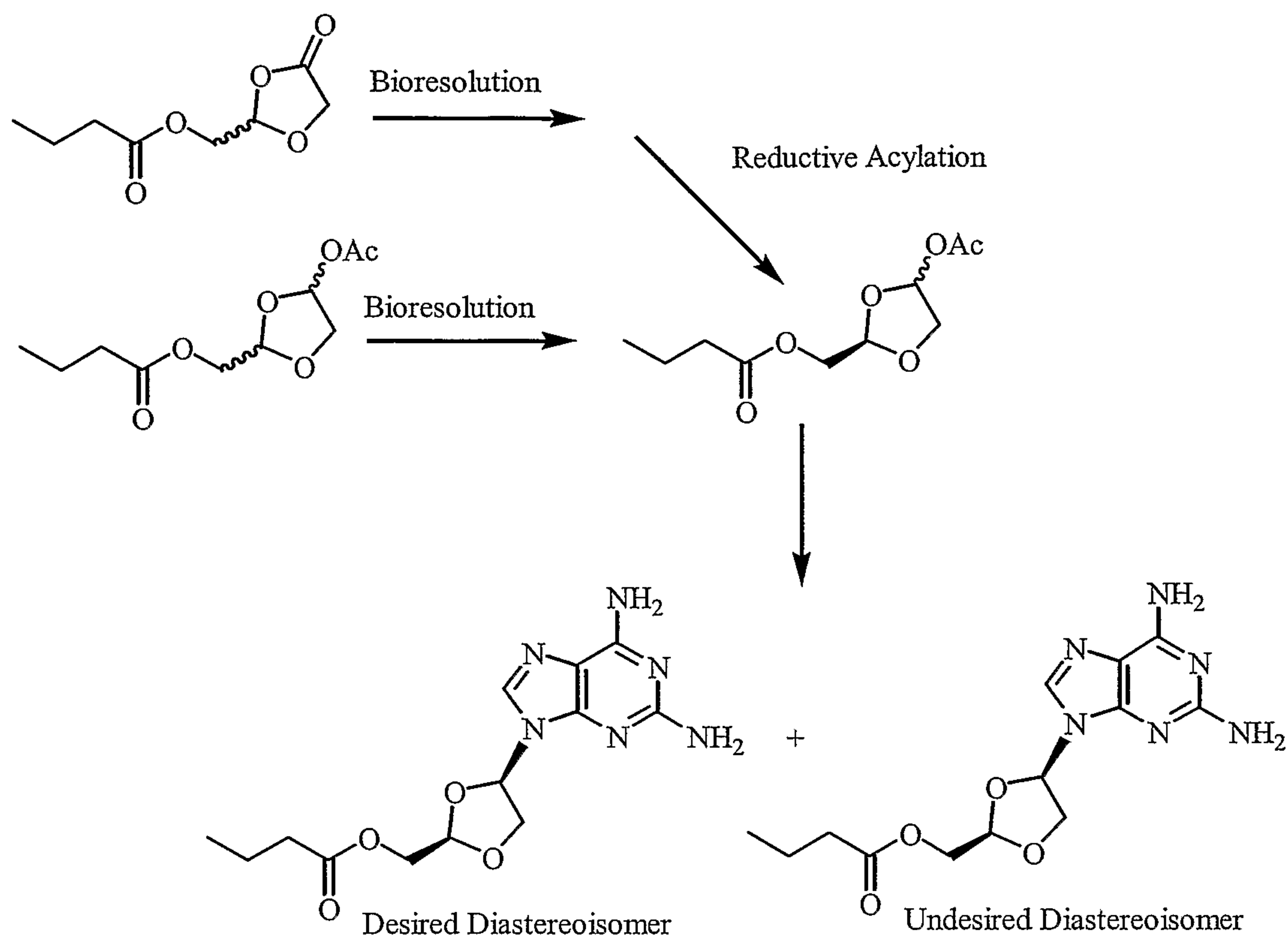
5 In a particular embodiment of the present invention, a process for preparing a substantially pure β-D-DAPD, is provided. See **Scheme 6**.



Scheme 6

10 In one embodiment, the invention provides processes for resolution of compounds that can be intermediates in the synthesis of 1,3-dioxolane nucleosides, such as 1-β-D-2,6-diaminopurine dioxolane (DAPD).

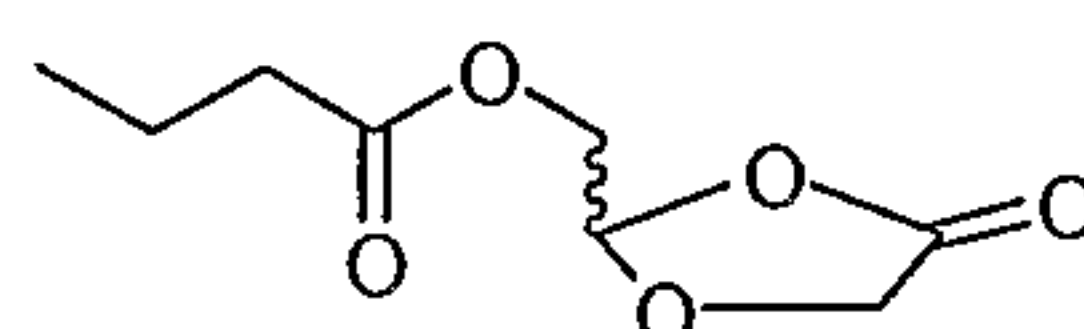
In one embodiment, the invention provides a biocatalytic method for the synthesis of DAPD intermediates as shown in **Scheme 7**.



Scheme 7: Exemplary Routes to DAPD

In one embodiment of the invention, the 1,3-dioxolane lactone of the formula (II) is the *sec*-butanoate ester (Compound 13).

In one embodiment of the present invention, if a (*S*)-enantiomer of the butyrate ester lactone (Compound 1 of the structure below) is desired,



Compound 1

PPL is used for the chiral resolution. As described in the examples, PPL was identified as the most selective enzyme (22% yield, 98% ee), and was found to leave the (*S*)-butyrate ester isomer in a full commercial enzyme screen of all available commercial enzymes and 200 microbial enzymes.

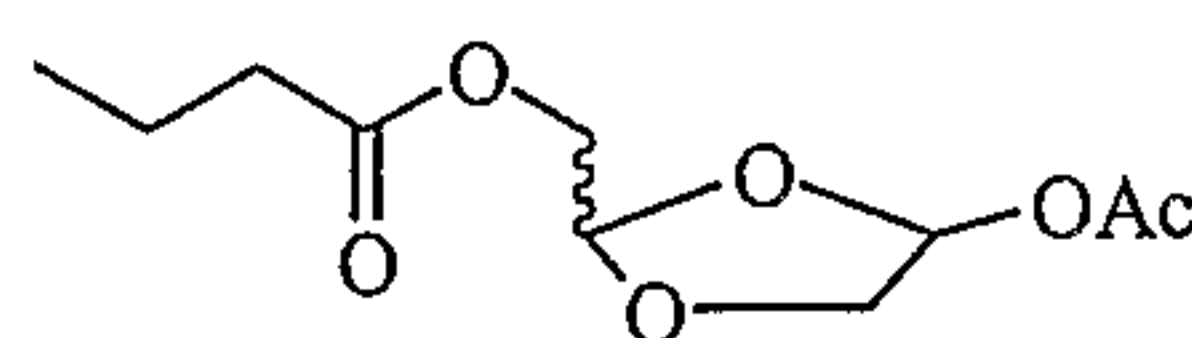
Although in general embodiments, any effective enzyme can be used, Lipase PS, immobilized lipase *Pseudomonas* and Lipase M were identified as selective enzymes that left the (*R*)-butyrate ester isomer, as described in the examples. Lipase PS was found to

be an efficient enzyme that possessed the required enantio-preference, giving 22% yield of 95% ee (*R*)-Compound 1. Therefore, in one alternative embodiment of the present invention, if a (*R*)-enantiomer is desired, then Lipase PS, Immobilised lipase *Pseudomonas* or Lipase M is used for the chiral resolution. In one particular
5 embodiment, the enzyme is Lipase PS.

Although the invention is not bound to particular conditions for resolution, in one embodiment, the temperature for resolution is about 0°C. In another embodiment, the temperature is between -5 °C and 10°C, or -2°C and +5°C or about -1°C to about 1°C. In another embodiment, the amount of substrate is about 100g/L. The amount of substrate
10 can vary, for example from 5 to 500, 20 to 200, 50 to 150, about 60, 70, 80, 90, 100, 110, 120, 130, 140, or more g/L of buffer or g/L of total reaction volume. In one embodiment, the buffer is 1:1 toluene buffer, which can be at about pH 6. In a specific embodiment, the conditions for resolution can be 0°C, 100g/L substrate in 1:1 toluene pH 6 buffer. As described in the examples, these conditions yield 22%, 95% ee (*R*)-
15 Compound 1 (E=5.3). The conditions can also vary slightly within experimental parameters for efficient resolution.

Microbial strains that resolve the compounds can vary. In one embodiment, the strains are from a species of *Acinetobacter*. The microbial screen identified 2 strains (*Acinetobacter* and *Acinetobacter junii*) which gave the (*R*)-butyrate in 80% ee. All
20 available commercial enzymes and 200 microbial enzymes were screened in order to identify an efficient resolution of the butyrate ester. The microbial screen revealed 2 promising strains (CMC 3419, *Acinetobacter* and CMC 3606, *Acinetobacter junii*) which gave (*R*)-butyrate in 80% ee.

In one embodiment, when resolution of the butyrate ester acetate (Compound 3)
25 of the structure below:



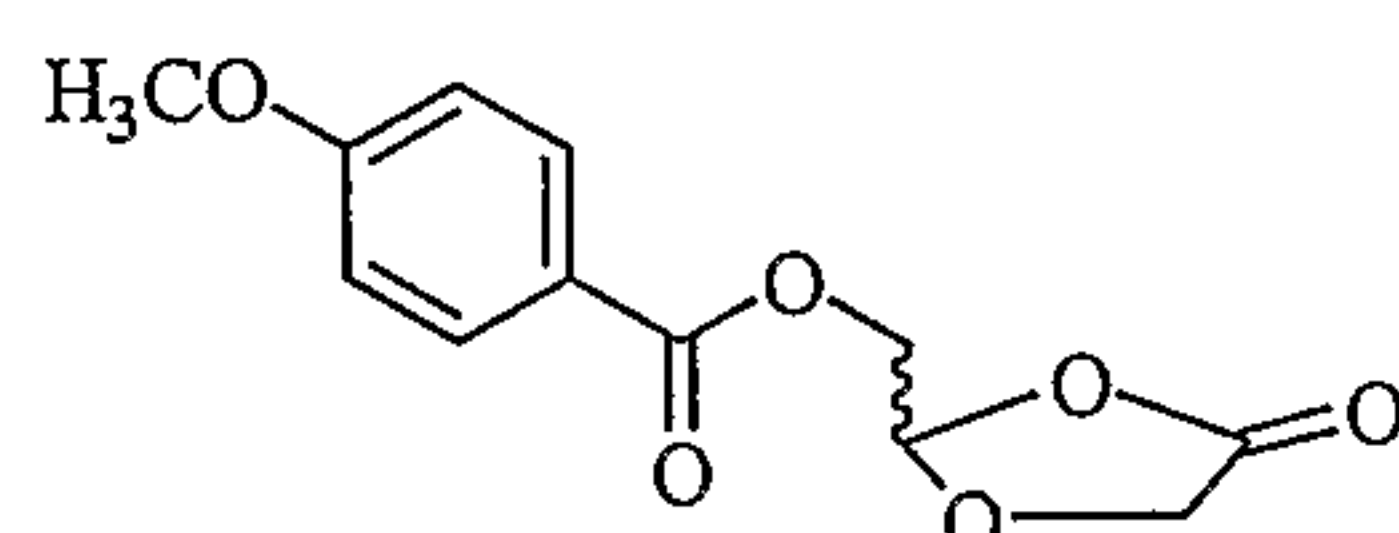
Compound 3

is desired, the enzyme used is Lipase PS. A full commercial enzyme screen of Compound 3 revealed that the majority of selective enzymes resolve at the acetate and
30 not the required butyrate centre. However Lipase PS, Lipase MY and Lipase AY possessed moderate selectivity, leaving the (*R*)-ester isomers. These enzymes were of similar selectivity to those for Compound 1. Further investigation, revealed Lipase PS to

be the most selective (effective $E=3.7$). Similarly, this is of comparable selectivity to the resolution of Compound 1 with Lipase PS.

In one embodiment of the invention, the resolution step occurs at an earlier stage of the synthesis, i.e. the lactone stage.

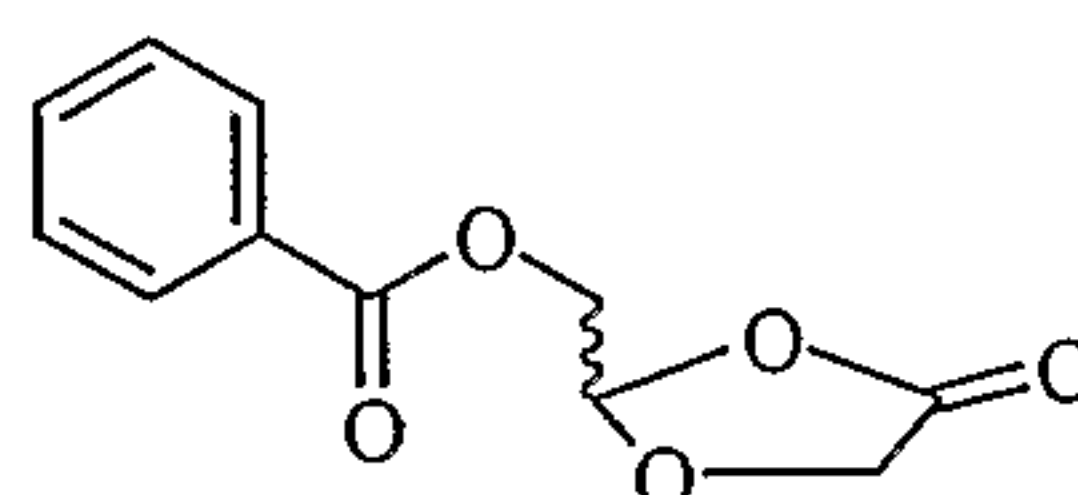
5 In one embodiment, when resolution of the (S)-enantiomer of *p*-methoxy-benzoyl lactone, 4-oxo-[1,3]dioxolan-2-ylmethyl 4'-methoxybenzoate (Compound 11).



Compound 11

10 is desired, then Chirazyme-L2 is used for the resolution of the lactone. A wide range of commercial and microbial enzymes were screened in order to identify an efficient resolution of Compound 11. Chirazyme-L2 was identified as the most selective enzyme giving residual ester in 39% yield, >98% ee of the (S)-enantiomer.

In another embodiment, when the resolution of the benzoyl lactone, 4-oxo-[1,3]dioxolan-2-ylmethyl benzoate (Compound 12).

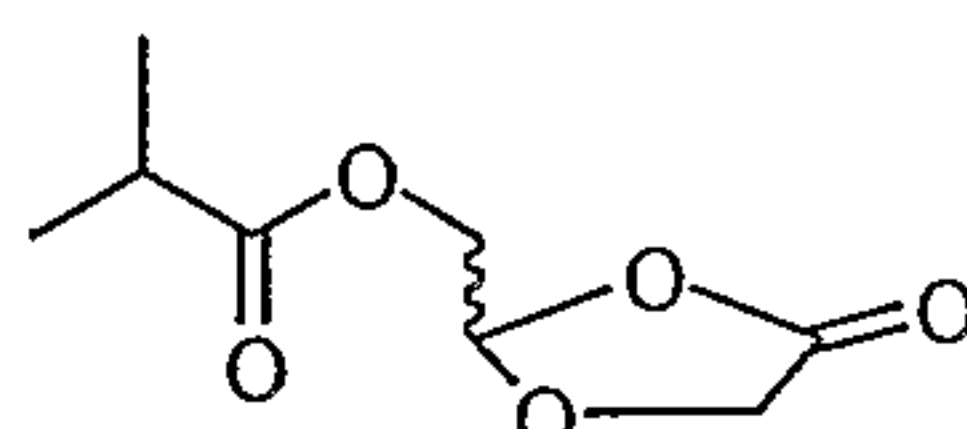


15

Compound 12

is desired, the enzyme used can be Chirazyme-L2. A commercial enzyme screen (ten enzymes) was undertaken for the benzoyl lactone (Compound 12). Chirazyme-L2 and Acid Protease-DS were identified as potential resolving agents in the screen.
20 Chirazyme-L2 gave comparable selectivity for the resolutions of Compounds 11 and 12. A limited commercial enzyme screen for Compound 12 found that Chirazyme-L2 was again the most efficient enzyme.

In another embodiment, when the resolution of the (R)-enantiomer of iso-butryl lactone, 4-oxo-[1,3]dioxolan-2-yl-methyl-2'-methylpropanoate (Compound 13).



25

Compound 13

is desired, the enzyme used can be Chirazyme-L2. A complete commercial and microbial enzyme screen was performed for Compound 13. Chirazyme-L2 was again

identified as the most selective enzyme. The configuration was determined to be the (*R*)-enantiomer by chemical correlation. Initial studies in MTBE-buffer gave the residual ester in 28% yield, 92% ee.

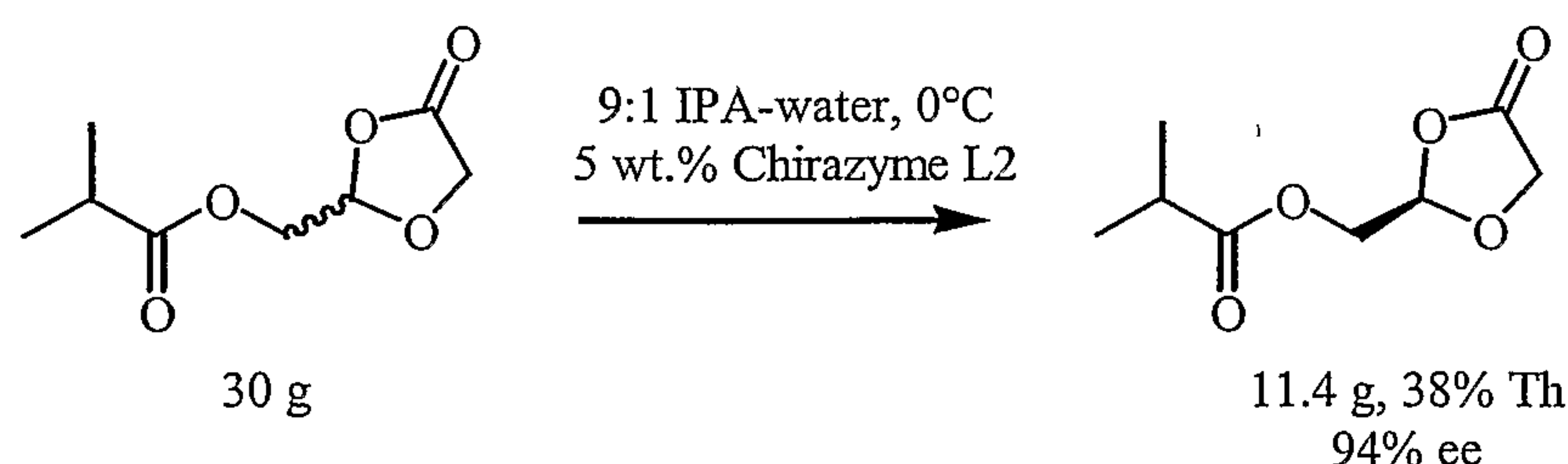
A full commercial enzyme screen for Compound 13 revealed that Chirazyme-L2, Lipase PS, Acid Protease DS and Chirazyme-L9 possessed moderate selectivity, leaving the (*R*)-enantiomer as the residual ester. A full microbial screen identified strains that were selective for either enantiomer. One microbe, CMC 103869, gave >95% ee lactone by running the resolution at 10°C.

In one embodiment, Compound 13 (iso-butryl lactone) was the substrate. In another embodiment, the substrate is one that affords simpler downstream chemistry, for example, simpler purification techniques. Chirazyme-L2 can be the enzyme used for resolution. In one embodiment, Chirazyme-L2 can give the (*R*)-configuration of residual ester.

The reaction was studied in MTBE-buffer, toluene-buffer and 2-propanol-water systems. Stability and selectivity problems were encountered with the MTBE and toluene systems. However the results in 2-propanol-water were excellent, giving a 38% yield of 94% ee Compound 13 from a 30 g input of racemate. Therefore, in one embodiment, the resolution is performed in 2-propanol-water.

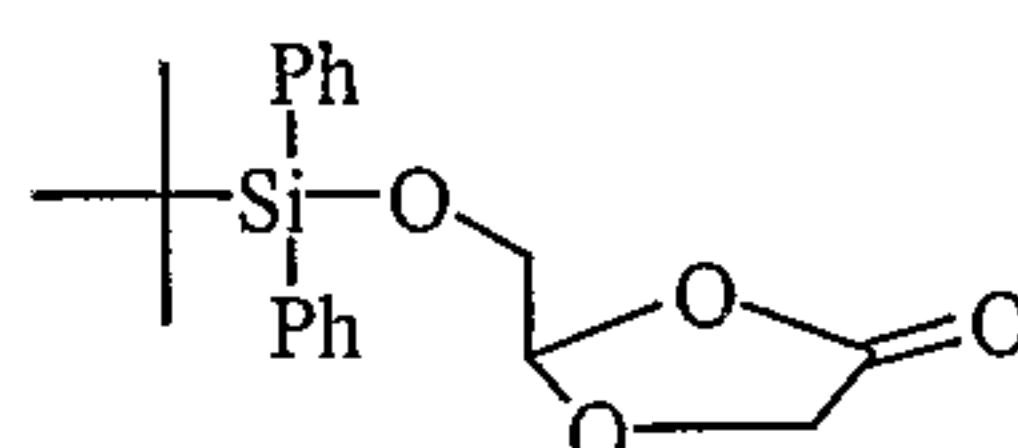
An optimization study was undertaken. The process was improved by employing a monophasic alcohol-water system as solvent and using Kugelrohr distillation to purify the product. Therefore, in one embodiment, the process involves a monophasic alcohol-water system and distillation.

The bioresolution of compound 13 with Chirazyme-L2 was scaled up to 30 g at 200 gL⁻¹ in 2-propanol:water (9:1) and gave 4-oxo-[1,3]dioxolan-2-yl-methyl-2'-methylpropanoate in 38% yield, 94% ee and 90% chemical purity. See **Scheme 8**.



Scheme 8: Conditions identified for the bioresolution of compound 13

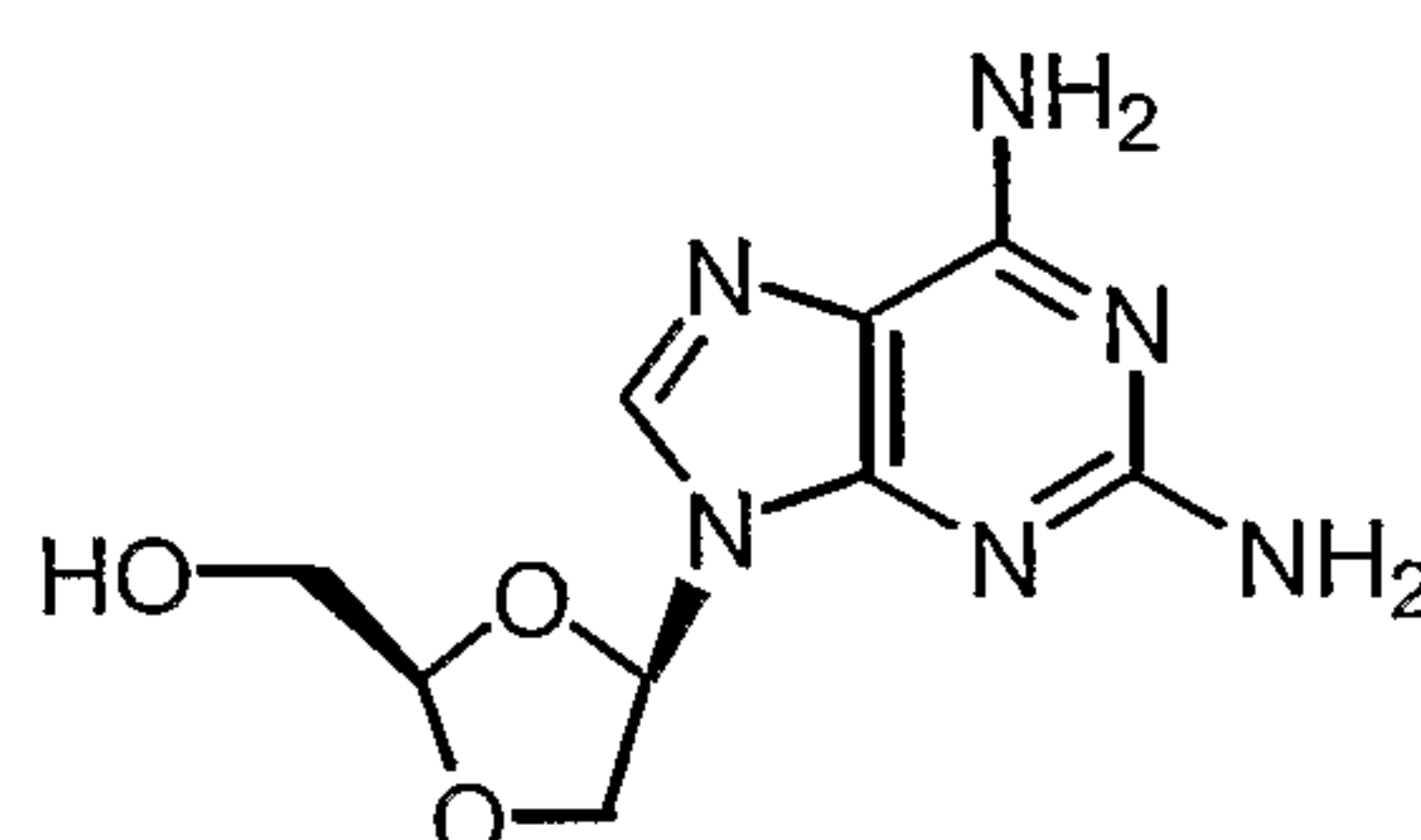
In contrast, the *t*-butyl-diphenyl silyl ether lactone (Compound 4) of the structure below:



Compound 4

5 was determined as a true racemate. The phase diagram for this compound was constructed which revealed the eutectic to be at 77.5% ee.

Racemic *cis*-DAPD was screened with several enzymes known for their acylation activity in the presence of vinyl butyrate, but they were found to give non-stereoselective reactions. PeptiCLEC-BL and Lipase AY gave fast reactions to the desired racemic butyrate and thus may be useful to regioselectively and mildly put an ester on the oxygen. In one embodiment, PeptiCLEC-BL is used. In a separate embodiment, Lipase AY is used.



15 **1-β-D-2,6-Diaminopurine Dioxolane (DAPD)**

Definitions

As used herein, the term “substantially pure,” “substantially free of,” “substantially in the absence of” or “isolated” refers to a nucleoside composition that includes at least 85, at least 90, at least 95%, or at least 99% to 100% by weight, of the designated enantiomer of that nucleoside. In one embodiment, the process produces compounds that are substantially free of enantiomers of the opposite configuration.

The term alkyl, as used herein, unless otherwise specified, refers to a saturated straight, branched, or cyclic, primary, secondary, or tertiary hydrocarbon of C₁ to C₁₀, and specifically includes methyl, trifluoromethyl, ethyl, propyl, isopropyl, cyclopropyl, butyl, isobutyl, *t*-butyl, pentyl, cyclopentyl, isopentyl, neopentyl, hexyl, isohexyl, cyclohexyl, cyclohexylmethyl, 3-methylpentyl, 2,2-dimethylbutyl, and 2,3-dimethylbutyl. The term includes both substituted and unsubstituted alkyl groups. Moieties with which the alkyl group can be substituted are selected from the group

consisting of hydroxyl, amino, alkylamino, arylamino, alkoxy, aryloxy, nitro, cyano, sulfonic acid, sulfate, phosphonic acid, phosphate, or phosphonate, either unprotected, or protected as necessary, as known to those skilled in the art, for example, as taught in Greene, *et al.*, Protective Groups in Organic Synthesis, John Wiley and Sons, Second Edition, 1991.

The term lower alkyl, as used herein, and unless otherwise specified, refers to a C₁ to C₄ saturated straight, branched, or if appropriate, a cyclic (for example, cyclopropyl) alkyl group, including both substituted and unsubstituted forms. Unless otherwise specifically stated in this application, when alkyl is a suitable moiety, lower alkyl is preferred. Similarly, when alkyl or lower alkyl is a suitable moiety, unsubstituted alkyl or lower alkyl is preferred.

The term aryl, as used herein, and unless otherwise specified, refers to phenyl, biphenyl, or naphthyl. The term includes both substituted and unsubstituted moieties. The aryl group can be substituted with one or more moieties selected from the group consisting of bromo, chloro, fluoro, iodo, hydroxyl, amino, alkylamino, arylamino, alkoxy, aryloxy, nitro, cyano, sulfonic acid, sulfate, phosphonic acid, phosphate, or phosphonate, either unprotected, or protected as necessary, as known to those skilled in the art, for example, as taught in Greene, *et al.*, Protective Groups in Organic Synthesis, John Wiley and Sons, Second Edition, 1991.

The term alkaryl or alkylaryl refers to an alkyl group with an aryl substituent. The term aralkyl or arylalkyl refers to an aryl group with an alkyl substituent.

The term halo, as used herein, includes bromo, chloro, fluoro, and iodo.

The term heteroatom, as used herein, refers to oxygen, sulfur, nitrogen, and phosphorus.

The term acyl refers to a carboxylic acid ester in which the non-carbonyl moiety of the ester group is selected from straight, branched, or cyclic alkyl or lower alkyl, alkoxyalkyl including methoxymethyl, aralkyl including benzyl, aryloxyalkyl such as phenoxymethyl, aryl including phenyl optionally substituted with halogen, C₁ to C₄ alkyl or C₁ to C₄ alkoxy, sulfonate esters such as alkyl or aralkyl sulphonyl including methanesulfonyl, the mono, di or triphosphate ester, trityl or monomethoxytrityl, substituted benzyl, trialkylsilyl (e.g. dimethyl-*t*-butylsilyl) or diphenylmethylsilyl. Aryl groups in the esters optimally comprise a phenyl group. The term "lower acyl" refers to an acyl group in which the non-carbonyl moiety is lower alkyl.

The term "protected" as used herein and unless otherwise defined refers to a group that is added to an oxygen, nitrogen, or phosphorus atom to prevent its further reaction or for other purposes. A wide variety of oxygen and nitrogen protecting groups are known to those skilled in the art of organic synthesis.

5 The term purine or pyrimidine base includes, but is not limited to, adenine, N⁶-alkyl-purines, N⁶-acylpurines (wherein acyl is C(O)(alkyl, aryl, alkylaryl, or arylalkyl), N⁶-benzylpurine, N⁶-halopurine, N⁶-vinylpurine, N⁶-acetylenic purine, N⁶-acyl purine, N⁶-hydroxyalkyl purine, N⁶-thioalkyl purine, N²-alkylpurines, N²-alkyl-6-thiopurines, thymine, cytosine, 5-fluoro-cytosine, 5-methylcytosine, 6-azapyrimidine, including
10 6-aza-cytosine, 2- and/or 4-mercapto-pyrimidine, uracil, 5-halouracil, including 5-fluorouracil, C⁵-alkylpyrimidines, C⁵-benzyl-pyrimidines, C⁵-halopyrimidines, C⁵-vinylpyrimidine, C⁵-acetylenic pyrimidine, C⁵-acyl pyrimidine, C⁵-hydroxyalkyl purine, C⁵-amido-pyrimidine, C⁵-cyanopyrimidine, C⁵-nitro-pyrimidine, C⁵-aminopyrimidine, N²-alkyl-purines, N²-alkyl-6-thiopurines, 5-azacytidinyl, 5-aza-
15 uracilyl, triazolopyridinyl, imidazolo-pyridinyl, pyrrolopyrimidinyl, and pyrazolopyrimidinyl. Purine bases include, but are not limited to, guanine, adenine, hypoxanthine, 2,6-diaminopurine, 2-(Br, Fl, Cl or I)-purine optionally with a substituent including an amino or carbonyl group in the 6-position, and 6-(Br, Cl, or I)-purine optionally with a substituent including an amino or carbonyl group in the 2-position.
20 Functional oxygen and nitrogen groups on the base can be protected as necessary or desired. Suitable protecting groups are well known to those skilled in the art, and include trimethylsilyl, dimethylhexylsilyl, *t*-butyldimethylsilyl, *t*-butyldiphenylsilyl, trityl, alkyl groups, and acyl groups such as acetyl and propionyl, methanesulfonyl, and *p*-toluenesulfonyl.

25 The term heteroaryl or heteroaromatic, as used herein, refers to an aromatic that includes at least one sulfur, oxygen, nitrogen or phosphorus in the aromatic ring. The term heterocyclic refers to a nonaromatic cyclic group wherein there is at least one heteroatom, such as oxygen, sulfur, nitrogen or phosphorus in the ring. Nonlimiting examples of heteroaryl and heterocyclic groups include furyl, furanyl, pyridyl, pyrimidyl, thienyl, isothiazolyl, imidazolyl, tetrazolyl, pyrazinyl, benzofuranyl, benzothiophenyl, quinolyl, isoquinolyl, benzothienyl, isobenzofuryl, pyrazolyl, indolyl, isoindolyl, benzimidazolyl, purinyl, carbazolyl, oxazolyl, thiazolyl, iso-thiazolyl, 1,2,4-thiadiazolyl, isooxazolyl, pyrrolyl, quinazolinyl, cinnolinyl, phthalazinyl, xanthinyl,
30

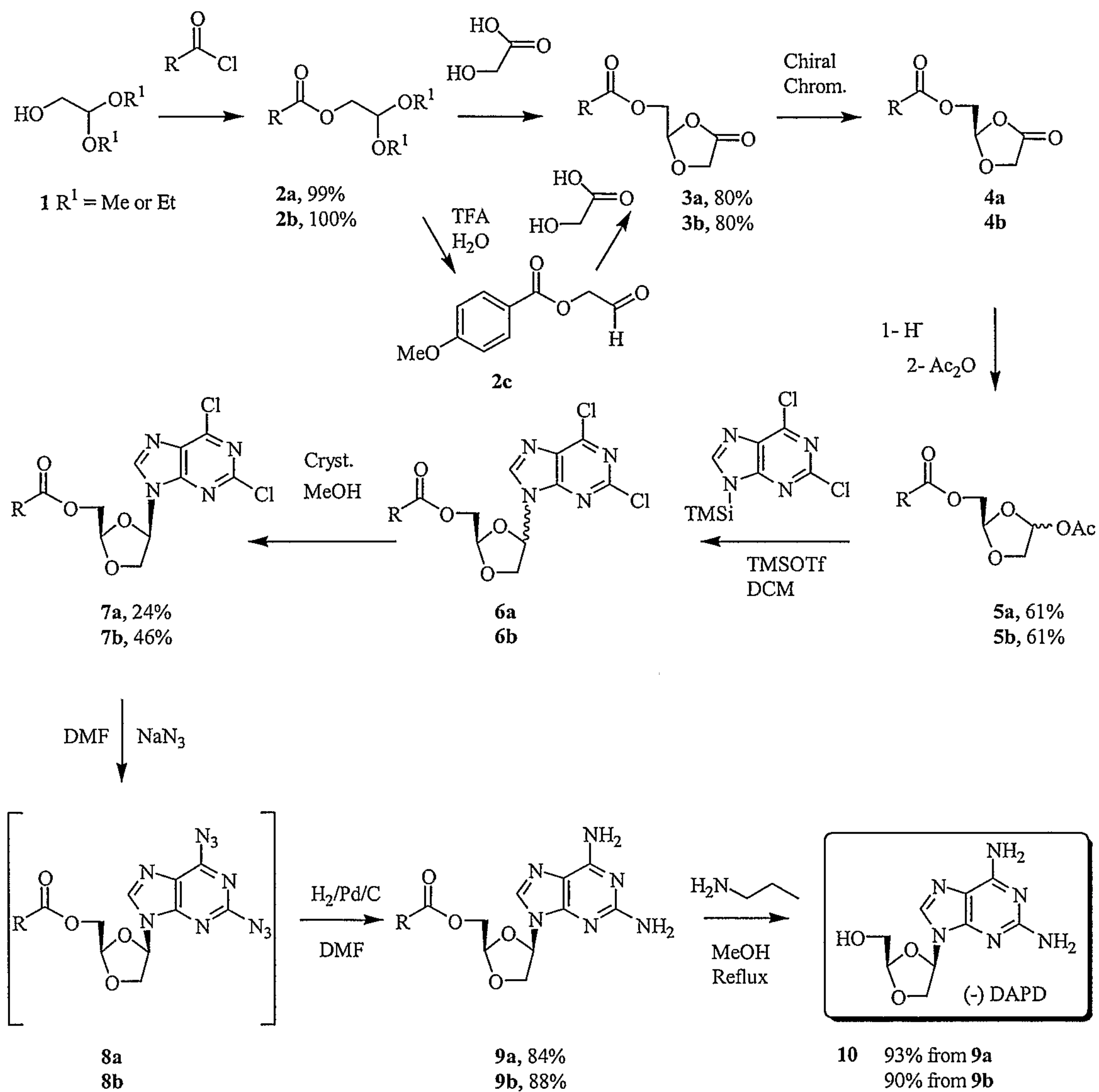
hypoxanthinyl, thiophene, furan, pyrrole, isopyrrole, pyrazole, imidazole, 1,2,3-triazole, 1,2,4-triazole, oxazole, isoxazole, thiazole, isothiazole, pyrimidine or pyridazine, and pteridinyl, aziridines, thiazole, isothiazole, 1,2,3-oxadiazole, thiazine, pyridine, pyrazine, piperazine, pyrrolidine, oxaziranes, phenazine, phenothiazine, morpholinyl, pyrazolyl, 5
 5 pyridazinyl, pyrazinyl, quinoxalinyl, xanthinyl, hypoxanthinyl, pteridinyl, 5-azacytidinyl, 5-azauracilyl, triazolopyridinyl, imidazolopyridinyl, pyrrolopyrimidinyl, pyrazolopyrimidinyl, adenine, N⁶-alkylpurines, N⁶-benzylpurine, N⁶-halopurine, N⁶-vinylpurine, N⁶-acetylenic purine, N⁶-acyl purine, N⁶-hydroxyalkyl purine, N⁶-thioalkyl purine, thymine, cytosine, 6-azapyrimidine, 2-mercaptopyrimidine, uracil, N⁵-
 10 alkylpyrimidines, N⁵-benzylpyrimidines, N⁵-halopyrimidines, N⁵-vinylpyrimidine, N⁵-acetylenic pyrimidine, N⁵-acyl pyrimidine, N⁵-hydroxyalkyl purine, and N⁶-thioalkyl purine, and isoxazolyl. The heteroaromatic group can be optionally substituted as described above for aryl. The heterocyclic or heteroaromatic group can be optionally substituted with one or more substituent selected from halogen, haloalkyl, alkyl, alkoxy,
 15 hydroxy, carboxyl derivatives, amido, amino, alkylamino, dialkylamino. The heteroaromatic can be partially or totally hydrogenated as desired. As a nonlimiting example, dihydropyridine can be used in place of pyridine. Functional oxygen and nitrogen groups on the heterocyclic or heteroaryl group can be protected as necessary or desired. Suitable protecting groups are well known to those skilled in the art, and
 20 include trimethylsilyl, dimethylhexylsilyl, *t*-butyldimethylsilyl, and *t*-butyl-diphenylsilyl, trityl or substituted trityl, alkyl groups, acyl groups such as acetyl and propionyl, methanesulfonyl, and *p*-toluenylsulfonyl.

These purine or pyrimidine bases, heteroaromatics and heterocycles can be substituted with alkyl groups or aromatic rings, bonded through single or double bonds
 25 or fused to the heterocycle ring system. The purine base, pyrimidine base, heteroaromatic, or heterocycle may be bound to the sugar moiety through any available atom, including the ring nitrogen and ring carbon (producing a C-nucleoside).

Abbreviations used throughout include ee: Enantiomeric excess for the starting material (s) or product (p); c: Conversion; and E: Enantioselectivity constant.

Detailed Description of Process Steps

a, R = -CH-(CH₃)₂
 b, R = p-MeO-Ph-



TOTAL YIELD OF 10 FROM: 5a, 11.4%
 5b, 22.2%

Scheme 9

Initially a 2,2-dialkoxy-ethanol (1) was esterified with the corresponding acid chloride in quantitative yields. In the case of the *iso*-butyryl (2a) ester, cyclization to the corresponding dioxolane lactone (3a), was affected with glycolic acid in the presence of a Lewis acid (BF₃·Et₂O) in 80% yield. However, under the same conditions, the *p*-methoxy benzoate ester (2b) afforded the corresponding lactone in low yield and purity. Much better results were achieved in a two step procedure in which the acetal was first

hydrolyzed to the corresponding aldehyde (**2c**), which then was subjected to similar cyclization conditions, affording the corresponding lactone (**3b**) in 80% yield as a solid. The lactones were resolved by chiral chromatography¹³. Selective reduction of the chiral lactones (**4a** and **4b**) with $\text{LiAlH}(\text{OtBu})_3$ and subsequent treatment with acetic anhydride afforded the corresponding acetates (**5a** and **5b**) in reasonable yields, after purification by flash column chromatography. Coupling with 2,6-dichloropurine under Vobruggen conditions afforded crude mixtures of $\alpha:\beta$ anomers of the corresponding nucleosides (**6a** and **6b**). The *iso*-butyrate ester was first purified by column chromatography and then crystallized from MeOH to afford the pure β anomer (**7a**) in a 24% yield. The *p*-methoxy benzoate ester nucleoside did not require chromatography, and was purified by crystallization from MeOH to afford pure β anomer (**7b**) in 46% yield. The chlorines were replaced with amino groups in a two-step procedure by treatment with sodium azide and then hydrogenation. The corresponding intermediates (**9a** and **9b**) were isolated and fully described. Removal of the protecting groups was achieved with *n*-butylamine in refluxing MeOH to afford (-)-DAPD (**10**) in 93% and 90% yield respectively.

The total overall yields from the corresponding chiral lactones (**5a** and **5b**) were: 11.4% for the *iso*-butyrate and 22.2 % for the *p*-methoxy benzoate.

EXAMPLES

Melting points were determined in open glass capillaries by use of a Melt-Temp II apparatus with a digital thermometer. ¹H-NMR spectra were recorded at 400 MHz with a Varian XL-400 spectrometer. Evaporations were performed under diminished pressure using a Buchi rotatory evaporator at 40 °C unless otherwise indicated. Solutions were dried over anhydrous Na_2SO_4 . TLC was performed on precoated glass plates (0.25 mm) with Silica Gel 60F₂₅₄ (E. Merck, Darmstad). Flash column chromatography was performed with Silica Gel 60 (230-400 mesh, E. Merck, Darmstad). Elemental analyses were performed by Atlantic Microlab (Atlanta, GA). High Resolution Mass Spectra (HRMS) were performed by Analytical Instrument Group Inc. (Raleigh, NC).

Gas Chromatography Analysis: Samples were prepared by dissolving 1 mg sample per 1 ml THF, or other suitable solvent. A 1 μl injection volume was used.

Samples were separated using a 30m HP-5 (crosslinked 5% PH ME siloxane) capillary column, Hewlett Packard part #19091J-413. The inlet temperature was set at 200 °C, and the oven temperature as follows: 35 °C for 1 minute, ramp to 250 °C at 12.5 °C/minute, hold at 250 °C for 1.8 minutes. The flame ionization detector temperature was set at 250 °C. The carrier gas was nitrogen set at a nominal flow of 8.0 ml/minute.

HPLC Analysis:

Method A: Column: Chiralpak AD-RH, 150 x 4.6 mm. Mobile phase: Methanol. Gradient: Isocratic. Flow rate: 0.5 ml/min. Run time: 30 min. Detection: UV at 280 nm.

Method B: Column: Aquasil C18, 150 x 4.6 mm. Mobile phase: solvent A: acetonitrile, solvent B: 50 mmol NH₄OAc, 0.1% AcOH in water. Gradient: time: 0 min., A: 1%, B: 99%; time: 17 min., A: 50%, B: 50%, then isocratic. Flow rate: 1.0 ml/min. Run time: 30 min. Detection: UV at 290 nm.

Method C: Column: Chiralpak AD, 250 x 4.6 mm. Mobile phase: Methanol. Gradient: Isocratic. Flow rate: 0.8 ml/min. Run time: 20 min. Detection: UV at 254 nm.

Example 1

Isobutyric acid 2,2-dimethoxy-ethyl ester (2a).

4-Methoxy-benzoic acid 2,2-diethoxy-ethyl ester (2b).

To a well-stirred solution of 2,2-diethoxy or 2,2-dimethoxy-ethanol (1, 100 mmol), DMAP (61 mg, 0.5 mmol) and Et₃N (16 ml, 11.64 g, 115 mmol) in EtOAc or *tert*-butylmethyl ether (50 ml) at 0 °C was slowly added the corresponding acid chloride (105 mmol). After stirring for 16 h at room temperature the reaction mixture was diluted with EtOAc (50 ml), and successively washed with: (c) NaHCO₃ (2 x 100 ml), brine (2 x 100 ml), dried, filtered and evaporated to afford:

Isobutyric acid 2,2-dimethoxy-ethyl ester (**2a**, 99%) as a yellow liquid that was used in the next step without any further purification. GC (*R*_t = 5.24 min., 98%); ¹H-NMR (CDCl₃) δ: 4.57 (1H, t, *J* = 5.2, (MeO)₂CHCH₂-), 4.11 (2H, d, *J* = 5.2, (MeO)₂CHCH₂-), 3.40 (6H, s, CH₃O-), 2.60 (1H, m, OCOCH(CH₃)₂), 1.54 (6H, d, *J* = 6.8, OCOCH(CH₃)₂).

4-Methoxybenzoic acid 2,2-diethoxy-ethyl ester (**2b**, 100%) as a syrup that was used in the next step without any further purification. GC (*R*_t = 13.7 min, 99%); ¹H-NMR (CDCl₃) δ: 7.98 (2H, d, *J* = 9.0, ArH), 6.89 (2H, d, *J* = 9.0, ArH), 4.79 (1H, t, *J* =

5.6, (EtO)₂CHCH₂-), 4.28 (2H, d, J = 5.6, (EtO)₂CHCH₂-), 3.82 (3H, s, CH₃O-), 3.73 (2H, m, CH₃CH₂O-), 3.59 (2H, m, CH₃CH₂O-), 1.22 (6H, t, J = 6.9, CH₃CH₂O-).

Example 2

Isobutyric acid 4-oxo-[1,3]-dioxolan-2-yl methyl ester (3a).

5 To a well stirred solution of the corresponding acetal (2a, 30 mmol) and α-hydroxy acetic acid (3.42 g, 45 mmol) in acetonitrile (30 ml) at 0 °C was slowly added BF₃·EtO₂ (6.38 g, 5.70 ml, 45 mmol). The solution was left at room temperature overnight with stirring. The solution was partitioned between EtOAc (150 ml) and (c) NaHCO₃ (150 ml). The organic solution was successively washed with (c) NaHCO₃
10 (150 ml), brine (2 x 150 ml), dried, filtered and evaporated to afford:

Isobutyric acid 4-oxo-[1,3]-dioxolan-2-yl methyl ester (3a, 80%) as a colorless syrup. GC (R_t = 7.89 min., 95%); ¹H-NMR (CDCl₃) δ: 5.83 (1H, s, H-2), 4.35-4.20 (4H, m, H-5, H-5' and -CH₂OCO-), 2.62 (1H, m, (CH₃)₂CHCOO-), 1.19 (6H, d, J = 7.0, (CH₃)₂CHCOO-).

15 *Calculated mass for C₈H₁₃O₅ (M + 1)⁺ : 189.0763. Found: (H.R.F.A.B.M.S.): 189.0763.*

Example 3

4-Methoxybenzoic acid 4-oxo-[1,3]-dioxolan-2-yl methyl ester (3b).

20 A solution of 4-methoxybenzoic acid 2,2-diethoxy-ethyl ester (2b, 7.5 g, 28 mmol) in Cl₂CH₂ (75 ml) was treated with TFA (16.7 g, 11.3 ml, 140 mmol) and water (7.5 g, 7.5 ml, 28 mmol). The homogeneous solution was stirred for 3.5 hours at room temperature until GC showed complete reaction. The solution was concentrated *in vacuo* at 40 °C and then diluted with hexane and concentrated *in vacuo* several times to remove traces of TFA. The product, 4-methoxybenzoic acid 2-oxo-ethyl ester (2c, 5.9 g, 28
25 mmol, 100%) was isolated as an amorphous white solid and was used in the next step without any further purification. GC (R_t = 11.0 min, 95%); ¹H-NMR (CDCl₃) δ: 9.72 (1H, s, HCO-), 8.06 (2H, d, J = 8.8, ArH), 6.95 (2H, d, J = 8.8, ArH), 4.87 (2H, s, HCOCH₂O-), 3.87 (3H, s, OCH₃).

30 To a well stirred solution of the crude aldehyde (2c, 5.9 g, 28 mmol) and α-hydroxy acetic acid (5.2 g, 68 mmol) in DME (100 ml) at 0 °C was slowly added BF₃·EtO₂ (12.3 g, 11.0 ml, 85 mmol). The solution was left at room temperature

overnight with stirring. The solution was partitioned between EtOAc (150 ml) and (c) NaHCO₃ (150 ml). The organic solution was successively washed with (c) NaHCO₃ (150 ml), brine (2 x 150 ml), dried, filtered and evaporated to afford a syrup. The syrup was treated with DME (15 ml) and a solid precipitated. After stirring for 30 minutes, the solid was filtered to afford 4-methoxybenzoic acid 4-oxo-[1,3]-dioxolan-2-yl methyl ester (**3b**, 5.7 g, 23 mmol, 80%) as a white granular solid; GC (R_t = 14.7 min, 99%); mp: 61 - 63 °C; ¹H-NMR (CDCl₃) δ : 7.97 (2H, d, J = 9.2, ArH), 6.91 (2H, d, J = 9.2, ArH), 5.95 (1H, t, J = 3.0, H-2), 4.57 (1H, dd, J = 3.0 and J = 12.6, -CH₂OCO-), 4.50 (1H, dd, J = 3.0 and J = 12.6, -CH₂OCO-), 4.41 (1H, d, J = 15.0, H-5), 4.31 (1H, d, J = 15.0, H-5), 3.87 (3H, s, OCH₃).

Calculated for C₁₂H₁₂O₆, C, 57.14; H, 4.80. Found: C, 57.40; H, 4.93.

Example 4

Isobutyric acid 4-oxo-[1,3]-dioxolan-2(R)-yl methyl ester (**4a**).

Racemic isobutyric acid 4-oxo-[1,3]-dioxolan-2-yl methyl ester (**3a**) was resolved by chiral chromatography¹³ to afford two fractions corresponding to each one of the enantiomers. Both fractions were colorless syrups. The first fraction corresponded to the R enantiomer (**4a**, isobutyric acid 4-oxo-[1,3]-dioxolan-2(R)-yl methyl ester); HPLC (Method A, R_t = 7.80 min., 100%); $[\alpha]_D^{20}$ = 20.20° (c 0.25, MeOH). The second fraction corresponded to the S enantiomer (isobutyric acid 4-oxo-[1,3]-dioxolan-2(S)-yl methyl ester); HPLC (Method A, R_t = 9.30 min., 100%); $[\alpha]_D^{20}$ = -14.40° (c 0.25, MeOH).

Example 5

4-Methoxybenzoic acid 4-oxo-[1,3]-dioxolan-2(R)-yl methyl ester (**4b**).

Racemic 4-methoxybenzoic acid 4-oxo-[1,3]-dioxolan-2-yl methyl ester (**3b**) was resolved by chiral chromatography⁷ to afford two fractions corresponding to each one of the enantiomers. Both fractions were white solids. The first fraction corresponded to the R enantiomer (**4b**, 4-methoxybenzoic acid 4-oxo-[1,3]-dioxolan-2(R)-yl methyl ester); mp: 76 - 78 °C; HPLC (Method A, R_t = 13.59 min., 99%); $[\alpha]_D^{20}$ = 12.20° (c 0.25, MeOH). The second fraction corresponded to the S enantiomer (4-methoxybenzoic acid 4-oxo-[1,3]-dioxolan-2(S)-yl methyl ester); mp: 76 - 78 °C; HPLC (Method A, R_t = 20.76 min., 99%); $[\alpha]_D^{20}$ = -13.50° (c 0.25, MeOH).

Example 6

Isobutyric acid 4-acetoxy-[1,3]-dioxolan-2(R)-yl methyl ester (5a).

4-Methoxybenzoic acid 4-acetoxy-[1,3]-dioxolan-2(R)-yl methyl ester (5b).

To a well stirred solution of the corresponding lactone (**4a** or **4b**, 15 mmol) in dry THF (45 ml) at -10°C , was slowly added a 1.0 M solution of $\text{LiAlH}(\text{OtBu})_3$ in THF (19.5 ml, 19.5 mmol) over a period of 20 minutes, maintaining the temperature between -15°C and -10°C . The reaction was followed by GC and was stirred for 30 minutes at room temperature (complete disappearance of starting material). The solution was again cooled to -10°C and DMAP (0.92 g, 7.50 mmol) was added in one portion followed by the dropwise addition of Ac_2O (15.3 g, 14.20 ml, 150 mmol). The reaction was further stirred for 1 h at -15°C , and then overnight at room temperature. The solution was cooled to -15°C and quenched with MeOH (40 ml). After stirring for 20 minutes at room temperature, the reaction was concentrated in vacuo to a red syrup that was purified by flash column chromatography (250 g silica, hexane:EtOAc 3:1) to afford:.

Isobutyric acid 4-acetoxy-[1,3]-dioxolan-2(R)-yl methyl ester (**5a**, 61%) as a yellow syrup. $^1\text{H-NMR}$ (CDCl_3) shows almost a 1:1 mixture of anomers, δ : 6.40 (d, $J = 3.8$) and 6.37 (d, $J = 3.8$) corresponding to H-4 α and H-4 β ; 5.41 (t, $J = 3.7$) and 5.32 (t, $J = 3.7$) corresponding to H-2 α and H-2 β .

4-Methoxybenzoic acid 4-acetoxy-[1,3]-dioxolan-2(R)-yl methyl ester (**5b**, 61%) as a yellow syrup. $^1\text{H-NMR}$ (CDCl_3) shows almost a 1:1 mixture of anomers, δ : 6.44 (dd, $J = 2.1$ and $J = 4.2$) and 6.37 (d, $J = 4.0$) corresponding to H-4 α and H-4 β ; 5.54 (t, $J = 3.6$) and 5.45 (t, $J = 4.0$) corresponding to H-2 α and H-2 β .

Example 7

Isobutyric acid 4(R)-(2,6-dichloro-purin-9-yl)-[1,3]-dioxolan-2(R)-yl methyl ester (7a).

4-Methoxybenzoic acid 4(R)-(2,6-dichloro-purin-9-yl)-[1,3]-dioxolan-2(R)-yl methyl ester (7b).

A suspension of 2,6-dichloropurine (1.27 g, 6.73 mmol), ammonium sulfate (38 mg, 0.29 mmol) and 1,1,1,3,3,3-hexamethyldisilazane (8.45 g, 11.04 ml, 52.3 mmol) was heated at reflux for 2.5 hs. The resulting solution was cooled to ambient temperature whereby a thick solid precipitated. The solids were redissolved through the addition of dry dichloromethane (14.9 ml). The solution was then cooled to -10°C , and a solution

of the corresponding acetate (**5a** or **5b**, 8.6 mmol) in dry dichloromethane (10 ml) was slowly added over a period of 20 minutes, maintaining the temperature between -10°C and -5°C . Then, TMSOTf (2.5 g, 2.08 ml, 10.4 mmol) was slowly added over a period of 20 minutes. The reaction was left overnight with stirring at room temperature. The solution was diluted with dichloromethane (120 ml) and quenched with water (150 ml). The organic layer was separated and successively washed with water (150 ml), (c) NaHCO_3 (2 x 150 ml), water (2 x 150 ml), dried, filtered and evaporated to a syrup (crude **6a**) or a yellow solid (crude **6b**).

Crude **6a** was further purified by column chromatography (Hexane:AcOEt 4:1) to afford **6a** as a 1.2:1 β : α mixture of anomers, according to ^1H -NMR. The mixture was slowly crystallized from MeOH (10 ml) to afford isobutyric acid 4(R)-(2,6-dichloro-purin-9-yl)-[1,3]-dioxolan-2(R)-yl methyl ester (**7a**, 24%) as a white solid and characterized as the β anomer; mp: $145 - 147^{\circ}\text{C}$; $[\alpha]_{\text{D}}^{20} = -47.67^{\circ}$ (c 0.25, MeOH); ^1H -NMR (CDCl_3) δ : 8.52 (1H, s, H-8), 6.55 (1H, d, $J = 4.9$, H-4), 5.34 (1H, t, $J = 5.7$, H-2), 4.58-4.30 (4H, m, $-\text{CH}_2\text{COO}-$, H-5 and H-5'), 2.61 (1H, m, $(\text{CH}_3)_2\text{CHCOO}-$), 1.19 (3H, d, $J = 6.8$, $(\text{CH}_3)_2\text{CHCOO}-$), 1.14 (3H, d, $J = 6.8$, $(\text{CH}_3)_2\text{CHCOO}-$).

Calculated for $\text{C}_{13}\text{H}_{14}\text{Cl}_2\text{N}_4\text{O}_4$: C, 43.23; H, 3.91; N, 15.51. Found: C, 43.39; H, 3.91; N, 15.58.

Crude **6b** was first washed with hot hexane to afford 4-methoxy-benzoic acid 4-(2,6-dichloro-purin-9-yl)-[1,3]-dioxolan-2(R)-yl methyl ester (**6b**) as a yellow solid. ^1H -NMR indicated a 2:1 mixture of β : α anomers. This mixture was slowly crystallized from MeOH (100 ml) to afford 4-methoxybenzoic acid 4(R)-(2,6-dichloro-purin-9-yl)-[1,3]-dioxolan-2(R)-yl methyl ester (**7b**, 46%) as a yellow solid and characterized as the β anomer; mp: $154 - 156^{\circ}\text{C}$; HPLC (Method B, $R_t = 20.73$ min.); $[\alpha]_{\text{D}}^{20} = -53.80^{\circ}$ (c 0.25, MeOH); ^1H -NMR (Cl_3CD) δ : 8.41 (1H, s, H-8), 7.91 (2H, d, $J = 8.5$, ArH), 6.92 (2H, d, $J = 8.5$, ArH), 6.53 (1H, d, $J = 4.8$, H-4), 5.44 (1H, bs, H-2), 4.70-4.30 (4H, m, H-5, H-5' and $-\text{CH}_2\text{OCO}-$), 3.85 (3H, s, OCH_3).

Calculated mass for $\text{C}_{17}\text{H}_{15}\text{Cl}_2\text{N}_4\text{O}_5$ ($\text{M} + 1$) $^+$: 425.0419. Found (H.R.F.A.B.M.S.): 425.0420.

Example 8

Isobutyric acid 4(R)-(2,6-diazido-purin-9-yl)-[1,3]-dioxolan-2(R)-yl methyl ester (**8a**).

4-Methoxybenzoic acid 4(R)-(2,6-diazido-purin-9-yl)-[1,3]-dioxolan-2(R)-yl methyl ester (8b).

To a well-stirred solution of the corresponding dichloropurine nucleoside (7a or 7b, 2.9 mmol) in dry DMF (13.5 ml) was added NaN₃ (390 mg, 6.0 mmol). The reaction mixture was left at room temperature with stirring for 4 h. The mixture was filtered through Celite™ to afford a solution of:

Isobutyric acid 4(R)-(2,6-diazido-purin-9-yl)-[1,3]-dioxolan-2(R)-yl methyl ester (8a) or 4-methoxybenzoic acid 4(R)-(2,6-diazido-purin-9-yl)-[1,3]-dioxolan-2(R)-yl methyl ester (8b) in DMF (70 ml) which was used in the next step without any further purification.

Example 9

Isobutyric acid 4(R)-(2,6-diamino-purin-9-yl)-[1,3]-dioxolan-2(R)-yl methyl ester (9a).

4-Methoxybenzoic acid 4(R)-(2,6-diamino-purin-9-yl)-[1,3]-dioxolan-2(R)-yl methyl ester (9b).

The solution from the previous reaction (8a or 8b) was hydrogenated (Parr apparatus, 50 psi) at room temperature, in the presence of 10% Pd/C (200 mg) overnight. The mixture was filtered through Celite and the filter cake washed with additional DMF.

In the case of 8a, the solution was concentrated to dryness and purified by column chromatography (Cl₃CH:MeOH 9:1) to afford a white solid that was crystallized from *iso*-propanol to afford isobutyric acid 4(R)-(2,6-diamino-purin-9-yl)-[1,3]-dioxolan-2(R)-yl methyl ester (9a, 84% after correction for the presence of one molecule of 2-propanol that co-crystallizes) as a white solid; mp: 143 - 145 °C; [α]_D²⁰ = -56.45° (c 0.25, MeOH); ¹H-NMR (CDCl₃) δ : 7.84 (1H, s, H-8), 6.33 (1H, dd, J = 1 and J = 5.1, H-4), 5.29 (3H, t, J = 3.2, H-2 and NH₂), 4.70 (2H, bs, NH₂), 4.50 (1H, dd, J = 1 and J = 9.0, H-5), 4.33 (2H, d, J = 3.2, -CH₂COO-), 4.23 (1H, dd, J = 5.1 and J = 9.0, H-5'), 2.60 (1H, m, (CH₃)₂CHCOO-), 1.18 (3H, d, J = 6.2, (CH₃)₂CHCOO-), 1.14 (3H, d, J = 7.2, (CH₃)₂CHCOO-).

Calculated for C₁₃H₁₈N₆O₄•2-propanol: C, 50.25; H, 6.85; N, 21.98. Found: C, 50.16; H, 6.84; N, 21.92.

In the case of 8b, the solution was concentrated to a final volume of 15 ml in vacuo at 60 °C. The solution was diluted with water (150 ml) and after a few minutes a

solid precipitated. The product was filtered, washed with water, dried overnight at 50 °C under vacuum to afford 4-methoxybenzoic acid 4(R)-(2,6-diamino-purin-9-yl)-[1,3]-dioxolan-2(R)-yl methyl ester (**9b**, 88%) as an amorphous solid; HPLC (Method B, R_t = 15.41 min.); $[\alpha]_D^{20}$ = -95.75° (c 0.25, MeOH); $^1\text{H-NMR}$ (DMSO- d_6) δ : 7.82 (2H, d, J = 8.8, ArH), 7.80 (1H, s, H-8), 7.02 (2H, d, J = 8.8, ArH), 6.77 (2H, bs, NH_2), 6.23 (1H, dd, J = 5.5 and J = 1.6, H-4), 5.85 (2H, bs, NH_2), 5.36 (1H, t, J = 3.3, H-2), 4.65 (1H, dd, J = 9.4 and J = 1.6, H-5), 4.46 (2H, d, J = 3.4, $-\text{CH}_2\text{OCO}-$), 4.26 (1H, dd, J = 9.4 and J = 5.5, H-5'), 3.84 (3H, s, OCH_3).

Calculated mass for $\text{C}_{17}\text{H}_{19}\text{N}_6\text{O}_5$: 387.1417. Found (H.R.F.A.B.M.S.): 387.1417.

Example 10

4(R)-[-(2,6-Diamino-purin-9-yl)-[1,3]-dioxolan-2(R)-yl]-methanol, [10, (-)-DAPD] from 9a.

A solution of isobutyric acid 4(R)-(2,6-diamino-purin-9-yl)-[1,3]-dioxolan-2(R)-yl methyl ester (**9a**, 100 mg, 0.31 mmol) and n-butylamine (0.45 g, 0.62 ml, 6.2 mmol) in MeOH (10 ml) was heated at reflux for 4 hs. The reaction was cooled to ambient temperature and concentrated in vacuo to afford a solid that was triturated with *tert*-butyl methyl ether and filtered to afford a solid that was crystallized from EtOH:water (1:4.5 ml) to afford (-)-DAPD (**10**, 75 mg, 0.29 mmol, 93%) as a white solid; mp: 237 – 239 °C (lit.² mp: 236 - 237 °C); HPLC (Method C, R_t = 8.7 min., an authentic sample of (-)-DAPD showed R_t = 8.7 min. and a sample of (+) DAPD showed R_t = 6.0 min.); DAPD; $^1\text{H-NMR}$ (DMSO- d_6) δ : 7.80 (1H, s, H-8), 6.74 (2H, bs, NH_2), 6.20 (1H, d, J = 5.5, H-4), 5.84 (2H, bs, NH_2), 5.16 (1H, t, J = 6.3, OH), 5.03 (1H, t, J = 2.9, H-2), 4.42 (1H, d, J = 9.7, H-5), 4.18 (1H, dd, J = 9.7 and J = 5.5, H-5'), 3.58 (2H, dd, J = 6.3 and J = 2.9, $-\text{CH}_2\text{OH}$).

Calculated for $\text{C}_9\text{H}_{12}\text{N}_6\text{O}_3$: C, 42.86; H, 4.80; N, 33.32. Found: C, 42.88; H, 4.79; N, 33.32.

Example 11

4(R)-[-(2,6-Diamino-purin-9-yl)-[1,3]-dioxolan-2(R)-yl]-methanol, [10, (-)-DAPD] from 9b.

A suspension of 4-methoxybenzoic acid 4(R)-(2,6-diamino-purin-9-yl)-[1,3]-dioxolan-2(R)-yl methyl ester (**9b**, 1.0 g, 2.6 mmol) and n-butylamine (3.7 g, 5.0 ml, 50

mmol) in MeOH (25 ml) was heated at reflux for 4 hours. The reaction was concentrated in vacuo to afford a yellow solid that was suspended in Cl_3CH at room temperature and filtered, to afford (-) DAPD (10, 590 mg, 2.3 mmol, 90%) as a white solid. Physical properties were identical to the sample of (-)-DAPD obtained above.

5

Example 12

Determination of the Enantioselectivity Constant

The enantioselectivity of a simple hydrolysis reaction may be characterized by the E value (C.S. Chen, C.J. Sih, Y. Fujimoto and G. Girdaukus, *J. Am. Chem. Soc.*, **1982**, *104*, 7294). This numerically represents the ratio of the specificity constants of the catalyst for the two enantiomers. The E value allows direct comparison of catalyst selectivity, even if the reactions have gone to different conversions.

To calculate the E value, the extent of conversion and the optical purity of the remaining starting material and/or the hydrolysis product are needed. If both ee_s and ee_p are known, one can calculate conversion (c) using Equation 1. If only ee_s or ee_p is known, c determined at the termination of the hydrolysis can be used to calculate E (Equation 2).

Equation 1:

$$c = \frac{ee_s}{ee_s + ee_p}$$

Equation 2:

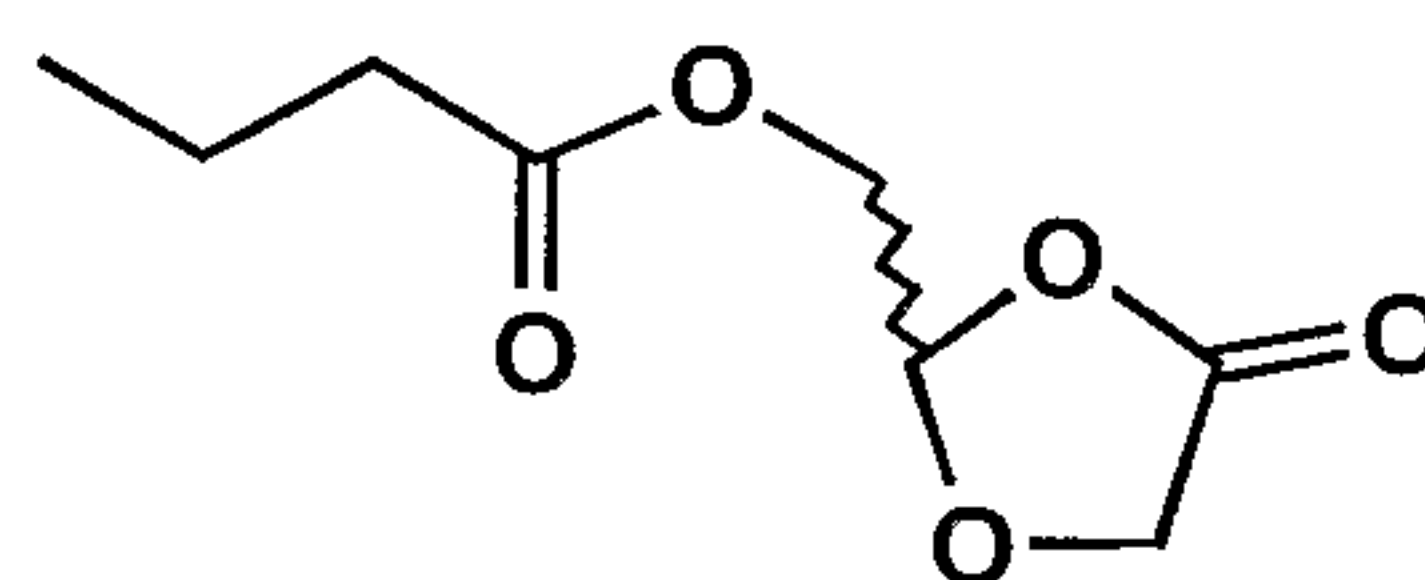
$$E = \frac{\ln[(1-c)(1-ee_s)]}{\ln[(1-c)(1+ee_s)]} = \frac{\ln[1-c(1+ee_p)]}{\ln[1-c(1-ee_p)]}$$

Abbreviations:

ee = Enantiomeric excess for the starting material (s) or product (p)

c = Conversion

E = Enantioselectivity constant

Example 13**Compound 1****5 Analytical Development for Compound 1**

A chiral assay was required for Compound 1. A baseline separation was achieved by chiral GC using the Chirasil DEX CB column. See **Figure 1A**.

GC Conditions:

10	Column:	Chirasil DEX CB
	Dimensions:	25m x 0.25mm
	Temp. Program:	140°C for 10 minutes then to 200°C at 15°C/min
	Carrier gas:	Helium @ 20 psi
	Detection:	FID @ 200°C
15	Retention times:	1 - 7.46 min (<i>R</i>)-enantiomer 2 - 7.70 min (<i>S</i>)-enantiomer

20 Enzymatic screening for Compound 1

Lipases: To each scintillation vial was added 50 μ L Compound 1, MTBE (5 mL) and 50 mM KH_2PO_4 , pH 7 (5 mL), followed by 25 mg enzyme. The vials were shaken in an incubator set at 30°C. Aliquots were removed periodically and assayed by TLC (50% EtOAc/heptane) and chiral GC.

25 *Proteases and esterases:* As above, except the reaction solvent was 5 mL, 50 mM KH_2PO_4 , pH 7 or 8 with no MTBE. Acid protease reactions were performed at pH 3 using 0.1 M lactic acid solution [pepsin, Acid protease A (Newlase A), and Acid protease II (Newlase II)].

30 In some cases 25 mg, 50% wt. of enzyme gave extremely fast reactions and so 100% conversion was reached before meaningful assays could be performed. In these cases the reactions were performed again with the appropriate amount of enzyme to give a reasonable rate of reaction (usually 5% wt.). Enzymes that required smaller loadings were Chirazyme-L2, -E1 and -E2, all the CLECs, Choline esterase, Candida esterase (Altus), PPL, Lipase PS and Lipase AK.

Two sets of conditions were used for the screen of racemic Compound 1, depending upon whether the enzyme used was a lipase or a protease/esterase. For lipase-catalyzed hydrolyses, the reaction solvent was 1:1 MTBE:50 mM potassium phosphate buffer pH 7. For protease and esterase-catalyzed hydrolyses, reactions were carried out in 50 mM potassium phosphate buffer, pH 7 or 8 (or pH 3 for acid proteases) with no immiscible organic co-solvent. Reactions were carried out in a shaker bath at 30°C and followed by TLC, with ee_s values determined by GC analysis (Chirasil Dex-CB column).

The alcohol product from hydrolysis of the butyrate ester appears to be unstable since several new spots on TLC were produced during the reactions. Thus with no chiral assay for the product possible, approximate conversions were estimated from the TLC analysis.

The results from the screens are shown in the tables below. **Table 1** shows the enzymes that selectively left peak 1 in the butyrate chiral assay, while **Table 2** shows the enzymes that selectively left peak 2 in the butyrate chiral assay. It is noteworthy that there appeared to be a very large number of enzymes that give non-stereoselective hydrolysis of Compound 1, which indicated that the substrate is unstable under the reaction conditions. When a control reaction was performed containing no enzyme, hydrolysis still occurred. Further investigation of the stability of the substrate is described herein.

Table 1: Enzymes that selectively leave **peak 1** in the butyrate ee assay

Enzyme	Time	ee _s	Approximate conversion
SAWA immobilised lipase <i>Pseudomonas</i>	6h	92%	>50%
	O/N	-	No S.M
Lipase M	6h	26%	
	O/N	91%	~75%
Lipase PS	1h	17%	
	3.75h	89%	>50%
	5h	-	No S.M.
Lipase F1 Biocon.	6h	13%	
	O/N	88%	>75%
Lipase AK	2.75h	32%	
	5h	64%	
	O/N	64%	>50%
Chirazyme-L9	1h	10%	
	5h	64%	>50%
	O/N	-	No S.M.

Enzyme	Time	ee _s	Approximate conversion
ChiroCLEC-PC	10 min	2%	
	1h	46%	>50%
Lipase MY	6h	11%	
	O/N	33%	>75%
Lipase DS	6h	2%	
	O/N	28%	
Candida Esterase (Altus)	10min	27%	>50%
	1h	-	No S.M.
Protease B	O/N	26%	
Lipase F-DS	6h	22%	
	O/N	-	No S.M.
Newlase F	6h	4%	
	O/N	20%	
Lipase F	6h	20%	
	O/N	-	No S.M.
Alcalase	1h	8%	
	5h	13%	
	O/N	18%	~50%
Lipase R	6h	9%	
	O/N	17%	

Table 2: Enzymes that selectively leave **peak 2** in the butyrate ee assay

Enzyme	Time	ee _s	Approximate conversion
PPL	5h	92%	
	O/N	96%	>50%
Lipase G	6h	3%%	
	O/N	41%	>50%
PLE, tech grade	1h	35%	>50%
Chirazyme-E1	30 min	13%	
	1h	21%	>50%
Chirazyme-L2	1h	8%	
	1.75h	22%	>50%

α -Chymotrypsin gave no reaction with Compound 1.

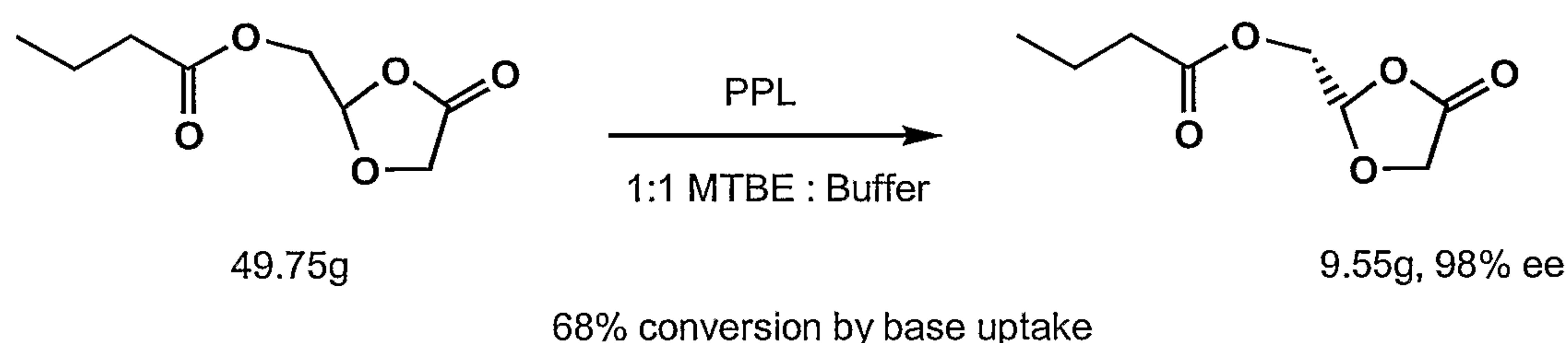
- 5 The following enzymes gave unselective reactions with Compound 1 (<10% ee_s):
- Lipase AY, Lipase A "Amano" 6, Lipase A "Amano" 12, Lipase N conc, Lipase AP6, Sigma CCL, Wheat germ lipase, Chirazyme-L5, ChiroCLEC-CR, Protease M, Peptidase R, Acid Protease A, Acid Protease II, Acid Protease DS, Protease A-DS, Protease N, Protease A2G, Protease NL, Protease DS, Protease S, Protease P "Amano" 6, Prozyme 6,
- 10 Proleather, Bromelain-F, Papain W-40 (Amano), Papain (Sigma), Protease X, Protease

XXXI, Savinase, Esperase, Pepsin, ChiroCLEC-BL, Ketoprofen Esterase and Chirazyme-E2.

Example 14

Scale-up of PPL Resolution of Compound 1

In order to determine which was the (*R*)-isomer, a quantity of resolved Compound 1 was resolved using the most promising enzyme from the screen set forth supra, and taken through the synthetic sequence to an intermediate where the isomer elution order by chiral assay was known. The most promising enzyme from the screen was PPL (porcine pancreatic lipase, Sigma), thus this reaction was scaled up to 50g racemic substrate input (**Scheme 10**).



Scheme 10: Large scale PPL resolution of Compound 1

Minimal optimization of the reaction conditions was performed, apart from increasing the substrate concentration to 50 g/L (a reaction on 5 g substrate was successfully run as a trial). The 50 g reaction was initiated with 5% wt PPL at 30°C and 3 M NaOH added as the reaction proceeded in an attempt to maintain the pH at 7. However the reaction was extremely fast and after 25 minutes was cooled to 15°C and the pH maintained at pH 6.

In particular, to a 2L jacketed vessel at 30°C was added MTBE (500 mL), 50 mM KH₂PO₄, pH 7 (500 mL) and racemic Compound 1 (49.75 g, 0.265 mol, TP-0257/98/D). To the stirred mixture (pH 7) was added PPL (2.5 g, 5 wt%), then 3M NaOH was added to maintain the pH at pH 6. Due to the speed of the reaction, after 25 min the vessel was cooled to 15 °C and the reaction run at this temperature. The ee_s was measured at time intervals by removing aliquots of MTBE, diluting with more MTBE, drying (MgSO₄), and analyzing by chiral GC. After 1h 10 min the ee_s had reached 98% and the reaction was worked-up. The mixture was filtered through celite and the celite washed with MTBE (200 mL). The mixture was allowed to separate and the organic layer retained.

The aqueous was then re-extracted with MTBE (500 mL). The MTBE extractions were combined, dried (MgSO₄) and evaporated *in vacuo* to give 22 g of a crude yellow oil. The crude product was purified by silica flash column chromatography (20% EtOAc:80% heptane) to give 9.55 g (*S*)-Compound 1 as a very pale yellow oil, 98% ee, 95% pure by GC-MS. A further 1.5 g of less pure material was also obtained. Combined yield was 22%.

GC-MS (CPSil8 CB/MS, 30m x 0.25mm, 60°C for 5 min, 10°C/min to 300°C) 13.98 min, M = 173 (M⁺ - CH₃), 95% pure by peak area.

¹H NMR (200 MHz, CDCl₃) δ 5.85 (t, 1H, CH-O), 4.35 (m, 4H, 2 x CH₂O), 2.35 (t, 2H, CH₂), 1.7 (sextet, 2H, CH₂), 0.95 (t, 3H, CH₃).

The ee_s was measured at time intervals (peak 2 in ee assay).

Table 3: PPL resolution of Compound 1

Time	Conversion by base uptake	Actual pH	ee _s
5 min	28%	6.5	-
10 min	57%	6.5	53%
55 min	63%	6.0	89%
1h 10 min	68%	6.0	98%

The reaction was worked up after 1h 10 min and the isolated resolved Compound 1 purified by silica column chromatography. This yielded 9.55g, 95% pure, 98% ee ester, along with 1.5g of less pure ester (22% overall yield). The impurity in the main batch of ester (9.55g) was butyric acid. This material was taken through the synthetic sequence to an intermediate where the isomer elution order by chiral assay was known, and was determined to be (*S*)-Compound 1. To obtain the (*R*)-isomer, an enzyme that selectively left peak 1 in the Compound 1 chiral assay was needed.

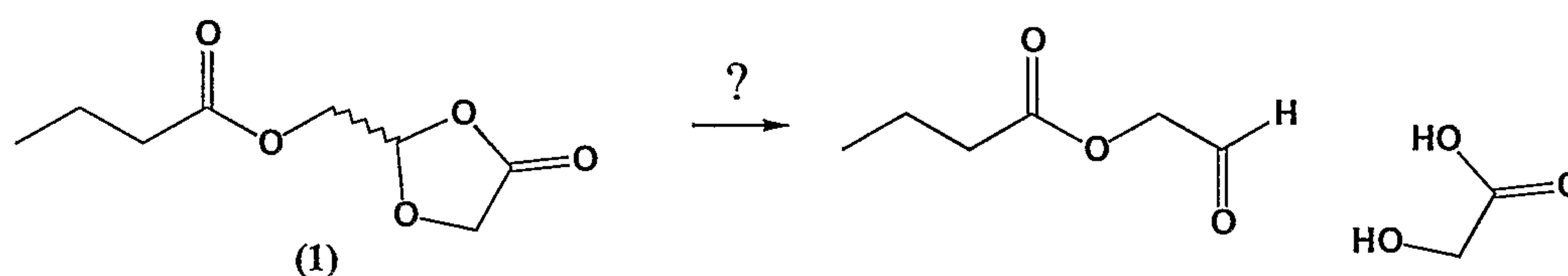
Example 15

Optimization of Commercial Enzyme Resolutions giving (*R*)-Compound 1

As can be seen from **Table 1**, the initial screen had shown SAWA immobilized lipase, Lipase M, Lipase PS and F1 Biocon. lipase to be potential candidates to efficiently give (*R*)-Compound 1. The resolutions were scaled up to determine their selectivities (F1 Biocon. lipase is no longer commercially available).

The enzyme was added to 2-5 g of ester in 15-20 mL each of solvent and buffer (0.2 N KH_2PO_4). Sodium hydroxide solution was added to control the pH. The work-up procedure was to filter (Celite), extract (MTBE), wash with saturated aqueous sodium bicarbonate, dry (MgSO_4) and concentrate. Analysis was by chiral GC (Chirasil Dex CB). The variables studied were enzyme, solvent, pH and temperature (see **Table 4**).

Initial trials with Lipase PS, Lipase M and SAWA immobilized *Pseudomonas* lipase in pH 7 phosphate buffer gave the remaining substrate with enantiomeric excesses of 90%, but the recovery of (*R*)-Compound 1 was low (<5%). The stability of the butyrate was investigated at pH 7 and pH 6 under standard reaction conditions but without enzyme present (entries 20 and 21). At pH 7 significant base was consumed to maintain pH and after work-up and isolation only 50% of (**1**) was recovered after 3.5 h. Stability is significantly better at pH 6. Possibly, the mode of breakdown is opening of the lactone-acetal to give an aldehyde and glycolic acid (**Scheme 11**).



Scheme 11: Possible decomposition pathway of Compound 1

Entry	Enzyme	Co-solvent	pH	Temp/ °C	mmol Base:SM	S.M. ee/%	Yield S.M./%	E ⁽ⁱ⁾	Comment
1	Lipase PS	MTBE	6-7	20	20:28	>95	0	—	10% enzyme, negligible S.M., 17 min
2	Lipase PS	MTBE	7	20	33:27	>95	0	—	Negligible S.M., 2 h
3	Lipase PS	MTBE	6	20	13:12	90	6.2	1.9	
4	Lipase PS	MTBE	7	10	28:26	>95	0	—	Negligible S.M., 2.5 h
5	Lipase PS	MTBE	5-7	10	10:13	82	0	—	Negligible S.M., 4.5 h
6	Lipase PS	heptane	6-7	0	8:17	88	NI	—	1M buffer
7	Lipase PS	heptane	6	0	10:24	88	14	3.1	
8	Lipase PS	toluene	6	0	10:19	54	45	4.3	
9	Lipase PS	MEK	6	0	2:20	19	70	3.1	
10	Lipase PS	THF	6	0	4:19	36	46	2.6	
11	Lipase PS	DCM	6	0	4:19	12	68	1.9	
12	Lipase PS	dioxane	6	0	6:18	50	22	1.9	
13	SAWA	MTBE	6	20	8.6:15	81	21	3.3	
14	SAWA	MTBE-EtOH	—	20	—	0	NI	—	No water, racemic
15	SAWA	toluene	6	0	13:19	37	41	2.4	
16	Lipase M	MTBE	6	20	20:15	—	0	—	No S.M. 20 h
17	Lipase M	MTBE	7	0-5	28:27	83	NI	—	1M buffer
18	Lipase M	toluene	6	0	14:19	39	42	2.5	
19	CLEC-PC	MTBE	6	20	—	—	0	—	No S.M. remaining
20	none	MTBE	7	20	9:9	—	48	—	3.5 h, S.M. decomposes
21	none	MTBE	6	20	no base	—	NI	—	2 h, no base consumed

(i) Enantioselectivity constant - see Example 12

Table 4: Optimization of resolution of Compound 1

Two other controls showed that starting ester of 98% ee is not racemized by the reaction conditions at pH 6 or pH 7. A further test reaction showed that after 24 hours at pH 6, 100 g/L substrate in 2:1 toluene:1 M buffer and 0 °C, greater than 90% yield of starting material was recovered with no loss of enantiomeric excess. Thus racemization and substrate stability under these reaction conditions is not a problem.

With these results in mind, all further enzyme reactions were performed at pH 6. The best results from the study are shown in entries 7, 8, 9 and 13. The most promising enzyme resolution was with Lipase PS in toluene/pH 6 buffer giving an E value of 4.3. This would give a yield of 17% at 95% ee. Resolution with Lipase PS was investigated further in the Examples herein. Variables studied for optimization were concentration, solvent:buffer volume ratio, enzyme loading, additives and temperature.

The optimal conditions were determined as 0°C, 100 g/L substrate in 1:1 toluene:potassium phosphate buffer (pH 6) which gives 95% ee, 22% yield of (*R*)-Compound 1. At 200 g/L substrate concentration, 95% ee (*R*)-Compound 1 was obtained in 17% yield.

Example 16

Microbial Enzyme Screen

From a screen of around 200 strains (using the new 96-well plate method), 7 positives were identified which left the (*R*)-isomer of Compound 1 (peak 1). These were classified as positive in so far as they hydrolyzed the ester and gave >10% ee of remaining substrate, thus having potential for generating high ee ester.

The screen was performed in 0.1M Na₂HPO₄/NaH₂PO₄, pH 6.0 with 20 g/l ester at 25°C for approximately 16 h. The results are depicted in **Figures 5A and 5B**.

The seven positive strains (peak 1) from the primary screen were grown up in TSB (tryptone soya broth) flasks and the cell pastes harvested. These were re-suspended at 10% w/v in 0.1 M Na₂HPO₄/NaH₂PO₄, pH 6.0 + 20 g/L ester and shaken in scintillation vials (at 1.0 mL per vial) for up to 92 hours at 25°C. The results are shown in **Table 5**.

Table 5: Further investigation of positive microbial strains

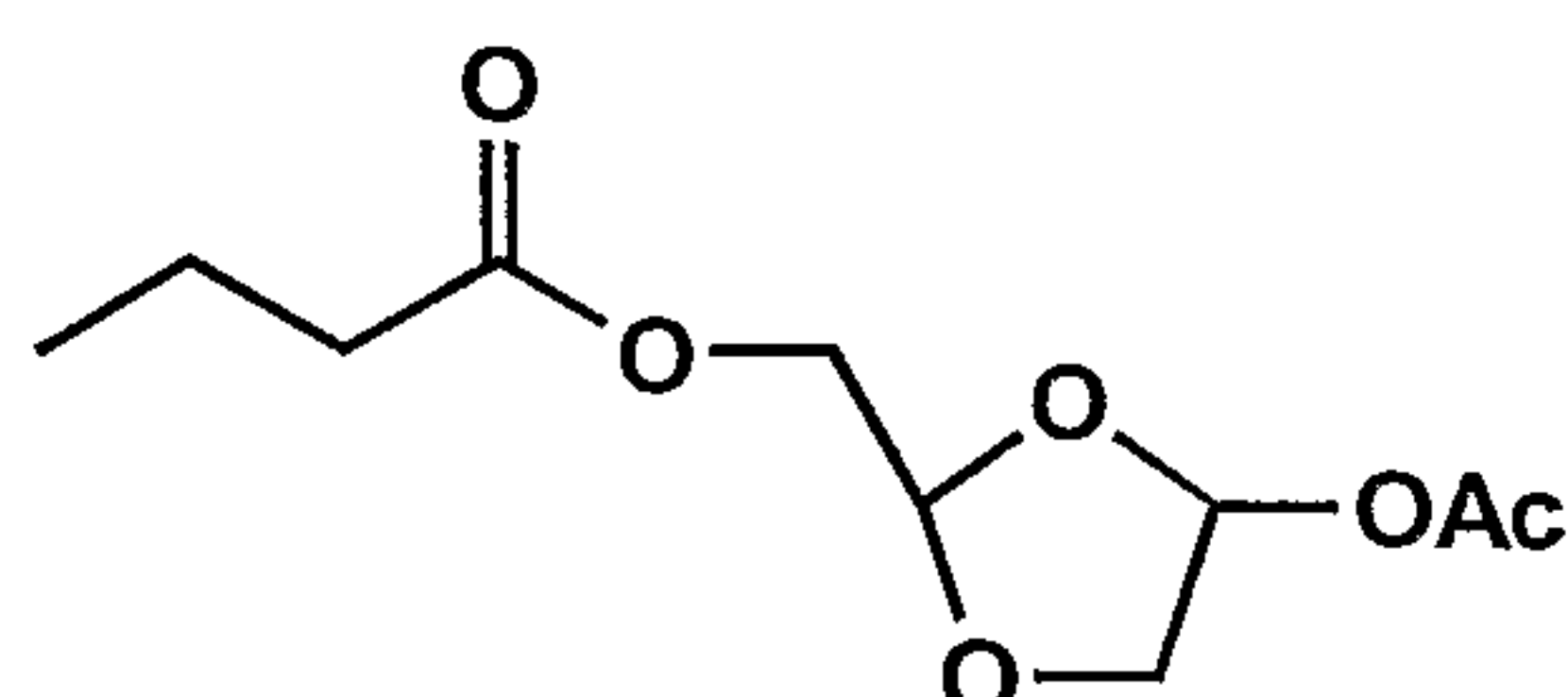
No.	CMC No.	Name	Time/h	Peak 1	Peak 2	ee _s
1	3127	<i>Serratia liquifaciens</i>	17.75	13291	10863	10.1%
			24	71866	67553	3.1%
			47			

No.	CMC No.	Name	Time/h	Peak 1	Peak 2	ee _s
			92			
2	3322	<i>P.alcaligenes</i>	17.75			
			24			
			47			
3	3373	<i>Acinetobacter sp</i>	17.75	67157	64051	2.4%
			24	9827	8169	9.2%
			47			
			92			
4	3418	<i>Unidentified</i>	17.75	14606	9430	21.5%
			24	11627	8948	13.0%
			47			
			92			
5	3419	<i>Acinetobacter</i>	17.75	69727	30271	39.5%
			24	55222	22993	41.2%
			47	18804	2077	80.1%
			92			
6	3606	<i>Acinetobacter junii</i>	17.75	114861	14557	77.5%
			24	93782	23728	59.6%
			47	45250	6326	75.5%
			92	7789	880	79.7%
7	3635	<i>Unidentified</i>	17.75	26812	23798	6.0%
			24	6685	4022	24.9%
			47	2134	1139	30.4%
			92			
8	Blank	<i>No Enzyme</i>	17.75	254977	256011	0.2%
			24	215295	216508	0.3%
			47	86300	85001	0.8%
			92	20053	19960	0.2%

The results from the control show that the ester is not stable over 24 hours at 25°C. There is a 97% decrease in peak area size between the peak areas at 17 and 92 hours. This clearly explains why all the samples peak areas show a dramatic drop. Of the strains re-screened, CMC 3606 (*Acinetobacter junii*) and CMC 3419 (*Acinetobacter*) showed good enantioselectivity (80% ee for peak one). These two strains were re-grown [CMC 3606 in flasks (as it was originally an unidentified bacteria) and CMC 3419 in

fermentation vessels] ready for further evaluation. For CMC 3322 (*P. alcaligenes*) there was no detectable ester after 17 hours. This may be due to a shift in pH (with the cells lysing) or from a high esterase activity.

Example 17



Compound 3

Analytical Development for Compound 3

A chiral GC assay was developed to separate the four possible diastereoisomers of Compound 3. Details of the assay conditions are as set forth below.

GC Conditions:

Column: Chiraldex GTA
Dimensions: 30m x 0.25mm
Temp. Program: 100°C for 20 minutes then to 160°C at 2°C/min
Carrier gas: Helium @ 14 psi
Detection: FID @ 200°C

Retention times: 1 - 41.74 minutes 2 - 41.96 minutes
3 - 43.07 minutes 4 - 43.82 minutes

Achiral GC-MS of the starting ester gave two peaks at 15.98 and 16.27 min (diastereoisomers) in a ratio of 1:1.4 respectively. See **Figure 1B**. Comparison of the four chiral GC ee/de assay peaks and GC-MS peaks indicated that peaks 1 and 2 were enantiomers and peaks 3 and 4 were enantiomers in the ee/de assay.

Enzyme Screen, Compound 3

Lipases: To each scintillation vial was added 50 µL Compound 3 (TP-0259/98/A), MTBE (5 mL) and 50 mM KH₂PO₄, pH 7 (5 mL), followed by 20 mg enzyme. The vials were shaken in an incubator set at 30°C, with aliquots removed periodically and assayed by TLC (50% EtOAc/heptane) and chiral GC.

Proteases and esterases: As above, except the reaction solvent was 5 mL, 50 mM KH₂PO₄, pH 7 or 8 with no MTBE. Acid protease reactions were performed at pH 3 using 0.1 M lactic acid solution [pepsin, Acid protease A (Newlase A), and Acid protease II (Newlase II)].

5 For some enzymes that were known to be very active for these substrates, the reactions were performed with an appropriate amount of enzyme to give a reasonable rate of reaction, usually 10-15 wt%. Enzymes that required these reduced loadings were Chirazyme-L2, -E1 and -E2, all the CLECs, Choline esterase and Candida esterase (Altus).

10 Racemic Compound 3 was screened for enzymatic hydrolysis with commercially available lipases, proteases and esterases. All lipase reactions were carried out in the presence of 50 µL Compound 3, 5 mL MTBE, 5 mL pH 7 phosphate buffer and 50% wt. enzyme (except Chirazyme-E1, Chirazyme-E2, Chirazyme-L2, Candida Esterase, Choline esterase and CLECs, 10% wt.). Esterase and protease reactions were performed
15 without MTBE present. The reactions were followed by TLC and chiral GC.

The stereochemistry at the acetate centre is not as important since it is set later in the synthesis of the bulk active DAPD. Thus it is desirable to resolve with respect to the butyrate centre. However, initially it was unknown which peak corresponded to which isomer. Control reactions, containing no enzyme, showed that the substrate was stable
20 under the reaction conditions. The results from the screen for resolution of Compound 3 are shown in **Tables 6 - 11**.

Table 6: Enzyme screen of Compound 3 - enzymes selective for peaks 1/3

Enzyme	Time	Peak 1	Peak 2	Peak 3	Peak 4	ee	Peaks	Conversion by TLC
Peptidase R	3.5h	32.0	27.5	40.0	0.5	8% 98%	1 3	~20%
	O/N	68.5	0.0	31.5	0.0	99% 99%	1 3	~50%
Protease M	1.25h	31.0	20.0	37.0	12.0	22% 51%	1 3	<20%
	6.5h	58.5	2.0	39.5	0.0	93% 99%	1 3	~50%
Acid Protease A (Newlase A)	4h	36.5	3	60.5	0	85% 99%	1 3	~50%

Enzyme	Time	Peak 1	Peak 2	Peak 3	Peak 4	ee	Peaks	Conversion by TLC
Protease B	7h	17.5	12.5	62.5	7.5	17% 79%	1 3	~50%
	24h	31	0	69	0	99% 99%	1 3	>80%
Chirazyme L2	45 min	28.0	21.0	35.5	16.0	14% 38%	1 3	~30%
	1.75	39.0	15.5	41.0	4.5	43% 80%	1 3	~50%
Protease A	3.5h	40.5	20.5	28.5	10.5	33% 46%	1 3	40-50%
	6.5h	51.5	18.0	26.5	4.0	48% 74%	1 3	~60%
Protease A 2G	3.5h	40.0	23.5	22.5	16.5	26% 15%	1 3	~50%
	O/N	70.5	5.0	15.0	10.0	87% 20%	1 3	>75%
Ketoprofen Esterase	3.5h	8.5	1.5	56.0	40.0	70% 17%	1 3	~60%
	6.5h	9.0	2.0	56.0	40.0	64% 17%	1 3	~60%
Protease N	1.25h	22.5	18.0	31.0	28.5	11% 4%	1 3	10-20%.
	6.5h	24.0	17.5	33.0	25.5	16% 13%	1 3	10-20%
PPL	3.5h	34.0	25.5	20.0	20.0	14% Rac	1 3/4	10-20%
	6.5h	43.5	26.0	16.0	14.0	25% 7%	1 3	~60%

These results show that Peptidase R, Protease M and Acid Protease A (Newlase A) have excellent selectivity for peaks 1 and 3; whilst Protease B, Chirazyme-L2, and Protease A have reasonable selectivity for peaks 1 and 3.

5

Table 7: Enzyme screen of Compound 3 - enzymes selective for peaks 1/4

Enzyme	Time	Peak 1	Peak 2	Peak 3	Peak 4	ee	Peaks	Conversion by TLC
PLE	1.25h	35.0	19.0	11.5	35.0	30% 50%	1 4	~50%
	6.5h	70.0	10.0	0.0	20.0	75% 99%	1 4	>80%
Chirazyme -E1	15min	33.0	19.0	17.0	31.0	27% 29%	1 4	~50%

	45min	52.0	12.0	8.0	28.0	63% 56%	1 4	~80%
Chirazyme -E2	1.25h	46.5	20.0	11.0	22.0	40% 33%	1 4	~60%
	3.5h	54.5	19.5	8.5	17.5	47% 26%	1 4	>80%
PLAP	24h	32.5	24.5	9.5	33.5	14% 56%	1 4	>50%

Thus PLE and Chirazyme-E1 are reasonably selective for peaks 1 and 4.

Table 8: Enzyme screen of Compound 3 - enzymes selective for peak 1

Enzyme	Time	Peak 1	Peak 2	Peak 3	Peak 4	ee	Peaks	Conversion by TLC
Papain (Sigma)	7h	64.5	19	16.5	0	54% 100%	1 3	~50%
	24h	93.5	5	1.5	0	90%	1	>50%
Protease P Amano 6	1h	44	23.5	14	18.5	30% 14%	1 4	~50%
	4h	84.5	15.5	0	0	69%	1	>80%
Prozyme 6	1h	44	26	12	18	26% 20%	1 4	~50%
	4h	78	22	0	0	56%	1	>80%

5

These results show that Papain displays very good selectivity for peak 1.

Table 9: Enzyme screen of Compound 3 - enzymes selective for peaks 2/3

Enzyme	Time	Peak 1	Peak 2	Peak 3	Peak 4	ee	Peaks	Conversion by TLC
Lipase AK	3.5h	22.0	52.5	24.0	2.0	41% 85%	2 3	~20%
	6.5h	14.0	73.5	12.5	0.0	68% 99%	2 3	~60%
Lipase MY	1.25h	19.0	31.5	36.0	13.5	25% 45%	2 3	30-40%
	6.5h	8.5	76.0	15.5	0.0	80% 99%	2 3	>80%
Lipase AY	3.5h	20.5	39.5	32.5	7.5	32% 63%	2 3	10-20%
	6.5h	17.5	56.0	25.0	1.5	52% 89%	2 3	~50%
CCL	3.5h	20.5	27.5	32.5	19.0	15% 26%	2 3	10-20%

Enzyme	Time	Peak 1	Peak 2	Peak 3	Peak 4	ee	Peaks	Conversion by TLC
	6.5h	20.5	33.5	33.0	13.0	24% 43%	2 3	30-40%
Acid Protease A	6.5h	21.5	24.0	29.0	25.5	5% 6%	2 3	~30%
	O/N	22.5	36.0	25.0	16.0	23% 22%	2 3	~50%
Protease NL	7h	22	24	33	21	Rac 22%	2/1 3	<50%
	24h	23.5	29.5	35.5	11.5	11% 51%	2 3	~50%

These results show that Lipase AK, Lipase MY and Lipase AY are reasonably selective for peaks 2 and 3.

5

Table 10: Enzyme screen of Compound 3 - enzymes selective for peaks 2/4

Enzyme	Time	Peak 1	Peak 2	Peak 3	Peak 4	ee	Peaks	Conversion by TLC
Protease XXXI	4h	25.5	29.5	19	26	7% 16%	2 4	~50%
	24h	31	46	7.5	15.5	19% 34%	2 4	>50%
Proleather	4h	25.5	30.5	18.5	25.5	9% 16%	2 4	~50%

Thus no enzyme that is selective for peaks 2 and 4 gives particularly high enantiomeric excesses.

Table 11: Enzyme screen of Compound 3 - enzymes selective for peak 2

Enzyme	Time	Peak 1	Peak 2	Peak 3	Peak 4	ee	Peaks	Conversion by TLC
Lipase PS	3.5h	18.5	65.5	3.5	12.5	56% 56%	2 4	~40%
	6.5h	7.5	89.0	0.0	3.5	84%	2	>80%
Chirazyme L5	1h	25	33	27	15	14% 29%	2 3	~50%
	4h	20	80	0	0	60%	2	>80%
Chirazyme L9	3.5h	34.0	40.0	5.5	21.0	8% 58%	2 4	~50%
	6.5h	38.0	57.0	0.0	5.5	20%	2	>80%

10

These results show that Lipase PS is reasonably selective for peak 2.

No reaction was observed with α -Chymotrypsin, Lipase GC, Lipase PGE, Lipase N conc, Lipase G, Lipase R, Lipase M, Lipase A "Amano" 12, Lipase AU miles 1988, Lipase F1 Biocon., Wheat Germ Lipase, Papain W-40, Protease S, Protease X and Pepsin.

5 Enzymes that gave unselective hydrolysis were Acid Protease II (Newlase II), Bromelain F, Alcalase, Savinase, Esperase, ChiroCLEC-BL, Trypsin, ChiroCLEC-PC, SAWA immobilised *Pseudomonas* lipase and Lipase F. Candida Esterase from Altus gave a reaction that was too fast to collect any enantiomeric excess data.

10 After this screen had been completed, a sample of resolved Compound 3 was prepared by reduction (*S*)-Compound 1 from a PPL resolution. This was run on the ee/de assay giving peaks 1 and 4. Since the PPL resolution gives (*S*)-Compound 1, peaks 2 and 3 were needed for the resolution of Compound 3. The results from the screen showed that the majority of enzymes resolved Compound 3 at the acetate centre, not the required butyrate centre. However Lipase AK, Lipase MY, Lipase AY and Lipase PS
15 give the required peaks with reasonable selectivity.

Resolutions of Compound 3 with Lipase PS and Lipase MY were investigated further (Lipase AK has been discontinued by Amano). Scale up and isolation of the product was need to determine if the resolutions were efficient (see **Table 12**). Lipase PS in toluene gave a 39% yield of recovered starting material with an effective ee of
20 58% and effective E of 3.7 (this gives a measure of the amount of correct (*R*)-butyrate that is present in the mixture). These selectivities were no better than what was obtained for Compound 1 with Lipase PS, E=5.3. Further, since in one embodiment, the resolution step is earlier in the reaction sequence, the following examples concentrate on optimizing the resolution of Compound 1.

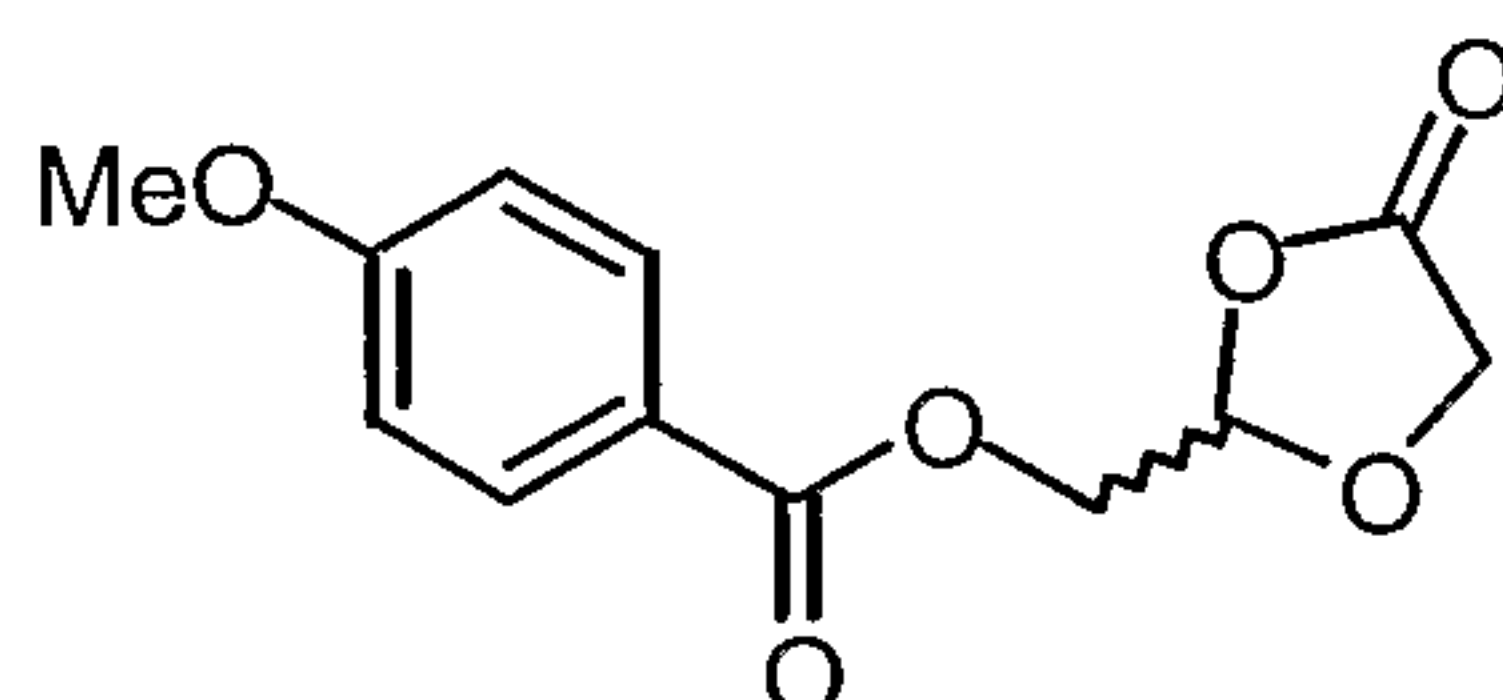
25 **Table 12:** Further investigations of resolutions of Compound 3

Scale/ g	Conc. g/l Solvent	Enzyme wt%	Peak 1	Pea k 2	Pea k 3	Pea k 4	Yield S.M.	Effective ee S.M.	Effective E
1.4	50 MTBE	PS, 3	23	45	13	19	52%	16%	1.6
1.3	50 toluene	PS, 6	15	65	14	6	39%	58%	3.7
1.3	50 MTBE	MY, 5	23	45	13	19	52%	16%	1.6

Scale/ g	Conc. g/l Solvent	Enzyme wt%	Peak 1	Pea k 2	Pea k 3	Pea k 4	Yield S.M.	Effective ee S.M.	Effective E
1.2	50 toluene	MY, 4	22	28	35	15	42%	26%	1.8

Conditions: 20 °C, 1:1 solvent:pH 7 buffer

Example 18



Compound 11

5 Analytical Development for Compound 11

A chiral assay was required for Compound 11. A baseline separation was achieved by chiral HPLC using the Chiralpak AS column. See **Figure 1C**.

HPLC Conditions:

10 **Column:** Chiralpak AS
Dimensions: 250mm x 4.6mm
Mobile Phase: 1:1 IPA:EtOH
Flow Rate: 0.6 ml/min
Detection: UV 254nm
Temperature: Ambient

15 **Retention times:** Peak 1 – 11.96 minutes Peak 2 – 13.17 minutes

As this compound was undergoing a microbial screen (>400 samples) as well as a commercial enzyme screen a sub 5 minute run time was required. The previous assay was taken to SFC where this was achieved. See **Figure 1D**.

SFC Conditions:

25 **Column:** Chiralpak AS
Dimensions: 250mm x 4.6mm
Mobile Phase: 95% CO₂:EtOH
Flow Rate: 3.0 ml/min, 3000 psi
Detection: UV 254nm
Temperature: 35 °C

30 **Retention times:** Peak 1 – 2.88 minutes Peak 2 – 3.95 minutes

Enzyme Screen for Compound 11

Lipases: In a scintillation vial 40 μ L Compound 11, MTBE (5 mL), 50 mM KH_2PO_4 , pH 6 (5 mL), and ~10 mg enzyme were shaken in an incubator at 30C. Aliquots were removed periodically and assayed by TLC (50% EtOAc/heptane) and chiral HPLC.

5 Proteases and esterases: as above, but without MTBE.

Two sets of conditions were used for the screen of Compound 11, the racemic methoxybenzoate ester, depending upon whether the enzyme used was a lipase or a protease/esterase. For lipase-catalysed hydrolyses, the reaction solvent was 1:1 methyl *tert*-butyl ether (MTBE):50 mM potassium phosphate buffer at pH 6. For protease and
10 esterase-catalysed hydrolyses, reactions were carried out in the phosphate buffer with no immiscible organic co-solvent. Reactions were carried out in scintillation vials in a shaker at 30°C and followed by thin layer chromatography (TLC). The enantiomeric excess of the residual substrate (ee_s values) was determined by HPLC analysis (Chiralpak AS). The alcohol product from hydrolysis of these esters was known to be
15 unstable from the Example above. Thus with no chiral assay for the product possible, conversions could not be calculated, so it was noted whether any substrate was still present by TLC analysis.

The results from the screens are shown in the tables below. **Table 13** shows the results for the enzymes that selectively left the enantiomer corresponding to peak 1 in the
20 chiral HPLC assay, and Error! Reference source not found. the results for the enzyme selective for peak 2.

Table 13: Enzymes that selectively leave peak 1 in the HPLC assay

<i>Enzyme</i>	<i>Time</i>	<i>ee_s/%</i>	<i>Comments</i>
Lipase M	72 hrs	24	Peak 1
	2 weeks	29	Substrate present
Lipase PS	72 hrs	7	Peak 2
	2 weeks	22	Substrate present
Chirazyme-L9	72 hrs	19	Peak 1
	2 weeks	56	Substrate present
Lipase MY	72 hrs	7	Peak 1
	2 weeks	21	Substrate present
Lipase DS	72 hrs	25	Peak 1
	2 weeks	66	Substrate present
Lipase F-DS	72 hrs	52	Peak 1
	2 weeks	80	Substrate present
Lipase F	72 hrs	37	Peak 1
	2 weeks	68	Substrate present
Chirazyme-L2	18hrs	100	Peak 1

<i>Enzyme</i>	<i>Time</i>	<i>ee_s /%</i>	<i>Comments</i>
Lipase AY	2 weeks	100	Substrate present
	72 hrs	31	Peak 1
Lipase A "Amano" 12	2 weeks	69	Substrate present
	72 hrs	16	Peak 1
Lipase N cone	2 weeks	45	Substrate present
	72 hrs	33	Peak 1
Lipase AP6	2 weeks	46	Substrate present
	72 hrs	17	Peak 1
Sigma CCL	2 weeks	43	Substrate present
	72 hrs	20	Peak 1
Acid Protease A	2 weeks	62	Substrate present
	48 hrs	24	Peak 1
Acid Protease DS	2 weeks	76	Substrate present
	48 hrs	66	Peak 1
	2 weeks	92	Substrate present

Table 14: Enzyme that selectively leaves peak 2 in the HPLC assay

<i>Enzyme</i>	<i>Time</i>	<i>ee_s /%</i>	<i>Comments</i>
Lipase PS	72 hrs	7	Peak 2
	2 weeks	22	Substrate present

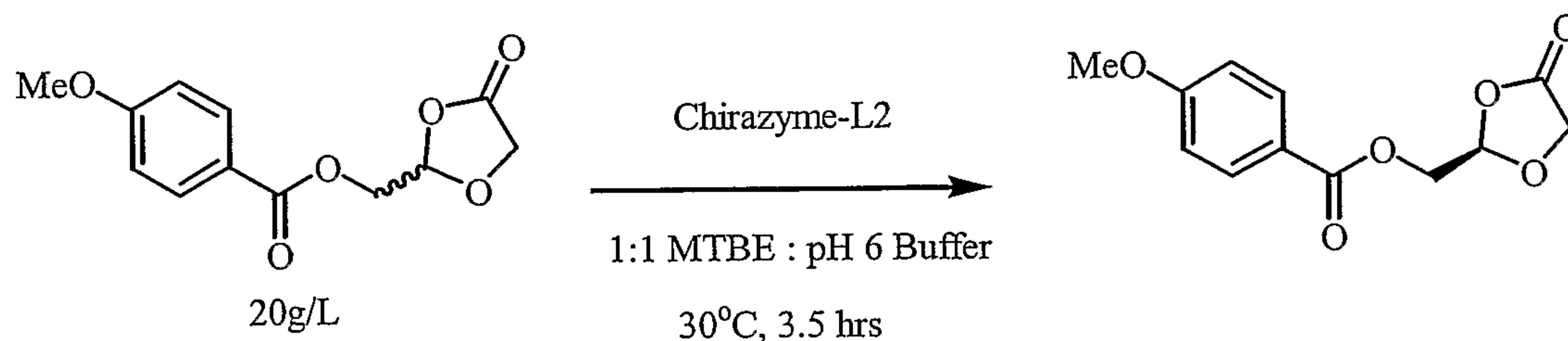
5 The following enzymes were unselective (<20% ee_s either peak): SAWA
 immobilised lipase; Chirocllec-PC; Protease B; Newlase F; Alcalase; Lipase R; PPL;
 Lipase G; PLE; Chirazyme-E1; Chirazyme-L5; ChiroCLEC-CR; Protease M; Peptidase
 R; Acid Protease A-DS; Protease N; Protease A2G; Protease NL; Protease DS; Protease
 S; Protease P "Amano" 6; Prozyme 6; Proleather; Bromelain-F; Papain W-40 (Amano);
 10 Papain (Sigma); Protease X; Protease XXXI; Savinase; Esperase; Pepsin; ChiroCLEC-
 BL; Choline Esterase; Chirazyme-E2; and α -Chymotrypsin.

Example 19

Scale-up of Chirazyme-L2 Resolution of Compound 11

15 During the initial studies of the resolution of Compound 11, it was not known
 how the enantiomers correlate to the peaks in the chiral HPLC assay. In order to
 determine the absolute configuration, a sample was taken through the entire synthetic
 sequence to a DAPD intermediate where the configuration could be determined. One of
 the most promising enzymes from the screen was Chirazyme-L2; thus this reaction was
 scaled up in order to prepare a sample (**Scheme 12**). The substrate concentration was
 increased to 20 g/L and the enzyme loading reduced, but otherwise optimization of the

process was minimal. With a 1 g input of racemate, 5 wt.% Chirazyme-L2 at 30°C with the pH maintained between 5 and 6, 0.321g (32%) of ester was obtained in >98% ee and good purity.



Scheme 12: Scale-up of Compound 11 resolution

The reaction was scaled-up further to 10 g; after 3.5 h, 3.9 g of Compound 11 was obtained in 39% yield, >99% ee as a white solid. This was determined to be the undesired (*S*)-enantiomer. Consequently the desired (*R*)-enantiomer must correspond to peak 2 in the chiral HPLC assay.

Therefore, a 500 mL jacketed vessel at 30°C was charged with MTBE (250 mL), racemic Compound 11 (10.00 g, 39.7 mmol, TP-43/100) and 50 mM KH₂PO₄, pH 6 (250 mL). Chirazyme-L2 (500 mg, 5 wt.%) was added to the mixture with stirring. The pH was maintained between pH 5 and 6 by addition of 2N NaOH. After 3.5 hours, the base uptake had stopped, and the mixture filtered through Celite[®] and separated. The aqueous was extracted with MTBE (2 × 200 mL), the organic layers were combined, washed with 2N NaOH (200 mL), sodium sulfite (200 mL), 2N HCl (200 mL) and dried (MgSO₄). Concentration *in vacuo* gave Compound 11 as a white solid. Yield 3.89 g, 39%; ee >99%; ¹H NMR (400MHz, CDCl₃) consistent with structure.

Example 20

Scale-up of Lipase PS Resolution of Compound 11

From the initial screen, only Lipase PS was a candidate to give the desired (*R*)-enantiomer of Compound 11 (see Error! Reference source not found.). This resolution was scaled up to 2 g in order to determine its selectivity. At approximately 50% conversion the enantiomeric excess of residual ester was only 8%. The enzyme is clearly not sufficiently selective for a viable process.

A 100 mL jacketed vessel at 30°C was charged with MTBE (25 mL), racemic Compound 11 (2.00 g, 7.94 mmol, TP-43/100) and 50 mM KH₂PO₄, pH 6 (25 mL).

Lipase-PS (100 mg, 5 wt.%) was added to the mixture with stirring. The pH was maintained between pH 5 and 6 by addition of 2N NaOH. At approximately 50% conversion (by base uptake), an aliquot was taken and analysed to be 8% ee. After a further 24 hours no substrate was detected.

5

Example 21

Microbial Enzyme Screen

General procedure for preparing 96 well culture plates.

2.2 mL 96 deepwell plates were used. 1.0 mL sterile TSB (Tryptone soya broth, Oxoid CM129) for bacteria, or YM (Yeast mould broth, Oxoid CM920B) for yeasts, was added per vial and inoculated with a stock of culture. The plates were shaken at 25°C for ≥ 48 hours. The cell pellets were harvested by centrifugation (1000g for 10 minutes at 4°C) and the supernatant removed. The cell pellets were stored at -20°C until required for screening.

15

Screening of 96 deepwell culture plates on Compound 11.

A 200 g/L stock solution of Compound 11 was prepared in acetone. 450 μ L 0.1M Tris-HCl + 0.1% Tween 80, pH 7.0 and 50 μ L Compound 1 stock solution were added per well of the 96 deepwell culture plate. These plates were shaken at 25°C for ≥ 72 hours. Samples were extracted into MTBE and assayed by chiral HPLC.

20

The screen was carried out against a range of bacteria and yeasts. These were grown up in 96 deep-well plates and their cell pellets harvested by centrifugation. This enables the pellets to be re-suspended in buffer with substrate for the screen. The plates were shaken at 25°C for ≥ 72 hours. Samples were extracted into MTBE and assayed by chiral HPLC. The results from the screen are summarized in **Figure 3C**. Several hits were identified as selective for leaving the enantiomer corresponding to peak 1 in the assay, however only a few strains gave the desired enantiomer (peak 2) at a low ee. This may be due to the low levels of enzyme expressed in the wild type micro-organisms, and hence low conversion. It must also be noted that the high throughput chiral HPLC method used can only give approximate values. The best results are collated in **Table 15**.

30

Table 15: Microbial screen of Compound 11

CMC No	Strain	Substrate ee /%	Selectivity*
103522	Unidentified	23	Peak 2
103978	<i>Lactobacillus plantarum</i>	12	Peak 2
103947	<i>Comamonas acidovorans</i>	11	Peak 2
103826	<i>Bacillus licheniformis</i>	10	Peak 2
103397	<i>Pseudomonas sp.</i>	10	Peak 2
103308	<i>Phaffia rhodozyma</i>	63	Peak 1
103115	<i>Rhodococcus sp.</i>	61	Peak 1
103188	<i>Pseudomonas putida</i>	58	Peak 1
103126	<i>Enterobacteriaceae sp.</i>	44	Peak 1

* major peak of residual ester in HPLC chromatogram.

5 The five strains that gave peak 2 selectively and three that gave peak 1 were selected for further trials to confirm the results from the initial screen; the results are shown in **Table 16**. Only CMC 103522, the best candidate for peak 2 from the primary screen, confirmed its selectivity at $\leq 10\%$ ee Peak 2.

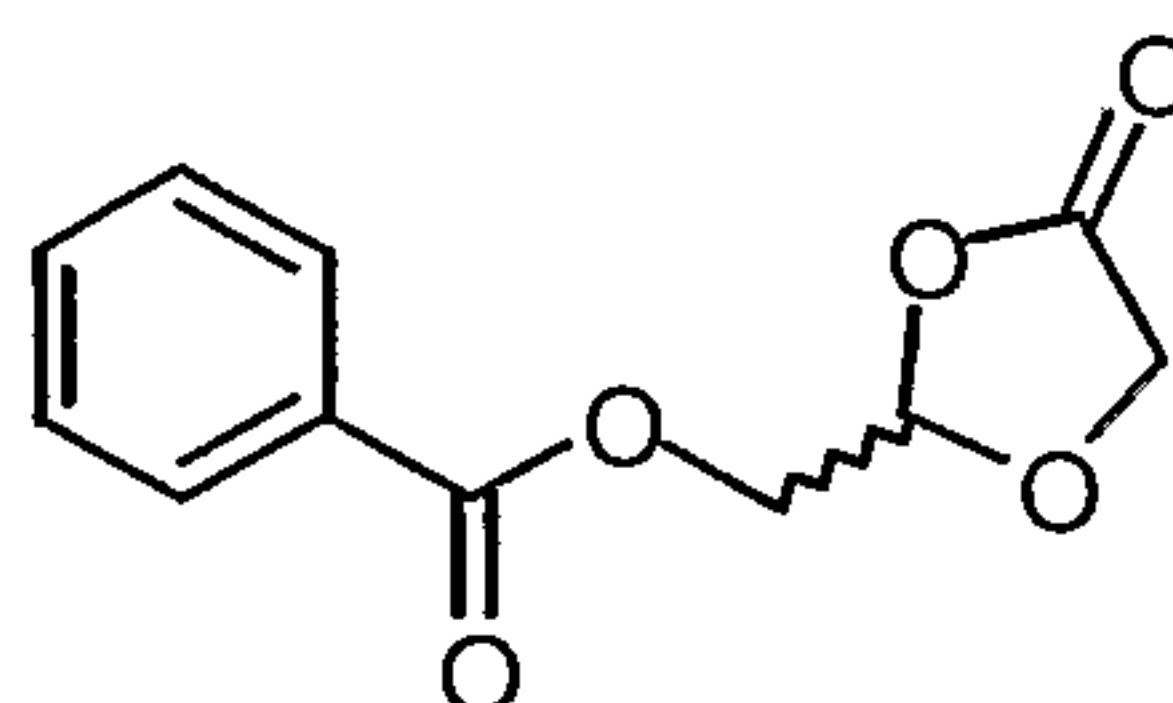
10

Table 16: Re-screen of strains against Compound 1

CMC No	Strain	Substrate ee /%	Selectivity*
103115	<i>Rhodococcus sp.</i>	5	Peak 1
103126	<i>Enterobacteriaceae sp.</i>	2	Peak 1
103188	<i>P.putida</i>	5	Peak 1
103397	<i>Pseudomonas sp.</i>	1	Peak 1
103522	Unidentified	9	Peak 2
103826	<i>B.licheniformis</i>	4	Peak 1
103947	<i>C.acidovorans</i>	2	Peak 1
103978	<i>L.planatarum</i>	2	Peak 1

* major peak of residual ester in HPLC chromatogram.

Example 22



Compound 12

15

Analytical Development for Compound 12

A chiral assay was also required for Compound 12. See **Figure 1E**. Details of the assay conditions are as follows:

HPLC Conditions:

Column: Chiralpak AS
Dimensions: 250mm x 4.6mm
Mobile Phase: 1:1 IPA:EtOH
Flow Rate: 0.8 ml/min
Detection: UV 254nm
Temperature: Ambient

Retention times: **Peak 1 – 6.91 minutes** **Peak 2 – 7.66 minutes**

Again this assay was taken to SFC to achieve a sub 5 minute run time to allow the large number of microbial screen samples to be analyzed in as short a time as possible. See **Figure 1F**.

SFC Conditions:

Column: Chiralpak AS
Dimensions: 250mm x 4.6mm
Mobile Phase: 95% CO₂:EtOH
Flow Rate: 3.0 ml/min, 3000 psi
Detection: UV 254nm
Temperature: 35 °C

Retention times: **Peak 1 – 3.06 minutes** **Peak 2 – 3.81 minutes**

Enzyme Screen.

Compound 12 was screened against the 96 well culture plates. The screen for Compound 12 was based on that developed for Compound 11, but limited to only ten enzymes. Two sets of conditions were used, depending upon whether the enzyme was a lipase or a protease/esterase. For lipase-catalyzed hydrolyses, the reaction solvent was 1:1 MTBE:50 mM potassium phosphate buffer at pH 6. For protease and esterase-catalyzed hydrolyses, reactions were carried out in the phosphate buffer with no immiscible organic co-solvent. In all reactions the amount of substrate utilized was 40 mg. Reactions were carried out in a shaker bath at 30°C and enantiomeric excess values determined by HPLC analysis (Chiralpak AS). The three positive results are summarized in **Table 17**.

Table 17: Commercial enzyme screen of Compound 2

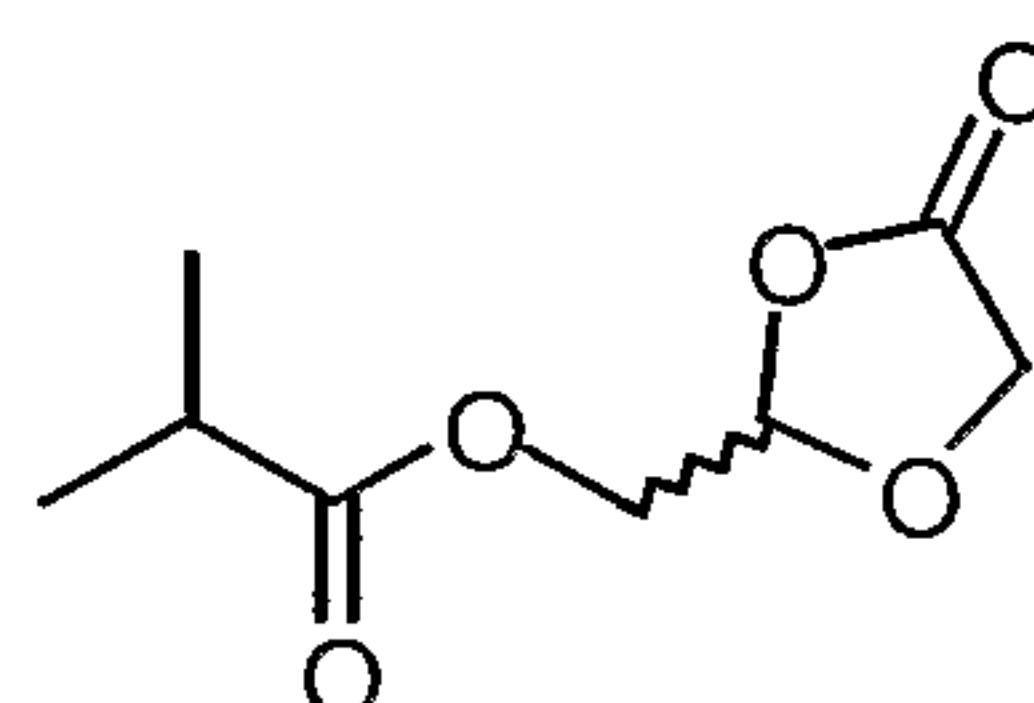
<i>Enzyme</i>	<i>Time</i>	<i>ee_s/%</i>	<i>Comments</i>
<i>Chirazyme-L2</i>	20 hrs	95	Peak 2

	1 week	100	Substrate present
<i>Acid Protease DS</i>	20 hrs	47	Peak 2
	1 week	-	No substrate
<i>Chirazyme-L9</i>	20 hrs	8	Peak 2
	1 week	25	Substrate present

The following enzymes gave less selective resolutions with Compound 2 (<20% ee_s): Lipase F-DS; Lipase PS; Lipase DS; Lipase AY; Lipase F; Acid Protease A; and Lipase N Cone.

5 Samples were assayed by chiral HPLC; the results are summarized in **Figure 3D**. As can be seen from **Figure 3D**, microbial strains were identified that are selective for both enantiomers. The conversions, and therefore the true selectivities are unknown. While Chirazyme-L2 was identified as an excellent enzyme for leaving the enantiomer corresponding to peak 2 in the HPLC assay, no enzymes were identified in this limited
10 screen that left peak 1. It is not known which peak in the chiral assay for Compound 2 corresponds to the required (*R*)-enantiomer.

Example 23



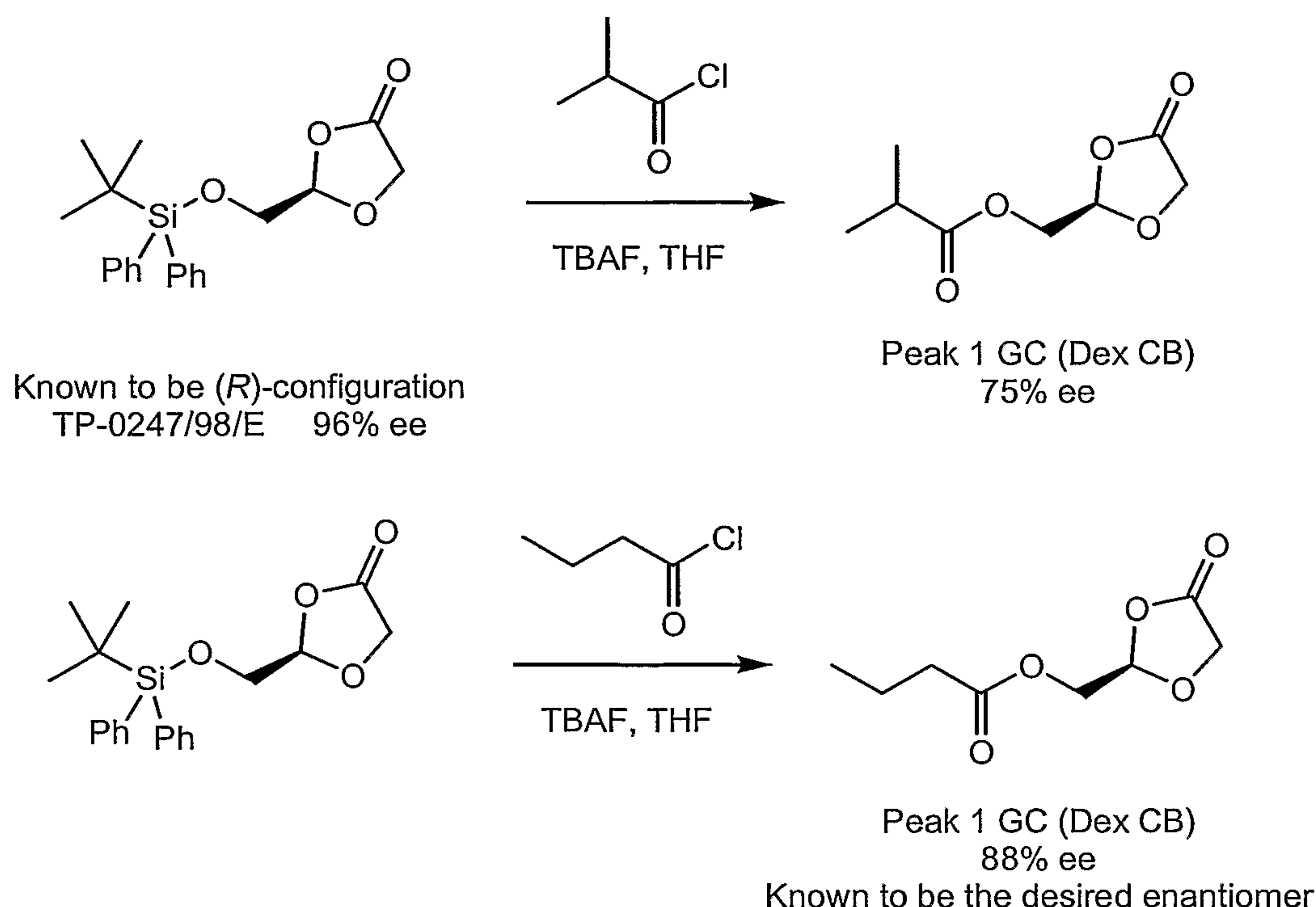
Compound 13

15

Chemical determination of configuration

As an expedient way of determining which of the peaks in the GC chromatogram correspond to the desired enantiomer, it was envisioned that authentic samples could be made from the silyl ether. The enantiomerically enriched silyl ether was heated with 2-methylpropanoyl chloride in tetrahydrofuran (THF) with tetrabutylammonium fluoride
20 (TBAF) (see **Scheme 13**).

63

**Scheme 13:** Determination of configuration for Compound 13

5 The goal was to cleave the silyl ether and trap the generated alcohol *in situ* with
 10 acid chloride. Although the intermediate alcohol is unstable, if only a small fraction
 reacts with acid chloride faster than decomposing, then ester of known configuration
 would be generated. Since the chiral assay is a GC method, the sensitivity is high and
 minute quantities can be detected. Starting from (*R*)-silyl ether, peaks were observed in
 15 the GC chromatograms for the product, enhanced in peak 1 in both cases. Spiking the
 samples with racemate confirmed that the peaks corresponded to the desired esters.
 Slight losses in enantiomeric excess were observed in both cases, possibly due to the
 acidity of the medium and the potential instability of the parent alcohol that is assumed
 to be an intermediate. The absolute configuration of the starting silyl ether is known to
 20 be the desired (*R*)-configuration. The enantiomer that gives peak 1 in the GC assay for
 the *n*-butanoate ester is also known to be the required enantiomer. Therefore, the
 reaction of the (*R*)-silyl ether with 2-methylpropanoyl chloride will give the required
 enantiomer of Compound 13, and that this corresponds to peak 1 in the GC assay. Since
 the other enantiomer of the silyl ether was available, for extra confirmation it was also
 demonstrated to give the peak 2 enantiomer in reaction with 2-methylpropanoyl chloride.

Experimental – procedure for preparation of esters from enantiomerically silyl ether

A mixture of 150 mg of silyl ether, 0.5 mL of acid chloride and 0.4 mL of TBAF (1M in THF), in 5 mL of THF, was heated to reflux under nitrogen for 3-4 hours. TLC showed a small amount of product formed. An aliquot was taken, passed through a plug of silica and analysed by chiral GC.

Example 24

Analytical Development for Compound 13

A chiral GC assay was developed to separate the enantiomers of Compound 13. See Figure 1G. Details of the assay conditions are as follows:

GC Conditions:

Column: Chirasil DEX CB
Dimensions: 25m x 0.25mm
Temp. Program: 140°C for 8 minutes then to 200°C at 15°C/min
Carrier gas: Helium @ 20 psi
Detection: FID @ 200°C

Retention times: Peak 1 – 6.5 minutes Peak 2 – 6.8 minutes

In addition, an achiral GC assay was developed to determine the purity of Compound 13. See Figure 1H. Details of the assay conditions are as follows:

GC Conditions:

Column: J&W Scientific DB5
Dimensions: 15m x 0.25mm
Film thickness: 0.25µm
Temp. Program: 40°C for 5 minutes then to 200°C at 10°C/min
Carrier gas: Helium @ 12 psi
Detection: FID @ 200°C
Retention time: Compound 3-17.80 minutes

Enzyme Screen

Stability of the *n*-butanoate ester was determined to be significantly better at pH 6. Therefore for the screening of the *sec*-butanoate ester, Compound 13, similar conditions were used. Racemic Compound 13 was screened for enzymatic hydrolysis with commercially available lipases, proteases and esterases. All lipase reactions were carried out in the presence of 40 µL Compound 13, 5 mL MTBE, 5 mL pH 6 phosphate

buffer and 20 wt.% enzyme. Esterase and protease reactions were performed without MTBE present. The mixtures were shaken in an incubator at 30°C and monitored by TLC and Chiral GC. The alcohol product, like the other esters, is unstable as previously described. Therefore no chiral assay for the product was possible and conversions could not be calculated. For the screen it was noted whether any substrate was still present by TLC analysis. The best results from the screens are shown in **Table 18** below.

Table 18: Commercial enzyme screen of Compound 13

<i>Enzyme</i>	<i>Time</i>	<i>ee_s/%</i>	<i>Comments</i>
Chirazyme-L2	1hr	81	Peak 1
	5hrs	-	Not enough substrate remaining
Lipase F-DS	1hr	10	Peak 1
	1week	46	Substrate present
Acid Protease DS	24hrs	49	Peak 1
	120hrs	-	Not enough substrate remaining
Lipase PS	5hrs	44	Peak 1
	24hrs	-	Not enough substrate remaining
Chirazyme L9	24hrs	42	Peak 1
	120hrs	-	Not enough substrate remaining
Acid Protease A	24hrs	23	Peak 1
	120hrs	-	No substrate
Lipase M	24hrs	28	Peak 1
	1week	30	Substrate present
Protease M	5hrs	19	Peak 1
	1 week	30	Not much substrate present
Protease P "Amano" 6	24hrs	21	Peak 1
	1 week	33	Not much substrate present

The following enzymes gave less selective reactions with Compound 13 (<20% ee_s): Lipase DS; Lipase AY; Lipase F; Lipase N Cone; SAWA immobilised lipase; Chiroclec-PC; Lipase MY; Protease B; Newlase F; Alcalase; Lipase R; PPL; Lipase G; PLE; Chirazyme-E1; Lipase A "Amano" 6; Lipase A "Amano" 12; Lipase AP6; Sigma CCL; Chirazyme-L5; ChiroCLEC-CR; Peptidase R; Acid Protease A-DS; Protease N; Protease A2G; Protease NL; Protease DS; Protease S; Prozyme 6; Proleather; Bromelain-F; Papain W-40 (Amano); Papain (Sigma); Protease X; Protease XXXI; Savinase; Esperase; Pepsin; ChiroCLEC-BL; Chirazyme-E2; and α-Chymotrypsin.

From **Table 19** it can be seen that all the enzymes selectively left peak 1 in the chiral GC assay, fortunately corresponding to the desired enantiomer. None of the enzymes screened were selective for peak 2. Four enzymes were chosen for re-screening

with lower loadings, the results of which can be seen in **Table** . The most promising of these was Chirazyme-L2, leaving residual ester in 90% ee after 5.5 hours.

Table 19: Re-screen of peak 1 selective enzymes for Compound 3

<i>Enzyme</i>	<i>Time</i>	<i>ee_s/%</i>	<i>Comments</i>
Chirazyme-L2	1hr	21	Peak 1
	5.5hrs	90	Substrate present
Lipase PS	16hrs	65	Peak 1
	24hrs	-	No substrate
Acid protease DS	24hrs	42	Peak 1
	48hrs	47	Not much substrate remaining
Chirazyme-L9	24hrs	21	Peak 1
	72hrs	64	Substrate present

Example 25

Scale-up of Chirazyme-L2 Resolution of Compound 13.

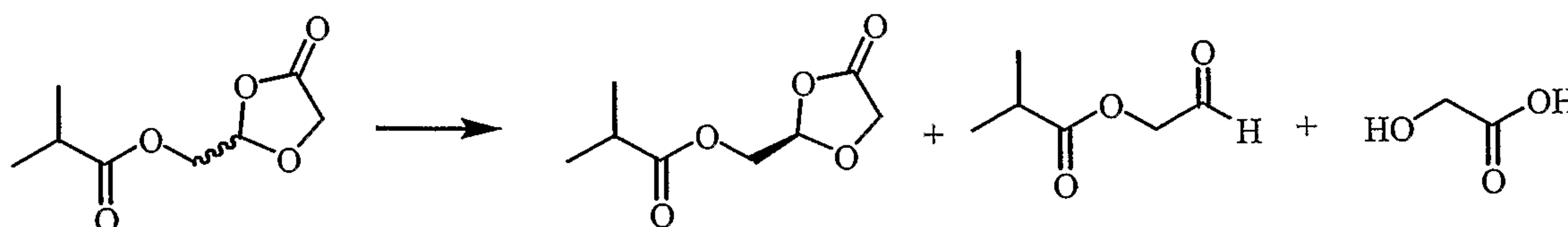
The Chirazyme-L2 resolution was scaled up to 1 g, 30 mg of enzyme and 25 mL each of pH 6 phosphate buffer and MTBE. After work-up the reaction yielded 0.212 g, 21% Th of Compound 13 as a colorless oil. The ¹H NMR spectrum showed a significant amount of impurities present, which had been carried through from the substrate.

On a 6 g scale, using the same conditions, at 20°C the yield was 1.52 g, 25% Th of 88% ee resolved Compound 3 as a pale yellow oil. Again ¹H NMR indicated some impurities were present. The selectivity was improved at a lower temperature, 0°C. Starting from 5.25 g of racemate, after 4.5 hours the resolution yielded 1.48 g, 28% Th of 92% ee Compound 3. The configuration was confirmed as the required (*R*)-enantiomer.

Initially the bioresolution was performed at a moderate concentration, 20 g/L. To study the effect of increasing the volume efficiency, the resolution was repeated on a 1 g scale at 50 g/L, 0°C in pH 6 phosphate buffer and MTBE. After 4.5hrs, the biotransformation yielded 0.44 g, 44% Th, of material as a pale yellow oil in 92% ee. From this it was concluded that the volume efficiency could be improved with no significant loss in selectivity.

The purity of product obtained from these processes was low. Several impurities were carried through from the racemate. For a bioresolution of moderate selectivity the conversion optionally can be increased to gain high enantiomeric purity of residual ester. One of the impurities, the 2-methylpropanoate ester of hydroxyacetaldehyde shown in

Scheme 14, was present in the racemate. However it was not clear whether this was also produced in the bioresolution, as outlined below.



Scheme 14: Formation of impurities

In order to determine if any additional aldehyde was generated during the biotransformation, the resolution was repeated on the same scale (1 g) using distilled lactone, which contained no of aldehyde. This reaction yielded 0.29 g, 29%, of 93% ee product. The ^1H NMR spectrum showed that the aldehyde was present; clearly this material is produced during the reaction or work up.

Changing the solvent to toluene resulted in a slower, but more selective resolution. On a 1 g scale at, 50 g/L, the reaction was worked-up after 22.5 hours to give 0.30 g, 30% yield of >99% ee ester. The concentration was further increased to 200 g/L, using 4 g of racemate, in 10 mL each of pH 6 phosphate buffer and toluene, at 0°C. The process was highly volume efficient, and no problems were encountered. After 17 hours the biotransformation yielded 1.11 g, 28%, of 95% ee product.

Several reactions were performed on a slightly larger scale (2-10 g) utilizing differing enzyme loadings and reaction times; the results are summarized in **Table 10** below. The rate of reaction was not entirely predictable. Several factors may be responsible for this, including different batches of racemate with different impurity profiles and differences in mixing of the biphasic solvent-immobilized enzyme system. The yields may be misleading due to impurities carried through from the racemate. It was found on this scale that thiosulfate, bicarbonate and acidic washes did not significantly reduce the amount of the aldehyde impurity. The work-up procedure required some clarification, but at this stage it was hoped that a combination of suitable wash and Kugelrohr distillation (wiped-film on scale) after resolution would be effective. This was investigated later on a larger scale. All these biotransformations used the toluene-buffer biphasic system at 200 g/L.

Table 10: Chirazyme-L2 resolution of Compound 3

<i>Entry</i>	<i>Enzyme Loading /wt. %</i>	<i>Scale /g</i>	<i>Time /hours</i>	<i>Yield /%</i>	<i>ee /%</i>
1	1	4	112	19	98
2	2	8	16	22	73
3	2	2	47	29	93
4	2.5	10	15	20	>99
5	10	10	4	ND	96

5 The process was scaled-up to 30 g using 10 wt.% enzyme at 0°C, 200 g/L. After a Kugelrohr distillation 4.2 g of >95% ee ester was recovered.

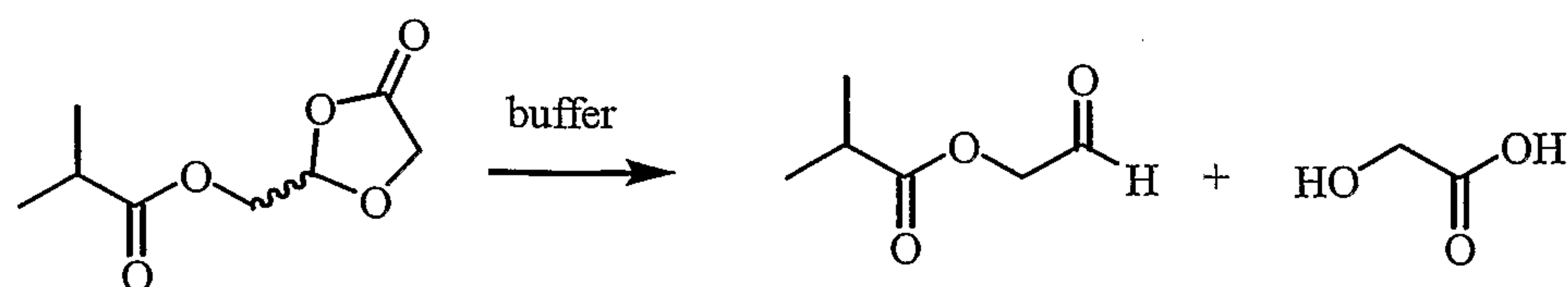
As discussed earlier, the purity of the racemate is vital to the quality and yield obtainable after biotransformation. Moreover it is difficult to determine the true selectivity of a resolution with accurate conversion. This is unavailable from
10 enantiomeric excess data since the product of the bioresolution cannot be isolated; the only information available is the enantiomeric excess of the residual substrate. An idea of selectivity is gained from the yield of the ester, but as it is contaminated with impurities, the accuracy is in doubt.

When considering the final process it may be advantageous to use a crude
15 racemate and purify after bioresolution, e.g. by wiped-film distillation. However, impurities may inhibit the enzyme. The possibility of one such process using very crude substrate was investigated; a mixture of racemate containing DME, water and other impurities, containing approximately 1 g of substrate was used in the bioresolution. During the biotransformation no base was consumed and so additional enzyme added.
20 An aliquot was removed after 16 hours and found to be racemic. These findings would suggest that the enzyme was being “killed” by the impurities present in this mixture. Thus, in one embodiment, purification of the substrate is preferable prior to bioresolution.

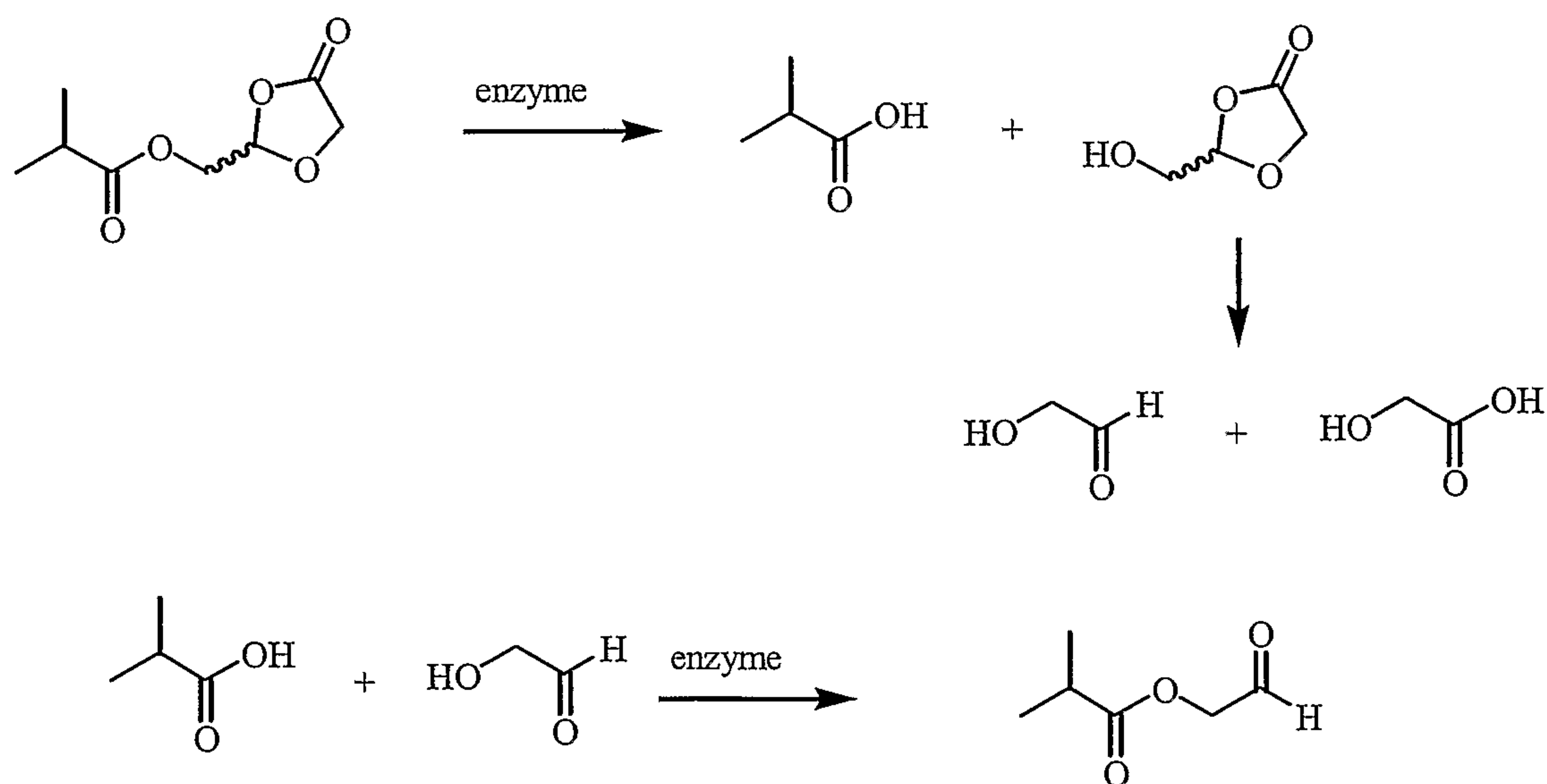
Example 26

25 Biotransformation in 2-Propanol-Water

As mentioned earlier the stability of the substrate was a potential problem. A background process in which lactone is opened to glycolic acid and the 2-methylpropanoate ester of hydroxyacetaldehyde cannot be ruled out (**Scheme 15**).

**Scheme 15:** Ring-opening of lactone

5 The mode of action of the enzyme on Compound 13 is also unknown. The only
 products detected after bioresolution are the 2-methylpropanoate ester of
 hydroxyacetaldehyde and glycolic acid. However the usual mode of action for a lipase is
 to hydrolyse the ester; this would give the lactone and 2-methylpropionic acid (**Scheme**
 16). The lactone alcohol product is not detected and breaks down to glycolic acid and
 10 hydroxyacetaldehyde. If this mechanism is correct then the esterified aldehyde must
 come from reaction of hydroxyacetaldehyde with 2-methylpropionic acid. An alternative
 mechanism is that the enzyme may act directly to open the lactone without first cleaving
 the ester. This mode of action whereby a lactone ring is opened has been observed for
 liver esterases (see for example: E. Fouque and G. Rousseau *Synthesis* **1989**, 661; and P.
 15 Barton and M. I. Page *J. Chem. Soc., Perkin Trans. 2*, **1993**, 2317), but is perhaps
 unlikely in this case.

**Scheme 16:** Possible reaction mechanism

20

The use of additives in biotransformations has been widely reported to enhance
 selectivities, but their effects on enantiomeric excess are usually unpredictable. See for
 example: T. V. Hansen, V. Waagen, V. Partali, H. W. Anthonsen and T. Anthonsen,

Tetrahedron Asymmetry **1995**, 6, 499; G. Duan and J. Y. Chen, *Biotechnology Letters* **1994**, 16, 1065; N. W. Boaz and R. L. Zimmerman, *Tetrahedron Asymmetry* **1994**, 5, 153; and K. Faber, G. Ottolina and S. Riva, *Biocatalysis* **1993**, 8, 91. The mode of action is also often unclear; the additive may be acting as a phase transfer catalyst, as an enzyme modifier, or as an alternative to water as a nucleophile. In the resolution of (-)-2',3'-dideoxy-5-fluoro-3'-thiacytidine (FTC, also known as Coviracil™ and emtricitabine), it was found that alcohol:water mixtures were a good alternative to the normal biphasic or mainly aqueous systems. It was envisioned that this might also be effective in the resolution of Compound 13, both with respect to enzyme selectivity and substrate stability. This hypothesis was tested using 1 g of Compound 13, with 3 wt.% enzyme, at 100 gL⁻¹ in an 8:2 2-propanol:water solvent system. After 47 hours the reaction yielded 0.47 g, 47%Th, of >98% ee material.

The initial result in 2-propanol-water looked excellent, although water and aldehyde may have bolstered the yield. More meaningful results were gained on a larger scale, with distillation to purify the product. The racemate was distilled using wipe film distillation. The conditions were 30 g of racemate in 9:1 2-propanol (IPA)-water, at 200 gL⁻¹ with an enzyme loading of 5 wt.%. This reaction was filtered after 10 hours and reduced *in vacuo*. The enantiomeric excess was determined to be 94%. This material was divided into two batches to investigate purification methods. The first portion was distilled using Kugelrohr apparatus at 133°C / 1.3 Torr to give two required fractions:

Fraction 1: 10.8 g contained 61% aldehyde, 31% Compound 3 and an undetermined amount of glycolic acid. Further distillation at 80°C/4-5 Torr to remove the aldehyde and a water wash gave 4.4 g of Compound 3 90% pure material containing 4% aldehyde.

Fraction 2: 2.7 g contained no aldehyde but a significant amount of glycolic acid and Compound 3. After a water wash, 1.8 g of Compound 3 was obtained, 91% pure (by GC).

The second portion of crude material was given a water wash prior to distillation at 140°C / 1.6 Torr followed by removal of the aldehyde at 60°C / 1.6 Torr, yielding 5.2 g of material in 89% purity, containing 6% aldehyde.

Therefore in total 11.4 g of the 2-methylpropanoate ester was produced in 90% purity, 94% ee, corresponding to an excellent 38% overall yield. This procedure clearly

out-performs that with toluene:buffer and with further optimization could provide an efficient route to the optically pure lactone.

Example 27

Experimental - Resolution of 30g of Compound 13 with Chirazyme-L2 in 2-propanol:water

A 500 mL jacketed vessel at 0°C was charged with 2-propanol (135 mL), water (15 mL), and racemic Compound 13 (30 g, DB/1005/85/1). The mixture was stirred and Chirazyme-L2 (1.5 g, 5 wt.%), added to this mixture. The ee_s was measured at intervals by removing aliquots of solution, extracting with ethyl acetate, drying (MgSO₄), and analysing by chiral GC. After 10h, the ee_s was 93%. Shortly after the mixture was filtered through Celite[®] and concentrated *in vacuo* to give 32.1 g of crude Compound 3 as a pale yellow oil. The oil was then dissolved in toluene and concentrated (2 × 250 mL) to remove any water azeotropically. Crude yield 29.4 g, 98%; ee 94%.

This oil was divided into two portions. The first was distilled using Kugelrohr apparatus at 133°C / 1.3 Torr, which gave three fractions overall. Fraction 1 (most volatile) contained 10.8 g of 61% aldehyde, 31% lactone and 8% of other impurities. Fraction 2 contained 2.7 g of lactone contaminated with a significant amount of glycolic acid and fraction 3 contained yellow low volatility impurities. Fraction 2 was dissolved in toluene (200 mL), washed with water (200 mL), dried (MgSO₄) and concentrated *in vacuo* to give 1.8 g of Compound 13 as a colourless oil in 91% purity by GC (no aldehyde observed). Most of the aldehyde in fraction 1 was then removed by Kugelrohr distillation at 80°C / 4-5 Torr to give, as the residue, 6.1 g of lactone containing ~12% aldehyde and an undetermined amount of glycolic acid. This material was then dissolved in toluene (200 mL), washed with water (200 mL), dried (MgSO₄) and concentrated *in vacuo* to give 4.4 g of Compound 3 as a colourless oil in 90% purity, 4% aldehyde by GC.

The second portion of material was dissolved in toluene (200 mL), washed with water (200 mL), dried (MgSO₄) and concentrated *in vacuo*. The resulting oil was then distilled using Kugelrohr apparatus at 140 °C / 1.6 Torr, to remove low volatility impurities, and then further distilled at 60°C / 1.3 Torr to remove any residual aldehyde. The material recovered from this distillation was determined to be 5.15 g of 89% purity, 6% aldehyde by GC.

Overall yield: 11.4 g, 38%; GC Purity: 90%

Example 28

Microbial enzyme screen.

As for Compounds 11 and 12, Compound 13 was screened against 96 well culture plates. Samples were assayed by chiral GC; it must be noted that the high through put chiral GC method used can only give approximate values. The results of the screen are represented in **Figure 3E**.

A large number of strains showed activity on Compound 13, with selectivities for both peaks 1 and 2 observed in the GC chromatograms. Those that gave an enantiomeric excess > 40% ee are shown in **Table 21**.

Table 21: Microbial Screen of Compound 13

CMC No	Strain	Substrate ee /%	Comments
103127	<i>Serratia liquifaciens</i>	42	Peak 1
103869	Unidentified	44	Peak 1
103355	Unidentified	54	Peak 1
103032	<i>Bacillus licheniformis</i>	43	Peak 1
103063	Unidentified	74	Peak 1
103071	Unidentified	75	Peak 1
103095	<i>Nocardia sp.</i>	54	Peak 1
103134	Unidentified	48	Peak 1
103146	<i>Pseudomonas sp.</i>	46	Peak 1
103188	<i>Pseudomonas putida</i>	46	Peak 2
103322	<i>Pseudomonas alcaligenes</i>	70	Peak 1
103419	<i>Acinetobacter sp.</i>	81	Peak 1
103422	Unidentified	79	Peak 1
103423	Unidentified	71	Peak 1
103669	Unidentified	51	Peak 1
103777	Unidentified	50	Peak 1
103780	<i>Streptomyces sp.</i>	49	Peak 1
103405	Unidentified	62	Peak 1
103785	<i>Pseudomonas fluorescens</i>	87	Peak 1
103869	Unidentified	92	Peak 1
103331	<i>Candida rugosa</i>	40	Peak 1
103587	<i>Staphylococcus sp.</i>	65	Peak 1
103774	<i>Streptoverticillium cinnamoneus</i>	87	Peak 2

From the results of the primary screen those strains that showed the best selectivity for peak 1 and good peak area were re-screened. The strains which reconfirmed their activities are shown in **Table 22**.

Table 22: Re-screen of strains against Compound 3

<i>CMC No</i>	<i>Strain</i>	<i>Substrate ee /%</i>
103063	Unidentified	45
103127	<i>S.liquifaciens</i>	41
103373	<i>Acinetobacter sp.</i>	41
103419	<i>Acinetobacter sp.</i>	14
103552	Unidentified	31
103606	<i>Acinetobacter junii</i>	21
103635	Unidentified	44
103661	<i>S.cerevisiae</i>	47
103777	Unidentified	18
103869	Unidentified	65

Example 29**Scale-up of biotransformations for CMC 103869 & 103661.**

5 From the screen, two strains were selected for scale-up. These were CMC 103869 (*Unidentified*) and CMC 103661 (*S. cerevisiae*). Cultures of both were grown up and their harvested cell pastes stored at -20°C . Biotransformations were set-up at 30 mL scale, using both pH and temperature control. Initial biotransformations were in 0.1M Tris-HCl, pH 7. Poor enantiomeric excesses and substrate stability were observed,

10 possibly due to the high pH. Lowering the pH to 6 partially increased selectivity, but there were still substrate stability problems. A change in buffer to 50 mM KH_2PO_4 , pH 6, further increased the enantiomeric excess. The results for both strains are summarized in **Figure 4**. Resolution of Compound 13 with CMC 103869 gave residual ester of 70% ee. The results with CMC 103361 were disappointing and therefore further studies with

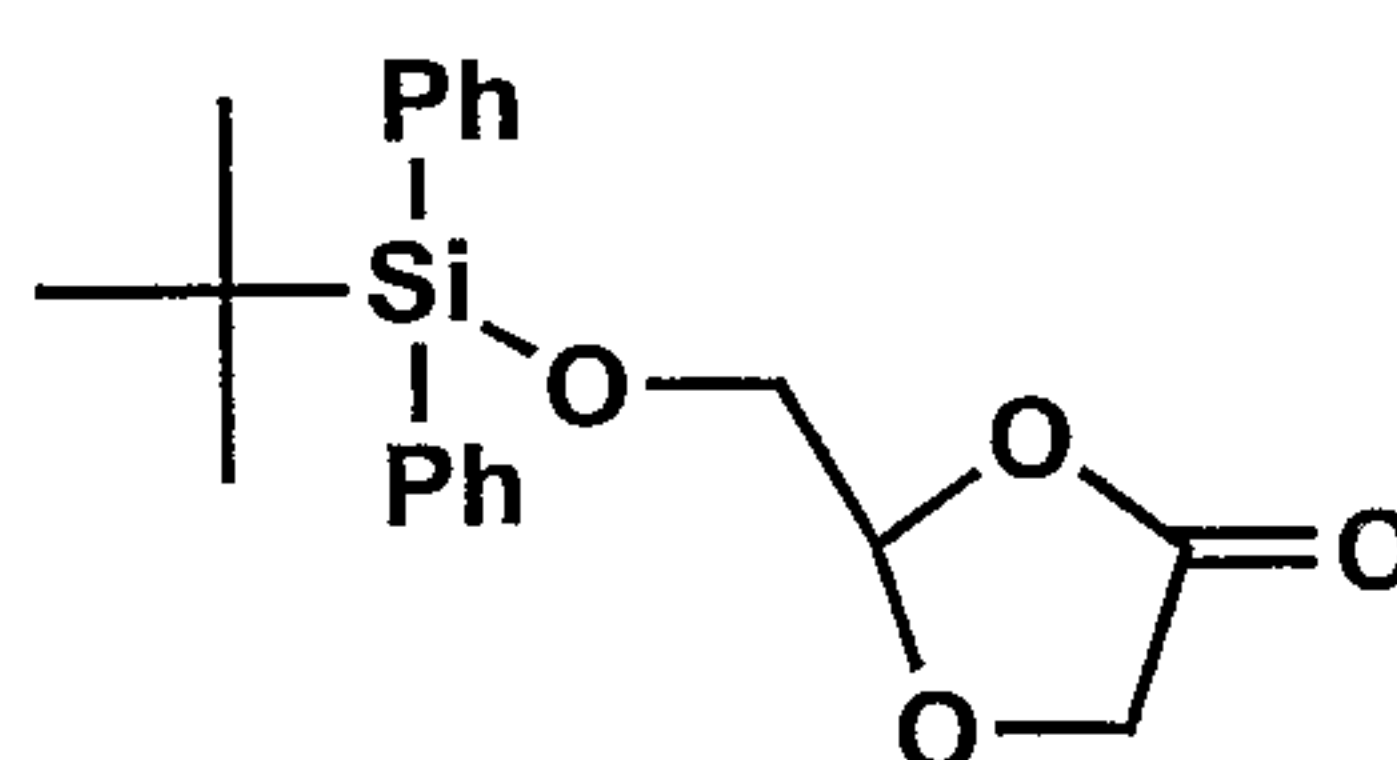
15 this strain were abandoned. For all the biotransformations a loss of residual substrate was observed, possibly due either to a second enzyme or poor substrate stability at 25°C . Finally by reducing the temperature to 10°C the selectivity increased to $\geq 95\%$ ee, however there was still a total loss of substrate over time. The conversion and hence absolute selectivity is not known.

20 CMC 103869 was grown up in TSB media and CMC 103661 was grown up in YM media in flasks. Both strains were grown at 25°C and cells harvested by centrifugation (2000g for 20 minutes at 4°C). Cell pastes were stored at -20°C . Biotransformations were run in 200 mL jacketed vessels with a magnetic stirrer, with temperature and pH control (with 1N NaOH). Cell pastes were re-suspended at 10% w/v

25 in either 0.1M Tris-HCl buffer (adjusted to the required pH), or 50 mM KH_2PO_4 , pH 6.

Substrate, buffer and cell paste were added to the vessel and samples taken for analysis by chiral GC by dilution into MTBE.

Example 30



Compound 4

5

Analytical Development for Compound 4

The conditions for the chiral assay HPLC method of analysis was reproduced to give the resolution shown in the chromatogram below:

10

HPLC Conditions:

Column: Chiralcel OJ
Dimensions: 250 x 4.6mm
Mobile phase: 85% Heptane
 15% EtOH
Flow rate: 1.0 mL/min
Detection: UV @ 254 nm

15

Retention times: 1 - 7.47 minutes
 2 - 11.16 minutes

20

Under the assay conditions the peak shapes of the enantiomers were a little broad (**Figure 1K**), thus an attempt was made to improve this assay. This was achieved by employing supercritical fluid chromatography (SFC), with both isomers giving sharp peaks that were fully resolved within 4 minutes. See **Figure 1L**.

25

Optimized SFC Conditions:

Column: Chiralcel OJ (250 x 4.6 mm)
Mobile phase: 95% CO₂, 5% MeOH
Flow rate: 3.0 mL/min
Pressure: 3000psi
Column temp.: 35°C
Retention times: 1 - 2.73 minutes [(-)-enantiomer]
 2 - 3.28 minutes [(+)-enantiomer]

30

35

Bioresolution of Compound 4

During the study of the biotransformation of Compound 1, it was discovered that the material was susceptible to non-enzymatic hydrolysis. This could be due to the high lability of Compound 1 towards hydrolysis and the instability of the postulated lactone-alcohol product, that a possible pathway for the biotransformation was opening of the lactone before saponification of the butyrate. If this was true then enzymatic resolution Compound 4 might be possible. Therefore the reactivity of Compound 4 was tested with lipases PS, PPL, M, MY and the SAWA immobilized lipase, in toluene-pH 7 buffer at 20°C. No reaction was observed (TLC, 24 hours). The enzymatic reaction probably proceeds by first cleavage of the butyrate and then possible lactone opening.

Conglomerate Studies

The IR spectra of the racemate and single enantiomer of Compound 4 were compared and found to be similar but not identical.

The melting points of each of these compounds were then determined by differential scanning calorimetry (DSC) and found to give an onset of melting of 91.7°C for the racemate and 81.1°C for the (-)-enantiomer (98.7% ee). Comparison of these melting points reveals that the racemate has a higher melting point than the single enantiomer. For a compound to be a conglomerate the IR spectra of the racemate and single enantiomer must be identical and the melting point of the racemate is required to be the lowest point on the phase diagram. Therefore, this compound is clearly not a conglomerate.

Subsequently, a phase diagram for Compound 4 was constructed. The position of the eutectic was determined, above which point the ee of this compound may be enhanced to enantiomeric purity by crystallization. This phase diagram information was to be obtained by analyzing the melting points (again by DSC) of samples of differing enantiomeric excess in 10% ee increments. These samples were initially prepared by dissolving the required amounts of racemate and single enantiomer in acetone and removing the solvent. This method was successful for the 10% ee, 80% ee and 90% ee samples, with the DSC traces containing just one peak. For all the other samples however, two peaks were observed by DSC (possibly due to polymorphs). A number of

methods for producing these samples were attempted to combat this problem. These included melting the sample; dissolving in acetone and heating O/N at 65°C; melting and heating overnight at 65°C and using dichloromethane as the solvent; but the DSC still gave two peaks. Therefore the phase diagram/eutectic could not be determined *via* this method. In addition since the compound was extremely soluble and only 1 g of the racemate and each of the single enantiomers was available, solubility studies were ruled out. However, since only one peak was given by the racemate and single enantiomer samples by DSC, the melting points and enthalpy values for these samples could be determined. This allowed the phase diagram to be constructed theoretically (J. Jacques, A. Collet and S.H. Wilden, *Enantiomers, Racemates and Resolutions*, New York: Wiley, 1981).

The Schroder-Van Laar equation can be used to calculate the liquidus curve between the pure enantiomer and the eutectic for a true racemate compound, using the melting point and enthalpy of fusion of the pure enantiomer:

$$\ln x = \frac{\Delta H_A^f}{R} \left(\frac{1}{T_A^f} - \frac{1}{T^f} \right)$$

Mole Fraction;	0.9	$T_f = 351.64 \text{ K}$	78.64°C
	0.8	$T_f = 346.60 \text{ K}$	73.60°C
	0.7	$T_f = 341.05 \text{ K}$	68.05°C
	0.6	$T_f = 334.86 \text{ K}$	61.86°C

Then the part of the curve below which the solid phase consists of pure racemic compound can be determined using the following equation (Prigogine-Defay):

$$\ln 4x(1-x) = \frac{2\Delta_R^f}{R} \left(\frac{1}{T_R^f} - \frac{1}{T^f} \right)$$

Mole Fraction;	0.9	$T_f = 349.26 \text{ K}$	76.26°C
	0.8	$T_f = 360.81 \text{ K}$	87.81°C
	0.7	$T_f = 366.53 \text{ K}$	93.53°C
	0.6	$T_f = 369.41 \text{ K}$	96.40°C

Where:

x = mole fraction of most abundant enantiomer ($0.5 \leq x \leq 1$) of a mixture whose melting terminates at T^f (K).

$$\Delta H_{\text{A}}^{\text{f}} = \text{enthalpy of fusion of single enantiomer (J.mol}^{-1}\text{)}.$$

5 ΔH_R^f = enthalpy of fusion of racemate (J.mol⁻¹).

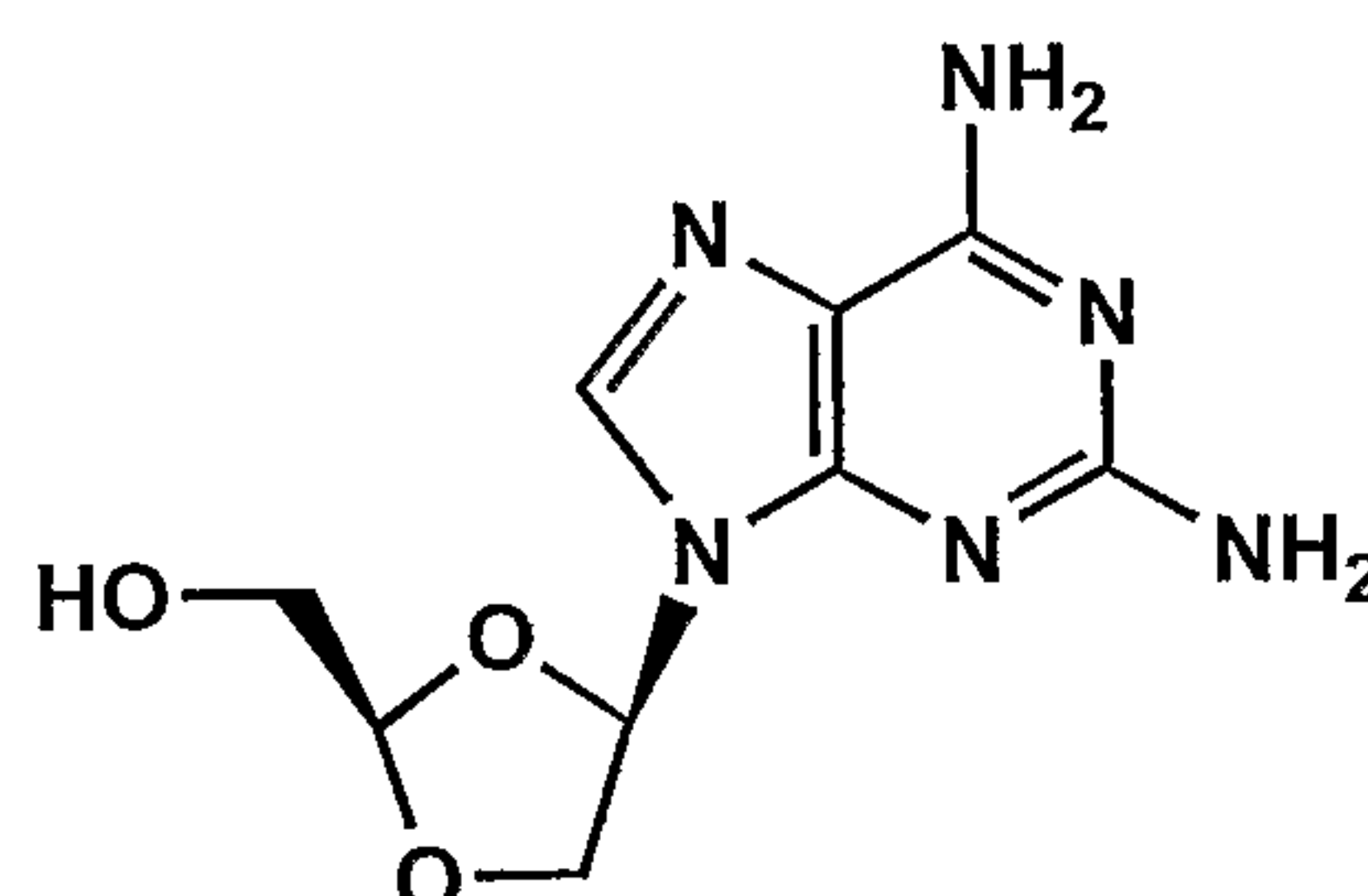
$$R = 8.31 \text{ (J.K}^{-1}.\text{mol}^{-1}\text{)}.$$

T_A^f = melting point of pure single enantiomer (K).

$$T_p^f = \text{melting point of racemate (K).}$$

10 Both of these curves were then plotted on the same graph to give the phase diagram. The point at which the lines cross is the eutectic, which in this case is 77.5% ee. See **Figure 4**.

Example 31



DAPD

Analytical Development for DAPD

Chiral assays were developed for *cis*-DAPD and its butyrate ester in order to analyze an enzymatic esterification screen of DAPD. See **Figure 1M**.

SFC Conditions for DAPD:

Column:	Chiralpak AD
Dimensions:	250 x 4.6 mm
Mobile phase:	70% CO ₂ , 30% MeOH with 0.1% TEA
Flow rate:	3.0 mL/min
Pressure:	3000psi
Column temp:	35°C
Detection:	UV @ 254 nm

Retention times: 1 - 3.57 minutes

2 - 5.64 minutes

In order to monitor the progress of the biotransformations for the DAPD material, the above assay was modified to include the baseline resolution of both the DAPD and the butyrate. See **Figure 1N**. The conditions and chromatogram are as follows:

SFC Conditions for DAPD and Butyrate ester:

Column: Chiralpak AD
Dimensions: 250 x 4.6 mm
Mobile phase: 80% CO₂, 20% MeOH with 0.1% TEA
Flow rate: 3.0 mL/min
Pressure: 3000psi
Column temp: 35°C
Detection: UV @ 254 nm

Retention times: 1 - 4.49 minutes 2 - 5.90 minutes
 3 - 9.18 minutes 4 - 11.0 minutes

Enzymatic Resolution of DAPD

A limited enzyme screen aimed at resolving the isomers of *cis*-DAPD by transesterification using vinyl butyrate was performed. Enzymes were chosen on the basis of known transesterification activity. Solvents investigated were toluene (substrate insoluble), DMF and pyridine (substrate soluble). The reactions were followed by TLC and the enantiomeric excesses measured by supercritical fluid chromatography (SFC) (Chiralpak AD). As confirmation, enantiomeric excesses were also measured using a HPLC assay (100% MeOH, Chiralpak AD) and were in agreement with the SFC results.

For each enzyme: To racemic *cis*-DAPD (TP0041/97/D-1, 10 mg, 0.04 mmol) was added solvent (1 mL) (toluene, DMF or pyridine, see Table 13), vinyl butyrate (0.1 mL, 0.8 mmol) and then enzyme (5-10 mg). The vials were shaken in an incubator at 30°C and assayed periodically by removing 50 µL aliquots, diluting with methanol and analysing by TLC (10:1:0.1 EtOAc: MeOH: H₂O) and chiral. **Table 23** shows the results obtained.

Table 23: Enzymatic screen for acylation of DAPD

Enzyme	Solvent	Time	ee _s	ee _p	Comments
Chirazyme-L2	Toluene	2h	4%	7%	1 product (<50% conversion)
		20h	8%	10%	2 products (~50%)

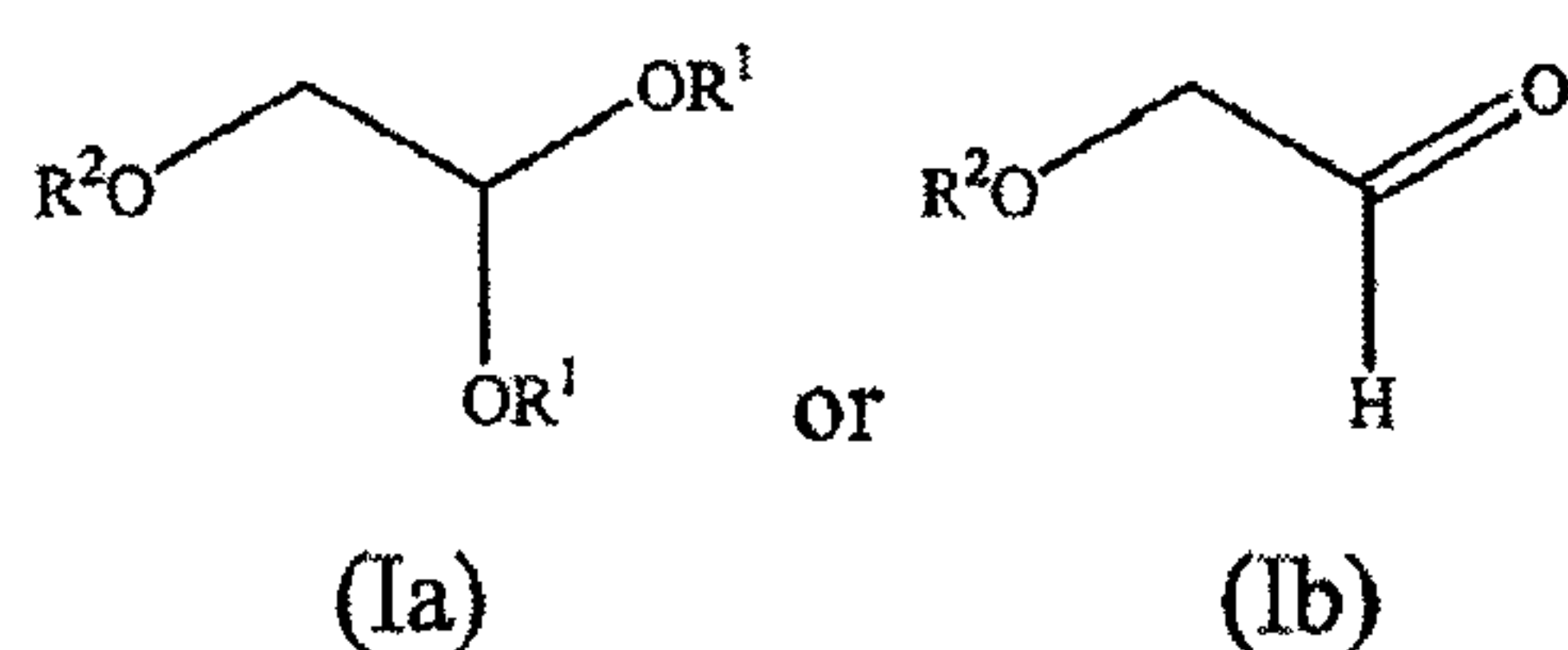
Enzyme	Solvent	Time	ee _s	ee _p	Comments
					conversion)
Chirazyme-L9	Toluene	2h	rac	18%	1 product (<50% conversion)
		20h	rac	10%	2 products (~50% conversion)
Lipase AY	Toluene	2h	7%	3%	1 product (~50% conversion)
		20h	11%	6%	1 product (>50% conversion)
Lipase AK	Toluene	2h			No reaction
		20h	rac	rac	1 product (<50% conversion)
Lipase PS	Toluene	2h	rac	12%	1 product (<50% conversion)
		20h	rac	14%	1 product (<50% conversion)
Alcalase (Immobilised)	DMF	2h			No reaction
		20h	9%	10%	1 product (<50% conversion)
Subtilisin carlsberg type VII	DMF	2h			No reaction
		20h	rac	rac	1 product (<50% conversion)
PeptiCLEC-BL	Pyridine	2h	-	rac	1 product (100% conversion)
	Pyridine (less enzyme than above)	2h	rac	10%	1 product (<50% conversion)
PeptiCLEC-BL	Toluene	4h	rac	rac	1 product (<50% conversion)
		24h	rac	12%	1 product
Protease N	Toluene	24h			No reaction
	DMF	24h	rac	11%	1 product (<50% conversion)
PLE	Toluene	24h			No reaction
PPL	Toluene	24h			No reaction

The results shown in **Table 23** show that although esterification of DAPD takes place, it is not an enantioselective reaction. TLC showed that more than one product was formed in some cases. However, for PeptiCLEC-BL and Lipase AY, only the desired butyrate was formed in a fast reaction, thus these would be good enzymes to regioselectively and mildly put an ester on the oxygen.

THE EMBODIMENTS OF THE INVENTION FOR WHICH AN EXCLUSIVE PROPERTY OR PRIVILEGE IS CLAIMED ARE DEFINED AS FOLLOWS:

1. A process for preparing a substantially pure β -D- or β -L-1,3-dioxolane nucleoside comprising:

- a) obtaining an esterified 2,2-dialkoxy ethanol of the formula (Ia) or (Ib);

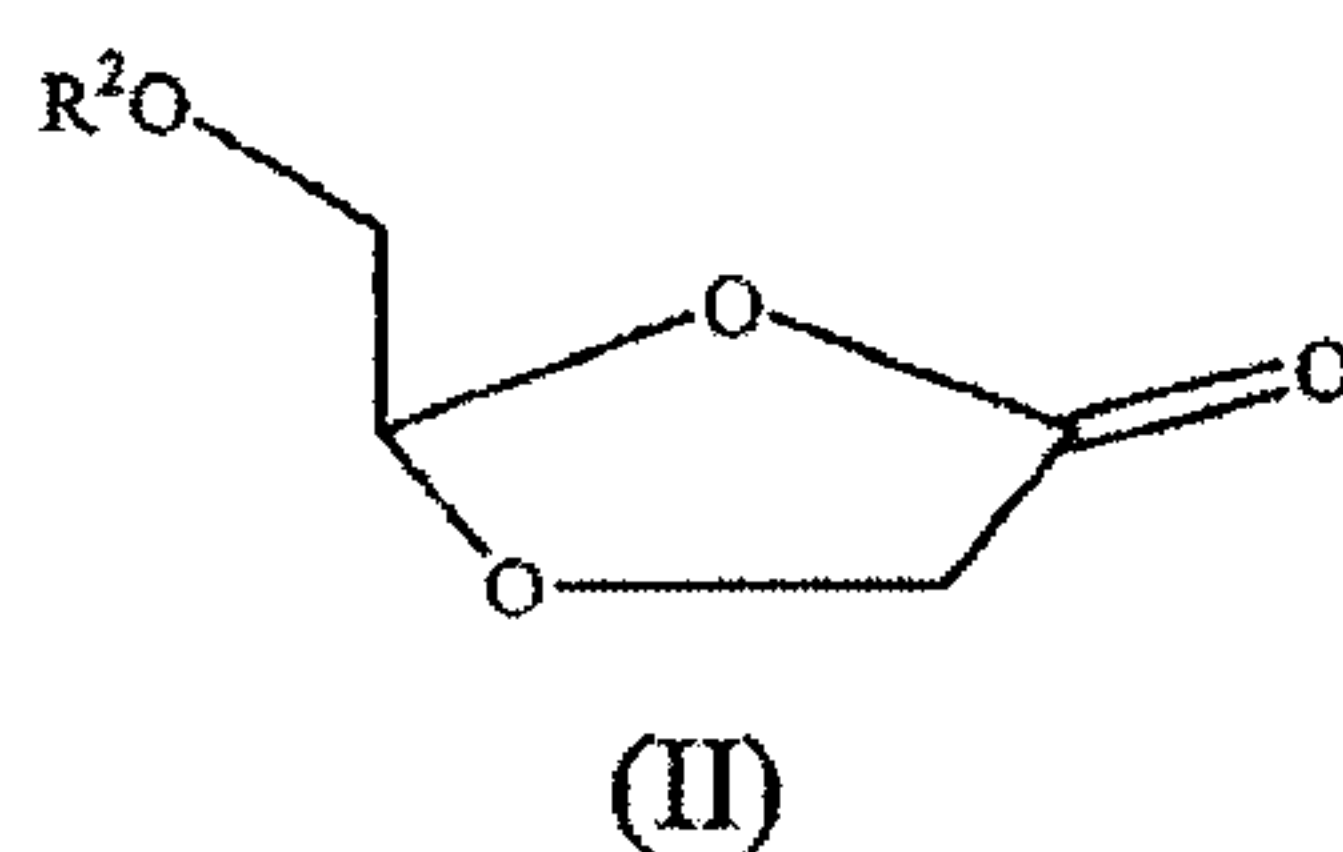


wherein:

each R^1 is independently alkyl, aryl, heteroaryl, heterocyclic, alkaryl, alkylheteroaryl, alkylheterocyclic, or aralkyl; and

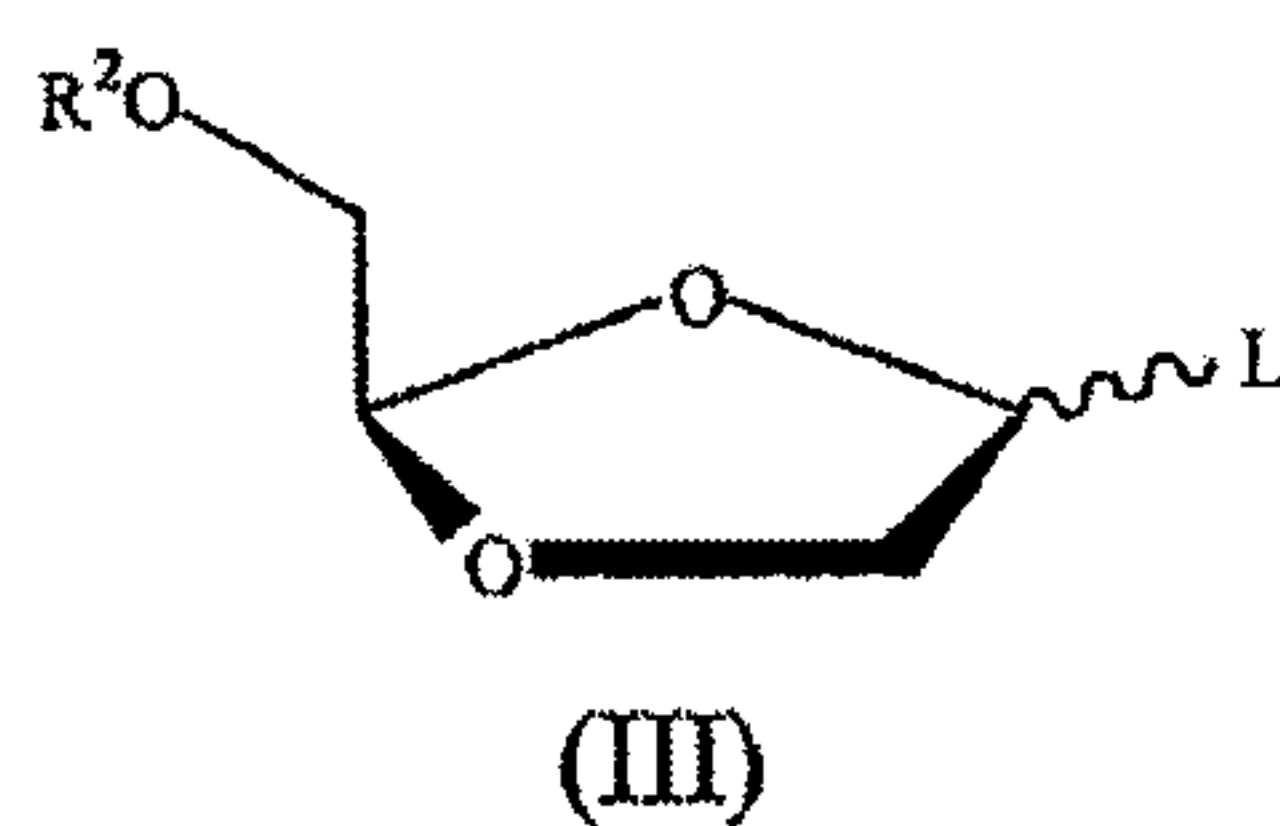
R^2 is any suitable removable group;

- b) cyclizing the esterified 2,2-dialkoxy ethanol of the formula (Ia) or (Ib) with glycolic acid to obtain a 1,3-dioxolane lactone of the formula (II):



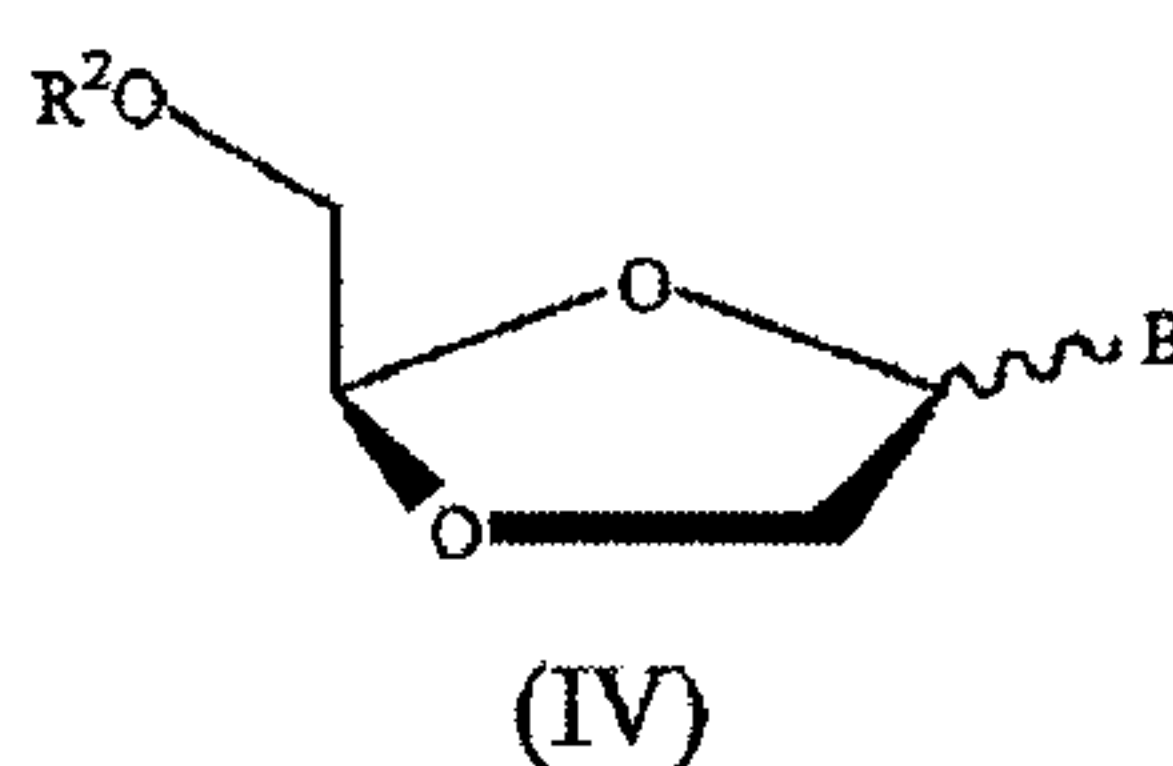
- c) resolving the 1,3-dioxolane lactone of the formula (II) to obtain a substantially pure D- or L-lactone;
- d) selectively reducing with a reducing agent and activating the substantially pure D- or L-chiral lactone to obtain a substantially pure D- or L-1,3-dioxolane of the formula (III):

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wherein L is a suitable leaving group;

- e) coupling the substantially pure D- or L-1,3-dioxolane of the formula (III) to an activated and/or protected purine or pyrimidine base or its derivative to obtain α : β mixture of substantially pure protected D- or L-1,3-dioxolane nucleosides of the formula (IV):

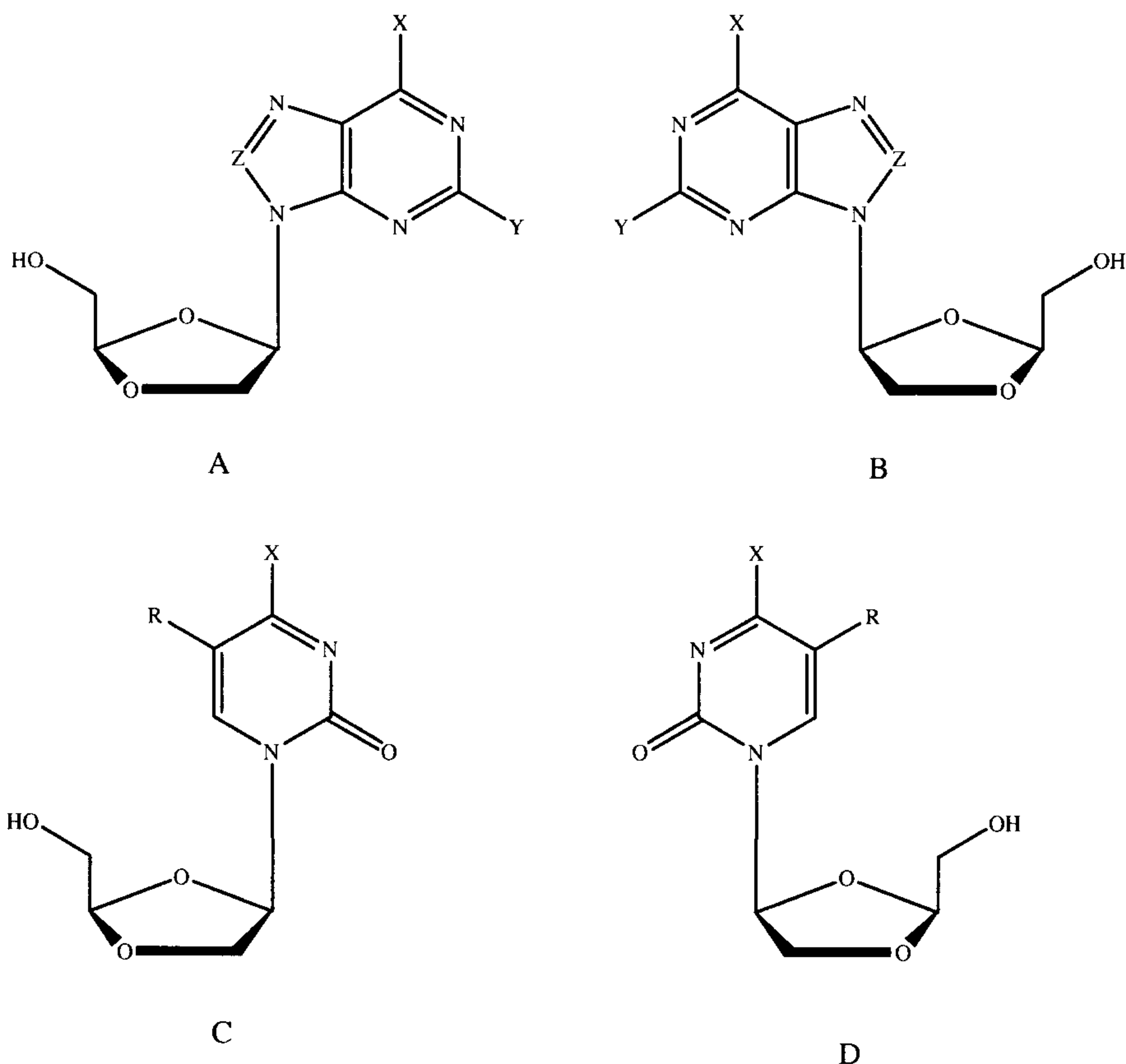


wherein B is a purine or pyrimidine base or its derivative;

- f) optionally purifying the α : β mixture of substantially pure protected D- or L-1,3-dioxolane nucleosides of the formula (IV) to obtain a substantially pure protected β -D- or β -L-1,3-dioxolane nucleoside; and
- g) optionally deprotecting the substantially pure protected β -D- or β -L-1,3-dioxolane nucleoside to obtain a substantially pure β -D- or β -L-1,3-dioxolane nucleoside.
2. The process of claim 1 wherein the substantially pure β -D- or β -L-1,3-dioxolane nucleosides is 9-(β -D-hydroxymethyl-1,3-dioxolanyl) aminopurine.
 3. The process of claim 1 wherein step (b) is in the presence of a Lewis acid.
 4. The process of claim 3 wherein the lewis acid is $\text{BF}_3 \cdot \text{Et}_2\text{O}$.

5. The process of claim 1 wherein the reducing agent in step (d) is $\text{LiAlH}(\text{OtBu})_3$.
6. The process of claim 1 wherein the suitable leaving group is selected from the group consisting of O-acyl, OAc, halogen, OMesylates and OToluates.
7. The process of claim 1 wherein R^2 is *iso*-butyryl or *p*-methoxy benzoyl.
8. The process of claim 1 wherein the activated and/or protected purine or pyrimidine base or its derivative is activated 2,6-dichloropurine.
9. The process of claim 1 wherein the substantially pure protected β -D- or β -L-1,3-dioxolane nucleoside is deprotected.
10. The process of claim 1 wherein the esterified 2,2-dialkoxy ethanol of the formula (Ia) is hydrolyzed to the corresponding aldehyde of formula (Ib).
11. The process of claim 1 wherein the esterified 2,2-dialkoxy ethanol of the formula (Ia) is hydrolyzed to the corresponding aldehyde of formula (Ib) when R^2 is *p*-methoxy benzoyl.
12. The process of claim 1 wherein the esterified 2,2-dialkoxy ethanol of the formula (Ia) is not hydrolyzed to the corresponding aldehyde of formula (Ib).
13. The process of claim 1 wherein the esterified 2,2-dialkoxy ethanol of the formula (Ia) is not hydrolyzed to the corresponding aldehyde of formula (Ib) when R^2 is *iso*-butyryl.
14. The process of claim 1 wherein the resolution of the 1,3-dioxolane lactone of the formula (II) is accomplished using chiral chromatography.
15. The process of claim 1 wherein the resolution of the 1,3-dioxolane lactone of the formula (II) is accomplished using enzymatic resolution.

16. The process of claim 1 wherein the substantially pure β -D- or β -L-1,3-dioxolane nucleosides is selected from the group consisting of formulas **A**, **B**, **C** and **D**:



and pharmaceutically acceptable salts or esters thereof, wherein:

R is independently H, halogen, OH, OR', OCH₃, SH, SR', SCH₃, NH₂, NHR', NR'₂, lower alkyl of C₁-C₄, CH₃, CH=CH₂, N₃C=CH₂, CO₂H, CO₂R', CONH₂, CONHR', CH₂OH, CH₂CH₂OH, CF₃, CH₂CH₂F, CH=CHCO₂H, CH=CHCO₂R', CH=CHCl, CH=CHBr, or CH=CHI;

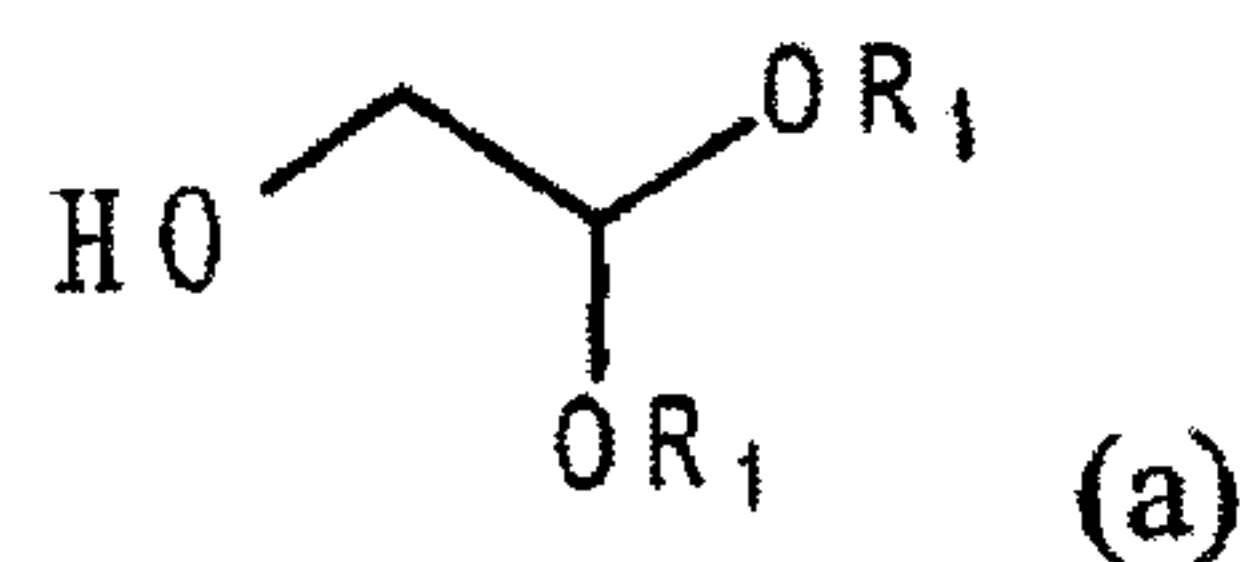
each R' is independently a lower alkyl of C₁-C₄;

Z is either CH or C-X; and

each X and Y are independently H, halogen, OH, OR', OCH₃, SH, SR', SCH₃, NH₂, NHR', NR'₂, or CH₃.

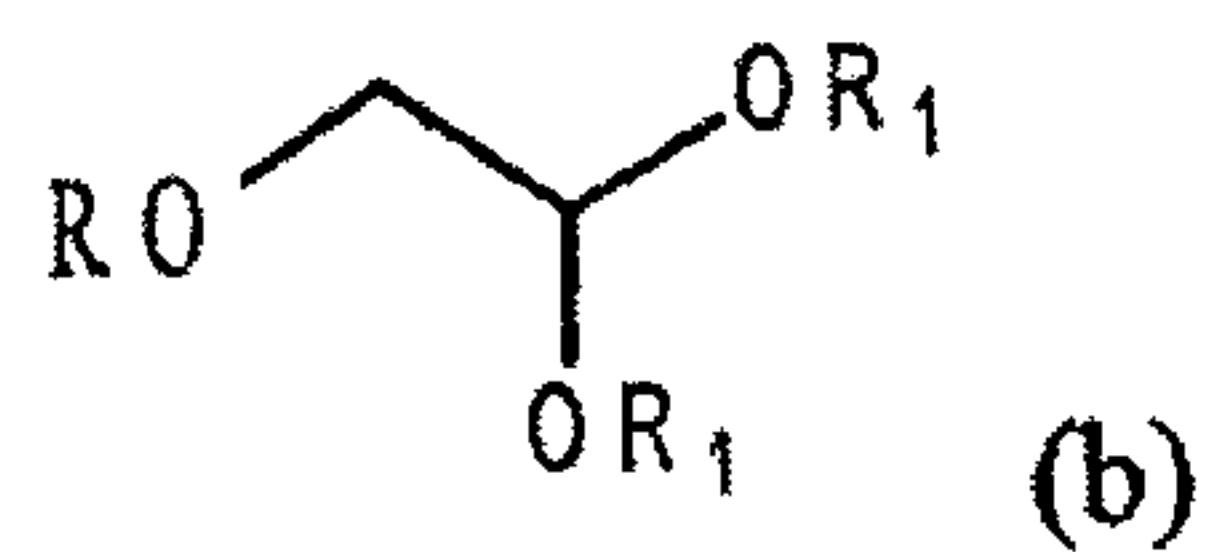
17. A process for preparing substantially pure 9-(β -D-hydroxymethyl-1,3-dioxolanyl) aminopurine, comprising:

- a) providing a compound of formula (a):



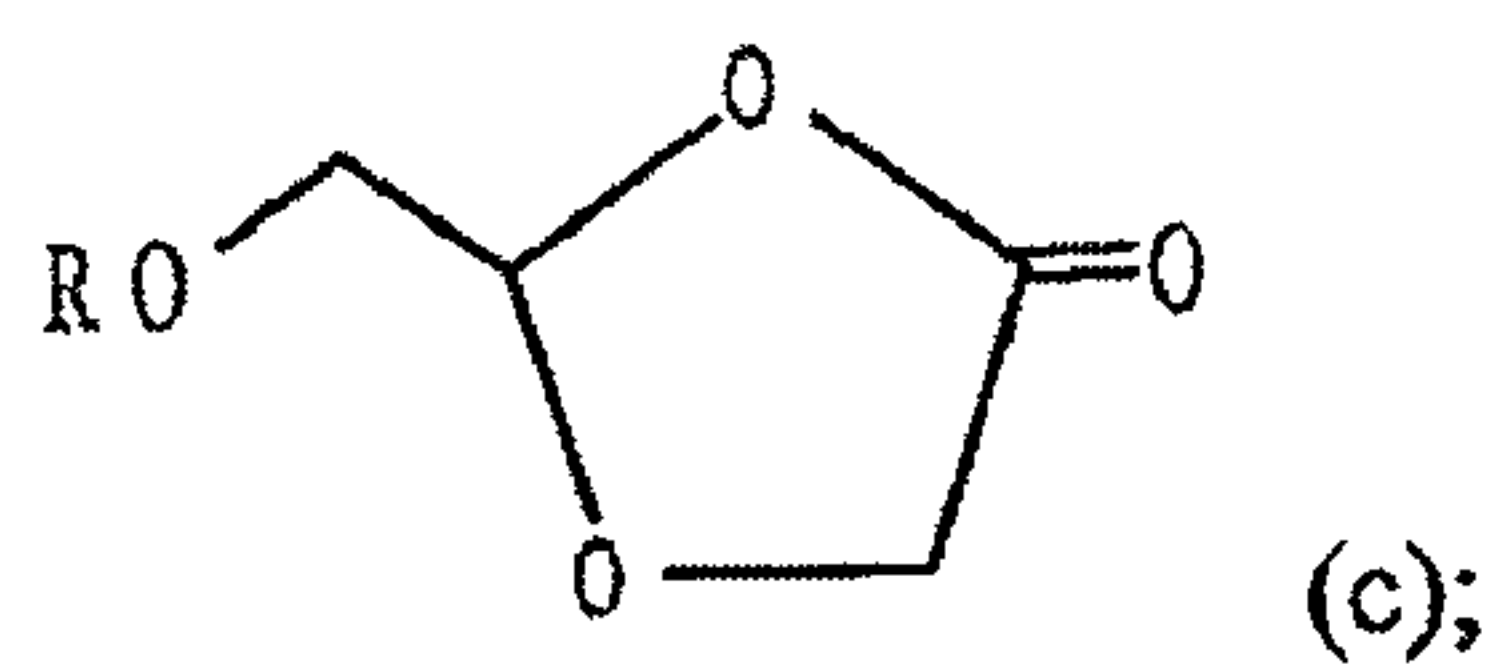
wherein each R^1 is independently alkyl, aryl, heteroaryl, heterocyclic, alkaryl, alkylheteroaryl, or alkylheterocyclic, or aralkyl;

- b) converting the compound of formula (a) to a compound of formula (b):

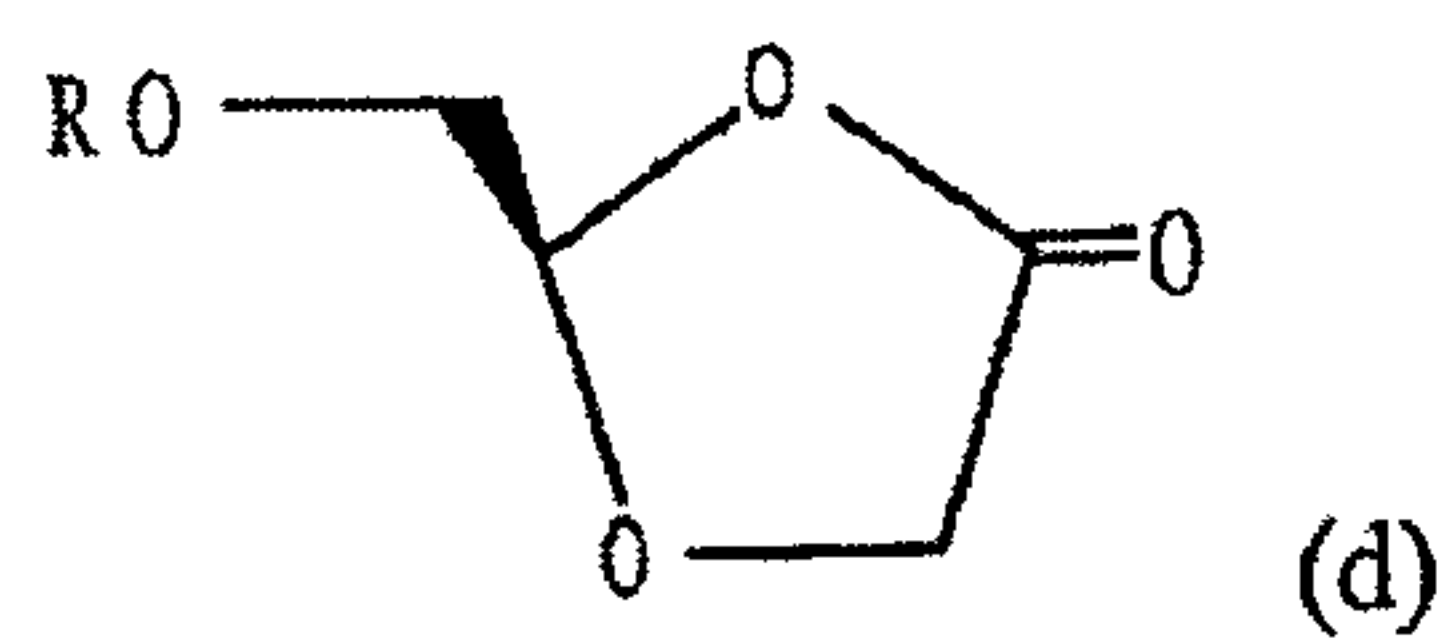


wherein OR is an ester;

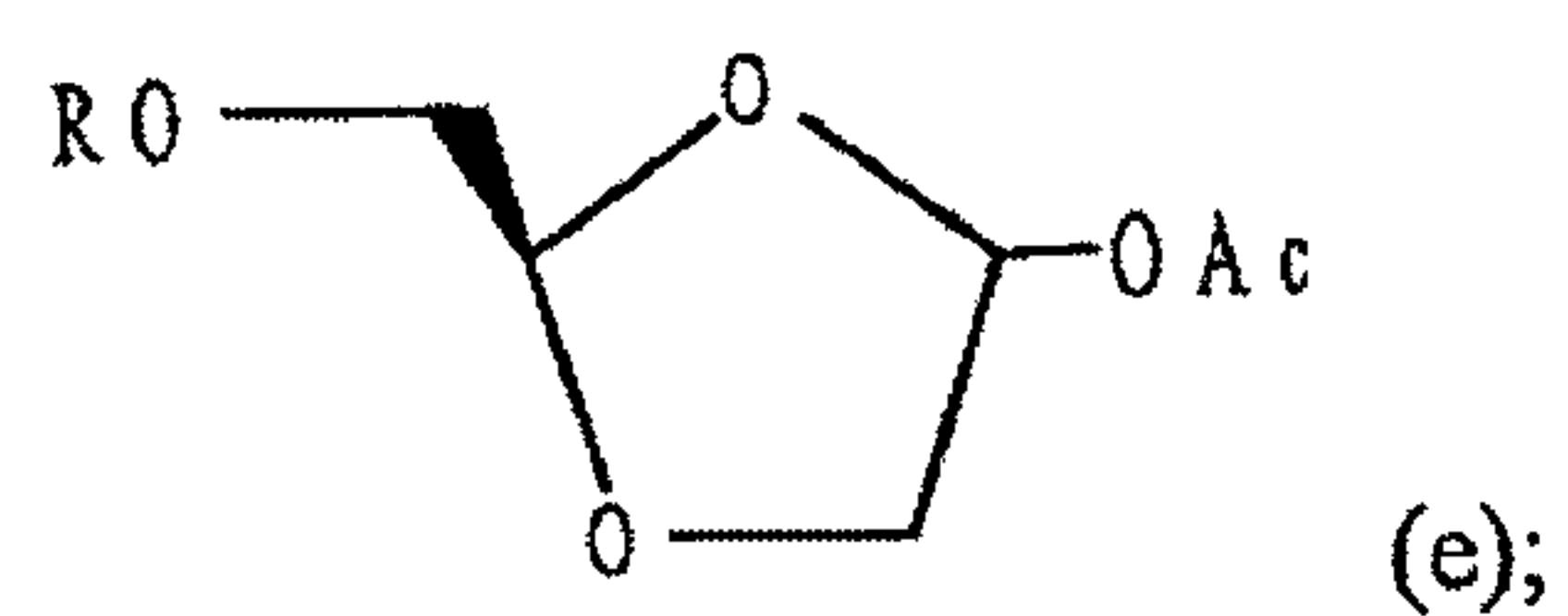
- c) cyclizing the compound of formula (b) to a racemic 1,3-dioxolone lactone of formula (c):



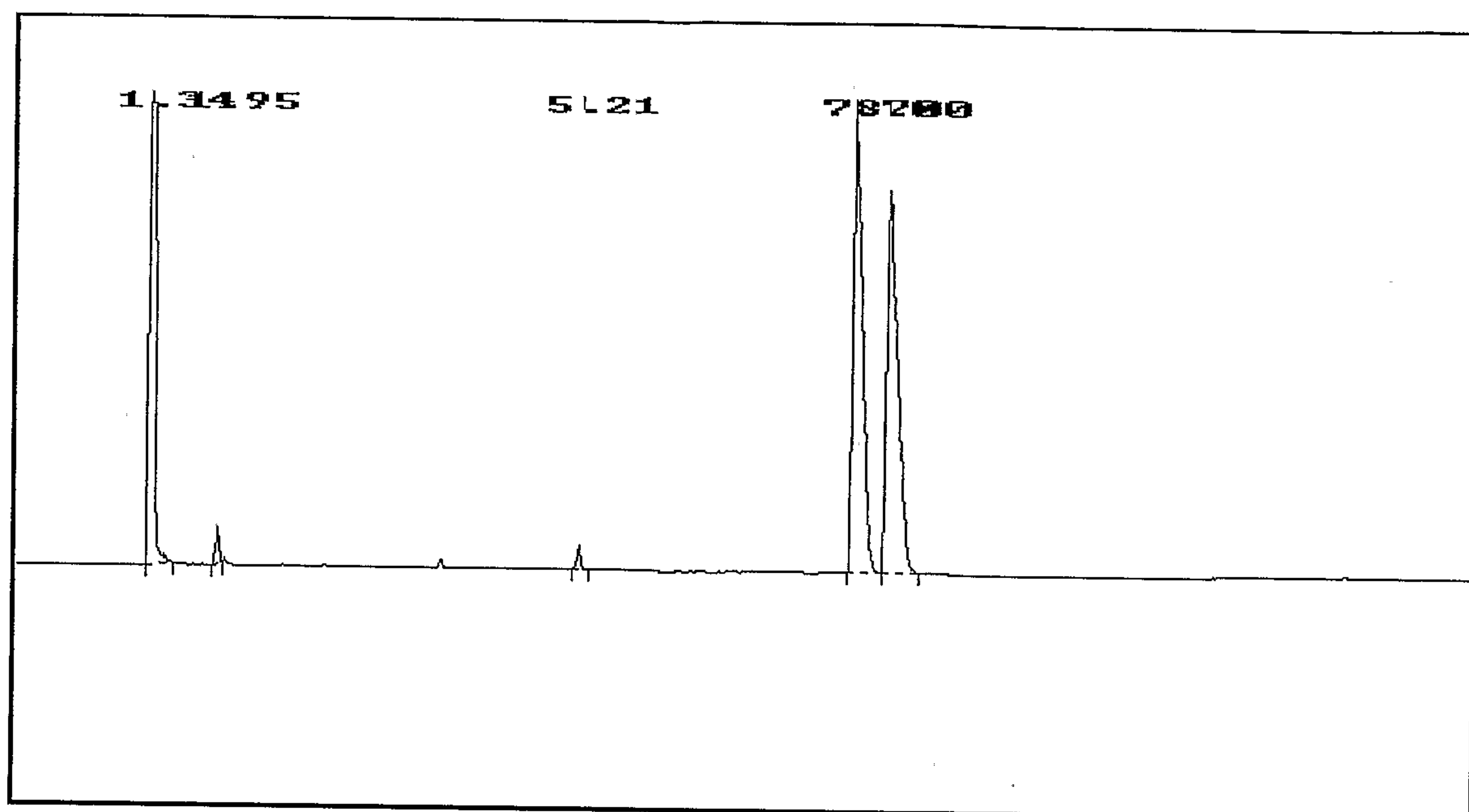
- d) resolving the compound of formula (c) to a chiral dioxolane lactone of formula (d);



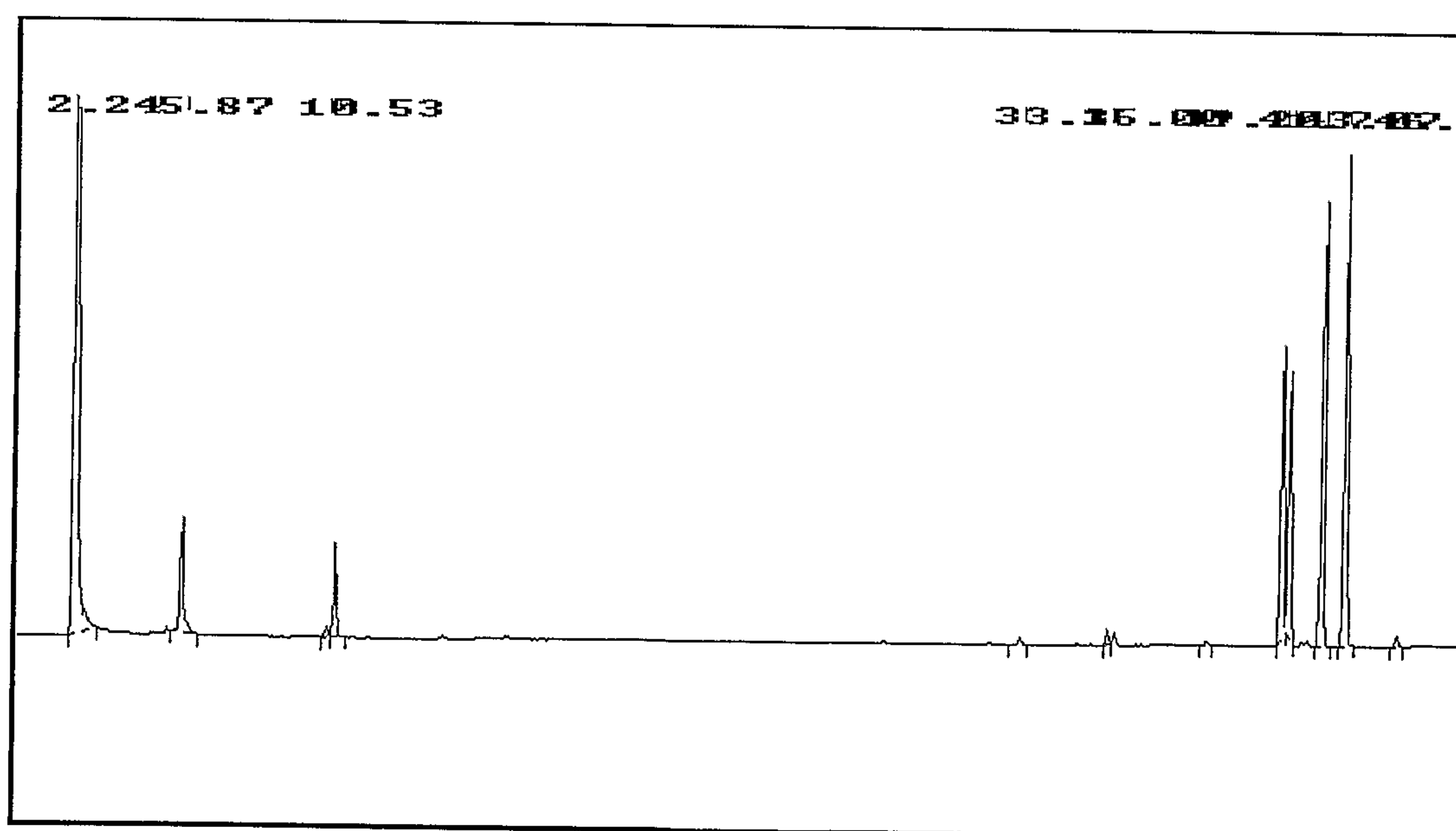
- e) acetylating the chiral dioxolane lactone of formula (d) to a chiral dioxolane acetate of formula (e); and



- f) coupling the compound of formula (e) to an activated and/or protected adenine base; and
- g) optionally purifying and deprotecting the compound of step (f) to obtain β -D-2,6-diaminopurine dioxolane.



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Figure 1A: Typical chromatogram for Compound 1

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Figure 1B: Typical chromatogram for Compound 3

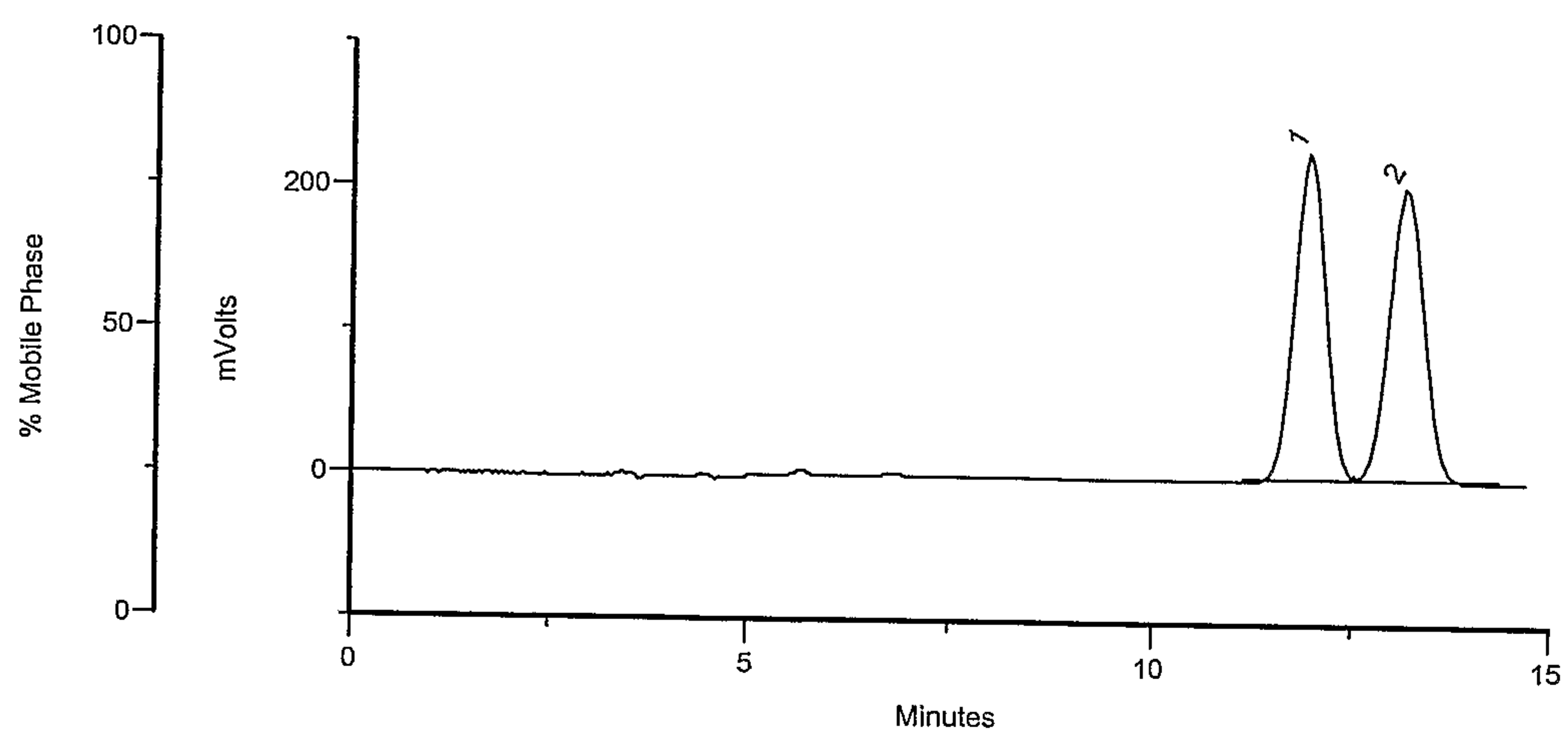


Figure 1C: HPLC method, Compound 11

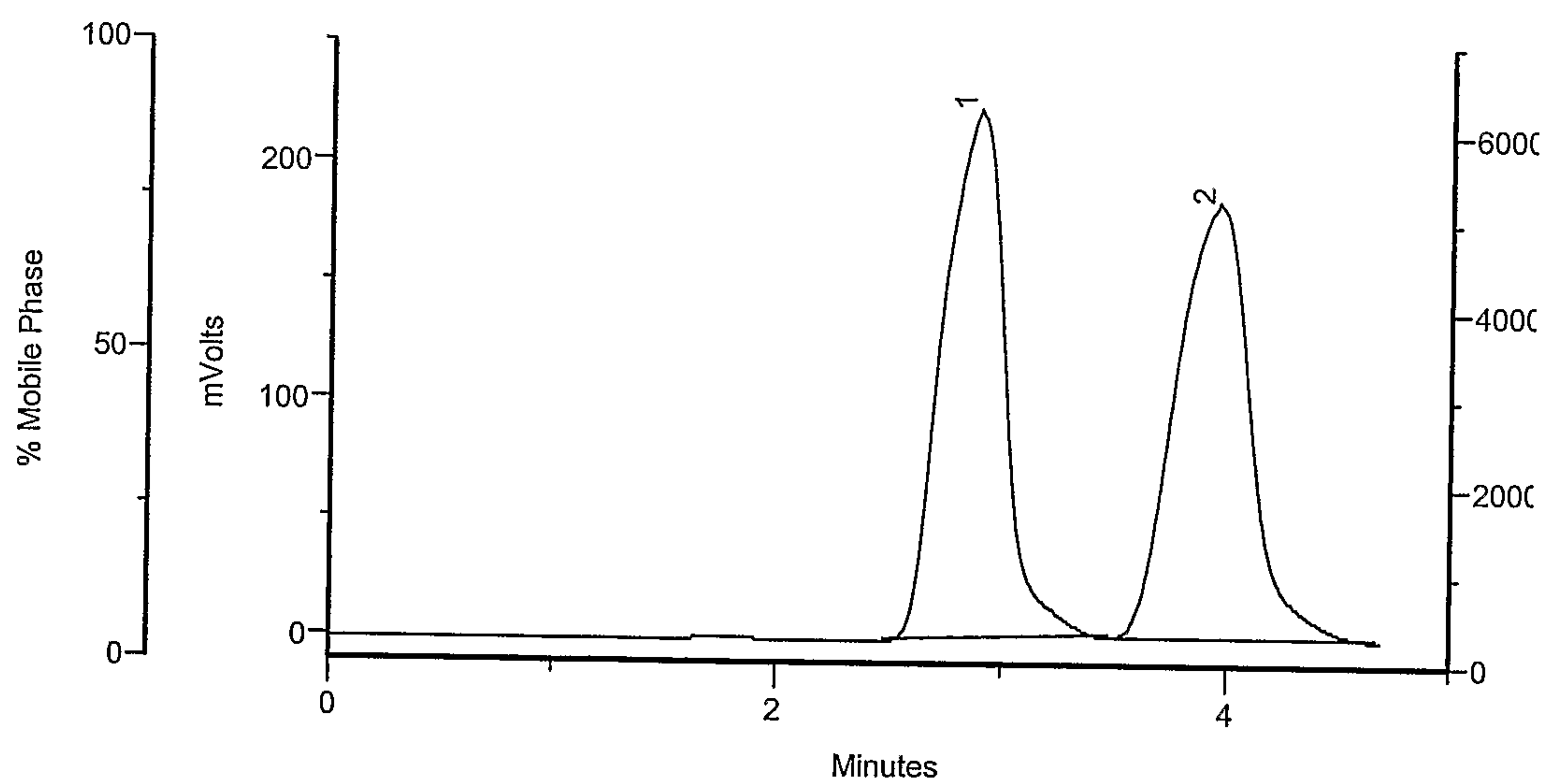


Figure 1D: SFC method, Compound 11

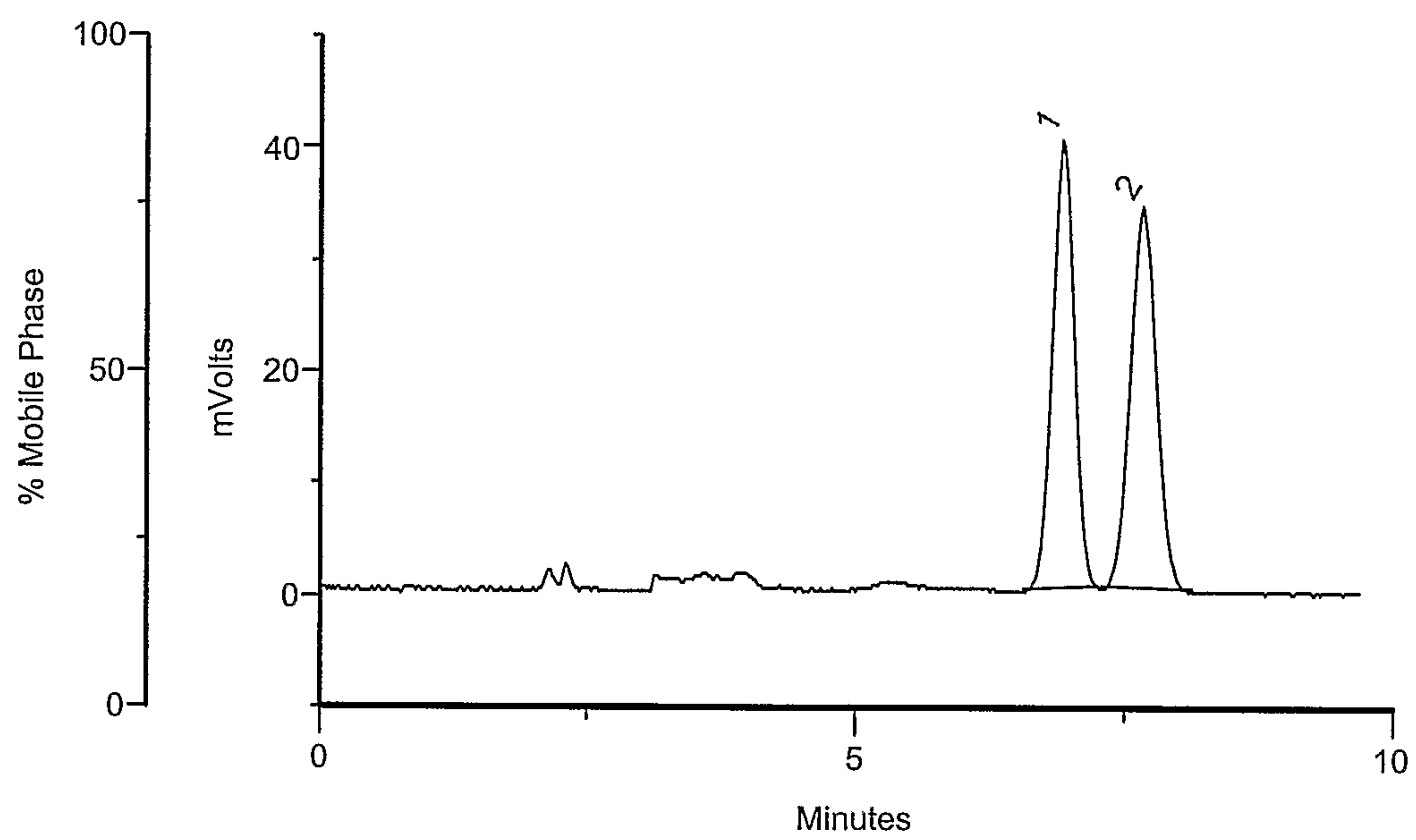


Figure 1E: HPLC method, Compound 12

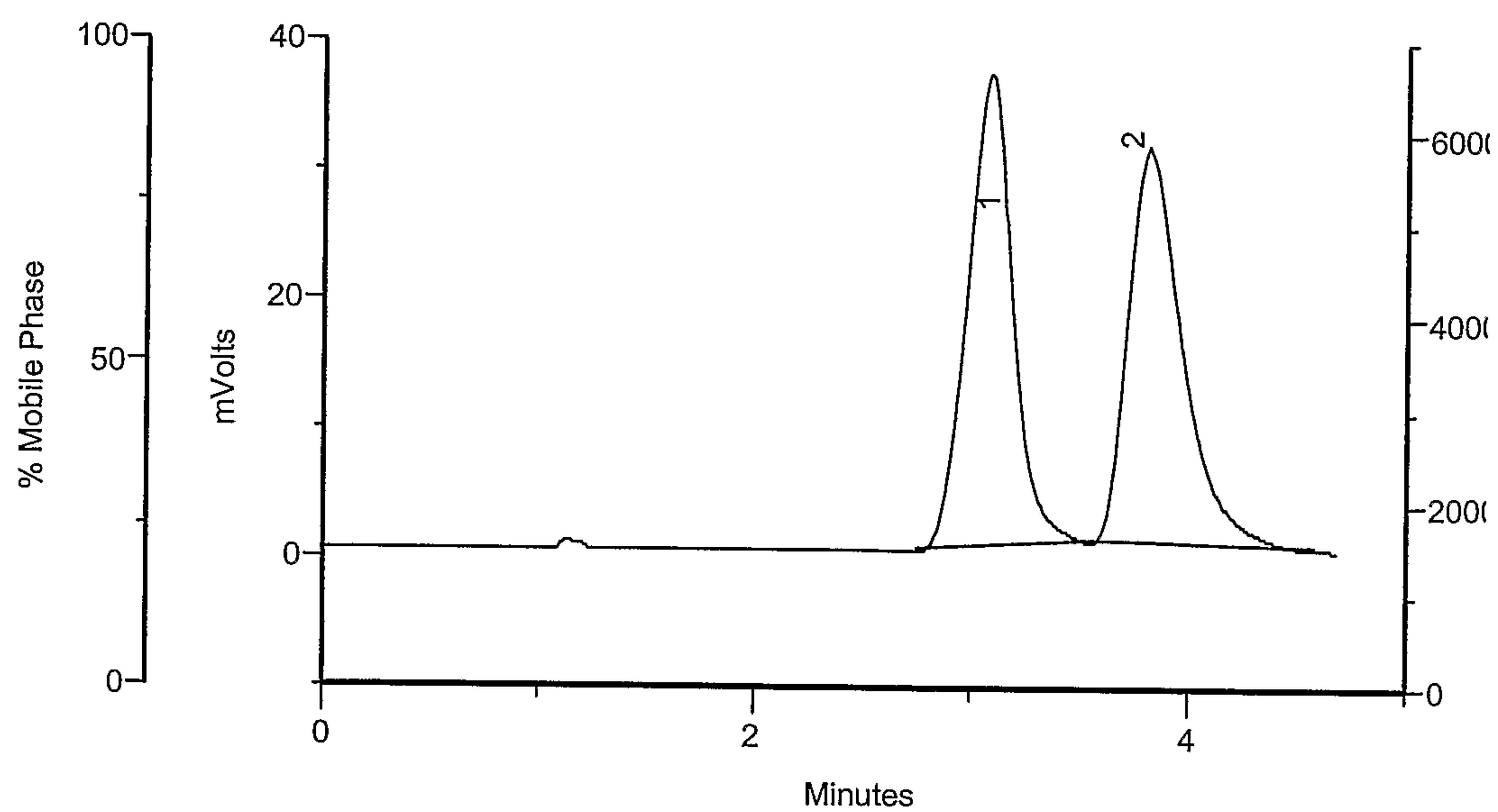


Figure 1F: SFC method, Compound 12

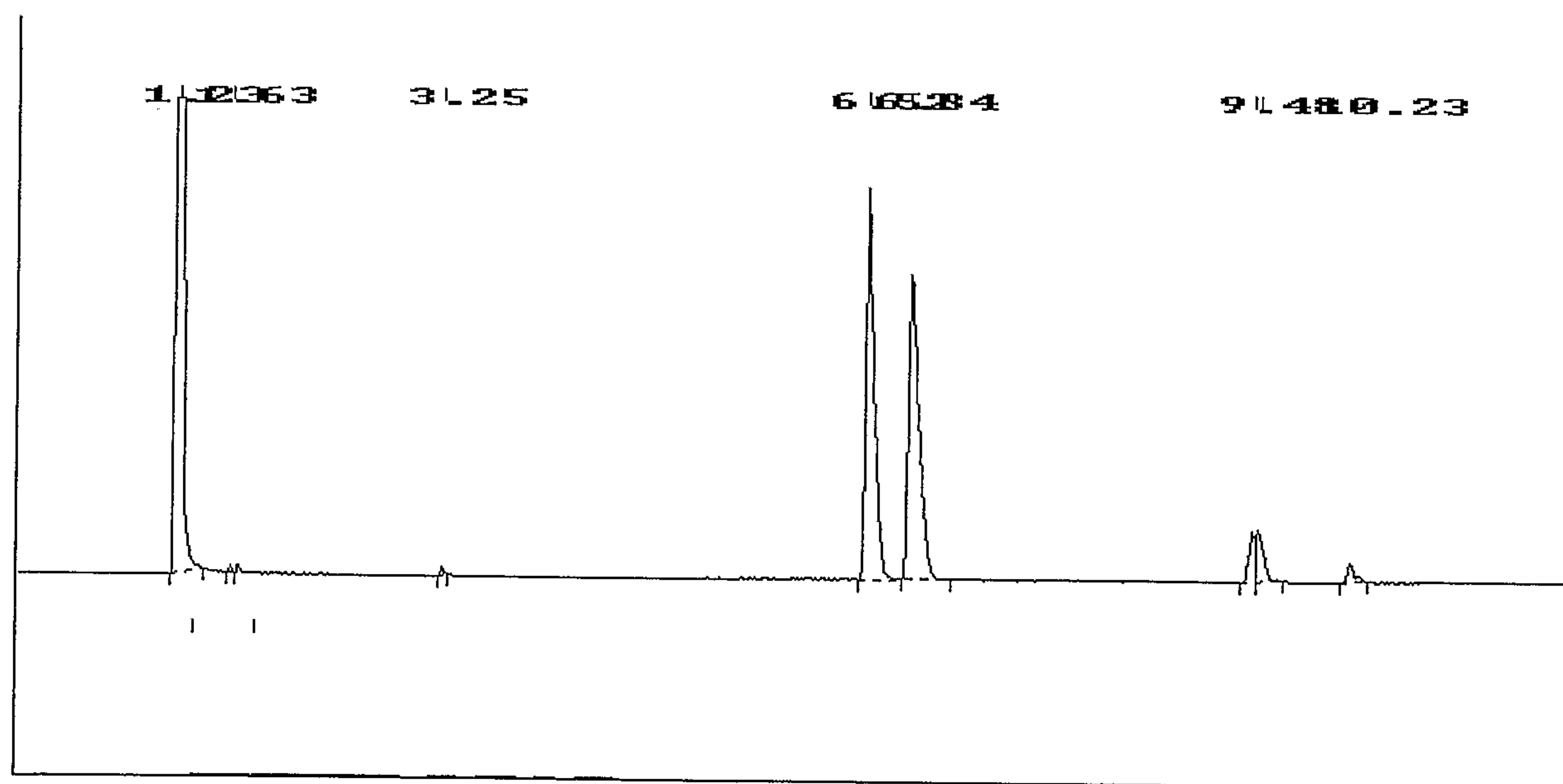


Figure 1G: Chiral GC method, Compound 13

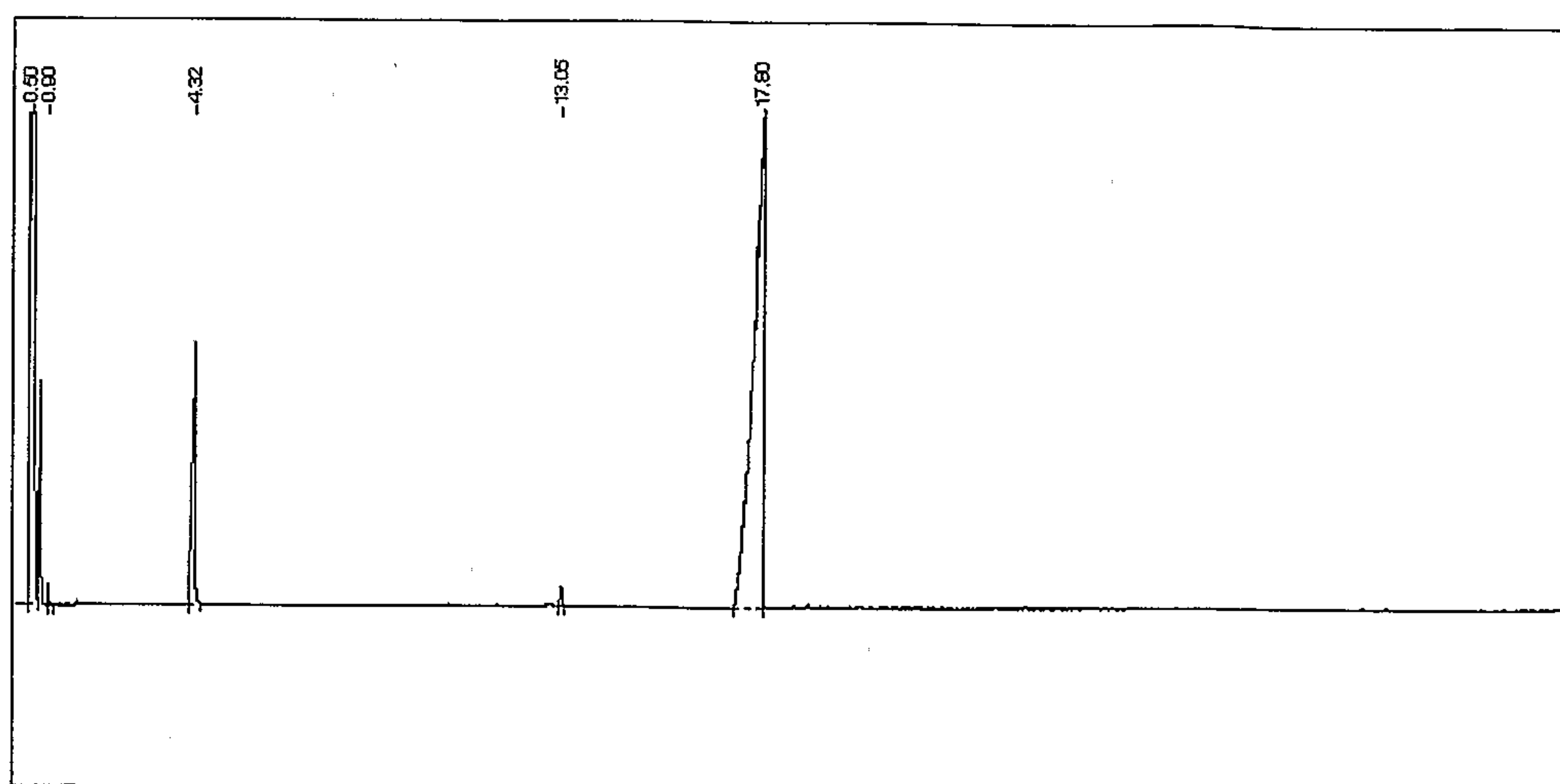


Figure 1H: GC purity method, Compound 13

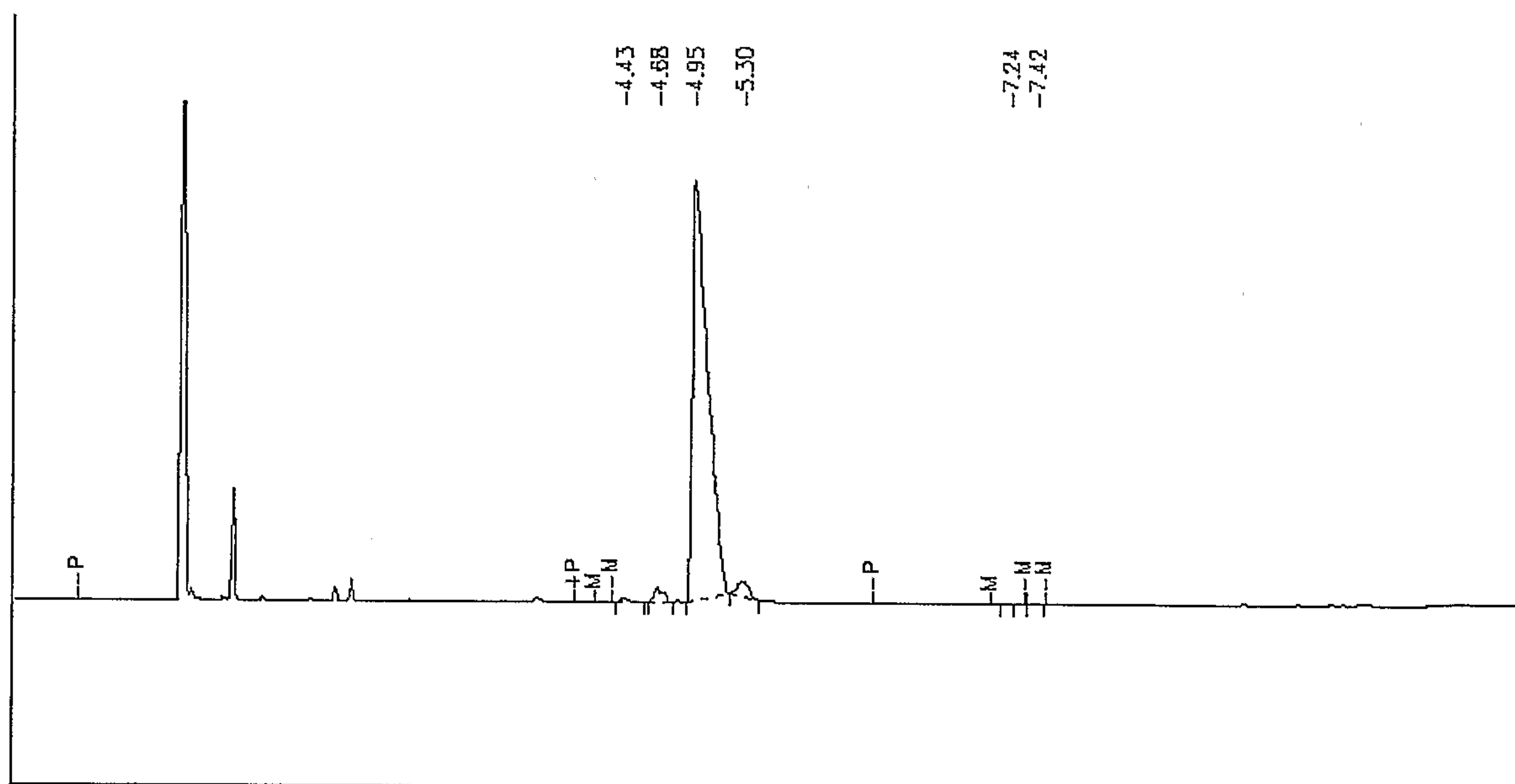
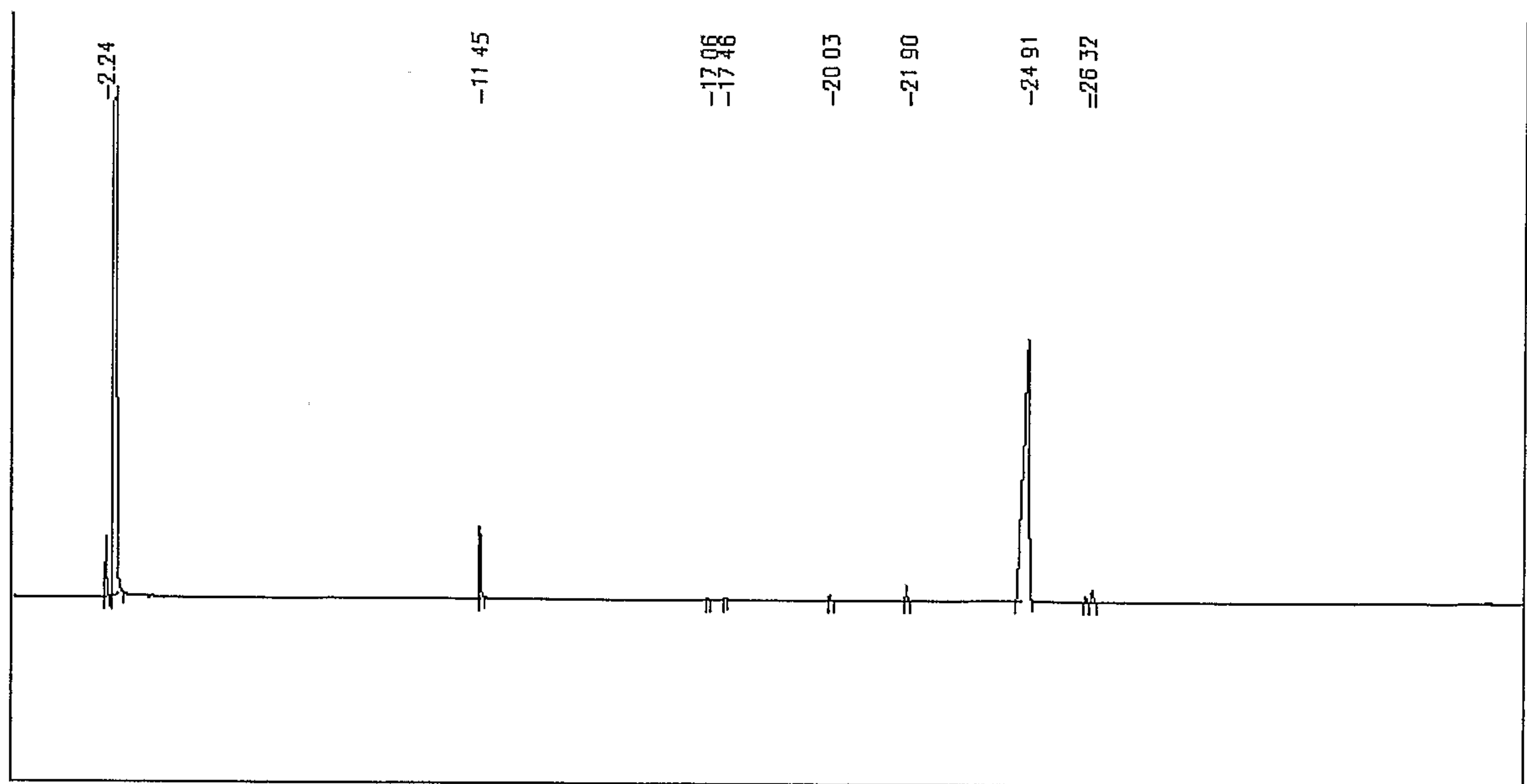
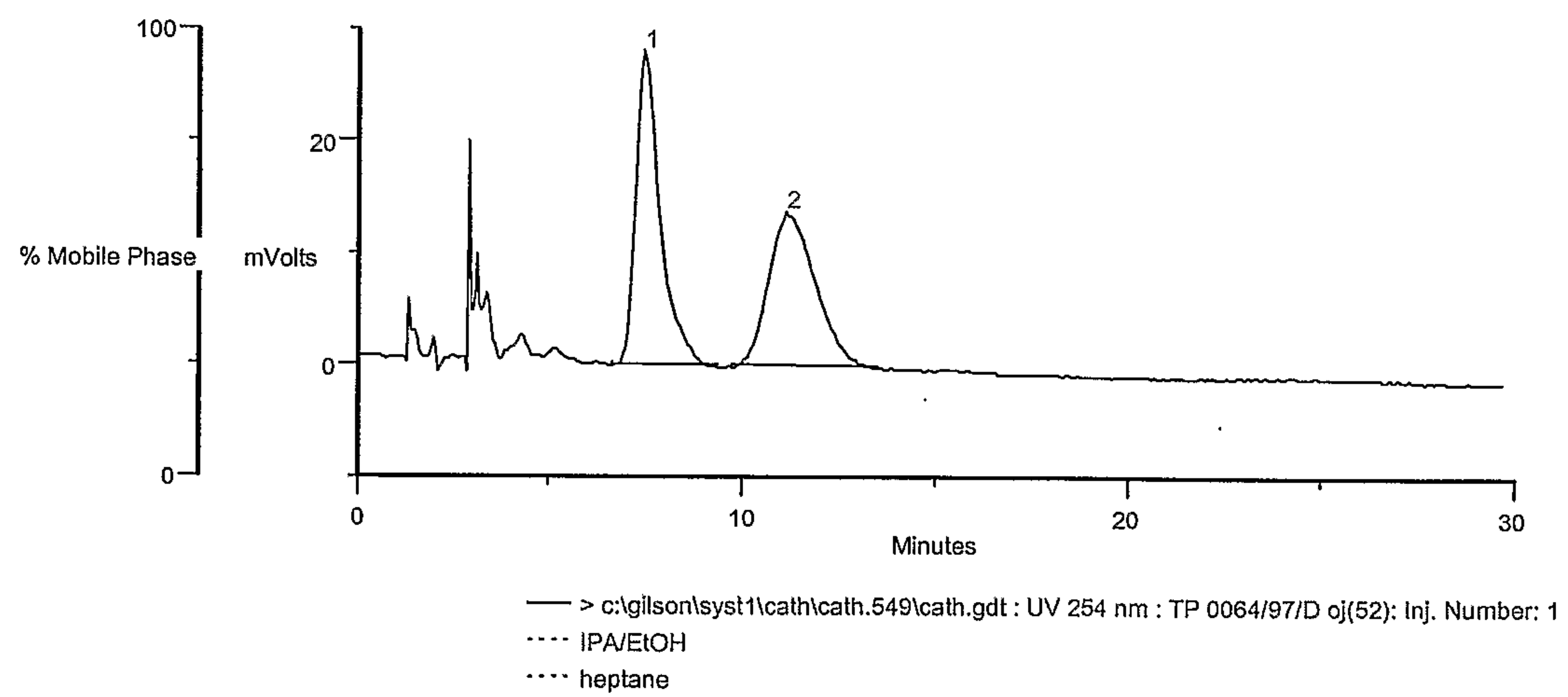


Figure 1I: Chiral GC Chromatogram, Compound 13

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**Figure 1J:** Achiral GC Chromatogram, Compound 13

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**Figure 1K:** Typical chromatogram for Compound 4

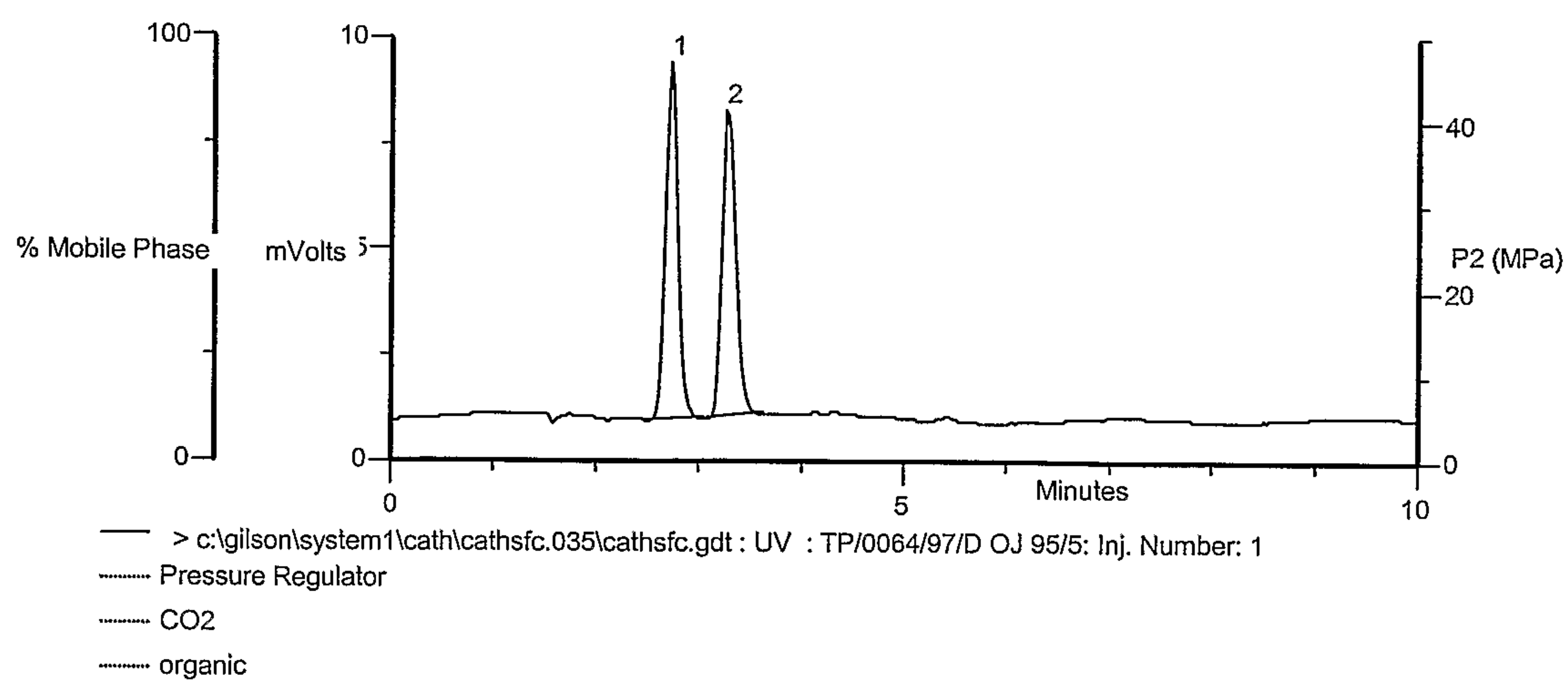


Figure 1L: Chromatogram of optimized assay for Compound 4

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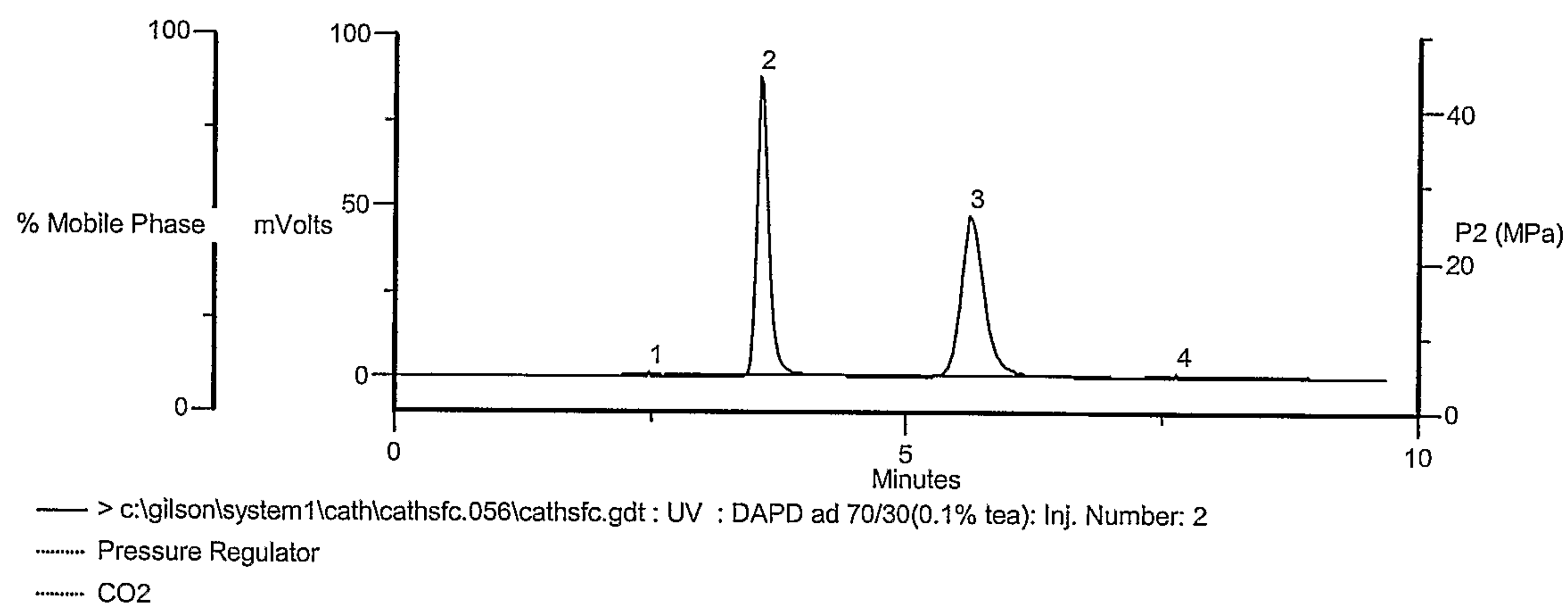
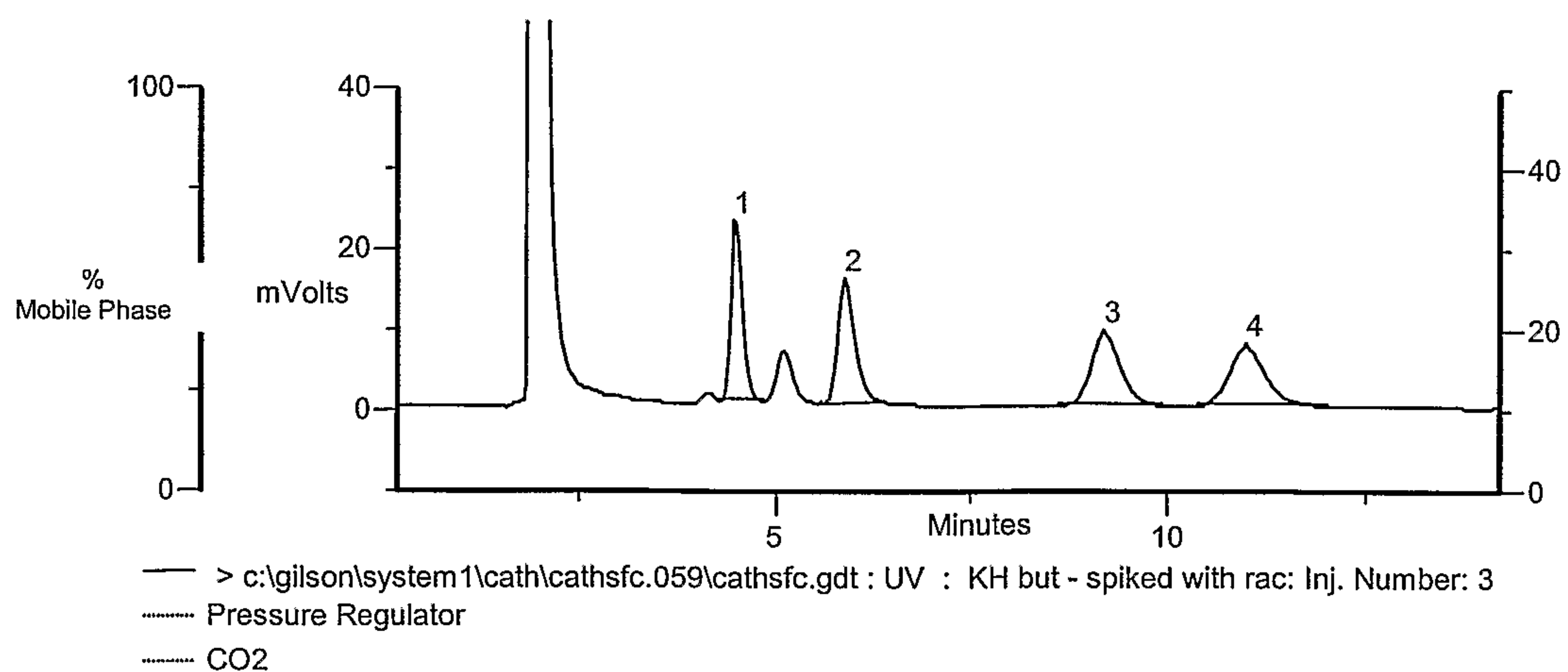


Figure 1M: Typical chromatogram for DAPD



Peaks 1 and 3 - DAPD butyrate

Peaks 2 and 4 - DAPD

5

Figure 1N: Typical chromatogram using SFC Conditions for DAPD and butyrate ester

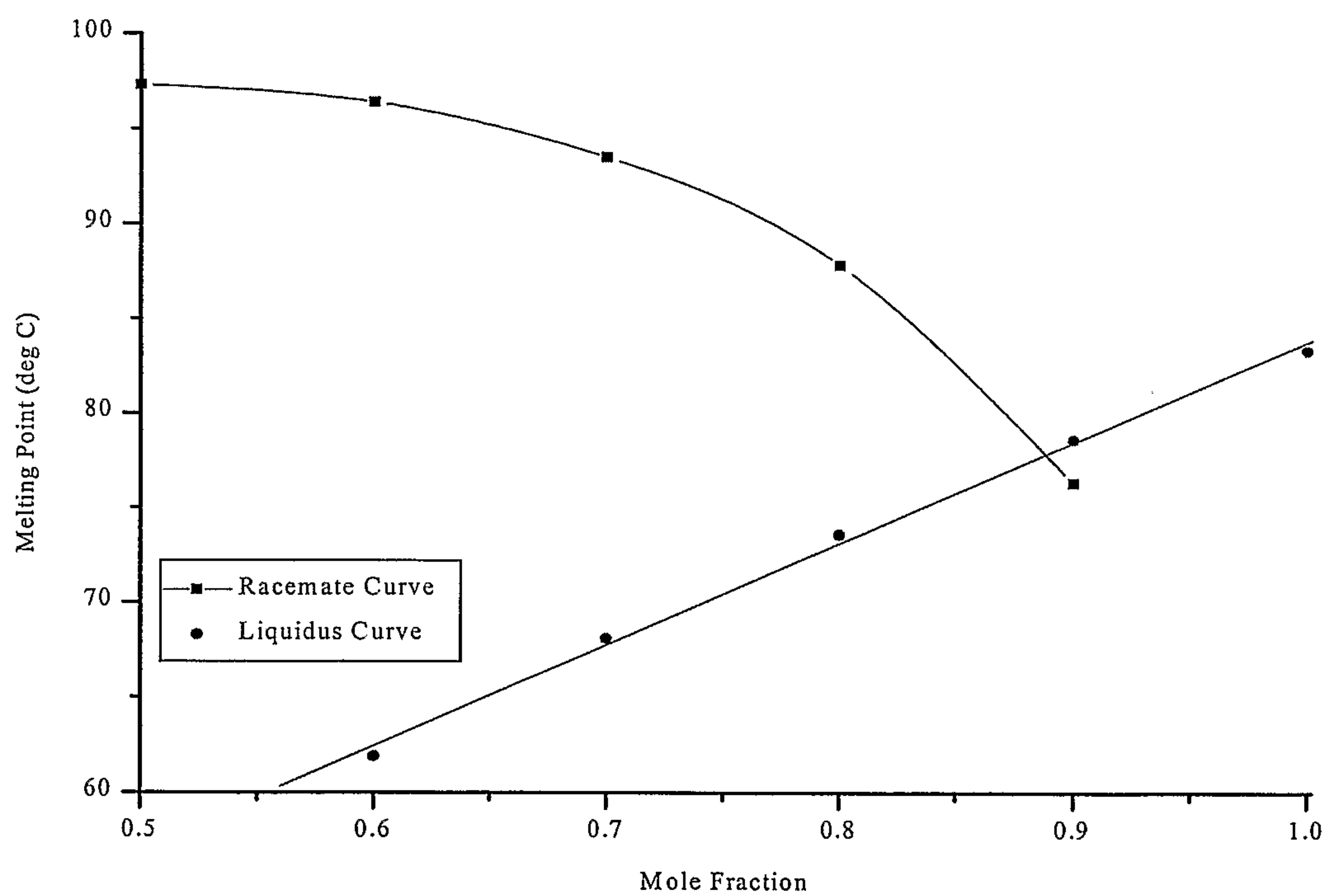


Figure 2: Theoretical phase diagram for Compound 4

10

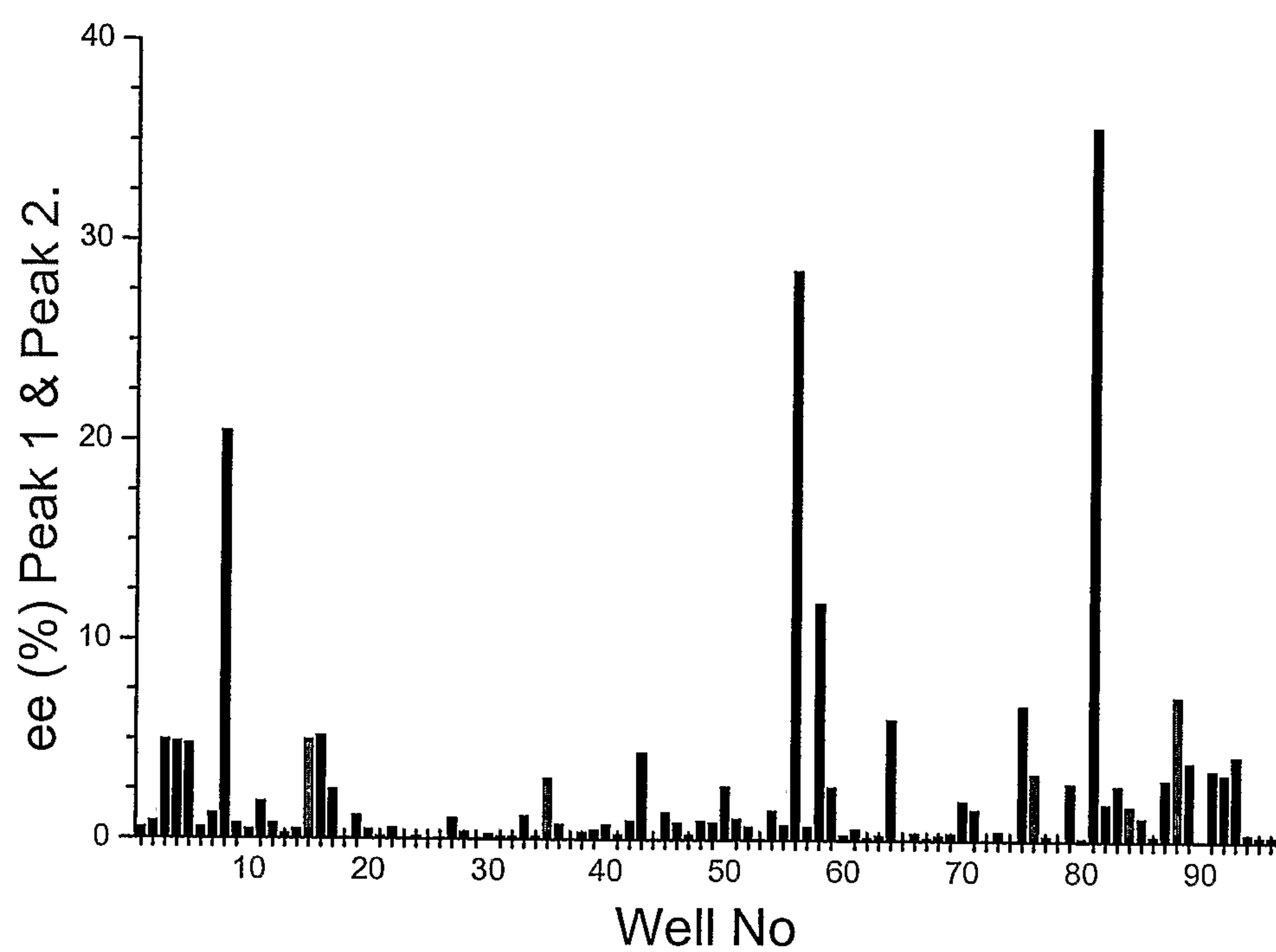


Figure 3A: Results from the screen of plate SCL0001 of Compound 1 (PK636/255)

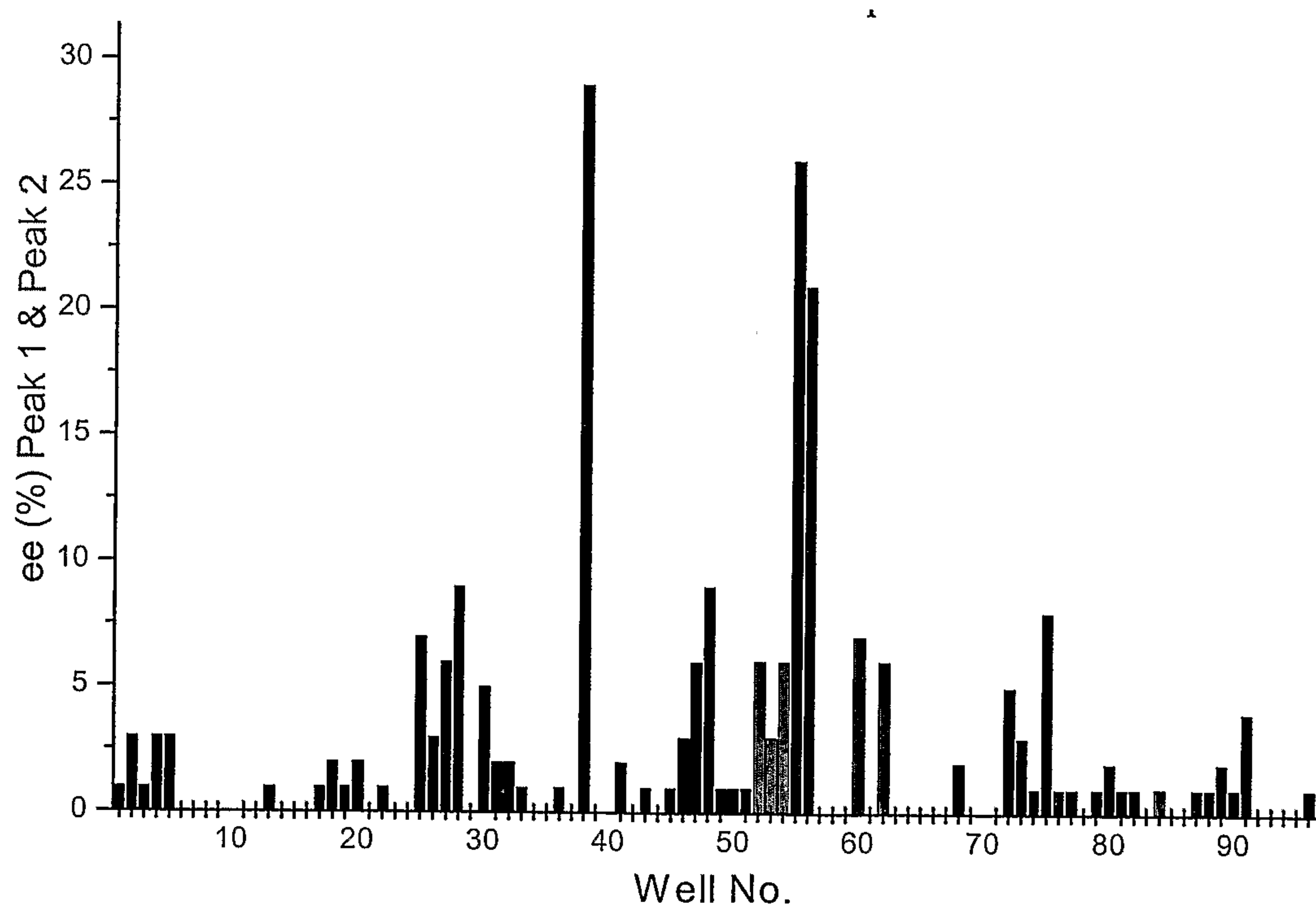
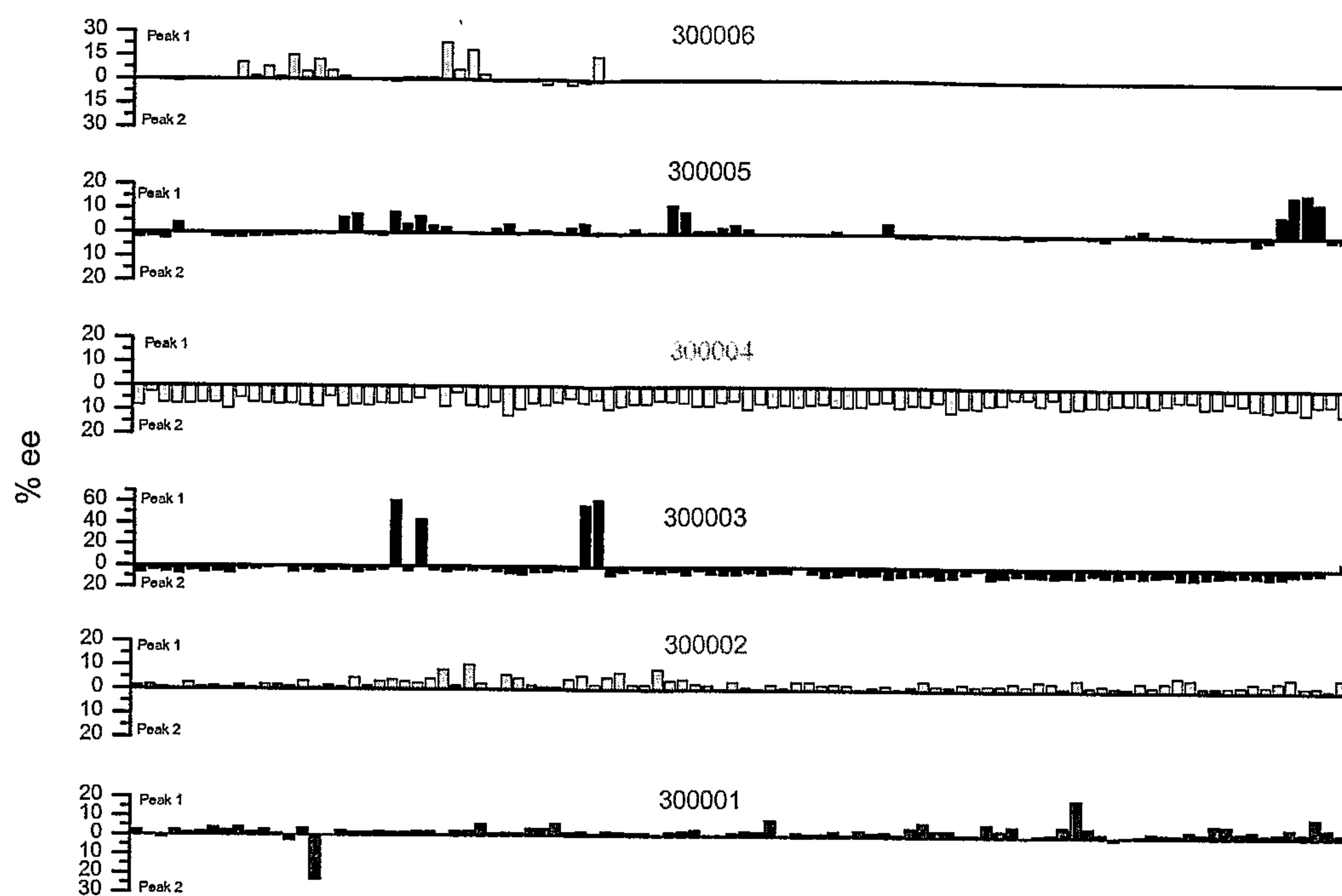
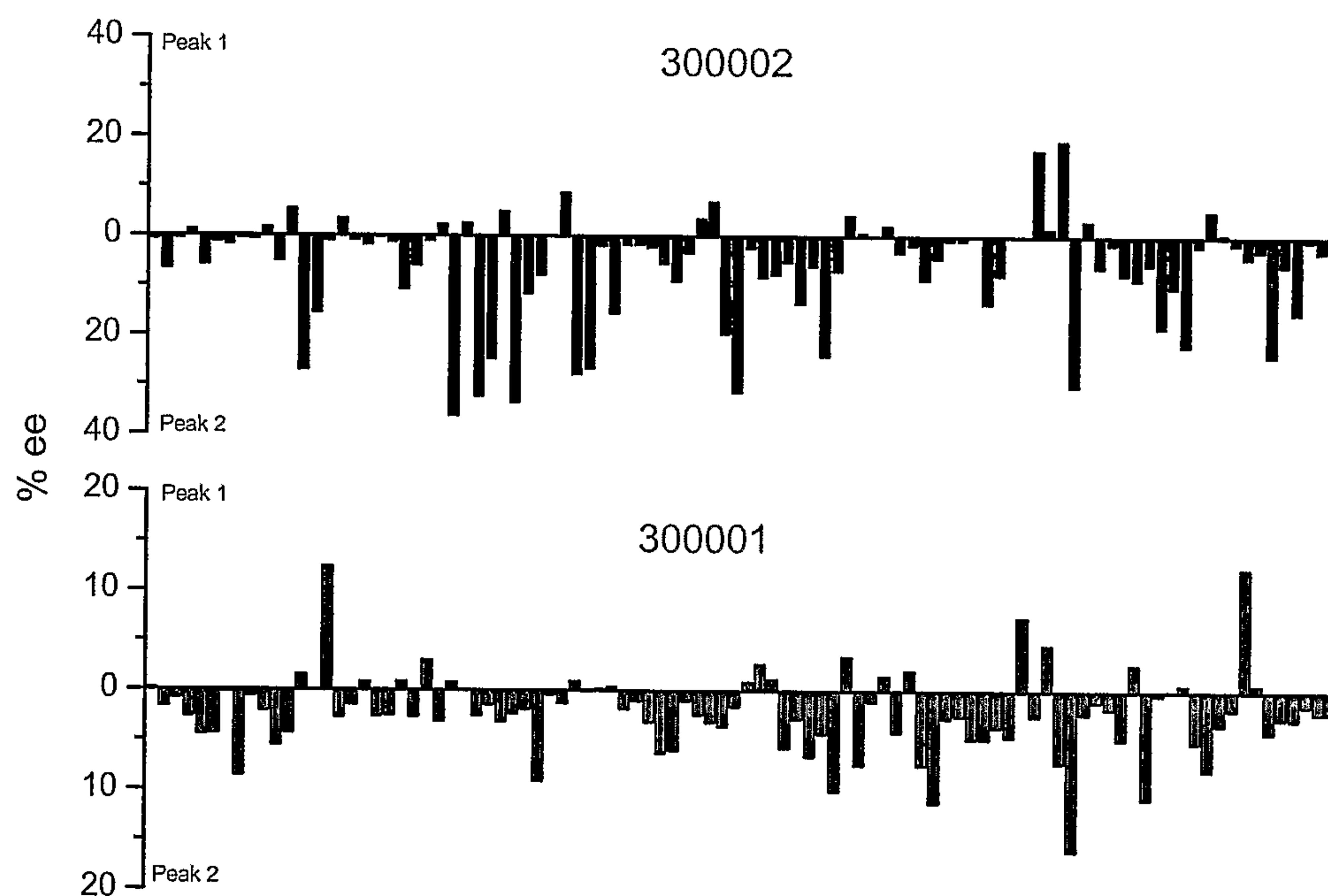


Figure 3B: Results from the screen of plate SCL0002 of Compound 1 (PK636/241)

10/13

**Figure 3C:** Microbial screen of Compound 11**Figure 3D:** Microbial Screen for Compound 12

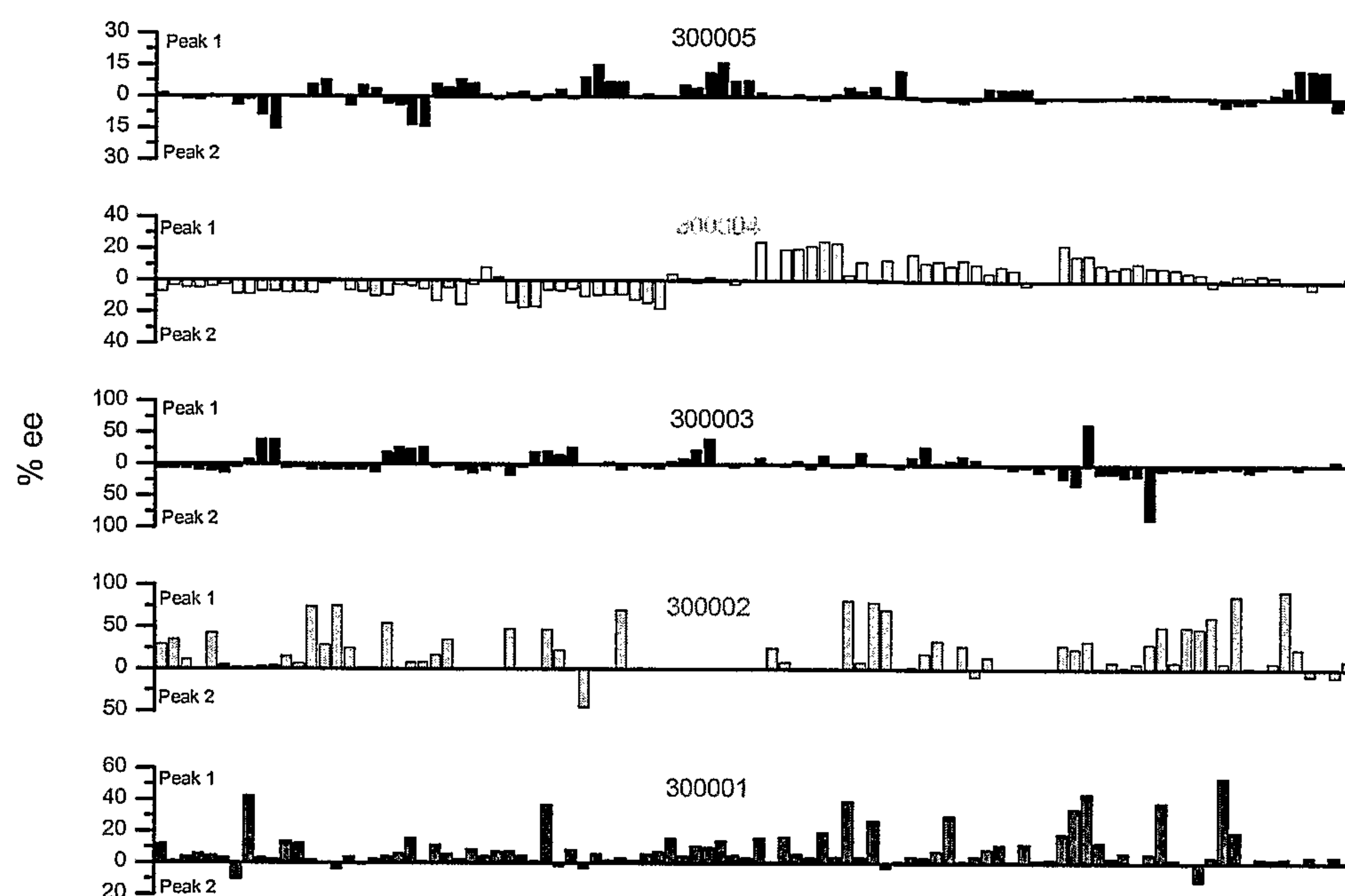


Figure 3E: Microbial Screen of Compound 13

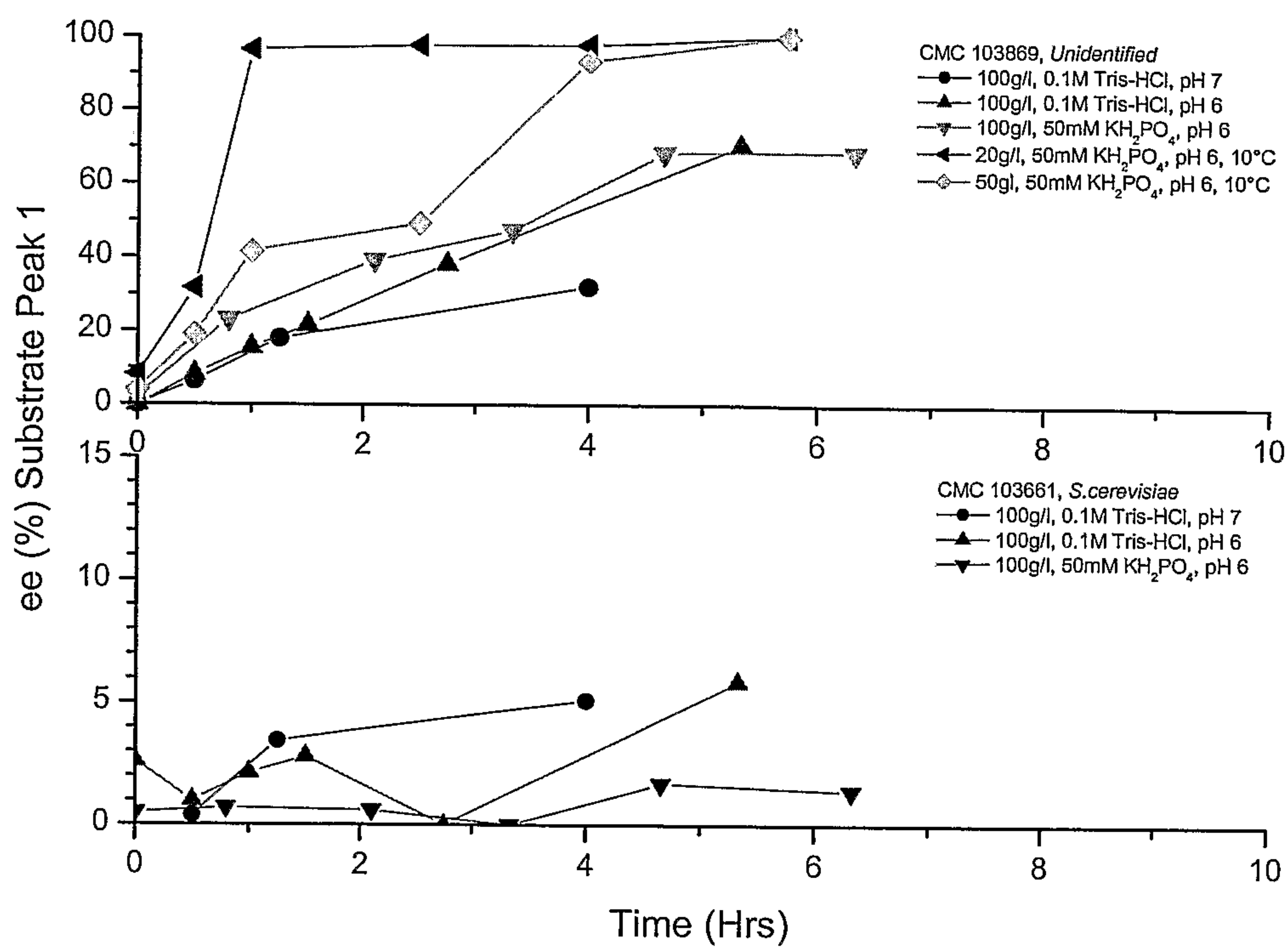


Figure 4: Microbial resolution of Compound 13

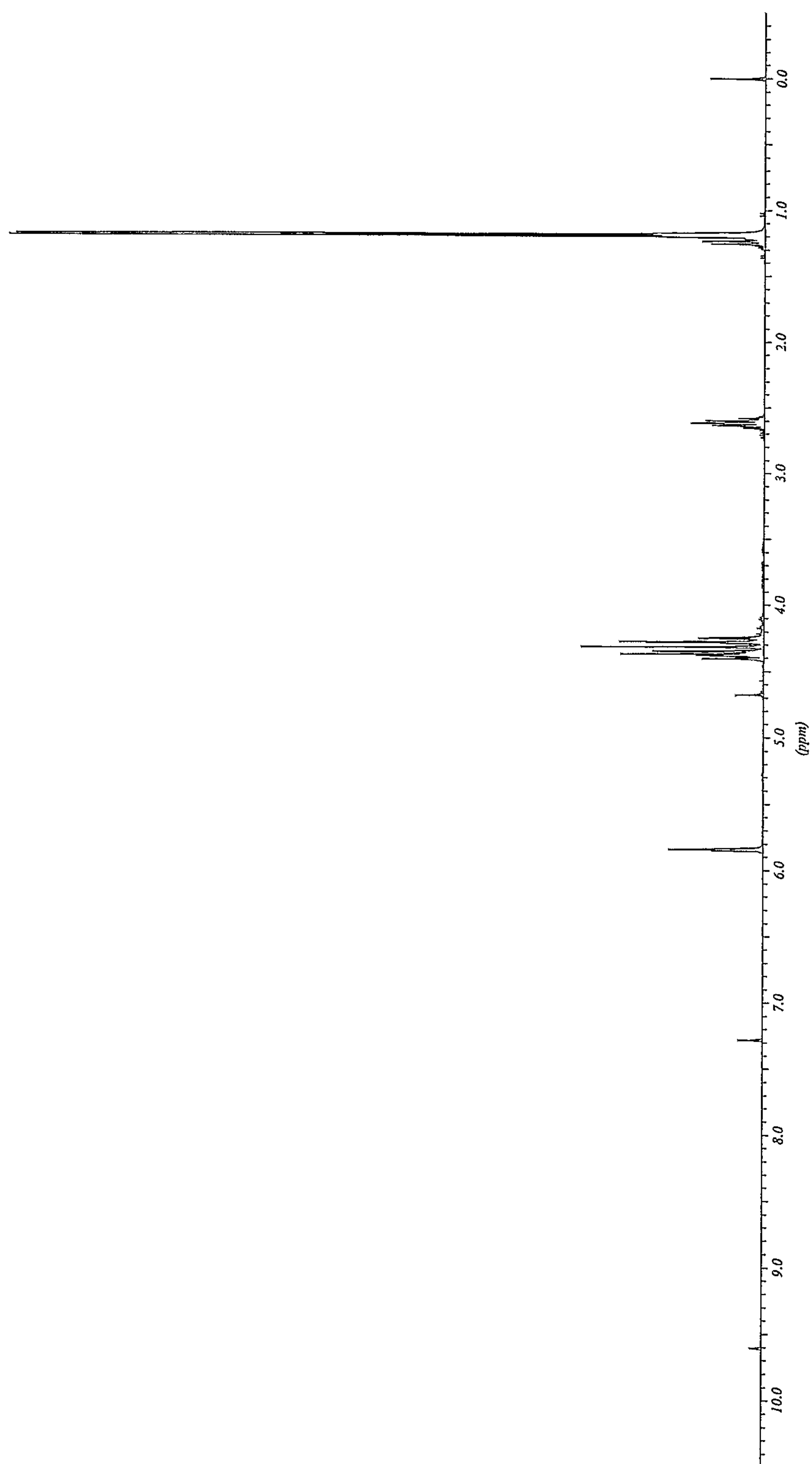
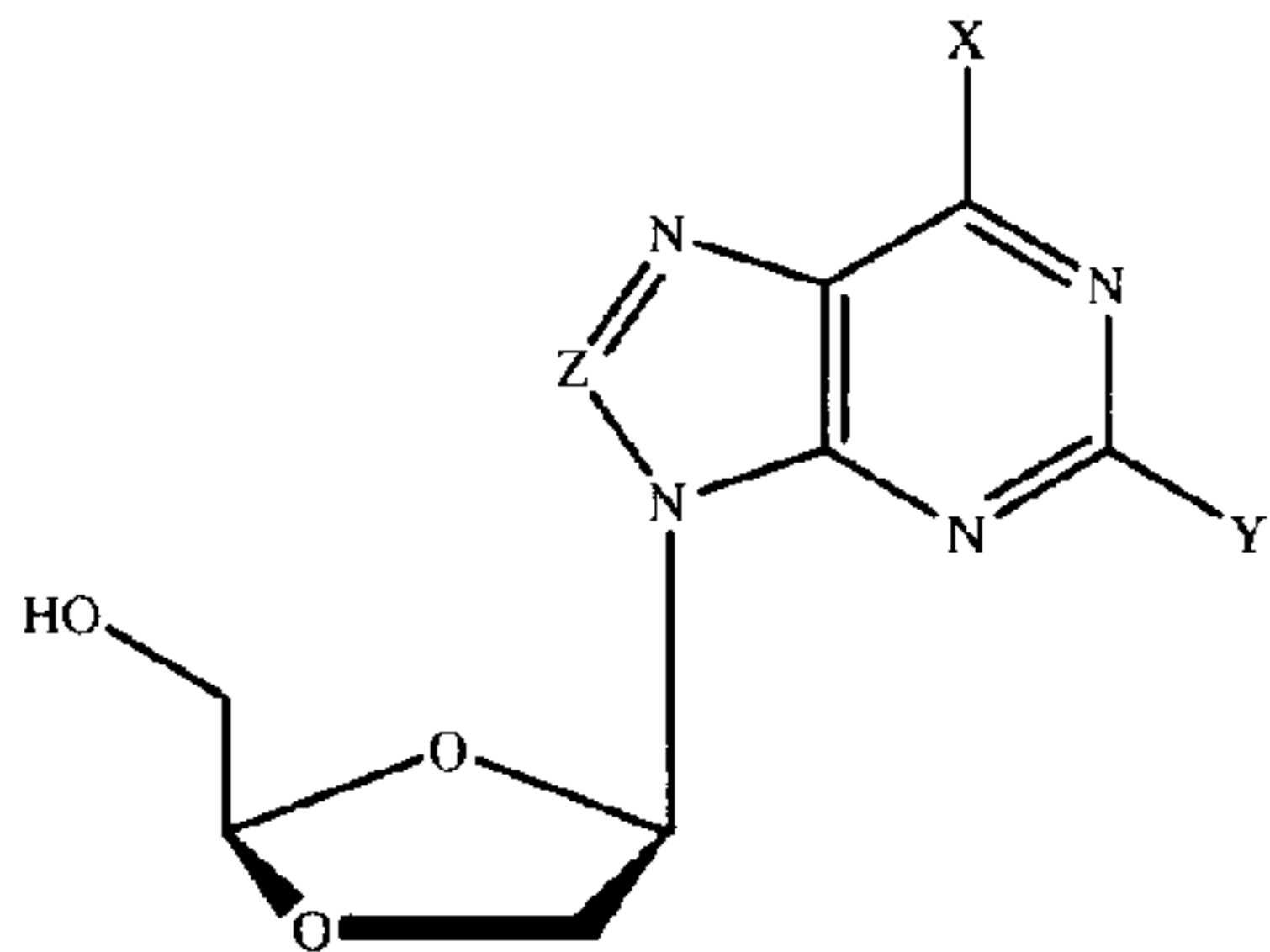
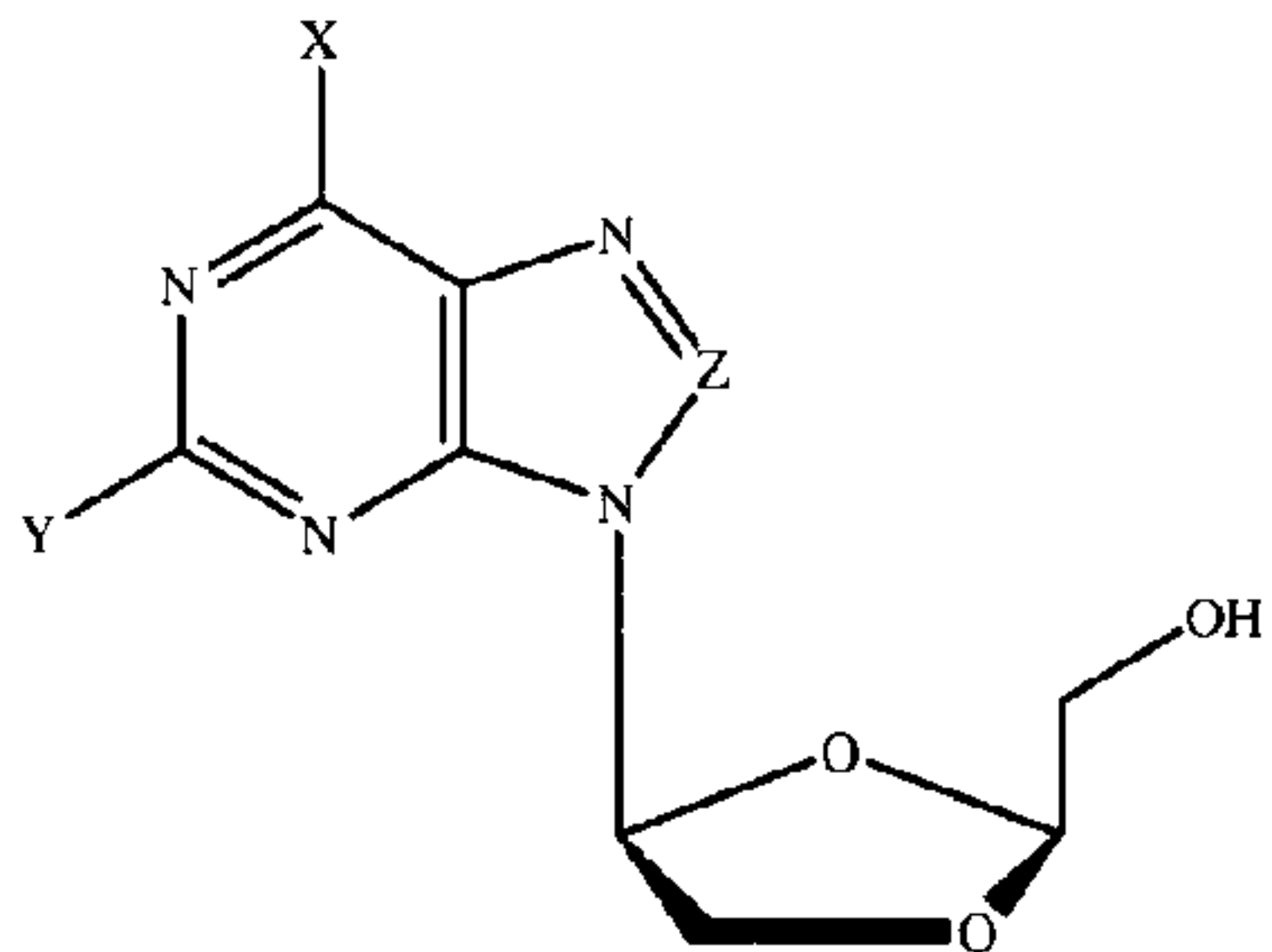


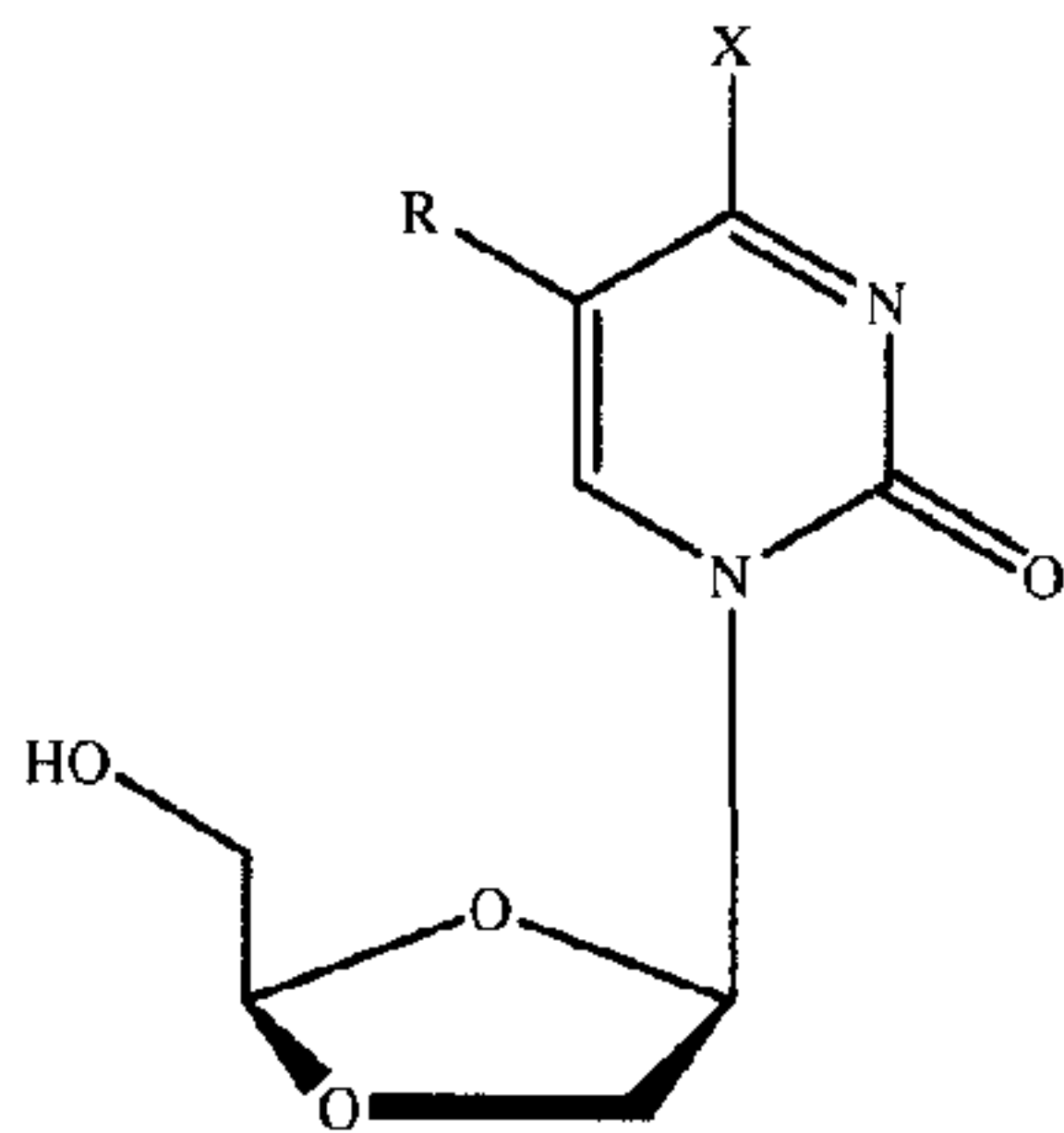
Figure 5: 400 MHz ^1H NMR Spectrum for Resolved Compound 13



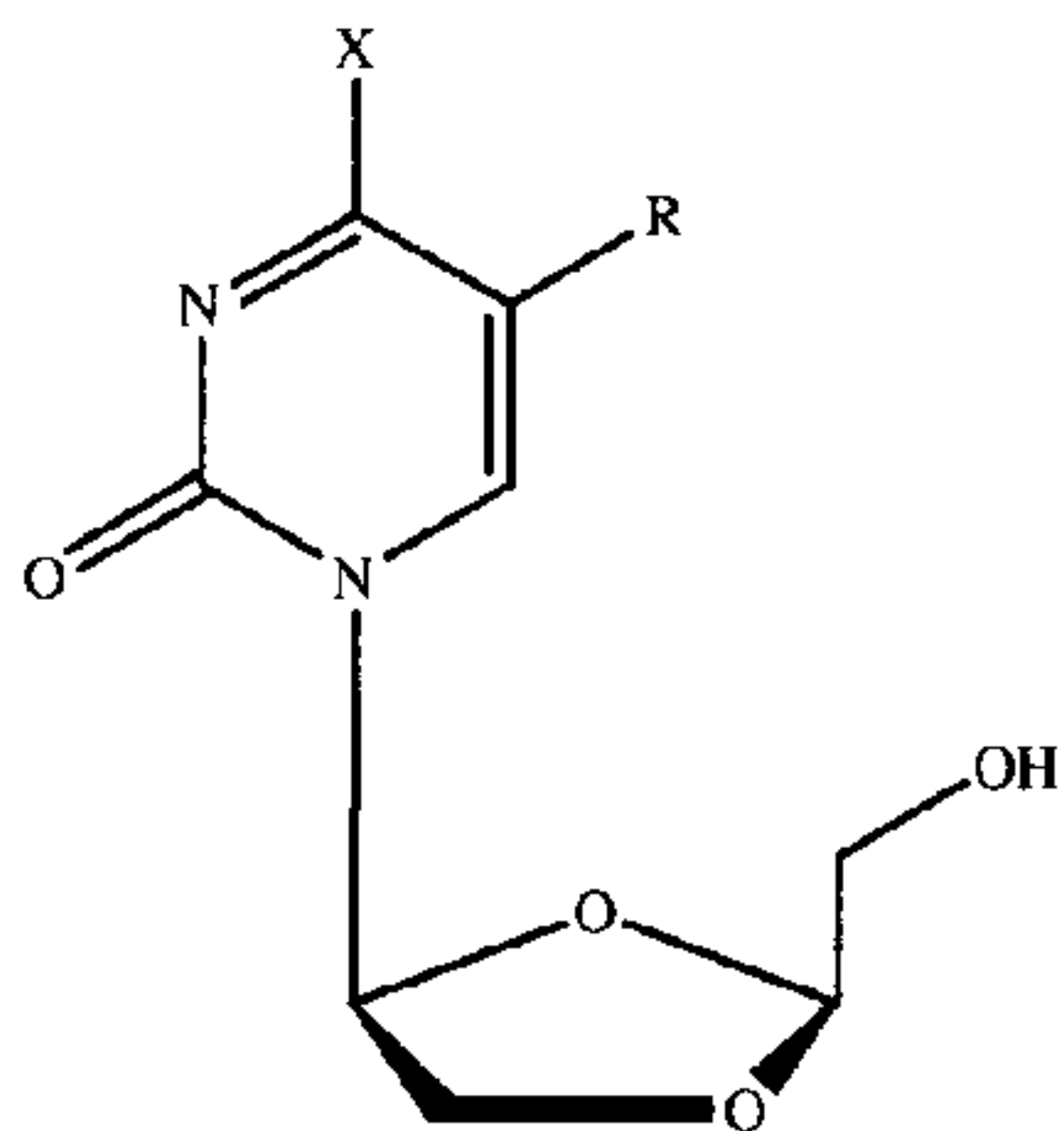
A



B



C



D