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# THE SHIKIMATE PATHWAY: ENZYMES AND ANTIMICROBIALS

A thesis submitted in part fulfilment  
of the requirements of the degree of  
Doctor of Philosophy

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“A definition of insanity:

Doing the same thing over and over again, and hoping for a different result.”

*Anon.*

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## Abbreviations

ABC	ATP-binding cassette
ADC	4-amino-4-deoxychorismate
ADC synthase	4-amino-4-deoxychorismate synthase
ADP	adenosine diphosphate
Ala	alanine
Amp	ampicillin
Arg	arginine
Asn	aspartamine
ATP	adenosine triphosphate
ATP-ase	adenosine triphosphate hydrolase
AU	absorbance units, measured at a given wavelength on an HP8453 diode array spectrophotometer
BBA	butane bisacetal
bisDHP	3,3',4,4'-tetrahydro-6,6'-spirobi-2 <i>H</i> -pyran
Boc	<i>tert</i> -butoxycarbonyl
BOP	benzotriazol-1-yloxy-tris(dimethylamino)phosphonium hexafluorophosphate
BTP	1,3-bis[tris-(hydroxy-methyl)methylamino]propane
Castro's reagent	see BOP
CD	circular dichroism
CSA	(±)-10-camphorsulfonic acid
DAHP	3-deoxy-D-arabino-heptulosonate-7-phosphate

DAHPS, DAHP synthase	phospho-2-hydro-3-deoxyheptonate aldolase
DAST	<i>N,N</i> -diethylaminosulfur trifluoride
DBU	1,8-diazabicyclo[5.4.0]undec-7-ane
DCM	dichloromethane
DHQ	3-dehydroquinic acid
5-DHQ	5-dehydroquinic acid
DHQase	3-dehydroquininate dehydratase
DHQS, DHQ synthase	7-phospho-3-deoxy-D- <i>arabino</i> -heptulosonate phosphate lyase
DHS	3-dehydroshikimic acid
DIC	di-isopropylcarbodiimide
DMAP	4,4-dimethylaminopyridine
DMF	<i>N,N</i> -dimethylformamide
Dpp	dipeptide permease
DTT	dithiothreitol
ElcB	unimolecular elimination reaction, involving the conjugate base
E4P	D-erythrose 4-phosphate
EDC	1-(3-dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride
EDC	1-(3-dimethylaminopropyl)-N-ethyl-carbodiimide hydrochloride
EPSP	enolpyruvyl shikimate 3-phosphate
EPSPS, EPSP synthase	phosphoenol pyruvate:3-phosphoshikimate 5- <i>O</i> -(1-carboxyvinyl) transferase

Et <sub>3</sub> N	triethylamine
F6P	D-fructose-6-phosphate
FMN	flavin mononucleotide
Fmoc	9-fluorenyl methoxycarbonyl
GATase	glutamine amidotransferase
β-glycerophosphate	glycerol-2-phosphate
glyphosate ®	N-(phosphonomethyl)-glycine
His	histidine
HOBt	1-hydroxybenzotriazole hydrate
HPLC	high performance liquid chromatography
IPTG	isopropyl-β-D-thioglucopyranoside
k <sub>cat</sub>	turnover number, number of molecules reacted per unit time
K <sub>i</sub>	dissociation constant of an enzyme inhibitor
k <sub>M</sub>	the substrate concentration at which the rate of reaction is half the maximal rate; approximates to the dissociation constant of the enzyme-substrate complex
Lys	lysine
Martin's reagent	[PhC(CF <sub>3</sub> ) <sub>2</sub> O] <sub>2</sub> SPh <sub>2</sub>
MIC	minimum inhibitory concentration
MOPS	4-morpholinepropanesulfonic acid
M <sub>r</sub>	relative molecular weight
MRSA	methicillin-resistant <i>Staphylococcus aureus</i>
ms	mass spectrometry
ms/ms	sequential mass spectrometry-mass spectrometry

Mtt	4-methyltrityl
NADH, NAD <sup>+</sup>	β-nicotinamide adenine dinucleotide
NADPH, NADP <sup>+</sup>	nicotinamide adenine dinucleotide phosphate
NMO	<i>N</i> -methylmorpholine <i>N</i> -oxide
NMR	nuclear magnetic resonance
Opp	oligopeptide permease
PABA	<i>para</i> -aminobenzoic acid
PD <sub>50</sub>	the dose of a pharmaceutical at which 50% of test subjects are protected against e.g. bacterial challenge
PDC	pyridinium dichromate
PEP	phospho <i>enol</i> pyruvate
Petrol	petroleum ether (40°C-60°C)
Phe	phenylalanine
PLP	pyridoxal-5'-phosphate
Pro	proline
<i>p</i> TsOH	<i>para</i> -toluenesulfonic acid
S3P	shikimate-3-phosphate
SDHase	shikimate dehydrogenase
SHK	shikimic acid
S <sub>N</sub> 1	unimolecular nucleophilic substitution
S <sub>N</sub> 2	bimolecular nucleophilic substitution
TBDMS	<i>tert</i> -butyldimethylsilyl
TBDMSCl	<i>tert</i> -butyldimethylsilyl chloride
TBDMSOTf	<i>tert</i> -butyldimethylsilyl triflate

Tf <sub>2</sub> O	trifluoromethanesulfonic anhydride
TFA	trifluoroacetic acid
THF	tetrahydrofuran
Thiobarbituric acid	4,6-dihydroxypyrimidine-2-thiol
TIPS	tri-isopropylsilane
TLC	thin layer chromatography
TMB	1,1,2,2-tetramethoxybutane
TMC	1,1,2,2-tetramethoxycyclohexane
TPAP	tetrapropylammonium perruthenate
TPP	thiamine pyrophosphate, cocarboxylase
Tpp	tripeptide permease
Tris.HCl	Tris(hydroxymethyl)aminomethane hydrochloride
Trp	tryptophan
Tyr	tyrosine
unit	one unit of enzyme activity is the amount of enzyme catalysing the formation of 1 $\mu$ mol of product per minute under specified conditions of pH, buffer composition, and temperature
UV	ultraviolet
Z	benzyloxycarbonyl

## Summary

(6*S*)-6-Fluoroshikimic acid **1** was developed as a potential antimicrobial which was converted to 6-fluorochorismate **2** and hence targeted the post-chorismate pathway to *p*-aminobenzoic acid **3** and folic acid (*fig. 1*). Although successful against microbial challenge, resistance developed rapidly. This resistance was found to be due to mutation at the *shiA* locus which codes for a transporter required for the entry of shikimic acid into bacterial cells.

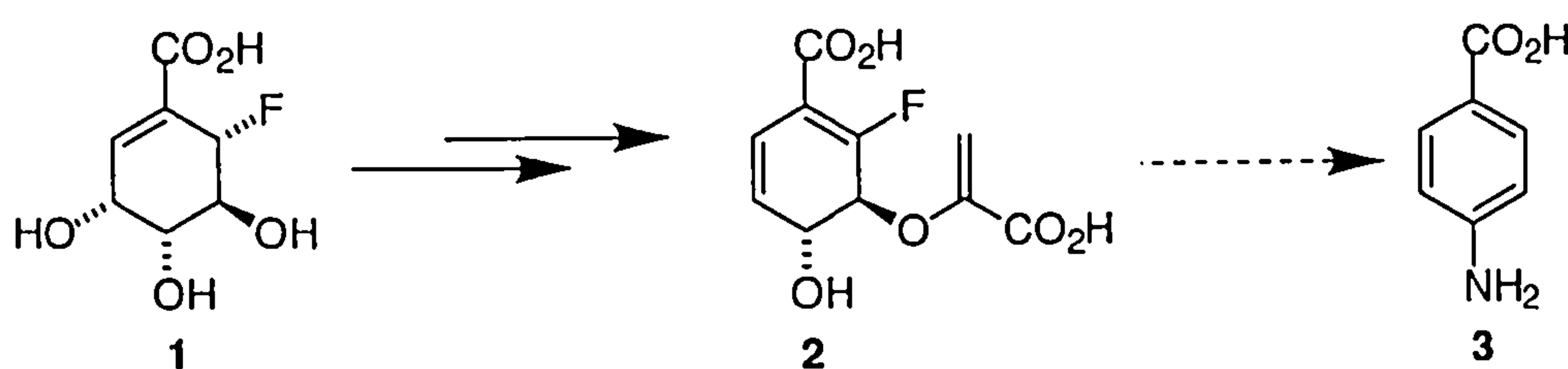
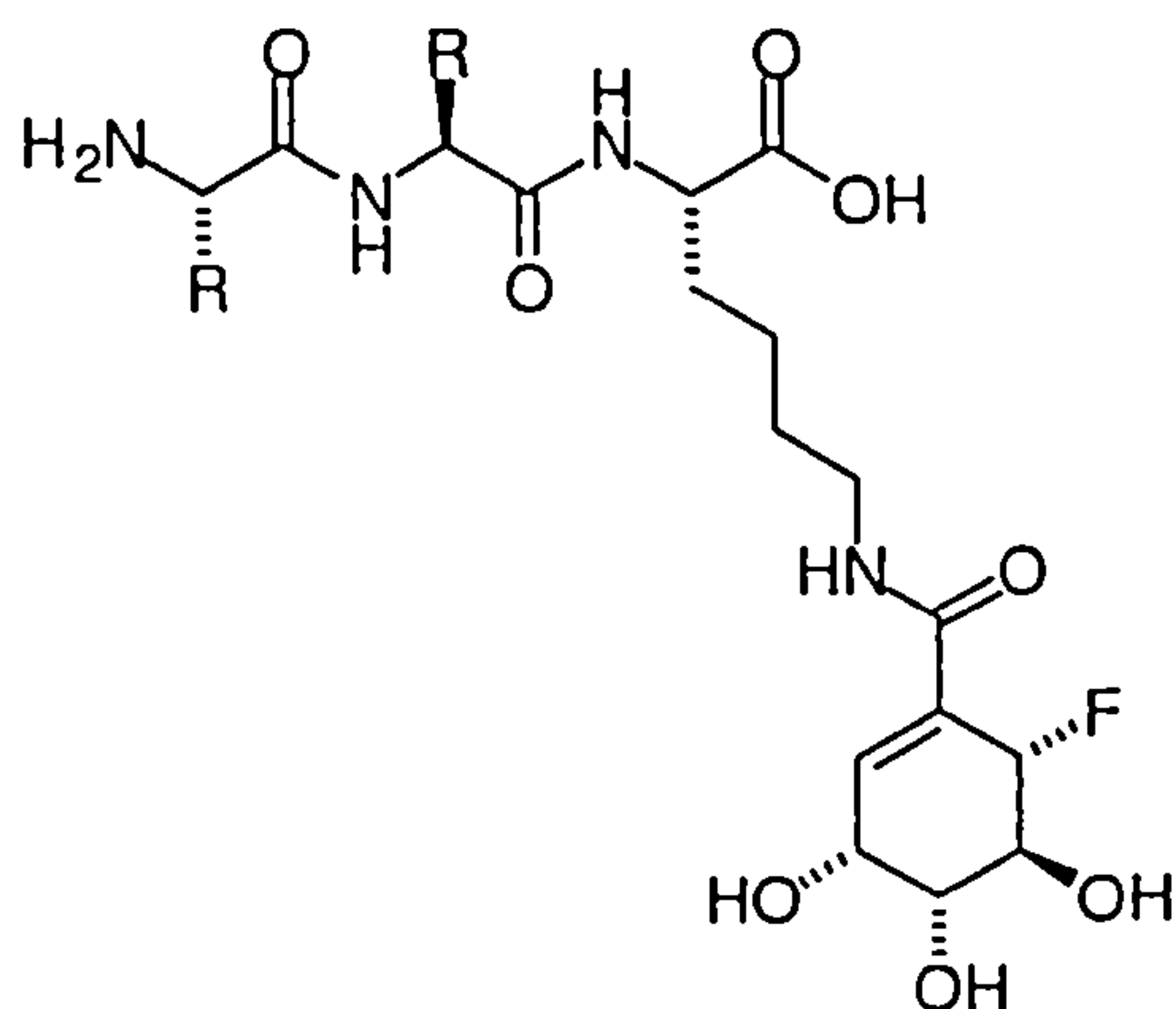


Figure 1: Mechanism of action of (6*S*)-6-fluoroshikimic acid

In the initial part of our research, we aimed to synthesise five peptide-based ‘smugglins’ **4 - 8** which would target the oligopeptide permease and hence provide a route for the entry of (6*S*)-6-fluoroshikimic acid into bacterial cells (*fig. 2*).



R = Me **4**, CH<sub>2</sub>CH(CH<sub>3</sub>)<sub>2</sub> **5**, CH<sub>2</sub>Ph **6**, CH<sub>2</sub>OH **7**, (CH<sub>2</sub>)<sub>2</sub>CO<sub>2</sub>H **8**

Figure 2: Smugglins designed to target the oligopeptide permease

Attempts to synthesise the required (6*S*)-6-fluoroshikimic acid **1** by biotransformation from erythrose -4-phosphate and fluoro-phosphoenolpyruvate were not successful.

However, a published route from shikimic acid **9** eventually provided sufficient material



for the synthesis of the test compounds (*fig. 3*). The details of these syntheses are described in Chapter 2.

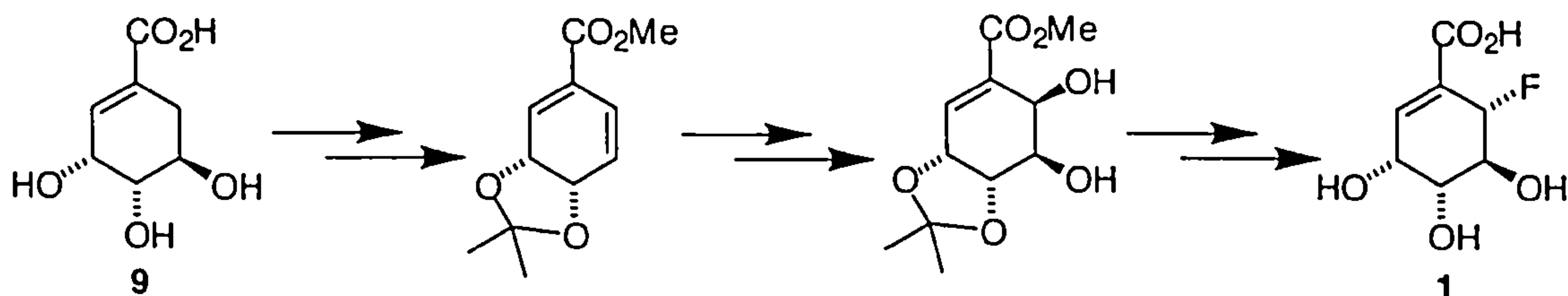


Figure 3: Published route to (6*S*)-6-fluoroshikimic acid from shikimic acid

The smugglins were synthesised using standard Fmoc-based solid-phase peptide synthesis using DIC/HOBt to catalyse peptide coupling and BOP/Et<sub>3</sub>N to catalyse the coupling of (6*S*)-6-fluoroshikimic acid to the peptide moiety. Testing of the phenylalanine and leucine-based smugglins **5** and **6** (*fig. 2*) for antibacterial activity against wild-type *Escherichia coli* has so far been unsuccessful. Details of these syntheses and investigations are described in Chapter 3.

As a secondary aim a route to pure 3-dehydroquinic acid (DHQ) **10**, required for the kinetic analysis of 3-dehydroquininate dehydratase (DHQase), was investigated. Initial attempts using acetal protecting groups developed to be selective for diequatorial diols were unsuccessful. However, modification of a published route to halogenated derivatives of dehydroquinic acid gave DHQ in good yield on a gram scale (*fig. 4*).

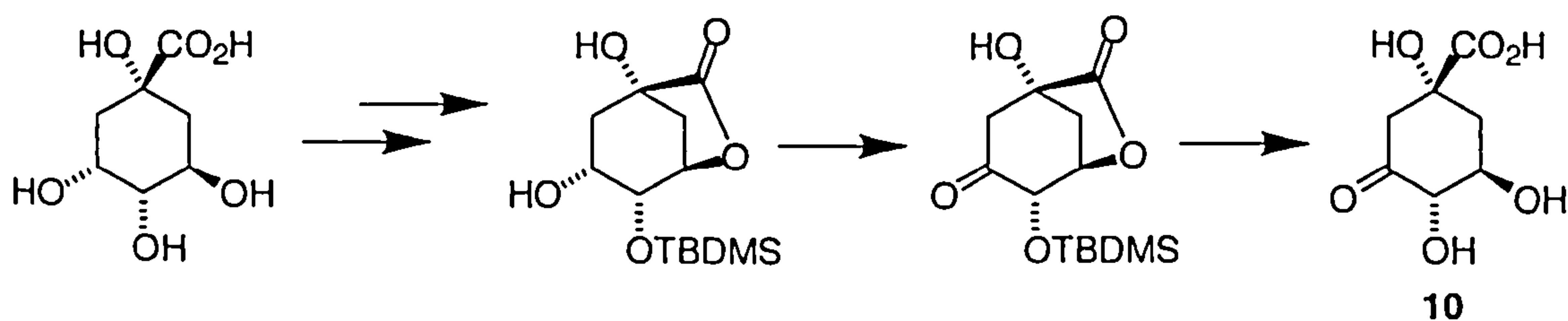


Figure 4: Route to DHQ from quinic acid

This pure DHQ was then used for the kinetic investigation of the type II DHQase from *Helicobacter pylori* and a mutant type II DHQase: *Bacillus subtilis* F28Y. Details of these syntheses and investigations are described in Chapter 4.

# 1 The Shikimate Pathway

## 1.1 Overview

Shikimic acid **9** was first isolated in 1885 by J.F. Eykmann from the fruits of *Illicium religiosum* and *Illicium anisatum* (Eijkman, 1885; Eykmann, 1891). Its fundamental role in the pathway which takes its name was eventually recognised by Davis (Davis, 1955).

The shikimate pathway (*fig. 5*) is a complex, branching metabolic pathway leading to the aromatic amino acids found in proteins (Phe, Trp, Tyr), *p*-aminobenzoic acid (PABA) which is the precursor of folic acid, the lipid-soluble isoprenoid quinones involved in electron transport, and the structural polymer lignin. The pathway is also a major source of secondary metabolites (those compounds which are produced by specific organisms for purposes other than the basic requirements of growth) such as phenols and alkaloids in plants and antibiotics in micro-organisms. For reviews on the biosynthesis of shikimate metabolites, see the series in *Natural Product Reports*, most recently authored by Andrew Knaggs (Knaggs, 2003). For an overview of the entire pathway, see Ronald Bentley's detailed study (Bentley, 1990) and Edwin Haslam's book (Haslam, 1993); along with two reviews which focus only on the 'main trunk' of the pathway to chorismate (Mousdale & Coggins, 1993; Herrmann & Weaver, 1999). Additionally, a review focussing on the chemical aspects of the shikimate pathway was published in 1998 (Jiang & Singh, 1998).

Depending on the organism studied, the central enzymes of the shikimate pathway (DHQ synthase to EPSP synthase) exist singly or in the form of multifunctional enzymes. In *Neurospora crassa*, *Aspergillus nidulans* and *Saccharomyces cerevisiae* as well as other

fungal species, the enzymes are found in the form of the *arom* multi-functional enzyme encoded by a single gene (Lumsden & Coggins, 1977; Coggins & Boocock, 1986; Duncan *et al.*, 1987). In contrast, the corresponding enzymes in *Escherichia coli* exist as five monofunctional enzymes encoded by genes dispersed throughout the genome (Coggins *et al.*, 1985). Many suggestions have been put forward as to why multifunctional enzymes exist, but the most convincing advantages would seem to be co-ordinate regulation of enzyme activity and of gene transcription, with protection of unstable reaction intermediates, and spatial organisation of enzyme functions as a secondary advantage (Hardie & Coggins, 1986).

As is the case with many other long biosynthetic pathways leading to amino acids, vitamins and nucleotides, the enzymes of the shikimate pathway have been lost by vertebrates. This is believed to be due to the high metabolic cost of maintaining the enzymes while the products can be obtained from the diet. As mammals are thus unable to synthesise aromatic compounds by this route, the enzymes of the shikimate pathway are attractive targets in the search for novel antimetabolites (Kishore & Shah, 1988; Payne *et al.*, 2000)

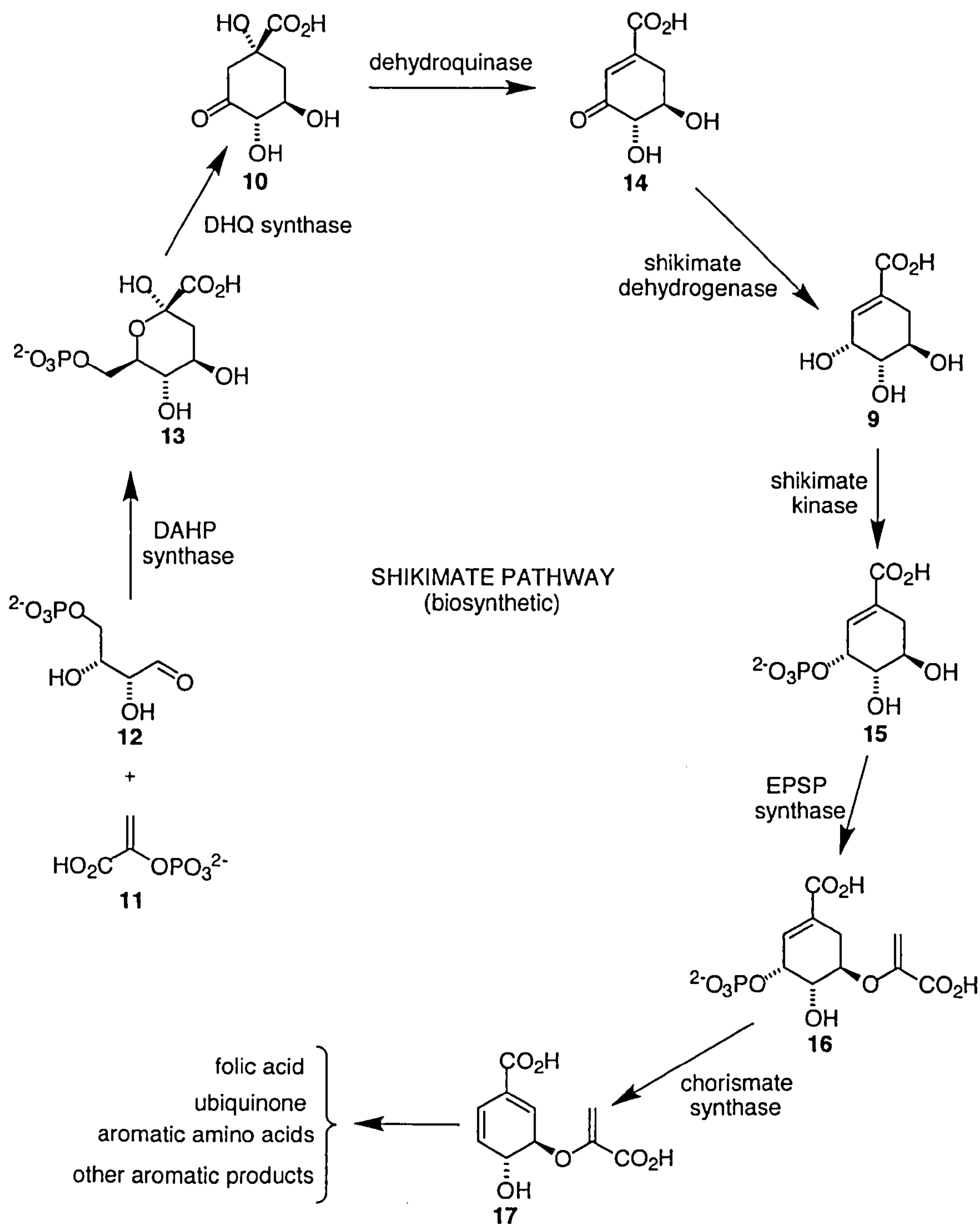


Figure 5: The Shikimate Pathway



The pathway has long been known to exist in plants, archaea, bacteria, and fungi (Bentley, 1990). The recent discovery of shikimate pathway enzymes in apicomplexan parasites, including the clinically important *Plasmodium* (malaria), *Cryptosporidium* and *Toxoplasma* species, has given further impetus to the search for effective enzyme inhibitors and hence possible pharmaceuticals (Roberts *et al.*, 1998; Keeling *et al.*, 1999; McConkey, 1999; Roberts *et al.*, 2002). It is believed that the apicomplexa are vulnerable to compounds which target bacterial enzymes due to the presence of a plastid, the apicoplast, which is thought to be the result of endosymbiosis of a cyanobacterial-like cell (McFadden & Roos, 1999).

Mutation or deletion of shikimate pathway genes in pathogenic species results in attenuated strains which are non-infective and non-viable *in vivo* (Poirer *et al.*, 1988). These have been used in the development of live oral vaccine candidates against *Salmonella typhimurium*, *Shigella flexneri* and the fish pathogen *Aeromonas salmonicida* (Hoiseth & Stocker, 1981; Dougan *et al.*, 1988; Edwards & Stocker, 1988; Verma & Lindberg, 1991; Vaughan *et al.*, 1993). Additionally, with the resurgence of tuberculosis world-wide, evidence that the shikimate pathway is essential for the viability of *Mycobacterium tuberculosis* has provided not only the possibility of new treatments, but also of new and more effective vaccines based on attenuated strains (Parish & Stoker, 2002).

1.2 The ‘main trunk’ enzymes of the shikimate pathway

1.2.1 3-Deoxy-D-*arabino*-heptulosonate 7-phosphate synthase

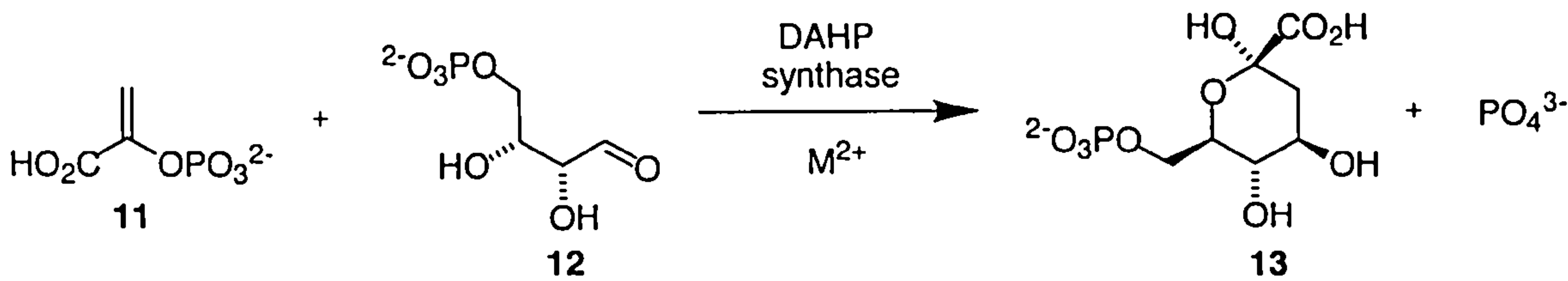


Figure 6: The reaction catalysed by DAHP synthase

3-Deoxy-D-*arabino*-heptulosonate 7-phosphate (DAHP) synthase catalyses the formation of 3-deoxy-D-*arabino*-heptulosonate 7-phosphate (DAHP) 13 from phosphoenolpyruvate (PEP) 11 and D-erythrose 4-phosphate (E4P) 12 and is properly referred to as phospho-2-hydro-3-deoxyheptonate aldolase, EC 4.1.2.15 (*fig. 6*). DAHP synthase activity was unequivocally detected in 1959 in *E. coli* cells and has since been observed in many microbial and plant sources (Srinivasan *et al.*, 1959).

Various different DAHP synthase isozymes have been identified in bacteria and plants due to the wide variety of feedback mechanisms involved in controlling carbon flow through the shikimate pathway by means of DAHP synthase regulation. The catalytic activity and actual concentration of certain DAHP synthase isozymes are affected by availability or otherwise of end-products such as the aromatic amino acids and caffeic acid (a secondary metabolite); or intermediates such as chorismic acid (Bentley, 1990).

In wild-type *E. coli* cells three DAHP synthase isozymes, each inhibited by a single aromatic amino acid, are encoded by the genes *aroF* (tyrosine sensitive), *aroG*



(phenylalanine-sensitive) and *aroH* (tryptophan-sensitive) (Jensen & Nasser, 1968).

DAHPS(Phe), which is a homotetramer, is the major isoform and constitutes around 75 % of activity in wild-type cells (McCandliss *et al.*, 1978). DAHPS(Trp), which contributes less than 1 % of activity, and DAHPS(Tyr), which contributes 25 %, are homodimers (Schoner & Herrmann, 1976; Akowski & Bauerle, 1997).

It was originally believed that DAHP synthases could be divided into two groups: those found in plants, and those found in microbes (Herrmann, 1995; Weaver *et al.*, 1993). It has since been shown that plant-like DAHP synthase enzymes ( $M_r \sim 54\,000$ ) exist in *Streptomyces spp.* as well as in *Neurospora crassa* (Walker *et al.*, 1996). The *E. coli*-like DAHP synthases ( $M_r \sim 40\,000$ ) have therefore been renamed 'type I', and the plant-like enzymes 'type II'.

The first DAHP synthase enzyme to be purified was the tryptophan-sensitive isozyme from *N. crassa* (Nimmo & Coggins, 1981). The first X-ray crystal structure obtained was of the phenylalanine-sensitive isozyme from *E. coli* (Shumilin *et al.*, 1999). The structure was confirmed as a dimer of two tight dimers, with the interesting proposal that four amino acid substitutions seen in DAHPS(Tyr) and DAHPS(Trp) would eliminate the hydrophobic interactions forming the tetramer to give the observed dimeric structures for these enzymes.

### 1.2.2 DHQ synthase

More properly known as 7-phospho-3-deoxy-D-*arabino*-heptulosonate phosphate lyase (EC 4.6.1.3), 3-dehydroquinate (DHQ) synthase catalyses the formation of DHQ 10 from DAHP 13 (*fig. 7*).

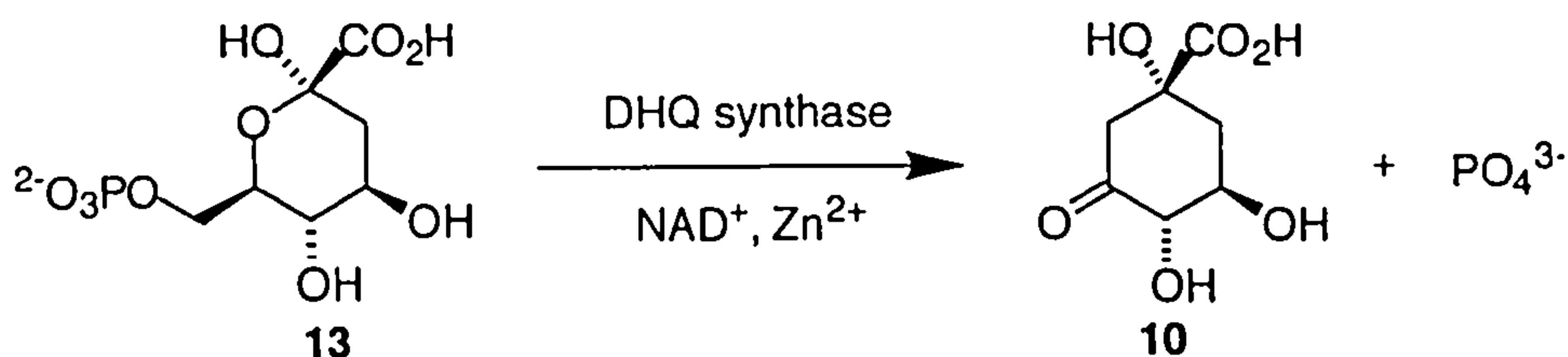


Figure 7: The reaction catalysed by DHQ synthase

DHQ synthase from *E. coli* has been purified by various groups with a particularly high-yielding overexpression system reported by Frost (Frost *et al.*, 1984; Mehdi *et al.*, 1987).

The enzyme has an absolute requirement for both  $NAD^+$  and a divalent metal cation generally believed to be  $Zn^{2+}$  (Lambert *et al.*, 1985)

The reaction catalysed by DHQ synthase was initially believed to be an extreme example of the capabilities of a single active site as it involves oxidation,  $\beta$ -elimination, reduction, and an intramolecular aldol reaction (Bartlett *et al.*, 1988). Later work demonstrated that the enzyme functions mainly as a dehydrogenase and structural template with elimination spontaneously following oxidation, and the aldol reaction occurring even in the absence of the enzyme (Widlanski *et al.*, 1989; Bartlett *et al.*, 1994).

The structure of the DHQ synthase domain of the AROM protein from *Aspergillus nidulans* has been determined by X-ray crystallography (Carpenter *et al.*, 1998). The enzyme is a homodimer with the active site located in the cleft between the two domains of each monomer. The authors propose that while many of the steps in DHQ synthesis may not be catalysed by the enzyme, reaction intermediates may be stabilised by interactions with the active site. This would explain the presence of reaction byproducts in the non-catalysed

formation of DHQ, and their absence in the enzyme-catalysed synthesis (Bartlett *et al.*, 1994).

### 1.1.3 Dehydroquinase

The enzyme which converts DHQ **10** to 3-dehydroshikimic acid (DHS) **14** is correctly referred to as 3-dehydroquinate dehydratase (EC 4.2.1.10) (*fig. 8*).

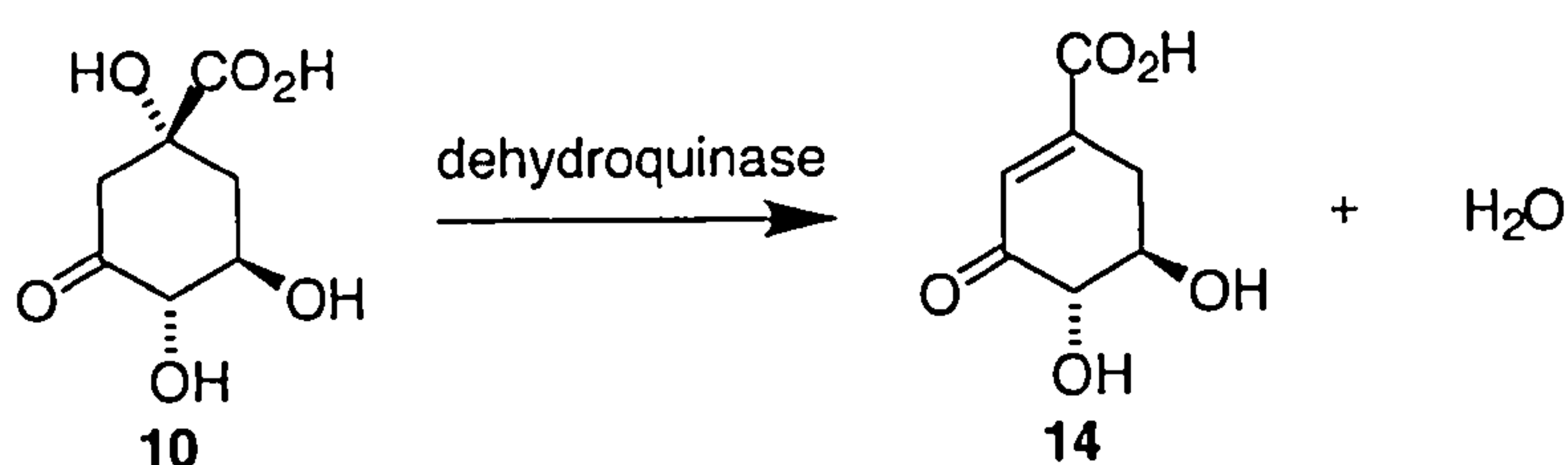


Figure 8: The reaction catalysed by dehydroquinase

Dehydroquinase (DHQase) enzymes occur in two distinct forms with differing chemical and biochemical properties (Kleanthous *et al.*, 1992). Type I enzymes occur in the shikimate pathway in fungi, plants and some bacteria. They are dimeric and catalyse a *syn*-elimination of water via an imine intermediate involving a conserved lysine residue (Butler *et al.*, 1986; Chaudhuri *et al.*, 1991; Smith *et al.*, 1970; Schneier *et al.*, 1991). Type II DHQases are found in the quinate pathway of fungi and the shikimate pathway of many bacteria. They are dodecameric and catalyse an *anti*-elimination of water via an enolate intermediate (Harris *et al.*, 1993; Harris *et al.*, 1996)

The two types of DHQase are unrelated at the sequence level, and X-ray crystal analysis has shown them to be structurally unrelated (Gourley *et al.*, 1999; Roszak *et al.*, 2002).

The dimeric type I enzyme possesses an  $\alpha/\beta$  barrel structure, while the type II enzyme has a

flavodoxin-like fold with subunits arranged as a tetramer of trimers. These differences are consistent with the differing stereochemical and mechanistic paths of the dehydration reactions catalysed by the two types of DHQase.

#### 1.1.4 Shikimate dehydrogenase

Shikimate dehydrogenase (EC 1.1.1.25) converts DHS **14** to shikimic acid (SHK) **9** using NADPH as the reducing agent (*fig. 9*). As a result, it has been termed shikimate:NADP<sup>+</sup> oxido-reductase, but is commonly abbreviated to SDHase.

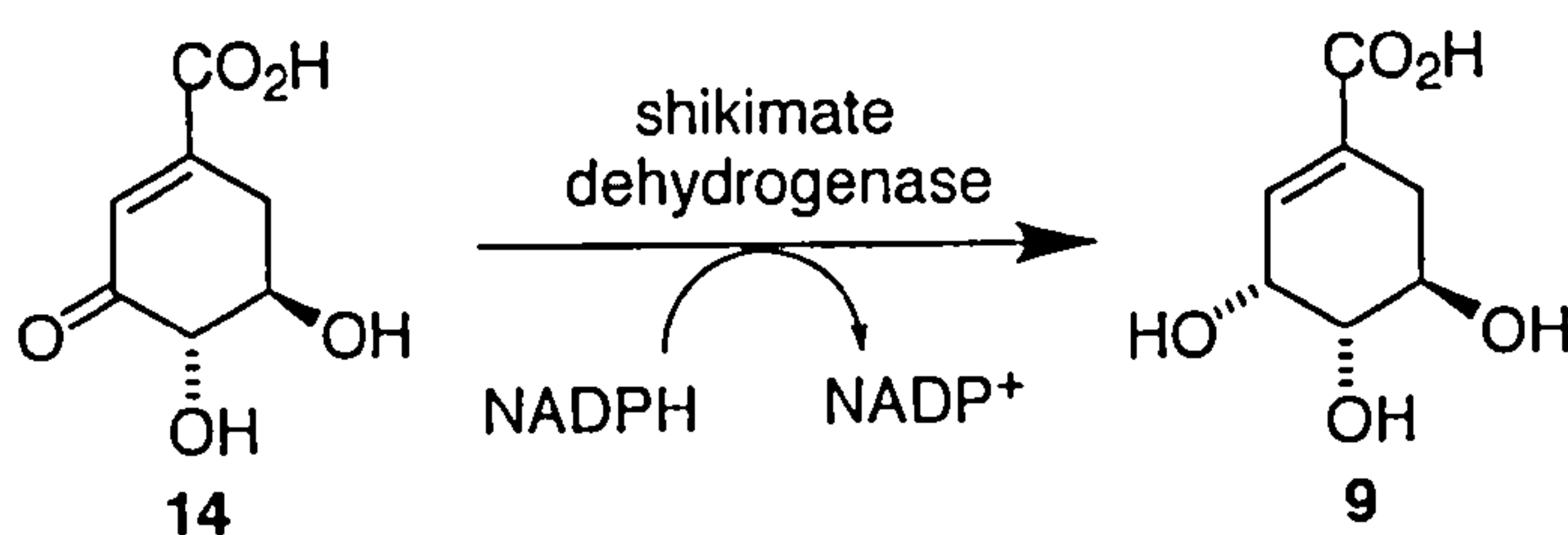


Figure 9: The reaction catalysed by shikimate dehydrogenase

In *E. coli* the enzyme is encoded by the *aroE* gene, and is monomeric with  $M_r \sim 32\,000$  (Chaudhuri & Coggins, 1985). In plants bifunctional DHQase:shikimate dehydrogenase enzymes have been described as well as the monofunctional shikimate dehydrogenases (Lourenco & Neves, 1984; Blume & McClure, 1980).

The enzyme has an absolute requirement for a C-4 hydroxyl in the substrate and shows a stereochemical bias towards compounds with the S configuration at both C-1 and C-4 (Bugg, 1988).

Shikimate dehydrogenase from *E. coli* has been crystallised (Michel *et al.*, 2003). The enzyme consists of two  $\alpha/\beta$  domains separated by a cleft in which NADP is bound in the



crystallised protein. The observation of protein molecules in ‘open’ and ‘closed’ forms in the crystal structure was taken as evidence of a conformational change on substrate binding in which the cleft between the domains closes.

### 1.1.5 Shikimate kinase

Shikimate kinase (EC 2.7.1.71) catalyses the transfer of phosphate from ATP to the C-3 -hydroxyl of shikimic acid **9** to form shikimate-3-phosphate (S3P) **15** (*fig. 10*).

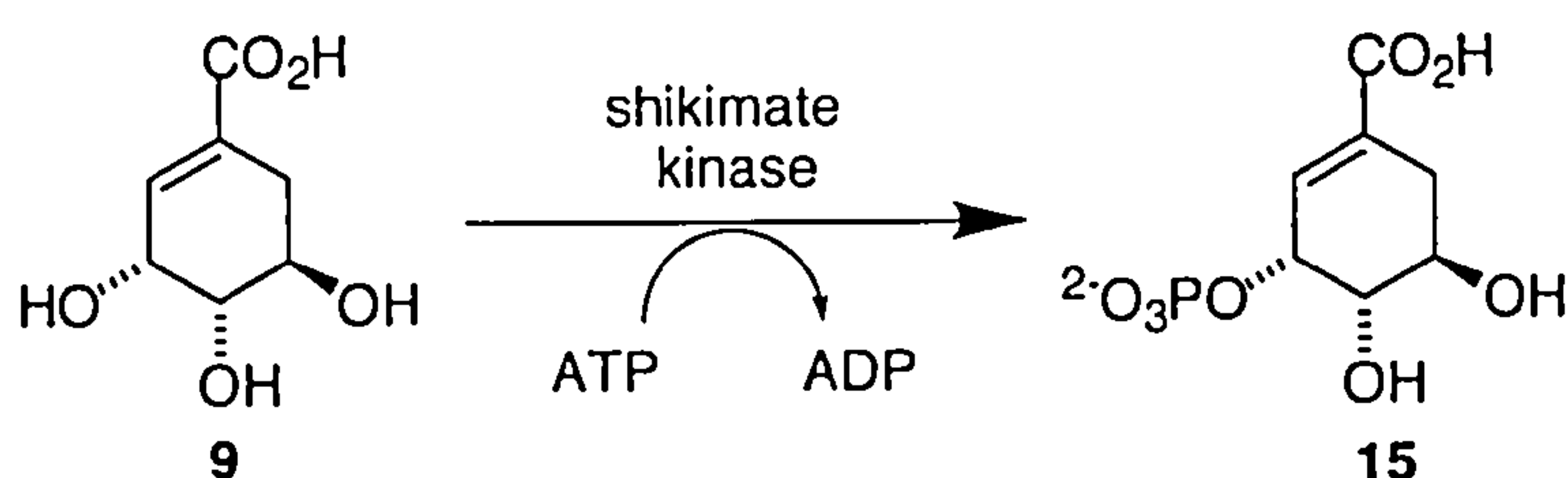


Figure 10: The reaction catalysed by shikimate kinase

Shikimate kinase exists in isozymic forms in both *E. coli* and *S. typhimurium* (Haslam, 1993; Pittard, 1996). In *E. coli*, shikimate kinase I is encoded by the *aroK* gene, and shikimate kinase II by the *aroL* gene (Millar *et al.*, 1986; Lobnerolesen & Marinus, 1992). The proteins are similar in length, and have 30 % sequence homology (Whipp, 1995). The enzymes do, however, have widely differing affinities for shikimic acid. Shikimate kinase I has an affinity in excess of 20 mM, while that of shikimate kinase II is 200  $\mu$ M (DeFeyter & Pittard, 1986; Millar *et al.*, 1986). This suggests that shikimate kinase II normally functions in the biosynthetic shikimate pathway while shikimate kinase I is believed to have an alternative biological role (DeFeyter & Pittard, 1986; Vinella *et al.*, 1996).

*E. coli* shikimate kinase II is monomeric with  $M_r \sim 19\,000$  and has an absolute requirement for a divalent metal cation, believed to be  $Mg^{2+}$  (Krell *et al.*, 1998). The type II enzyme from *Erwinia chrysanthemi*, which has 53 % amino acid sequence identity with the *E. coli* enzyme, has been crystallised in the presence of Mg-ADP (Krell *et al.*, 1998). The enzyme is an  $\alpha/\beta$  protein which resembles adenylate kinase in that it has a central nucleotide-binding core with two flexible domains. Evidence for significant structural change on substrate and cofactor binding due to induced-fit movement was observed using circular dichroism (CD) spectra.

### 1.1.6 EPSP synthase

With such a lengthy formal name, it is unsurprising that phosphoenolpyruvate:3-phosphoshikimate 5-*O*-(1-carboxyvinyl)transferase (EC 2.5.1.19) should more commonly be referred to as EPSP synthase. Catalysing the reaction between shikimate 3-phosphate **15** and PEP (*fig. 11*) this enzyme is the target of the post-emergence herbicide glyphosate (Steinrücken & Amrhein, 1980; Boocock & Coggins, 1983; Krell *et al.*, 1988). As a result, the structure and mechanism have been intensively studied.

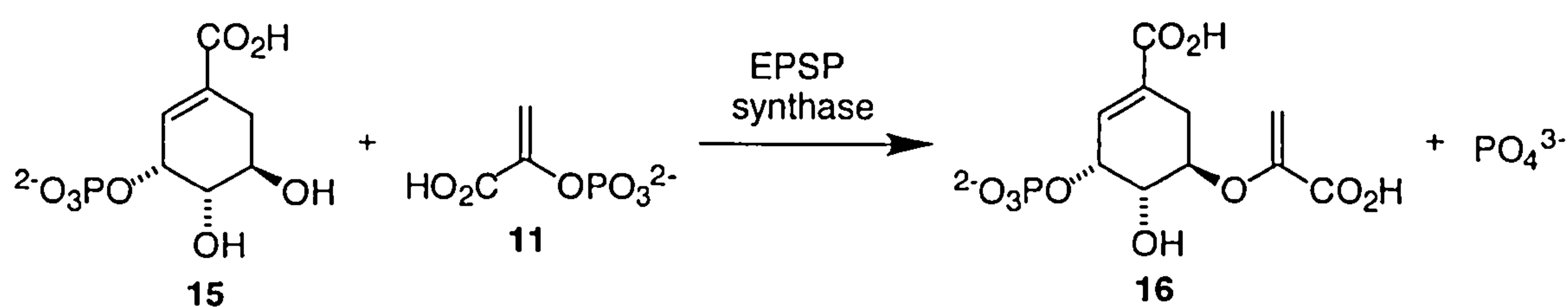


Figure 11: The reaction catalysed by EPSP synthase

In *E. coli*, monofunctional EPSP synthase is encoded by the *aroA* gene and has  $M_r \sim 46\,000$  (Lewendon & Coggins, 1983; Duncan *et al.*, 1984). In *S. typhimurium*, the causative agent



of typhoid fever, EPSP synthase has only 11 % amino acid sequence divergence from the *E. coli* enzyme (Stalker *et al.*, 1985). In both organisms, expression of *aroA* is linked to that of *serC*, which codes for 3-phosphoserine aminotransferase, in an unusual mixed-function operon (Duncan & Coggins, 1986; Hoiseth & Stocker, 1985). Overexpression of EPSP synthase in *E. coli* results in resistance to glyphosate (Duncan *et al.*, 1984).

In plants, EPSP synthase is found in the stromal fraction of chloroplasts (Mousdale & Coggins, 1984). Plant EPSP synthases show significant homology with bacterial enzymes, which can function in plants provided the chloroplast transit sequence has been added to the enzyme (della-Cioppa *et al.*, 1987). A great deal of research effort has been concentrated on the development of glyphosate-resistant lines. This has involved the overexpression of EPSP synthase, modification of the enzyme to reduce sensitivity to the inhibitor, or modification to increase the specific activity of the enzyme (Rogers *et al.*, 1983; Amrhein *et al.*, 1983; Shah *et al.*, 1986; Comai *et al.*, 1983; Comai *et al.*, 1985; Stalker, 1985; Steinrücken, 1986).

EPSP synthase has been crystallised in the presence and absence of glyphosate. (Abdel-Meguid *et al.*, 1985; Stallings *et al.*, 1991; Schönbrunn *et al.*, 2001). The enzyme consists of two globular domains which are roughly equal in size. The ligand binding site is situated at the interface between the domains where the macrodipole effect of multiple  $\alpha$ -helices is believed to result in the accumulation of positive charge (Stallings *et al.*, 1991). Substrate binding induces a radical change in conformation as the cleft between the domains closes.

### 1.1.7 Chorismate synthase

The enzyme catalysing the 1,4-elimination of phosphate from EPSP **16** to give chorismate **17** is correctly named  $O^5$ -(1-carboxyvinyl)-3-phosphoshikimate phosphate lyase (EC 4.6.1.4) but more commonly referred to as chorismate synthase (*fig. 12*).

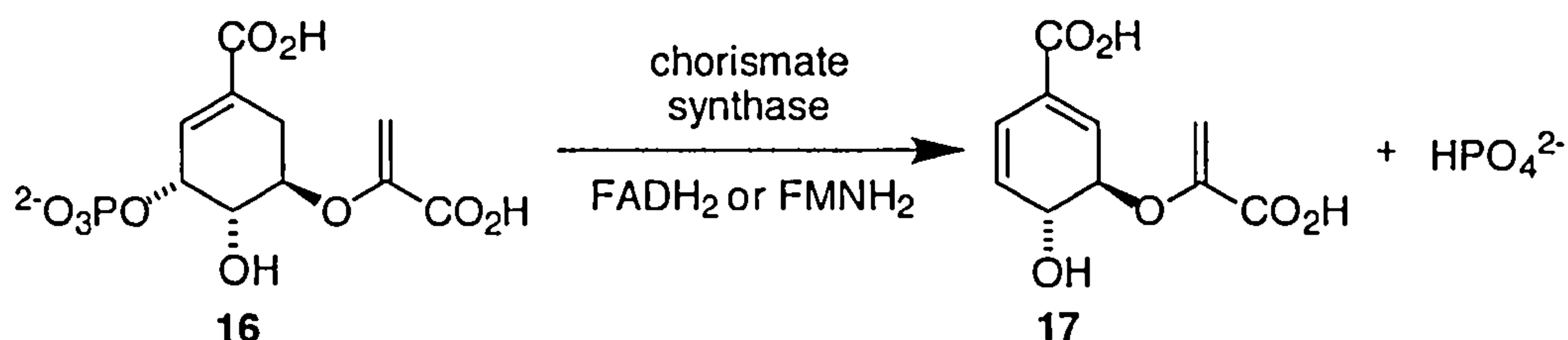


Figure 12: The reaction catalysed by chorismate synthase

The reaction is unusual in two respects. Firstly, elimination is *anti* with loss of the C-6H<sub>R</sub> proton, and secondly, there is an absolute requirement for a reduced flavin cofactor in catalysis even though there is no change in the overall oxidation state of the substrate (Hill & Newkome, 1969; Onderka & Floss, 1969; Welch *et al.*, 1974). In addition, despite intensive study, the mechanism of the reaction has not yet been conclusively proven (Macheroux *et al.*, 1999; Bornemann, 2002)

Chorismate synthases can be divided into two groups based on the way in which the reduced flavin cofactor is obtained. Monofunctional chorismate synthases such as those found in *E. coli* and plants require the presence of reduced FMN in the environment. Bifunctional chorismate synthases, generally fungal, possess an intrinsic flavin reductase activity which uses NADPH to reduce FMN (White *et al.*, 1988). There is a third type, exemplified by the *B. subtilis* enzyme, which associates with a separate NADPH:FMN oxidoreductase (Hasan & Nester, 1978). Early data suggested that large chorismate

synthase enzymes ( $M_r \sim 50\,000$ ) were bifunctional, while smaller ones ( $M_r \sim 40\,000$ ) were monofunctional (White *et al.*, 1988). However, the small bifunctional enzyme from *Saccharomyces cerevisiae* ( $M_r\,40\,800$ ) runs counter to this classification (Henstrand *et al.*, 1995; Henstrand *et al.*, 1996).

The x-ray crystal structure of chorismate synthase has not been published.

### 1.1.8 PABA synthase

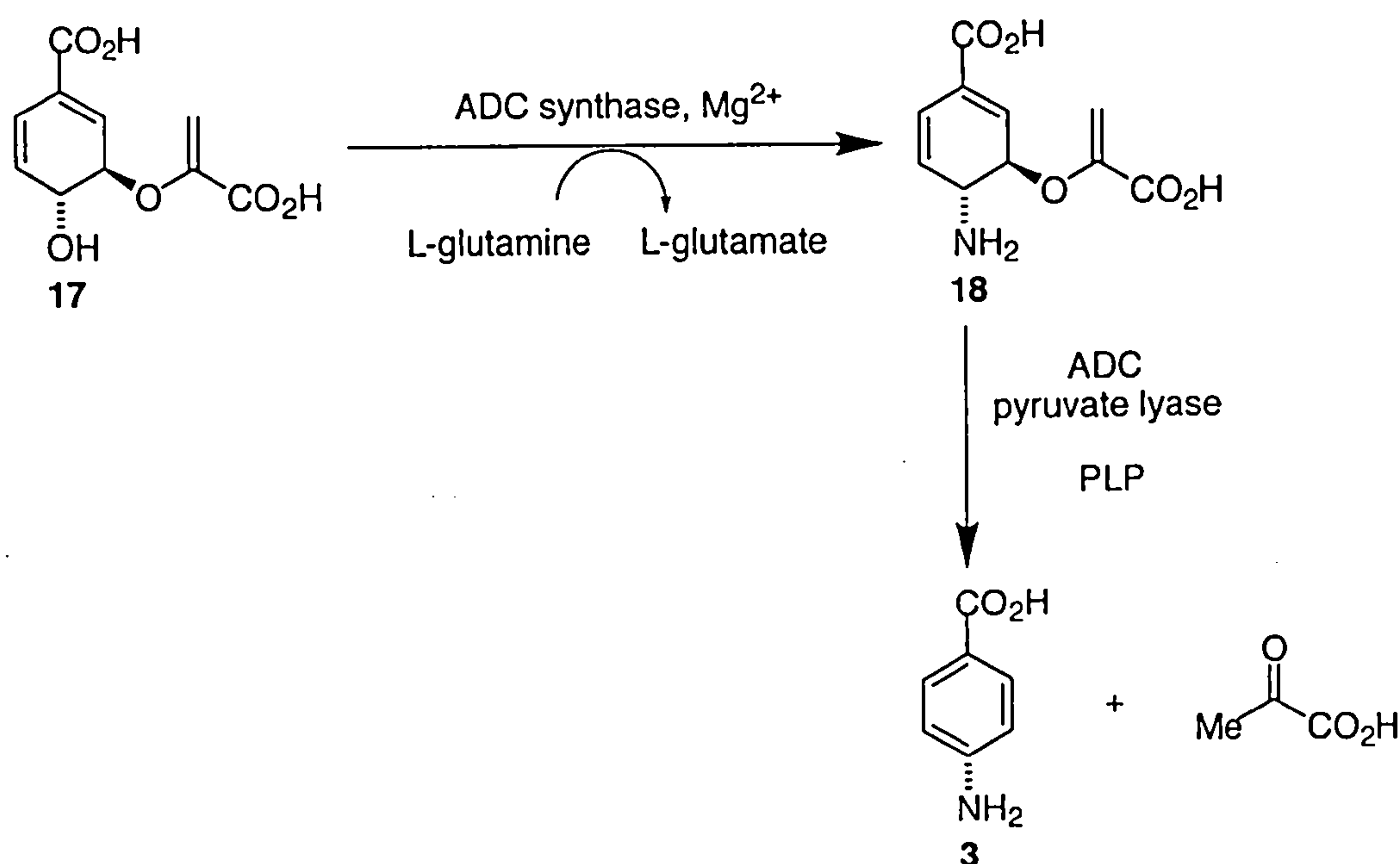


Figure 13: The formation of PABA *via* 4-amino-4-deoxychorismate (ADC)

4-Aminobenzoic acid (*p*-aminobenzoic acid, PABA) 3 is an essential component of dihydrofolate which acts as a 1-C carrier in biosynthesis. The conversion of chorismate 17 to PABA is a two-step process (*fig. 13*) (Nichols *et al.*, 1989). The first step, catalysed by ADC synthase, is the reversible amination of chorismate to give ADC 18 (Anderson *et al.*, 1991). ADC synthase consists of two non-identical subunits: a glutamine amidotransferase

(GATase) encoded by the *pabA* gene, and the ADC synthase proper, encoded by the *pabB* gene (Kaplan & Nichols, 1983; Ye, *et al.*, 1990). ADC synthase requires both glutamine and  $Mg^{2+}$  for activity. The GATase activity is conditional on complex formation, while ADC synthase activity is increased fourfold in the complex compared to the isolated subunit (Roux & Walsh, 1992; Viswanathan *et al.*, 1995)

The second step is the cleavage of pyruvate from ADC to give PABA 3. This is carried out by ADC pyruvate lyase which is encoded by the *pabC* gene (Greene & Nichols, 1991; Green *et al.*, 1992). Pyridoxal-5'-phosphate (PLP) acts as a cofactor in this reaction. ADC pyruvate lyase has been crystallised and the structure determined using X-ray crystallography (Nakai *et al.*, 2000). The enzyme is a homodimer with  $M_r$  60 000. Each subunit consists of a small N-terminal domain and a larger C-terminal domain, with the active site of the enzyme (and the site of PLP binding) situated at the domain and subunit interface.



1.2 The Quinate Pathway

Quinic acid esters are common storage compounds in plants and quinic acid may consist of up to 10 % by weight of decaying leaves (Hawkins *et al.*, 1993). This abundant source of carbon is utilised by saprophytic organisms by means of a group of inducible catabolic enzymes.

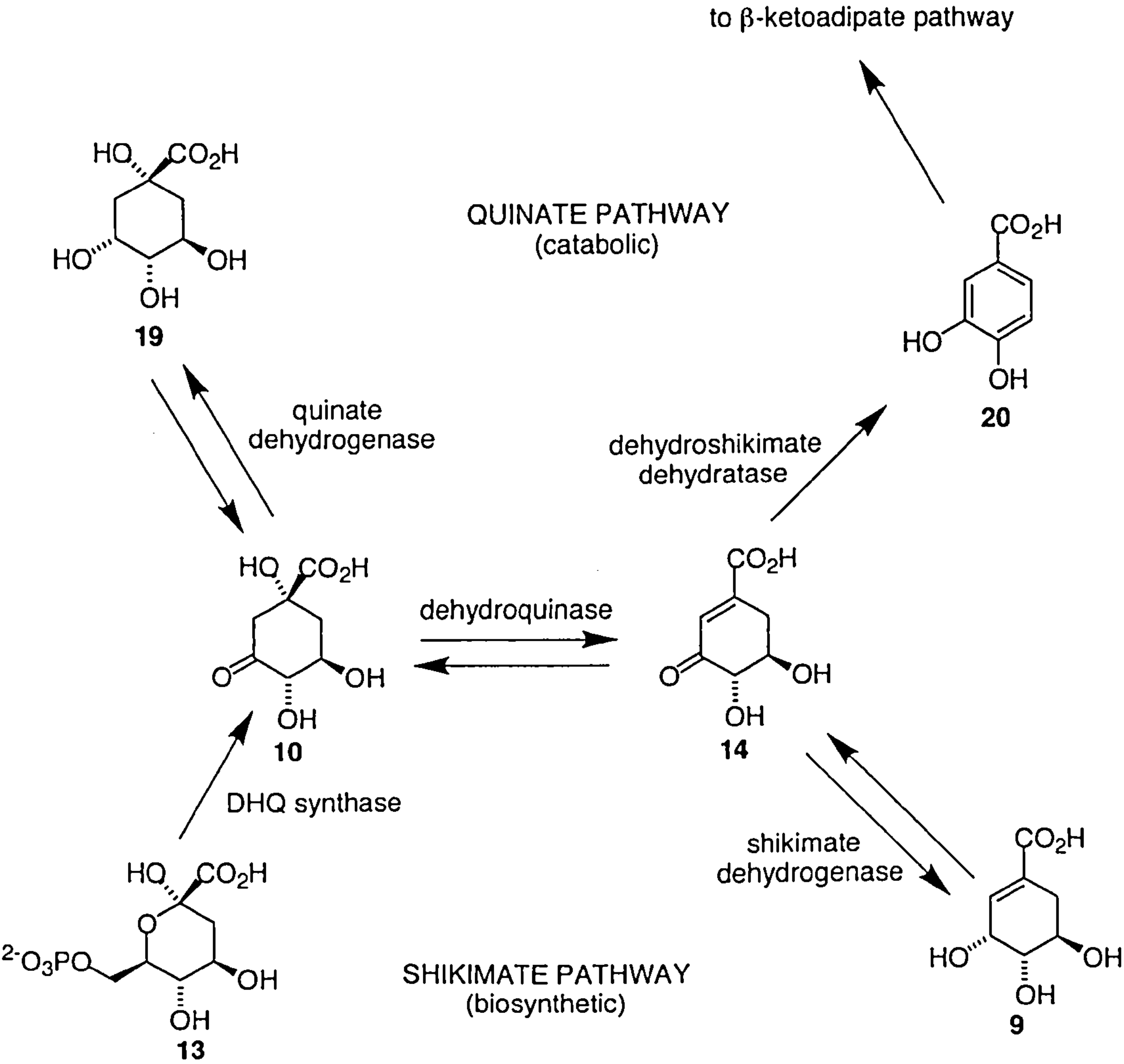


Figure 14: The inducible catabolic quinate pathway

Quinic acid **19** is initially converted to protocatechuic acid **20** and hence, *via* the  $\beta$ -ketoadipate pathway and oxidative cleavage of the aromatic ring, to acetyl CoA and succinic acid (*fig. 14*) (Gross, 1958; Ornston & Stanier, 1966).

The enzymes required for quinate utilisation are tightly regulated, being induced by the presence of quinic acid but inhibited by other, preferred, sources of carbon such as glucose (Giles *et al.*, 1985; Grant *et al.*, 1988). Of interest to our work is the fact that the DHQase isoenzyme found in the quinate pathway is entirely separate from that found in the shikimate pathway, having a different primary sequence, structure and mechanism of action (*see Section 1.2 above*) (Kleanthous *et al.*, 1992).



1.3 Known antimetabolites targeting the shikimate pathway

The best-known inhibitor of a shikimate pathway enzyme is the non-specific post-emergence herbicide N-(phosphonomethyl)-glycine (glyphosate) which has been commercialised under the trade name Roundup®. The herbicidal action is due to inhibition of the enzyme EPSP synthase. EPSP synthase catalyses the transfer of the enolpyruvyl group from PEP 11 to the 5-hydroxyl of shikimate-3-phosphate 15 to give EPSP 16 and inorganic phosphate (Steinrücken & Amrhein, 1980; Mousdale & Coggins, 1984; Mousdale & Coggins, 1985; Kishore & Shah, 1988).

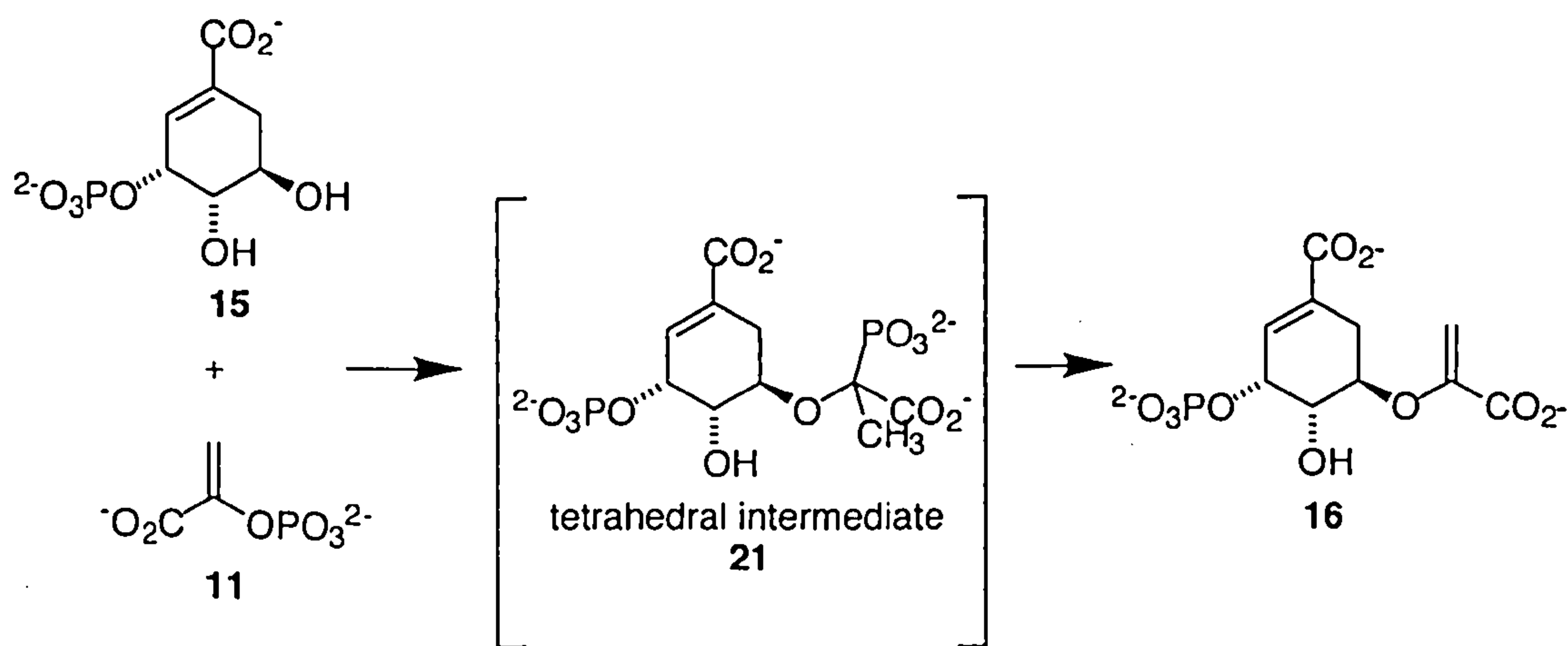


Figure 15: EPSP synthase reaction

The reaction proceeds via a tetrahedral intermediate 21 (*fig. 15*), and it is this intermediate which is believed to be mimicked by the formation of a ternary complex 23 between shikimate-3-phosphate 15 and glyphosate 22 on the enzyme (*fig. 16*) (Boocock & Coggins, 1983; Anderson & Johnson, 1990; Schönbrunn *et al.*, 2001).

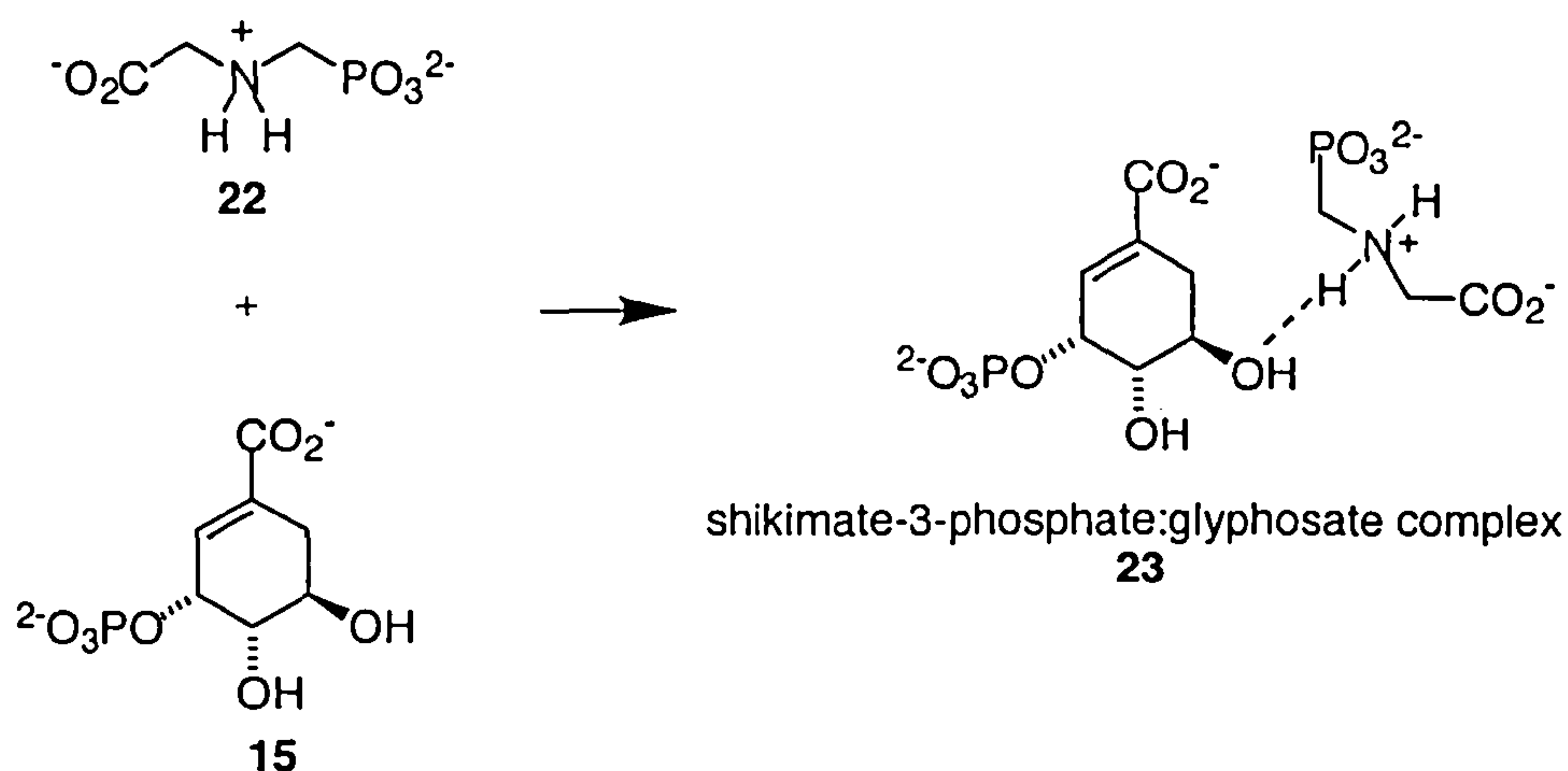


Figure 16: Proposed mechanism for inhibition of EPSP synthase by glyphosate

Various structural analogues of shikimate pathway substrates have been synthesised as potential inhibitors (*fig. 17*). The homophosphonate analogue of DAHP **24** inhibits DHQ synthase with  $K_i = 2.5 \mu M$  (Reimer *et al.*, 1986). The carbocyclic analogue **25** is more potent still with  $K_i = 1.7 \mu M$  and the phosphonate equivalent **26** is the best known inhibitor of the enzyme with  $K_i \sim 0.8 nM$  (Widlanski *et al.*, 1989). The carbocyclic malonate **27** analogue ( $K_i = 0.7 \mu M$ ) is inhibitory in the sub-micromolar range (Tian *et al.*, 1996) while the cyclohexenyl equivalent **28**, which was designed to mimic an E1cB-like transition state on the enzyme, is also inhibitory at nanomolar concentrations ( $K_i = 8.6 nM$ ) (Montchamp and Frost, 1997).

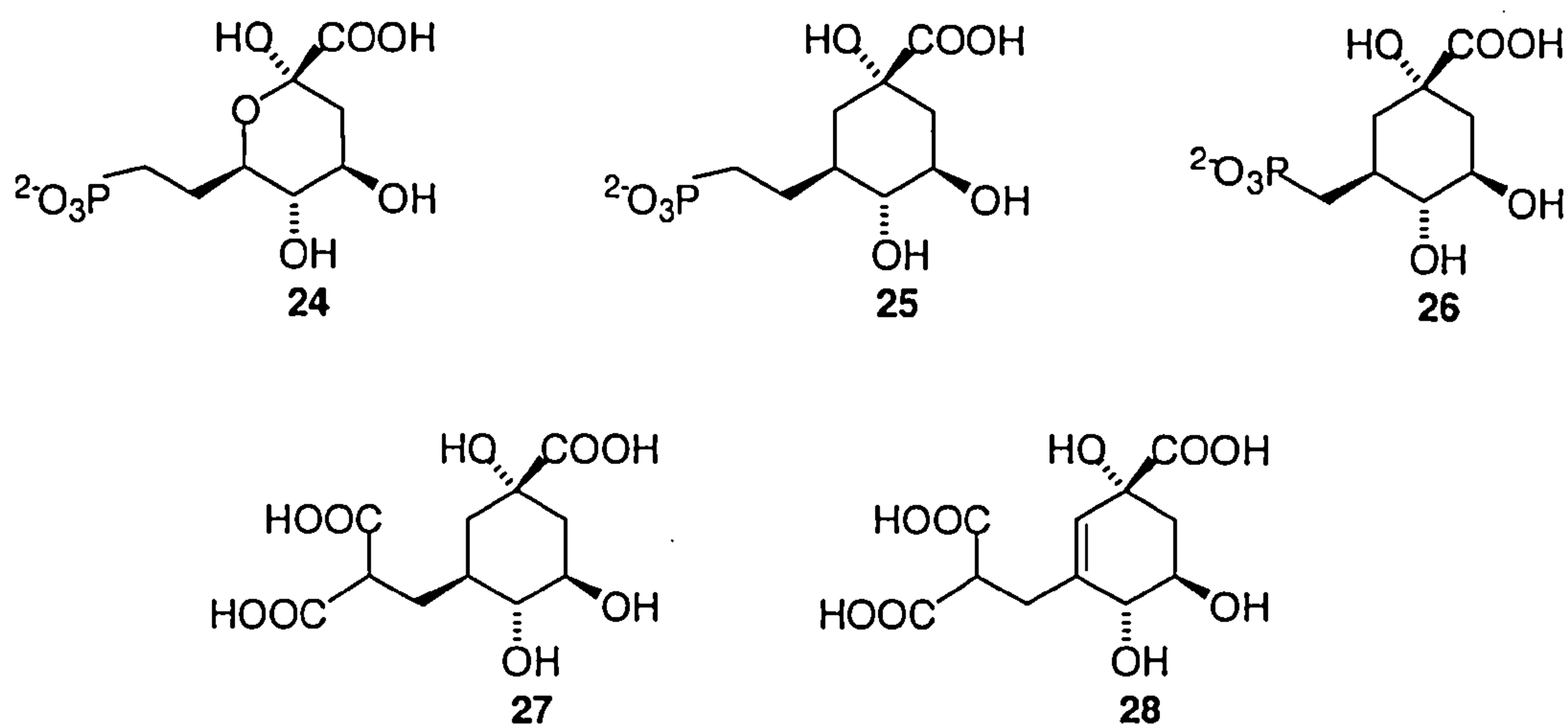


Figure 17: Potent inhibitors of DHQ synthase

The structural and mechanistic differences between type I and type II DHQases have been exploited in the design of specific inhibitors. The substrate analogues (2*R*)-2-bromodehydroquinate **29** and (2*R*)-2-fluorodehydroquinate **30** were designed as inhibitors of type I DHQase as they lacked the C-2-*pro-R* hydrogen while still being substrates for type II DHQases which require a C-2-*pro-S* hydrogen (*fig. 18*) (Gonzalez-Bello *et al.*, 2000).

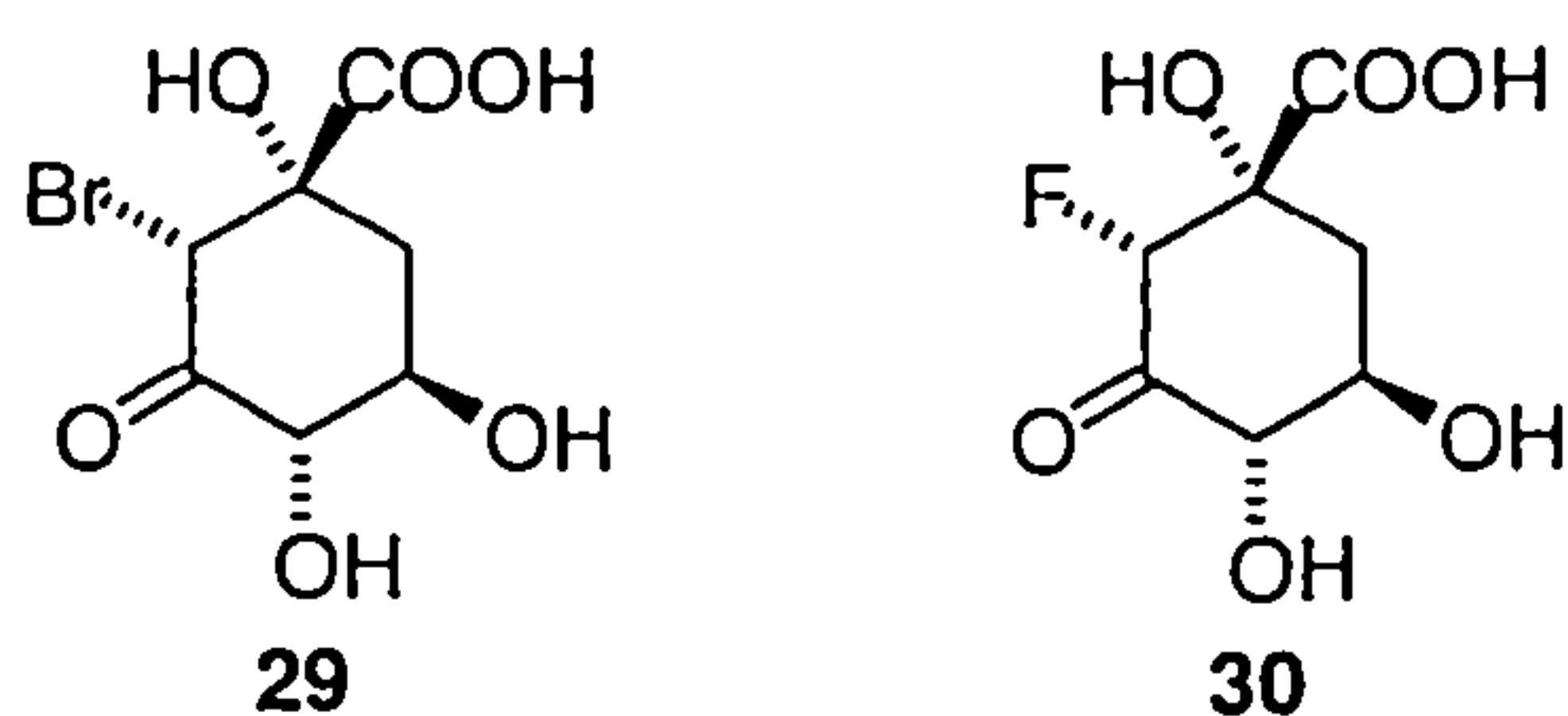


Figure 18: Irreversible selective inhibitors of type I DHQase

Compounds designed to selectively inhibit type II DHQases have been designed based on the structure of the *M. tuberculosis* DHQase (Gourley *et al.*, 1999) and the type II enzyme from *Streptomyces coelicolor* (Roszak *et al.*, 2002). Based on the fact that the elimination

catalysed by type II DHQases proceeds via an E1cB mechanism and is thought to involve an enolate intermediate four compounds modified at C-3 were tested against DHQases from a variety of organisms (Coggins *et al.*, 2003). The carbonyl at C-3 is required for imine formation with type I enzymes, so it was expected that both 2,3-anhydroquinic acid **31** and the reduced equivalent **32** (Frederickson *et al.*, 1999) would be selective for type II enzymes (*fig. 19*). The oxime **33** (Frederickson *et al.*, 1999) and fluorinated analogue **34** (Frederickson *et al.*, 2002) were designed to mimic the enolate intermediate. All were significantly better competitive inhibitors of the type II enzymes than of the type I enzyme tested (Coggins *et al.*, 2003).

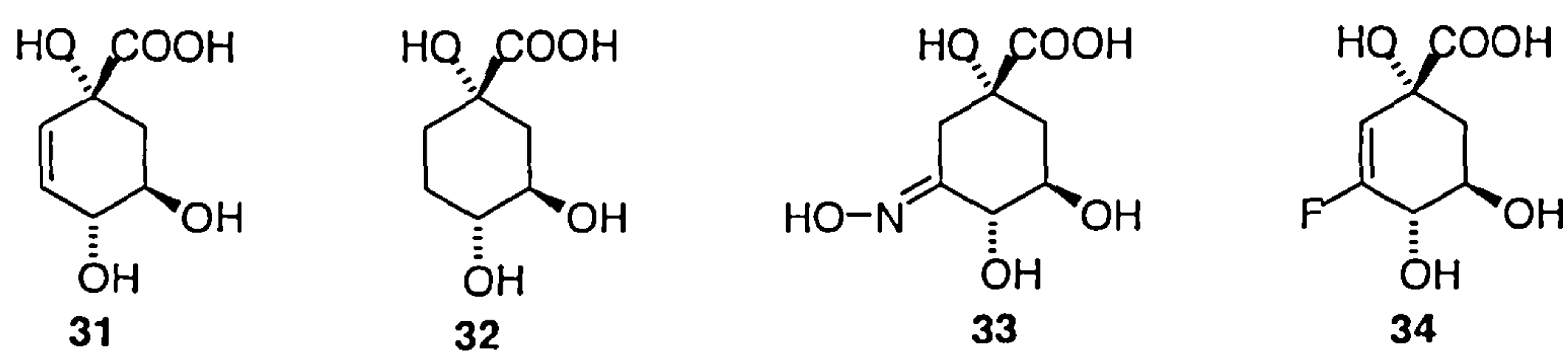


Figure 19: Selective inhibitors of Type II DHQase

Halogenated derivatives of shikimic acid have also been investigated as possible inhibitors of shikimate pathway enzymes from shikimate kinase onwards (*fig. 20*). Modification at C-2 to give 2-bromo- **35** and 2-fluoroshikimic acids **36** was carried out using a combination of synthesis and biotransformation (Gonzalez-Bello *et al.*, 1998). Modification at C-3 gave the (3*R*)- **37** and (3*S*)-fluoro **38** analogues of shikimic acid as well as the (3*S*)-chloro analogue **39** in syntheses starting from shikimic acid (Brettle *et al.*, 1996a; Brettle *et al.*, 1996b). The difluoro-substituted shikimic acid **40** was synthesised from quinic acid (Jiang *et al.*, 1999). None of these compounds was tested against shikimate pathway enzymes.



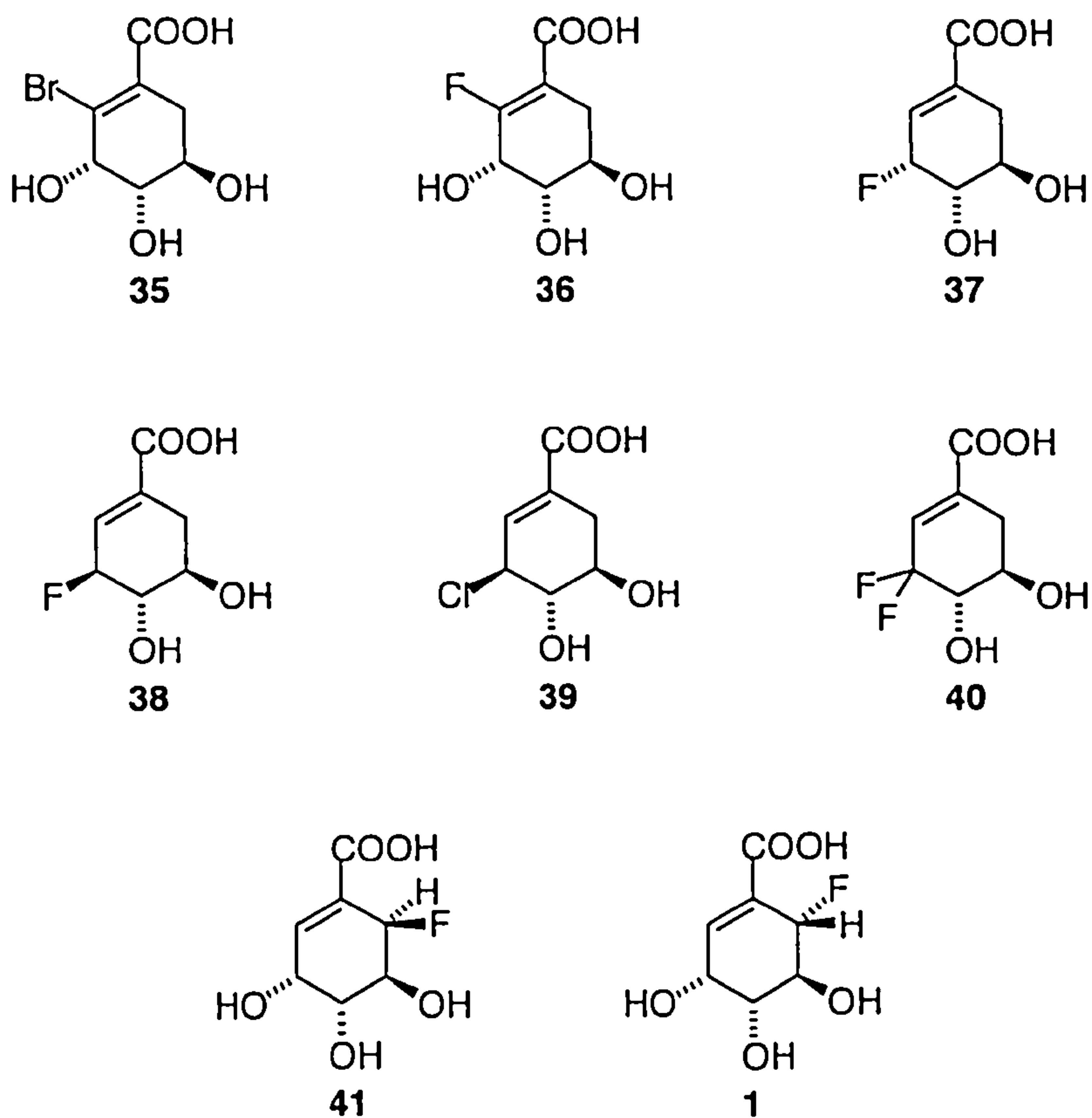


Figure 20: Halogenated derivatives of shikimic acid

By contrast, the 6-fluoroshikimic acids **41** and **1** were designed based on, amongst other considerations, the fact that the C-6 *pro-R* hydrogen is lost during the transformation of EPSP to chorismate (Davies *et al.*, 1994).



## 1.4 (6S)-6-fluoroshikimic acid

### 1.4.1 Design of a drug candidate

In considering an enzyme inhibitor as a potential antimetabolite, a crucial issue is whether the targeted organism is able to obtain the end-products of the inhibited pathway from external sources or alternative biosynthetic routes. Mutations of enzymes in the shikimate pathway have been shown to result in severely attenuated strains of pathogenic organisms which have potential use as live vaccines (Hoiseth & Stocker, 1981; Poirer *et al.*, 1988; Verma & Lindberg, 1991; Vaughan *et al.*, 1993). These ‘*aro*’ strains are unable to synthesise PABA, which is not imported into the cells, and hence have no source of the folate coenzymes involved in C-1 transfer in biosynthesis. In targeting the shikimate pathway, the ideal compound would therefore either be, or be converted into, a derivative of chorismate which could not be converted into PABA.

### 1.4.2 Modifications chosen/reasons

Shikimic acid was selected as a suitable candidate for modification as it is small, stable, and is known to be transported into bacterial cells (Pittard & Wallace, 1966; Pittard & Wallace, 1966b). Modification at C-6 was chosen as the conversion of EPSP to chorismate was known to proceed with the loss of the C-6 *pro-R* hydrogen. In substituting fluorine for hydrogen, the overall size and shape of the derivatives would be largely unchanged from the parent compound due to the similar atomic radii of the two atoms. As a result the modified compounds would be expected to bind to enzymes in a similar way to the natural substrate (Davies *et al.*, 1994). The substitution would, however, have a significant effect

on the ability of enzymes to catalyse the normal sequence of reactions. The fluorine-carbon bond (552kJ/mol) is almost twice as strong as the hydrogen-carbon bond (338 kJ/mol) making any elimination far less likely to occur, and the electronegativity difference between F and H suggested that enzyme-substrate interactions at this position would be disrupted.

Both (6*R*)- **41** and (6*S*)-6-fluoroshikimic acids **1** were synthesised. The authors stated that they expected the (6*R*)-6-fluoro- compound to be converted to (6*R*)-6-fluoro-shikimate-3-phosphate **42** and hence to (6*R*)-6-fluoro-EPSP **43**. This was expected to inhibit chorismate synthase as the normal elimination reaction would not be catalysed (*fig. 21*) (Davies *et al.*, 1994).

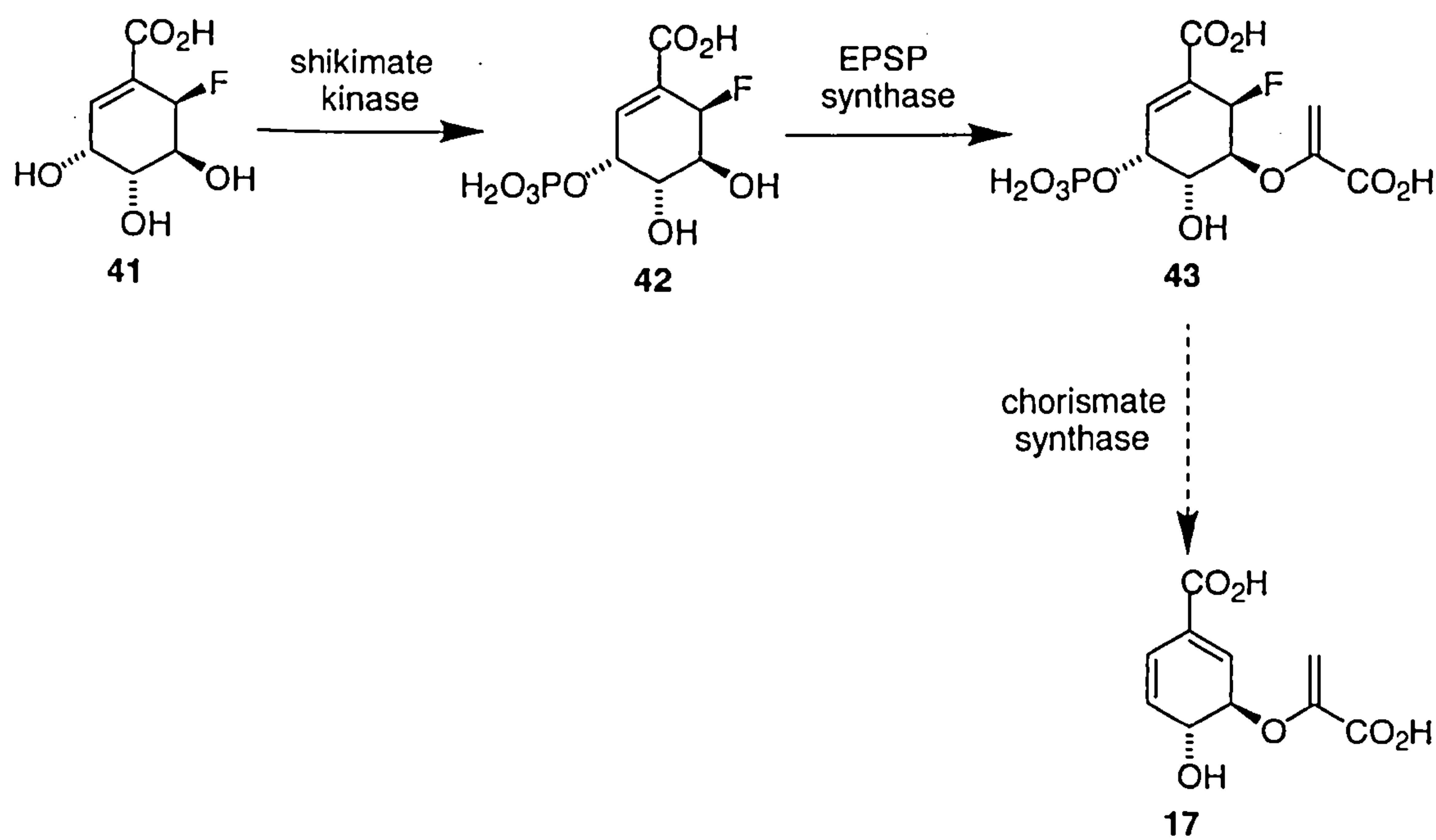


Figure 21: Expected transformation of (6*R*)-6-fluoroshikimic acid

The authors also hoped that the (6*S*)-6-fluoro- derivative **1** would be converted to 2-fluorochorismate **2** via (6*S*)-6-fluoro-shikimate-3-phosphate **44** and (6*S*)-6-fluoro-EPSP **45**. 2-Fluorochorismate **2** was expected to inhibit PABA synthase (*fig. 22*).

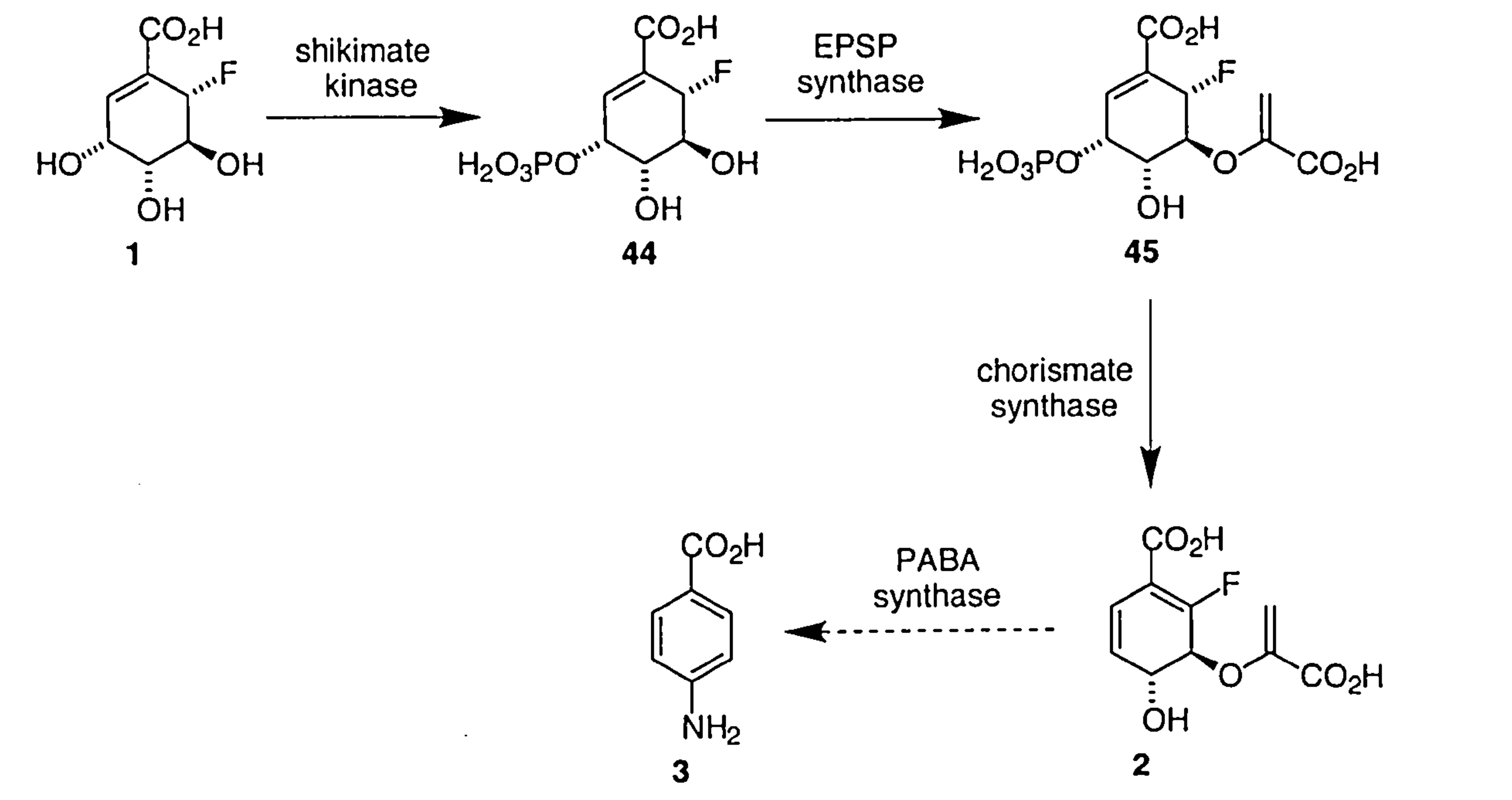


Figure 22: Expected transformation of (6*S*)-6-fluoroshikimic acid

### 1.4.3 Antibacterial action

*In vitro* testing showed that (6*R*)-6-fluoroshikimic acid **41** was weakly antibacterial against wild-type *E. coli* grown on minimal medium with a minimum inhibitory concentration (MIC) of 64  $\mu\text{g.mL}^{-1}$ . (6*S*)-6-Fluoroshikimic acid **1** was more potent, displaying MICs of between 0.25 and 0.1  $\mu\text{g.mL}^{-1}$  depending on the strain of *E. coli* tested. The inhibition of growth caused by (6*S*)-6-fluoroshikimic acid was reversed by the addition of *p*-aminobenzoic acid **3** to the growth medium, but not by the addition of any or all of the

aromatic amino acids phenylalanine, tyrosine or tryptophan. The slow formation of 2-fluorochorismate **2** in the growth medium, along with the reversal of inhibition detailed above were consistent with the theory that inhibition of PABA synthesis was crucial for the antibacterial activity of (6*S*)-6-fluoroshikimic acid.

Earlier studies had already shown that both (6*R*)- and (6*S*)-6-fluoro-EPSP **43** and **44** are inhibitors of chorismate synthase (Balasubramanian, 1991) but their conversion to 2-fluorochorismate was not observed. More detailed studies, however, showed that (6*S*)-6-fluoro-EPSP is converted to 6-fluorochorismate by the enzyme chorismate synthase (Bornemann, 1995).

As (6*S*)-6-fluoroshikimic acid **1** was more effective as an antimicrobial *in vitro* it was tested against bacterial challenge in mice. Immunocompromised mice (cyclophosphamide-treated) were infected with ten times the lethal dose of the bacterial strains used, and then injected peritoneally with the antibacterial compound being tested. The PD<sub>50</sub> (mg.kg<sup>-1</sup>) of (6*S*)-6-fluoroshikimic acid against the wild-type *E. coli* B was below 3.1, while against *Pseudomonas aeruginosa* and *Staphylococcus aureus* it was 28. Cefotaxime was protective at an almost identical dosage against *S. aureus* challenge, and while (6*S*)-6-fluoroshikimic acid could not compare with vancomycin in the treatment of MRSA infection it was effective at a four-fold lower dose than methicillin.

Under exactly the same test conditions, mice infected with a spontaneously resistant strain of *E. coli* B (designated *E. coli* B12) were not protected by treatment with (6*S*)-6-



fluoroshikimic acid, confirming that the test compound was acting directly against the bacteria.

#### 1.4.4 Microbial resistance to (6*S*)-6-fluoroshikimic acid

As resistance to (6*S*)-6-fluoroshikimic acid arose spontaneously after overnight incubation of a surface innoculum in the presence of the compound, further studies were undertaken in an attempt to determine the frequency and mechanism of resistance to (6*S*)-6-fluoroshikimic acid (Ewart, 1995). Four species of enterobacteria were investigated, and found to spontaneously develop resistance at frequencies between  $10^{-6}$  and  $10^{-2}$  with most being around  $10^{-5}$ . Resistant variants of *E. coli* K-12 were used for the biochemical and genetic study of resistance to (6*S*)-6-fluoroshikimic acid.

Uptake of [ $^{14}\text{C}$ ]shikimate by the resistant strains was negligible, and all strains resistant to (6*S*)-6-fluoroshikimic acid were also resistant to (6*R*)-6-fluoroshikimic acid. This suggested that resistance was due to a change in the transport of shikimate into the bacterial cells. More detailed genetic analysis suggested that mutation at the *shiA* locus had occurred (Ewart, 1995). *ShiA* is one of the two known loci controlling shikimate uptake (Pittard & Wallace, 1966b; Brown & Doy, 1976; Whipp, 1998) and these results confirmed that resistance was most likely caused by a lack of uptake of shikimate in resistant strains.

Further work using various shikimate analogues in exchange-diffusion experiments with [ $^{14}\text{C}$ ]shikimate showed that (6*S*)-6-fluoroshikimic acid is a substrate for the shikimate transport system (Jude, 1996). The authors also speculated that their experiments with



respiration inhibitors and uncouplers suggested that the shikimate transport system was in some way kinetically gated, and could be used to transport a variety of toxic shikimate analogues into cells. They cautioned, however, that the high frequency at which the transport system appeared to be lost under selective pressure meant that alternative transport mechanisms would have to be used along with the shikimate transport system in order to introduce these drugs into the bacterial cytoplasm.

## **1.5 A possible solution to transport resistance: peptide based prodrugs**

### **1.5.1 Prodrugs: an introduction**

In the development of a new drug, chemical modification of the basic structure is used to achieve a compound with the desired pharmacological activity. If this optimised compound shows undesirable physicochemical characteristics, reversible derivatives can be used to optimise the clinical application of the drug. These derivatives are known as prodrugs and are chemical derivatives of drugs which are pharmacologically inactive but can be converted into the active drug molecule *in vivo*.

Prodrugs can be designed to target specific enzymes or carriers. Both enzymes and membrane-based carrier proteins are targeted to improve oral bioavailability of a drug or to allow site-specific delivery of the active compound (Kearney, 1996; Sherwood, 1996).

Activation may then be by means of an enzyme found only in the tissue of interest, or more generally by such ubiquitous enzymes as peptidases and esterases.

Peptide transporters are an attractive target in prodrug design as they have both broad substrate specificity and generally high capacity. They have also been extensively studied, providing useful leads as to the types of modifications which will be tolerated by each carrier system (Payne, 1994).

## **1.5.2 Transport in bacterial cells**

### **1.5.2.1 The need for transport systems**

Bacterial cells, like all other cells, are surrounded by a semi-permeable cell membrane which limits the entry and/or exit of substances from the cell. The membrane thus provides a separate compartment in which the conditions are kept optimal for biological reactions, as well as controlling the exchange of material and information between the cell and its environment.

The phospholipid bilayer of the cell membrane is an extremely effective barrier to the diffusion of most polar solutes. As a result, bacteria have developed methods for the uptake and export of biologically important compounds. Most of these methods involve coupling energy expenditure to the transport of a specific compound required for growth or defence.

The situation is further complicated in Gram negative bacteria by the presence of an outer cell membrane beyond the cell wall. While this membrane is relatively permeable to small solutes, it is a barrier to larger molecules (Lugtenberg & Van Alpen, 1983; Nikaido & Vaara, 1985; Nakae, 1986). Channel-forming proteins, the porins, in this membrane allow the diffusion of solutes with molecular weights up to approximately 600 Da (the limiting factor is the hydrodynamic or “Stokes radius”) into the periplasm (Benz, 1988; Nikaido, 1992). Thus the outer membrane acts as a molecular sieve, limiting the size of molecule which can reach the cell membrane and the transport systems associated with it (Payne & Gilvarg, 1968; Nakae & Nikaido, 1975; Decad & Nikaido, 1976). Some porins do show limited substrate specificity, and thus certain solutes will only be taken up by bacterial cells

with functioning porins of the correct specificity e.g. OmpF and OmpC in *E. coli* are required for the movement of peptides across the outer membrane (Andrews & Short, 1985; Alves, 1985).

#### **1.5.2.2 Why peptides are transported**

Amino-acid synthesis is extremely energetically demanding. Atkinson used an ATP-currency to estimate the relative metabolic costs of various metabolites including  $\alpha$ -amino acids (Atkinson, 1977). These range from 12 ATP-equivalents for glycine to 78 ATP-equivalents for tryptophan. Mammals no longer have the ability to synthesise the more costly of these amino acids, obtaining them instead from the diet. Only the few enzymes required to synthesise those amino acids which can be made in a few steps from intermediates in the citrate cycle or glycolysis have been retained.

Bacteria have the ability to import and hydrolyse short-chain peptides in order to obtain amino acids for protein synthesis. The metabolic cost of *de novo* amino acid synthesis far outweighs the energy expended in active transport. For a review of these non-specific bacterial peptide permeases as well as other microbial peptide transport mechanisms, see Payne & Smith, 1994.

#### **1.5.3 Peptide transport in *E. coli* and *S. typhimurium***

The peptide uptake systems of *E. coli* and *S. typhimurium* have been studied in some detail. There are three main routes by which small peptides of between two and five amino-acid residues in length are imported into the bacterial cells. The dipeptide permease (Dpp) transports dipeptides, and to a limited extent tripeptides; the tripeptide permease (Tpp)



transports tripeptides, and to a limited extent dipeptides; and the oligopeptide permease (Opp) transports di- and oligopeptides with up to five amino-acid residues. The Opp is by far the best characterised of these systems and, as a binding protein-dependent transport system, it has been studied as a model of this type of transporter.

#### **1.5.3.1 Binding-protein-dependent transport systems**

Binding-protein-dependent transport systems are part of the ATP-binding cassette (ABC) superfamily of proteins (Holland & Blight, 1999). Typically, ABC transporters consist of a highly conserved ABC-ATPase and at least one membrane domain which provides the transport pathway, although the basic unit is generally accepted to be a dimer containing two of each. In this way, the energy of ATP hydrolysis is coupled to the transport of solutes across cellular membranes.

ABC transporters are involved in a wide variety of transport processes in both eukaryotes and prokaryotes such as solute uptake and antibiotic resistance in bacteria, multidrug resistance systems in eukaryotes, and the excretion of various compounds. Their importance is illustrated by the fact that the genome sequence of *E. coli* K-12 contained at least 80 identifiable ABC proteins, which equates to 1 - 2 % of total proteins (Blattner *et al.*, 1997). For reviews on ABC transporters, see Ames *et al.*, 1990; Higgins, 1992; Linton & Higgins, 1998; Holland, 1999; Jones, 1999 and Schmitt, 2002.

Periplasmic binding-protein dependent systems are a type of ABC transporter found in Gram-negative bacteria. They generally consist of four different protein components which can be either subunits or domains of a single multifunctional peptide associated with the cell membrane. The defining feature, however, is the presence of a soluble substrate-



binding protein in the periplasm. These systems transport a wide range of substrates and include the transporters for maltose, histidine and oligopeptides. Binding-protein dependent systems have also been called 'shock-sensitive transport systems' as osmotic shock leads to the loss of the soluble periplasmic binding protein and hence the loss of transport activity (Ames, 1986).

It was initially believed that these transport systems were unique to Gram-negative bacteria, as Gram-positive bacteria lack an outer membrane and periplasm. However, analogous systems have been found in Gram-positive bacteria and archaea where the binding protein is a secreted glycolipid which is anchored to the outer surface of the cell membrane, e.g. the trehalose/maltose transport system of the hyperthermophilic archaeon *Thermococcus litoralis* (Horlacher *et al.*, 1998). Obviously, these anchored binding proteins will not be lost if the cell is subjected to osmotic shock.

#### 1.5.3.2 The oligopeptide permease

The oligopeptide permease (Opp) is a periplasmic binding protein-dependent system which transports peptides ranging in length from two to five amino acid residues (Matthews & Payne, 1980; Payne, 1978; Payne, 1980). The upper size limit for transport by the Opp is controlled by the outer membrane porins. These have a channel diameter of ~1nm which equates to a molecular weight of about 600 Da for the peptides being transported.

Transport assays of the oligopeptide permease showed that it possesses greatest affinity for tripeptides, but will transport any  $\alpha$ -amino peptide up to a pentapeptide (Alves *et al.*, 1985; Payne, 1980.; Payne, 1986).

The *opp* locus consists of four genes, *oppA*, *oppB*, *oppC*, and *oppD* which are organised as a single operon and cotranscribed. OppA is a 60 kDa protein which makes up ~ 8% of the total protein in osmotic shock fluid from *E. coli* (Guyer, 1985). It preferentially binds tri- and tetrapeptides, with limited binding to pentapeptides and none to isolated amino acids or dipeptides (Guyer, 1986). Binding to oppA does not appear to be greatly influenced by the amino acyl side chains of peptides. In contrast, modification of the amino or carboxy termini of a peptide greatly reduced binding to oppA (Tame, 1994).

OppA has been crystallised with a variety of natural and unnatural peptide substrates (Tame, 1994; Tame, 1995; Davies, 1999; Sleight, 1999). Unlike most other periplasmic binding proteins, which consist of two hinged domains which enclose substrates like a Venus' fly-trap closing, OppA is organised into three domains, but the relative organisation of the outer two domains resembles that of the two lobes found in the common binding proteins (Tame, 1995).

Peptides bound to OppA do so in an extended conformation, with tight interactions between the peptide backbone of the substrate and  $\beta$ -sheets in the binding protein (Tame, 1995). The amino terminus of the substrate is bound to an aspartate residue, and the carboxy terminus to one of several charged side chains depending on the length of the peptide bound, thus explaining the loss of binding affinity to N-acylated or C-esterified peptide substrates (Tame, 1994). Amino-acid side chains are accommodated in large hydrated pockets within OppA, with highly-ordered water molecules within these pockets satisfying hydrogen-bond requirements without imposing binding specificity (Tame *et al.*, 1996; Sleight, 1999; Rostom, 2000).

#### 1.5.4 Examples of peptide-based prodrugs

In the design of prodrugs, oral administration is generally a desirable outcome. As a result, peptide-based prodrugs have not been widely used. Those peptide prodrugs which do exist have often been targeting specific mammalian transporters such as the proton-coupled transporters PEPT1 and PEPT2 which are found in the human gut (Nielsen & Brodin, 2003).

Prodrugs utilising bacterial transporters to target antimicrobial compounds have been referred to as 'smugglins' (Payne, 1976). Toxic amino acids have been incorporated in peptides, e.g. alafosphalin (Allen, 1978) while impermeant non-peptide molecules have been attached to amino-acyl side chains e.g. 5-fluorouracil (Kingsbury *et al.*, 1984). Importantly, extensive *in vivo* studies have shown that the development of transport resistance to compounds targeting peptide permeases is unlikely (Ringrose, 1985).

## 2 (6S)-6-fluoroshikimic acid

### 2.1 Published syntheses of 6-fluoroshikimic acids

#### 2.1.1 From quinic acid

A chiral synthesis of both (6*R*)- and (6*S*)-6-fluoroshikimic acid from quinic acid was published by the Davies group (*fig. 23*) (Sutherland *et al.*, 1989). The known lactone **46** was prepared in 85 % yield from quinic acid (Elliott *et al.*, 1983). The tertiary hydroxyl of **46** was then protected using benzyl chloroformate to give **47** and the lactone cleaved to give the ester **48**. Sequential treatment with trifluoromethanesulfonic anhydride (Tf<sub>2</sub>O) and 1,8-diazabicyclo[5.4.0] undec-7-ane (DBU) gave the alkene **49**. Hydrolysis followed by acetylation gave the acid **50** which yielded the β-lactone **51** on bromolactonisation. The lactone was cleaved with simultaneous removal of the acetate protecting group to give bromohydrin **52**. Epoxidation of the alkene gave **53** which was then dehydrated to give **54**. This dehydration was problematic and was only successful when the sulfurane [PhC(CF<sub>3</sub>)<sub>2</sub>O]<sub>2</sub>SPh<sub>2</sub> (Martin's Reagent) was used. Epoxide ring opening with HF-pyridine was unfortunately non-specific and gave the epimeric fluorohydrins **55** and **56** along with the regioisomer **57**. These were partially separated using silica-gel column chromatography, and purified using a C<sub>18</sub> HPLC column. Hydrolysis of the ester and cleavage of the cyclohexylidene group gave the desired shikimate derivatives **1** and **41** along with the regioisomer **58**. The synthesis requires six steps from quinic acid, with an approximate overall yield of 3 % of (6*S*)-6-fluoroshikimic acid **1**.

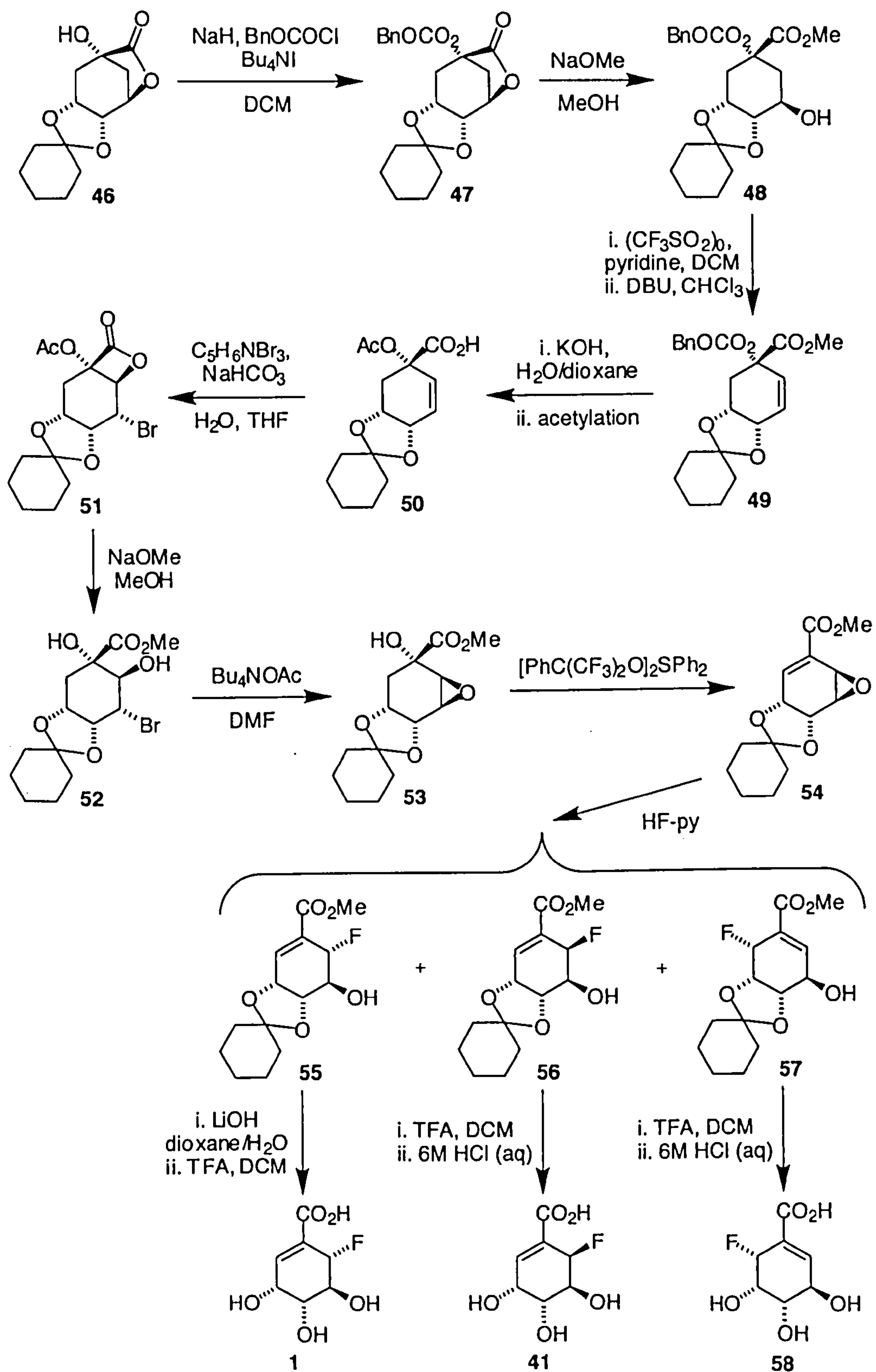


Figure 23: Route to 6-fluoroshikimic acids from quinic acid



The lack of specificity of the epoxide ring opening on reaction of **54** is the major problem with this synthetic approach. Normally, attack by fluoride at an epoxide would be expected to occur in an S<sub>N</sub>2-like manner, giving **55**. However, in this case, a significant proportion of an S<sub>N</sub>1-like reaction appears to have occurred. The positive charge formed on opening of the epoxide ring may be stabilised by delocalisation, allowing attack by fluoride from the opposite face to the bulky cyclohexylidene group at both C-2, giving **56**; and C-6, giving **57**.

An improvement to this synthesis was outlined a few years later in a communication published by the same group (*fig. 24*) (Sutherland *et al.*, 1993).

The known diene **49** was made in five steps from quinic acid (*fig. 23*) (Sutherland *et al.*, 1989). Osmium catalysed dihydroxylation using *N*-methylmorpholine *N*-oxide (NMO) as the stoichiometric oxidant gave **59** which was selectively protected as the C-5 triflate to give **60**. Treatment with NaH in dichloromethane resulted in a *trans*-transesterification to give the regioisomer **61**. Dehydration using Martin's reagent and hydrogenolysis gave the hydroxyshikimic acid derivative **62**. Reaction with *N,N*-diethylaminosulfur trifluoride (DAST) then gave the desired (6*S*)-6-fluoro derivative **63** in approximately 16 % yield over 11 steps from quinic acid. If similar deprotection methods to those used in the 1989 synthesis were used to generate (6*S*)-6-fluoroshikimic acid **1** (*fig 23*), the overall yield for this route from quinic acid would be in the 6 - 10 % range.

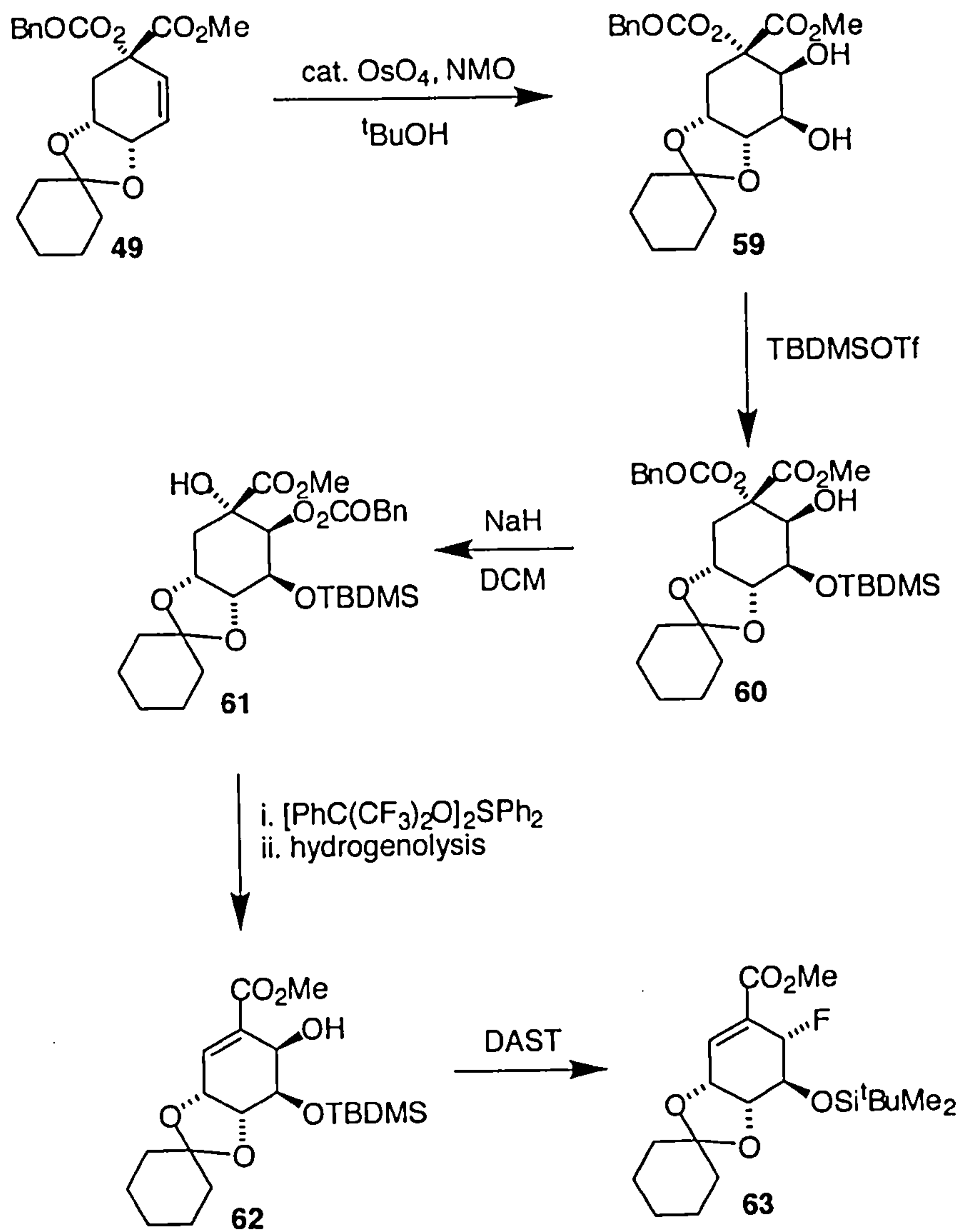


Figure 24: Improved route to (6*S*)-6-fluoroshikimic acid from quinic acid

### 2.1.2 By biotransformation from 3-fluoro-phosphoenolpyruvate

Multistep enzymatic synthesis had been used successfully to generate isotopically labelled intermediates in vitamin B<sub>12</sub> biosynthesis (Scott, 1994). As a result, the Abell group decided to look for an enzymatic route to both (6*R*)- and (6*S*)-6-fluoroshikimic acids (Duggan *et al.*, 1995).

The conversion of 3-fluoroPEP to 3-fluoroDAHP was known (Pilch & Somerville, 1976). 3-fluoroDAHP had also been used as a substrate for DHQ synthase in the presence of DHQase (Le Maréchal *et al.*, 1986). Additionally, (6*S*)-6-fluoroshikimic acid was known to be converted to (6*S*)-6-fluoroEPSP and 2-fluorochorismate by the relevant enzymes (Bornemann *et al.*, 1995). It therefore seemed a reasonable assumption that purified enzymes along with the relevant cofactors could be used to convert both isomers of 3-fluoroPEP **64** and **65** to the 6-fluoroshikimic acids **1** and **41** (*fig. 25*).

A test reaction using a mixture of the four enzymes required together with their cofactors was followed spectrophotometrically by monitoring the consumption of NADPH at 340 nm. The results suggested that a shikimate derivative was being formed. On a preparative scale, the reaction was carried out in 50 mM MOPS buffer at pH 7 with sequential addition of the enzymes and cofactors as each step was completed. An initial decrease in absorbance at 234 nm was indicative of the consumption of 3-fluoroPEP **64** and **65**. An increase in absorbance at the same wavelength showed the formation of the 6-fluorodehydroshikimates **70** and **71** on the addition of DHQ synthase, DHQase, Co<sup>2+</sup> and NAD<sup>+</sup>. The reaction was considered complete when the addition of further aliquots of SDHase had no effect on the levels of NADPH in the reaction mixture.

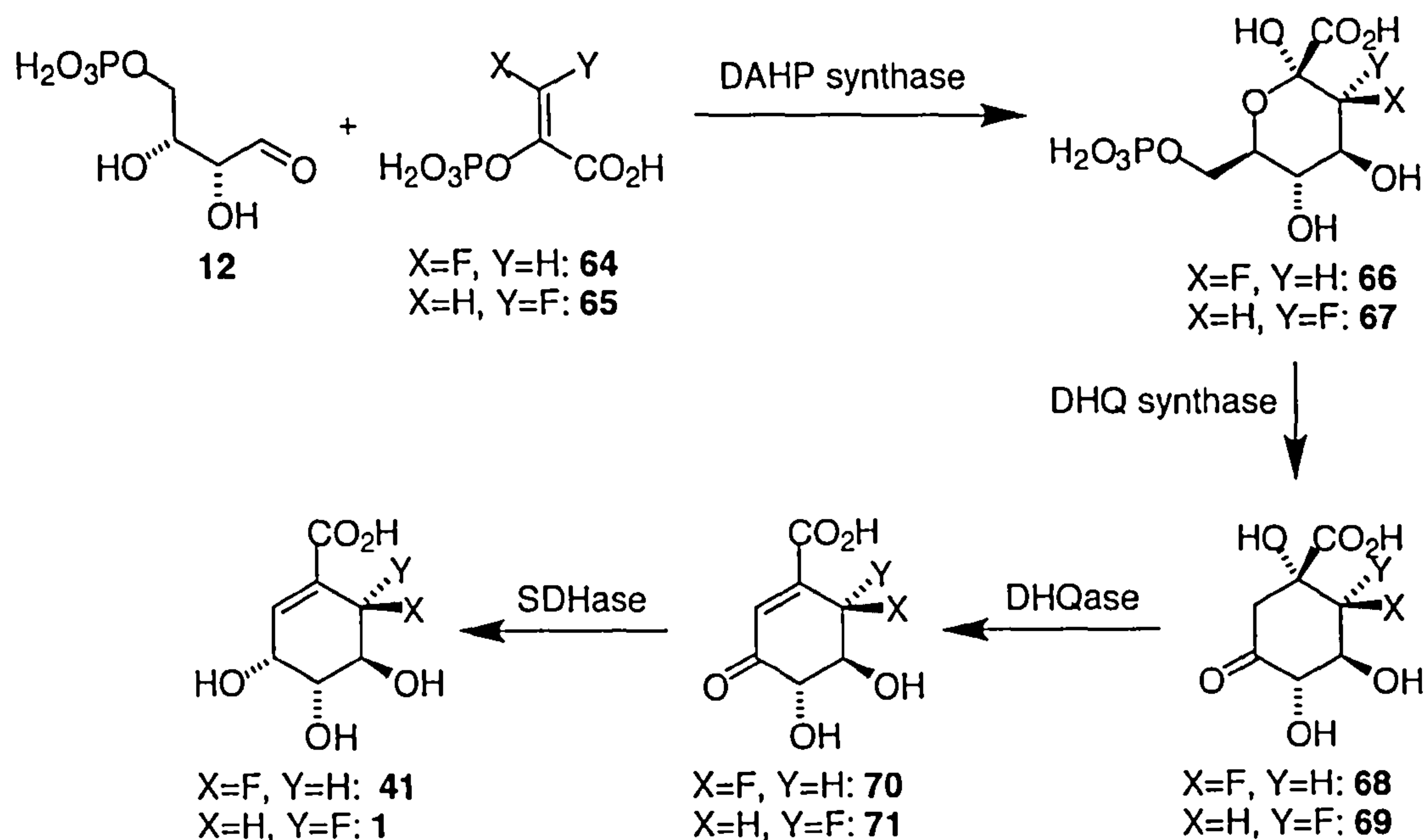


Figure 25: Proposed biotransformation route to the 6-fluoroshikimic acids

A combination of anion exchange and ion-exclusion HPLC was used to purify the 6-fluoroshikimates **1** and **41** giving isolated yields of approximately 17 % of each epimer. Unexpectedly, despite the ratios of the 3-fluoroPEP substrate **64** and **65** being 88:12 the 6-fluoroshikimates were isolated in equimolar amounts.  $^{19}\text{F}$ -NMR analysis was used to show that this was at least partly due to the very slow conversion of (3*S*)-3-fluoroDAHP **67** to (6*S*)-6-fluoroDHQ **69** compared to the reaction of (3*R*)-3-fluoroDAHP **66**. A suggestion put forward was that the rate of the spontaneous reaction of (3*S*)-3-fluoroDAHP **67** was comparable to that of the enzyme-catalysed reaction, and that this led to the loss of stereospecificity in the conversion (C. Abell, Fluorine Research Conference, 2002). An alternative product was formed from **67** during the course of the  $^{19}\text{F}$ -NMR reaction. This further reduced the yield of the desired shikimate derivatives but was not characterised by the authors (Duggan *et al.*, 1995).

Compared with the synthetic routes (*fig. 23* and *fig. 24*), this biotransformation route has two significant advantages. The yield is at least double that of the best total synthesis, and the total time required for the reaction is 26 h followed by HPLC purification as compared to the weeks or months required for some challenging synthetic chemistry. Disadvantages are that the method requires experience in enzyme purification and in the use of enzymes in transformations. Also, the substrate erythrose-4-phosphate is both unstable and very expensive.



2.1.3 From shikimic acid

In late 2001, the Singh group published a shorter synthesis of (6*S*)-6-fluoroshikimic acid **1** from shikimic acid **9** (fig. 26) (Song *et al.*, 2001). By extracting the starting material from Chinese star anise (*Illicium verum*) on a large scale in the laboratory, the authors avoided the major disadvantage of this route which is the high cost of commercially available shikimic acid (Aldrich, 1 g = £38.80).

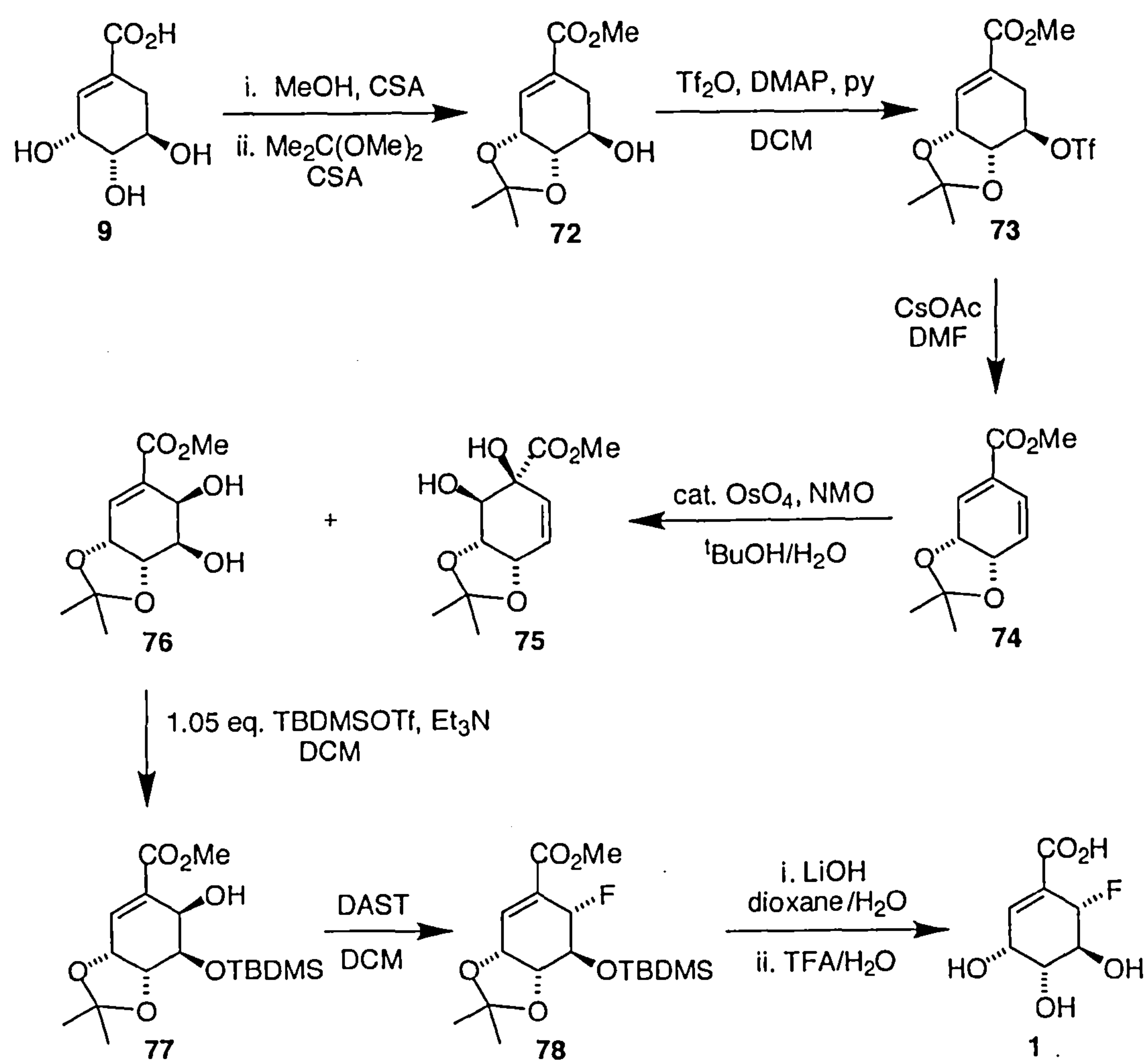


Figure 26: Synthesis of (6*S*)-6-fluoroshikimic acid from shikimic acid

Shikimic acid **9** was esterified by heating in methanol under acid catalysis. The resulting ester was further protected using 2,2-dimethoxypropane and camphorsulfonic acid (CSA) to give the acetonide **72**. Reaction with  $\text{TiCl}_4$  formed the triflate **73** which was then treated with cesium acetate in DMF to yield the diene **74**. Osmium-catalysed dihydroxylation of the diene using NMO as secondary oxidant gave the diols **75** and **76** in approximately equal amounts. After separation, the C-5 hydroxyl of diol **76** was selectively protected as the *t*-butyldimethylsilyl (TBDMS) ether, forming the protected hydroxylshikimic acid derivative **77**. Treatment with an excess of DAST then gave the fluorinated derivative **78**. Hydrolysis of the ester using lithium hydroxide was followed by treatment with aqueous trifluoroacetic acid (TFA) to remove the silyl and isopropylidene protecting groups. The desired (6*S*)-6-fluoroshikimic acid **1** was thus synthesised in nine steps and 15 % overall yield from shikimic acid.

## 2.2 Results: attempted syntheses of (6S)-6-fluoroshikimic acid

### 2.2.1 Biotransformation from 3-fluoroPEP

The published biotransformation from 3-fluoroPEP to both epimers of 6-fluoroshikimic acid (*fig. 25*) (Duggan *et al.*, 1995) is both higher-yielding and less time-consuming than the chiral syntheses from quinic acid (*fig. 23 & 24*) (Sutherland *et al.*, 1989; Sutherland *et al.*, 1993). Initial attempts to synthesise (6S)-6-fluoroshikimic acid **1** were therefore made using the biotransformation method.

E-4-P **12** is very expensive and is also known to be unstable in solution where it dimerises to give compounds which are not substrates for DAHP synthase (Duke & MacLeod, 1981).

It was therefore decided to attempt to generate E-4-P *in situ* from D-fructose-6-phosphate (F-6-P) **79** using transketolase from bakers' yeast (*fig. 27*) (Reimer *et al.*, 1986). This method has been used to convert 3-fluoro-PEP to the corresponding DAHP derivatives **66** and **67** (Parker, 1996) and had been validated in our laboratories (C-A. Deans, Senior Honours project, 1999, University of Glasgow). The generation of E-4-P was carried out in the presence of DAHP synthase, its cofactors and an excess of 3-fluoro-PEP, thus avoiding the high concentrations of E-4-P which result in the formation of unreactive dimers.

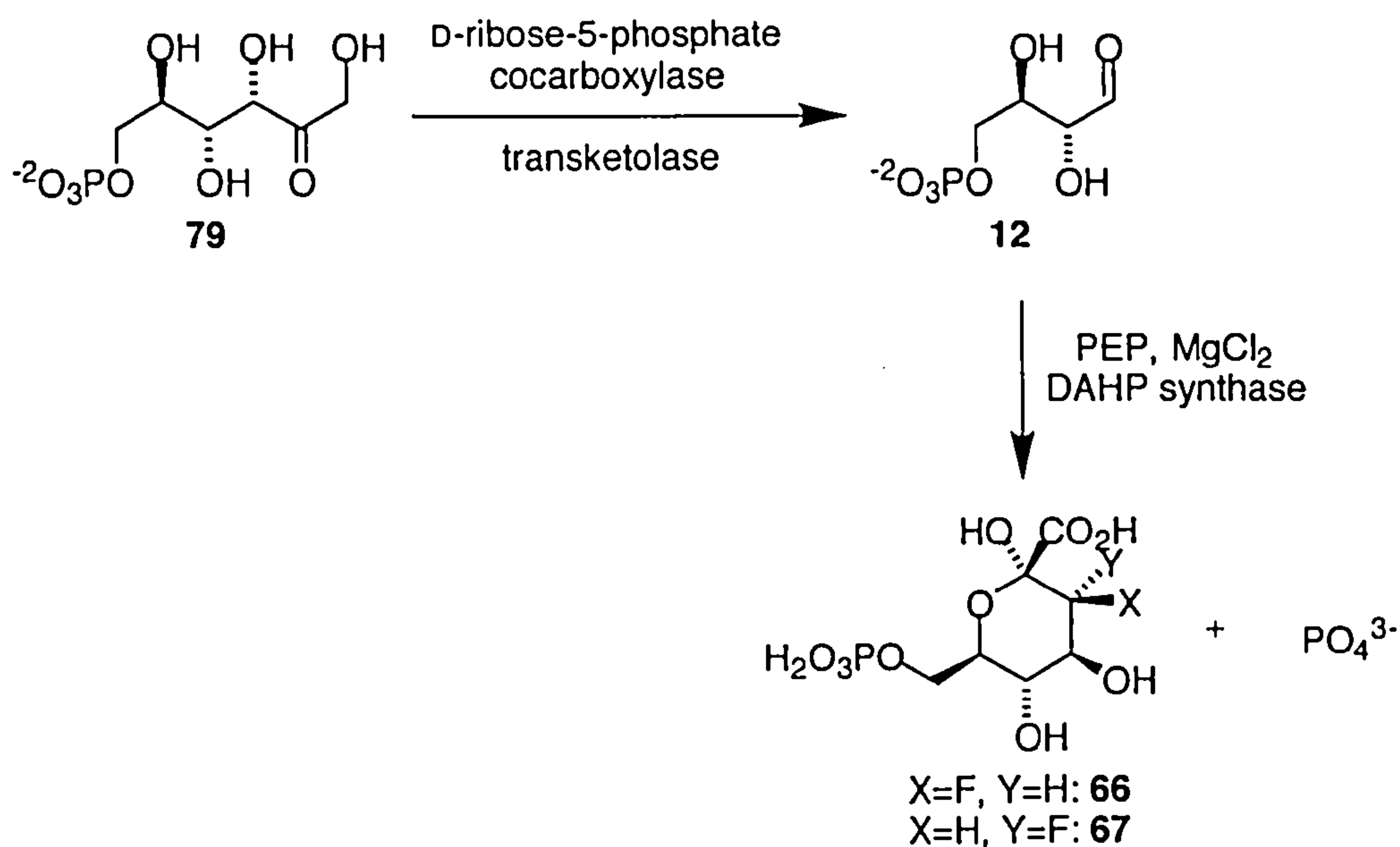


Figure 27: Proposed biotransformation to 3-fluoro-DAHP using *in situ* generated E-4-P

This route had not been extended beyond 3-fluoro-DAHP to the shikimate derivatives desired. As significant modifications to the published route were proposed, it was decided to attempt the initial biotransformation reaction using the natural substrates.

#### 2.2.1.1 Enzyme preparation

An overproducing strain of *E. coli* (*E. coli* W3110/pJB14) was used to obtain fresh stocks of dehydroquinate synthase, which converts DAHP to DHQ. As the enzyme had been extensively studied in the group, a protocol was available both for the purification of the enzyme and for assaying its activity (Mousdale & Coggins, 1993). All other enzymes required for the biotransformation were available in sufficient quantity in the group. For enzyme preparation methods and assay conditions, see Chaudhuri *et al.*, 1987a,b; Coggins *et al.*, 1987; Mehdi *et al.*, 1987; Gourley *et al.*, 1999; Maclean *et al.*, 2000 and Michel *et al.*, 2003.

2.2.1.2 Model studies using PEP

The proposed biotransformation involved the conversion of PEP to shikimic acid **9** in a single pot with *in situ* generation of E-4-P **12** (fig. 28). It was therefore necessary to find a buffer system which allowed a reasonable rate of reaction for all the enzymes involved, as well as ensuring that the conditions required for the formation of E-4-P were compatible with the remainder of the proposed route.

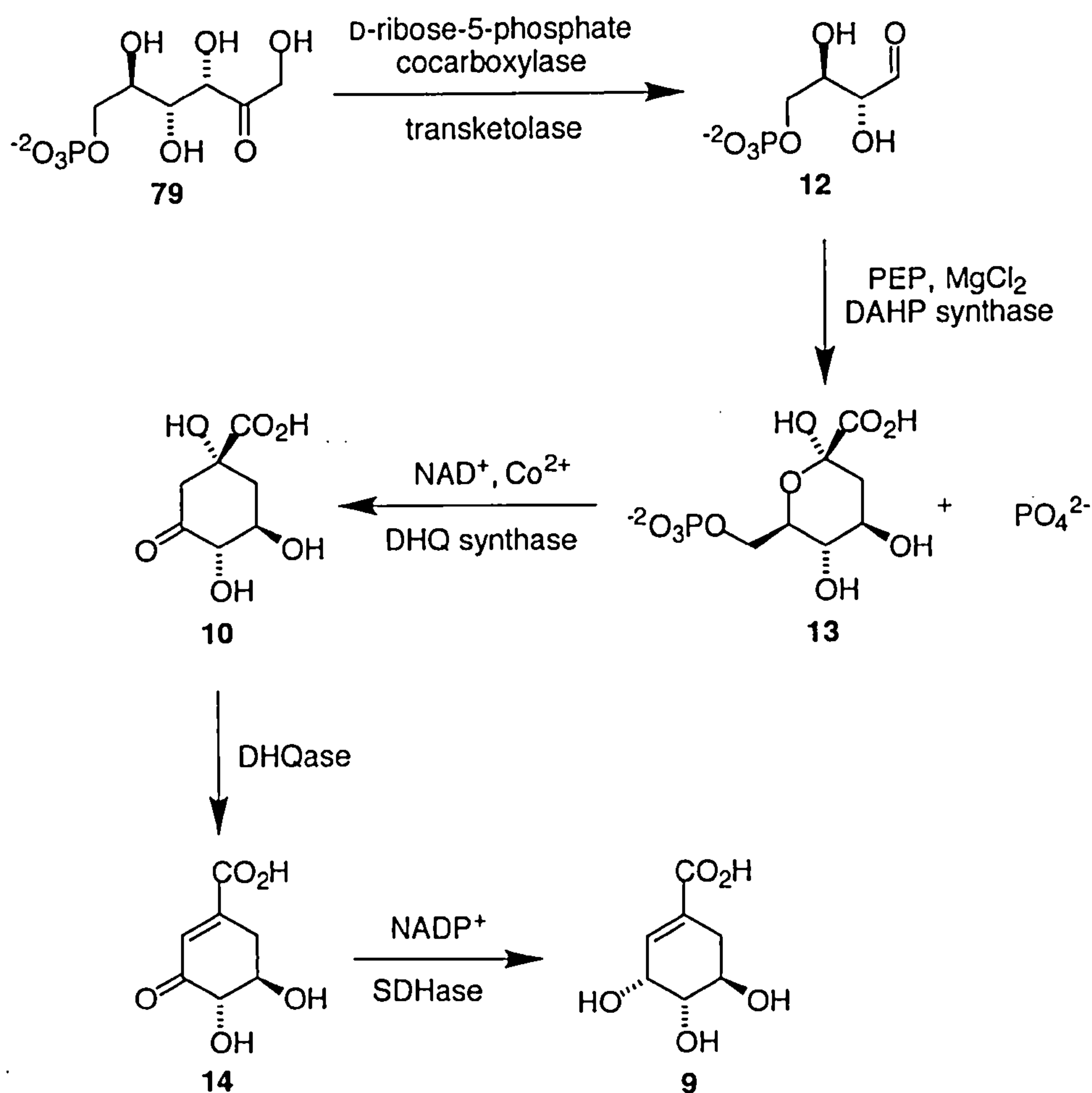


Figure 28: Proposed biotransformation from PEP to shikimic acid



The published biotransformation route to both 6-fluoroshikimic acids was carried out in 50 mM MOPS at pH 7 (Duggan *et al.*, 1995). By contrast, the synthesis of DAHP 13 using *in situ* generation of E-4-P was carried out in unbuffered aqueous solution under anaerobic conditions with the pH adjusted to pH 7 prior to reaction initiation (Parker, 1996).

Transketolase, either from bakers' yeast (commercial) or *Leishmania mexicana* (gift, N. Veitch) and its cofactor cocarboxylase were added to a mixture of F-6-P 79 and an excess of PEP in either water or 4-morpholinepropanesulfonic acid (MOPS) buffer at pH 7. The formation of DAHP 13 was followed using the thiobarbituric acid assay of sialic acids developed by Warren (Warren, 1959). In this assay, a pink chromagen absorbing at 549 nm is formed by the reaction of oxidised DAHP with thiobarbituric acid (4,6-dihydroxy-2-mercaptopyrimidine).

In MOPS buffer, DAHP formation was markedly decreased compared to reaction in unbuffered aqueous solution (*fig. 29*). Strict anaerobic conditions were not maintained during these reactions, as the cofactors required for the later stages of the biotransformation reacted adversely with the dithiothreitol and sodium azide which would normally have been used. Dissolved oxygen was, however, removed from the reaction mix by displacement with nitrogen.

	A <sub>549</sub> 50 mM MOPS pH 7 (AU)	A <sub>549</sub> H <sub>2</sub> O pH7 (AU)
T = 0	0.14	0.09
T = 1.5 h	0.24	0.48
T = 18 h	0.18	0.97

Figure 29: Formation of DAHP in 50 mM MOPS vs. H<sub>2</sub>O both at pH 7

The conditions of the *E. coli* DHQ synthase assay were then used to investigate the formation of DHS 14 from PEP (Coggins *et al.*, 1987). The reaction was monitored spectrophotometrically, with an increase in absorbance at 234 nm corresponding to the formation of DHS. When the mixture of DHQ synthase,  $\text{Co}^{2+}$ ,  $\text{NAD}^+$  and DHQase were added to purified DAHP 13 in water with the pH adjusted to pH 7, the initial reaction rate was  $1.4 \times 10^{-2} \text{ AU.s}^{-1}$ . However, when the enzymes and cofactors were added to the biotransformation mix containing DAHP, no measurable change in absorbance at 234 nm was observed over several hours. This lack of reaction was also observed if cocarboxylase was added to purified DAHP 13 along with DHQ synthase, DHQase and their cofactors. This result is consistent with the mechanism of action of transketolase, as the initial step is the addition of a ketone to thiamine pyrophosphate (cocarboxylase) (Stryer, 1988). The presence of a reactive carbonyl on both DHQ 10 and DHS 14 which could be attacked by the activated nitrogen of cocarboxylase was thought to be the reason for the failure of this approach.

A two-pot approach was thus decided on. DAHP 13 was synthesised from PEP using transketolase to generate E-4-P 12 *in situ* and purified on a DEAE Sephacel column eluted with an ammonium hydrogen carbonate gradient. After lyophilisation, the purified DAHP was converted to shikimic acid 9 according to the literature protocol (Duggan *et al.*, 1995). At this stage, the shikimic acid was not purified, but changes in the UV spectrum were consistent with those reported in the literature.

The first two steps in the biotransformation were monitored at 234 nm, where an increase in absorbance corresponded to the formation of 3-dehydroshikimate 14. The final conversion

to shikimic acid **9** was monitored indirectly, as the consumption of NADPH gave a decrease in absorbance at 340 nm.

**2.2.1.3 Biotransformation from 3-fluoroDAHP**

As 3-fluoroDAHP which had been prepared previously in the group was available, it was decided to attempt the biotransformation to the 6-fluoroshikimic acids **1** and **41** using this material as substrate (*fig. 30*). Again, the conditions described in the literature were used for this biotransformation (Duggan *et al.*, 1995).

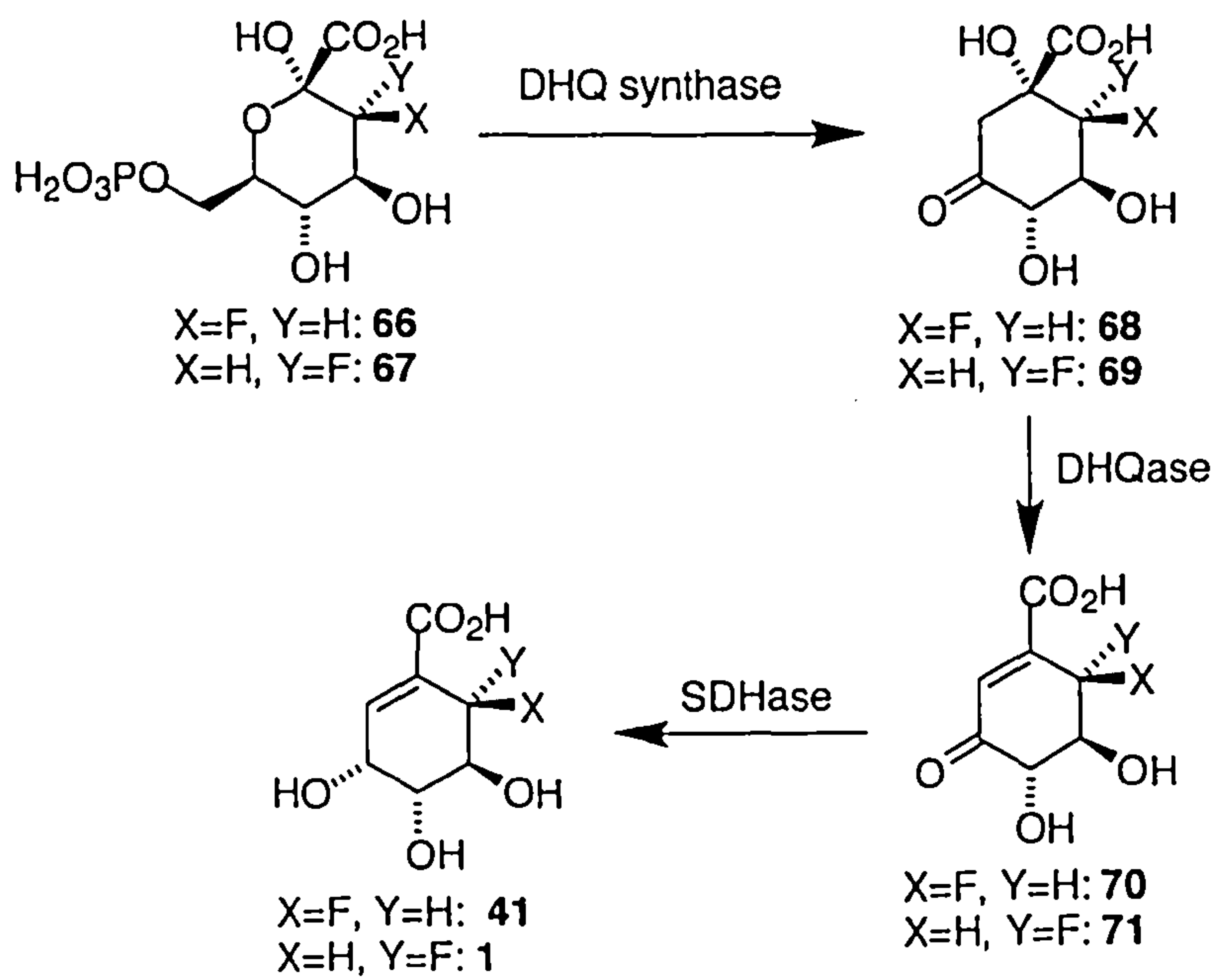


Figure 30: Proposed route to the 6-fluoroshikimic acids from 3-fluoroDAHP

Analysis of the biotransformation mix using a BioRad Organic Acids HPLC column as specified by the authors provided no evidence for the formation of the desired fluorinated products. <sup>19</sup>F-NMR analysis of the starting material then showed that errors in characterisation had been made in the original synthesis, and that the compound provided

was in fact 3-fluoroPEP. Attention was therefore turned to attempts to form 3-fluoroDAHP by biotransformation from 3-fluoroPEP with *in situ* generation of E-4-P.

2.2.1.4 Biotransformation from 3-fluoroPEP

The conditions used to synthesise DAHP 13 from PEP with *in situ* generation of E-4-P (fig. 28) were used in this attempt to synthesise 3-fluoroDAHP 66 and 67 from 3-fluoroPEP (fig. 31). Unfortunately, the sialic acid assay used to follow the formation of DAHP is not suitable for assaying the fluorinated equivalents, and we had previously found changes in absorbance at 234 nm to be less reliable as an indicator of reaction progress.

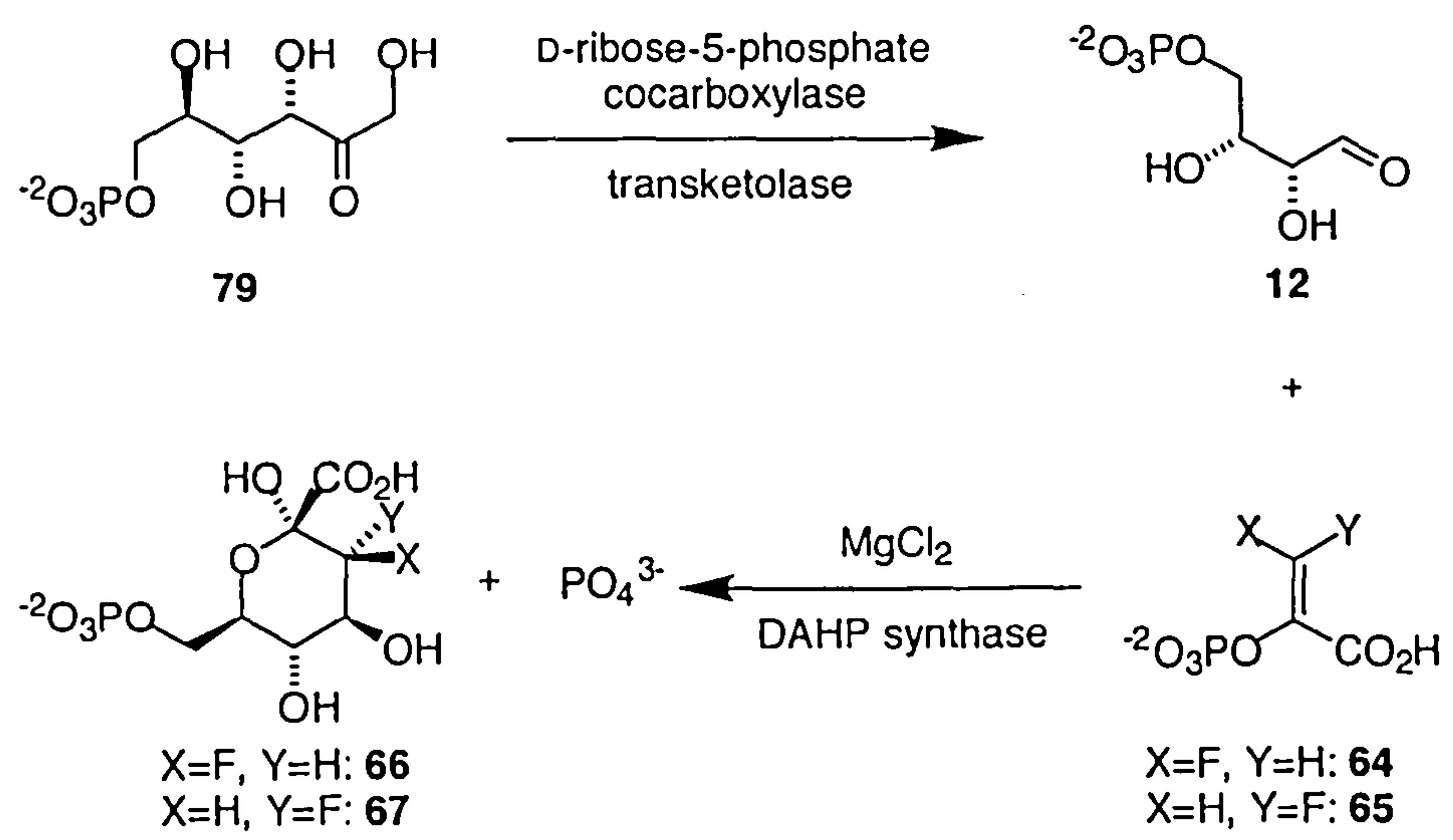


Figure 31: Attempted biotransformation to 3-fluoroDAHP

The decrease at 234 nm which would correspond to the consumption of 3-fluoroPEP and hence the formation of 3-fluoroDAHP 66 and 67 was not observed over a series of reactions. As a result, it was decided to attempt to follow the reaction indirectly.

A larger-scale biotransformation was carried out using the same conditions. The reaction mix was purified as before on a DEAE Sephacel column eluted with an ammonium hydrogen carbonate gradient. The column fractions were assayed for the presence of inorganic phosphate after treatment with alkaline phosphatase (Lanzetta *et al.*, 1979).

Again, the formation of 3-fluoroDAHP was not detected. There was also no reaction when the column fractions were assayed using dehydroquinase synthase and dehydroquinase along with the relevant cofactors.

In case a contaminant of the 3-fluoro-PEP used was inhibiting the enzymes, it was decided to follow the reaction using  $^{19}\text{F}$ -NMR to look for anomalous product formation. A greatly increased concentration of 3-fluoroPEP **64** and **65** was required compared to that used in the earlier biotransformation reactions in order to obtain meaningful  $^{19}\text{F}$ -NMR spectra. The literature conditions were modified in that the MOPS buffer was omitted, and the E-4-P was provided by *in situ* generation from F-6-P, but otherwise were as published (Duggan *et al.*, 1995). Unfortunately, over 12 h, the only fluorinated compounds observed were the original isomers of 3-fluoroPEP and the trace fluorinated impurities present in the starting material. Changes in the relative height of the peaks in the spectrum were not observed over the course of the reaction, indicating that the failure of the biotransformation was not due to breakdown of the starting materials under the reaction conditions.

The published  $^{19}\text{F}$ -NMR experiment was then replicated precisely, using freshly regenerated, commercially available, E-4-P in 50 mM MOPS buffer at pH 7. Again, no sign of the desired 3-fluoroDAHP derivatives **66** and **67** were observed over 42 h. All the enzymes had been assayed immediately prior to use in the experiment, and all reagents were as specified in the original paper (Duggan *et al.*, 1995). The 3-fluoroPEP used,



however, had been a specially commissioned synthesis by D. Picken (Link Technology, ML4 3BF) and although the major compounds present were the desired isomers of 3-fluoroPEP, trace fluorinated impurities were visible in the  $^{19}\text{F}$ -NMR signal. It is possible that an impurity was inhibiting DAHP synthase activity which was not present in the original synthesis. This route to (6*S*)-6-fluoroshikimic acid was therefore abandoned.

### 2.2.2 Synthesis from shikimic acid

As the paper detailing this synthesis was a communication and therefore gave minimal experimental detail, the methods I used (*fig. 32*) may differ slightly from those which yielded the published results.

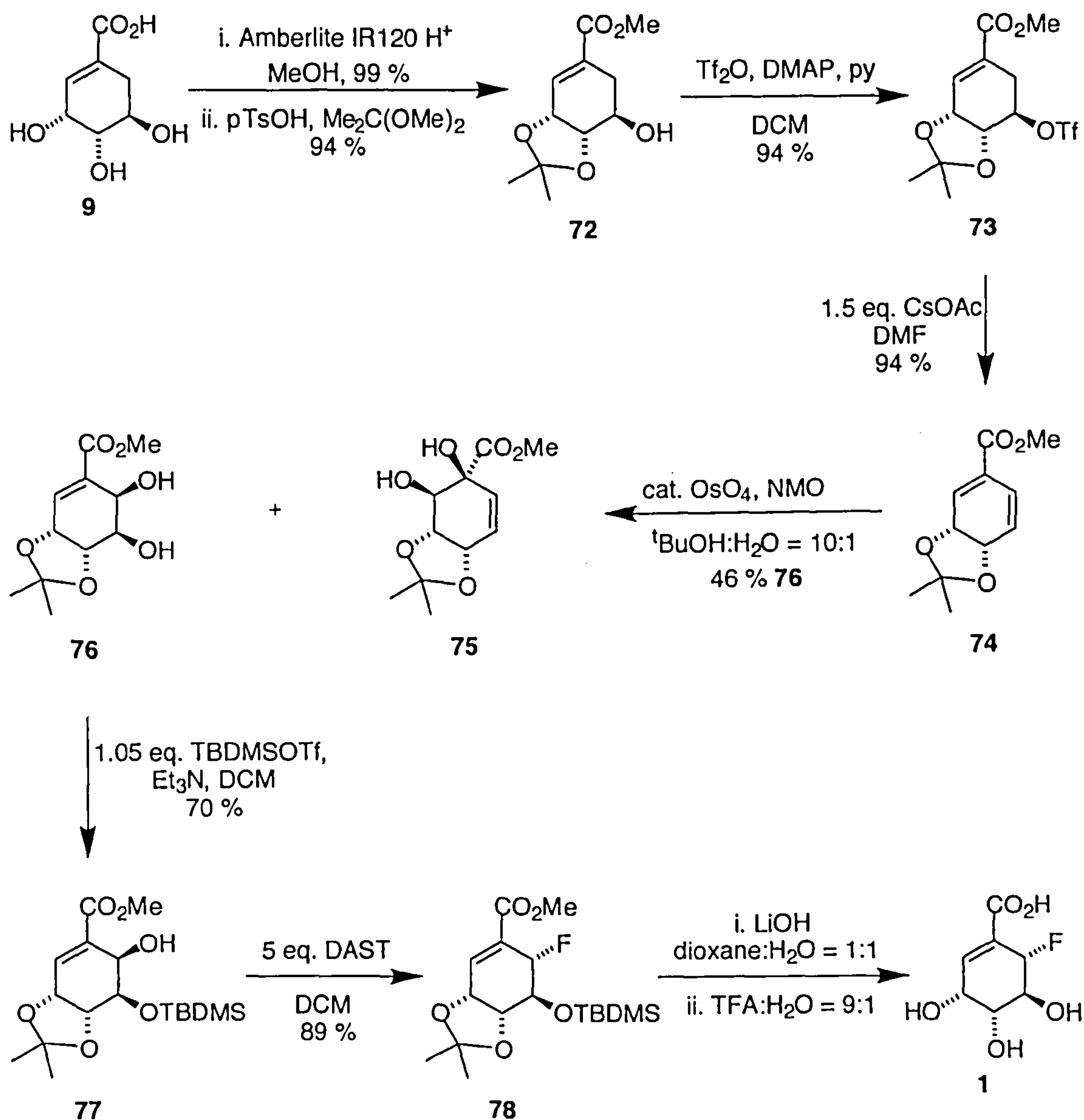


Figure 32: Synthesis of (6*S*)-6-fluoroshikimic acid from shikimic acid

Initial attempts focussed on replicating the published conditions (*fig. 26*; Song *et al.*, 2001) but it was immediately apparent that the synthesis of methyl shikimate could not be carried out using CSA as catalyst if it was to be purified prior to acetal formation. The desired methyl ester was formed, and could be converted into the protected shikimate derivative **72** without purification, but the yields obtained were not as high as those quoted. The original reference (Chahoua *et al.*, 1992) used amberlite ion-exchange resin to catalyse the formation of the methyl ester, and this alternative method was used to form methyl shikimate in excellent yield.

Freshly distilled 2,2-dimethoxypropane was used to form the acetal **72** using *p*TsOH as the acid catalyst. The distillate was less pure than 2,2-dimethoxypropane as purchased, however, and higher yields were obtained using the commercially available material.

After extensive experimentation it was found that the triflate **73** was unstable and had a tendency to eliminate partially during workup. If the entire workup was kept cold (Ross *et al.*, 2001) including removal of the solvent under reduced pressure, and the material was used immediately after purification, both high yields and purity were achieved.

Initial attempts to form the diene **74** by elimination of **73** were unsuccessful as CsOAc is hygroscopic and the reaction appears to be unpredictable if conditions are not strictly anhydrous. This was achieved by drying the CsOAc under high vacuum for 30 min before adding a solution of the triflate **73** in DMF. Further difficulties were experienced due to the instability of the diene. Even at -20°C the material decomposed completely within 10 h, and at 25°C evidence of decomposition was observed within a few hours. Excellent yields were eventually achieved by using an excess of dry CsOAc, carrying out the workup using

solvents and aqueous materials cooled to 4°C, and by using the diene **74** immediately after purification.

Formation of the diol **76** by osmium catalysed dihydroxylation using NMO as the stoichiometric oxidant was carried out using a combination of the conditions given in the communication (Song *et al.*, 2001) and the proportion of reagents used for the dihydroxylation of a similar quinic acid-derived compound (Barco *et al.*, 1998). Separation of **75** and **76** was achieved using a gradient elution on a silica-gel column and the desired compound was eventually isolated in moderate yield. The use of OsO<sub>4</sub> which had not been in solution for longer than six weeks was found to be crucial as dihydroxylation of both double bonds of diene **74** was found to occur more readily when the OsO<sub>4</sub> catalyst had been stored for an extended period in aqueous solution.

Treatment of the diol **76** with 1.1 equivalents of TBDMSOTf gave selective protection of the C-5 hydroxyl as a silyl ether in good yield. Using a greater excess of TBDMSOTf reduced the yield of the desired compound as formation of the bis-silylated product was found to predominate. The difficulties associated with purification were also increased under these conditions. The crystalline product **77** was then treated with DAST (Middleton, 1974) in DCM. The protected 6-fluoroshikimic acid derivative **78** was obtained in excellent yield after purification.

Deprotection was carried out in two steps. The methyl ester was cleaved by treatment with LiOH in a water/dioxane solvent mix. The crude material from this reaction was then treated with a TFA/water mix to simultaneously cleave the silyl ether and acetal protecting groups. By <sup>1</sup>H-NMR three fluorine-containing compounds were formed, with the major

product being the desired (6*S*)-6-fluoroshikimic acid **1**. Only a small amount of material was deprotected in order to confirm that the correct diastereomer had been formed as the protected derivative **78** was required for further syntheses.

Purification of (6*S*)-6-fluoroshikimic acid **1** was attempted on an Organic Acids HPLC column eluting with 50 mM formic acid at 0.6 mL.min<sup>-1</sup>. The major peak, believed to be (6*S*)-6-fluoroshikimic acid by <sup>1</sup>H-NMR analysis, eluted at 10.2 min as compared with the literature value of 10.8 min during a series of analytical runs on a small sample of the crude material (Duggan, 1995). However, when the remainder of the product was dissolved in water and a preparative run was attempted, the compound was found to have decomposed. No further purification was possible.



## 2.3 Conclusions and future work

The decision to synthesise (6*S*)-6-fluoroshikimic acid by biotransformation from E-4-P and F-PEP was based on a number of factors. Firstly, the chemistry involved in the syntheses from quinic acid (*fig. 23 & 24*) was not only likely to be time-consuming, but also required a degree of experience in the reactions carried out which was not available in our laboratory. Secondly, the biotransformation route (*fig. 25*) was both higher yielding and apparently less time-consuming. Members of our laboratory had experience of working with the enzymes of the shikimate pathway and the purification methods required for the enzyme substrates.

As detailed in this chapter, attempts to synthesise (6*S*)-6-fluoroshikimic acid by biotransformation were unsuccessful. We believe that this was due to an inorganic contaminant of the F-PEP substrate which was not apparent during <sup>1</sup>H-NMR or <sup>19</sup>F-NMR analysis and which was inhibiting DAHP synthase. It is possible that purification of the substrate on an ion-exchange column would have eliminated this contaminant.

The synthesis from shikimic acid (*fig. 26 & 32*) is a significant improvement over those from quinic acid as it is both shorter and higher-yielding. It is unfortunate that the (6*S*)-6-fluoroshikimic acid decomposed unexpectedly before full purification could be carried out, as this resulted in the lack of a positive control for the biological testing of the smugglins.

### **3 Peptide-shikimate conjugates as prodrugs**

#### **3.1 Prodrugs**

As detailed in Chapter 1 (*section 1.6*), prodrugs are pharmacologically inactive derivatives of drug molecules which are converted into the active form *in vivo*. Changes to the structure of the molecule may be made in order to increase bioavailability by modifying e.g. solubility, but prodrugs can also be designed to target specific enzymes or carriers e.g. peptide transporters.

#### **3.2 The oligopeptide permease as a prodrug target**

As detailed in Chapter 1, the oligopeptide permease (Opp) is a periplasmic binding protein-dependent system which preferentially transports tri- and tetrapeptides into bacterial cells (Matthews & Payne, 1980; Payne, 1978; Payne, 1980). Amino-acid side chains are accommodated in large hydrated pockets within OppA, with highly-ordered water molecules within these pockets satisfying hydrogen-bond requirements without imposing binding specificity (Tame *et al.*, 1996; Sleight, 1999; Rostom, 2000).

This ability to accommodate a wide variety of natural and unnatural amino-acyl side chains suggested the possibility of constructing a ‘smugglin’ which would target the Opp as a means of transporting (6*S*)-6-fluoroshikimic acid into bacterial cells.

### 3.3 Peptides conjugated to (6S)-6-fluoroshikimic acid

#### 3.3.1 Design

As (6S)-6-fluoroshikimic acid has been shown to be an effective antimicrobial both *in vitro* and *in vivo*, the prodrug approach to increasing bioavailability is unnecessary. (Davies *et al.*, 1993) However, the rapid development of resistance to the compound due to mutation of the specific bacterial transporter involved suggested that a ‘smugglin’ approach might be viable (Ewart *et al.*, 1995).

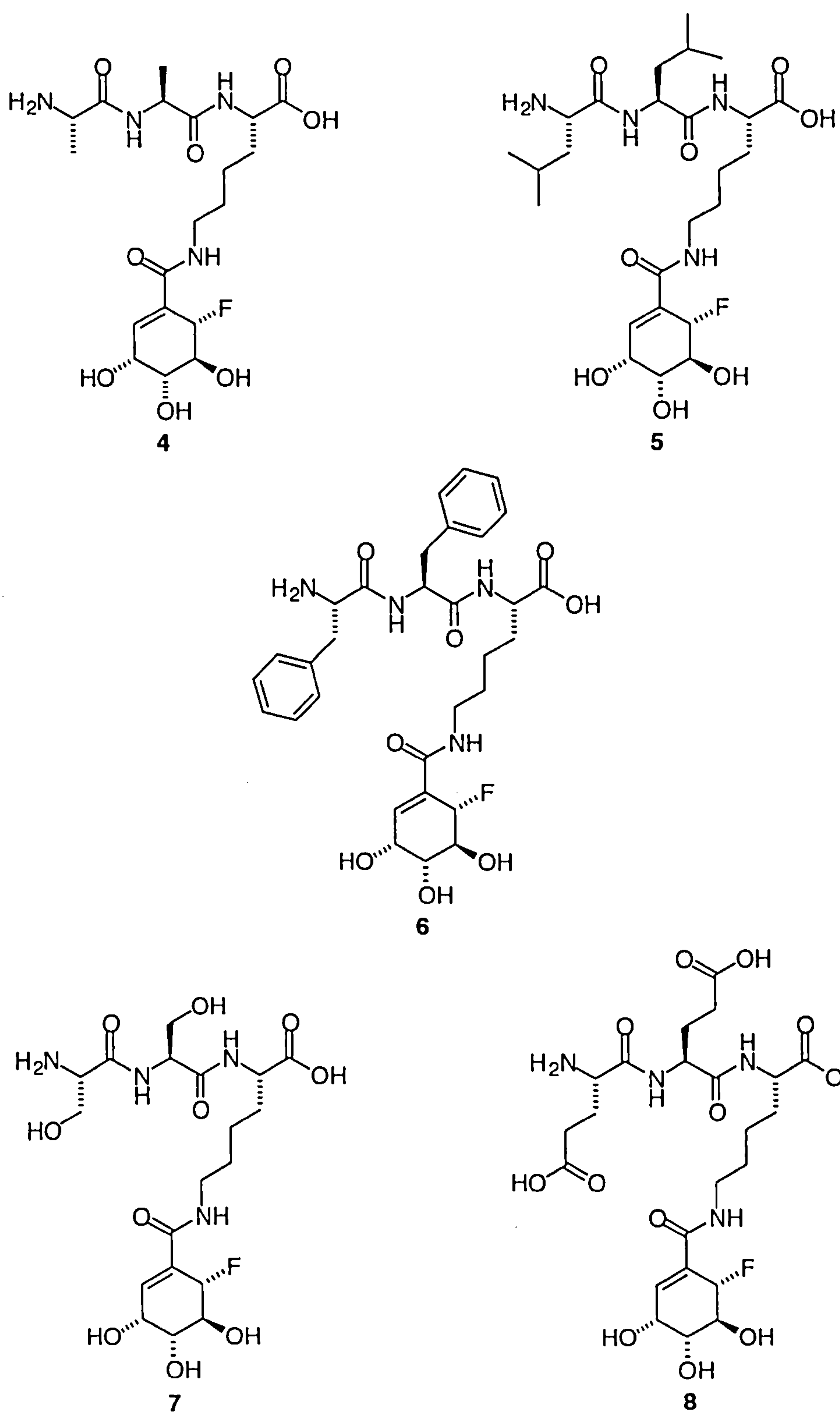
As all three peptide permeases in *E. coli* and *S. typhimurium* transport tripeptides to some extent, it was decided to attempt an initial synthesis of tripeptide conjugates of (6S)-6-fluoroshikimic acid. The tripeptides were chosen based on the known specificity of the oligopeptide permease, the crystallographic data available on binding to OppA, and the need for a cleavable linkage from the peptide to (6S)-6-fluoroshikimic acid.

Modification of the N- or C-terminal ends of a tripeptide eliminates, or at least greatly reduces, binding to OppA and transport by the oligopeptide permease. As a result, it was decided to attach (6S)-6-fluoroshikimic acid to an amino-acyl side chain using a linkage which would be readily cleaved within bacterial cells. Both an ester linkage to the hydroxyl of serine and an amide linkage to the  $\epsilon$ -amino group of lysine were considered. The amide linkage was chosen as it is more stable, but likely to be readily cleaved by intracellular peptidases to release the active compound.

Various binding studies of OppA have suggested that positively charged side chains in a peptide reduce binding to OppA, while hydrophobic residues are preferred. (Guyer *et al.*,

1986) The detail of crystal structures also showed that the C-terminal amino acyl side chain was often disordered, and suggested that modifications at this position might be better tolerated than at either of the first two. (Davies *et al.*, 1999) The results obtained from co-crystallising a series of unnatural amino acids in tripeptides of the form Lys-X-Lys with OppA (Davies *et al.*, 1999) also suggested that relatively large moieties could be accommodated in the side-chain binding pockets.

In order to maximise the potential information on the transport of peptide-shikimate conjugates, it was decided to synthesise tripeptides of the form XXLys where X could be aliphatic, aromatic, polar, or charged. The amino acids chosen were alanine, leucine, phenylalanine, serine and glutamic acid. (6*S*)-6-Fluoroshikimic acid would then be linked to the side-chain of the C-terminal lysine in each tripeptide to give compounds **4** through **8** (*fig. 33*).



**Figure 33: Structure of the smugglins designed to target the Oligopeptide permease**



### 3.3.2 Proof of concept: N- $\epsilon$ -shikimoyl-L-lysyl-L-phenylalanine

As an initial proof-of-concept, it was decided to attempt the synthesis of an easily-purified dipeptide-shikimate conjugate. Using shikimic acid instead of (6*S*)-6-fluoroshikimic acid would allow method development while the synthesis of the fluorinated derivative was ongoing, while also reducing the cost of the model synthesis. Synthesising a dipeptide conjugate rather than the tripeptide conjugates would facilitate purification, as solution-phase peptide synthesis could be used rather than the solid-phase methods preferred for longer-chain peptides.

The dipeptide conjugate chosen was N- $\epsilon$ -shikimoyl-L-lysyl-L-phenylalanine **83**. The phenylalanyl moiety was chosen to facilitate purification by increasing the hydrophobicity of the dipeptide and peptide conjugate, as well as to give a clearly distinguishable UV-active TLC. A further advantage of the phenylalanyl moiety was that  $^1\text{H}$ -NMR signals from the dipeptide would be unlikely to overlap with those from the shikimate moiety.

#### Results and discussion:

The hydroxyl groups of shikimic acid were protected as acetates by reaction with acetic anhydride in pyridine. The reaction was carried out on both a gram and milligram scale to give the tri-acetate **80** in excellent yield.

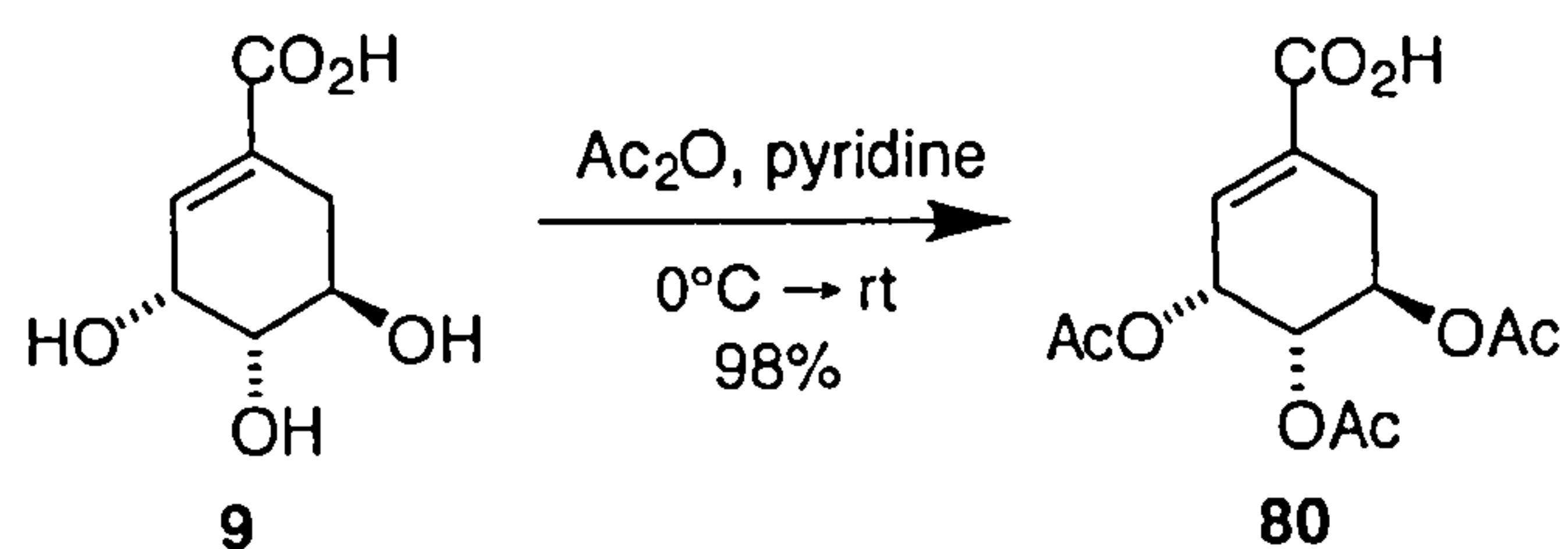


Figure 34: Protection of shikimic acid as the tri-*O*-acetate

The protected dipeptide Boc-Lys(Z)-Phe-OMe **81** (Boc is butoxycarbonyl; Z is benzyloxycarbonyl) was synthesised using standard solution-phase methodology and was obtained in excellent yield. The coupling reagent 1-(3-dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride (EDC) was used with the addition of 1-hydroxybenzotriazole (HOBt) to minimise racemisation. After deprotection of the  $\epsilon$ -amino group of the lysine moiety by hydrogenolysis, a standard peptide coupling reaction with protected shikimic acid **80** gave the fully protected peptide conjugate **82** in good yield. Deprotection was carried out by sequential treatment with NaOH and TFA to give the desired dipeptide-shikimate conjugate **83** as the major product by  $^1\text{H}$ -NMR analysis. Analysis of the crude isolate by HPLC using a combined UV and conductivity detection system showed a single major peak.

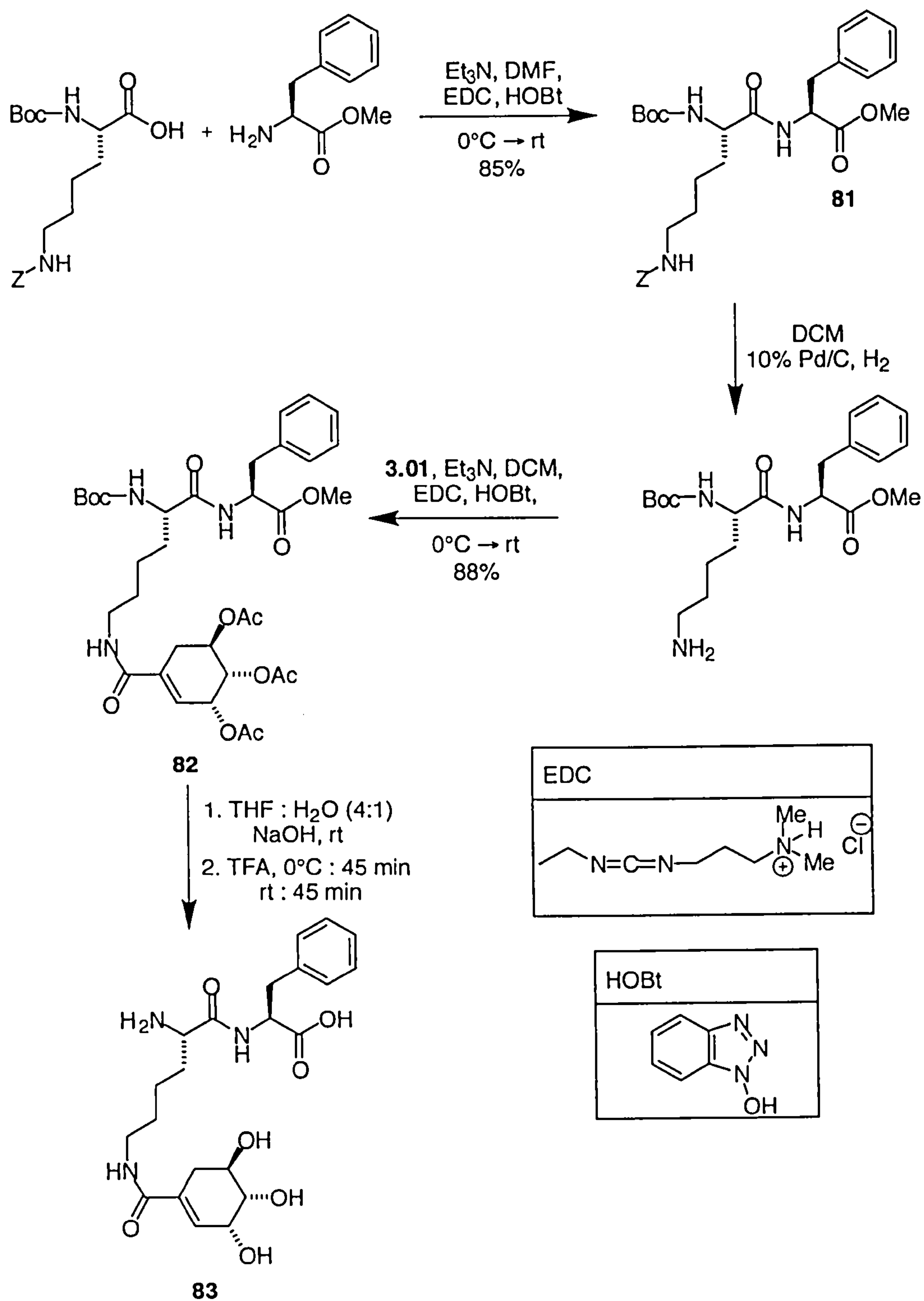


Figure 35: Solution-phase synthesis of the dipeptide-shikimate conjugate

### 3.3.3 Model syntheses: tripeptide shikimate conjugates on solid phase:

As previously discussed, five tripeptide conjugates of the form XXLys (where X is one of alanine, leucine, phenylalanine, serine or glutamic acid) were chosen for the initial investigation of the oligopeptide permease as a means of targeting (6*S*)-6-fluoroshikimic acid to bacterial cells. It was decided to develop the solid-phase methodology using shikimic acid to reduce the cost of the model syntheses and to allow method development to be carried out in tandem with attempts to synthesise (6*S*)-6-fluoroshikimic acid.

The substitution of a fluorine atom for a hydrogen atom at C-6, while having a significant effect on the biological activity of shikimic acid, was not thought likely to have any major effect on either the reactivity or solubility of the compound. As a result, it was believed that the methods developed in the syntheses of the five model conjugates would be directly applicable to the final target syntheses.

N- $\alpha$ -Fmoc-amino acid residues (Fmoc is 9-fluorenylmethoxycarbonyl) were used to build up the tripeptides on Wang resin in all syntheses. The resin was purchased pre-derivatised with an orthogonally protected lysine residue **84**.

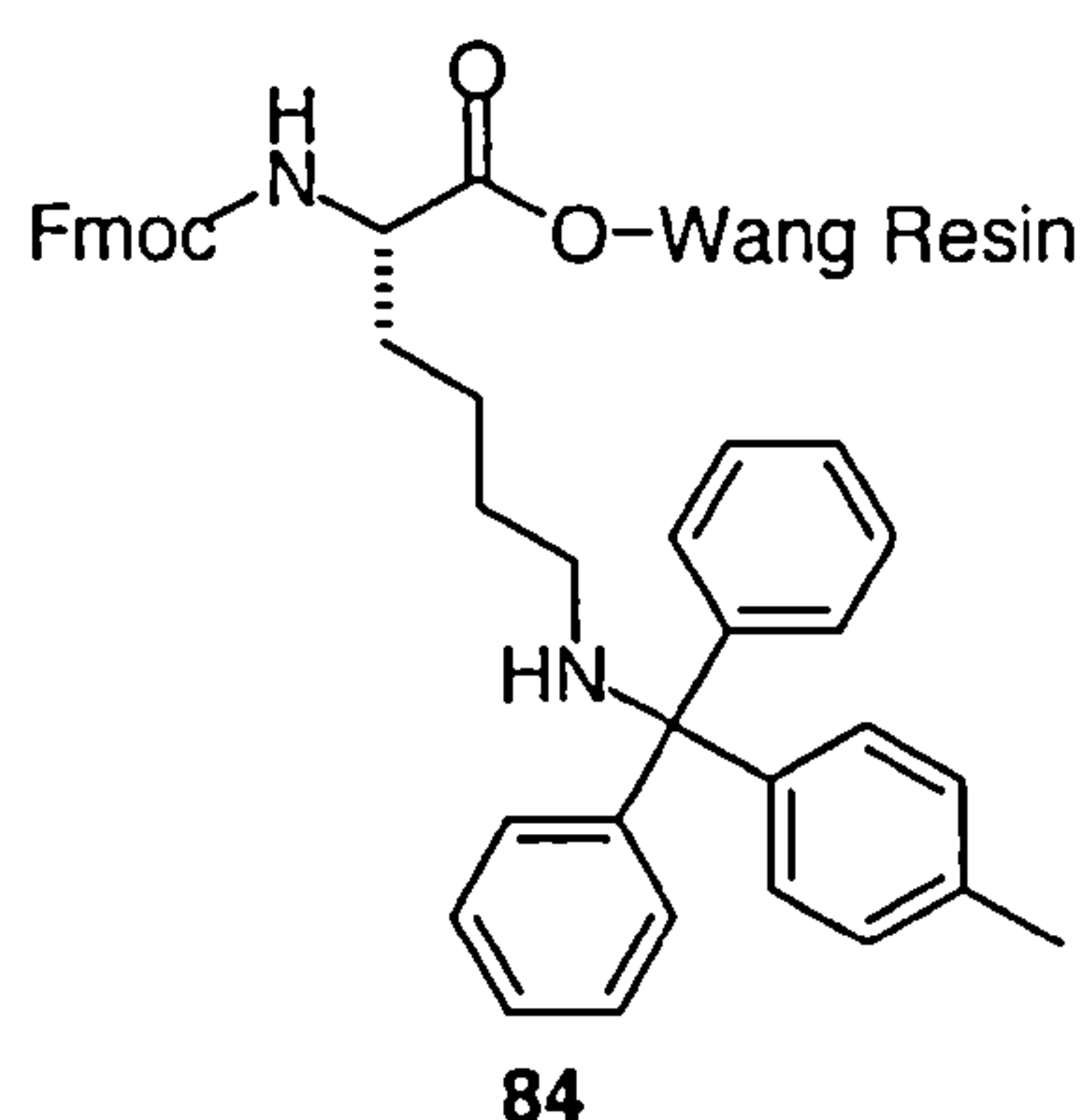


Figure 36: Wang resin coupled to an orthogonally protected lysine residue.



As shikimic acid was to be coupled to the  $\epsilon$ -amino group of the C-terminal lysine the protecting group for this amino group needed to be stable to standard Fmoc solid phase chemistry. It also needed to be readily cleavable under conditions which would not cause cleavage of the peptide from the resin, or cleavage of the *t*-butyl protecting groups on the side chains of the glutamic acid and serine residues. The 4-methyltrityl (Mtt) protecting group, which is base stable, but readily cleaved using 1 % TFA, 5 % tri-isopropyl silane (TIPS) in DCM, was a logical choice (Aletras *et al.*, 1995).

## Results and Discussion:

The basic method of synthesis was invariant, and is summarised in figure 37. Later syntheses involved the coupling of shikimic acetate **80** to preformed tripeptide chains as in the synthesis of the (6*S*)-6-fluoroshikimate conjugates **4 - 8** (*fig. 42*).

The 4-methyltrityl protecting group on the resin-bound lysine residue **84** was removed by treatment with 1 % TFA in dry DCM with the addition of 5 % tri-isopropylsilane (TIPS) to trap the trityl cations and reduce the likelihood of side-reactions. The resin was then dried under a steady air stream at reduced pressure as the presence of residual methanol used to wash the resin during deprotection completely inhibited coupling to shikimic acetate. The dried resin was then coupled to shikimic acetate **80** using a large excess of DIC and HOBt in dry DCM and extended reaction times to ensure coupling occurred. If the standard 3 eq. of DIC was used in this step, coupling was either extremely limited or non-existent, depending on the tripeptide involved.



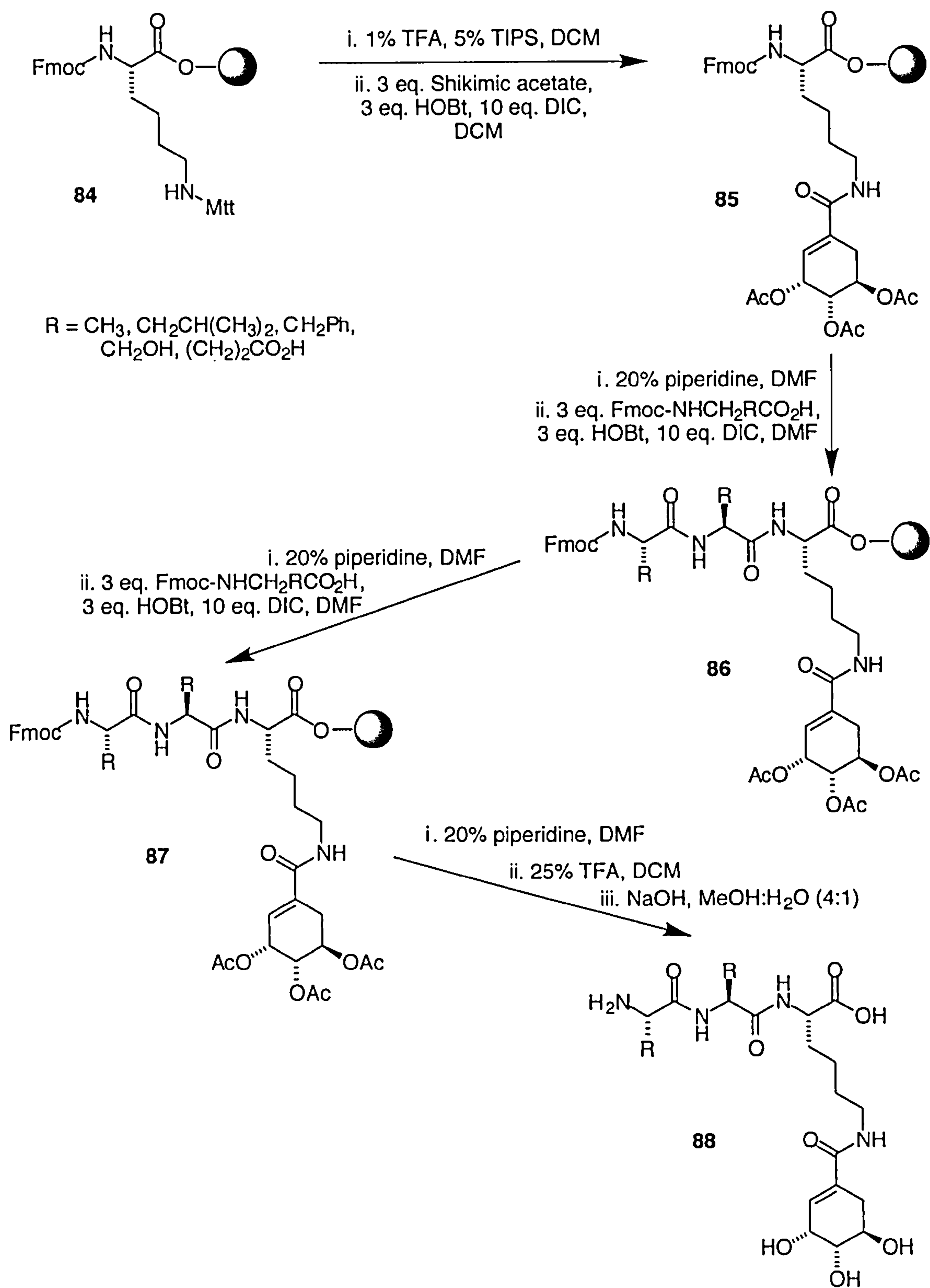


Figure 37: Solid-phase synthesis of shikimate tripeptide conjugates

The Fmoc protecting group on the now-derivatised resin-bound lysine residue **85** was cleaved by treatment with 20 % piperidine in dry DMF. Bromophenol blue was initially used to test for the presence of free amino groups on the resin, but the inaccuracy of the results meant that this approach was abandoned. All cleavage reactions were then carried out a set number of time, over a fixed minimum reaction time. The deprotected residue was then coupled to the relevant Fmoc protected amino acid derivative using a large excess of di-isopropyl carbodiimide (DIC) and HOBt in dry DMF to give the protected, resin-attached dipeptide-shikimate conjugate **86**. Again, the use of bromophenol blue to test for complete reaction gave unsatisfactory results and all coupling reactions were allowed a minimum reaction time of two hours. The deprotection and coupling steps were repeated to give the desired resin-attached tripeptide-shikimate conjugate **87**.

Cleavage from the resin using 25 % TFA in DCM gave the desired tripeptides conjugated to shikimic acid protected as the triacetate. At this stage, <sup>1</sup>H-NMR was used to confirm that coupling had occurred. The characteristic doublet of doublets at ~2.5 ppm due to the CH<sub>2</sub> protons on C-6 of shikimic acid was generally not obscured by peptidic protons, and provided a marker for successful coupling reactions.

<sup>1</sup>H-NMR analysis was followed by deprotection of the shikimate moiety by treatment with NaOH in MeOH:H<sub>2</sub>O (1:4). As the product tripeptide conjugates **88** were only soluble in methanol or acid, it was found impossible to separate them from the NaOAc released in this deprotection step without recourse to HPLC. HPLC-Mass spec analysis was used to confirm the presence of the desired compounds in the crude material, with ms/ms

confirming the identity of selected peaks, before the method was applied to the synthesis of the target tripeptide-(6*S*)-6-fluoroshikimic acid conjugates.

The conjugates were not purified at this stage, as it was felt that the limited time remaining for experimentation would be better used in attempting the synthesis of the final target compounds. Crude yields are noted in the experimental section (*Chapter 5*)

### 3.3.4 Tripeptide (6*S*)-6-fluoroshikimic acid conjugates on solid phase:

As <sup>1</sup>H-NMR analysis had shown the successful formation of all five model peptide-shikimate conjugates, an attempt was made to transfer the methodology to the synthesis of the same five tripeptides conjugated to (6*S*)-6-fluoroshikimic acid.

Problems were immediately encountered, as attempts to protect the hydroxyl of (6*S*)-6-fluoroshikimic acid **1** by reaction with acetic acid in pyridine led to total decomposition and the formation of 3,5-diacetoxybenzoic acid **89** (*fig. 38*). The substitution of a fluorine for a hydrogen on C-6 gave only a 0.1 ppm change in the <sup>1</sup>H-NMR signal for the protons on both C-3 and C-5, characteristic of a slight increase in acidity at these positions. Surprisingly, therefore, base-catalysed elimination of the components of acetic acid, followed by those of hydrofluoric acid, was observed. Fluoride is normally an extremely poor leaving group, but the driving force of aromatisation was apparently sufficiently strong as to overcome this.

It would be possible to overcome this difficulty by using a base such as 2,6-lutidine which is more sterically hindered than pyridine, and hence less nucleophilic. However, as the tripeptide conjugates are all protected at the N-terminus with an Fmoc group, and this is removed using 20 % piperidine in DMF (which is likely to have the same effect as

pyridine), an alternative protection strategy for the hydroxyl groups of (6*S*)-6-fluoroshikimic acid was required.

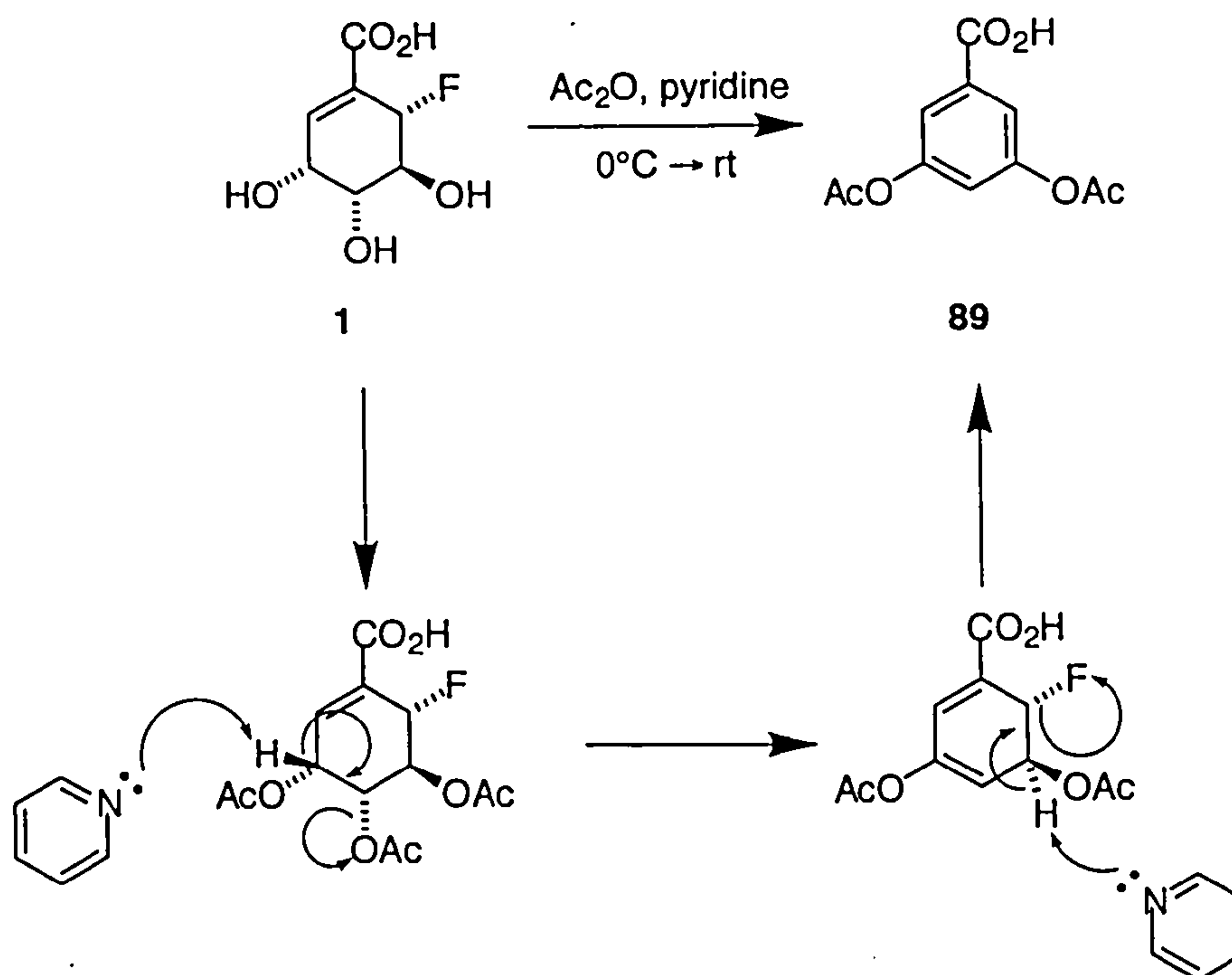


Figure 38: Base-catalysed aromatisation of (6*S*)-6-fluoroshikimic acid

The obvious solution was to use the product of the penultimate step in the synthesis of (6*S*)-6-fluoroshikimic acid detailed in Chapter 2 (*fig. 32*), where the C-5 hydroxyl is protected as a silyl ether, and the C-3 and C-4 hydroxyls as a ketal (**90**). As this compound has already been shown to be stable to LiOH hydrolysis during the saponification of a methyl ester, deprotection of Fmoc groups should not be problematic. Additionally, as these groups are removed by treatment with 90 % TFA in DCM, purification of the peptide conjugates after cleavage from the resin should be simplified relative to the model compounds.

As the phenylalanine-containing tripeptide-shikimate conjugate had shown the most complete coupling by  $^1\text{H}$ -NMR analysis (conjugate:tripeptide = 1:3 by visual inspection), it was decided to attempt the synthesis of this tripeptide conjugated to (6*S*)-6-fluoroshikimic acid in the first instance **91** (*fig. 39*).

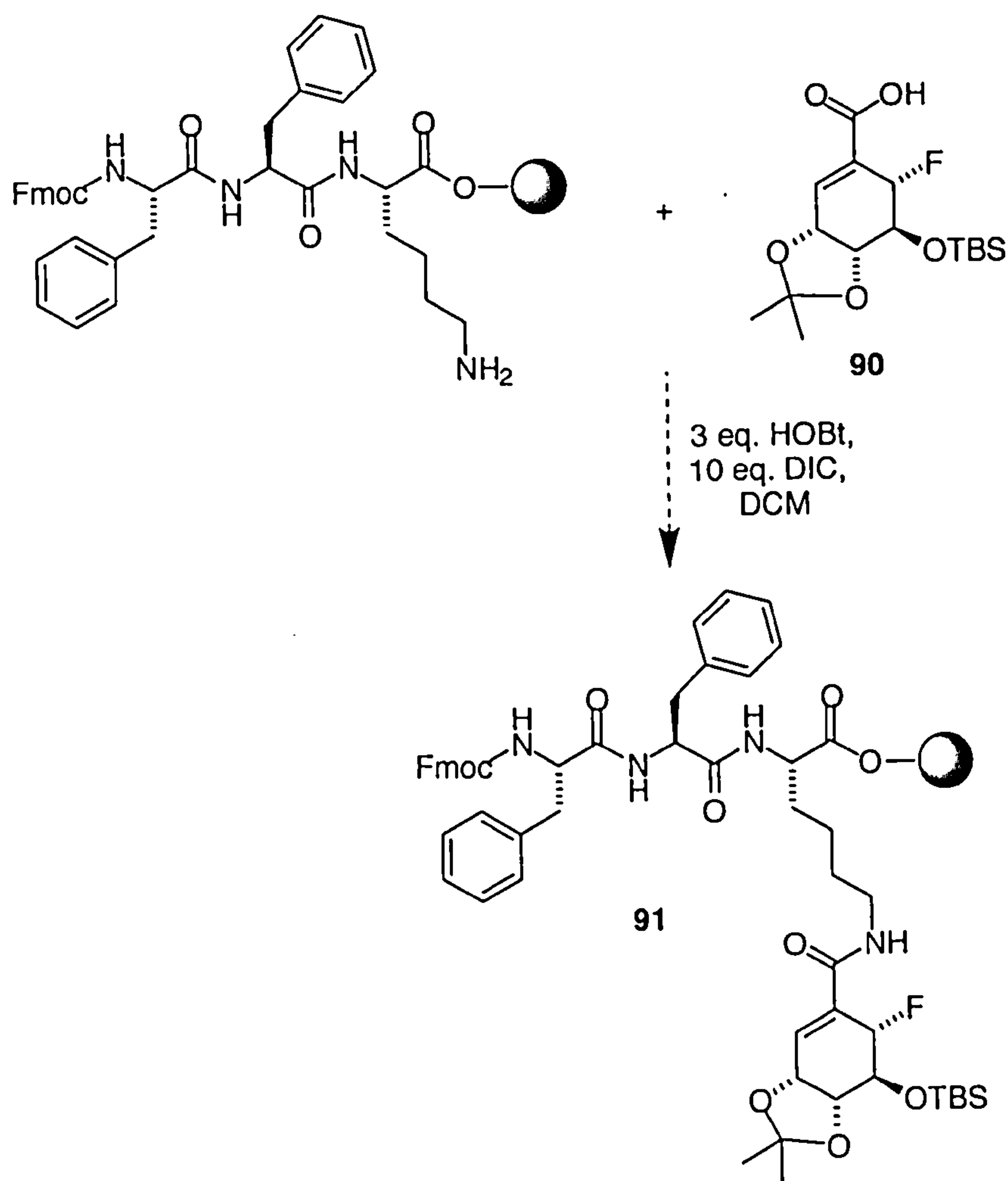


Figure 39: Proposed coupling of fully protected (6*S*)-6-fluoroshikimic acid to resin-bound Fmoc-Phe-Phe-Lys



Again, direct transfer of the methods developed in the model synthesis was not possible. After cleavage of the products from the resin, <sup>1</sup>H-NMR analysis showed no coupling between the ε-amino group of lysine and the carboxylic acid of the (6*S*)-6-fluoroshikimic acid derivative **90**. Addition of triethylamine to the coupling mix, in an attempt to increase the proportion of deprotonated carboxylic acid, was also unsuccessful.

We now believe that the increased electronegativity at C-6 due to the presence of the fluorine in (6*S*)-6-fluoroshikimic acid reduces the reactivity of the carboxylic acid moiety towards electrophiles. As a result, addition of the carboxylic acid to DIC in the initial step of peptide bond formation is less likely to occur, and hence the lack of coupling observed.

The formation of 'difficult' peptide bonds, such as those involved in synthesising cyclic dipeptides, is often facilitated by benzotriazol-1-yloxy-tris(dimethylamino)phosphonium hexafluorophosphate (BOP) **92**, also known as Castro's Reagent (El Mahdi *et al.*, 2000). The initial step in BOP-coupled peptide bond formation is attack by the carboxyl moiety onto a charged phosphorus atom with the strong thermodynamic driving force of P-O bond formation favouring reaction. In contrast, carbodiimide coupling reagents involve attack by the carboxyl moiety on a neutral carbon atom. As a result, the deactivated carboxyl of the acid derivative **90** should react to give the activated HOBt ester **93** and hence peptide bond formation.

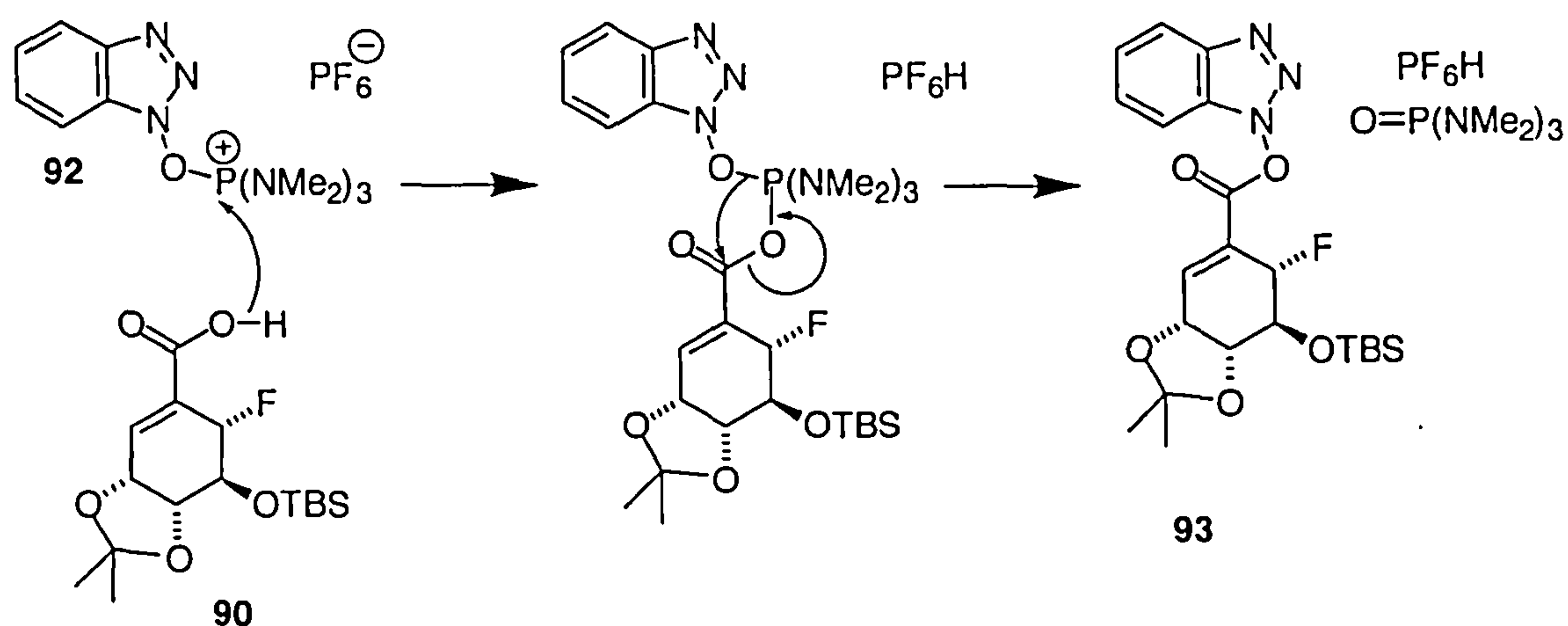


Figure 40: Suggested initial step of BOP-facilitated peptide bond formation

Test reactions on the derivatised resin **84** showed that the desired amino-acid conjugate **94** was formed, although a tertiary amine was also required to be present in excess. The coupling did not proceed to completion, reaching ~45 % after 24 h reaction by  $^1\text{H}$ -NMR analysis of the crude material cleaved from the resin.

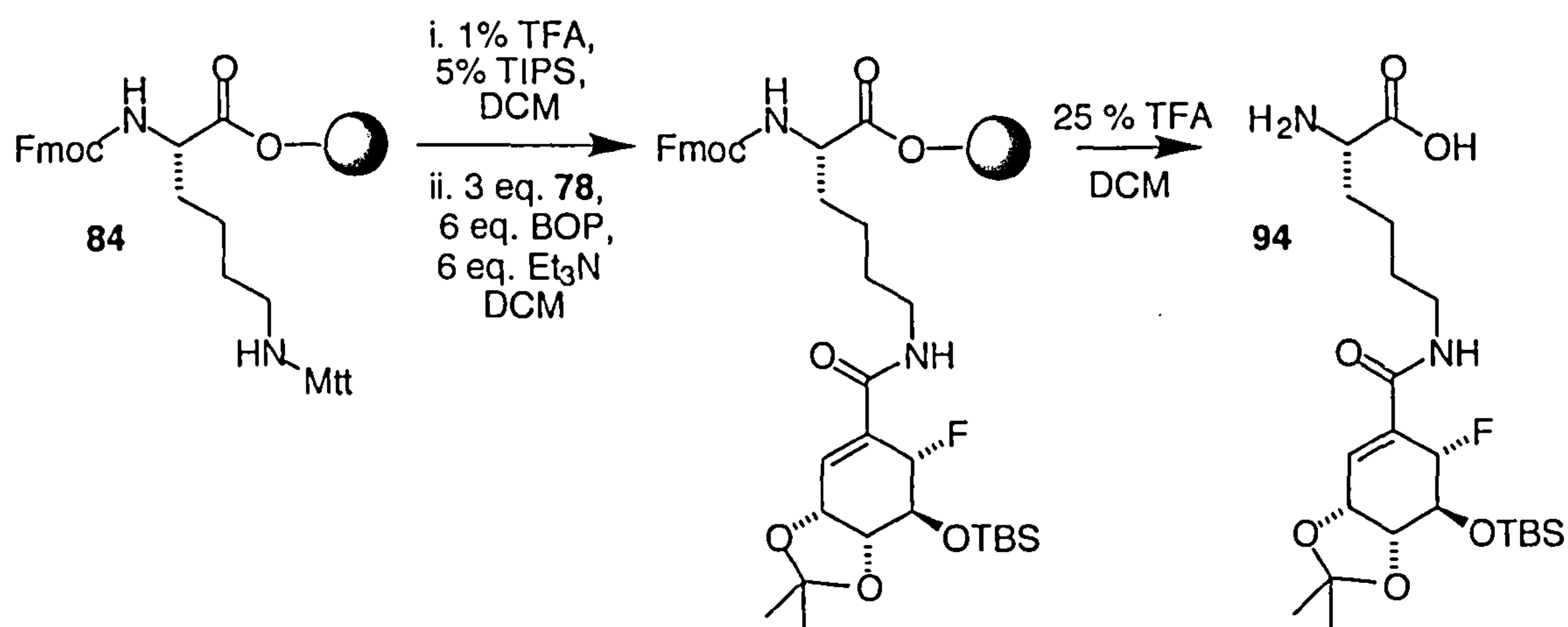


Figure 41: Test coupling of (6S)-6-fluoroshikimic acid to resin-bound lysine

Synthesis of the five tripeptide-(6*S*)-6-fluoroshikimic acid conjugates **4** to **8** (Chapter 3, *fig. 33*) was then undertaken. It was believed that steric hindrance at the resin would favour carrying out the challenging coupling to lysine before building the peptide chain. However, as complete coupling could not be achieved even over 24 h reaction and with a 6-fold excess of BOP (*fig. 41*), it was decided to avoid the formation of a wide variety of branched peptides by coupling the (6*S*)-6-fluoroshikimic acid derivative to the completed tripeptide chains (*fig. 42*).

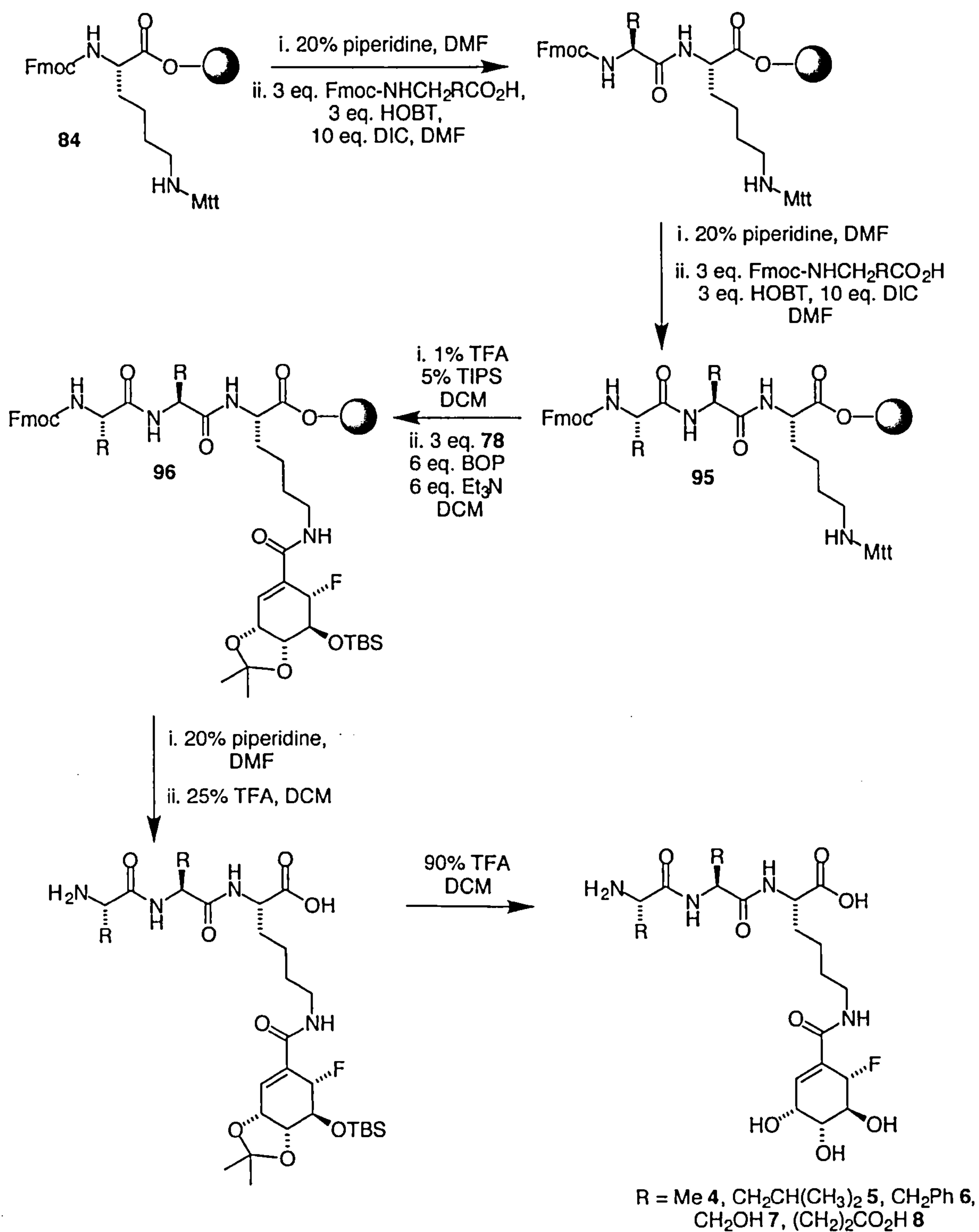


Figure 42: Solid-phase synthesis of tripeptide-(6*S*)-6-fluoroshikimic acid conjugates

The conditions developed for the synthesis of the model compounds were used in order to synthesise the tripeptide chains, i.e. standard Fmoc-based solid phase synthesis using DIC/HOBt coupling. After cleavage of the Mtt group, BOP with the addition of an excess of Et<sub>3</sub>N was used to couple the (6*S*)-6-fluoroshikimic acid derivative to the ε-amino group of lysine of the tripeptide **95** to give the protected resin-bound conjugates **96**. The conjugates were then cleaved from the resin and partially deprotected by treatment with 3:1 TFA:DCM. Protection was completed by treatment of the crude material with 9:1 TFA:H<sub>2</sub>O. Crude yields are given in figure 43.

Compound synthesised	<b>4</b> (Ala)	<b>5</b> (Leu)	<b>6</b> (Phe)	<b>7</b> (Ser)	<b>8</b> (Glu)
resin-bound lysine: starting material (μmol)	72	72	72	72	72
crude material isolated (mg)	43	62	86	67	81

Figure 43. Crude yields of tripeptide-(6*S*)-6-fluoroshikimate conjugates

HPLC/Ms-ms analysis was used to confirm the presence of the desired compounds **4** to **8** in the crude material cleaved from the resin. All five compounds were present and could be separated from the crude mixture using an RP Max HPLC column eluting with a 3 to 50 % gradient of 50 mM formic acid in MeCN.

It was decided to attempt the purification of the leucine **5** and phenylalanine **6** containing conjugates as these gave the most clearly separated peaks. A preparative RP Max HPLC column was used for the separation. The fractions containing the desired compounds were selected by analysing a small sample of the eluent by Ms-ms. These fractions were combined, lyophilised, and stored at -20°C.



### 3.1.5 Biological testing of (6*S*)-6-fluoroshikimic acid conjugates

Initial biological testing was carried out by Dr R. Aitken at the University of Glasgow. A lawn of *E. coli* K12 (the strain used for the original testing of (6*S*)-6-fluoroshikimic acid) was grown on solid medium based on a minimal glucose medium with thiamine supplementation (buffer 56) first described in 1951 (Monod *et al.*, 1951; Davies *et al.*, 1994; Ewart *et al.*, 1995).

A control experiment, spotting a solution of ampicillin into the centre of the lawn, gave inhibition in the 50 - 100 µg range. This suggested that the chosen test method was fairly insensitive. However, as (6*S*)-6-fluoroshikimic acid displayed MICs below 0.5 µg.mL<sup>-1</sup> against this strain of *E. coli* (Davies *et al.*, 1994) it was decided to test the leucine- 5 and phenylalanine-containing 6 conjugates in the same way.

The conjugates were dissolved in 50 mM formic acid, which had previously been shown to have no effect on bacterial growth, and spotted onto the centre of a lawn of *E. coli* K12 on a solid buffer 56 plate. No evidence of antibacterial activity was observed after overnight incubation at 37°C.

A second method of testing was then attempted. *E. coli* K-12 (wild type) bacteria were grown at 37°C in a 96-well plate in buffer 56 liquid medium (Monod *et al.*, 1951).

Ampicillin (20 mg/mL), formic acid (50 mM) and phosphate buffered saline were used as controls against six- and two-fold serial dilutions of the test compounds in 50 mM formic acid. Growth was monitored by measuring the absorbance at 620 nm.

The leucine containing tripeptide conjugate appeared to have a very slight inhibitory effect on the growth of *E. coli* K-12 as compared to the negative controls, and this effect appeared to decrease with increasing dilution (fig. 44). However, the effect was not comparable with that of ampicillin (fig. 45), and the variation in absorbance values obtained suggested that growth inhibition by the conjugate was marginal.

The concentrations given below are based on an assumed maximum yield of 2 mg of active compound after HPLC purification. This is due to the fact that although compound was clearly present after lyophilisation, a negative mass was obtained by difference using a four decimal-place balance.

formic acid (negative control)			6 (Phe)			5 (Leu)		
mM	A <sub>620</sub> T=2h	A <sub>620</sub> T=4.5h	μM	A <sub>620</sub> T=2 h	A <sub>620</sub> T=4.5h	μM	A <sub>620</sub> T=2 h	A <sub>620</sub> T=4.5h
12.5	0.432	0.460	407	0.362	0.470	457	0.257	0.269
12.5	0.440	0.555	68	0.415	0.494	76	0.257	0.343
12.5	0.471	0.578	11	0.532	0.593	13	0.405	0.516
			1.9	0.500	0.582	2.1	0.365	0.569
			0.3	0.378	0.479	0.4	0.306	0.458
			0.05	0.390	0.563	0.06	0.342	0.538

Figure 44: Growth of *E. coli* K-12 (A<sub>620</sub>): six-fold serial dilution of active compounds

ampicillin (positive control)			PheFshik 6			LeuFshik 5		
mM	A <sub>620</sub> T=2 h	A <sub>620</sub> T=5.5h	μM	A <sub>620</sub> T=2 h	A <sub>620</sub> T=5.5h	μM	A <sub>620</sub> T=2 h	A <sub>620</sub> T=5.5h
23	0.088	0.069	650	0.163	0.186	732	0.148	0.176
11	0.087	0.072	325	0.166	0.197	366	0.143	0.168
5.7	0.089	0.071	163	0.175	0.212	183	0.156	0.198
2.9	0.097	0.066	81	0.178	0.219	91	0.150	0.189
1.4	0.097	0.073	41	0.189	0.288	46	0.164	0.199
0.7	0.102	0.073	20	0.194	0.222	23	0.181	0.217
0.4	0.104	0.073	10	0.205	0.228	11	0.239	0.221
0.2	0.117	0.078	5.1	0.233	0.228	5.7	0.243	0.240

Figure 45: Growth of *E. coli* K-12 (A<sub>620</sub>): two-fold serial dilution of active compounds

### 3.4 Conclusions/Future Work

Although five ‘smugglins’ consisting of (6*S*)-6-fluoroshikimic acid conjugated to tripeptides were synthesised, only two have been tested for biological activity. These were chosen for their ease of purification and as such are the least hydrophilic of the five compounds synthesised.

This hydrophobicity may be a reason for the lack of biological activity observed when the conjugates were tested against *E. coli* K12. However, an alternative explanation is that while the oligopeptide permease will tolerate a wide variety of peptide side-chains, the addition of the (6*S*)-6-fluoroshikimic acid moiety to the side chain of lysine exceeds the space available in the binding pocket.

The crystal structures of tripeptides containing one of a variety of unnatural amino acids bound to OppA were published in 1999 (Davies *et al.*, 1999) and suggest that while a side-chain as bulky as naphthalene can be accommodated, it is tending towards the limit of what is possible.

A modification of the smugglin design to take this into account could be easily made, with the substitution of 2,3-diaminopropionic acid for the C-terminal lysine. This could make the coupling of the protected (6*S*)-6-fluoroshikimic acid to the resin-bound tripeptide more challenging due to steric interactions between BOP and the resin. It would, however, greatly reduce the bulk of the ‘side chain’ at position three of the tripeptide conjugates, and might therefore increase the likelihood of the smugglins being transported by OppA.

## 4 Dehydroquinase and 3-dehydroquinic acid

### 4.1 Brief Introduction

Dehydroquinase (3-dehydroquinate dehydratase EC 4.2.1.10; DHQase) catalyses the conversion of DHQ 10 to DHS 14 (*fig. 46*). This reaction is not only part of the shikimate pathway, as described in Chapter 1 (*fig. 5*), but is also part of a catabolic pathway found mainly in fungi: the quinate pathway (Chapter 1, *fig. 14*). In this catabolic pathway, quinate is converted to *p*-hydroxybenzoate which can be used as a carbon-source via the  $\beta$ -ketoadipate pathway (Gross, 1958; Ornston & Stanier, 1966).

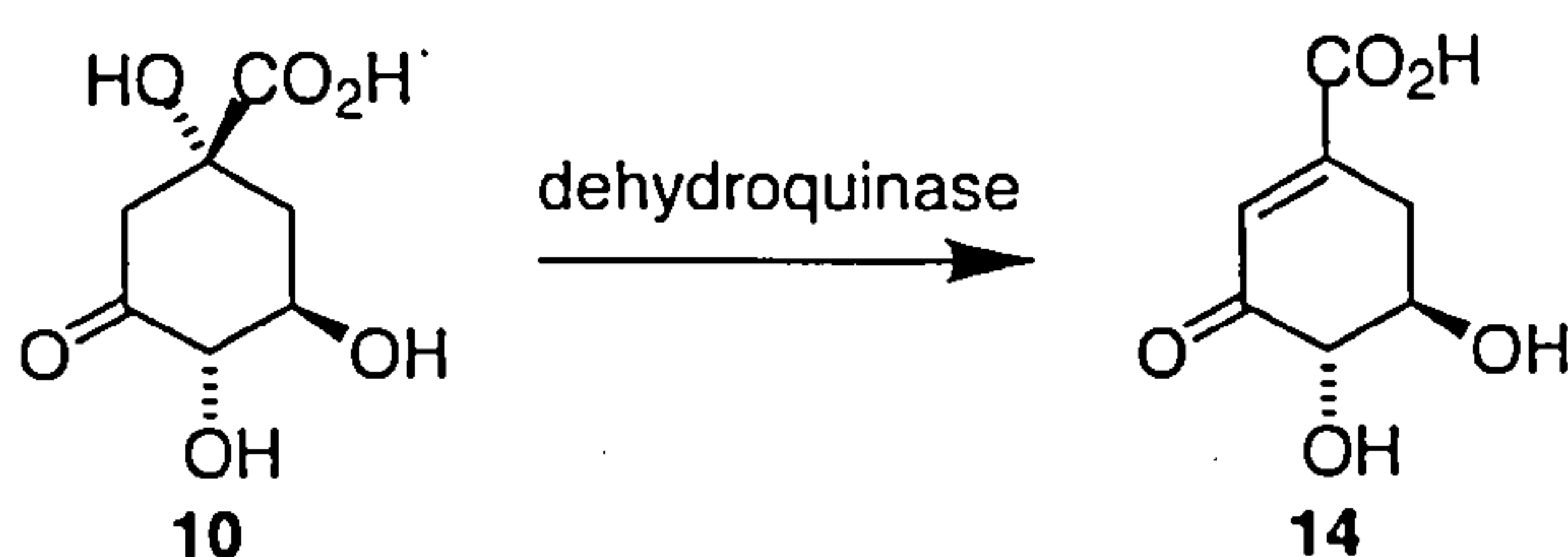


Figure 46: The reaction catalysed by DHQase

Two types of enzyme exhibiting DHQase activity have been described. Type I enzymes, which involve a *syn* elimination, generally catalyse the biosynthetic reaction. Type II enzymes can catalyse either the biosynthetic or catabolic reaction, and involve an *anti* elimination. The two enzyme types differ in terms of their amino-acid sequence, their 3-dimensional structure, and their catalytic mechanism (Gourlay *et al.*, 1999; Roszak *et al.*, 2002). Type I enzymes are dimers of 27 kDa subunits which contain an  $(\alpha/\beta)_8$  fold while type II enzymes are dodecameric, with the 16 kDa subunits arranged as tetramers of trimers. Each type II subunit adopts a flavodoxin-like fold consisting of a 5-stranded



parallel  $\beta$ -sheet core flanked by 4  $\alpha$ -helices. The reaction catalysed by the type I DHQases is known to proceed via an imine intermediate (Butler *et al.*, 1974; Chaudhuri *et al.*, 1991), while that catalysed by type II enzymes is believed to involve an enol intermediate (Roszak *et al.*, 2002).

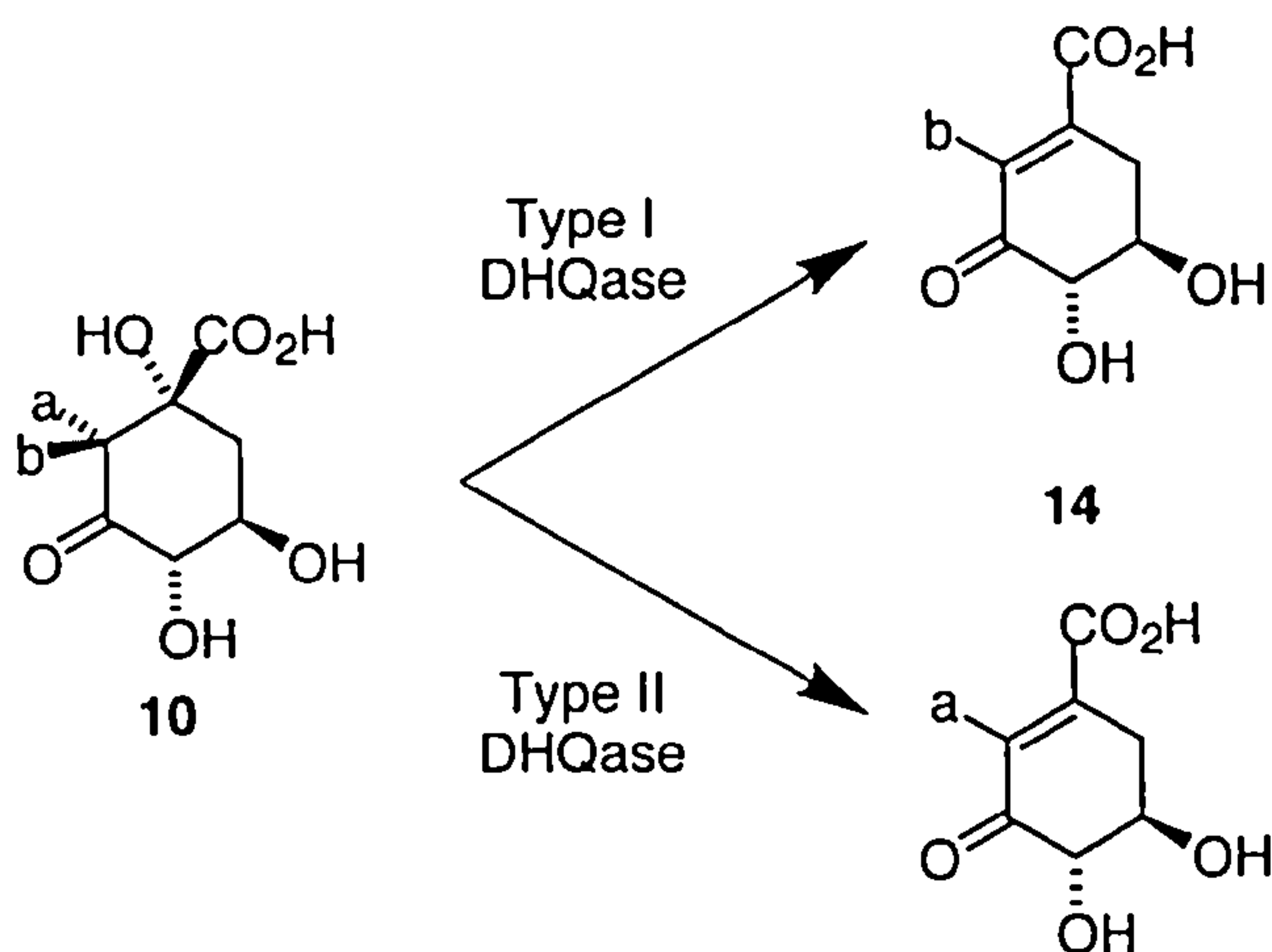


Figure 47: The differing outcomes of catalysis by type I and type II DHQases

Kinetic characterisation of the type II DHQases appears to divide them into two groups. The first group, consisting of the enzymes from organisms such as *Streptomyces coelicolor* and *Aspergillus nidulans*, has relatively high values of  $k_{\text{cat}}$  at between 100 and 1000 s<sup>-1</sup> at pH 7.0 and 25°C. The second group, which includes the enzymes from *Helicobacter pylori*, *Mycobacterium tuberculosis* and *Neurospora crassa*, has much lower values of  $k_{\text{cat}}$  in the range 10 s<sup>-1</sup> or lower. Unusually, there appears to be no obvious correlation between the values of  $k_{\text{cat}}$  and  $K_{\text{m}}$  for these enzymes (Evans *et al.*, 2002).

## **4.2 Rationale for a new synthesis of 3-dehydroquinic acid**

DHQ is required in quantity for the kinetic investigation of type II DHQases in our laboratory. In particular, stopped-flow kinetic analysis requires equimolar concentrations of enzyme and substrate in order to study the first few milliseconds of a reaction. At these concentrations, any minor impurity in the substrate can greatly affect both the interaction of enzyme and substrate and the UV absorbance of the reaction mixture.

In addition, several versions of type II DHQases with very low rates of reaction have been obtained either by isolation or by genetic modification in the laboratory. Even steady-state kinetic investigation of these mutants involves very high concentrations of the substrate DHQ. As a result, the elimination of background absorbance from the substrate used was crucial for the observation of activity.

A source of uncontaminated DHQ is therefore essential for unambiguous experimental results.

## 4.3 Published syntheses of 3-dehydroquinic acid

### 4.3.1 Nitric acid

The synthetic method of choice in our laboratory has been the classical synthesis in which quinic acid **19** was oxidised by concentrated nitric acid over 45 h to give DHQ **10** (*fig. 48*)(Grewe & Jeschke, 1966).

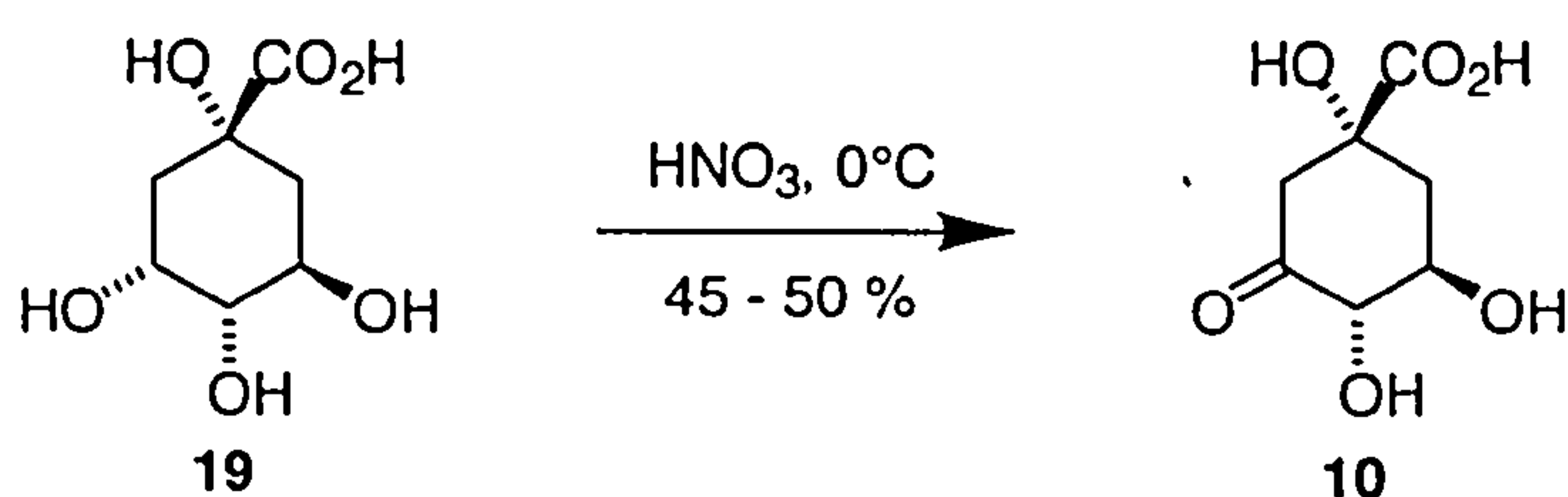


Figure 48: Synthesis of DHQ by nitric acid oxidation of quinic acid

The reaction products were separated by lengthy ion-exchange chromatography using the acetate form of basic Amberlite CG-400/I and the DHQ further purified by a difficult crystallisation as the ammonium salt (55 - 60 %). The free acid was obtained by ion-exchange chromatography using Amberlite IR-120 and crystallised from isopropanol/pet. ether (45 - 50 %)

In our hands, complete separation of the 3- and 5-dehydroquinic acids formed was not achieved, and the product was further contaminated by a highly UV-absorbing aromatic compound. These contaminants severely limited the utility of the product as 5-dehydroquinic acid is thought to be an inhibitor of DHQase, and at the concentrations required for kinetic analysis, the spectrophotometer was overloaded by the UV absorbance of the aromatic impurity.

### 4.3.2 Pt catalysed oxidation

An alternative preparation was reported in 1961 (Heyns & Gottschalk, 1961). Their method was based on the observation that under catalytic conditions, an axial hydroxyl will be oxidised to the corresponding ketone while any equatorial hydroxyls are unaffected.

The six-membered ring of quinic acid **19** exists in a chair conformation, with the C1-carboxyl group equatorial. As a result, both the C-4 and C-5 hydroxyl groups are equatorial, while the C-1 and C-3 hydroxyl groups are axial. Platinum thus catalyses the exclusive oxidation of the C-3 hydroxyl to the ketone (*fig. 49*).

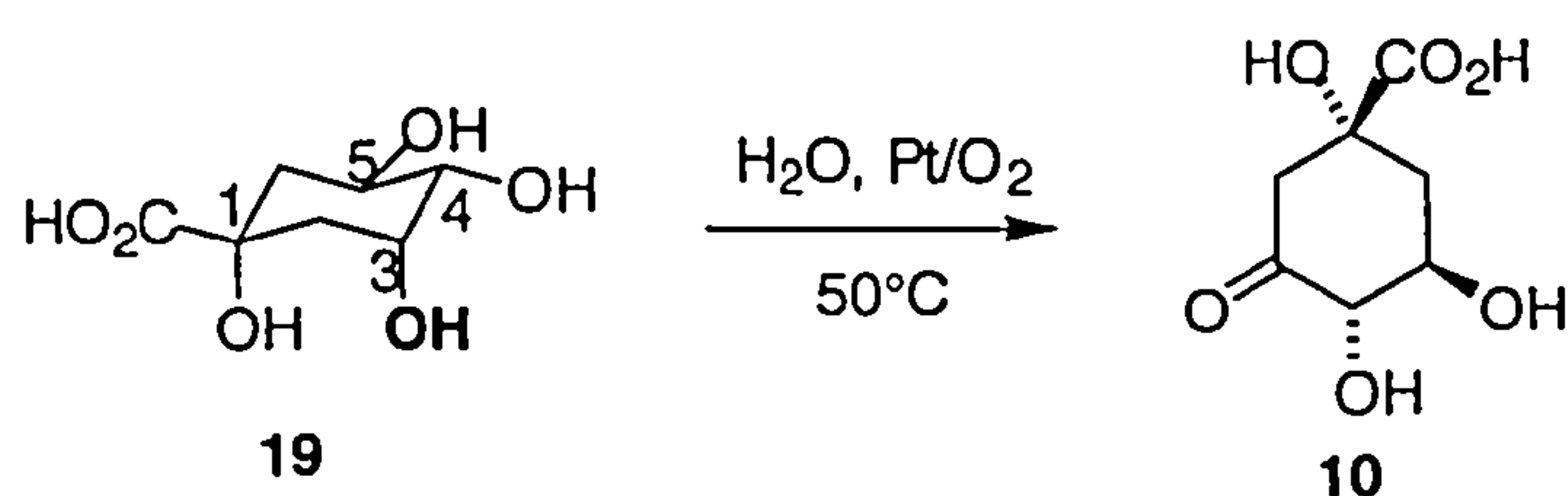


Figure 49: The Pt-catalysed oxidation of quinic acid to DHQ

The published reaction was carried out in water on a 5 g scale using a platinum catalyst in an oxygen atmosphere. The product was isolated as the brucine salt in 45 % yield after repeated recrystallisation.

### 4.3.3 Formal synthesis to methyl ester

In their investigation of inhibitors of DHQ synthase, the Frost group utilised the butane 2,3-bisacetal (BBA) protecting group to selectively protect the trans diequatorial diol of quinic acid (Tian *et al.*, 1996). BBA was developed to avoid problems associated with the earlier *trans*-diol selective protecting groups 3,3',4,4'-tetrahydro-6,6'-spirobi-2*H*-pyran (bisDHP)

and 1,1,2,2-tetramethoxycyclohexane (TMC) (Montchamp *et al.*, 1996). It is easily prepared, does not require chromatographic purification, unlike bisDHP it can be used in methanol, and unlike TMC it does not give a complex NMR signal (Ziegler, 1994).

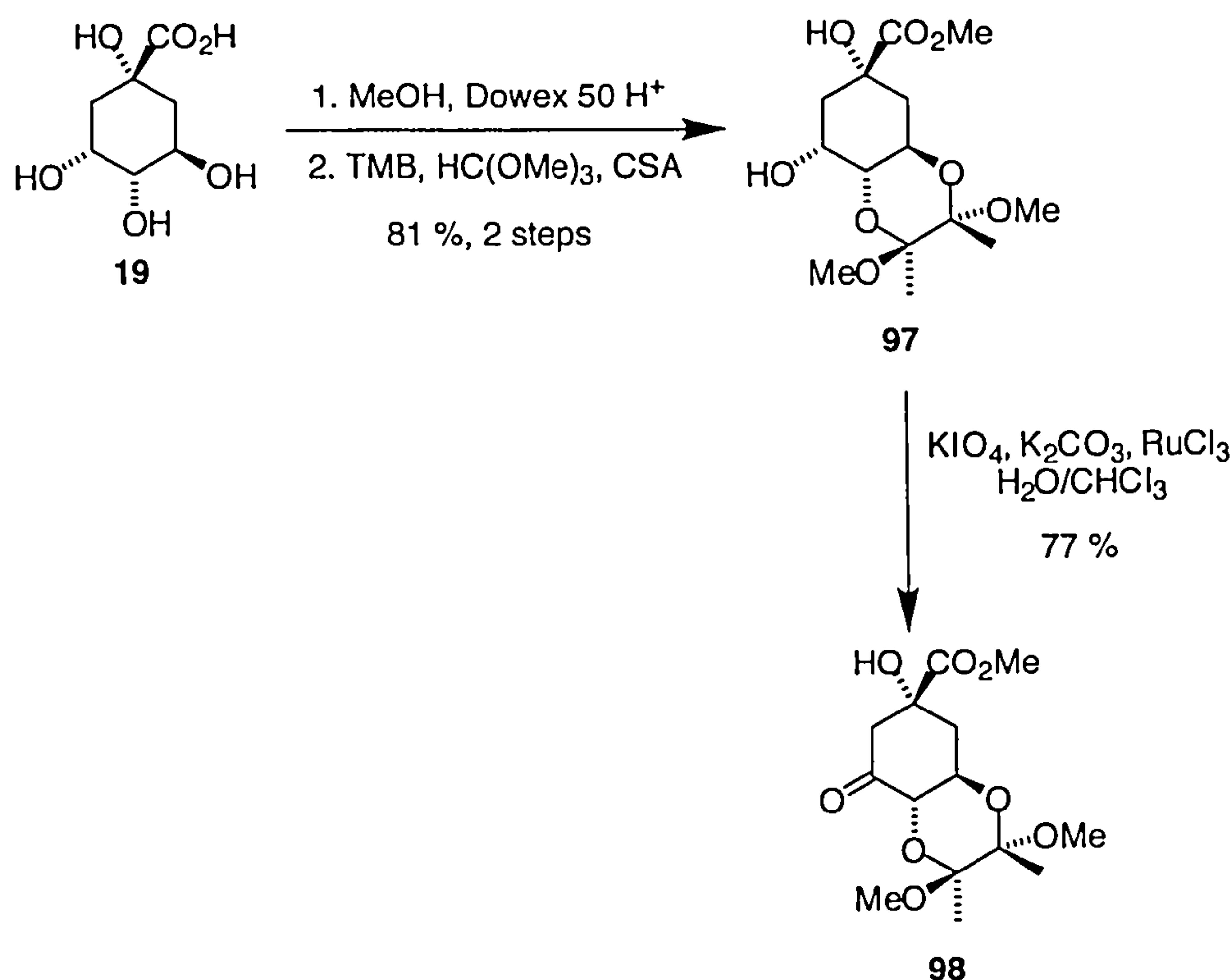


Figure 50: Formal synthesis of DHQ

Quinic acid **19** was protected as the methyl ester by refluxing in acidified methanol. The ester was then reacted with TMB in the presence of trimethyl orthoformate and catalytic (±)-10-camphorsulfonic acid (CSA) to give the protected compound **97** in 87 % yield. Ruthenium-catalysed oxidation of the unprotected C-3 hydroxyl then gave the protected 3-DHQ derivative **98** in 77 % yield (*fig. 50*).

In their route to (-)-methyl shikimate and various derivatives modified at C-3, the Payne group used a modification of this synthesis (Alves *et al.*, 1999). Starting from methyl



quinate **99**, the BBA protection step was catalysed by *p*-toluenesulfonic acid (*p*-TSA). The reaction time for this protection was also shortened from 22 h to 1 h, increasing the yield from 81 % to 85 %. Swern oxidation of the quinate derivative **97** then afforded the fully protected 3-DHQ derivative **98** in 74 % yield with the corresponding protected shikimate derivative **100** as a significant byproduct (*fig. 51*).

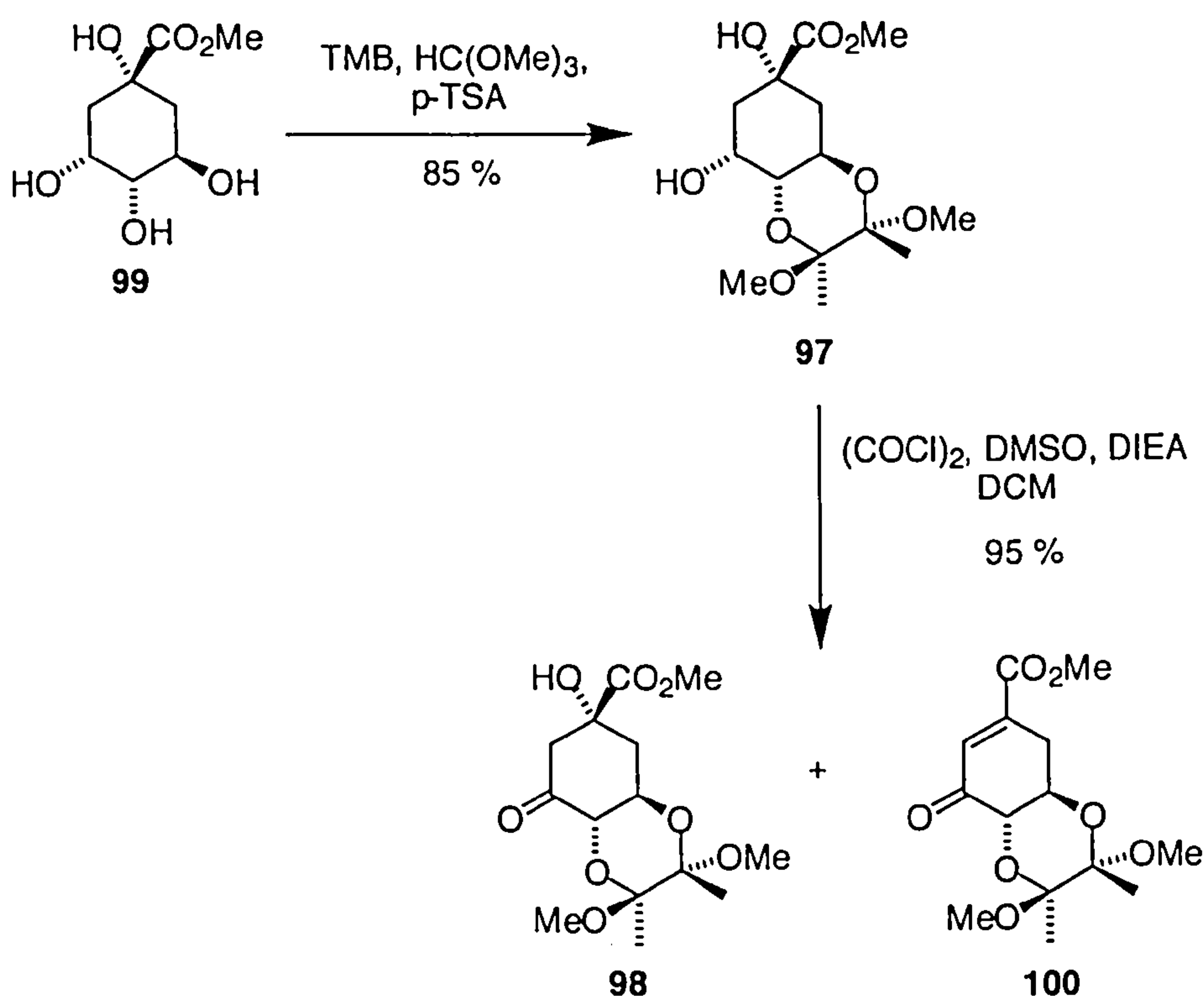


Figure 51: Formal synthesis of DHQ and DHS

The DHQ derivative **98** was not deprotected in either of these synthetic routes.

#### 4.3.4 Benzyldiene acetal/lactone protection

A key intermediate in the synthesis of (2*R*)-2-bromodehydroquinic acid and (2*R*)-2-fluorodehydroquinic acid published by the Abell group was a protected form of 3-

dehydroquinic acid (Manthey *et al.*, 1997). Reaction of quinic acid **19** with benzaldehyde under acid catalysis with azeotropic removal of water gave the benzylidene acetal **101**, which was then hydrogenated to give quinic acid lactone **102** in excellent yield. Reaction of the lactone with *t*butyldimethylsilyl chloride (TBDMSCl) in DMF gave a 1:2 mixture of the C-5 protected compound **103** and the desired C-4 protected lactone **104**. The authors noted that the undesired compound **103** could be converted to a mixture of **103** and **104** on re-exposure to the reaction conditions. The C-3 secondary hydroxyl was then oxidised using pyridinium dichromate (PDC) to give the acid-sensitive DHQ derivative **105** in excellent yield (*fig. 52*).

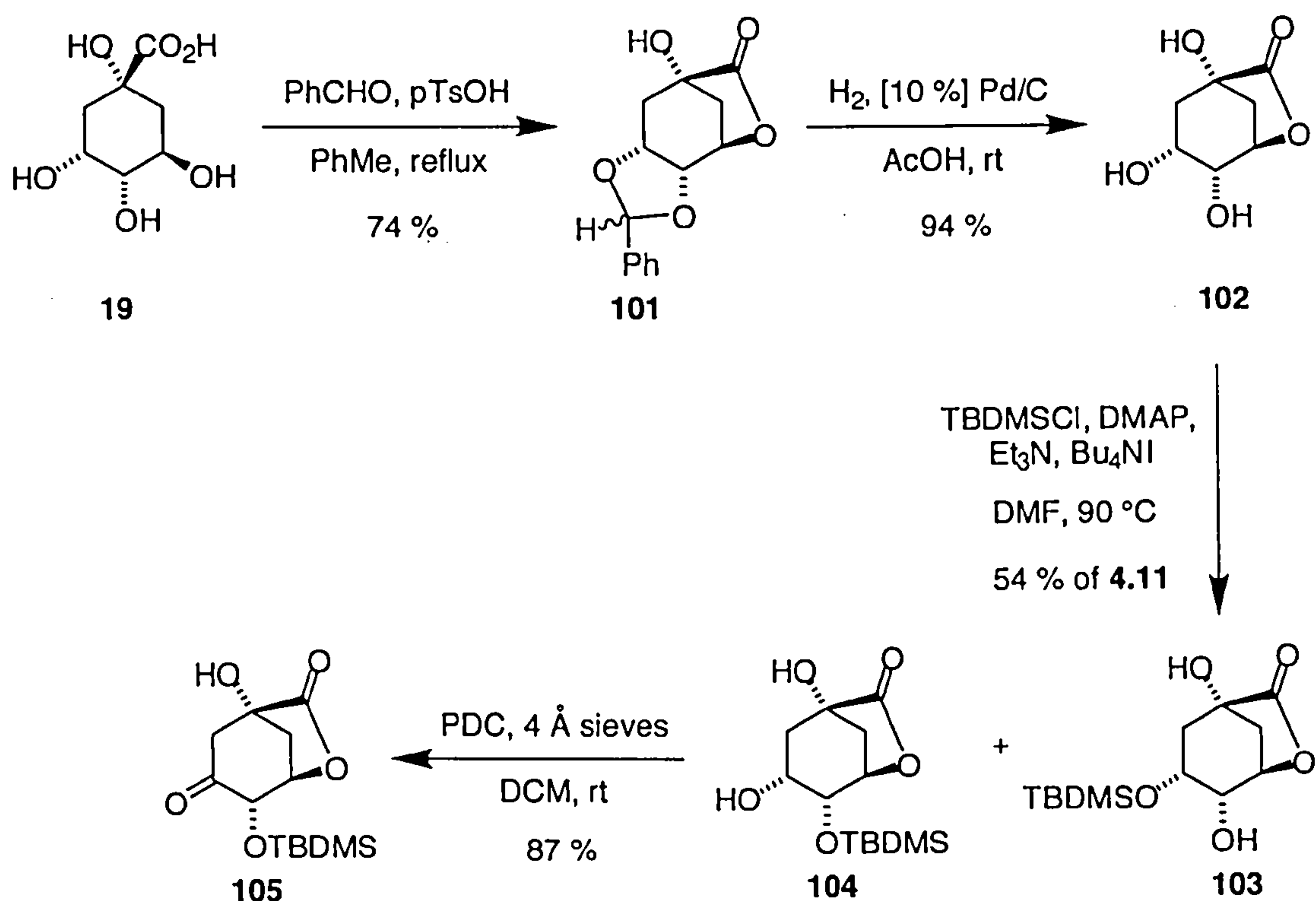


Figure 52: Formal synthesis of DHQ via the quinide.

The alternative method of direct lactonisation published by in 1954 (Panizzi, 1954) was mentioned in this paper, but the authors found it to be experimentally inferior to the two-step procedure. The one-step procedure involved refluxing quinic acid **19** in a large volume of dry dioxane with 0.3 eq. dry HCl for 6 - 7 hours, and neutralising with dry  $K_2CO_3$  (*fig. 53*).

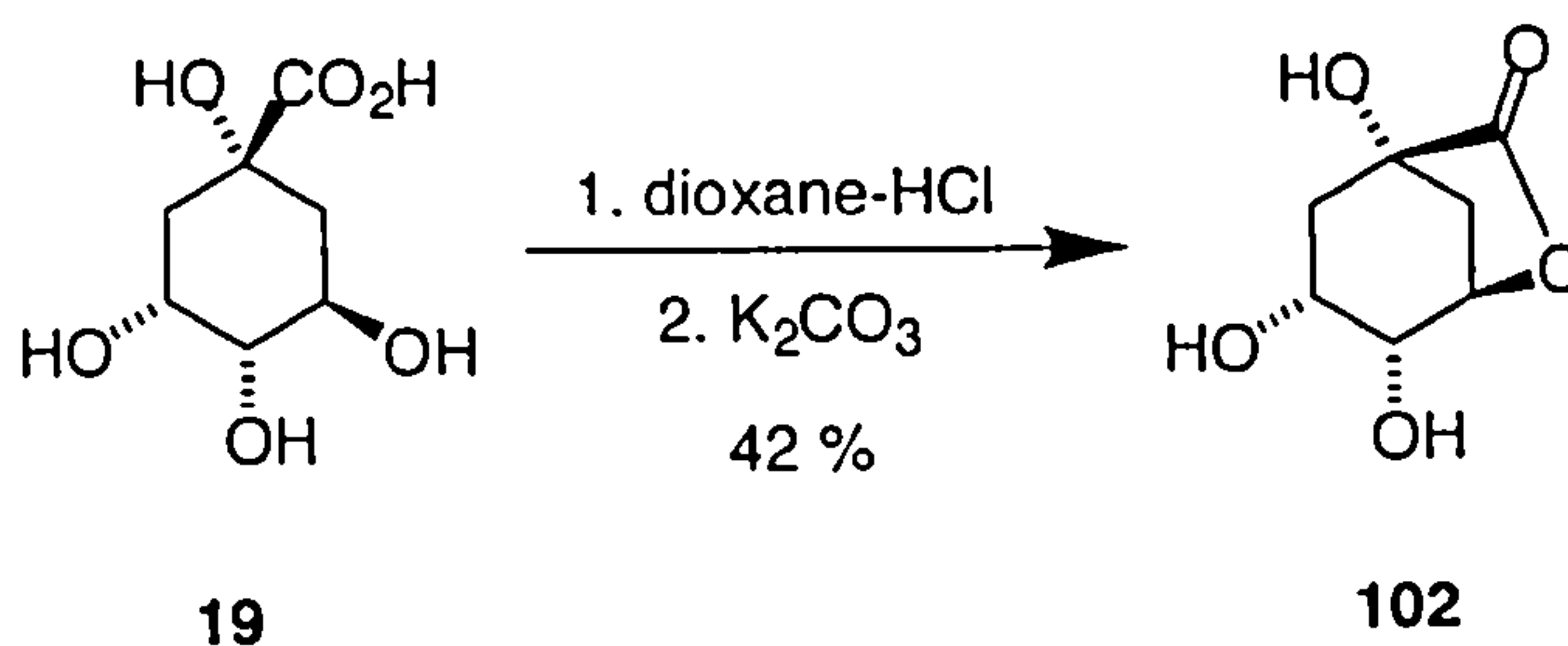


Figure 53. Direct lactonisation of quinic acid to give the quinide

The quoted yield was 3.8 g of the lactone **102** from 10 g of quinic acid, or 42 % as compared to 70 % quoted for the two-step procedure.

## 4.4 Results and Discussion

### 4.4.1 BBA

The route to BBA-protected methyl-DHQ (Tian *et al.*, 1996) (*fig 50*) provided the logical starting point for an attempted synthesis of DHQ. The only novel steps involved would be removal of the acid-sensitive BBA moiety, and saponification of the ester.

#### Attempts to replicate the literature protocol

TMB **108** was prepared according to the literature protocol (Montchamp *et al.*, 1996). 2,3-Butanedione **106** and trimethyl orthoformate **107** were treated with concentrated  $\text{H}_2\text{SO}_4$  in MeOH to give TMB **108** in a 45 % yield after distillation under high vacuum.

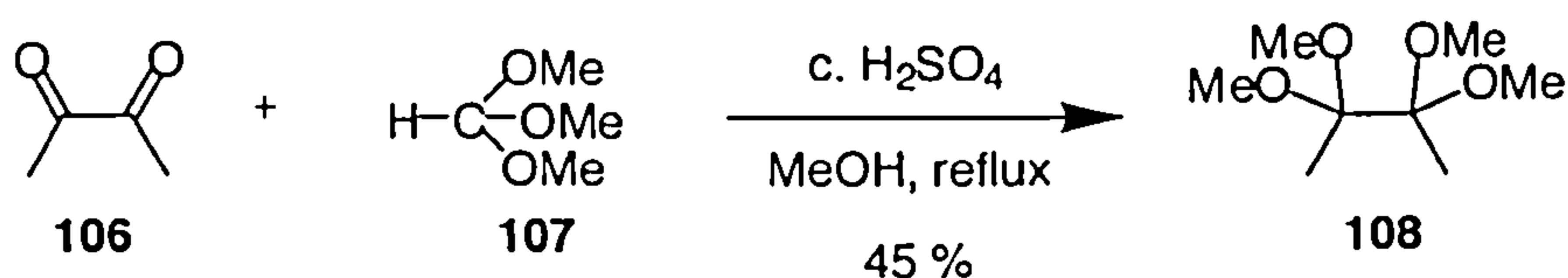


Figure 54: Synthesis of TMB

Methyl quinate was synthesised by the acid catalysed esterification of quinic acid **19** in dry MeOH in a yield of 90 % on a 5 g scale. The diequatorial diol was then protected as the butane bis-acetal by reaction with TMB in refluxing dry MeOH. *p*-TSA was used instead of CSA as the catalyst, giving the desired protected quinate derivative **97** in 64 % yield (*fig. 55*).

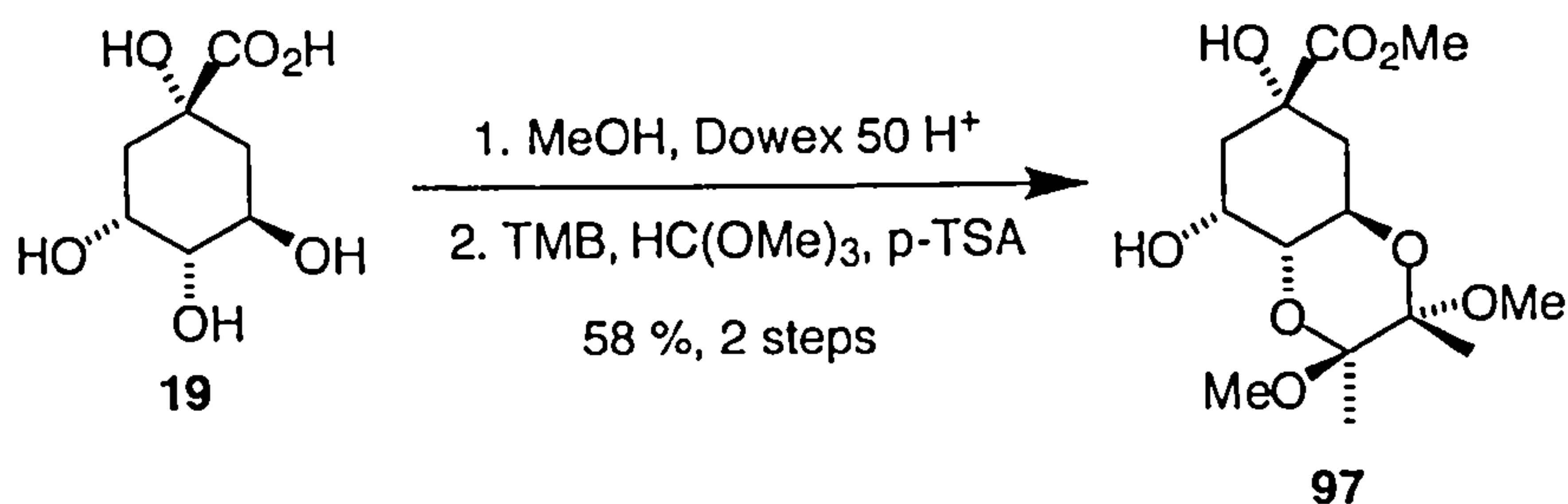


Figure 55: Protection of quinic acid as the methyl ester and butane bis-acetal

The authors state that it is unnecessary to distil TMB before use, although they routinely did so (Montchamp *et al.*, 1996). Our attempts to use the crude products of the reaction of 2,3-butanedione and trimethyl orthoformate to protect the diequatorial diol of methyl quinate were not successful. A second modification to the procedure, suggested by the Ley group (Hense *et al.*, 1997), involves the *in situ* generation of TMB from 2,3-butanedione and trimethyl orthoformate under acid catalysis in MeOH at reflux. This was slightly more successful, but still only gave 24 % of the desired compound 97 after repeated silica-gel column chromatography purification and crystallisation attempts.

We did, however, find that the first two steps could be carried out in a single pot without intermediate purification. The reaction of quinic acid 19 with TMB under acid catalysis in MeOH at reflux gave the BBA-protected methyl quinate derivative 97 in an improved yield compared to the two-step procedure in our hands (*fig. 57*). We were also able to shorten the reaction time relative to the one-pot, two-step procedure (Montchamp *et al.*, 1996).

Ruthenium-catalysed oxidation of the C-3 hydroxyl group of 97 using potassium periodate as the secondary oxidant in a chloroform/water two-phase system was then attempted.

While the authors of the paper quote a 77 % yield, the reaction time required to reach



complete oxidation was not given. We found that after 3 days of exposure to the oxidation conditions the reaction had only reached 95 % completion, and the desired 3-DHQ derivative **98** was obtained in less than 50 % yield (*fig. 56*).

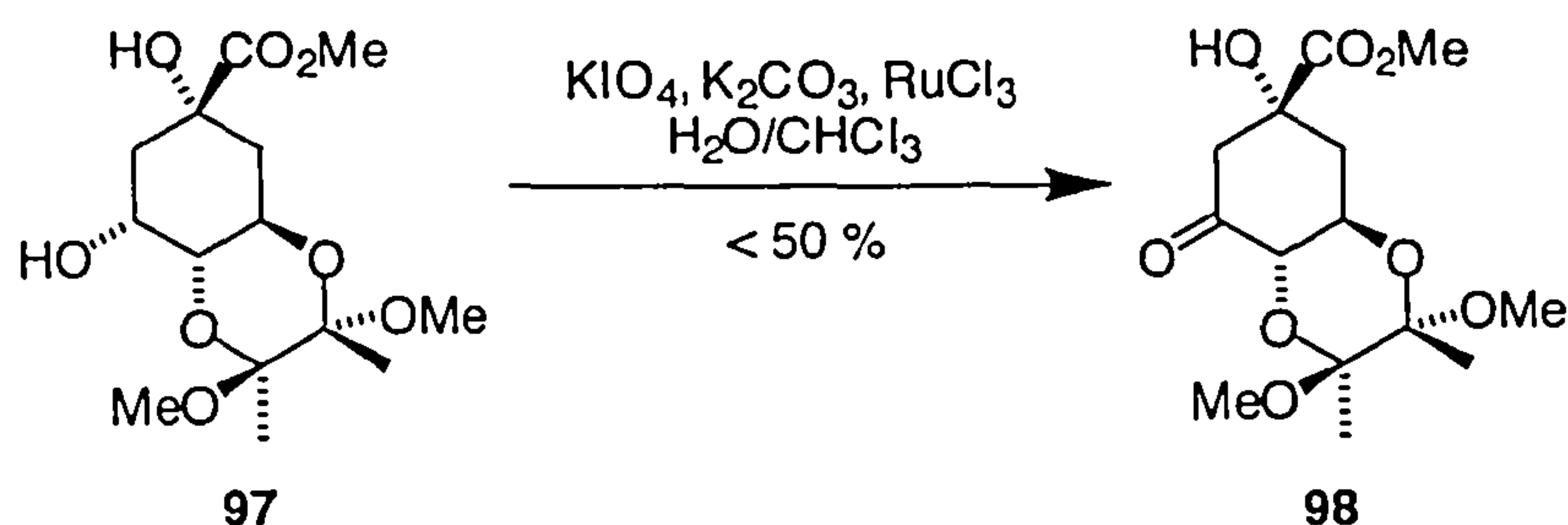


Figure 56: Attempted Ru-catalysed oxidation to give DHQ

The authors stated that they carried out small-scale oxidations using tetrapropylammonium perruthenate (TPAP) and 4-methylmorpholine *N*-oxide (NMO) rather than  $\text{RuCl}_3/\text{KIO}_4$  which they used for large-scale (30 g) reactions. The difficulties we encountered may thus have been due to the absence of a phase-transfer catalyst (Morris & Kiely, 1987).

Under aqueous alkaline conditions, the non-selective oxidant  $\text{RuO}_4$  is converted to the less reactive  $\text{RuO}_4^{2-}$  (ruthenate). Classically, a solution of  $\text{RuO}_4$  in  $\text{CCl}_4$  was extracted into aqueous 1 M NaOH to give a dark orange solution which was then used stoichiometrically to oxidise alcohols in the presence of alkenes and alkynes (Coates *et al.*, 1982). Later, persulfate ( $\text{K}_2\text{S}_2\text{O}_8$ ) was used as a secondary oxidant, again in aqueous solution, to carry out the same oxidations using catalytic ruthenium (Green *et al.*, 1984). When reagent solubility necessitated a two-phase solvent system of water/ $\text{CCl}_4$  a phase-transfer catalyst was introduced along with either persulfate or periodate ( $\text{KIO}_4$ ) as secondary oxidant. Both

benzyltriethylammonium chloride and tetrabutylammonium chloride are mentioned in this regard (Morris & Kiely, 1984).

At the time, we believed that our problems were due to a loss of catalyst activity as an insoluble black particulate material rapidly accumulated in the reaction vessel with a loss of the characteristic yellow-orange colour. An extensive literature search eventually led to the original Sharpless paper (Carlsen *et al.*, 1981) on “Ruthenium tetroxide catalysed oxidations”. In this paper, the authors speculated that the presence of carboxylic acids in the reaction mixture was leading to the formation of low-valent ruthenium carboxylate complexes, and hence to the loss of catalyst activity. Using the then-standard mixed solvent system of  $\text{CCl}_4\text{:H}_2\text{O}$  (1:1), ruthenium carboxylate complexes were inactive as oxidation catalysts, but the addition of MeCN to the reaction mixture rapidly restored the catalytic activity. The authors speculated that this was due to the ability of MeCN to coordinate low-valent transition metal ions, and hence break up the insoluble ruthenium carboxylate complexes.

Using the Sharpless conditions of a  $\text{CCl}_4\text{:MeCN:H}_2\text{O}$  (2:2:3) mixed solvent system, but with the addition of  $\text{K}_2\text{CO}_3$  as specified in the Montchamp paper to generate the more selective ruthenate species (Lee *et al.*, 1984), the oxidation proceeded to completion within 20 h (*fig. 57*). The desired protected DHQ derivative **98** was isolated in 79 % yield, which was an improvement over the literature yield (*fig. 50*). The explanation proposed by Sharpless, where carboxylic acids lead to catalyst inactivation cannot hold true for this reaction, as the C-1 carboxyl of quinic acid had been protected as the methyl ester. However, as  $\text{RuO}_4^{2-}$  is water-soluble, and the protected quinic acid derivative is not,

perhaps the addition of MeCN to the solvent mixture allowed sufficient mixing of the aqueous and organic phases to allow oxidation to occur in the absence of a phase-transfer catalyst.

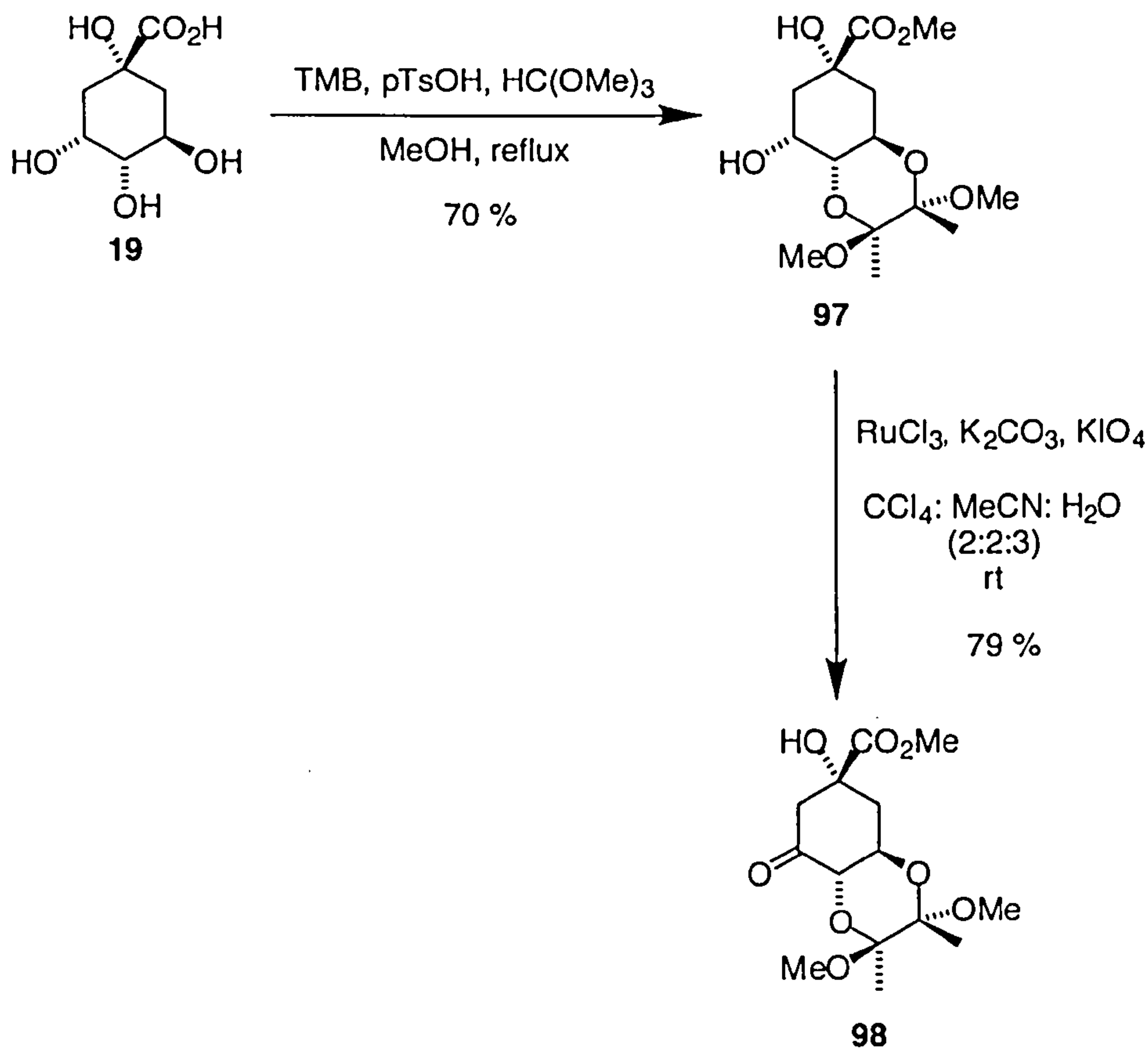


Figure 57: Synthesis of DHQ protected as the methyl ester and butane bis-acetal using the Sharpless conditions for Ru-catalysed oxidation

Substituting CHCl<sub>3</sub> for CCl<sub>4</sub> i.e. a CHCl<sub>3</sub>:MeCN:H<sub>2</sub>O (2:2:3) solvent system, gave a 78 % isolated yield of the desired compound **98**. Interestingly, the reaction required 3 days of stirring at room temperature to reach 95 % completion as determined by <sup>1</sup>H-NMR peak heights as opposed to the 20 h sufficient for complete reaction using CCl<sub>4</sub>.

## Extension of the literature protocol: cleavage of the BBA and methyl ester groups

The acid-sensitive BBA protecting group of **98** was readily cleaved by treatment with trifluoroacetic acid (TFA) in a dichloromethane/water mix at -5°C (Riley, 1999) to give methyl dehydroquinate **109**. Removal of the methyl ester, however, was to prove more challenging.

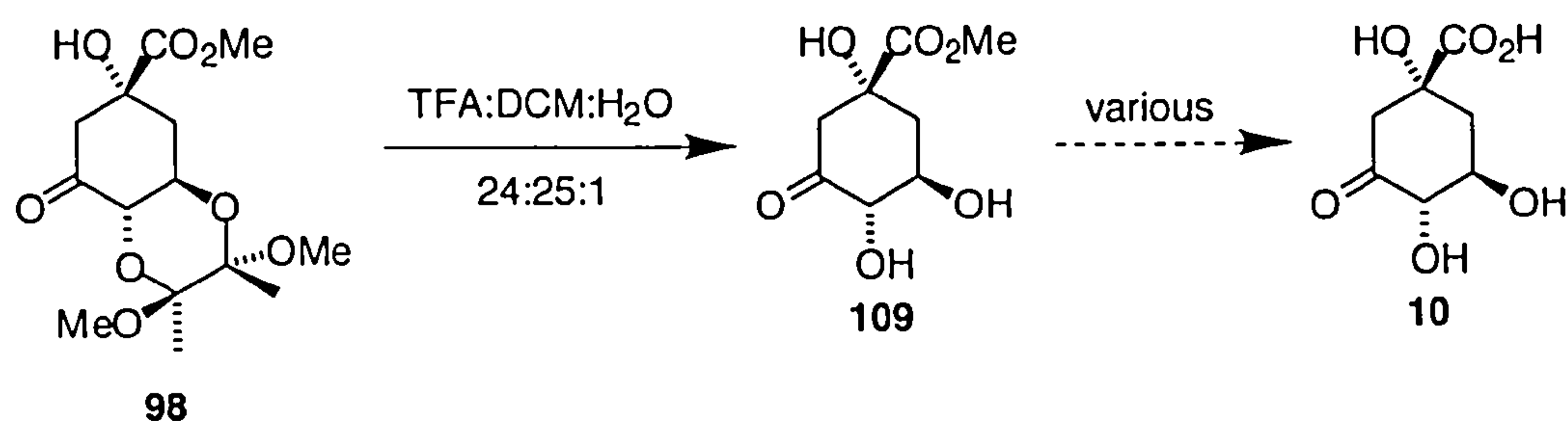


Figure 58: Attempted saponification of the methyl ester of methyl-DHQ

Initial attempts to cleave the methyl ester using 1M NaOH in a THF-water mix gave no recognisable products by <sup>1</sup>H-NMR or mass spectrometry. Exposure of **109** to α-chymotrypsin in an ammonium bicarbonate buffer for 44 h gave only returned starting material, but at least showed that the compound was not decomposing at room temperature in this mildly acidic buffer.

DHQ **10** is known to be base-sensitive, slowly forming 3,4-dihydroxybenzoic acid on exposure to even slightly elevated pH. It was therefore decided to attempt the cleavage of the methyl ester before removal of the BBA protecting group, hopefully reducing the likelihood of aromatisation (*fig. 59*).

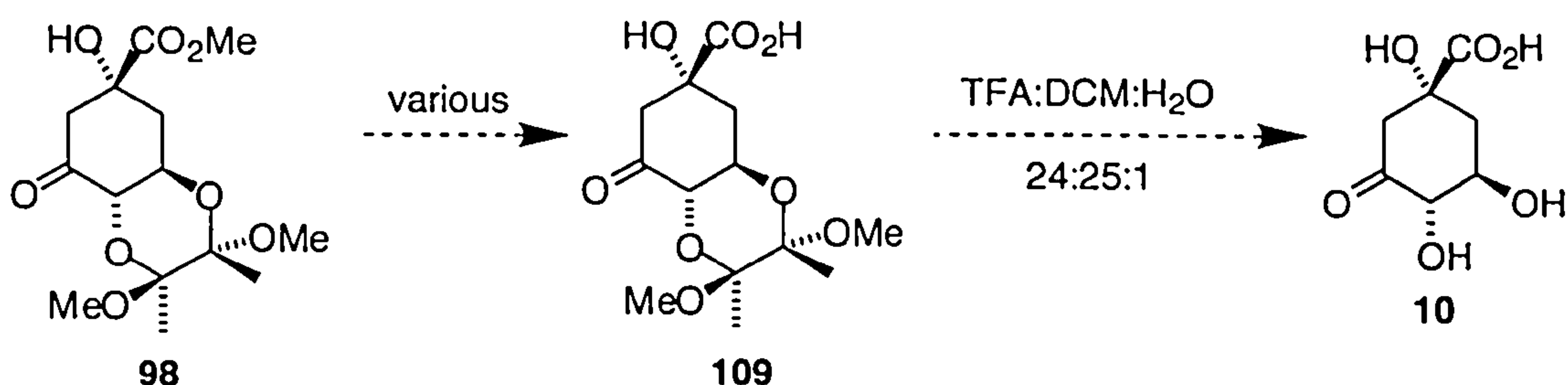


Figure 59: Proposed alternative route for deprotection to give DHQ

Standard base-catalysed hydrolysis of the DHQ derivative **98** [1M NaOH (aq.):MeOH = 10:1 at room temperature, followed by an acidic workup] gave a brown material which could not be purified. <sup>1</sup>H-NMR analysis showed the presence of the starting material **98**, BBA-protected methyl dehydroshikimic acid **100** and 3,4-dihydroxybenzoic acid **111** in an approximate ratio of 1:2:5 (*fig. 60*). This suggested that the methyl ester was only cleaved after elimination of the C-1 hydroxyl had formed the dehydroshikimate derivative.

Substituting THF or DCM for the MeOH, in an attempt to reduce the likelihood of the ester reforming, gave traces of 3,4-dihydroxybenzoic acid, and no other recognisable organic compounds by <sup>1</sup>H-NMR analysis. The milder base LiOH in a THF-water mix at 0°C also gave an inseparable mixture of aromatic products.





of transferring the THF-H<sub>2</sub>O slurry to a second flask after 40 min reaction, leaving the yellow 3,4-dihydroxybenzoic acid **111** in the original flask as an oil. Attempts to separate the compounds obtained when the transferred THF-H<sub>2</sub>O was removed under reduced pressure (**111**:**110**:**112** = 1:2:2 by <sup>1</sup>H-NMR analysis of the crude material) by silica-gel column chromatography were not successful, and only 3,4-dihydroxybenzoic acid **111** was isolated.

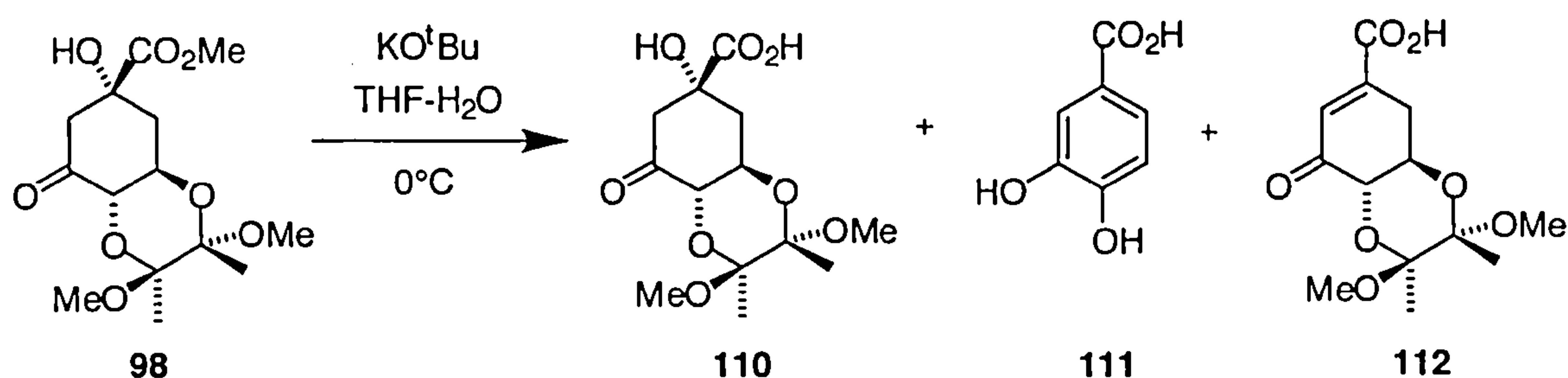


Figure 61: Attempted saponification of the methyl ester of protected DHQ

LiI in dry pyridine is considered to be a mild form of the standard ‘basic’ ester cleavage method. The fully protected methyl DHQ derivative **98** was stirred in dry pyridine at reflux with 4 eq. of LiI (Magnus, 1984). After 30 min, 2 h and 3 h, a portion was acidified and extracted into CHCl<sub>3</sub>. <sup>1</sup>H-NMR showed the gradual conversion of the BBA-protected methyl DHQ **98** into the corresponding dehydroshikimate derivative **100** such that after 3 h 60 % conversion had occurred (*fig. 62*). The same elimination reaction was seen when the DHQ derivative **98** was stirred in pyridine at rt without the addition of LiI.

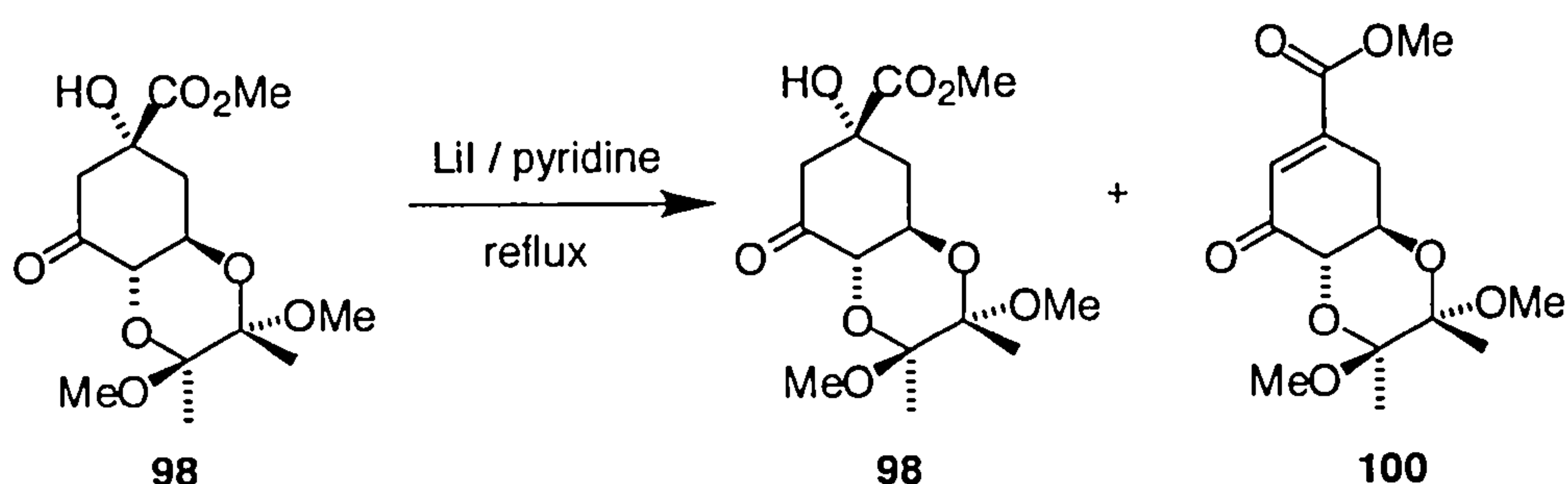


Figure 62: Attempted saponification of the methyl ester of protected DHQ

The potassium salt of trimethylsilanol (TMSOK) has been used to convert esters to carboxylic acid salts under mild conditions. The authors noted that TMSOK appeared to act as a nucleophile rather than a base under the conditions given, as  $\alpha$ -protons did not affect the desired reaction. (Laganis, 1984) Subsequently, TMSOK in THF was used to cleave the methyl ester of a base-sensitive shikimate derivative. (Jiang, 1999)

In a similar manner, the protected dehydroquininate derivative **98** was stirred in a slurry of TMSOK in toluene for 4 h (*fig. 63*). After removing the toluene, the residue was treated with 5 % aq.  $\text{NaHCO}_3$  and extracted into  $\text{Et}_2\text{O}$  in an attempt to separate any acid-containing products from the ester-containing starting materials without causing elimination. The aqueous fraction was then acidified and the solvent removed to give a yellow oil along with solid  $\text{NaCl}$ . The aqueous fraction was found to contain the BBA-protected dehydroshikimate derivative **112** along with a trace of 3,4-dihydroxybenzoic acid **111**, while the etherial layer consisted of a 2:1 mixture of BBA protected methyl dehydroshikimate **100** and the starting material **98**. Again, this suggested that the methyl ester was only removed after elimination of the C-1 hydroxyl.

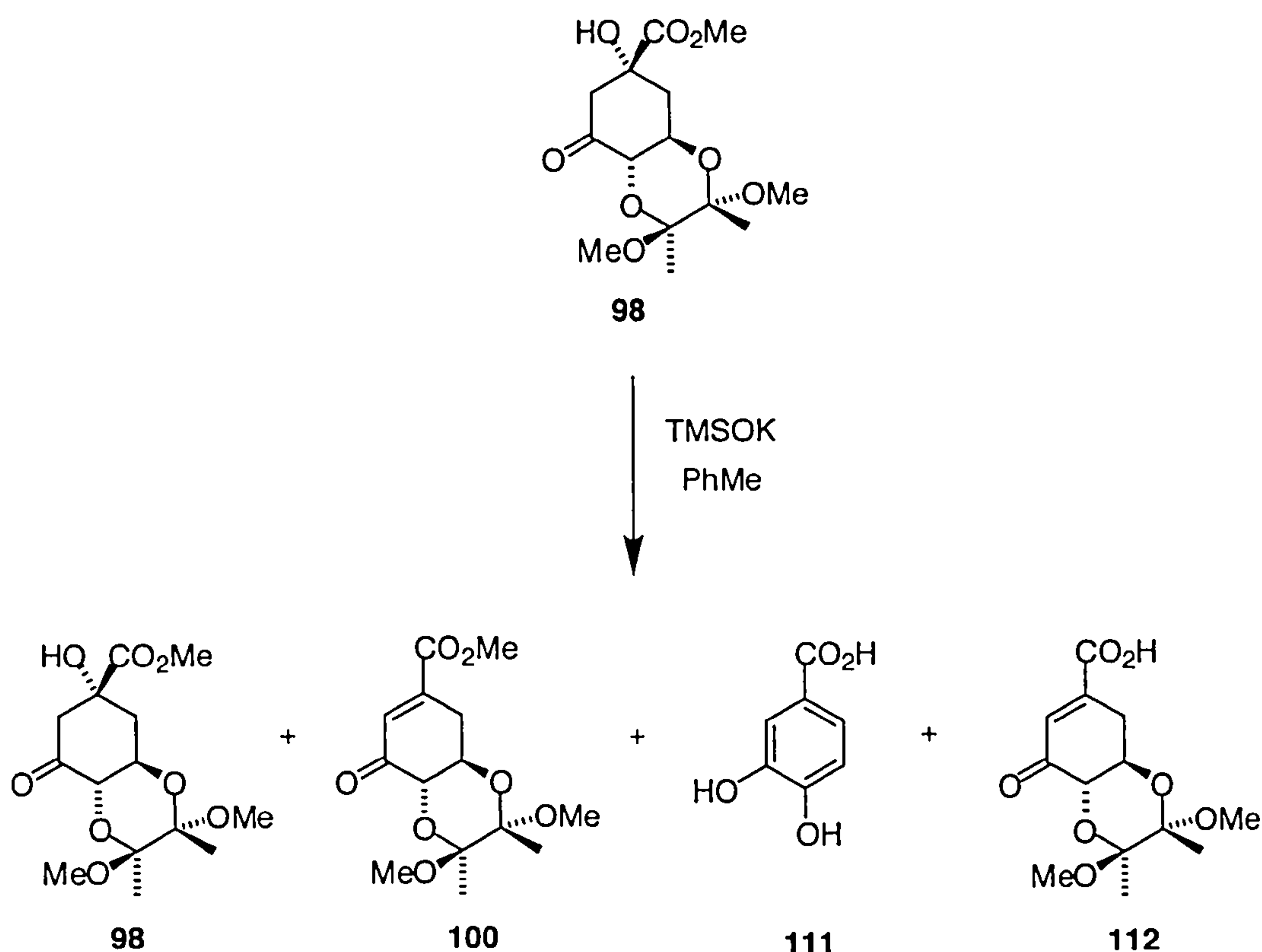


Figure 63: Attempted saponification of the methyl ester of protected DHQ

By this stage it had become apparent that derivatives of DHQ are extremely sensitive to base, eliminating water to give DHS derivatives in the first instance, and aromatising under more forcing conditions. As a result, a neutral to acidic method of ester hydrolysis was sought. Enzymatic hydrolysis using chymotrypsin in a 5 % ammonium bicarbonate buffer was attempted, but after 72 h no reaction had occurred. Chymotrypsin, a protease, had been recommended by an enzymologist for this transformation. It was decided that investigation of all the esterases and lipases available would be unrealistic, and the enzymatic approach was abandoned.

Bis(tributyltin) oxide [(Bu<sub>3</sub>Sn)<sub>2</sub>O] has been used for the cleavage of simple esters (Mata & Mascaretti, 1988). The authors speculated that the mechanism involved an organotin ester intermediate which was converted into the free acid during workup. As the method is both mild and non-basic, it was hoped that the methyl ester would be cleaved without concomitant conversion to the dehydroshikimate derivative **112**.

After reaction with (Bu<sub>3</sub>Sn)<sub>2</sub>O in Et<sub>2</sub>O at rt, silica-gel column chromatography gave approximately 50 % returned starting material **98** despite TLC analysis of the reaction mixture suggesting that the reaction had proceeded to completion (*fig. 64*). Removal of Sn residues was not successful and as heavy metals are known to inhibit many enzymes this approach was abandoned.

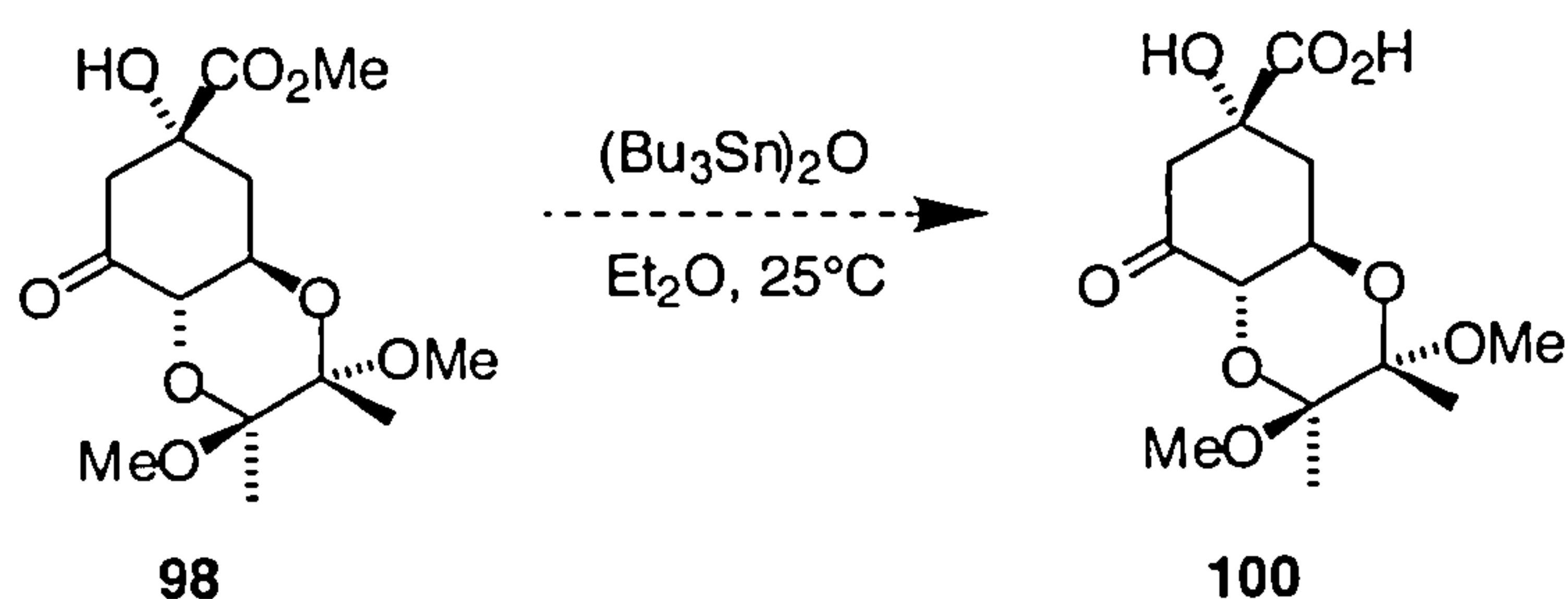


Figure 64: Attempted cleavage of the methyl ester of protected DHQ

BCl<sub>3</sub> and BBr<sub>3</sub> are also 'neutral' ester cleavage reagents (Barton *et al.*, 1971; Felix, 1974), but they were thought to be far too reactive to provide a viable solution to this problem.



### 4.1.2 Dispoke

The dispiroketal (Dispoke) protecting group was developed by the Ley group for the selective protection of *trans* diequatorial vicinal diols in carbohydrate systems (Ley *et al.*, 1992). The standard conditions for protection are treatment of the carbohydrate with bis-DHP in dry chloroform at reflux with catalytic CSA. Unlike BBA, therefore, it seemed likely that the dispoke protecting group could be used to protect the C-4 and C-5 hydroxyl groups of quinic acid without forming the methyl ester, solving the major problem with the previous synthetic route (*fig. 65*).

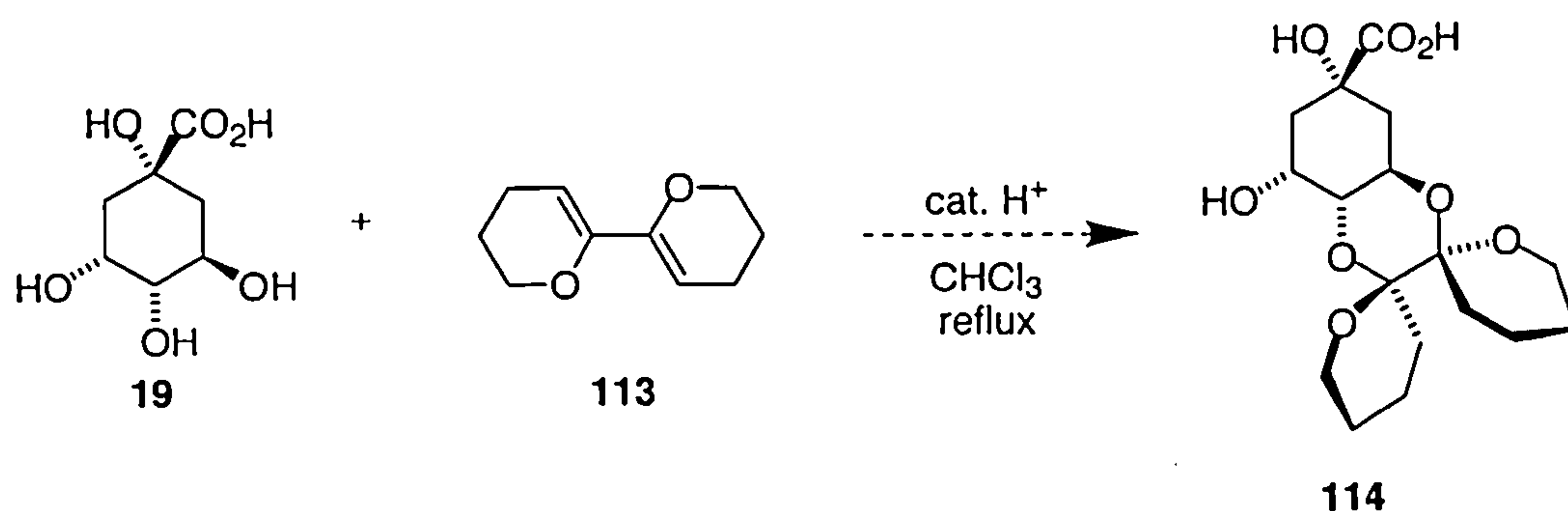


Figure 65: Proposed protection of quinic acid using Dispoke

Bis-DHP **113** was synthesised via a palladium catalysed oxidative dimerisation reaction according to the method provided for large-scale reactions (Ley *et al.*, 1992) (*fig 66*). The organolithium compound **116** was generated by adding <sup>t</sup>BuLi to dry distilled 3,4-dihydro-2*H*-pyran **115**, and dimerisation was initiated by adding the solution of **116** to a slurry of the palladium catalyst in THF. After quenching with an ammonium chloride/ammonia solution and extracting into ether, the product was purified by silica-gel column chromatography and crystallised from pet. ether. Due to difficulties experienced in

purification and consequent losses of material, the yields for these syntheses were poor, but sufficient pure bis-DHP **113** was obtained to test the protection method.

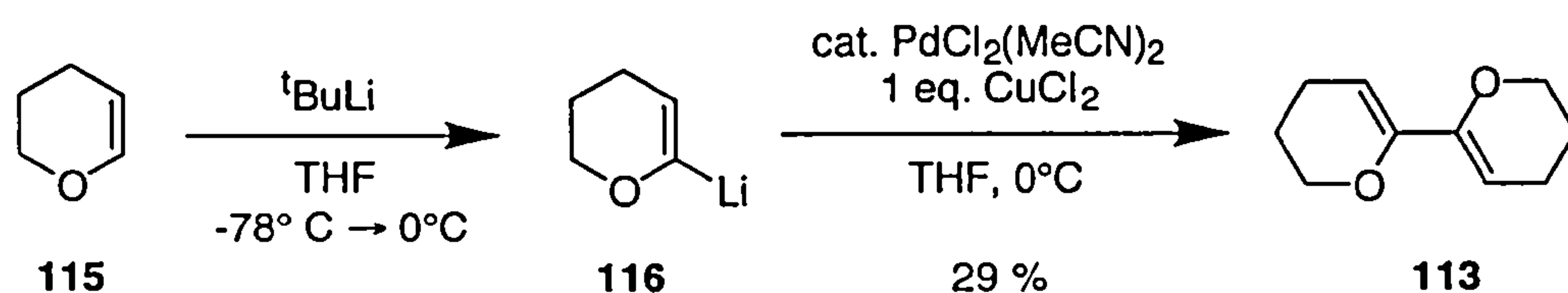


Figure 66: Synthesis of bis DHP

Treatment of quinic acid **19** with 2 eq. bis-DHP **113** in refluxing  $\text{CHCl}_3$  with catalytic  $p\text{TsOH}$  gave a black mixture after 1 h. Removal of the solvent after 5 h gave mostly unreacted bis-DHP in the chloroform-soluble fraction, where any of the desired protected quinate derivative **114** (fig. 65) would have been expected to be found.

As quinic acid is completely insoluble in  $\text{CHCl}_3$ , but soluble in warm DMF, the same reaction was attempted using triphenylphosphine hydrobromide ( $\text{PPh}_3\cdot\text{HBr}$ ) as catalyst in DMF.

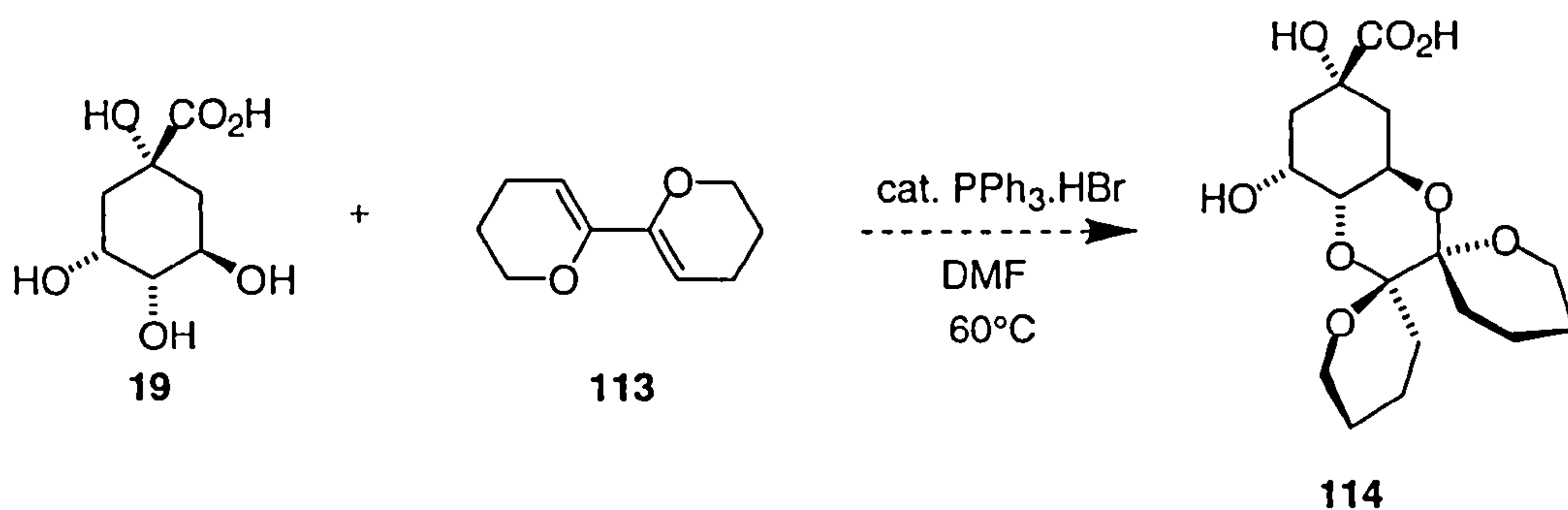


Figure 67: Attempted protection of quinic acid using bisDHP under modified protection conditions

This method was used by the Ley group to protect a glucopyranoside which gave unsatisfactory yields using the standard protection conditions (Hughes *et al.*, 1994). The authors commented that the low solubility of the sugar derivative in  $\text{CHCl}_3$ , and the instability of bis-DHP were probably both to blame for the low yields.

After four days reaction the  $\text{CHCl}_3$ -soluble fraction of the crude reaction mix showed  $^1\text{H}$ -NMR peaks consistent with the decomposition of bis-DHP. The authors (Hughes *et al.*, 1994) did mention that the use of DMF as solvent generally gave breakdown of bis-DHP rather than the desired ketal formation under most circumstances, and this appears to be in accordance with our observations.

This approach was abandoned as the insolubility of quinic acid in aprotic solvents meant that it was unlikely that any further modifications of the protection protocol would be successful.

### 4.1.3 Benzylidene acetal

As mentioned previously, a key intermediate in the synthesis of fluorinated derivatives of DHQ (Manthey *et al.*, 1997) is a protected form of DHQ **105**. This route was initially disregarded as the third step in the reaction scheme involves distinguishing between the two secondary hydroxyls of the lactone **102** by means of TBDMS protection with a quoted yield of 54 %. However, the DHQ derivative formed by this route is protected solely using acid-sensitive groups and thus offers the possibility of a global deprotection step which should not lead to elimination of water and hence the aromatisation which dogged previous synthetic attempts.

#### Attempts to replicate the literature protocol

Using the method of Manthey *et al.*, 1997 attempts were made to protect quinic acid as the benzylidene acetal. The use of toluene at reflux, however, led to extensive charring of the reagents after 2 h reaction. The literature is fairly evenly divided between those who use toluene for this reaction and those who use benzene, so the reaction was repeated using benzene at reflux. The lower reaction temperature of around 90 °C gave a much cleaner reaction and the desired acetal **101** was isolated in a 35 % yield (*fig. 68*).

Hydrogenolysis under acidic conditions gave the lactone **102** in 54 % yield after crystallisation from acetic acid. In contrast to the published procedure, we found this step required an average of three days to reach completion, while the crystallisation step was difficult and low-yielding even when the pure compound could be isolated.

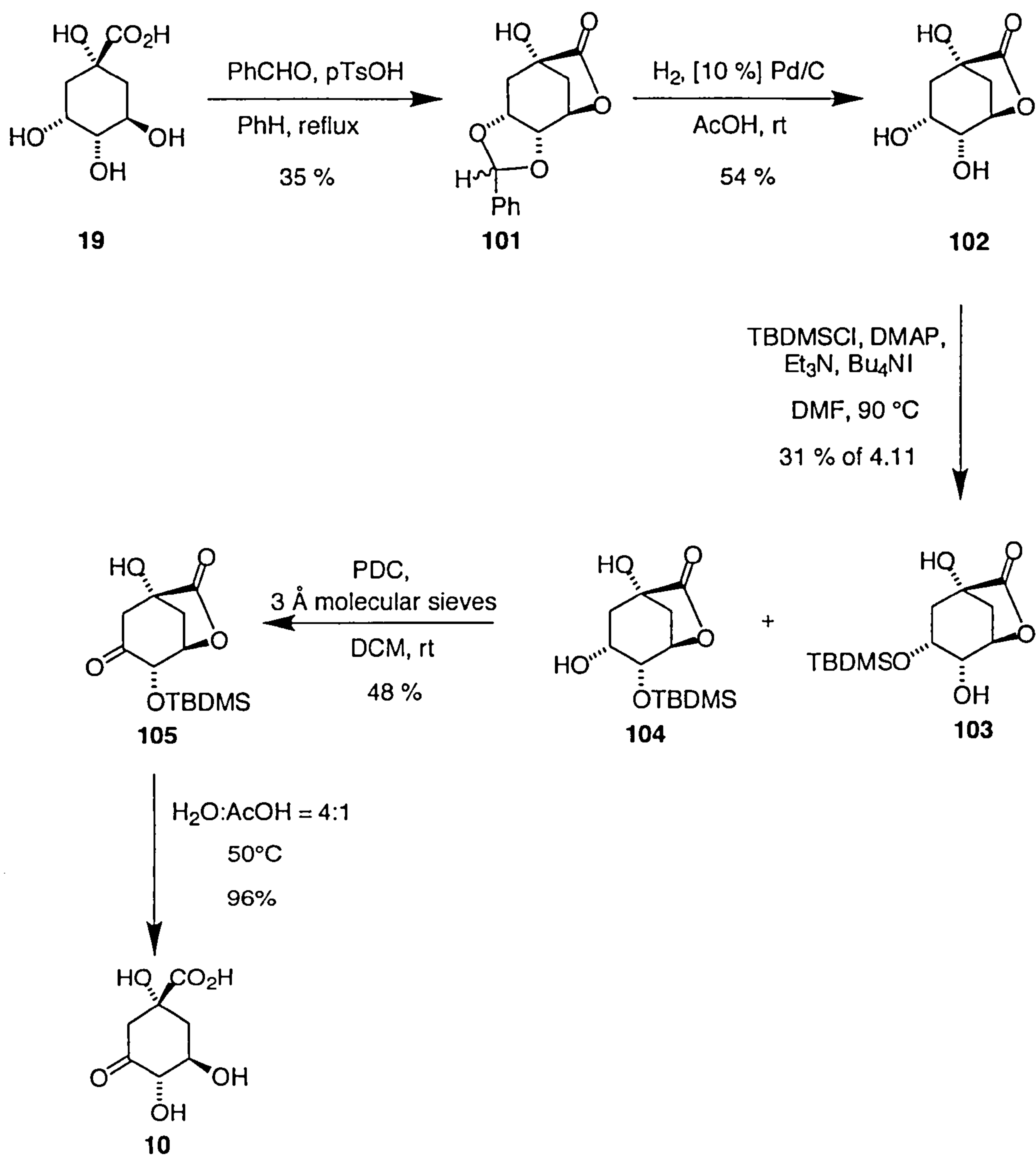


Figure 68: Attempted replication of the literature protocol towards the synthesis of DHQ

Selective protection of the C-4 hydroxyl of **102** as a TBDMS ether was carried out under thermodynamic conditions to give **104** in 31% yield after silica-gel column chromatography and repeated recrystallisations. The reaction time of this step appears to be critical, as any shortening of the reaction time gives an increase of the C-3 protected



compound **103** while an increase in the reaction time gives the bis-silylated compound as the major product. Successful separation of the products by silica-gel column chromatography is essential, as the C-3 **103** and C-4 **104** protected compounds are co-crystalline when they are present in roughly equimolar quantities.

Oxidation of the free C-3 hydroxyl using PDC in DCM with freshly activated, powdered, 3Å molecular sieves gave the protected DHQ derivative **105** in 48 % yield after crystallisation. Powdered molecular sieves were used as the oxidation occurred extremely slowly with pelleted 4Å molecular sieves, and did not reach completion despite the use of a large excess of PDC. Using a two-fold excess (by mass) of freshly activated powdered sieves, complete oxidation was achieved with only 2 eq. PDC after 5 h reaction.

Pure DHQ **10** was obtained by stirring the protected derivative **105** in an AcOH-H<sub>2</sub>O mix at 50°C for two days followed by repeated lyophilisation.

The main problem with this approach was the low-yield obtained for formation of the lactone **102**. It was therefore decided to attempt direct lactonisation rather than the two-step approach recommended by the authors (Manthey *et al.*, 1997).

#### 4.1.4 Direct lactonisation and deprotection

The lactone **102** has been formed by heating under acid catalysis with simultaneous removal of water in 42 % yield (Panizzi *et al.*, 1954), and also by direct heating (Wolinsky *et al.*, 1964; Elliott *et al.*, 1981) in up to 55 % yield. The step following the formation of the lactone requires the presence of an acid catalyst, and the lactone is formed in refluxing benzene during formation of the benzylidene acetal **101**. It was therefore decided to attempt direct lactonisation in refluxing benzene with catalytic *p*TsOH in a Dean-Stark apparatus to facilitate removal of the water formed on lactonisation (*fig. 69*).

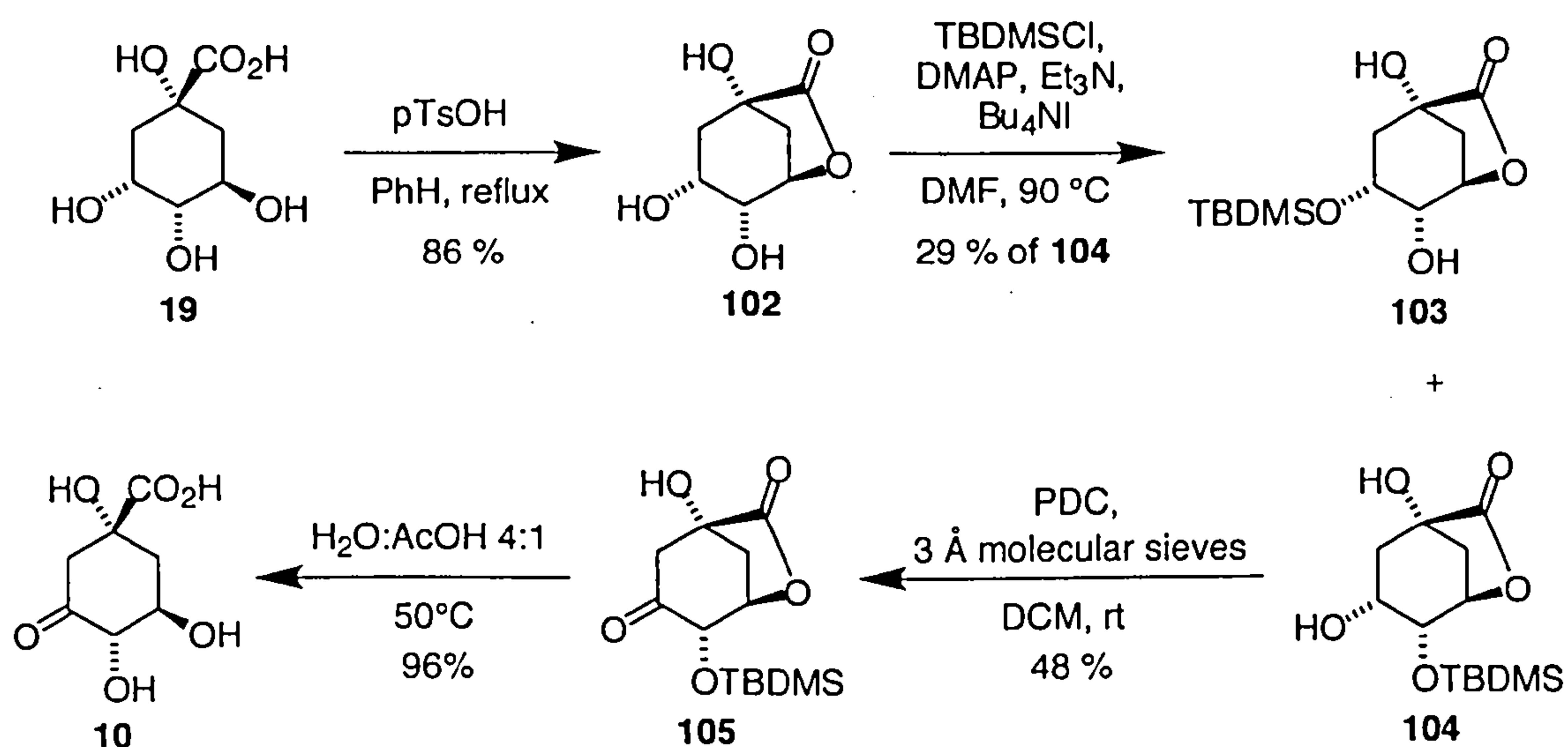


Figure 69: Modification of the literature protocol towards the synthesis of DHQ

The desired lactone **102** was formed by stirring a suspension of quinic acid and catalytic *p*TsOH in refluxing benzene (*fig. 69*). The lactone is extremely moisture sensitive, and the yield given was determined by first dissolving a fixed mass of the crude material in dry MeOH, removing any insoluble material by filtration, and removing the solvent to give the

proportion of soluble compounds. The same procedure was carried out using CD<sub>3</sub>OD and <sup>1</sup>H-NMR analysis used to determine the proportion of the desired lactone **102** to *p*TsOH and any other impurities. The crude material from the lactonisation was then treated with TBDMSCl under thermodynamic conditions as before to give the C-4 silylated derivative **104** in only slightly reduced yield compared to the literature method in our hands (*fig. 68*). Unfortunately, we were not able to even approach the literature yield for this step.

The remaining steps in the synthesis were carried out as before on a gram scale to give 1.04 g of 3-dehydroquinic acid **10** which has been used for detailed kinetic analysis of both naturally occurring and mutant type II dehydroquinase enzymes.

## 4.2 Kinetic studies using the 3-dehydroquinic acid synthesised

### 4.2.1 Background

Stopped-flow kinetic analysis is used to study the first few microseconds of a reaction, where any lag in the observed reaction course may be evidence of subtle mechanistic differences between enzymes. In order to give an accurate picture of the early stages of an enzyme-catalysed reaction, enzyme and substrate are used in equimolar amounts. The result is that the observed reaction is equivalent to the reaction of a single molecule of substrate on a single enzyme molecule.

Steady-state kinetic analysis, where the enzyme is present at less than 1 % of the concentration of the substrate, is used to give information on the specificity of an enzyme for a particular substrate and the general type of reaction mechanism involved.

As discussed in Chapter 4, section 4.1, kinetic characterisation of the type II DHQases appears to divide them into two groups based on  $k_{\text{cat}}$  with no obvious correlation between  $k_{\text{cat}}$  and  $K_{\text{m}}$  (Evans *et al.*, 2002). The first group, which includes the enzymes from organisms such as *Streptomyces coelicolor* and *Aspergillus nidulans*, has relatively high values of  $k_{\text{cat}}$  at between 100 and 1000  $\text{s}^{-1}$  at pH 7.0 and 25°C. The second group, which includes the enzymes from *Helicobacter pylori*, *Mycobacterium tuberculosis* and *Neurospora crassa*, has values of  $k_{\text{cat}}$  in the range 10  $\text{s}^{-1}$  or lower.

As part of the research effort within the group, I carried out steady-state and stopped-flow kinetic analysis on the type II dehydroquinase enzyme from *Helicobacter pylori* which is a

‘low kcat’ enzyme. I also investigated a putative type II dehydroquinase enzyme from *Bacillus subtilis*.

The YqhS protein of *B. subtilis*, which shows 47% identity to *S. coelicolor* DHQase, is completely inactive towards DHQ. However, when the substitution of F28Y was introduced by site-specific mutagenesis activity was restored albeit to a very limited extent. Tyr28 is believed to be the residue involved in deprotonation at C-2 of DHQ and as such is thought to be essential for the catalytic activity of the enzyme. This mutant F28Y enzyme is an ideal candidate for the investigation of the mechanism of type II DHQase enzymes. The ‘slow’ enzyme allows for more detailed investigation of the early part of the reaction, where mechanistic differences might be apparent.

As has been mentioned previously, the source of DHQ for the kinetic analysis of DHQase enzymes in our group had been the classical synthesis involving nitric acid oxidation of quinic acid (*fig. 48*). We had reason to believe that this material was contaminated with 5-dehydroquinic acid, and there had been some concern that this compound was a potential inhibitor of DHQase. In addition, the presence of highly UV-absorbing contaminants in the substrate meant that the investigation of enzymes with low activity or binding affinity was not possible, as the small changes in absorbance were swamped by the background.

The availability of an improved synthesis of DHQ (*fig. 69, Chapter 4*) which was known to be free from contamination made it possible to compare the historic values for  $k_{\text{cat}}$  and  $K_{\text{m}}$  with those obtained using ‘clean’ substrate.



#### 4.2.2 The reaction catalysed by type II dehydroquinases

Type II dehydroquinases catalyse the *anti* elimination of water with the loss of the more acidic axial *pro-S* hydrogen of DHQ (Roszak *et al.* 2002). The reaction is thought to involve an enolate intermediate but recent work has suggested the possibility of an enol (Harris *et al.*, 1996; Roszak *et al.* 2002). The structure of the *S. coelicolor* type II DHQase has been published both in the apo form and complexed with a variety of ligands (Roszak *et al.* 2002). This structural information was used to devise a detailed explanation of the possible reaction mechanism (*fig 70*).

The axial C-2 *pro-S* hydrogen is abstracted by the side chain of Tyr 28 which is deprotonated due to the close proximity of Arg 113. The enolate intermediate thus formed is stabilised by a proton from a conserved water molecule held in the active site by Asn 19, Pro 15 and Ala 81. Elimination of the C-1 hydroxyl follows, with His 106 acting as the proton donor. The intermediate formed undergoes a structural change and becomes planar as the product dehydroshikimate is formed, and this is proposed to drive the release of product from the active site.

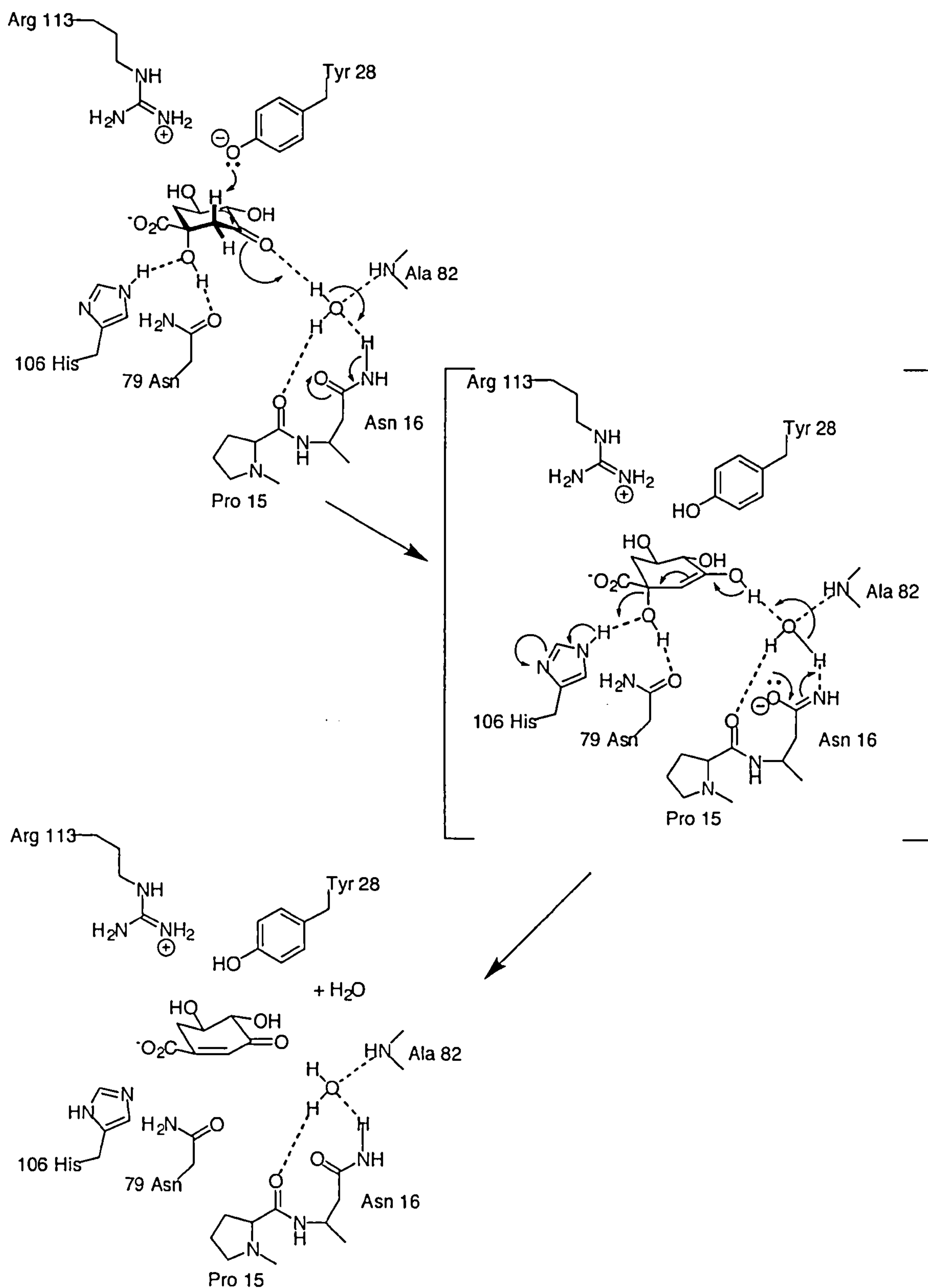


Figure 70: Proposed reaction mechanism of type II DHQases

### 4.2.3 Stopped-flow kinetic analysis: Results

Stopped-flow analysis of both enzymes showed no sign of a lag phase suggesting that analogous mechanisms were operating in both cases. This was confirmed by work carried out on a number of type II DHQases from other organisms in our laboratory by L. Evans. However, work investigating the binding of polyanions to the type II DHQases of *S. coelicolor* and *M. tuberculosis* can be correlated with the existence of a single binding site in the *S. coelicolor* enzyme and multiple sites in the *M. tuberculosis* enzyme (Evans *et al.*, 2002). These differences in binding may provide a structural explanation for the observed differences in  $k_{\text{cat}}$ .

### 4.2.4 Steady state kinetic analysis: Results

Steady-state kinetic analysis of the two enzymes was carried out at 20°C in Tris-acetate buffer at pH 7 in a total volume of 1 mL. The assays were carried out at the lower temperature rather than the 25°C which had become routine in our laboratory to enable comparison with historical data (White *et al.*, 1990). The formation of DHS was monitored spectrophotometrically at 234 nm. The values of  $K_m$  and  $V_{\text{max}}$  were obtained by fitting the raw data to the Michaelis-Menten equation by non-linear regression using Microcal Origin software (*fig. 71 & fig. 72*). The values of  $k_{\text{cat}}$  were calculated based on  $M_r = 20\ 000$  for *H. pylori* DHQase and  $M_r = 18\ 000$  for *B. subtilis* F28Y.

Analysis of *B. subtilis* F28Y at 20°C in Tris.acetate buffer pH 7.0 gave  $K_m = 622 \mu\text{M}$  and  $k_{cat} = 0.22 \text{ s}^{-1}$ .

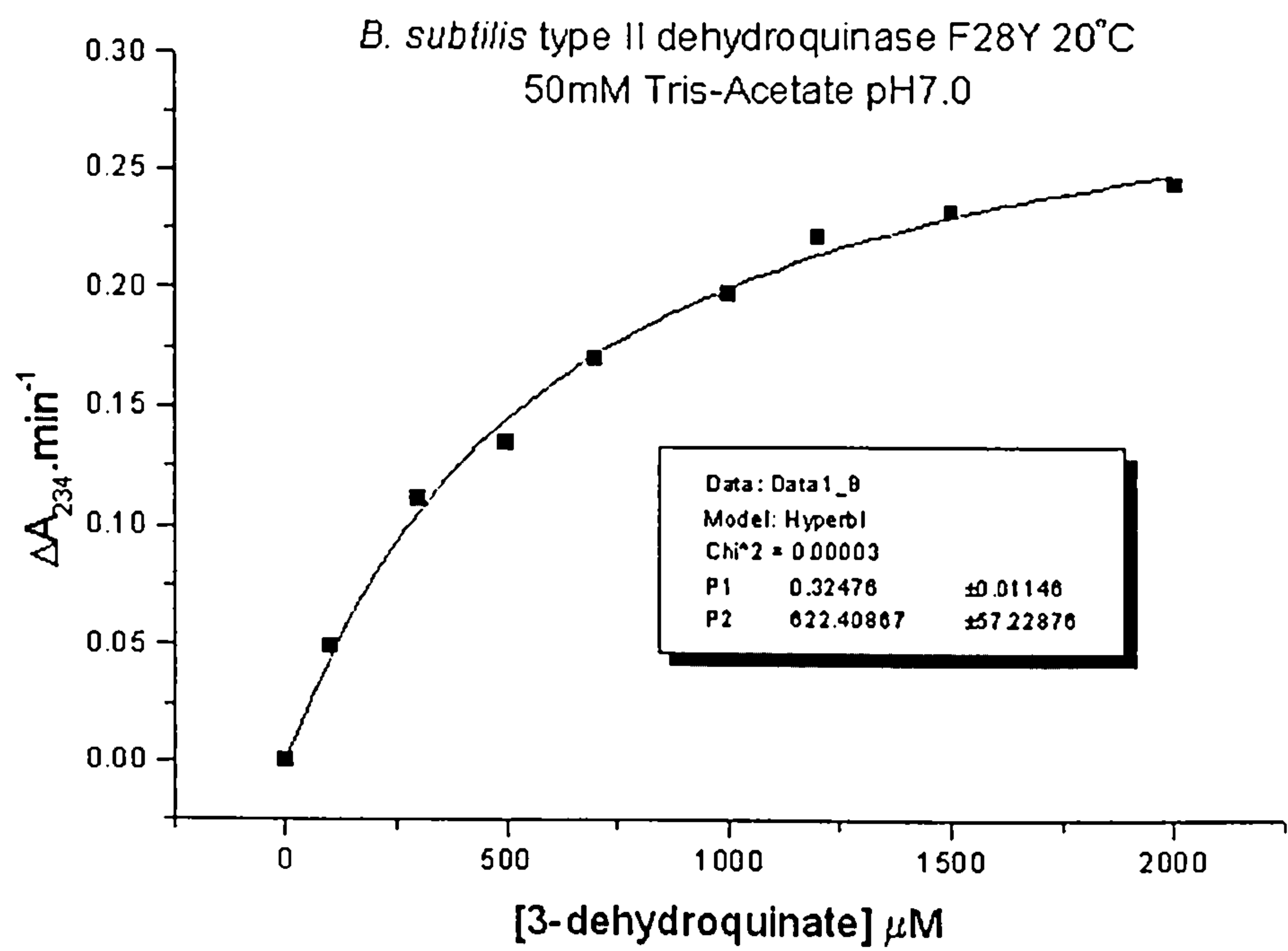


Figure 71: Data obtained by steady-state kinetic analysis of *B. subtilis* F28Y

Analysis of *H. pylori* DHQase at 20°C in Tris.acetate buffer pH 7.0 gave  $K_m = 251 \mu\text{M}$  and  $k_{cat} = 19.4 \text{ s}^{-1}$ . This is similar to the values of  $K_m = 205 \mu\text{M}$  and  $k_{cat} = 0.9 \text{ s}^{-1}$  obtained by L. Evans at 25°C (Evans *et al.*, 2002).

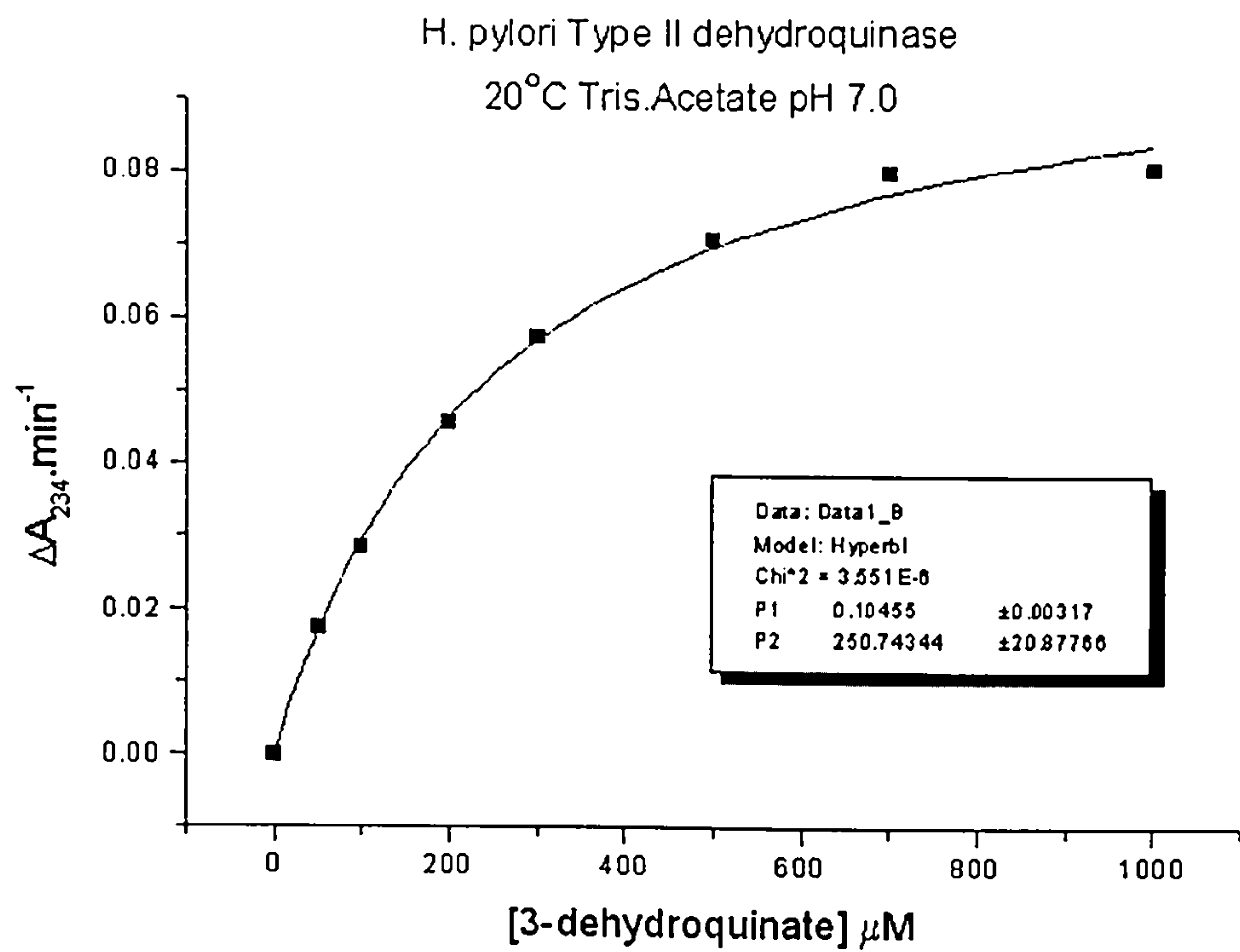


Figure 72: Data obtained by steady-state kinetic analysis of *H. pylori* type II DHQase



### 4.3 Conclusions and future work

The synthesis of DHQ from quinic acid via the lactone (*fig. 69*), despite its length and relatively low overall yield, is still an improvement on the classical nitric acid oxidation synthesis (*fig. 48*). The product is pure by  $^1\text{H}$ -NMR analysis and has a negligible absorbance at 234 nm, facilitating the kinetic investigation of DHQase enzymes with extremely low values of  $k_{\text{cat}}$ .

The attempted synthesis of DHQ from quinic acid using the diol-selective protecting group BBA was unsuccessful. However, if methyl dehydroquinic acid could be maintained at a steady pH sufficiently high to saponify the ester without leading to dehydration, this method would be a great improvement over the synthesis used to generate DHQ. The synthesis of methyl quinate by this method was high-yielding, and the chemistry was less challenging than the route eventually followed.

The comparison of historic values of  $k_{\text{cat}}$  and  $K_{\text{m}}$ , determined using substrate which may have been contaminated with enzyme inhibitors and those determined using the clean substrate, is still ongoing in the group. However, the availability of large amounts of pure DHQ has already facilitated the use of stopped-flow kinetic analysis to probe the early reaction mechanism of type II DHQase enzymes. The pure substrate has also been used in the investigation of polyanion binding to type II DHQase enzymes (Evans *et al*, 2002) providing further insight into the structural basis for the observed differences in  $k_{\text{cat}}$ .

## Experimental

### 5.1 Starting materials and solvents:

AcOH was distilled from quinoline under reduced pressure and stored over 4Å molecular sieves at 4°C.

PhH was stirred over CaH for a minimum of 2 h, distilled under reduced pressure and stored over 4Å molecular sieves

Butan-2-ol was dried by standing over activated 4Å molecular sieves for at least 24 h.

CHCl<sub>3</sub> was distilled from, and stored over, molecular sieves.

DCM was distilled from calcium hydride.

DMF was stirred for 18h over CaH<sub>2</sub>, then distilled under reduced pressure and stored over 4Å molecular sieves.

DMAP was crystallised from toluene and dried under high vacuum for at least 12h.

Et<sup>3</sup>N was distilled from calcium hydride, and stored over potassium hydroxide.

MeOH was distilled from magnesium methoxide and used immediately (storage over activated 3Å molecular sieves was unsatisfactory).

Molecular sieves were routinely dried and activated by heating for at least 12 h in an oven at 140°C.

Petrol was distilled from 4Å molecular sieves.

Piperidine was distilled from, and stored over, potassium hydroxide.

Pyridine was distilled from, and stored over, potassium hydroxide.

THF was distilled from sodium benzophenone.

All enzyme buffers were prepared using distilled water and sterile glassware.

Water used for HPLC was purified using a Millipore UV purification system, filtered and degassed for 20 minutes per litre. All HPLC buffers were stored for a maximum of 48 hours prior to use.

Unless stated otherwise, all starting materials were used as purchased.

Unless stated otherwise, all compounds are known.

## 5.2 Apparatus:

Glassware was dried for at least 30 minutes in an oven at approximately 140°C. Analytical thin layer chromatography was performed on precoated silica gel plates with visualisation under UV<sub>254</sub> light and by dipping in phosphomolybdic acid solution (phosphomolybdic acid (12g) in EtOH (200 mL) . <sup>1</sup>H NMR and <sup>13</sup>C NMR were measured on a Bruker DPX 400 (400 and 100 MHz respectively). All chemical shifts are given in ppm relative to residual CHCl<sub>3</sub> (δ = 7.27 and δ = 77.0) in CDCl<sub>3</sub> solutions, residual HOD in D<sub>2</sub>O solutions (δ = 4.60), or residual CHD<sub>2</sub>OD in CD<sub>3</sub>OD (δ = 3.35). <sup>19</sup>F NMR was carried out on a Bruker 360 with chemical shifts given in ppm relative to external CF<sub>2</sub>Cl<sub>2</sub>. Coupling constants are given in Hz and are uncorrected. Mass spectra were determined on a JMS 700 spectrometer. Infra red spectral analysis was carried out on a Perkin Elmer 410 spectrometer. Melting points were carried out using a Stuart Scientific capillary apparatus SMP2 and are uncorrected. HPLC analysis carried out in the laboratory was on a Dionex system using UV and amperometric detection. Enzyme assays were carried out using an HP8453 diode array spectrophotometer.

### 5.3 Enzyme sources and specific activity

#### Transketolase

The N-terminal His tagged recombinant transketolase used was a gift of Ms Nicola Veitch. The protein was expressed in *E. coli* BL21(DE3) cells using pET16b. The enzyme was provided fresh as required. Details of the activity of each preparation were provided by Ms Veitch with a typical value being 0.2 units.mg<sup>-1</sup>.

#### DAHPSynthase

The DAHPS(phe) used was a gift of Mr John Greene. The protein was expressed in *E. coli* CB198 using pttG1(phe). The activity of a typical enzyme preparation as provided by Mr Greene was 59 units.mg<sup>-1</sup>.

#### Dehydroquinase

The DHQ synthase used was a gift of Mr John Greene. The protein was expressed in *E. coli* W3110 using pJB14. The activity of a typical enzyme preparation as provided by Mr Greene was 36 units.mg<sup>-1</sup>.

#### Dehydroquinase dehydratase

The type II DHQase used in the biosynthesis was a gift of Mr John Greene. The protein was expressed in *E. coli* AB2848 using pKD203. The activity of a typical enzyme preparation as provided by Mr Greene was 747 units.mg<sup>-1</sup>.

The *B. subtilis* wild type and F28Y DHQase enzymes, as well as the *S. coelicolor* and *H. pylori* enzymes used in the kinetic analysis experiments were a gift of Mr Lewis Evans.



## **Shikimate dehydrogenase**

The shikimate dehydrogenase used was a gift of Mr John Greene. The protein was expressed in *E. coli* BL21 using pTB361. The activity of a typical enzyme preparation as provided by Mr Greene was 854 units.mg<sup>-1</sup>.

5.4 General procedures:

5.4.1 Enzyme assays

DAHP synthase (Schoner & Hermann, 1976)

The assay was carried out at 25°C, pH 7.0 in a 1 mL, 1 cm path length cuvette.

The assay mixture, total volume 1 mL, consisted of 10 mM 1,3-bis[tris-(hydroxymethyl)methylamino]propane (BTP) buffer pH 7.0, 100 µM PEP and 300 µM E4P.

Reaction was initiated by the addition of DAHP synthase.

The rate of change of absorbance at 234 nm is related to the enzyme activity in units per mL as follows:

$$\text{Activity} = \frac{\Delta A_{232} \cdot \text{min}^{-1} \cdot \text{mL}^{-1}}{\epsilon} \qquad \epsilon = 2.8 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$$

Dehydroquinate synthase (Lambert *et al.*, 1985)

The assay was carried out at 25°C, pH 7.0 in a 1 mL, 1 cm path length cuvette.

The assay mixture, total volume 1 mL, consisted of 50 mM potassium phosphate buffer pH 7, 40 µM NAD<sup>+</sup>, 0.2 µM CoCl<sub>2</sub>, 2 units *E. coli* dehydroquinase and 4 units *E. coli* dehydroquinate synthase. Reaction was initiated by the addition of 200 µM DAHP.

The rate of change of absorbance at 234 nm is related to the enzyme activity in units per mL as follows:

$$\text{Activity} = \frac{\Delta A_{234} \cdot \text{min}^{-1} \cdot \text{mL}^{-1}}{\epsilon} \qquad \epsilon = 12 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$$

### Dehydroquate dehydratase (dehydroquinase) (White *et al.*, 1990)

Assays were carried out at 20°C in a 1 mL, 1 cm path length cuvette. The limiting change in  $A_{234}$  on the addition of *S. coelicolor* type II dehydroquinase (1  $\mu$ g) to a solution of DHQ in the reaction buffer was used to calculate the concentration of substrate. Calculations took into account the fact that the equilibrium constant for the reaction is 15 (Kleanthous *et al.*, 1991).

The assay mixture, total volume 1 mL, consisted of 50 mM Tris.acetate buffer pH 7.0 and 1 mM DHQ (variable for kinetic determination). Reaction was initiated by the addition of DHQase.

The activity of the enzyme in units per mL was calculated as follows:

$$\text{Activity} = \frac{\Delta A_{234} \cdot \text{min}^{-1} \cdot \text{mL}^{-1}}{\epsilon} \quad \epsilon = 12 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$$

### Shikimate dehydrogenase (Chaudhuri & Coggins, 1985)

The assay was carried out at 25°C, pH 10.6 in a 1 mL, 1 cm path length cuvette. The enzyme was assayed in the reverse direction, monitoring the reduction of NADP<sup>+</sup> at 340 nm. The assay mixture, total volume 1 mL, consisted of 100 mM Na<sub>2</sub>CO<sub>3</sub> buffer pH 10.6, 4 mM shikimic acid and 2 mM NADP<sup>+</sup>. Reaction was initiated by the addition of SDHase.

$$\text{Activity} = \frac{\Delta A_{340} \cdot \text{min}^{-1} \cdot \text{mL}^{-1}}{\epsilon} \quad \epsilon = 6.2 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$$

### 5.4.2 Chemical assays

#### Sialic acid (thiobarbituric acid) assay for DAHP (Warren, 1959)

The test solution (10  $\mu\text{L}$ ) was added to 0.2M  $\text{NaIO}_4$  in 9M  $\text{H}_3\text{PO}_4$  (25  $\mu\text{L}$ ) and left at room temperature for 20 min. 0.75 M  $\text{NaAsO}_2$  in 0.5 M  $\text{Na}_2\text{SO}_4$ :50 mM  $\text{H}_2\text{SO}_4$  (100  $\mu\text{L}$ ) was added and the solution mixed. 40 mM Thiobarbituric acid (750  $\mu\text{L}$ ) was added and the solution incubated at 100°C for 15 min. A pink chromagen absorbing at 549 nm was formed and the absorbance related to the concentration of DAHP by comparison with standard solutions.

#### Phosphate assay (Lanzetta *et al.*, 1979)

Solutions required:

0.045% malachite green hydrochloride

4.2% ammonium molybdate in 4M HCl

34% sodium citrate.2H<sub>2</sub>O (w/v)

10 mM  $\text{KH}_2\text{PO}_4$  for use as standard

A 3:1 mixture of the malachite green and ammonium molybdate solutions was stirred for 20 min and filtered to form the 'colour reagent'.

To 100  $\mu\text{L}$  of the assay sample was added 800  $\mu\text{L}$  of the colour reagent. After 1 min, 100  $\mu\text{L}$  of the citrate solution was added, and the colour left to develop for 30 min at rt.

### 5.4.3 Fmoc-based solid-phase peptide synthesis

Fmoc-Lys(Mtt)-wang resin was used as the basis for these syntheses. The DCM used was freshly distilled, while the DMF and piperidine had been distilled and stored over activated 4Å molecular sieves. The MeOH used was reagent grade but otherwise unpurified.

#### **Fmoc deprotection:**

The resin was swelled in DMF (2 mL) for 5 min and drained. The swelled resin was treated with 20 % piperidine in DMF (2 mL, 4 × 15 min), washing with DMF (5 × 1 mL) after each iteration. The deprotected resin was then washed with DCM (5 × 1 mL) and MeOH (5 × 1 mL) and dried under reduced pressure in an air stream for 1 hour.

#### **Coupling of amino acid residues:**

The resin was swelled in DMF (2 mL) for 5 min and drained. The desired Fmoc-protected amino acid derivative (3 eq.) was added to the swelled resin along with HOBt (3 eq.) and DIC (10 eq.). DMF (2 mL) was added and the mixture was agitated at room temperature for 3 h. The reaction mix was drained from the resin, and the resin washed with DMF (5 × 1 mL).

#### **Mtt deprotection:**

The resin was swelled in DCM (2 mL) for 5 min and drained. The swelled resin was treated with 1 % TFA, 5 % TIPS in DCM (2 mL, 5 × 10 min), washing with DCM (5 × 1 mL) after each iteration. This treatment was continued until no further yellow coloration was observed on the addition of the deprotection mix. The deprotected resin was then washed with MeOH (5 × 1 mL) and dried under reduced pressure and an air stream for 1 hour.



### **Coupling of shikimic acetate derivative:**

The resin was swelled in DCM (2 mL) for 5 min and drained. The swelled resin was treated with shikimic acetate **80** (3 eq.), HOBt (3 eq.) and DIC (10 eq.). DCM (2 mL) was added and the mixture was agitated at room temperature for between 12 and 18 hours. The reaction mix was drained and the resin was washed with DCM (5 × 1 mL) and MeOH (5 × 1 mL) and dried in a steady air stream under reduced pressure for 30 min.

### **Coupling of (6*S*)-6-fluoroshikimic acid derivative:**

The resin was swelled in DCM (2 mL) for 5 min and drained. The swelled resin was treated with the (6*S*)-6-fluoroshikimic acid derivative **90** (3 eq.), BOP (6 eq.) and Et<sub>3</sub>N (3 eq.). DCM (2 mL) was added and the mixture was agitated at room temperature for 24 hours. The reaction mix was drained into sat. NaHCO<sub>3</sub> (aq.) to neutralise the HMPA formed. The resin was then washed with DCM (5 × 1 mL) and MeOH (5 × 1 mL) and dried in a steady air stream under reduced pressure for 30 min.

### **Cleavage of peptides from resin:**

The resin was swelled in DCM (2 mL) for 5 min and drained. The swelled resin was treated with TFA (1.5 mL) and DCM (0.5 mL) and the mixture was agitated at room temperature for 45 min. The solvent was drained into a suitable flask. The resin was washed with DCM (5 × 1 mL) and these washings were added to the flask. The solvent was removed under reduced pressure.

### **Final deprotection:**

#### **a. *t*Bu ether and ester cleavage**

Serine and glutamic acid-containing conjugates (protected as the *t*Bu ether and ester respectively) were cooled to 0°C. TFA (2 mL) was added and the mixture stirred for 45

min at 0°C and a further 45 min at rt. The TFA was then removed under reduced pressure to give the crude product.

**b. acetate cleavage**

Conjugates containing shikimic acetate were treated with THF (2 mL) and 1M NaOH (aq.) (0.5 mL). The mixture was stirred at rt for 90 min and the solvent removed under reduced pressure. The crude material obtained was analysed by <sup>1</sup>H-NMR and HPLC-mass-spectrometry.

**c. TBDMS ether cleavage**

Conjugates containing the protected (6*S*)-6-fluoroshikimic acid derivative **90** were treated with 100 % TFA (2 mL) for 90 min [occasionally, 90 % TFA in DCM was used]. The TFA was removed under reduced pressure to give the crude product which was analysed by <sup>1</sup>H-NMR and HPLC-mass-spectrometry.

## 5.5 Experimental for Chapter 2

### Formation of DAHP 13 by biotransformation from D-fructose-6-phosphate 79:

Reactions were carried out using conditions based on those given in Duggan *et al.*, 1995.

Reactions were carried out on a 2.5 mL scale, in a variety of buffers:

The standard reaction mix contained: fructose-6-phosphate (6.5 mg, 21  $\mu\text{mol}$ ), D-ribose-5-phosphate (14.2 mg, 52  $\mu\text{mol}$ ), phosphoenol pyruvate (7.6 mg, 28  $\mu\text{mol}$ ),  $\text{MgCl}_2$  (5.5 mg, 27  $\mu\text{mol}$ ), and cocarboxylase (trace).

Dissolved oxygen was removed by bubbling  $\text{N}_2$  through the solution for 2 min. The reaction was initiated by adding transketolase (0.2 units) and DAHP synthase (1 unit). 10  $\mu\text{L}$  aliquots were taken at 30 min intervals and assayed for DAHP formation using the thiobarbituric acid assay (5.4.2).

### Formation of 3-dehydroshikimic acid 14 by biotransformation from PEP 11:

Reactions were carried out in a 1 mL, 1 cm path length cuvette for continuous monitoring.

To 1 mL of the DAHP biotransformation reaction mix was added:  $\text{CoSO}_4$  to a final concentration of 50  $\mu\text{M}$  and  $\text{NAD}^+$  to a final concentration of 0.2  $\mu\text{M}$ . The reaction was initiated by the addition of *E. coli* dehydroquinase (2 units) and *E. coli* dehydroquinase synthase (4 units), and monitored for the formation of DHS at 234 nm.

### **Formation of 3-dehydroshikimic acid 14 by biotransformation from DAHP 13:**

Reactions were carried out in a 1 mL, 1 cm path length cuvette for continuous monitoring. The conditions used for the assay of *E. coli* DHQ synthase were utilised with the rate of reaction monitored spectrophotometrically at 234 nm in various buffers. The effect of the constituents of the DAHP biotransformation from F-6-P was investigated by the addition of these components to the reaction mix.

### **Formation of shikimic acid 9 by biotransformation from DAHP 13:**

Reactions were carried out in a 1 mL, 1 cm path length cuvette for continuous monitoring. The conditions used for the assay of *E. coli* DHQ synthase were utilised in order to obtain a reaction mix containing dehydroshikimic acid. This was treated with NADPH and *E. coli* shikimate dehydrogenase at varying concentrations and the rate of reaction monitored by the consumption of NADPH at 340 nm and the consumption of dehydroshikimic acid at 234 nm.

Reactions used for HPLC method development involved higher concentrations of reagents: To 6 mL of 50 mM MOPS pH 7, 50  $\mu$ M CoSO<sub>4</sub> was added DAHP (10 mg, 30  $\mu$ mol), NAD<sup>+</sup> (0.15  $\mu$ mol by dilution), dehydroquinase (4 units) and dehydroquinase synthase (16 units). The reaction was incubated at 25°C overnight. To a 1 mL aliquot of this biotransformation mix was added NADPH (30 mg, 36  $\mu$ mol) and shikimate dehydrogenase (8 units) and the mixture was incubated at 25°C. 2  $\mu$ L Aliquots of the biotransformation mix were taken at regular intervals. The formation of shikimic acid was monitored using a Dionex Carbopac HPLC column and amperometric detection, eluting with a 5 mM - 100 mM NaOH gradient.

## **Biotransformation to form (6*S*)-6-fluoroshikimic acid 1 and (6*R*)-6-fluoroshikimic acid 41**

3-fluoro-PEP.C<sub>6</sub>H<sub>11</sub>NH<sub>3</sub> (126 mg, 0.4 mmol), fructose-6-phosphate (140 mg, 0.45 mmol), D-ribose-5-phosphate (180 mg, 0.65 mmol), cocarboxylase (1 mg) and MnSO<sub>4</sub> (0.4 mg, 50 μM) were added to 15 mL H<sub>2</sub>O. The pH was adjusted to pH 6.8 using 10% NaOH and the total volume made up to 50 mL with H<sub>2</sub>O. The reaction was initiated by the addition of transketolase (10 u) and DAHP synthase (32 u). After 10 hours reaction, a further aliquot of DAHP synthase (16 u) was added and the biotransformation continued for 16 hours. The biotransformation was purified using an ammonium bicarbonate gradient (5 to 500 mM over 400 mL) on a DEAE sephacel column pre-equilibrated with 5 mM NH<sub>4</sub>.HCO<sub>3</sub> (600 mL) taking 5 mL fractions. These fractions were assayed for inorganic phosphate using the Lanzetta phosphate assay after overnight equilibration of 200 μL aliquots with alkaline phosphatase.

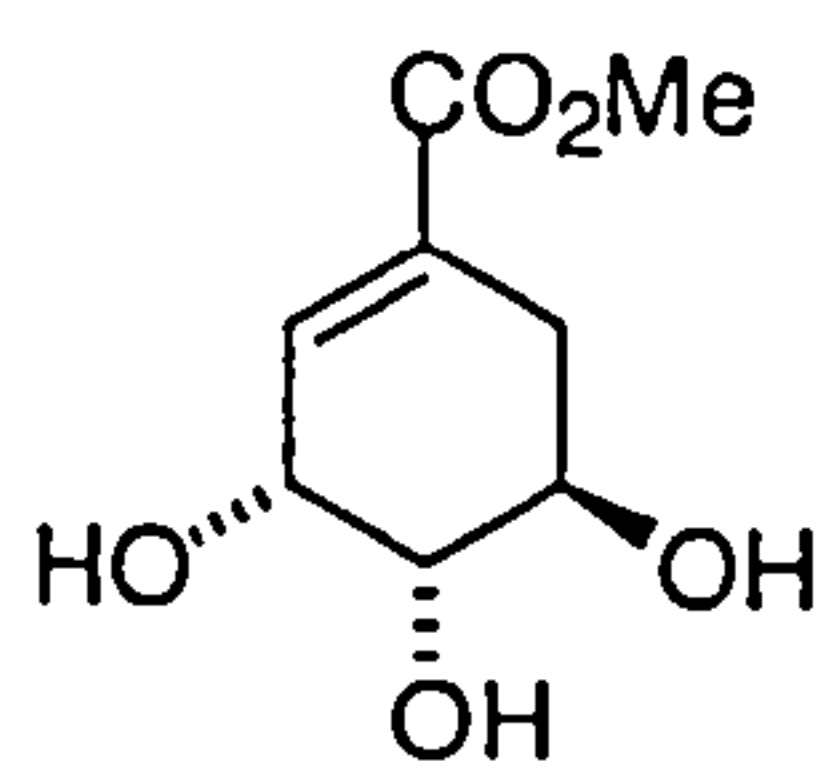
### **Investigation of the transformation of F-PEP to F-DAHP by <sup>19</sup>F-NMR:**

3-fluoro-PEP.C<sub>6</sub>H<sub>11</sub>NH<sub>3</sub> (30 mg, 0.064 mmol) was dissolved in 1 mL D<sub>2</sub>O. Fructose-6-phosphate (30 mg, 0.064 mmol), D-ribose-5-phosphate (20 mg, 0.064 mmol), a trace amount of cocarboxylase, and MgCl<sub>2</sub> (2.0 mg, 0.01 mmol) was added. The solution was degassed by bubbling a stream of N<sub>2</sub> through for 2 min. The pH was adjusted to pH 7.1 using 0.1 M NaOH solution. Transketolase (1 unit) and DAHP synthase (3 units) were added. The <sup>19</sup>F-NMR spectrum was taken at regular intervals. There was no change from the observed spectrum of the substrate 3-fluoro-PEP. δ<sub>F</sub>(188.3 MHz; D<sub>2</sub>O): -140.8 (1F, dm, Z-F), -150.0 (1F, dm, E-F).

Literature values (Duggan *et al.*, 1995): δ<sub>F</sub>(235 MHz; D<sub>2</sub>O): -141.4 (Z-F), -152.7 (E-F)



**Methyl shikimate** (Chahoua *et al.*, 1992)

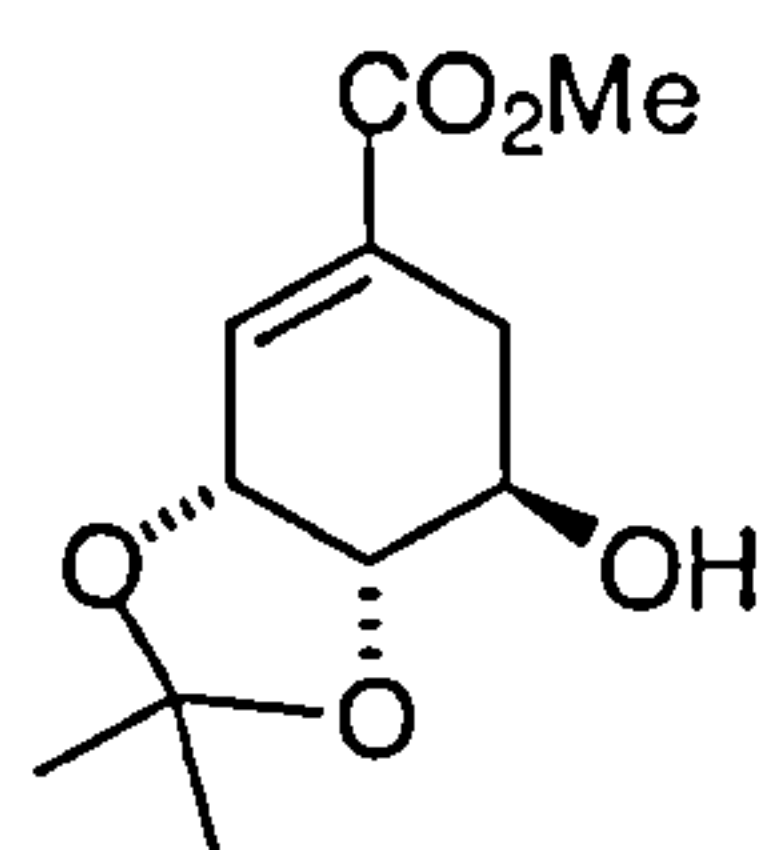


Shikimic acid **9** (1.00 g, 5.74 mmol) was heated to reflux in dry MeOH (40 mL) in the presence of Amberlite IR120 H<sup>+</sup> ion-exchange resin (1.0 g). After 18 h the resin was removed by filtration and the solvent evaporated to give the desired ester (1.07 g, 5.69 mmol, 99 %) as a white solid.

$\delta_{\text{H}}$ (400 MHz; CDCl<sub>3</sub>) 6.84 - 6.82 (1H, m, H-2), 4.41 (1H, br m, H-3), 4.03 (1H, td, *J* 5.2 and 1.8, H-4), 3.78 (3H, s, OMe), 3.73 (1H, dd, *J* 7.3 and 4.2, H-4), 2.75 (1H, ddt, *J* 18.2, 4.9 and 1.8, H-6) 2.24 (1H, ddt, *J* 18.2, 5.4 and 1.6, H-6);  $\delta_{\text{C}}$ (100 MHz; CDCl<sub>3</sub>) 169.1 (CO<sub>2</sub>CH<sub>3</sub>), 139.5 (C=CH), 130.6 (CH=C), 73.0 (C-OH), 68.8 (C-OH), 67.7 (C-OH), 52.8 (CO<sub>2</sub>CH<sub>3</sub>), 32.0 (CH<sub>2</sub>).

lit. (Chahoua *et al.*, 1992)  $\delta_{\text{H}}$ (250 MHz; CD<sub>3</sub>OD) 6.79, 4.37, 4.00, 3.74, 3.69, 2.70, 2.20 (abbreviated data quoted).

**Methyl 3,4-*O*-isopropylidene-shikimate 72** (Chahoua *et al.*, 1992)



Methyl shikimate (1.04 g, 5.53 mmol) was dissolved in 2,2-dimethoxypropane (20 mL). *p*TsOH (105 mg, 0.55 mmol, 10 %) was added and the solution stirred at rt. After 50 min the reaction was quenched with sat. NaHCO<sub>3</sub> (aq.) (10 mL) and extracted into Et<sub>2</sub>O (3 × 40 mL). The etherial extracts were dried (Na<sub>2</sub>SO<sub>4</sub>), filtered and evaporated under reduced

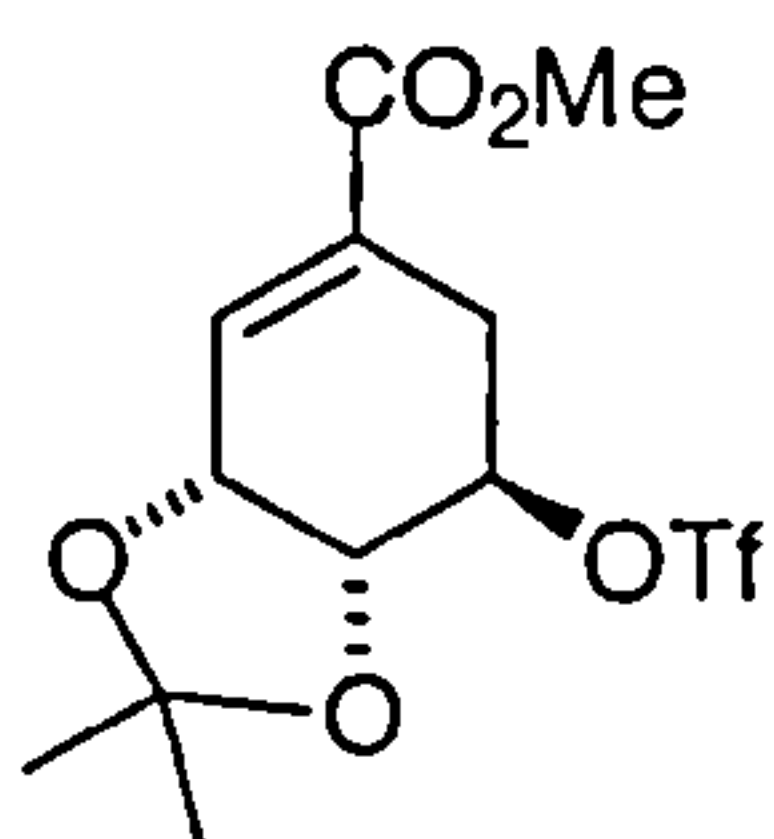
pressure to give the crude product (1.18 g) as a pale yellow oil. Silica-gel column chromatography (EtOAc:Petrol = 1:1) gave the desired acetal **72** (1.16 g, 5.08 mmol, 92 %) as a colourless oil.

$\delta_{\text{H}}$ (400 MHz;  $\text{CDCl}_3$ ) 6.92 (1H, m, H-2), 4.75 (1H, m, H-3), 4.09 (1H, t,  $J$  7.2, H-4), 3.93 - 3.87 (1H, m, H-5), 3.77 (3H, s, OMe), 2.73 (1H, dd,  $J$  17.4 and 4.6, H-6), 2.37 (1H, d,  $J$  2.7, OH), 2.18 (1H, ddt,  $J$  17.4, 8.3 and 1.9, H-6), 1.45 (3H, s, CMe), 1.40 (3H, s, CMe);  
 $\delta_{\text{C}}$ (100 MHz;  $\text{CDCl}_3$ ) 166.5, 133.9, 130.6, 109.7, 77.9, 72.2, 68.8, 52.1, 29.3, 27.9, 25.7;  
 $m/z$  ( $\text{CI}^+$ ) 229.18 ( $[\text{M}+\text{H}^+]$ , 10%), 213.16 (100%), 197.17 (20%), 171.15 (20%), 153.14 (40%), 139.13 (30%).

lit. (Chahoua *et al.*, 1992)  $\delta_{\text{H}}$ (200 MHz;  $\text{CDCl}_3$ ) 6.90, 4.73, 4.08, 3.88, 3.75, 2.79, 2.22, 1.44, 1.39.

(abbreviated data quoted).

### Methyl 3,4-*O*-isopropylidene-5-*O*-trifluoromethanesulfonate-shikimate **73**

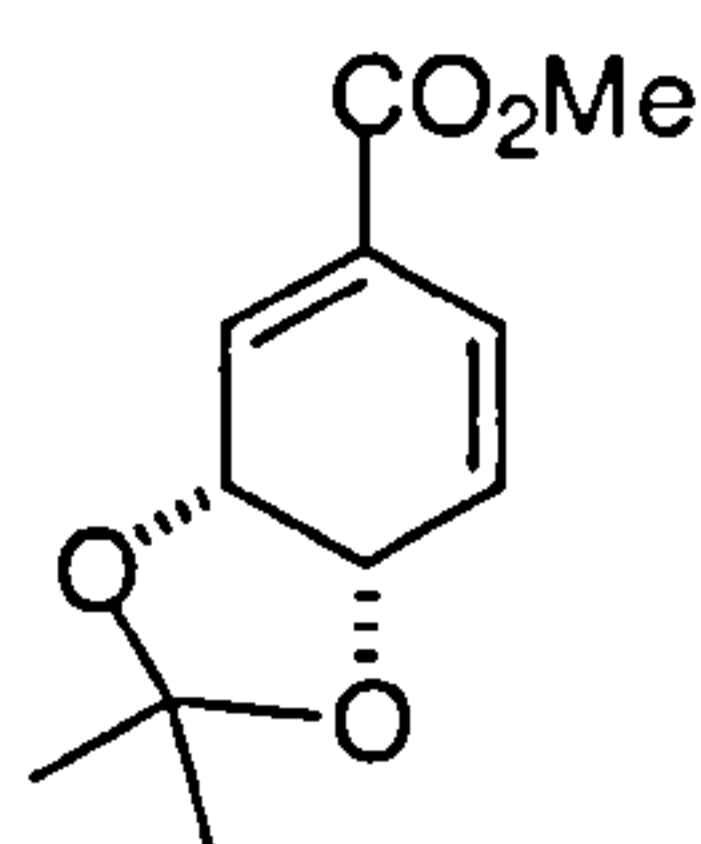


The protected shikimate compound **72** (2.92 g, 12.80 mmol) was dissolved in dry DCM (50 mL) and the solution cooled to  $-20^{\circ}\text{C}$ . DMAP (156 mg, 1.28 mmol, 10%) was added to the stirred solution. Pyridine (4.14 mL, 51.2 mmol, 4 eq.) was added dropwise with stirring.  $\text{Tf}_2\text{O}$  (4.33 mL, 25.6 mmol, 2 eq.) was added dropwise with stirring over a period of 50 min, during which the temperature was kept below  $-10^{\circ}\text{C}$ . The reaction was quenched by pouring the reaction mixture into ice-cold sat.  $\text{NaHCO}_3$  (aq.) (70 mL) and the aqueous and organic layers were separated. The organic layer was washed with 0.2 M citric acid ( $3 \times 70$

mL), sat. NaHCO<sub>3</sub> (aq.) (70 mL) and brine (70 mL). The organic layer was then dried (MgSO<sub>4</sub>), filtered and evaporated under reduced pressure to give the desired triflate (4.35 g, 12.08 mmol, 94 %) as a pale yellow oil.

$\delta_{\text{H}}$ (400 MHz; CDCl<sub>3</sub>) 7.00 (1H, m), 5.01 (1H, dt, *J* 8.1 and 4.9), 4.84 (1H, br dd, *J* 5.0 and 4.8), 4.32 (1H, t, *J* 6.9), 3.81 (3H, s), 3.05 (1H, dd, *J* 17.5 and 4.8), 2.62 (1H, br ddt, *J* 17.5 and 8.5), 1.47 (3H, s), 1.43 (3H, s); *m/z* (CI<sup>+</sup>) 361.1 ([M+H<sup>+</sup>], 100%), 345.1 (20%), 153.1 (90%), *m/z* HRMS found: [M+H<sup>+</sup>] 361.0568, C<sub>12</sub>H<sub>16</sub>O<sub>7</sub>F<sub>3</sub>S requires 361.0569

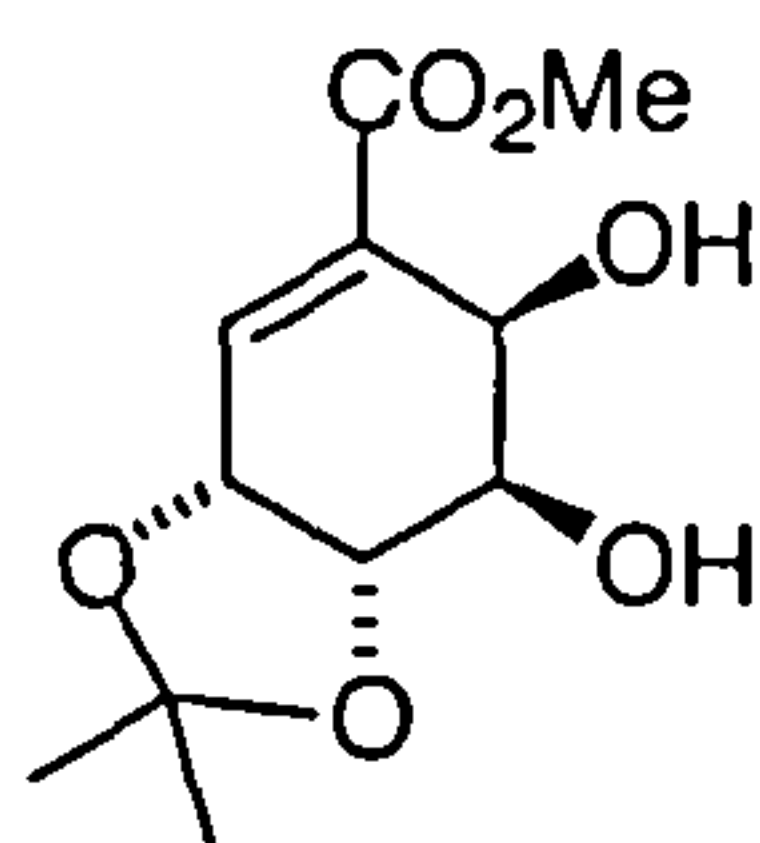
#### Methyl (3*R*,4*S*)-*O*-isopropylidenedioxycyclohexa-1,5-diene carboxylate **74**



The protected triflate **73** (4.12 g, 11.45 mmol,) was dissolved in dry DMF (15 mL). CsOAc (3.47 g, 18.08 mmol, 1.58 eq.) was dried under high vacuum with vigorous stirring for 30 min. Dry DMF (5 mL) was added to the dry CsOAc and the mixture stirred at rt for 10 min. The solution of **73** in DMF was added to the CsOAc/DMF mixture and the resulting pink solution stirred for 2.5 h. The CsOAc did not fully dissolve during this time. The reaction mix was partitioned between iced water (100 mL) and petrol (100 mL). The aqueous layer was extracted into petrol (3 × 100 mL). The organic extracts were combined, dried (MgSO<sub>4</sub>) and the solvent removed under reduced pressure to give the desired protected diene **74** (2.26 g, 10.76 mmol, 94 %) as a pale yellow oil. The temperature of the workup was maintained below 10°C at all times.

$\delta_{\text{H}}$ (400 MHz;  $\text{CDCl}_3$ ) 6.87 (1H, d,  $J$  3.7, C=CH), 6.55 (1H, d,  $J$  6.9, C=CH), 6.05 (1H, dd,  $J$  9.9 and 4.0, C=CH), 4.82 (1H, dd,  $J$ , 8.8 and 3.7), 4.66 (1H, dd,  $J$  8.8 and 4.0), 3.81 (3H, s, OMe), 1.42 (3H, s, CMe), 1.40 (3H, s, CMe)

**Methyl 3,4-*O*-isopropylidene-(6*R*)-hydroxy-shikimate 76** (Song *et al.*, 2001)



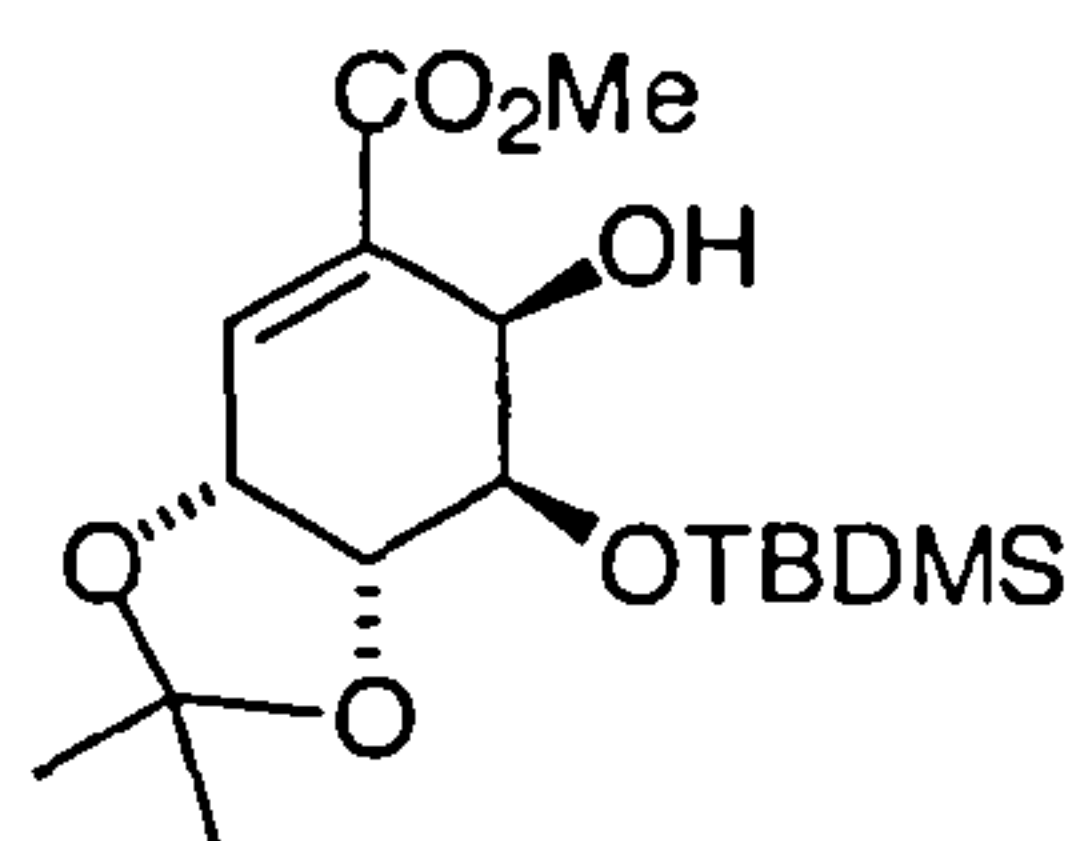
The fully protected diene **74** (2.15 g, 10.20 mmol) was dissolved in *t*BuOH. NMO (1.25 g, 10.71 mmol, 1.05 eq.) was added to the solution.  $\text{OsO}_4$  (51.9 mg, 0.20 mmol, 2 %) in  $\text{H}_2\text{O}$  (5.2 mL) was added and the solution was stirred at rt in the dark. After 14 h sat.  $\text{Na}_2\text{S}_2\text{O}_3$  (aq.) (50 mL) was added to quench the reaction and the mixture stirred for a further 5 h. The aqueous and organic layers were separated and the organic layer was stored overnight at  $-20^\circ\text{C}$ . The product was adsorbed onto silica and placed on top of a plug of the same material. A flash column (EtOAc) yielded a pale yellow oil (2.33 g). Silica-gel column chromatography (Hexane, 60 % EtOAc:1 column volume; then Hexane: 80 % EtOAc) gave partial separation of the dihydroxylated compounds **75** and **76**. The fractions containing the compound **76** were pooled and the solvent removed under reduced pressure. Silica-gel column chromatography (DCM, 10 % acetone: 1 column volume; then DCM, 20 % acetone) gave the desired compound **76** (841 mg, 3.44 mmol, 34 %) as a clear colourless oil.

$\delta_{\text{H}}$ (400 MHz;  $\text{CDCl}_3$ ) 6.93 (1H, d,  $J$  3.4), 4.80 (1H, dd,  $J$  5.0 and 3.4), 4.67 (1H, d,  $J$  3.0), 4.46 (1H, t,  $J$  6.0), 4.13 - 4.11 (1H, br s), 3.83 (3H, s), 3.78 (1H, br s), 2.80 (1H, br s), 1.40 (6H, s);  $\delta_{\text{C}}$ (100 MHz;  $\text{CDCl}_3$ ) 166 ( $\text{CO}_2\text{CH}_3$ ), 137.1 (CH=C), 130.3 (CH=C), 110.0 ( $\text{CMe}_2$ ), 71.7 (CHOR), 70.0 (CHOR), 65.1 (CHOR), 52.4 (CHOR), 27.8 ( $\text{CCH}_3$ ), 25.8 ( $\text{CCH}_3$ ).

lit. (Song *et al.*, 2001)  $\delta_{\text{H}}$ (270 MHz;  $\text{CDCl}_3$ ) 6.95, 4.83, 4.68, 4.45, 4.14 - 4.10 , 3.98 - 3.93, 3.81, 3.55 - 3.62, 1.43, 1.40

(abbreviated data quoted)

**Methyl 3,4-*O*-isopropylidene-5-*O*-*t*-butyldimethylsilyl-(6*R*)-hydroxy-shikimate 77**

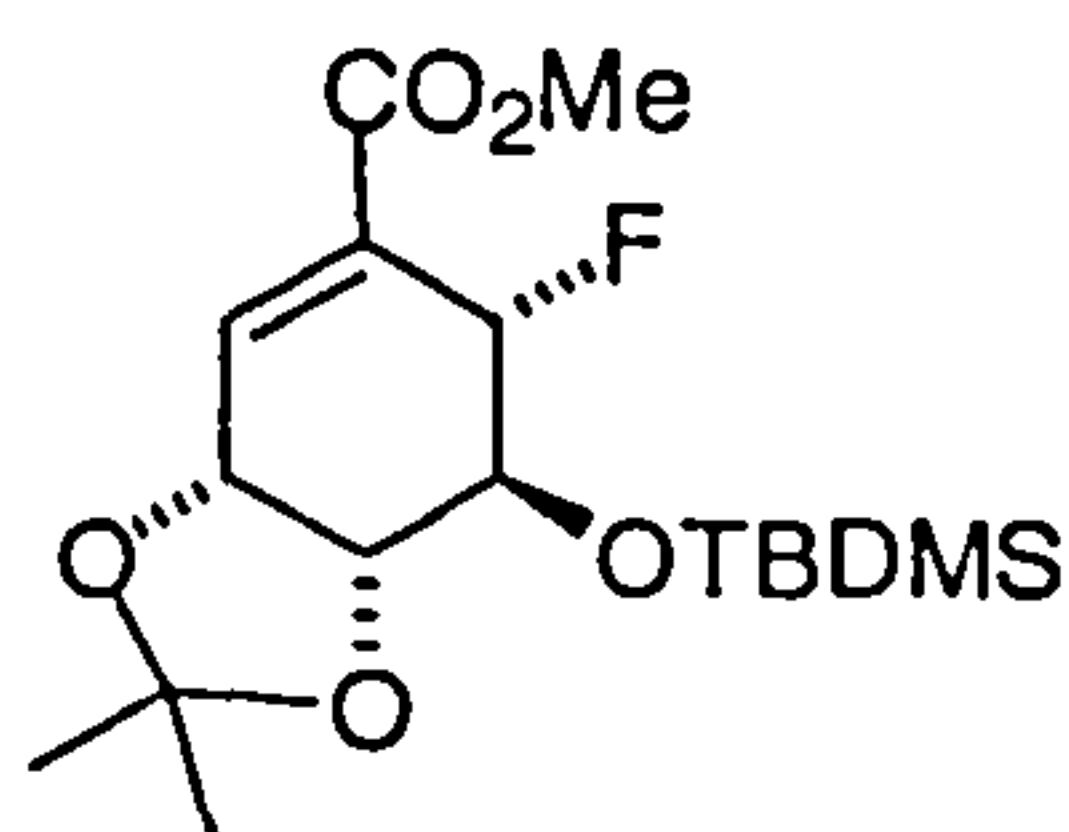


The diol **76** (841 mg, 3.44 mmol) was dissolved in dry DCM (40 mL) and cooled to between -20°C and -30°C.  $\text{Et}_3\text{N}$  (2.40 mL, 17.22 mmol, 5 eq.) was added dropwise with stirring. TBDMSOTf (0.83 mL, 3.62 mmol, 1.05 eq.) was added dropwise with vigorous stirring while the reaction mix was maintained at -20°C. Vigorous stirring at -20°C was continued for a further 30 min and the reaction was then quenched by the addition of ice-cold sat.  $\text{NaHCO}_3$  (aq.) (40 mL). The aqueous and organic layers were separated and the organic layer was extracted into DCM (4  $\times$  40 mL). The combined organic extracts were dried ( $\text{Na}_2\text{SO}_4$ ), filtered and evaporated under reduced pressure to give the crude product (1.54 g). Silica-gel column chromatography (hexane: $\text{Et}_2\text{O}$  = 2:1) yielded the desired protected diol **77** (868 mg, 2.42 mmol, 70 %) as a colourless oil. Crystallisation from hexane at -20°C gave flat square colourless crystals, but these returned to an oil at rt.

$\delta_{\text{H}}$ (400 MHz;  $\text{CDCl}_3$ ) 6.90 (1H, d,  $J$  3.1), 4.87 (1H, dd,  $J$  6.8 and 3.1), 4.55 (1H, d,  $J$  3.2), 4.39 (1H, t,  $J$  7.2), 3.80 (3H, s), 3.69 (1H, dt,  $J$  7.4 and 3.2), 2.21 (1H, d,  $J$  7.1), 1.47 (3H, s), 1.41 (3H, s), 0.87 (9H, s), 0.19 (3H, s), 0.05 (3H, s).



**Methyl 3,4-*O*-isopropylidene-5-*O*-*t*-butyldimethylsilyl-(6*S*)-fluoro-shikimate 78** (Song *et al.*, 2001)



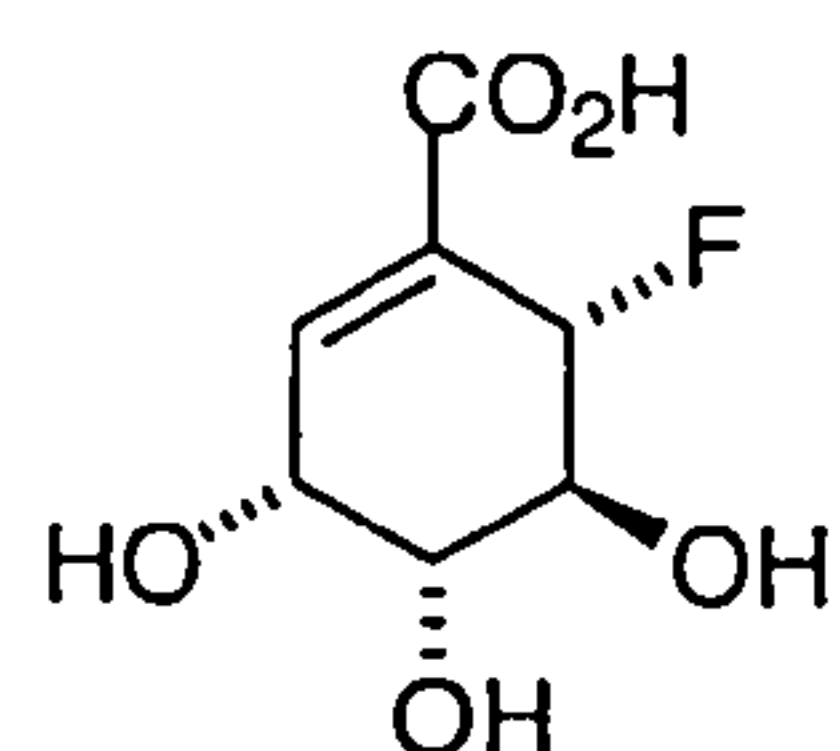
The protected diol **77** was dissolved in dry DCM (20 mL) and cooled to -78°C. DAST (1.02 mL, 7.75 mmol, 5 eq.) was added to the cooled solution with vigorous stirring. The solution was allowed to warm gradually to rt and stirred for a further 4 h before being cooled to -78°C. The reaction was quenched by the addition of 1M NaHCO<sub>3</sub> (40 mL) and the stirred mixture allowed to warm to rt. Stirring was continued until no further gas was evolved. The aqueous layer was extracted into DCM (3 × 40 mL) and the combined organic extracts were washed with 0.2M citric acid (120 mL) and brine (120 mL). The organic layer was then dried (Na<sub>2</sub>SO<sub>4</sub>), filtered and the solvent removed under reduced pressure to give an orange oil. Silica-gel column chromatography (hexane:Et<sub>2</sub>O = 3:1) yielded the desired protected (6*S*)-6-fluoroshikimic acid derivative **78** (461 mg, 1.28 mmol, 83 %) as a pale yellow oil.

$\delta_{\text{H}}$ (400 MHz; CDCl<sub>3</sub>) 7.00 (1H, t, *J* 3.3), 5.02 (1H, dd, *J* 46.2 and 3.2), 4.73 - 4.70 (1H, m), 4.41 (1H, dt, *J* 11.3 and 3.4), 4.25 (1H, t, *J* 4.5), 3.84 (3H, s), 1.41 (6H, s), 0.87 (9H, s), 0.14 (3H, s), 0.13 (3H, s);  $\delta_{\text{C}}$ (100 MHz; CDCl<sub>3</sub>) 165.6 (CO<sub>2</sub>Me), 139.4 (CH=C, d, *J* 6.5), 127.3 (CH=C, d, *J* 17.5), 111.1 (CMe<sub>2</sub>), 84.5 (CF, d, *J* 173.3), 74.7 (CHOR, d, *J* 1.6), 71.2 (CHOR, d, *J* 2.6), 69.1 (CHOR, d, *J* 25.4), 52.2 (CO<sub>2</sub>CH<sub>3</sub>), 27.9 (CCH<sub>3</sub>), 26.4 (CCH<sub>3</sub>), 25.6 (CMe<sub>3</sub>), 17.9 (SiCMe<sub>3</sub>), -5.04 (SiMe<sub>2</sub>).

lit. (Song *et al.*, 2001)  $\delta_{\text{H}}$ (270 MHz;  $\text{CDCl}_3$ ) 7.02 - 6.95, 5.15, 4.74 - 4.68, 4.45 - 4.37, 4.24, 3.84, 1.40, 0.86, 0.13.

(abbreviated data quoted)

#### (6*S*)-6-fluoroshikimic acid **1**



Fully protected (6*S*)-6-fluoroshikimic acid **78** (461 mg, 1.28 mmol) was dissolved in dioxane (10 mL). LiOH (aq.) (1.1 eq., 1.41 mmol, 1.4 mL of a 1M solution) and  $\text{H}_2\text{O}$  (8.6 mL) were added and the solution was stirred at rt for 2 h. The pH was adjusted to pH 4 with 1M HCl and the product extracted into EtOAc (3 x 30 mL). The EtOAc was dried ( $\text{Na}_2\text{SO}_4$ ), filtered and concentrated under reduced pressure to give a pale yellow oil (399 mg) which was used without further purification.

Selected NMR peaks:  $\delta_{\text{H}}$ (400 MHz;  $\text{CDCl}_3$ ) 7.02 (1H, t, *J* 2.9), 5.02 (1H, dd, *J* 46.2 and 3.0), 4.65 - 4.62 (1H, m), 4.34 (1H, dt, *J* 10.5 and 3.8), 4.16 (1H, t, *J* 4.8), 1.27 (6H, s), 0.77 (9H, s), 0.04 (6H, s).

A small amount of this free acid **90** (73 mg, impure) was dissolved in TFA (1.8 mL) and  $\text{H}_2\text{O}$  (0.2 mL) and the mixture was stirred at rt for 2.5 h. The solvent was removed under reduced pressure to give an orange oil which was partitioned between  $\text{H}_2\text{O}$  (10 mL) and  $\text{Et}_2\text{O}$  (10 mL). The aqueous layer was washed with  $\text{Et}_2\text{O}$  (2 x 10 mL) and lyophilised to give a yellow/brown foaming oil (42 mg).

Selected NMR peaks:  $\delta_{\text{H}}$ (400 MHz;  $\text{CD}_3\text{OD}$ ) 6.95 (1H, d, *J* 4.3), 5.12 (1H, dd, *J* 47.7 and 5.1), 4.41 - 4.39 (1H, m), 4.17 - 4.09 (1H, m), 3.67 (1H, dd, *J* 8.4 and 4.1); *m/z* 192.0 ( $\text{M}^+$ ,

10%), 191.0 (100%), 171.1 (25%), 113.0 (37%), 69.0 (45%).

Analytical HPLC using a BioRad Organic Acids column eluting with 50 mM formic acid (0.6 mL/min) gave the major peak eluting at 10.65 min [lit. (Duggan *et al.*, 1995) 10.8 min]. However, when a preparative run was attempted the compound was found to have decomposed.

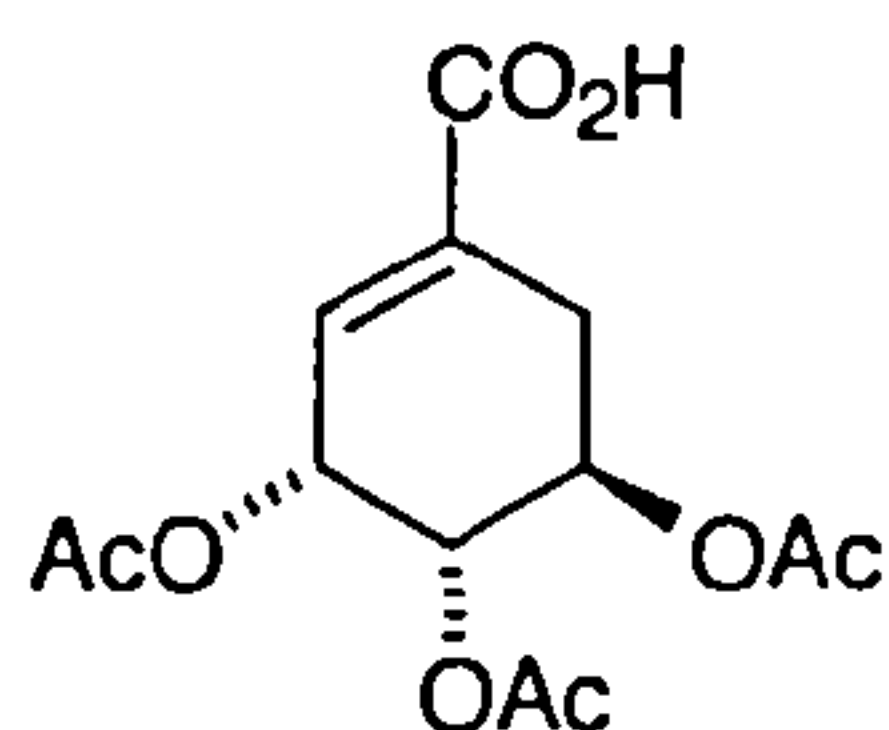
Literature characterisation data for (6*S*)-6-fluoroshikimic acid were not obtainable, however, data for the methyl ester has been published (Bowles *et al.*, 1990).

$\delta_{\text{H}}$ (400 MHz;  $\text{CDCl}_3$ ) 6.95 (1H, dd, *J* 5.0 and 1.0, 2-H), 5.23 (1H, br dd, *J* 48.0 and 6.0, 6-H), 4.49 (1H, br dd, *J* 5.0 and 4.0, 3-H), 4.23 (1H, ddd, *J* 17.0, 9.0 and 6.0, 5-H), 3.82 (3H, s,  $\text{OCH}_3$ ), 3.69 (1H, dd, *J* 9.0 and 4.0, 4-H), 2.70 (3H, br s, 3OH)

This data, particularly the coupling constant for the hydrogen on C-6, suggested that desired enantiomer had indeed been synthesised.

## 5.6 Experimental for Chapter 3

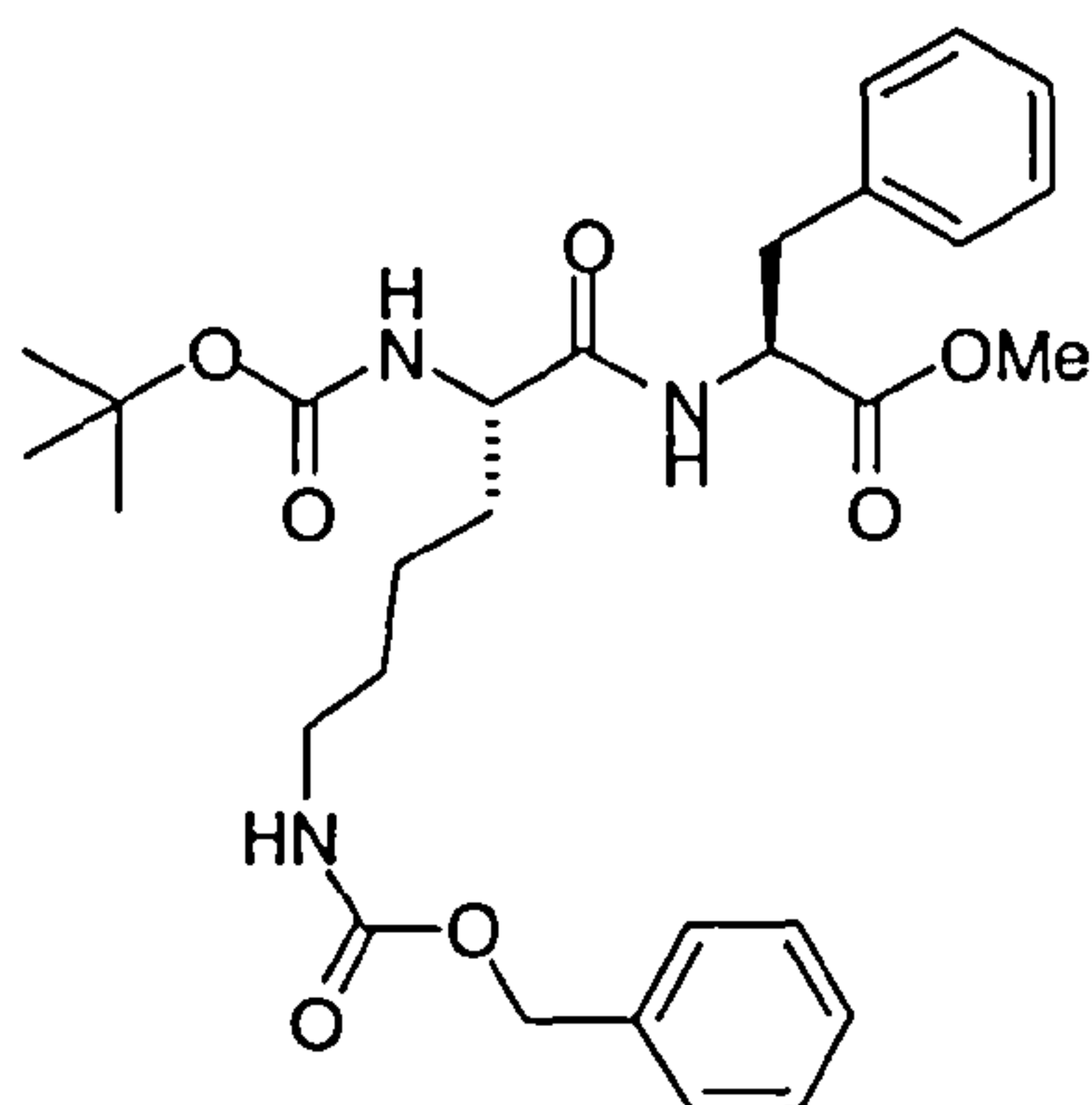
### Synthesis of tri-*O*-acetyl shikimic acid **80**



Shikimic acid **9** (1.00 g, 5.72 mmol) was dissolved in dry pyridine (20 mL) and the stirred solution cooled to 0°C. Ac<sub>2</sub>O (2.4 mL, 25 mmol) was added drop-wise and the solution left stirring overnight at room temperature. The solution was then evaporated under reduced pressure and the product dissolved in DCM (30 mL). Saturated NaHCO<sub>3</sub> aq. (200 mL) was added, and the organic and aqueous phases separated after it was ascertained that the pH of the aqueous layer was above pH 8. The aqueous layer was acidified to pH 3 using aqueous HCl (2M) and extracted using CHCl<sub>3</sub> (4 × 300 mL).. The combined organic extracts were dried (MgSO<sub>4</sub>) and evaporated under reduced pressure to give the tri-acetate **80** (1.68 g, 5.61 mmol, 98%) as a white amorphous solid which was used without further purification.

$\nu_{\text{max}}(\text{CaF}_2)/\text{cm}^{-1}$  3676, 3518, 3172, 3023, 2966, 2927, 2853, 2677, 2629, 2520, 1745, 1701, 1657, 1508, 1429, 1372, 1232, 1149, 1110, 1079, 1043;  $\delta_{\text{H}}(400 \text{ MHz}; \text{CDCl}_3)$  9.55 (1H, broad s, CO<sub>2</sub>H), 6.81 (1H, m, HCCCCO<sub>2</sub>H), 5.69 (1H, m, CHCHOCOCH<sub>3</sub>) 5.26-5.20 (2H, m, 2 × CHOCOCH<sub>3</sub>), 2.83 (1H, broad d, J 17) and 2.37 (1H, broad d, J 18), HO<sub>2</sub>CCCH<sub>2</sub>, 2.05 (3H, s, COCH<sub>3</sub>), 2.03 (3H, s, COCH<sub>3</sub>), 2.00 (3H, s, COCH<sub>3</sub>);  $\delta_{\text{C}}(100 \text{ MHz}; \text{CDCl}_3)$  170.8 (CO<sub>2</sub>H), 170.4 (2 × COCH<sub>3</sub>), 170.3 (COCH<sub>3</sub>), 135.4 (CHCCO<sub>2</sub>H), 131.0 (CCO<sub>2</sub>H), 67.8 (CHOCOCH<sub>3</sub>), 67.1 (CHOCOCH<sub>3</sub>), 66.4 (CHOCOCH<sub>3</sub>), 28.4 (CH<sub>2</sub>), 21.3 (OCH<sub>3</sub>), 21.1 (2 × OCH<sub>3</sub>);  $m/z$  318.1 ([M+NH<sub>4</sub>]<sup>+</sup>, 100%.

**Synthesis of N- $\alpha$ -*t*-butoxycarbonyl-N- $\epsilon$ -benzyloxycarbonyl-L-lysyl-L-phenylalanine methyl ester **81****



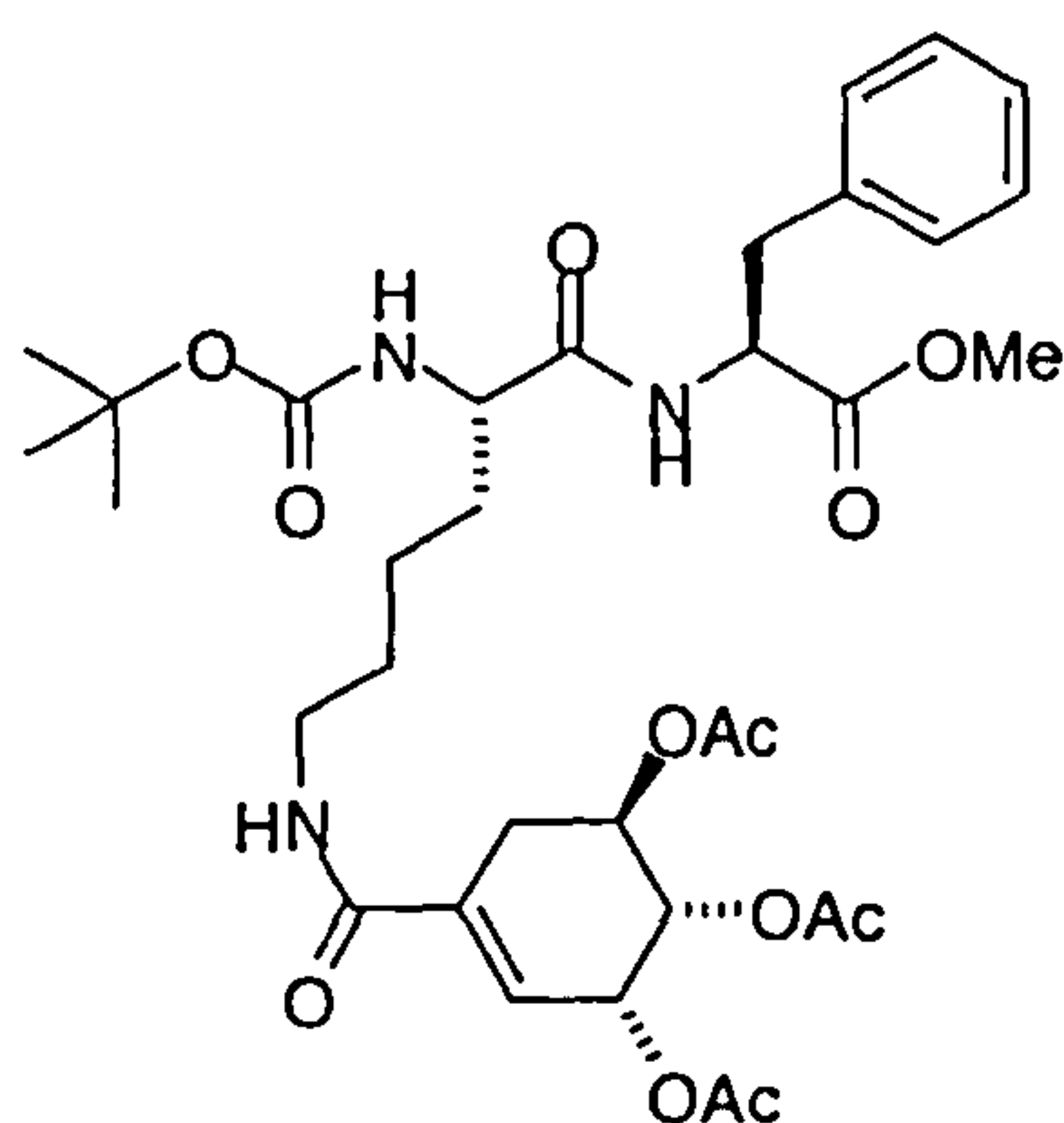
L-Phe methyl ester hydrochloride (260 mg, 1.21 mmol) was dissolved in dry DMF (20 mL) and the stirred solution cooled to 0°C after which Et<sub>3</sub>N (0.67 mL, 4.8 mmol.) was added. Boc-Lys-(Z)-OH (460 mg, 1.21 mmol) was then dissolved in dry DMF (20 mL) and the solution added to the reaction mixture. EDC (280 mg, 1.51 mmol) and HOBt (192 mg, 1.42 mmol) were added and the solution left stirring for 16 h during which time the temperature returned to rt. The solution was evaporated under reduced pressure to dryness to give the crude product. Silica-gel column chromatography (ethyl acetate:petrol, 1:4 – ethyl acetate gradient elution) gave the pure dipeptide **81** (555 mg, 1.02 mmol, 85%) as a white solid.

m.p. 98-100°C (lit. 105-106°C; Visser, 1968);  $\nu_{\max}$ (CaF<sub>2</sub>)/cm<sup>-1</sup> 3435 (broad, NH), 3163 (weak, CH), 2979 (weak, CH), 2939 (weak, CH), 1709 (CO), 1679 (CO), 1500 (Ph), 1499 (Ph);  $\delta_{\text{H}}$ (400 MHz; CDCl<sub>3</sub>) 7.27-7.02 (10H, m, 2 × Ph), 6.54 (1H, broad d, *J* 7.6, NH), 5.1, 1H, broad d, *J* 6.8, NH), 5.03 (2H, s, OCH<sub>2</sub>Ph), 4.98 (1H, m, NH), 4.76 (1H, m, CH), 3.98 (1H, m, CH), 3.58 (3H, s, OCH<sub>3</sub>), 3.10 (4H, m, CH<sub>2</sub>Ph and CH<sub>2</sub>N), 1.7-1.23 (6H, m, 3 × CH<sub>2</sub> Lys), 1.35 (9H, s, C(CH<sub>3</sub>)<sub>3</sub>);  $\delta_{\text{C}}$ (100 MHz; CDCl<sub>3</sub>) 172.2 (CO<sub>2</sub>), 157.0 (CO<sub>2</sub>), 156.0



(CO<sub>2</sub>), 137.0 (C-Ar), 136.2 (C-Ar), 129.6 (CH), 129.0 (CH), 128.9 (CH), 128.5 (2 × CH), 127.5 (CH), 80.4 (CMe<sub>3</sub>), 67.0 (CH<sub>2</sub>), 54.6 (OCH<sub>3</sub>), 53.5 (CH), 52.7 (CH), 40.7 (CH<sub>2</sub>), 38.2 (CH<sub>2</sub>), 32.3 (CH<sub>2</sub>), 29.7 (CH<sub>2</sub>), 28.7 (C(CH<sub>3</sub>)<sub>3</sub>), 22.7 (CH<sub>2</sub>); *m/z* 542.2 ([M+H]<sup>+</sup>, 32%), 442.2 ([M+H-Boc]<sup>+</sup>, 100%), 91.5 (Z<sup>+</sup>, 83%).

**Synthesis of N- $\alpha$ -*t*-butoxycarbonyl-N- $\epsilon$ -(tri-*O*-acetyl-shikimoyl)-L-lysyl-L-phenylalanine methyl ester **82****



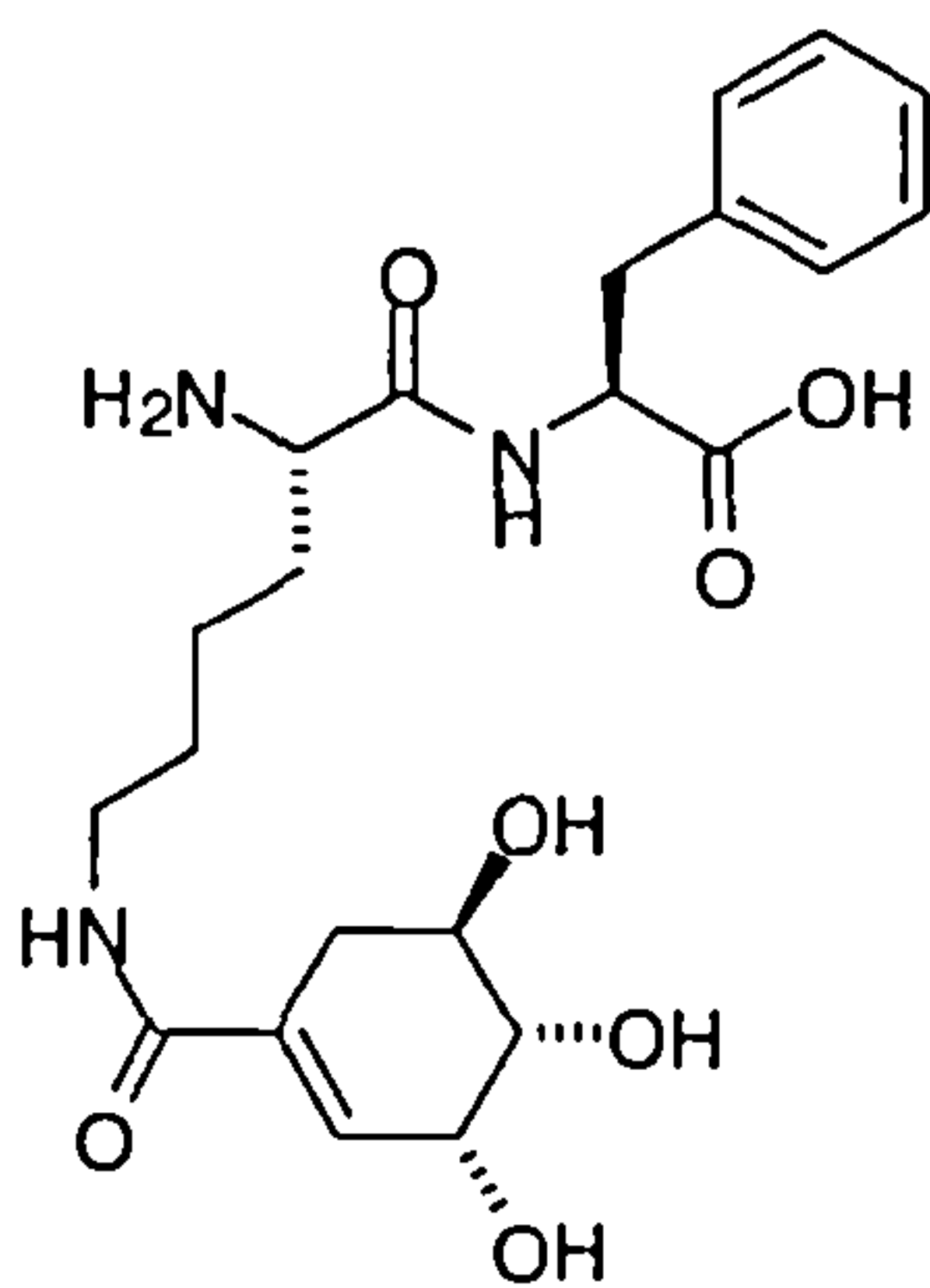
Boc-Lys(Z)-Phe-OMe **81** (126 mg, 0.23 mmol) was dissolved in dry DCM (15 mL). 10% Pd/C (0.1 g) was added and the flask evacuated and flushed with H<sub>2</sub> gas three times. The suspension was then stirred under H<sub>2</sub> for 42 h, filtered through celite and concentrated under reduced pressure to give the crude deprotected dipeptide (107 mg). This was then dissolved in dry DCM (10 mL) and the stirred solution cooled to 0°C after which Et<sub>3</sub>N (0.20 mL, 1.43 mmol) was added. Tri-*O*-acetyl shikimic acid **80** (43 mg, 0.14 mmol) was dissolved in dry DCM (5 mL) and added to the reaction mixture. EDC (70 mg, 0.36 mmol) and HOBt (50 mg, 0.36 mmol) were added and the solution left stirring under N<sub>2</sub> for 16 h during which time the temperature returned to rt.

The solution was evaporated under reduced pressure to give the crude product as a brown mass. Silica-gel column chromatography (ethyl acetate:petrol, 1:5 – ethyl acetate gradient

elution) gave the pure shikimate derivative **82** (85 mg, 0.12 mmol, 88%) as a white amorphous solid.

m.p. 76-84°C;  $\nu_{\text{max}}(\text{CaF}_2)/\text{cm}^{-1}$  3435 (broad), 3330 (broad), 3089, 3067, 3032, 3006, 2984, 2953, 2936, 2861, 2261, 2248, 1745, 1712, 1644, 1517, 1499, 1455, 1437;  $\delta_{\text{H}}(400 \text{ MHz}; \text{CDCl}_3)$  7.21-7.05 (5H, m, Ph), 6.87 (1H, broad d,  $J$  6.5, NH), 6.50 (1H, m), 6.28 (1H, m, NH), 5.61 (1H, m), 5.33 (1H, d,  $J$  7.7,  $\text{CHOCOCH}_3$ ), 5.26-5.13 (2H, m,  $2 \times \text{CHOCOCH}_3$ ), 4.73 (1H, m, CH), 4.05 (1H, m, CH), 3.61 (3H, s,  $\text{OCH}_3$ ), 3.19 (2H, m,  $\text{CH}_2\text{Ph}$ ), 3.08-2.90 (3H, m, CH and  $\text{CH}_2\text{N}$ ), 2.33 (1H, dd,  $J$  6.3 and 18.2, CH), 1.99 (3H, s,  $\text{OCOCH}_3$ ), 1.97 (3H, s,  $\text{OCOCH}_3$ ), 1.96 (3H, s,  $\text{OCOCH}_3$ ), 1.68-1.15 (7H, m, CH and  $\text{CH}_2$ ), 1.34 (9H, s,  $\text{C}(\text{CH}_3)_3$ );  $\delta_{\text{C}}(100 \text{ MHz}; \text{CDCl}_3)$  172.4 (C=O), 172.1 (C=O), 170.5 (C=O), 170.3 (C=O), 166.7 (C=O), 156.0 (C), 136.2 (C-Ar), 136.2 (C), 129.5 (CH), 128.9 (CH), 127.4 (CH), 126.0 (CH), 80.2 ( $\text{OCH}_3$ ), 77.8 (C), 68.8 (CH), 67.1 (CH), 66.4 (CH), 60.7 ( $\text{CH}_2$ ), 54.5 (CH), 53.6 (CH), 52.6 (CH), 39.5 ( $\text{CH}_2$ ), 38.1 ( $\text{CH}_2$ ), 32.1 ( $\text{CH}_2$ ), 29.6 ( $\text{CH}_2$ ), 29.2 ( $\text{CH}_2$ ), 28.6 ( $\text{CH}_3$ ), 22.9 ( $\text{CH}_2$ ), 21.3 ( $\text{O}_2\text{CCH}_3$ ), 21.1 ( $\text{O}_2\text{CCH}_3$ ), 21.0 ( $\text{O}_2\text{CCH}_3$ ), 14.5 (CH);  $m/z$  (FAB<sup>+</sup>) 712.5 ( $[\text{M}+\text{Na}]^+$ , 100%), 590.4 (16%), 176.1 (12%), 121.2 (15%).

## Synthesis of N- $\epsilon$ -shikimoyl-L-lysyl-L-phenylalanine **83**

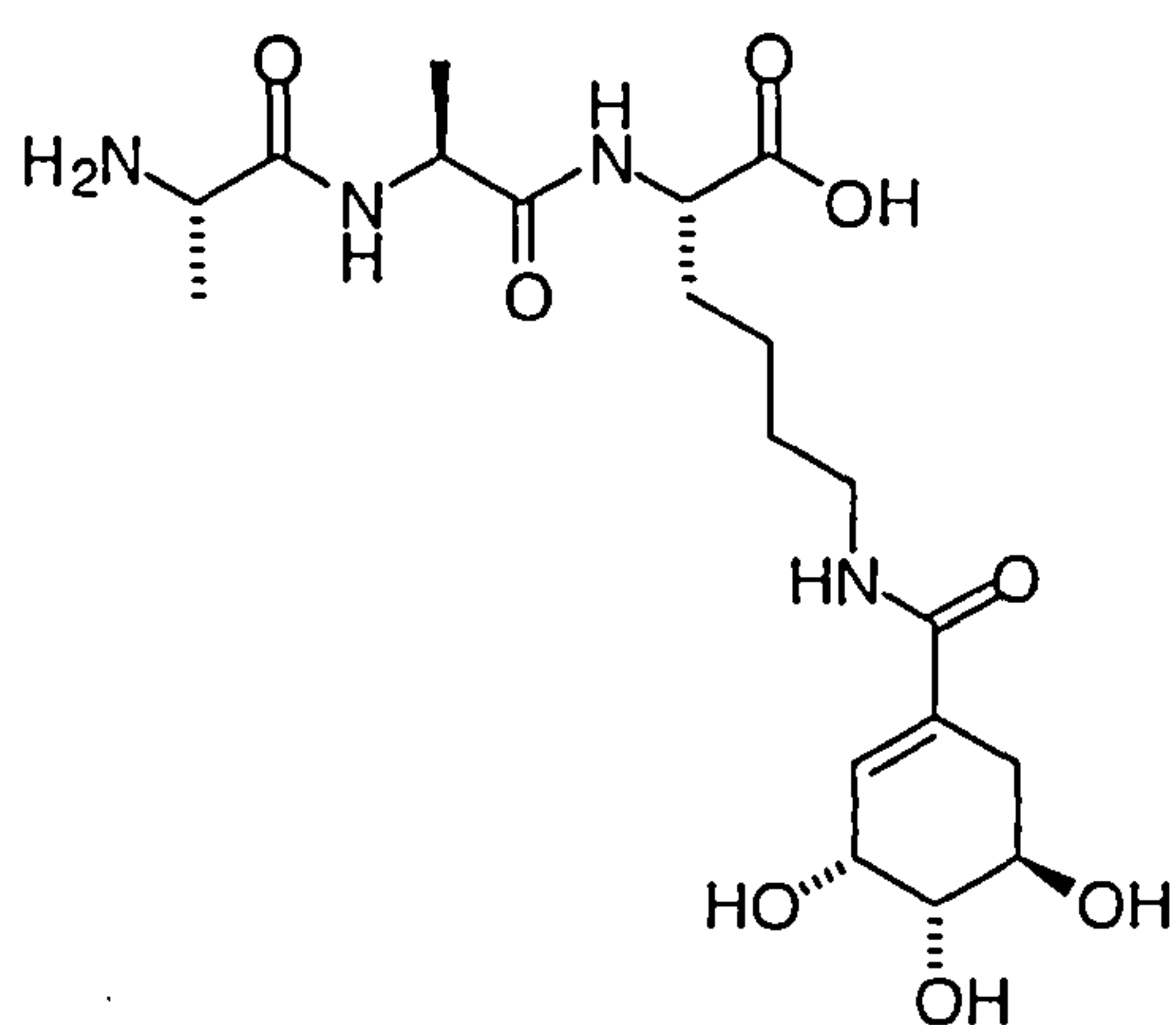


The shikimate conjugate **82** (312 mg, 0.43 mmol) was dissolved in THF:H<sub>2</sub>O = 4:1. NaOH (190 mg, 4.72 mmol) was added with stirring. The stirring was continued for 23 h until no trace of the starting material could be detected by TLC (petrol). The solution was evaporated to dryness and the solid residue dissolved in distilled H<sub>2</sub>O (50 mL). The aqueous solution was washed with Et<sub>2</sub>O (4 × 50 mL) and the resulting aqueous layer stirred with ion exchange resin (Amberlite IR 120, hydrogen form, 1 g) until it reached pH 2. The solution was then filtered through celite and concentrated to give the crude product (212 mg) as a pale yellow viscous oil. This oil was dissolved in TFA (15 mL) and stirred at 0°C for 45 min. The solution was allowed to return to room temperature and stirred for a further 45 min after which it was evaporated to dryness to give the crude product **83** as a white solid. No further purification was carried out.

$\delta_{\text{H}}$ (400 MHz; CDCl<sub>3</sub>) 7.06-6.96 (5H, m, Ph), 6.61 (1H, s, CHCON), 4.48-4.44 (1H, m, CH), 4.19 (1H, s, CH), 3.79 (2H, s, CH<sub>2</sub>NH), 3.73-3.70 (1H, m, CH), 3.51-3.49 (1H, m, CH), 3.01-2.97 (2H, m, CH<sub>2</sub>Ph), 2.89-2.79 (1H, m, CH), 2.50 (1H, dd, *J* 4.5 and 17.4, OCCCH<sub>A</sub>), 1.98 (1H, dd *J* 6.5 and 17.4, OCCCH<sub>B</sub>), 1.63-1.58 (2H, m, CH<sub>2</sub>-Lys), 1.36-1.28 (2H, m, CH<sub>2</sub>-Lys), 1.10 (2H, broad d, *J* 6.1, CH<sub>2</sub>-Lys);  $\delta_{\text{C}}$ (100 MHz; CDCl<sub>3</sub>) 174.4 (C),

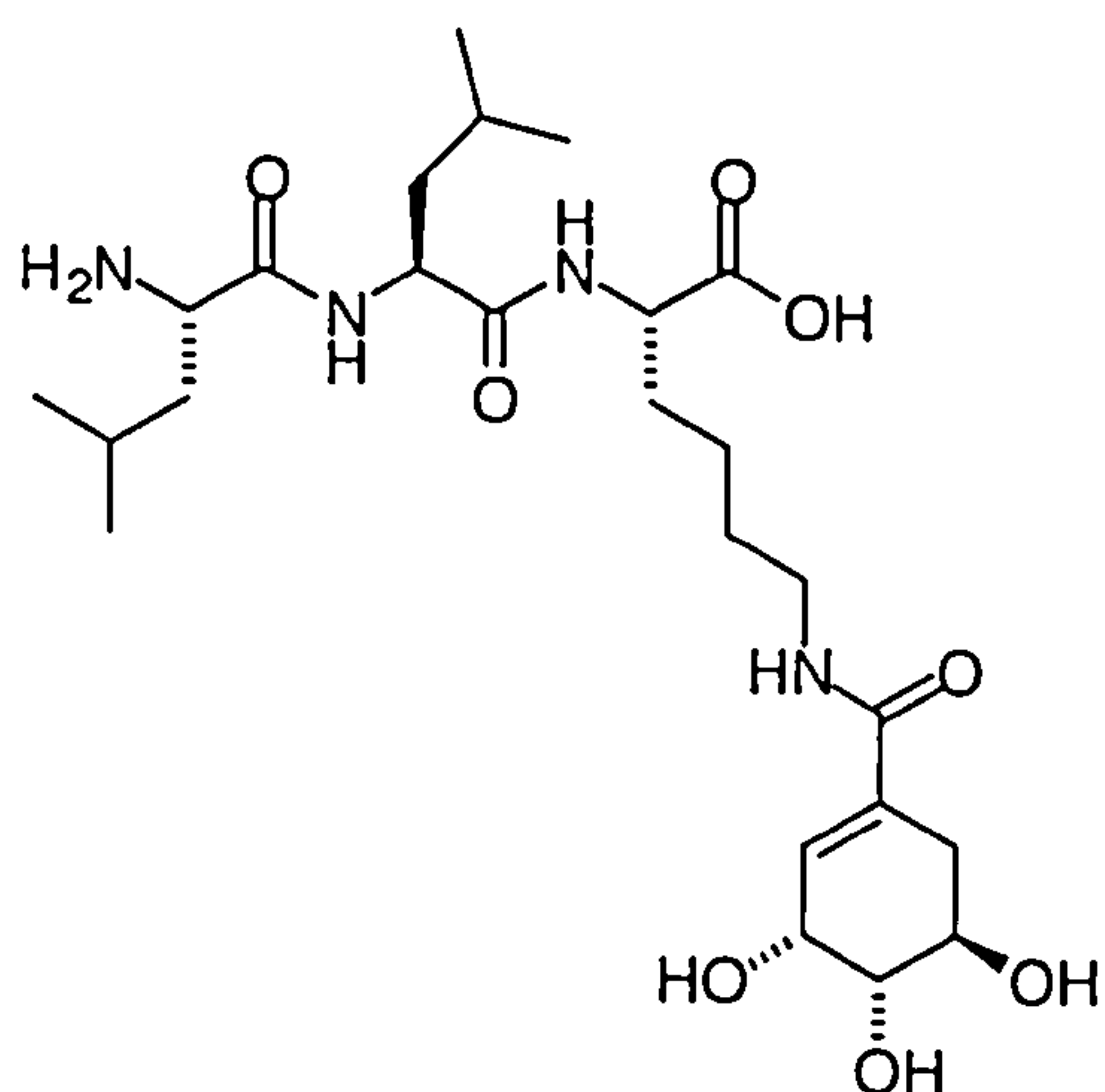
171.9 (C), 170.3 (C), 169.8 (C), 163.4 (C), 138.5 (C), 136.7 (C), 133.4 (C), 131.1 (CH), 129.4 (CH), 129.3 (CH), 129.0 (CH), 127.5 (CH), 71.7 (CH), 66.6 (CH), 66.1 (CH), 54.5 (CH), 54.0 (CH), 53.1 (CH), 39.3 (CH), 36.4 (CH<sub>2</sub>), 31.3 (CH<sub>2</sub>), 30.8 (CH<sub>2</sub>), 28.2 (CH<sub>2</sub>), 27.0 (CH<sub>2</sub>), 21.5 (CH<sub>2</sub>); *m/z* (FAB<sup>+</sup>) 593.2 (7%), 493.2 (12%), 456.3 ([M+7H]<sup>+</sup>, 51%), 455.3 ([M+6H]<sup>+</sup>, 40%), 341.1 (18%), 340.1 (15%), 247.0 (23%), 246.0 (16%), 169.1 (16%), 121.2 (57%).

### Synthesis of L-alanyl-L-alanyl-N-ε-shikimoyl-L-lysine 88a on solid phase



Using the method described in General Procedures (*Section 5.4.3*), tri-*O*-acetyl shikimic acid (65 mg, 0.22 mmol) **80** was coupled to Fmoc-Lys(Mtt)-Wang Resin (100 mg, 72 μmol) using HOBt (30 mg, 0.22 mmol) and DIC (91 mg, 0.113 mL, 0.72 mmol). Fmoc-Ala-OH (67 mg, 0.22 mmol) was coupled to the amino-acid conjugate to give the resin-bound tripeptide after the second iteration. The desired tripeptide-shikimate conjugate **88a** was obtained as a crude mixture after cleavage from the resin and deprotection (crude product 43 mg; max. theoretical yield 32 mg). *m/z* (ES) 445.20 ([M+H]<sup>+</sup>, 100%)

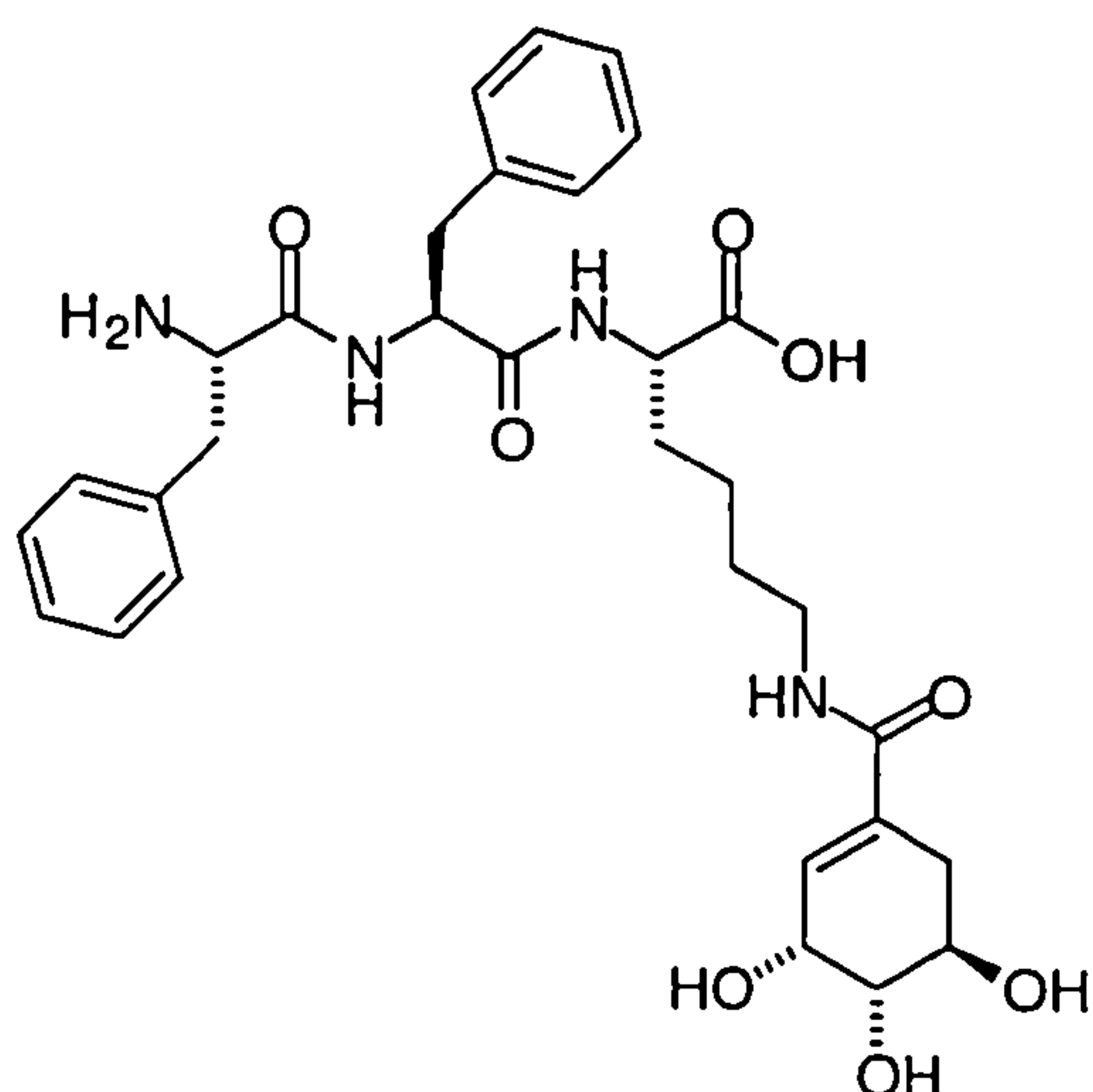
## Synthesis of L-leucyl-L-leucyl-N- $\epsilon$ -shikimoyl-L-lysine **88b** on solid phase



Using the method described in General Procedures (*Section 5.4.3*), tri-*O*-acetyl shikimic acid (65 mg, 0.22 mmol) **80** was coupled to Fmoc-Lys(Mtt)-Wang Resin (100 mg, 72  $\mu$ mol) using HOBt (30 mg, 0.22 mmol) and DIC (91 mg, 0.113 mL, 0.72 mmol). Fmoc-Leu-OH (76 mg, 0.22 mmol) was coupled to the amino-acid conjugate to give the resin-bound tripeptide conjugate after the second iteration. The desired tripeptide-shikimate conjugate **88b** was obtained as a crude mixture after cleavage from the resin and deprotection (crude product 62 mg; max. theoretical yield 38 mg).  $m/z$  (ES) 529.30 ( $[M+H]^+$ , 95%)

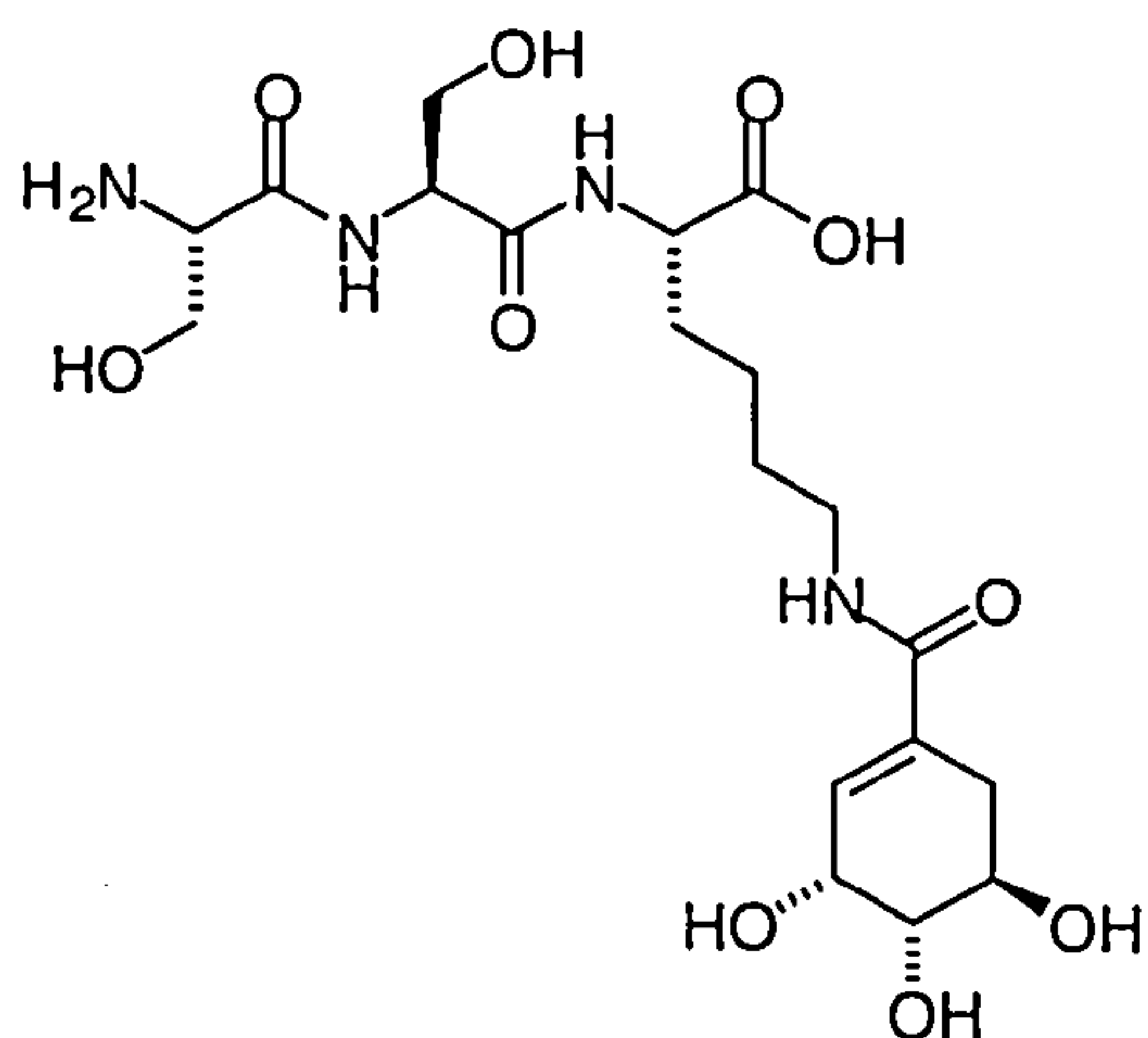


## Synthesis of L-phenylalanyl-L-phenylalanyl-N- $\epsilon$ -shikimoyl-L-lysine **88c** on solid phase



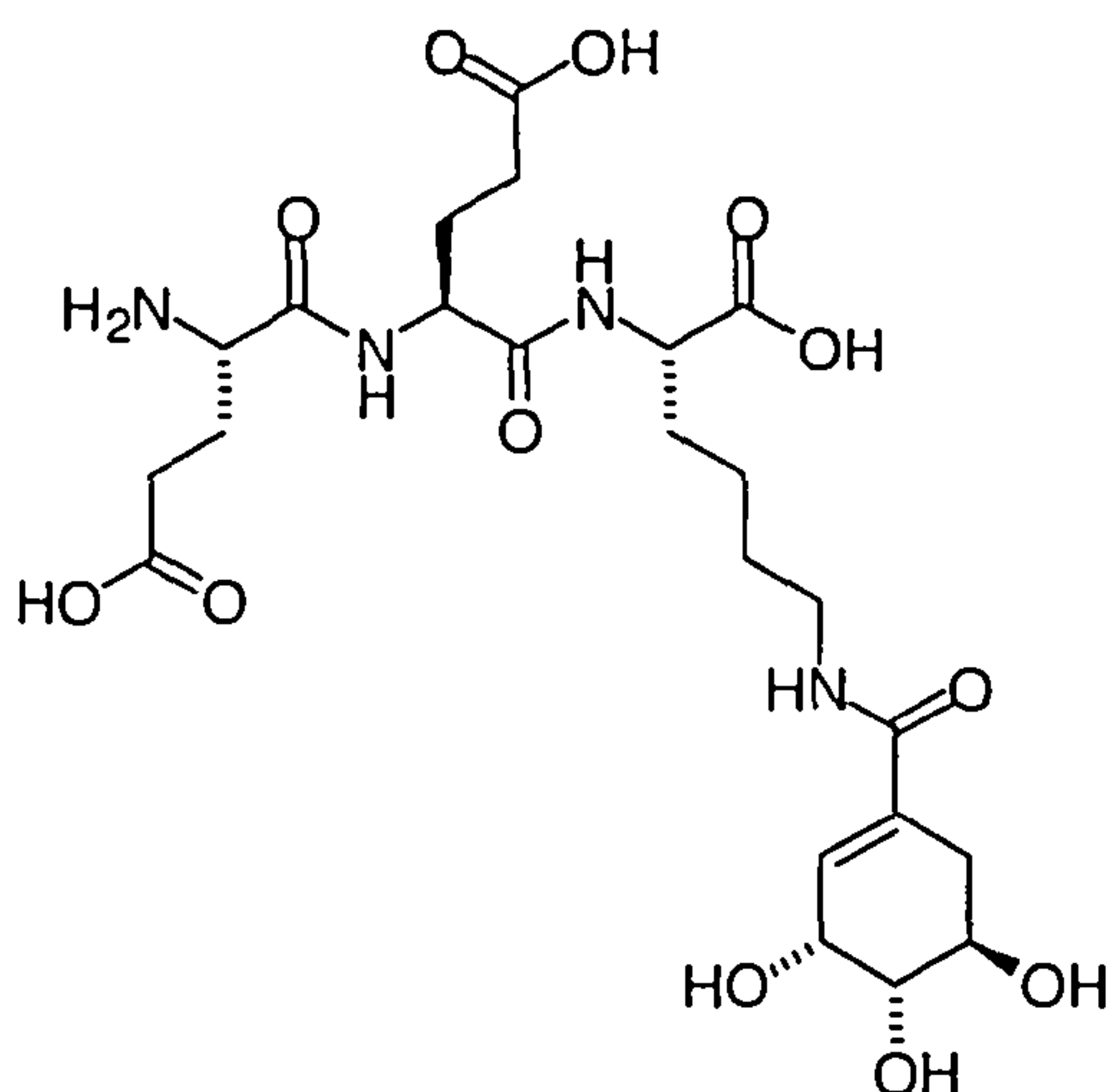
Using the method described in General Procedures (*Section 5.4.3*), tri-*O*-acetyl shikimic acid (65 mg, 0.22 mmol) **80** was coupled to Fmoc-Lys(Mtt)-Wang Resin (100 mg, 72  $\mu$ mol) using HOBt (30 mg, 0.22 mmol) and DIC (91 mg, 0.113 mL, 0.72 mmol). Fmoc-Phe-OH (84 mg, 0.22 mmol) was coupled to the amino-acid conjugate to give the resin-bound tripeptide conjugate after the second iteration. The desired tripeptide-shikimate conjugate **88c** was obtained as a crude mixture after cleavage from the resin and deprotection (crude product 86 mg; max. theoretical yield 43 mg). *m/z* (ES) 597.3 ( $[M+H]^+$ , 100%)

## Synthesis of L-seryl-L-seryl-N- $\epsilon$ -shikimoyl-L-lysine **88d** on solid phase



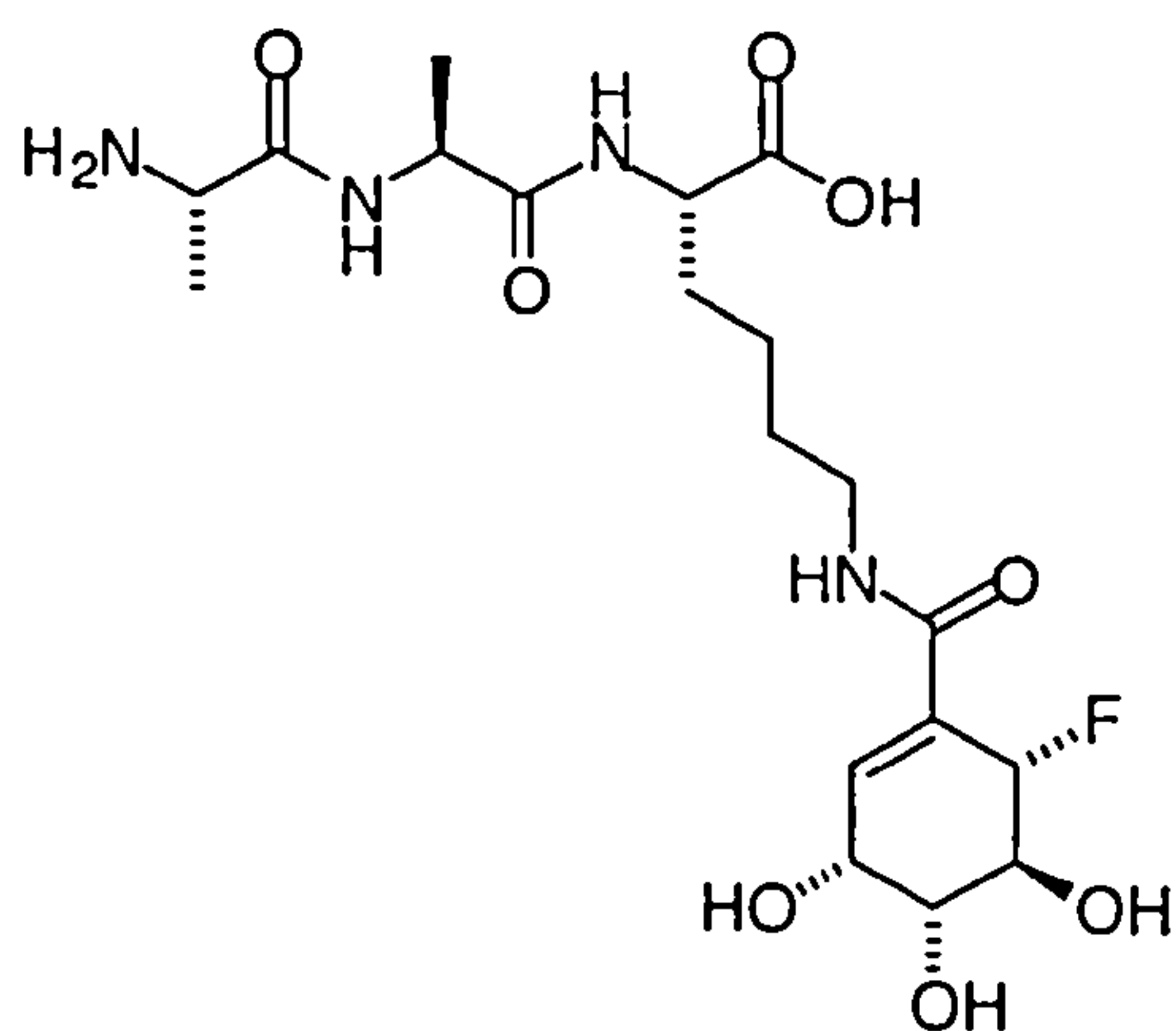
Using the method described in General Procedures (*Section 5.4.3*), tri-*O*-acetyl shikimic acid (65 mg, 0.22 mmol) **80** was coupled to Fmoc-Lys(Mtt)-Wang Resin (100 mg, 72  $\mu$ mol) using HOBt (30 mg, 0.22 mmol) and DIC (91 mg, 0.113 mL, 0.72 mmol). Fmoc-Ser(*t*Bu)-OH (83 mg, 0.22 mmol) was coupled to the amino-acid conjugate to give the resin-bound tripeptide conjugate after the second iteration. The desired tripeptide-shikimate conjugate **88d** was obtained as a crude mixture after cleavage from the resin and deprotection (crude product 67 mg; max. theoretical yield 34 mg).  $m/z$  (ES) 477.27 ( $[M+H]^+$ , 100%)

## Synthesis of L-glutamoyl-L-glutamoyl-N- $\epsilon$ -shikimoyl-L-lysine **88e** on solid phase



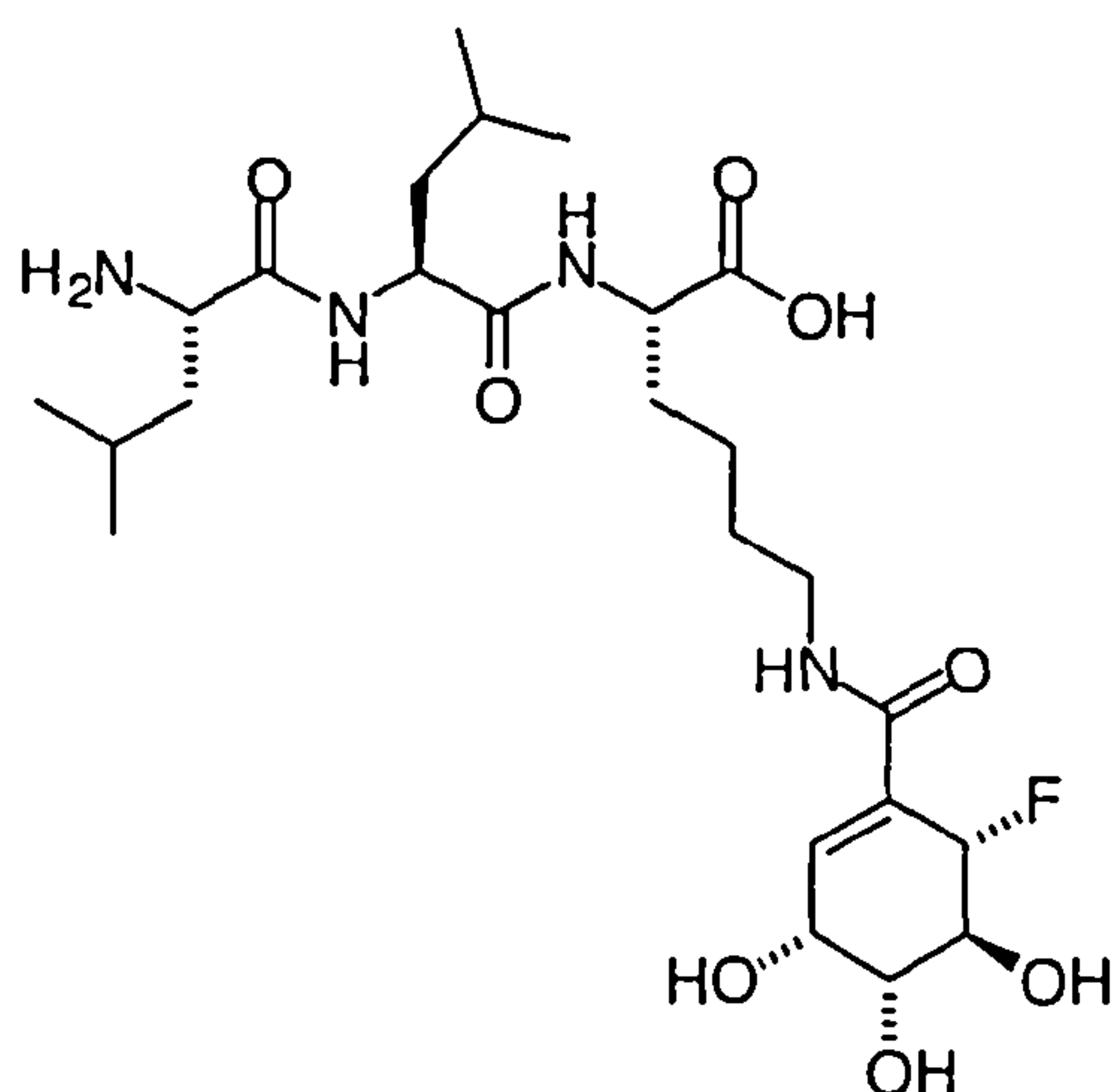
Using the method described in General Procedures (*Section 5.4.3*), tri-*O*-acetyl shikimic acid (65 mg, 0.22 mmol) **80** was coupled to Fmoc-Lys(Mtt)-Wang Resin (100 mg, 72  $\mu$ mol) using HOBt (30 mg, 0.22 mmol) and DIC (91 mg, 0.113 mL, 0.72 mmol). Fmoc-Glu(*O**t*Bu)-OH (91 mg, 0.22 mmol) was coupled to the amino-acid conjugate to give the resin-bound tripeptide conjugate after the second iteration. The desired tripeptide-shikimate conjugate **88d** was obtained as a crude mixture after cleavage from the resin and deprotection (crude product 81 mg; max. theoretical yield 40 mg). *m/z* (ES) 477.27 ( $[M+H]^+$ , 100%)

## Synthesis of L-alanyl-L-alanyl-N-ε-(6S)-6-fluoroshikimoyl-L-lysine 4 on solid phase



Using the method described in General Procedures (*Section 5.4.3*), Fmoc-Ala-OH (35 mg, 0.11 mmol) was coupled to Fmoc-Lys(Mtt)-Wang Resin (50 mg, 36  $\mu$ mol) using HOBt (15 mg, 0.11 mmol) and DIC (45 mg, 0.05 mL, 0.36 mmol) to give the resin-bound tripeptide after the second iteration. The protected (6*S*)-6-fluoroshikimic acid derivative **90** (38 mg, 0.11 mmol) was coupled to the tripeptide using BOP (120 mg, 0.28 mmol) and Et<sub>3</sub>N (0.28 mmol). A crude mixture containing the tripeptide-(6*S*)-6-fluoroshikimic acid conjugate **4** was obtained after cleavage from the resin and deprotection (crude yield 61 mg; max. theoretical yield ). The desired conjugate eluted at 5.09 min on an RP Max HPLC column eluting with 3 - 50 % 50 mM formic acid in MeCN. *m/z* (ES) 463.30 ([M+H]<sup>+</sup>, 100%)

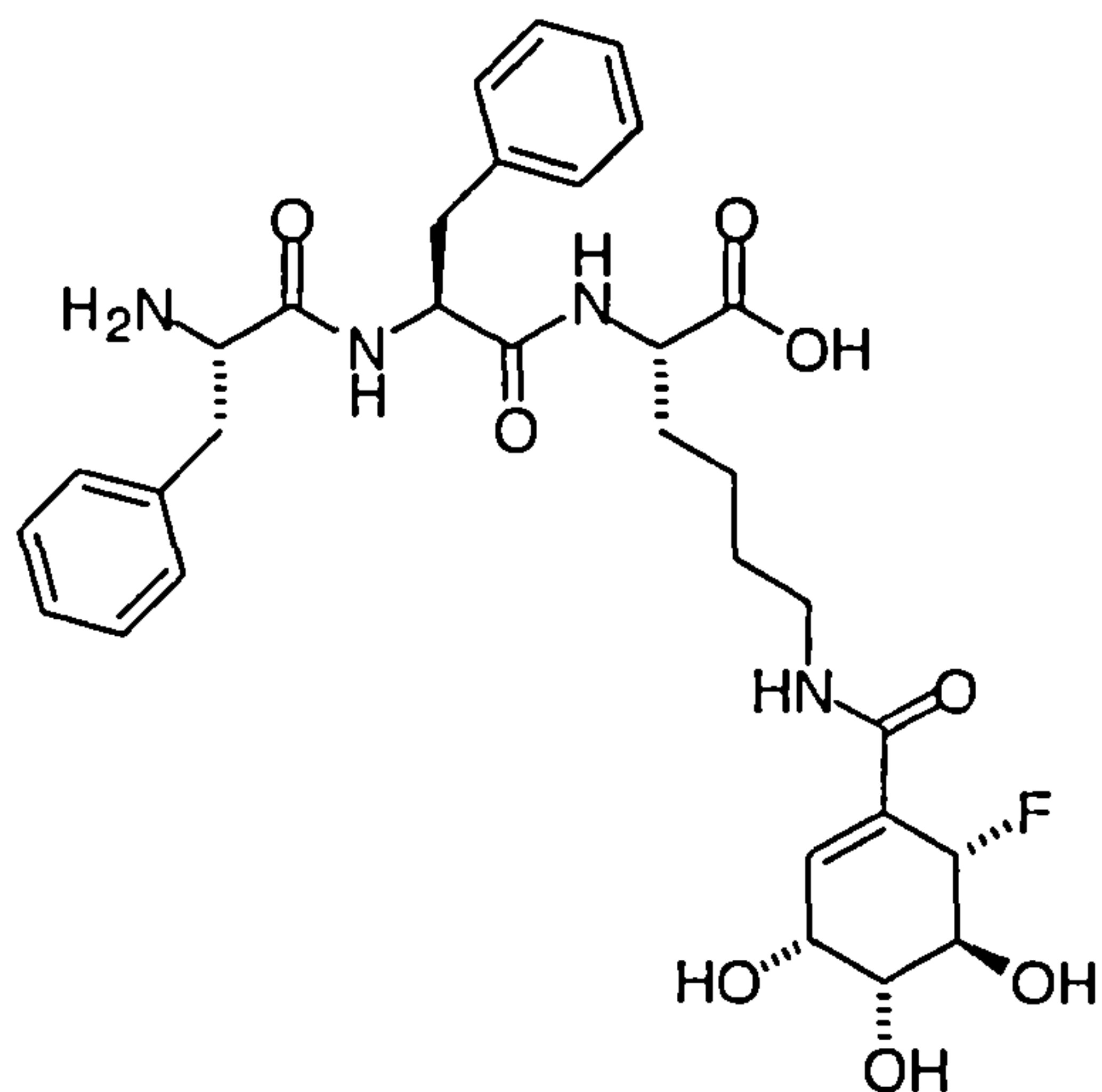
## Synthesis of L-leucyl-L-leucyl-N-ε-(6S)-6-fluoroshikimoyl-L-lysine **5** on solid phase



Using the method described in General Procedures (*Section 5.4.3*), Fmoc-Leu-OH (36 mg, 0.11 mmol) was coupled to Fmoc-Lys(Mtt)-Wang Resin (50 mg, 36  $\mu$ mol) using HOBt (15 mg, 0.11 mmol) and DIC (45 mg, 0.05 mL, 0.36 mmol) to give the resin-bound tripeptide after the second iteration. The protected (6*S*)-6-fluoroshikimic acid derivative **90** (38 mg, 0.11 mmol) was coupled to the tripeptide using BOP (120 mg, 0.28 mmol) and Et<sub>3</sub>N (0.28 mmol). A crude mixture containing the crude tripeptide-(6*S*)-6-fluoroshikimic acid conjugate **5** was obtained after cleavage from the resin and deprotection (crude yield: 65 mg). The desired conjugate eluted at 10.9 min on an RP Max HPLC column eluting with 3 - 50 % 50 mM formic acid in MeCN. The fractions containing the desired compound **5** were lyophilised and stored at -20°C. *m/z* (ES) 543.3 ([M+H]<sup>+</sup>, 100%)

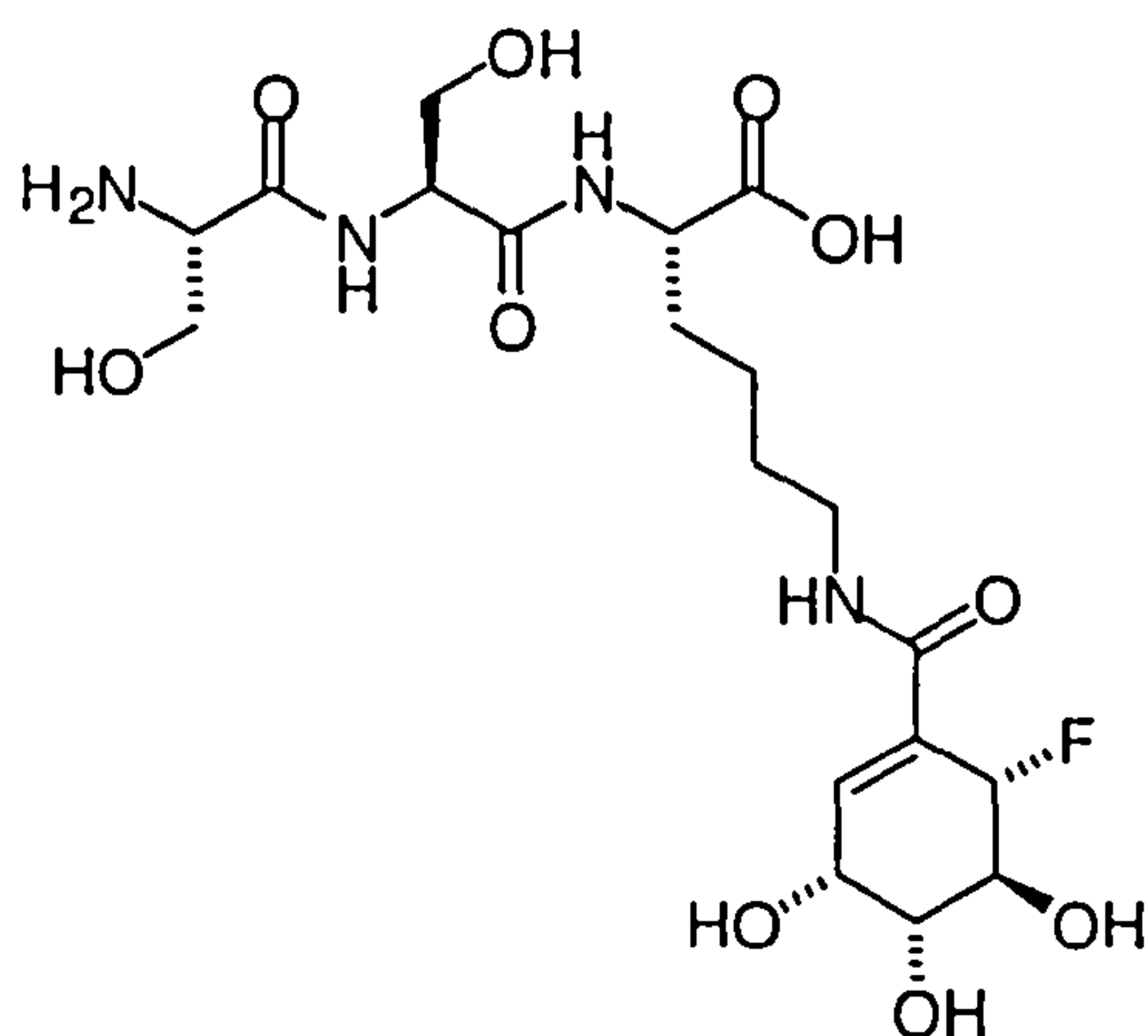


## Synthesis of L-phenylalanyl-L-phenylalanyl-N- $\epsilon$ -(6*S*)-6-fluoroshikimoyl-L-lysine **6** on solid phase



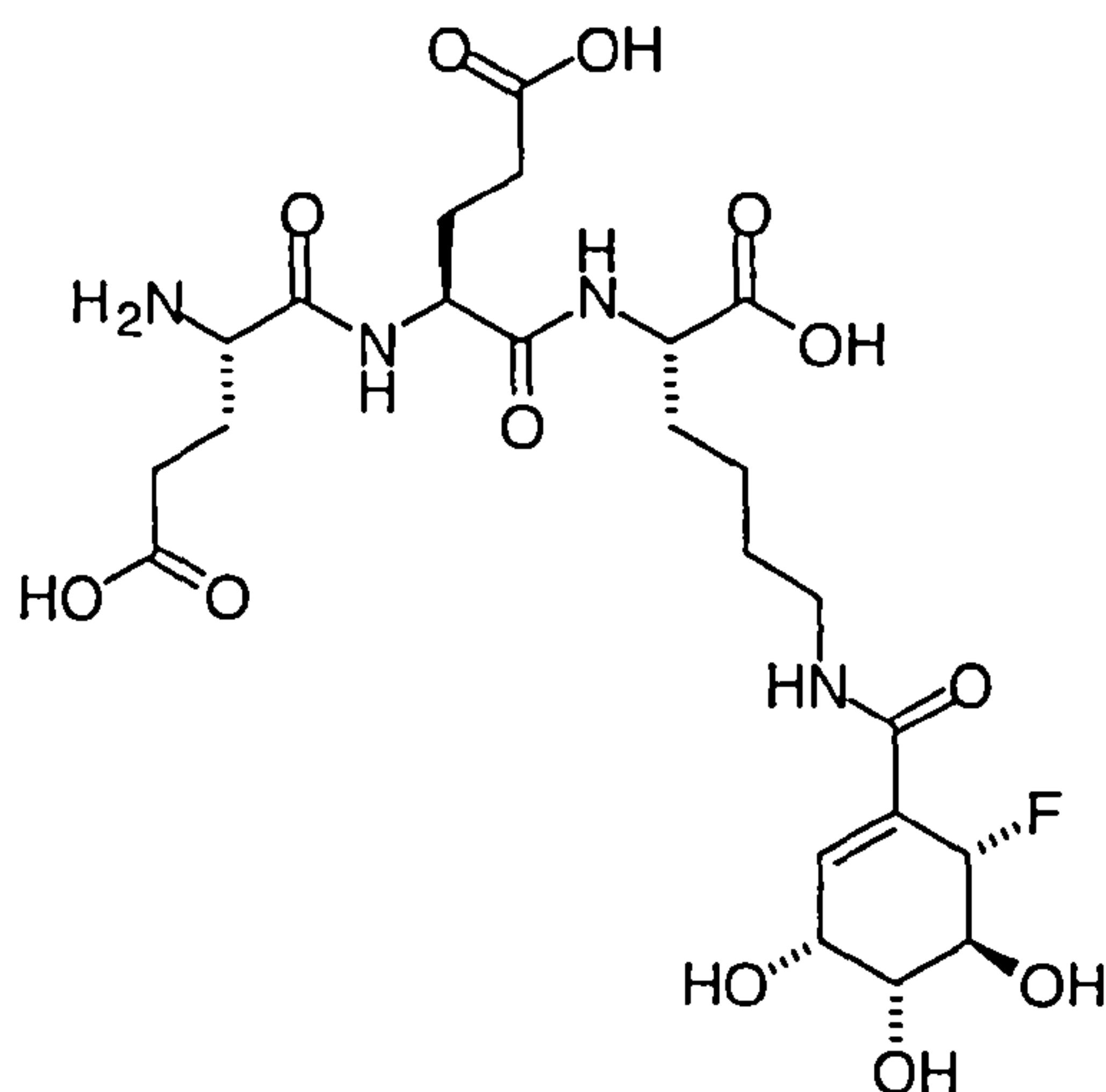
Using the method described in General Procedures (*Section 5.4.3*), Fmoc-Phe-OH (42 mg, 0.11 mmol) was coupled to Fmoc-Lys(Mtt)-Wang Resin (50 mg, 36  $\mu$ mol) using HOBt (15 mg, 0.11 mmol) and DIC (45 mg, 0.05 mL, 0.36 mmol) to give the resin-bound tripeptide after the second iteration. The protected (6*S*)-6-fluoroshikimic acid derivative **90** (38 mg, 0.11 mmol) was coupled to the tripeptide using BOP (120 mg, 0.28 mmol) and Et<sub>3</sub>N (0.28 mmol). A crude mixture containing the crude tripeptide-(6*S*)-6-fluoroshikimic acid conjugate **6** was obtained after cleavage from the resin and deprotection (crude yield: 87 mg). The desired conjugate eluted at 11.5 min on an RP Max HPLC column eluting with 3 - 50 % 50 mM formic acid in MeCN. The fractions containing the desired compound **6** were lyophilised and stored at -20°C. *m/z* (ES) 543.3 ([M-3H]<sup>+</sup>, 100%), 546.3 (M<sup>+</sup>, 5%)

## Synthesis of L-seryl-L-seryl-N- $\epsilon$ -(6*S*)-6-fluoroshikimoyl-L-lysine **7** on solid phase



Using the method described in General Procedures (*Section 5.4.3*), Fmoc-Ser(*t*Bu)-OH (41 mg, 0.11 mmol) was coupled to Fmoc-Lys(Mtt)-Wang Resin (50 mg, 36  $\mu$ mol) using HOBt (15 mg, 0.11 mmol) and DIC (45 mg, 0.05 mL, 0.36 mmol) to give the resin-bound tripeptide after the second iteration. The protected (6*S*)-6-fluoroshikimic acid derivative **90** (38 mg, 0.11 mmol) was coupled to the tripeptide using BOP (120 mg, 0.28 mmol) and Et<sub>3</sub>N (0.28 mmol). A crude mixture containing the crude tripeptide-(6*S*)-6-fluoroshikimic acid conjugate **7** was obtained after cleavage from the resin and deprotection (crude yield: 99 mg). *m/z* (ES) 495.3 ([M+H]<sup>+</sup>, 15%)

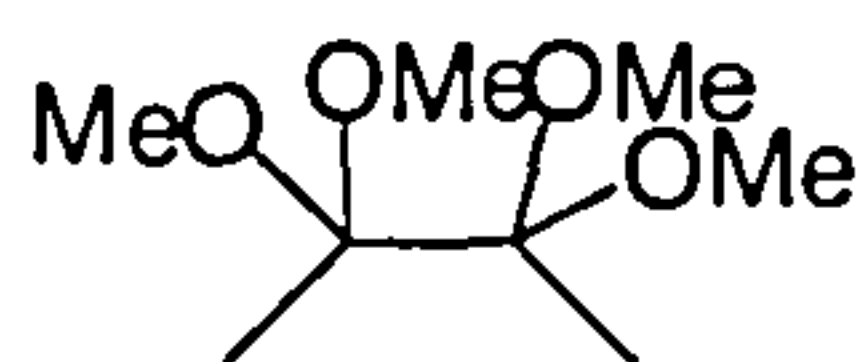
## Synthesis of L-glutamoyl-L-glutamoyl-N- $\epsilon$ -(6*S*)-6-fluoroshikimoyl-L-lysine **8** on solid phase



Using the method described in General Procedures (*Section 5.4.3*), Fmoc-Glu(O*t*Bu)-OH (46 mg, 0.11 mmol) was coupled to Fmoc-Lys(Mtt)-Wang Resin (50 mg, 36  $\mu$ mol) using HOBt (15 mg, 0.11 mmol) and DIC (45 mg, 0.05 mL, 0.36 mmol) to give the resin-bound tripeptide after the second iteration. The protected (6*S*)-6-fluoroshikimic acid derivative **90** (38 mg, 0.11 mmol) was coupled to the tripeptide using BOP (120 mg, 0.28 mmol) and Et<sub>3</sub>N (0.28 mmol). A crude mixture containing the crude tripeptide-(6*S*)-6-fluoroshikimic acid conjugate **8** was obtained after cleavage from the resin and deprotection (crude yield: 109 mg). *m/z* (ES) 579.3 ([*M*+*H*]<sup>+</sup>, 100%)

## 5.7 Experimental for Chapter 4

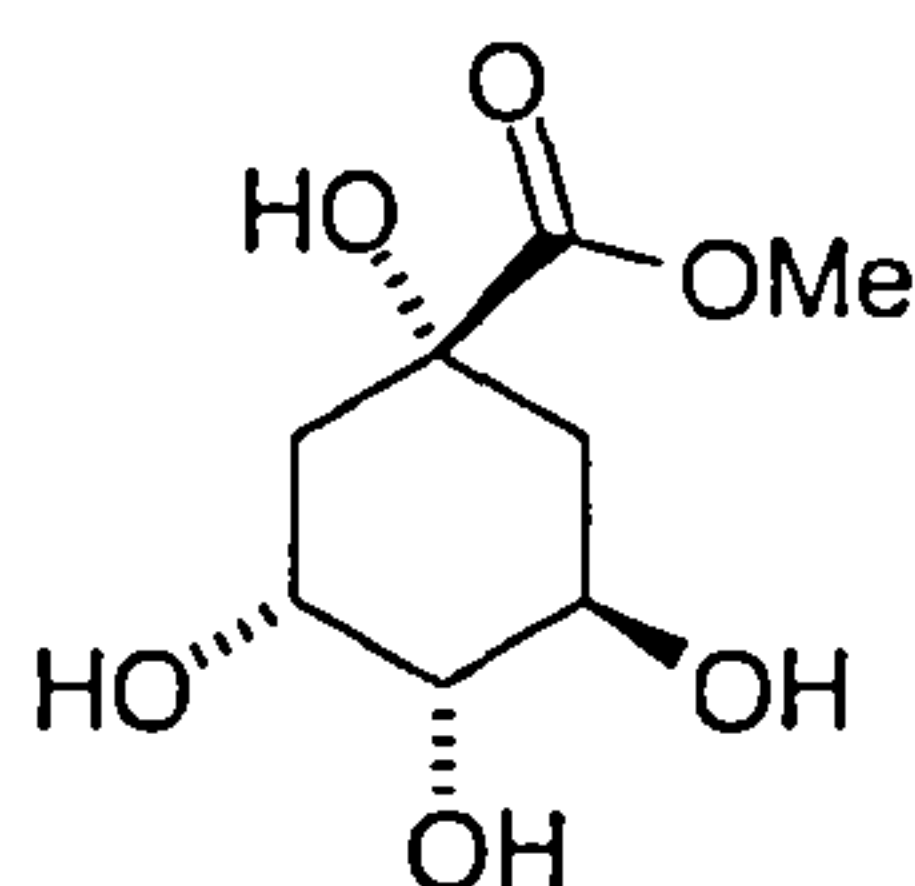
### 2,2,3,3-Tetramethoxybutane **108** (Montchamp *et al.*, 1996)



According to the method of Montchamp *et al.*, 2,3-butanedione **106** (22 mL, 0.25 mol), trimethyl orthoformate **107** (65 mL, 0.59 mol) and MeOH (32 mL) were treated with sulfuric acid (3 drops). The resulting solution was refluxed under N<sub>2</sub> for 20 h. Powdered NaHCO<sub>3</sub> (710 mg) was added to the cool reaction mixture which was then concentrated under reduced pressure to give a reddish-brown oil. This liquid was diluted with Et<sub>2</sub>O (150 mL) and washed with sat. NaHCO<sub>3</sub> (3 × 100 mL). The organic layer was dried (Na<sub>2</sub>SO<sub>4</sub>) and concentrated under reduced pressure to give an orange oil. The oil was distilled (T<sub>B</sub> 22°C, 0.2 mm Hg) to give a clear colourless liquid identical by <sup>1</sup>H- and <sup>13</sup>C-NMR to the literature compound **108** (20.0 g, 0.11 mol, 45%).

$\delta$ H(400 MHz; CDCl<sub>3</sub>) 3.32 (12H, s, 4 × OCH<sub>3</sub>), 1.33 (6H, s, 2 × CH<sub>3</sub>);  $\delta$ C(100 MHz; CDCl<sub>3</sub>) 103.3 (C), 49.6 (OCH<sub>3</sub>), 19.1 (CH<sub>3</sub>).

### Methyl quinate (Montchamp & Frost, 1994)

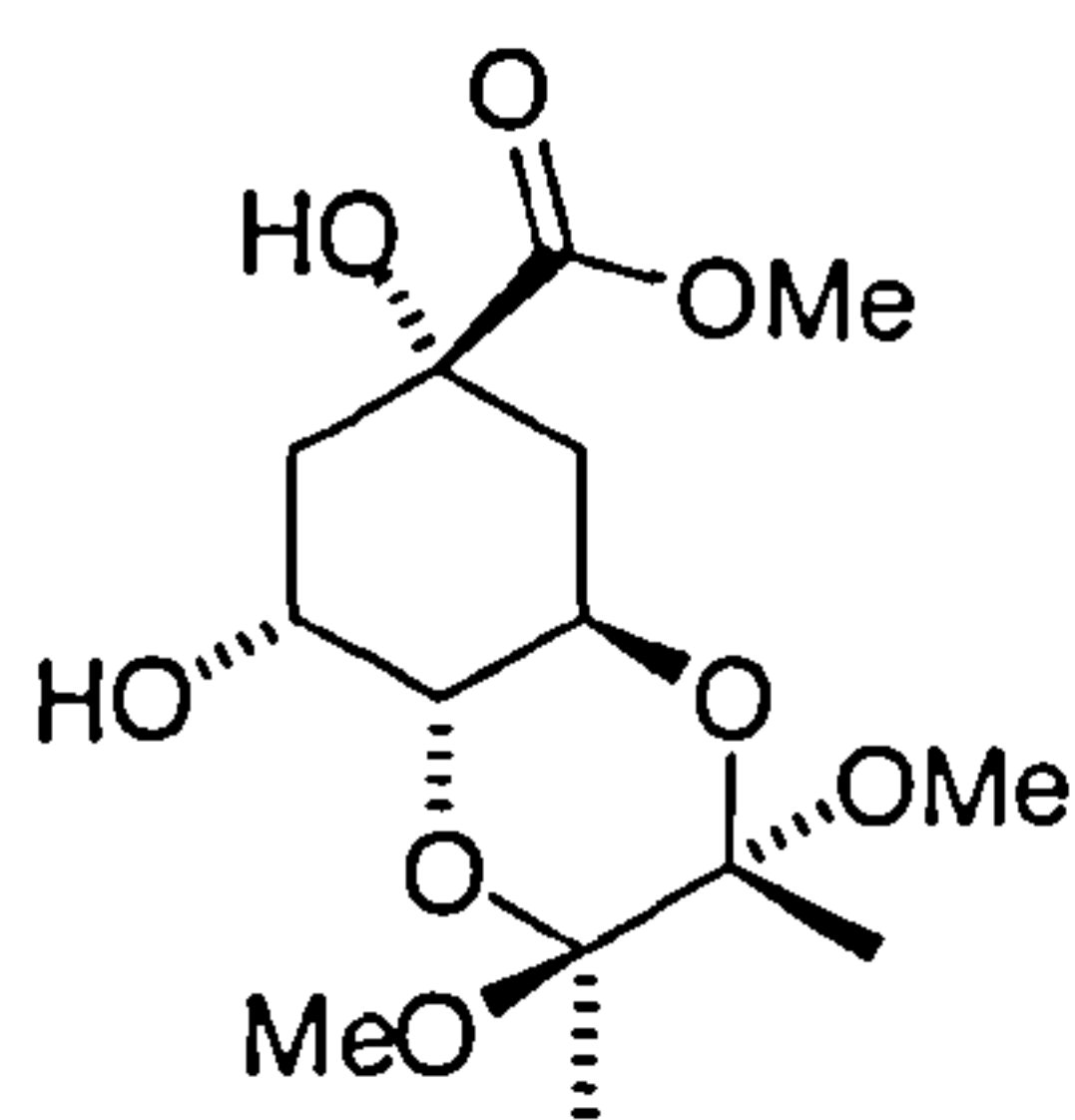


A mixture of (-)-quinic acid **19** (5.0 g, 26.0 mmol) and Dowex 50 H<sup>+</sup> (1 g) was refluxed for 14 h in dry MeOH (50 mL). After cooling, the mixture was filtered and concentrated under

reduced pressure to give a white solid (5.12 g, 24.5 mmol). This was recrystallised from EtOH to give the product as white crystals (4.87 g, 23.6 mmol, 90%)

m.p. 121-124°C [lit. (Montchamp & Frost, 1994) 127°C];  $\delta_{\text{H}}$ (400 MHz; CD<sub>3</sub>OD) 4.07-4.06 (1H, m, CH), 4.01-3.95 (1H, m, CH), 3.72 (3H, s, OMe), 3.41 (1H, dd, *J* 3 and 9, CH), 2.12-1.82 (4H, m, 2-CH<sub>2</sub> and 6-CH<sub>2</sub>);  $\delta_{\text{C}}$ (100 MHz; CD<sub>3</sub>OD) 176.3 (C=O), 77.2 (CCO<sub>2</sub>CH<sub>3</sub>), 76.9 (CO<sub>2</sub>CH<sub>3</sub>), 71.8 (CH), 68.6 (CH), 53.3 (CH), 42.4 (CH<sub>2</sub>), 38.7 (CH<sub>2</sub>); *m/z* (FAB<sup>+</sup>) 435.1 ([2M+Na]<sup>+</sup>, 7%), 413.1 ([M+Na]<sup>+</sup>, 18%), 229 ([M+Na]<sup>+</sup>, 17%), 207.0 (100%), 171.0 (23%), 111.2 (14%).

#### Methyl 3,4-O-(2,3-dimethoxybutan-2,3-diyl) quinate **97**



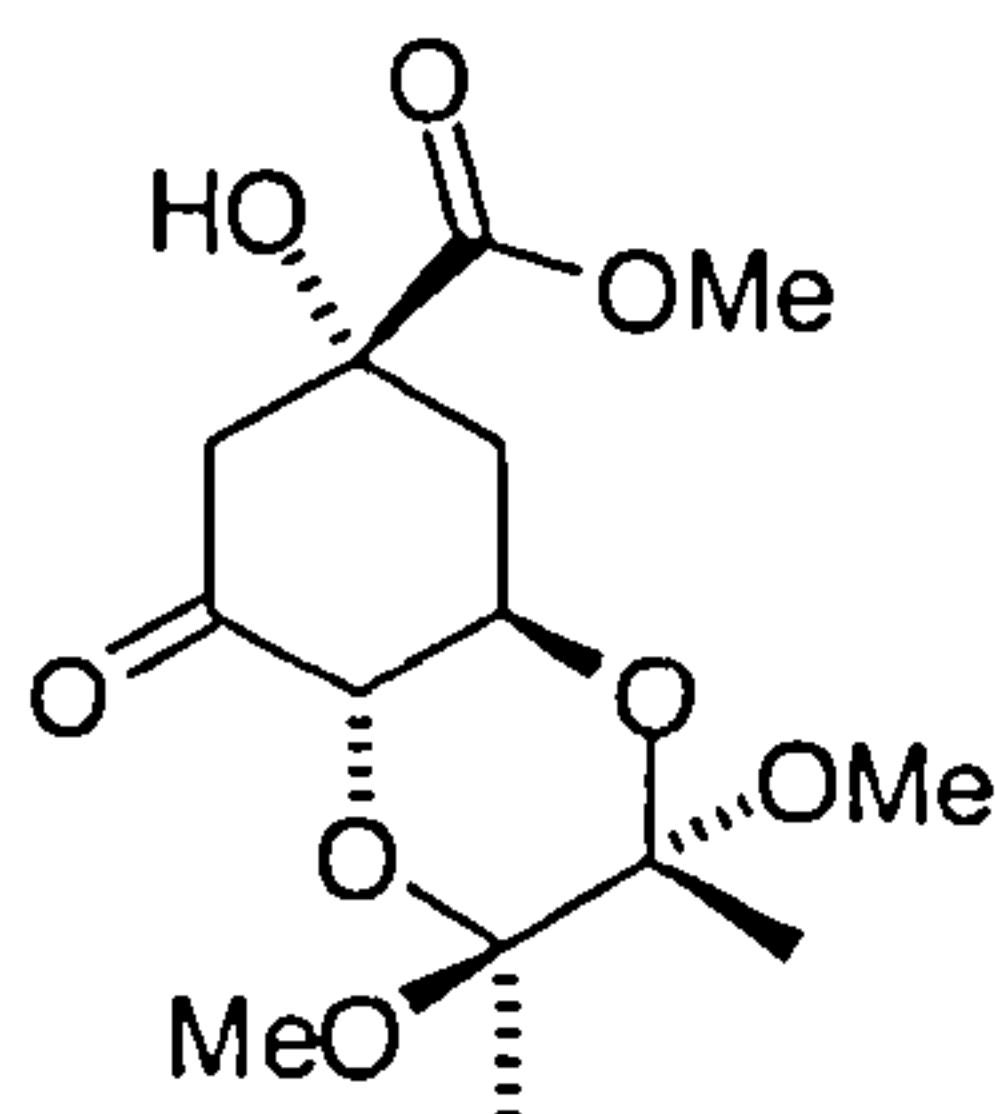
In a modification of the procedure detailed by Montchamp & Frost in 1994, quinic acid **19** (2.7 g, 14.1 mmol), trimethyl orthoformate **107** (7.2 mL, 6.98 g, 65.8 mmol), TMB **108** (2.54 g, 14.3 mmol) and *p*TsOH (135 mg, 0.71 mmol, 5 mol%) were dissolved in dry MeOH (40 mL). The mixture was refluxed under N<sub>2</sub> for 20 h, allowed to cool, and treated with powdered NaHCO<sub>3</sub> (500 mg). The solvent was removed under reduced pressure to give an orange oil. This oil was partitioned between EtOAc (100 mL) and sat. NaHCO<sub>3</sub> (100 mL) and the aqueous layer extracted with EtOAc (2× 100 mL). The combined organic extracts were dried over MgSO<sub>4</sub>, filtered through silica, and concentrated under reduced pressure to give a cream solid (3.83 g). The product was dissolved in hot EtOAc, cooled, and crystallised by the dropwise addition of hexane to give white needles (3.15 g, 9.85



mmol, 70 %).

m.p. 131-133°C [lit. (Montchamp & Frost, 1994) 139-140°C];  $\nu_{\max}(\text{NaCl})/\text{cm}^{-1}$  3577 (broad), 3503 (broad), 2997 (sharp), 2955 (broad), 2905 (broad), 2835, 1736 (C=O), 1451;  $\delta_{\text{H}}(400 \text{ MHz}; \text{CDCl}_3)$  4.31 (1H, ddd  $J$  4.6, 10.2, 12.2, CH), 4.25 (1H, s, CH), 4.19 (1H, q  $J$  3.1, CH), 3.79 (3H, s, OCH<sub>3</sub>), 3.60 (1H, dd  $J$  2.9 and 10.1, CH), 3.26 (2 × 3H, s, OCH<sub>3</sub>), 3.15 (1H, dd  $J$  0.7 and 3.8, CH), 2.19 (1H, dt  $J$  3.0 and 14.9, CH), 2.10 (1H, ddd  $J$  2.9, 4.6, 12.8, CH<sub>2</sub>), 2.04 (1H, m, CH<sub>2</sub>), 1.95 (1H, t  $J$  12.4, CH<sub>2</sub>), 1.83 (1H, s, CH<sub>2</sub>), 1.34 (3H, s, CCH<sub>3</sub>), 1.30 (3H, s, CCH<sub>3</sub>);  $\delta_{\text{C}}(100 \text{ MHz}; \text{CDCl}_3)$  174.0 (C=O), 138.5 (OCCO<sub>2</sub>Me), 100.4 (OCOMe), 99.8 (OCOMe), 75.8 (C), 72.7 (CO<sub>2</sub>CH<sub>3</sub>), 69.2 (OCH<sub>3</sub>), 62.4 (OCH<sub>3</sub>), 53.0 (OCH), 47.9 (OCH), 38.6 (CH<sub>3</sub>), 37.4 (CH<sub>3</sub>), 17.8 (CH<sub>2</sub>), 17.7 (CH<sub>2</sub>);  $m/z$  (Cl<sup>+</sup>) 338.2 ([M+NH<sub>4</sub>]<sup>+</sup>, 100%), 306.2 ([M+NH<sub>4</sub>-OMe]<sup>+</sup>, 80%), 289.2 ([M-OMe]<sup>+</sup>, 55%), 274.1 (20%).

**Methyl 3,4-*O*-(2,3-dimethoxybutan-2,3-diyl) 5-dehydroquinate 98** (Montchamp *et al.*, 1996b)

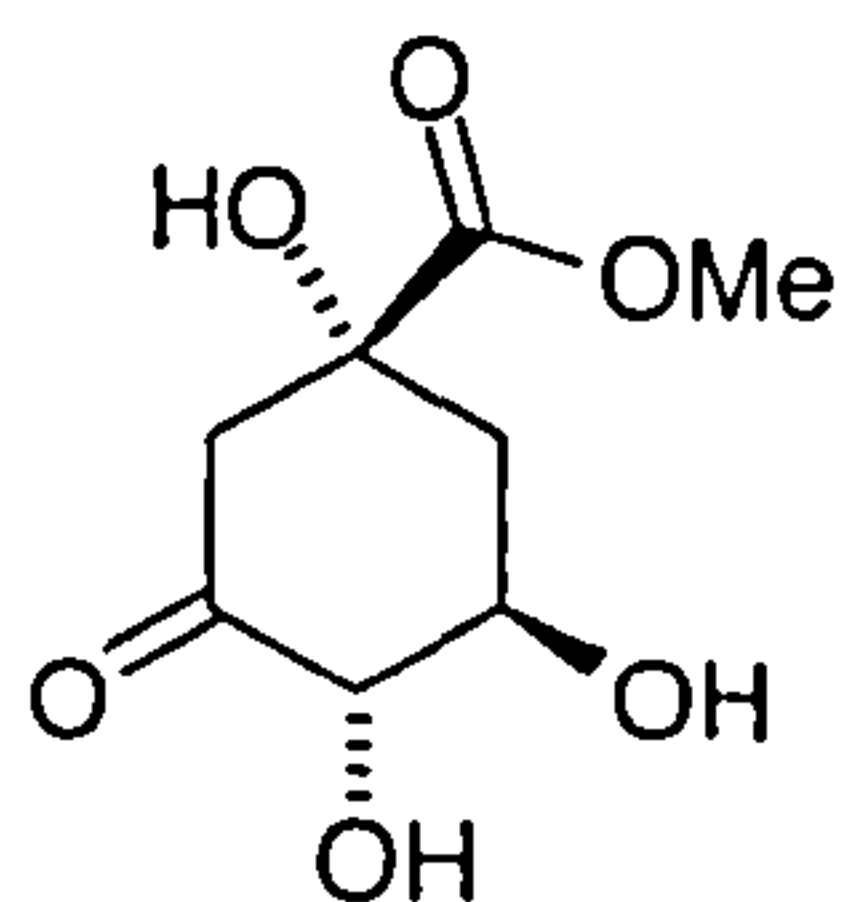


The protected methyl quinate derivative **97** (3.12 g, 9.74 mmol) was dissolved in CCl<sub>4</sub> (20 mL). To this was added MeCN (20 mL), H<sub>2</sub>O (30 mL), KIO<sub>4</sub> (9.0 g), and RuCl<sub>3</sub>·H<sub>2</sub>O (150 mg). The suspension was stirred vigorously at room temperature for 20 h. The reaction mixture was filtered through celite, the solid material washed with CCl<sub>4</sub> (20 mL) and the aqueous and organic layers were separated. The aqueous layer was saturated with NaCl and extracted with EtOAc (3 × 80 mL). The combined organic extracts were dried

(MgSO<sub>4</sub>) and concentrated. The crude material was redissolved in CHCl<sub>3</sub> (70 mL) and filtered through celite to remove all remaining ruthenium species. The solution was dried (MgSO<sub>4</sub>), filtered and concentrated to give a white foaming solid **98** (2.45 g, 7.69 mmol, 79 %).

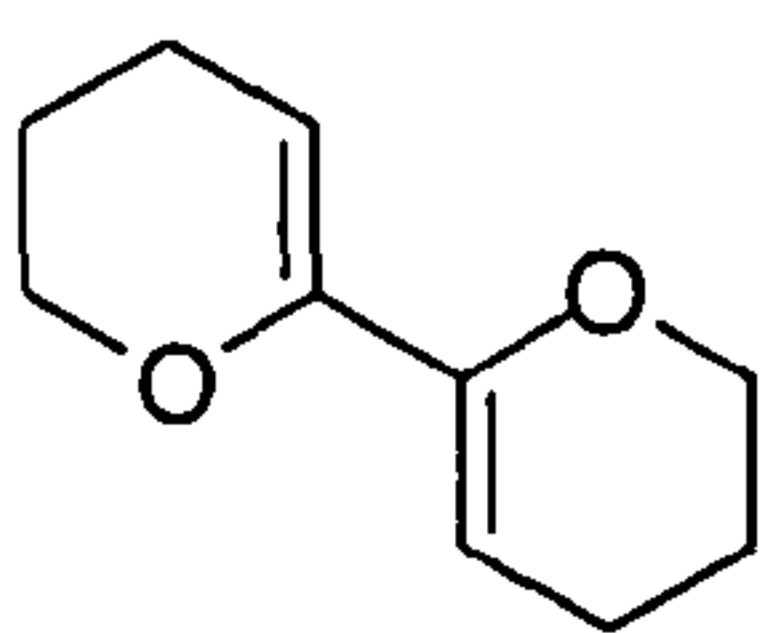
m.p. 209-213°C [lit (Montchamp *et al.*, 1996b). 212-214°C];  $\delta_{\text{H}}$ (400MHz; CDCl<sub>3</sub>) 4.42 (1H, dd, *J* 0.8, 10, CHO), 4.27 (1H, ddd *J* 4.4, 10, 12, CHO), 3.85 (3H, s, CO<sub>2</sub>Me), 3.27 (3H, s, COMe), 3.23 (3H, s, COMe), 2.90 (1H, dd *J* 1.2 and 14.4, CH<sub>2</sub>), 2.51 (1H, dd *J* 2.8 and 14.4, CH<sub>2</sub>), 2.36 (1H, t *J* 12.6, CH<sub>2</sub>), 2.12 (1H, ddd *J* 3.2, 4.4, 13.2, CH<sub>2</sub>), 1.40 (3H, s, CCH<sub>3</sub>), 1.30 (3H, s, CCH<sub>3</sub>);  $\delta_{\text{C}}$ (100MHz; CDCl<sub>3</sub>) 199.5 (C=O), 174.0 (C=O), 100.4 (OCOMe), 99.6 (OCOMe), 77.2 (CO<sub>2</sub>CH<sub>3</sub>), 74.0 (HOCCO<sub>2</sub>Me), 67.0 (CH), 53.6 (CH), 49.0 (CH<sub>2</sub>), 48.3 (CH<sub>3</sub>), 48.0 (CH<sub>3</sub>), 38.2 (CH<sub>2</sub>), 17.6 (CH<sub>3</sub>), 17.3 (CH<sub>3</sub>); *m/z* (Cl<sup>+</sup>) 336.2 ([M+NH<sub>4</sub>]<sup>+</sup>, 100%), 304.1 ([M+NH<sub>4</sub>-OMe]<sup>+</sup>, 60%), 287.1 ([M-OMe]<sup>+</sup>, 35%), 272.1 (15%).

### Methyl 3-dehydroquinate 109



The protected dehydroquinate derivative **98** (100 mg, 0.3 mmol) was dissolved in CH<sub>2</sub>Cl<sub>2</sub> (5 mL) and H<sub>2</sub>O (0.2 mL). The mixture was stirred at -5°C for 5 min. CF<sub>3</sub>CO<sub>2</sub>H (4.8 mL) was added and the mixture stirred for a further 15 min to give a yellow solution. This solution was concentrated under reduced pressure to give a colourless oil (91 mg). The oil was extracted into CD<sub>3</sub>OD and <sup>1</sup>H NMR showed the presence of the desired compound.  $\delta_{\text{H}}$ (400MHz; CD<sub>3</sub>OD) 4.16 (1H, d *J* 9.2), 3.99-3.90 (1H, m), 3.82 (3H, s, OCH<sub>3</sub>), 3.07 (1H, dd *J* 0.8 and 14), 2.56 (1H, dd *J* 3.2 and 14.4), 2.33-2.20 (2H, m).

**3,3',4,4'-Tetrahydro-6,6'-bi-2*H*-pyran 113** (Ley *et al.*, 1992)

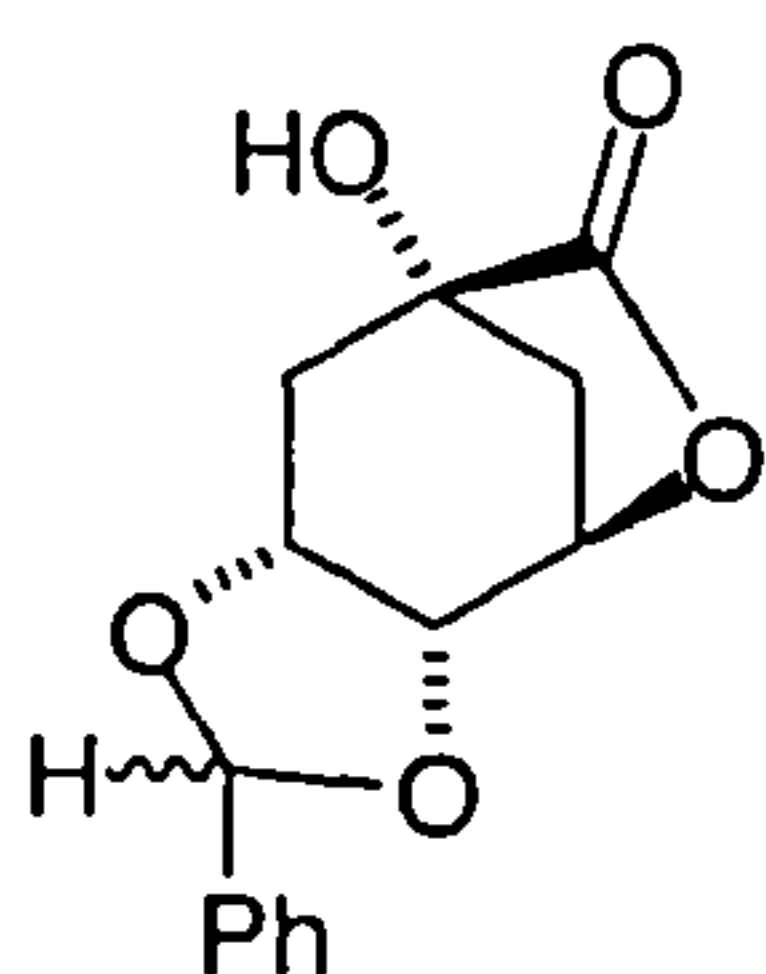


*t*BuLi (1.7M in pentanes, 14 mL, 24 mmol) was added dropwise to a solution of dry, distilled 3,4-dihydro-2*H*-pyran 115 (2 mL, 22 mmol) in THF (4 mL) at  $-78^{\circ}\text{C}$  under  $\text{N}_2$ . The cloudy mixture was stirred for 1 h at  $0^{\circ}\text{C}$  to give a clear, pale-yellow solution. This solution was added via cannula to a cooled ( $0^{\circ}\text{C}$ ), rapidly stirred slurry of  $\text{Pd(II)Cl}_2(\text{MeCN})_2$  (140 mg, 0.54  $\mu\text{mol}$ ) and anhydrous  $\text{CuCl}_2$  (3.1 g, 24 mmol) in THF (20 mL). The resulting black slurry was stirred at  $0^{\circ}\text{C}$  for 1 h. The reaction was quenched by addition of sat.  $\text{NH}_4\text{Cl}/0.88$  ammonia solution (4:1, pH 10, 50 mL) and extracted into ether ( $3 \times 100$  mL). The combined ether extracts were dried ( $\text{MgSO}_4$ ), filtered and concentrated under reduced pressure to give a yellow solid (500 mg). The crude material was purified by silica gel chromatography (10%  $\text{Et}_2\text{O}$ -hexane) and crystallised from petroleum ether to give white crystals (342 mg, 2.1 mmol, 19%).

m.p.  $47-49^{\circ}\text{C}$  [lit (Ley *et al.*, 1992)  $49-50^{\circ}\text{C}$ ];  $\delta_{\text{H}}$ (400 MHz;  $\text{CDCl}_3$ ) 5.18 (2H, t, *J* 4, 5-H and 5'-H), 4.06-4.03 (4H, m, 2- $\text{CH}_2$  and 2'- $\text{CH}_2$ ), 2.18-2.10 (4H, m, 4- $\text{CH}_2$  and 4'- $\text{CH}_2$ ), 1.87-1.57 (4H, m, 3- $\text{CH}_2$  and 3'- $\text{CH}_2$ );  $\delta_{\text{C}}$ (100 MHz;  $\text{CDCl}_3$ ) 148.0 (C), 97.2 (CH), 66.6 ( $\text{CH}_2$ ), 22.8 ( $\text{CH}_2$ ), 20.6 ( $\text{CH}_2$ ).



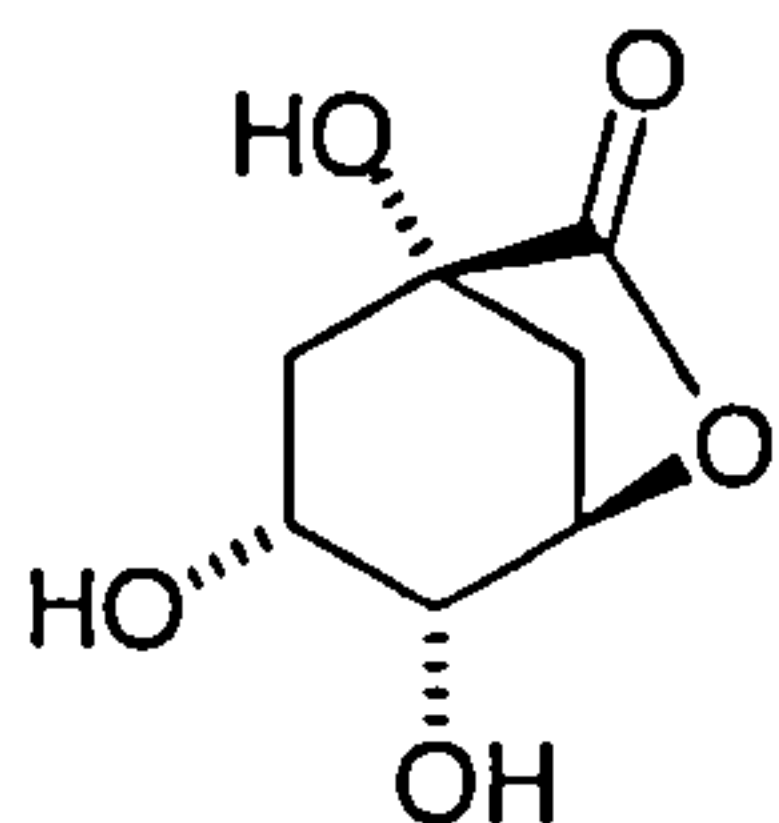
**(1*S*,3*R*,4*R*,5*R*)-4,5-benzylidenedioxy-1-hydroxycyclohexane-1,3-carbolactone 101**



In a modification of the procedure detailed by Manthey *et al.*, 1997; benzene (125 mL) was heated to reflux in a Dean-Stark apparatus for 2 h and allowed to cool to rt. To this dried solvent was added freshly distilled benzaldehyde (4 mL, 39.4 mmol), quinic acid **19** (5.0 g, 26 mmol) and *p*TsOH (250 mg, 1.31 mmol) and the mixture was heated to reflux. After 7 h the solvent was removed under reduced pressure. The residue was diluted with Et<sub>2</sub>O (200 mL) and decanted from the solid. The solvent was removed under reduced pressure to give the crude product as a clear oil (5.31 g). The mixture was purified by silica gel column chromatography on 1 L silica (gradient Et<sub>2</sub>O: Petrol, 1:1 to neat Et<sub>2</sub>O to give the desired compound **101** as a mixture of epimers at the benzylic centre in the form of a white crystalline solid in a colourless oil (2.38 g, 9.1 mmol, 35 %). The major epimer was obtained by recrystallisation from Et<sub>2</sub>O.

m.p. 105-107°C [lit. (Manthey *et al.*, 1997) 100–101°C];  $\delta_{\text{H}}$ (400 MHz; CDCl<sub>3</sub>) 7.52-7.42 (5H, m, Ph), 5.78 (1H, s, PhCHO<sub>2</sub>), 4.83 (1H, dd, *J* 6.3 and 2.2), 4.56 (1H, td, *J* 7.0 and 2.5), 4.40 (1H, ddd, *J* 6.9, 2.1 and 1.5), 3.02 (1H, s), 2.80 (1H, d, *J* 12.0), 2.48 (1H, ddd, *J* 15.1, 7.5 and 2.1), 2.38 (1H, dd, *J* 15.1 and 2.6), 2.37 (1H, ddt, *J* 11.9, 6.3 and 1.9);  $\delta_{\text{C}}$ (100 MHz; CDCl<sub>3</sub>) 178.8 (C), 135.4 (C–Ar), 129.9 (CH–Ar), 128.6 (CH–Ar), 126.6 (CH–Ar), 103.8 (OCHPh), 75.6 (3-CH), 73.0 (CHOH), 72.7 (CHOH), 71.4 (1-COH), 37.7 (CH<sub>2</sub>), 34.4 (CH<sub>2</sub>); *m/z* (EI<sup>+</sup>) 262 (M<sup>+</sup>, 70%), 261.1 ([M–H]<sup>+</sup>, 100%), 105.1 ([PhCHO]<sup>+</sup>, 50%).

**(1S,3R,4R,5R)-1,4,5-trihydroxycyclohexane-1,3-carbolactone 102**



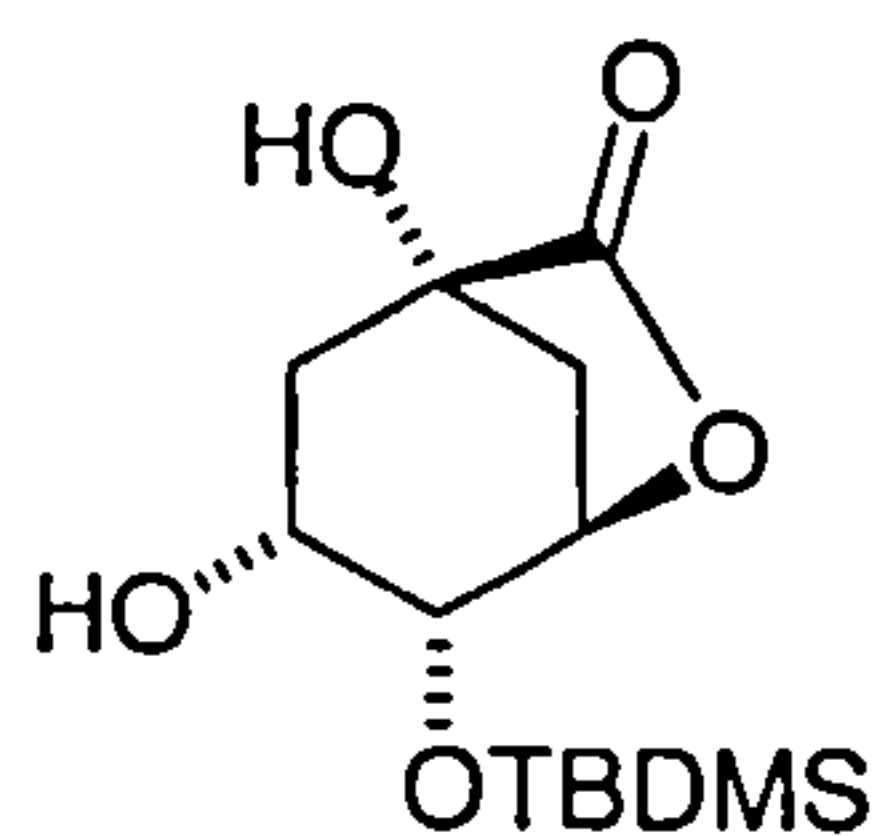
Benzene (150 mL) was heated to reflux in a Dean-Stark apparatus for 2h and allowed to cool to rt. Quinic acid **19** (10.0 g, 52.0 mmol) and *p*TsOH (1.0 g, 5.8 mmol) were added and the suspension heated to reflux for 20 h. The benzene was removed under reduced pressure and the residue washed with EtOAc (200 mL). The EtOAc was removed by filtration and the residue dried under high vacuum to give a yellow solid (8.75 g). NMR analysis showed that 89 % of the material was the desired lactone, giving a yield of 7.79 g (44.8 mmol, 86 %).

m.p. 181-183°C [lit. (Manthey *et al.*, 1997) 184-185°C];  $\delta_H$ (400 MHz; CD<sub>3</sub>OD) 4.90 (3H, s, 3 × OH), 4.74 (1H, dd, *J* 5.12), 4.02 (1H, t, *J* 4.59), 3.74 (1H, ddd, *J* 11.44, 6.57 and 4.48), 2.55 (1H, d, *J* 11.40), 2.26 (1H, ddd, *J* 11.36, 6.02 and 2.90), 2.07 (1H, dddd, *J* 11.75, 6.61, 2.99 and 0.51), 1.91 (1H, t, *J* 11.60);  $\delta_C$ (100 MHz; CD<sub>3</sub>OD) 179.87 (CO<sub>2</sub>), 78.27 (CH), 73.50 (C[OH]CO<sub>2</sub>), 67.73 (CHOH), 67.23 (CHOH), 40.50 (CH<sub>2</sub>), 38.23 (CH<sub>2</sub>); *m/z* (Isobutane Cl<sup>+</sup>) 175.08 ([M+H], 100%), 157.07 (9%), 139.07 (8%), 111.07 (11%), 79.02 (10%).



**(1*S*,3*R*,4*R*,5*R*)-4-*tert*-butyldimethylsiloxy-1,5-dihydroxycyclohexane-1,3-carbolactone**

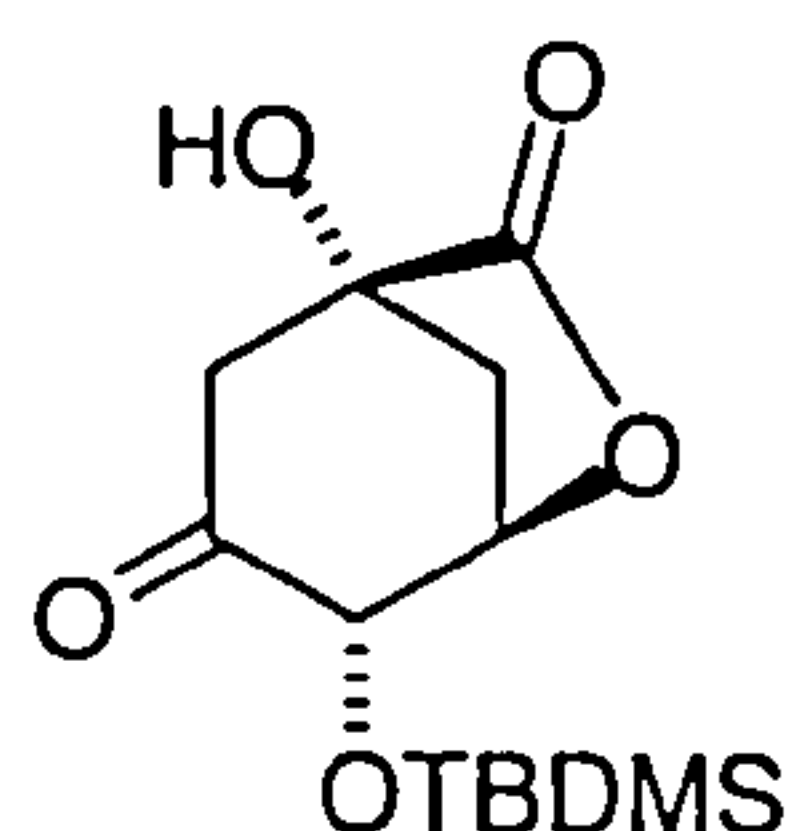
**104** (Manthey *et al.*, 1997)



The lactone **102** (1.68 g, 9.6 mmol) was dissolved in dry DMF (20 mL). To this solution was added DMAP (215 mg, 1.8 mmol), Bu<sub>4</sub>NI (220 mg, 0.6 mmol), Et<sub>3</sub>N (2.0 mL, 14.3 mmol) and TBDMSCl (1.68 g, 11.1 mmol). The mixture was heated to 90°C for 22 h, and allowed to cool to rt. The solution was diluted with EtOAc (200 mL) and filtered through celite. The filtrate was washed with 2M HCl (200 mL) and brine (3 × 200 mL), dried (MgSO<sub>4</sub>), filtered and concentrated to give the crude product as a yellow oil (3.46 g). The material was purified by silica gel column chromatography (800 mL silica, gradient elution DCM-2% acetone to 10% acetone) to give the desired compound **104** as a white solid (1.23 g, 4.3 mmol, 45%).

m.p. 148-152°C [lit. (Manthey *et al.*, 1997) 154-155°C];  $\delta_{\text{H}}$ (400 MHz; CDCl<sub>3</sub>) 4.68 (1H, dd, *J* 5.7 and 5.1), 4.11 (1H, t, *J* 4.6), 3.87–3.78 (1H, m), 2.8 (1H, s), 2.53 (1H, d, *J* 11.5), 2.30 (1H, ddd, *J* 11.4, 6.1 and 3.0), 2.18 (1H, ddd, *J* 12.2, 6.6 and 3.0), 1.85 (1H, t, *J* 11.6), 0.95 [9H, s, SiC(CH<sub>3</sub>)<sub>3</sub>], 0.17 (3H, s, SiCH<sub>3</sub>), 0.14 (3H, s, SiCH<sub>3</sub>);  $\delta_{\text{C}}$ (100 MHz; CDCl<sub>3</sub>) 177.6 (CO<sub>2</sub>), 76.5 (CH), 71.9 [C(OH)CO<sub>2</sub>], 66.7 (CH), 66.1 (CH), 40.6 (CH<sub>2</sub>), 36.3 (CH<sub>2</sub>), 25.6 [SiC(CH<sub>3</sub>)<sub>3</sub>], 18.0 (SiCMe<sub>3</sub>), -4.6 (SiCH<sub>3</sub>), -4.9 (SiCH<sub>3</sub>)

**(1S,3R,4R,5R)-4-tert-butyldimethylsiloxy-1-hydroxy-5-oxocyclohexane-1,3-carbolactone 105** (Manthey *et al.*, 1997)

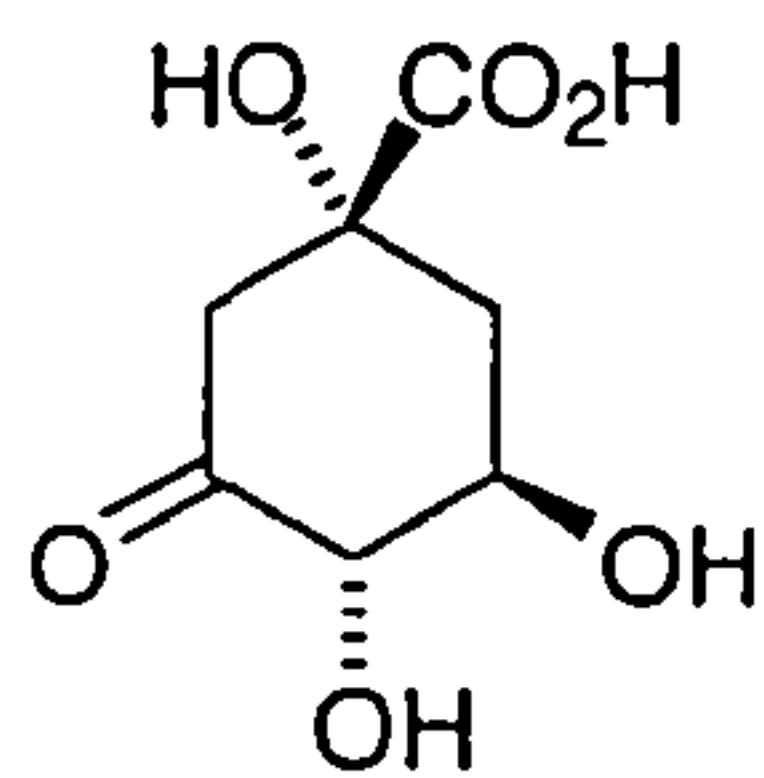


3Å molecular sieves (2.0 g) were heated under high vacuum for 10 min and allowed to cool to rt. Dry DCM (20 mL), PDC (2.5 g, 6.65 mmol) and the protected lactone **104** (0.90 g, 3.12 mmol) were added and the suspension stirred under N<sub>2</sub> at rt. After 5h no further change in TLC was visible and the mixture was diluted with Et<sub>2</sub>O (150 mL) and filtered (celite). The resulting solution was washed with 1M HCl (150 mL) and brine (2 × 150 mL), dried (MgSO<sub>4</sub>), filtered and evaporated to give the crude product. A flash column (2 cm × 6 cm silica, 400 mL Et<sub>2</sub>O) gave a white solid in a yellow oil (0.75 g). Crystallisation (petrol) yielded the desired protected 3-DHQ **105** as fine white needle-shaped crystals (0.41 g, 1.45 mmol, 46%)

m.p. 93-94°C, [lit. (Manthey *et al.*, 1997) 92-93°C];  $\delta_{\text{H}}$ (400 MHz; CDCl<sub>3</sub>) 4.71 (1H, dd, *J* 6.21 and 3.93), 3.99 (1H, d, *J* 3.68), 3.23 (1H, s), 3.03 (1H, d, *J* 17.06), 2.79 (1H, d, *J* 12.16), 2.74 (1H, dd, *J* 17.11 and 2.35), 2.61 (1H, ddd, *J* 12.10, 6.05 and 2.83), 0.89 [9H, s, SiC(CH<sub>3</sub>)<sub>3</sub>], 0.16 (3H, s, SiCH<sub>3</sub>), 0.11 (3H, s, SiCH<sub>3</sub>);  $\delta_{\text{C}}$ (100 MHz; CDCl<sub>3</sub>) 203.17 (CO), 177.51 (CO<sub>2</sub>), 75.47 (CH), 71.81 [C(OH)CO<sub>2</sub>], 71.26 (CH), 50.36 (CH<sub>2</sub>), 36.20 (CH<sub>2</sub>), 25.91 [SiC(CH<sub>3</sub>)<sub>3</sub>], 18.42 (SiCMe<sub>3</sub>), -4.49 (SiCH<sub>3</sub>), -4.85 (SiCH<sub>3</sub>); *m/z* (Isobutane Cl<sup>+</sup>) 287.1 ([M+H], 100%), 269.1 (10%), 241.1 (8%), 229.1 (85%), 201.1 (10%), 157.1 (30%), 129.1 (7%), 75.0 (10%).

**(1*S*,3*R*,4*R*,5*R*)-1,4-hydroxy-5-oxocyclohexane-carboxylic acid (3-dehydroquinic acid)**

**10**



The protected DHQ derivative **105** (1.73 g, 6.0 mmol) was dissolved in water (40 mL) and acetic acid (10 mL) and heated to 50°C. After 48 h the solution was cooled to rt and the solvent removed by freeze-drying overnight. The crude material was dissolved in H<sub>2</sub>O (200 mL) and washed with EtOAc (2 × 200 mL). The water was removed by freeze-drying overnight. The washing and freeze drying was repeated to finally give 3-dehydroquinic acid **10** as a white solid/glass (1.04 g, 5.47 mmol, 91%). The material was dissolved in water and aliquoted out to give 20 mg portions after freeze-drying.

$\delta_{\text{H}}$ (400 MHz; D<sub>2</sub>O) 4.12 (1H, dd, *J* 9.53 and 0.59), 3.75 (1H, dt, *J* 9.43 and 7.14), 2.99 (1H, dd, *J* 14.37 and 0.70), 2.42 (1H, dt, *J* 14.34 and 1.56), 2.17-2.14 (2H, m);  $\delta_{\text{C}}$ (100 MHz; D<sub>2</sub>O) 208.44 (CO), 176.79 (CO<sub>2</sub>), 80.95 (CH), 74.38 (C[OH]CO<sub>2</sub>), 71.64 (CH), 47.84 (CH<sub>2</sub>), 39.86 (CH<sub>2</sub>).

### **Steady-state kinetic investigation of *B. subtilis* F28Y type II dehydroquinase**

The assays were carried out as described in 'Enzyme Assays' (Section 5.4.1).

The concentration of enzyme in the stock solution was determined by calculation based on an extinction coefficient of 0.465 at 280 nm for the enzyme. All reactions involved 40  $\mu$ g of the enzyme.

Kinetic data was obtained by varying the concentration of substrate between 100  $\mu$ M and 2 mM. The initial rate data obtained was fitted to the Michaelis-Menten equation by non-linear regression using Microcal Origin software. The value of  $k_{cat}$  was calculated using  $M_r$  18 000 for the *B. subtilis* F28Y enzyme subunit.

At 20°C in 50 mM Tris.acetate buffer pH 7.0,  $K_m$  622  $\mu$ M= and  $k_{cat}$  = 0.22 s<sup>-1</sup>

### **Steady-state kinetic investigation of *H. pylori* type II dehydroquinase**

Assays were carried out at 20°C in 50 mM Tris.acetate buffer pH 7.0.

The assays were carried out as described in 'Enzyme Assays' (Section 5.4.1).

The concentration of enzyme in the stock solution was determined by calculation based on an extinction coefficient of 0.25 at 280 nm for the enzyme. All reactions involved 150  $\mu$ g of the enzyme.

Kinetic data was obtained by varying the concentration of substrate between 50  $\mu$ M and 1 mM. The initial rate data obtained was fitted to the Michaelis-Menten equation by non-linear regression using Microcal Origin software. The value of  $k_{cat}$  was calculated using  $M_r$  20 000 for the *H. pylori* enzyme subunit.

At 20°C in 50 mM Tris.acetate buffer pH 7.0,  $K_m$  251  $\mu$ M= and  $k_{cat}$  = 19.4 s<sup>-1</sup>

## Stopped-flow kinetic investigation of *B. subtilis* F28Y and *H. pylori* type II dehydroquinases

Assays were carried out at 13°C in 50 mM Tris.acetate buffer pH 7.0.

The concentration of DHQ was determined according to the published method (Kleanthous *et al.*, 1991) using *S. coelicolor* type II dehydroquinase to convert DHQ to DHS.

The type II dehydroquinase (either 1 mg or 2 mg in 1 mL buffer) was mixed rapidly with DHQ (approx. 0.1 mM, 10 mL) in the stopped flow apparatus.

Observation of the graphical output of the apparatus showed no lag phase in the reaction involving either *B. subtilis* F28Y or *H. pylori* type II dehydroquinase.

The apparatus and results were validated by calculating the  $K_m$  and  $k_{cat}$  for the *B. subtilis* F28Y enzyme.

At 13°C in 50 mM Tris.acetate buffer pH 7.0  $K_m = 300 \mu\text{M}$  and  $k_{cat} = 0.085 \text{ s}^{-1}$ . This is within the expected range for the enzyme taking into account the low temperature at which the assay was carried out.



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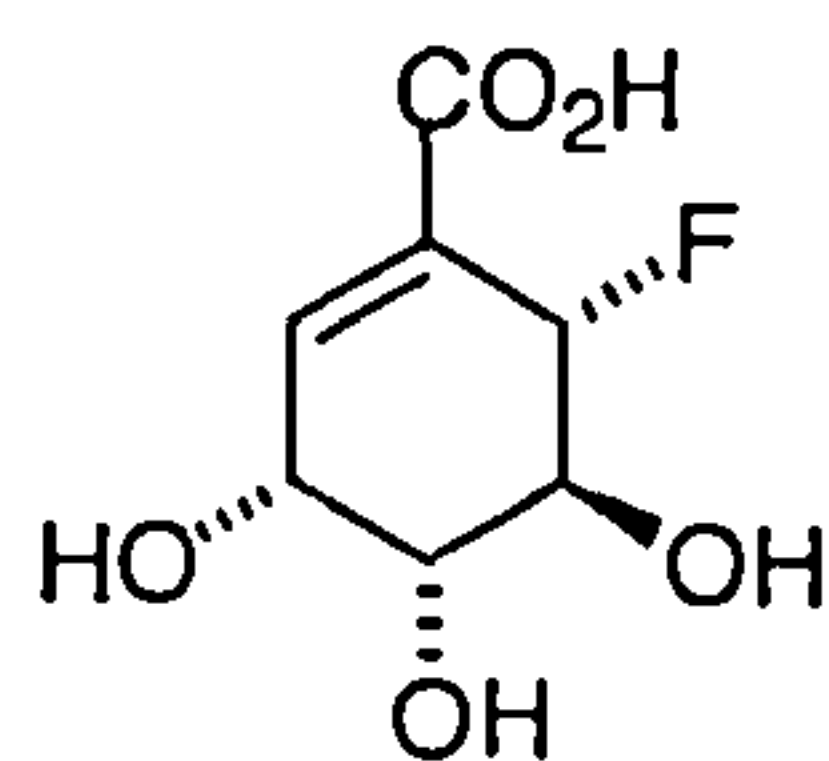
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## Appendices

### Appendix 1: (6S)-6-Fluoroshikimic acid 1



$^1\text{H-NMR}$  ( $\text{CD}_3\text{OD}$ , 400 MHz)

$m/z$  (electrospray)

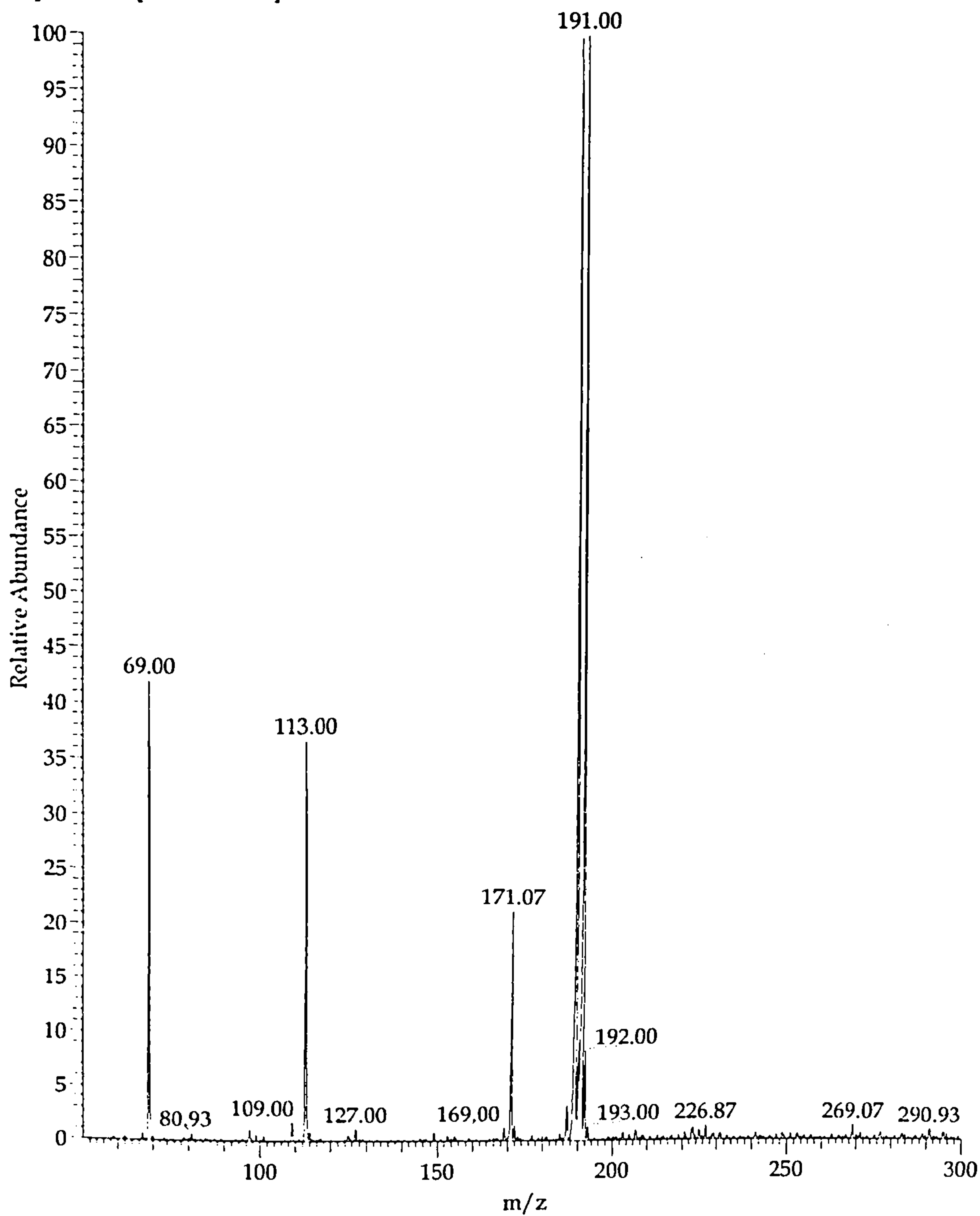
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HPLC method program

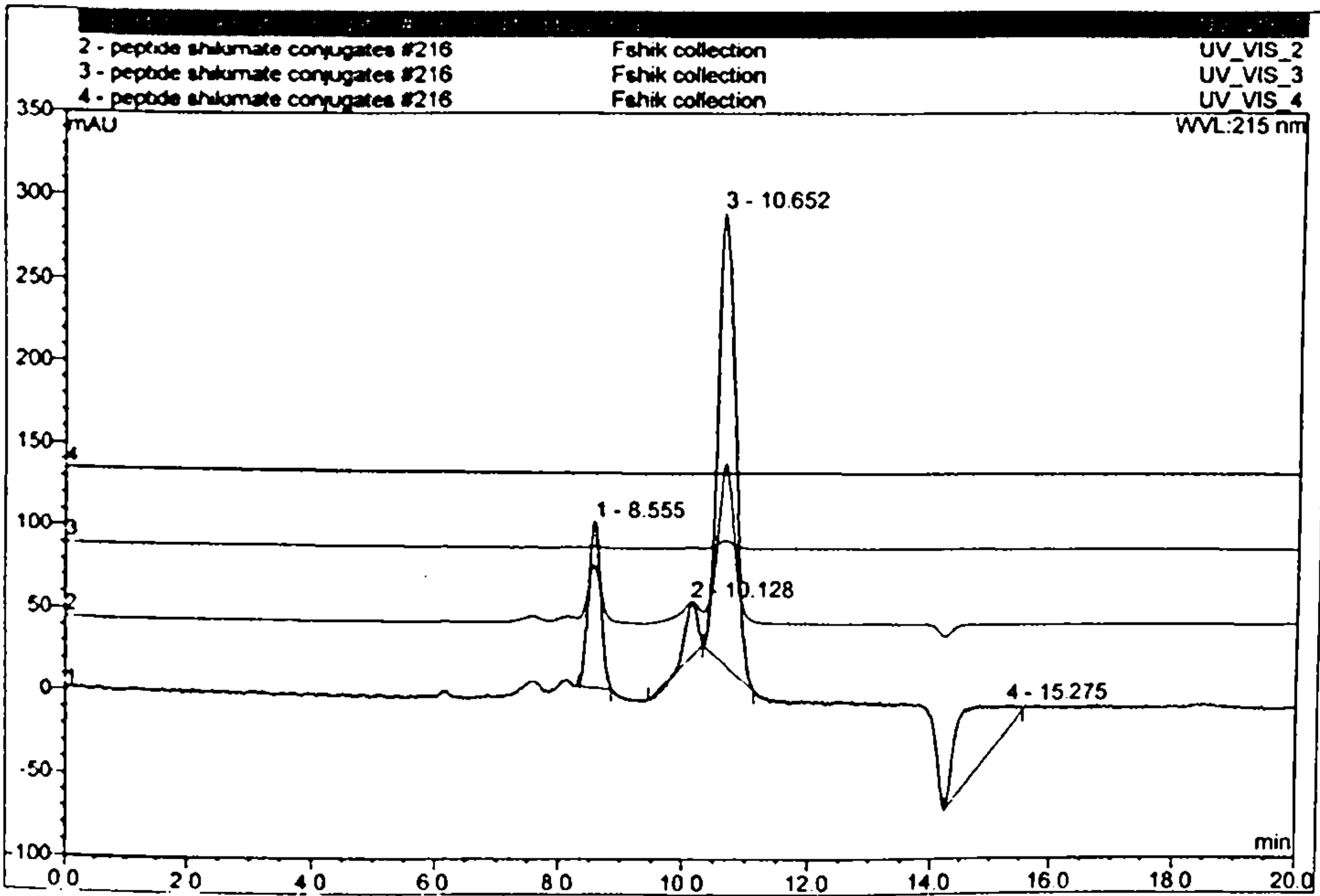


LN277a #2-46 RT: 0.01-0.44 AV: 45 NL: 1.03E5

T: - p Full ms [ 50.00-300.00]



216 Fshik collection			
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Sample Type:	unknown	Wavelength:	215
Control Program:	Organic Acids 20 min fraction 1 min 50mM	Bandwidth:	1
Quantif. Method:	default	Dilution Factor:	1.0000
Recording Time:	9/7/03 16:36	Sample Weight:	1.0000
Run Time (min):	20.00	Sample Amount:	1.0000



No.	Ret.Time min	Peak Name	Height mAU	Area mAU*min	Rel.Area %	Amount	Type
1	8.56	n.a.	100.320	22.644	14.93	n.a.	BMB
2	10.13	n.a.	32.441	6.486	4.28	n.a.	BMB*
3	10.65	n.a.	272.362	90.389	59.60	n.a.	BMB
4	15.27	n.a.	14.438	32.135	21.19	n.a.	BMB
Total:			419.560	151.654	100.00	0.000	

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Operator: Lorna

Page 1 of 2  
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Program File: Organic Acids 20 min 50mM formic 0\_6mL\_min  
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Page 2 of 2  
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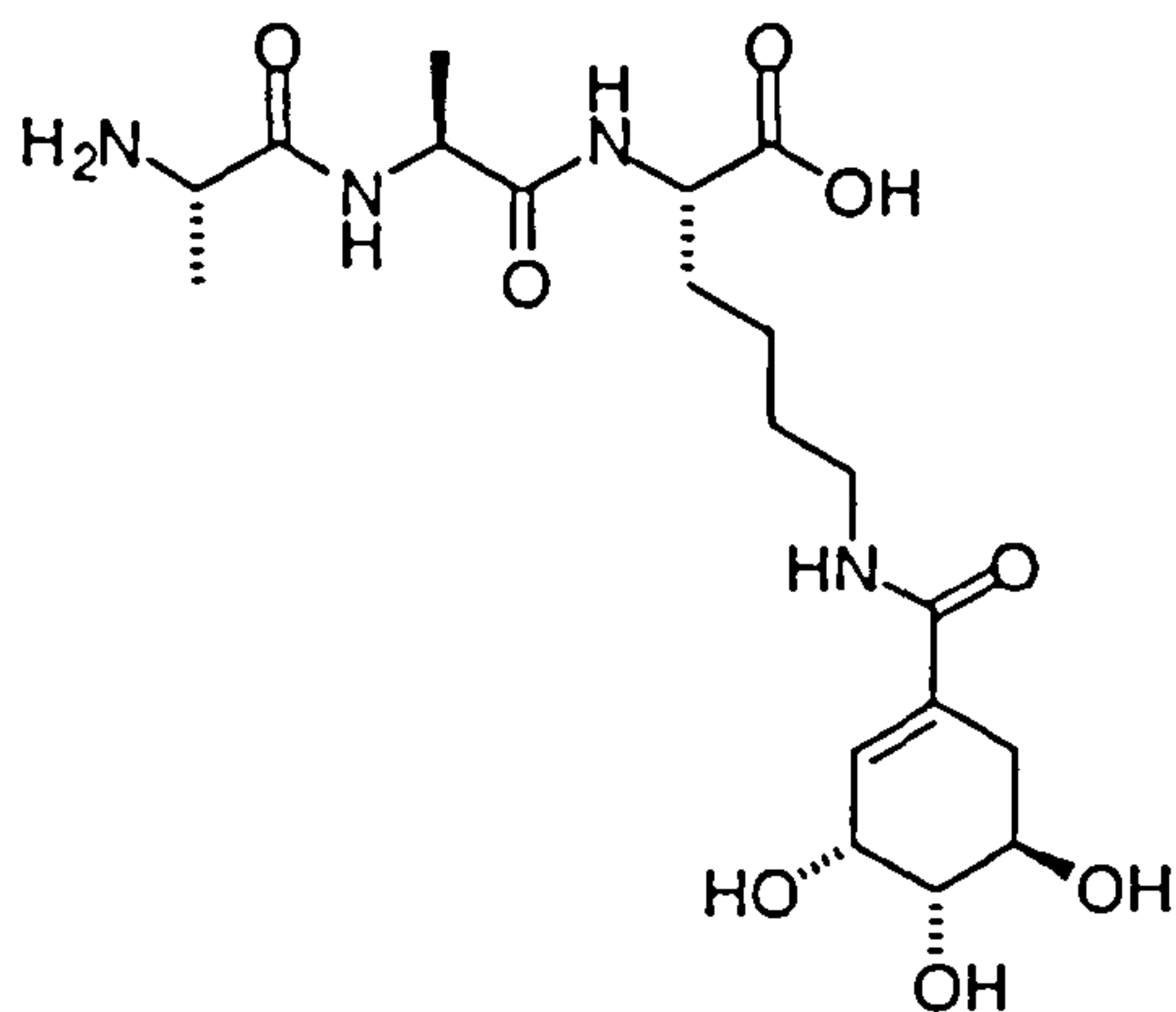
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Wait InjectState  
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UV\_VIS\_1.AcqOn  
UV\_VIS\_2.AcqOn  
UV\_VIS\_3.AcqOn  
UV\_VIS\_4.AcqOn  
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## Appendix 2: Model compounds - Peptide shikimate conjugates (88a - e)

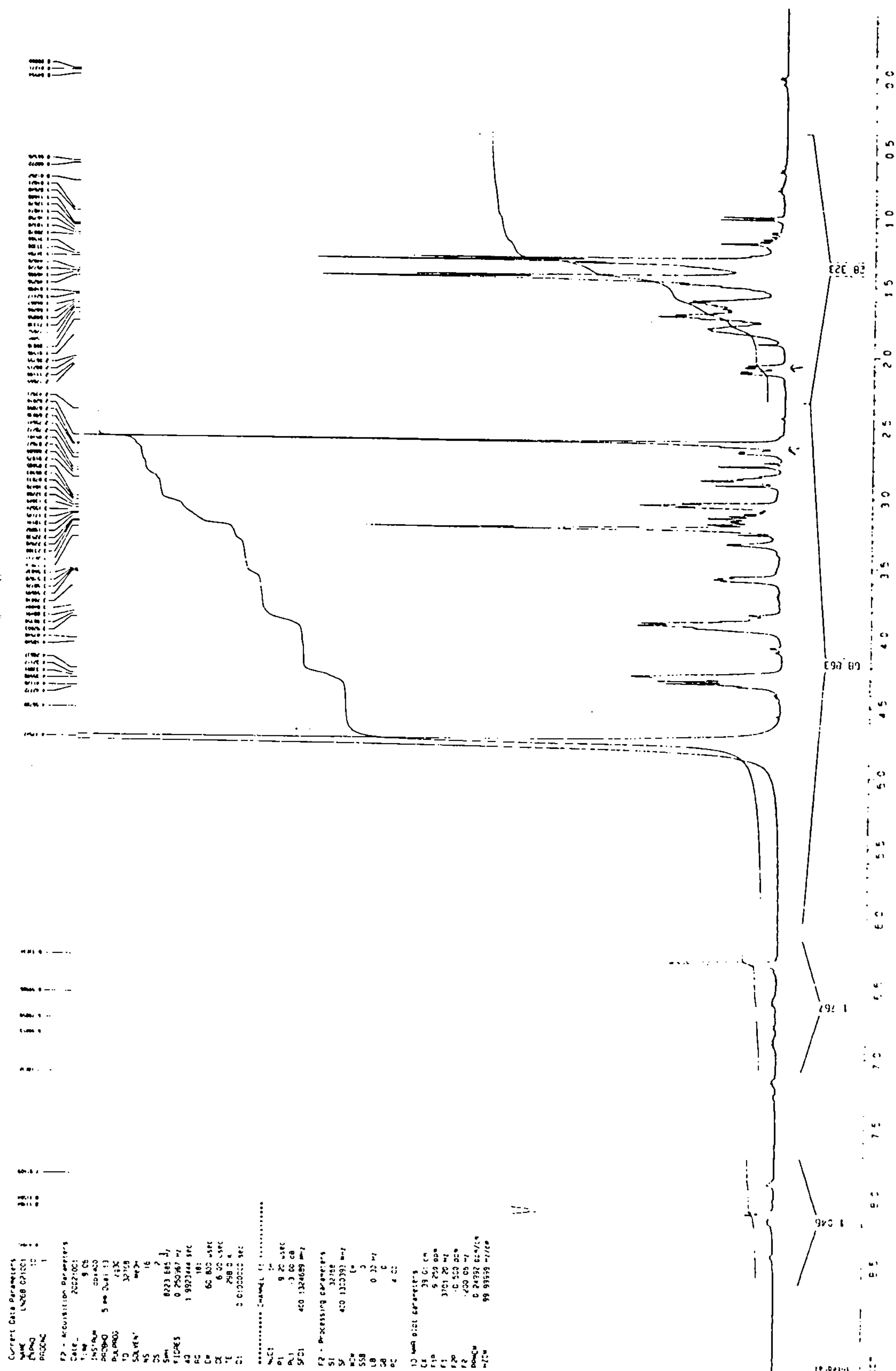
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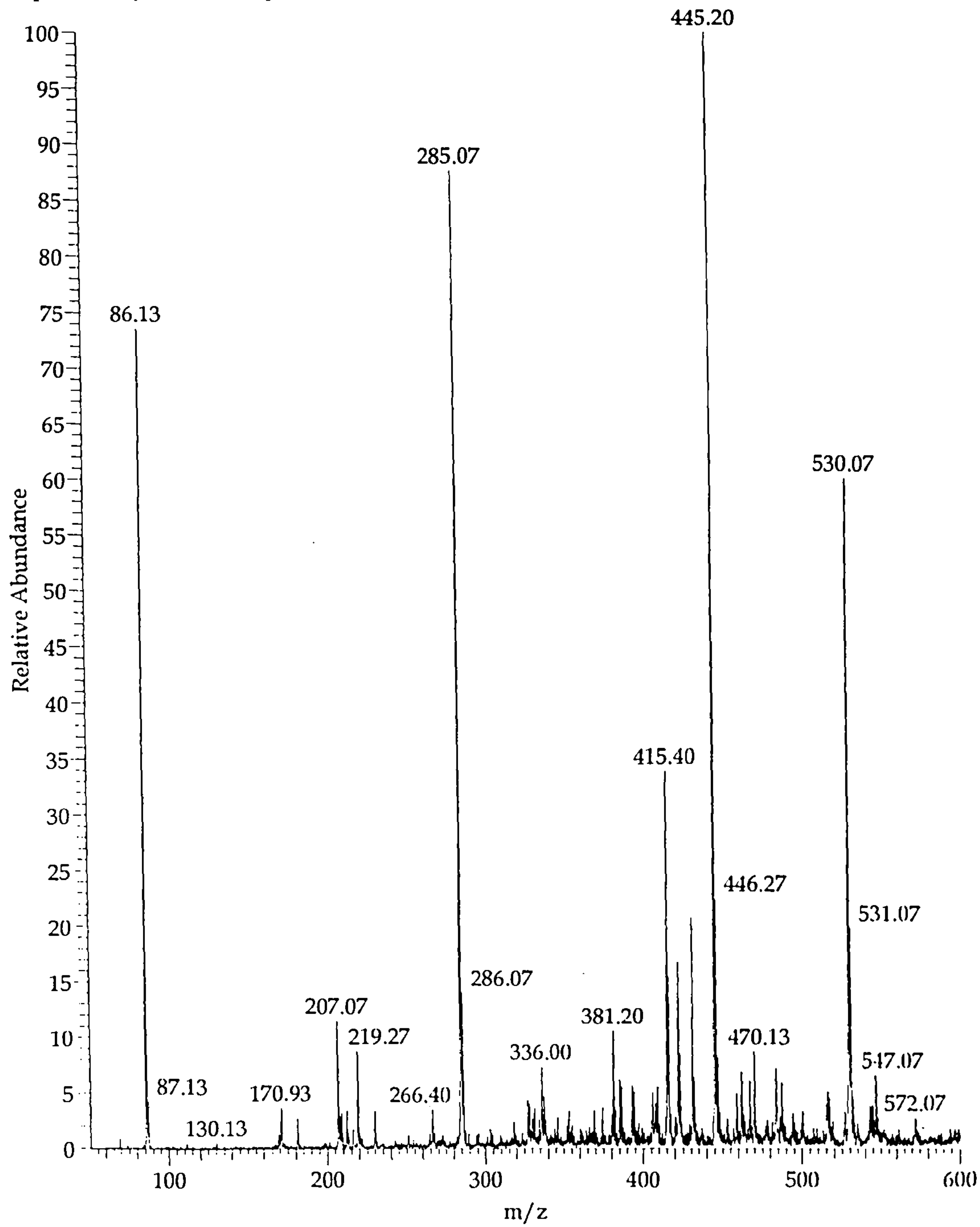
$^1\text{H}$ -NMR of crude product from resin ( $\text{CD}_3\text{OD}$ , 400 MHz)

$m/z$  (electrospray)

$m/z$  (electrospray) msms

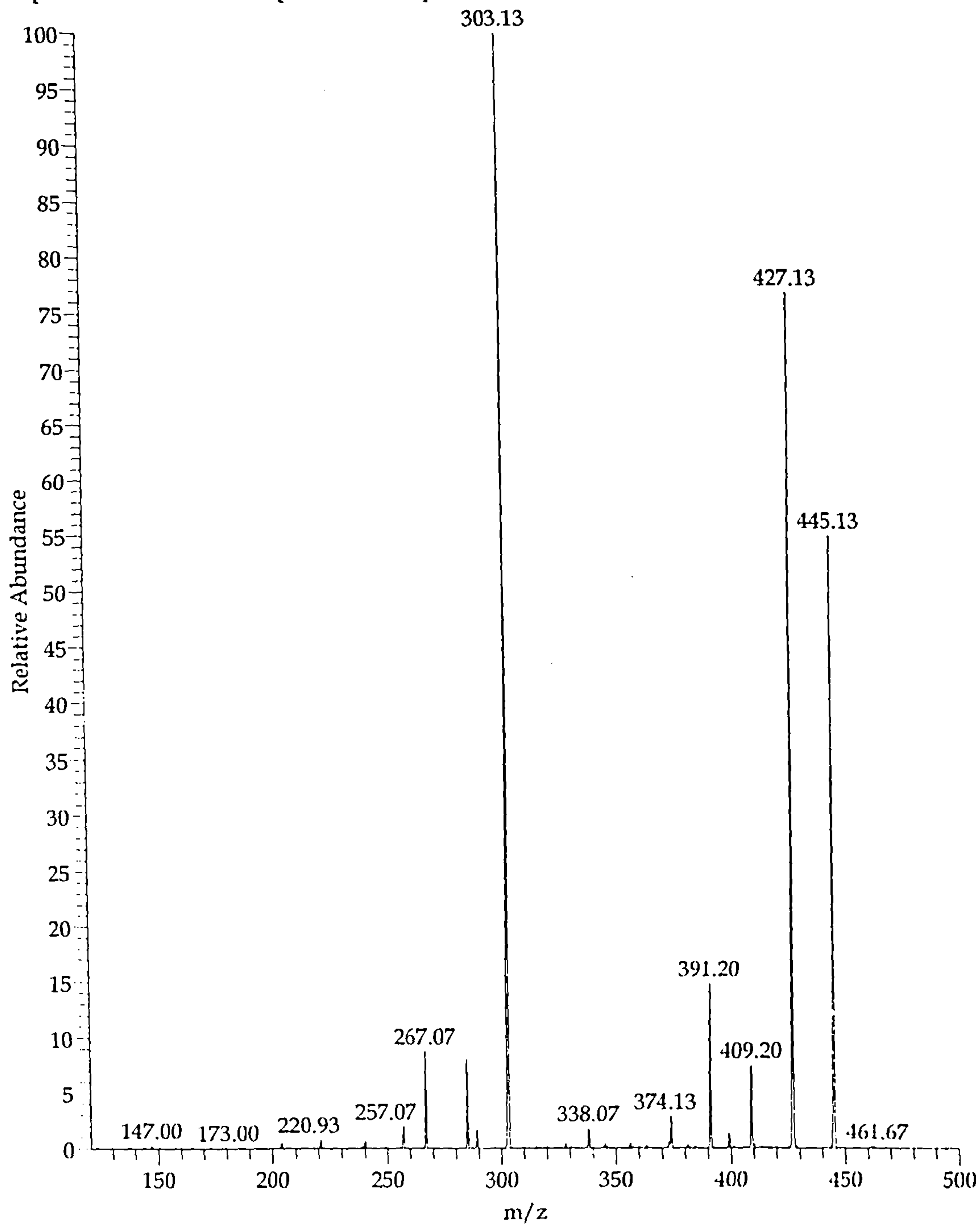


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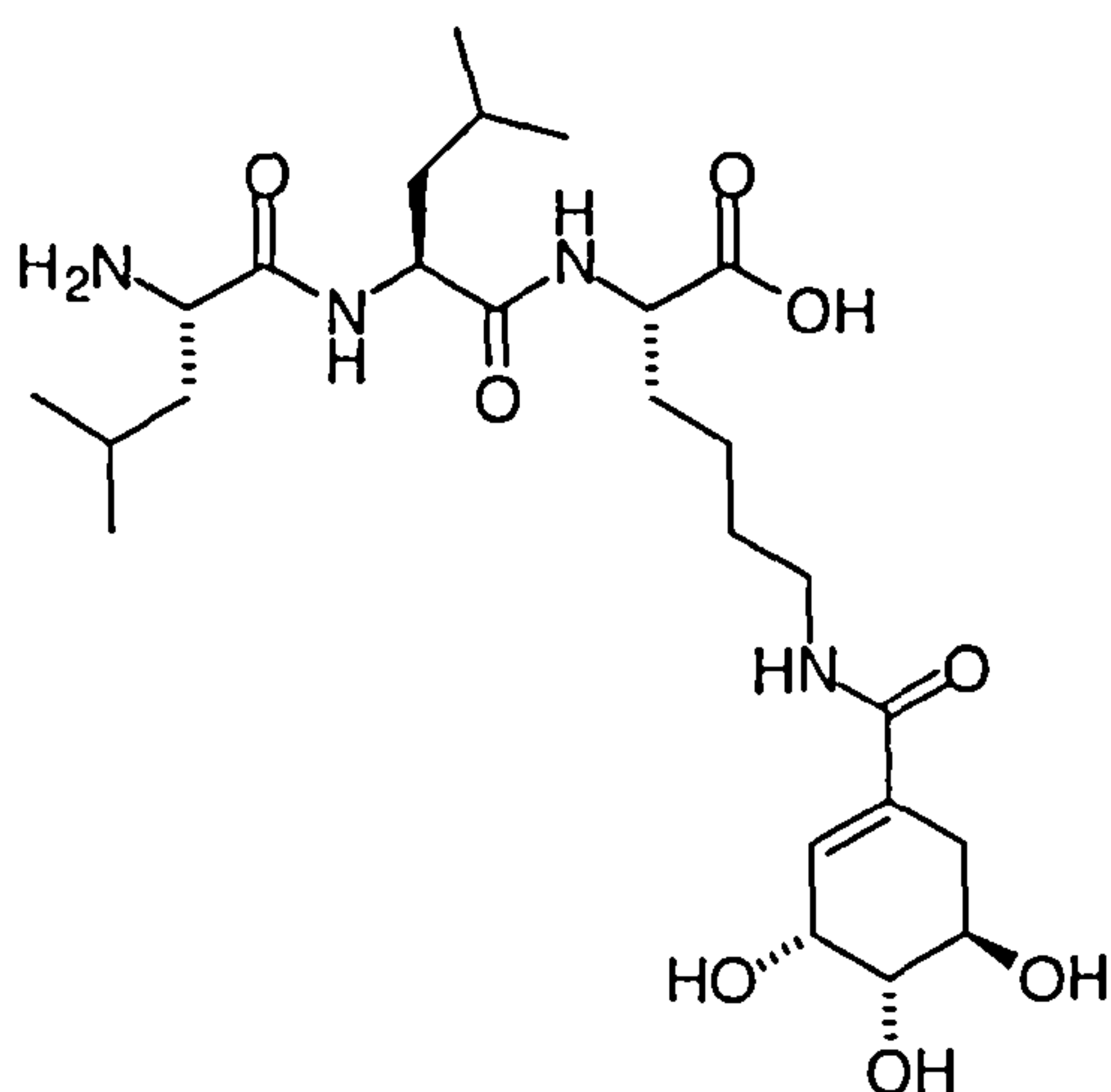
LN223msms #1-45 RT: 0.01-0.68 AV: 45 NL: 2.33E5

T: + p Full ms2 445.10@23.00 [120.00-500.00]





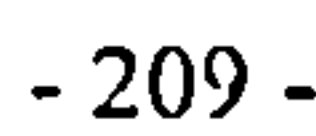
**L-leucyl-L-leucyl-N- $\epsilon$ -shikimoyl-L-lysine 88b**



$^1\text{H}$ -NMR of crude product from resin ( $\text{CD}_3\text{OD}$ , 400 MHz)

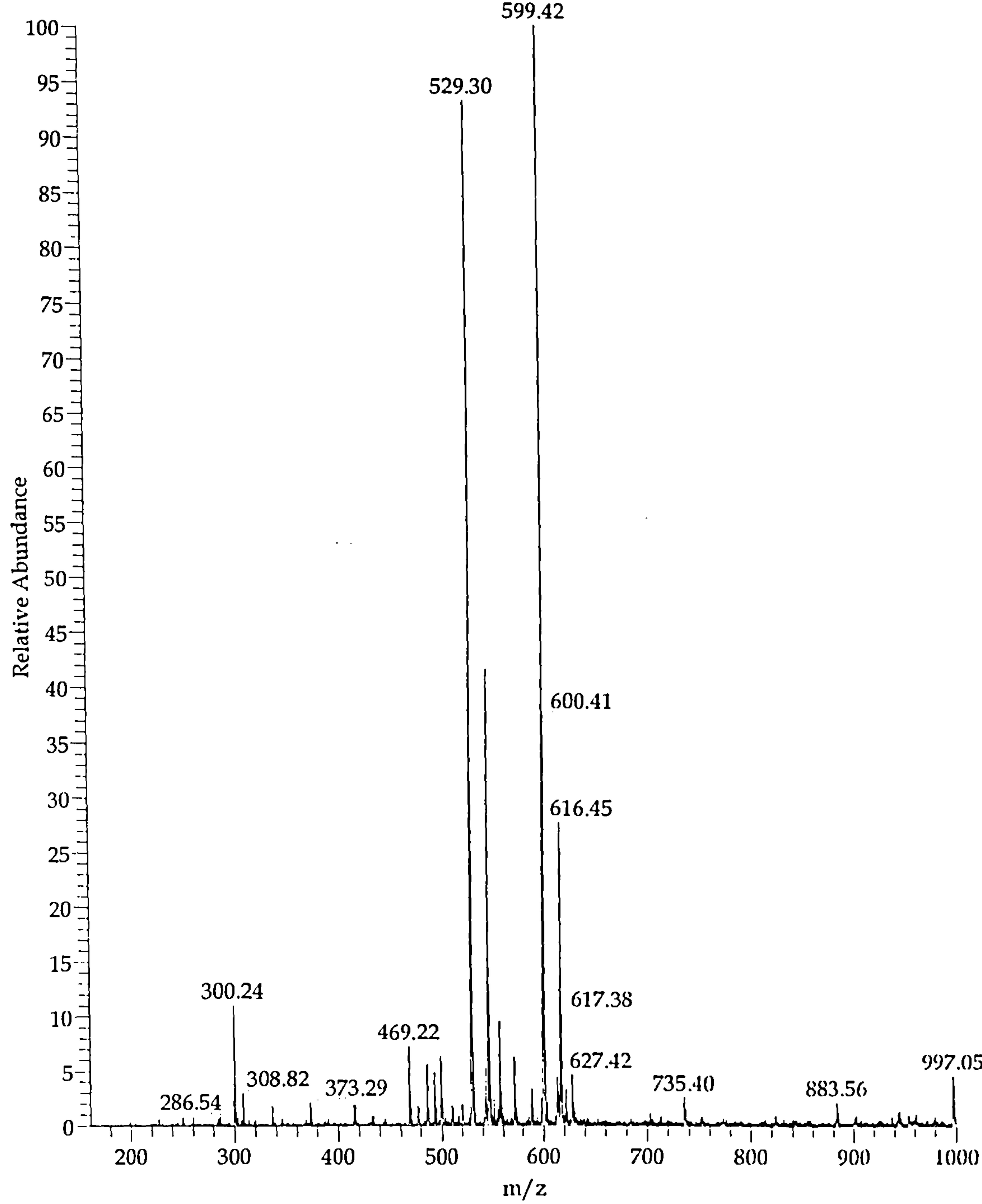
$m/z$  (electrospray)

$m/z$  (electrospray) msms

[illegible]

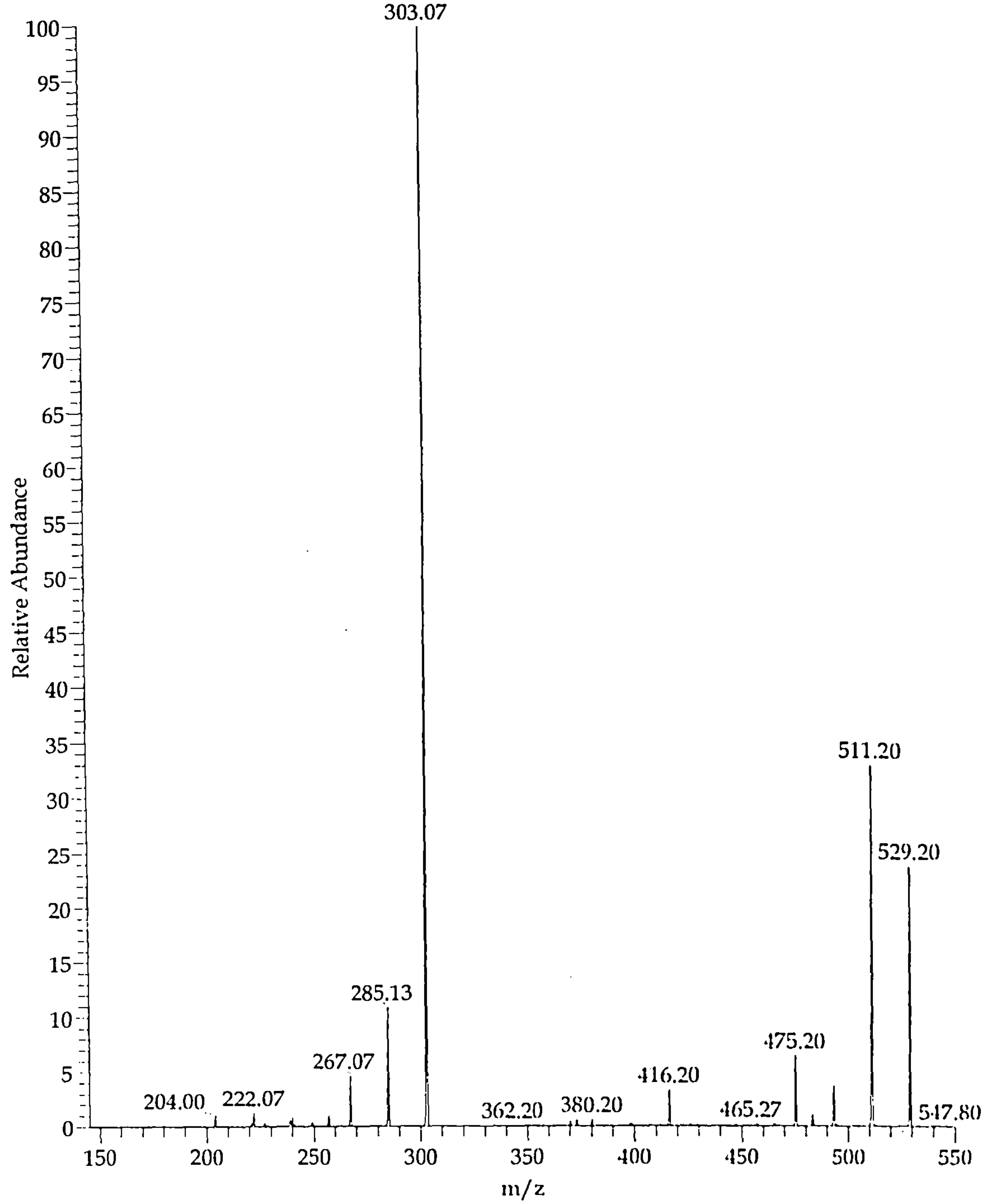
LN225 #1-39 RT: 0.00-0.58 AV: 39 NL: 3.42E7

T: + p Full ms [160.00-1000.00]

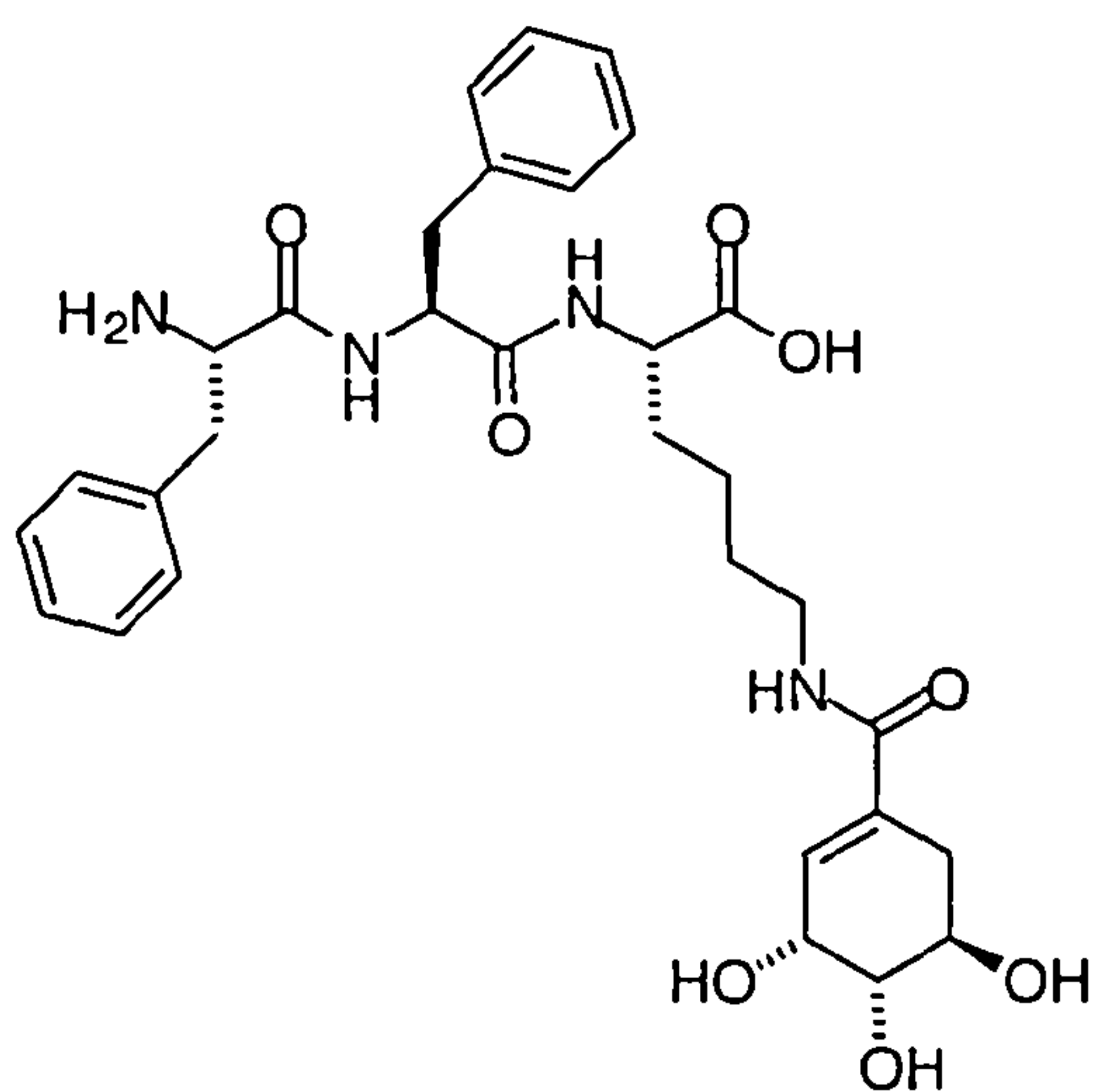


LN225msms #1-41 RT: 0.01-0.53 AV: 41 NL: 1.75E7

T: + p Full ms2 529.30@26.00 [ 145.00-550.00]



**L-phenylalanyl-L-phenylalanyl-N-ε-shikimoyl-L-lysine 88c**



<sup>1</sup>H-NMR of crude product from resin (CD<sub>3</sub>OD, 400 MHz)

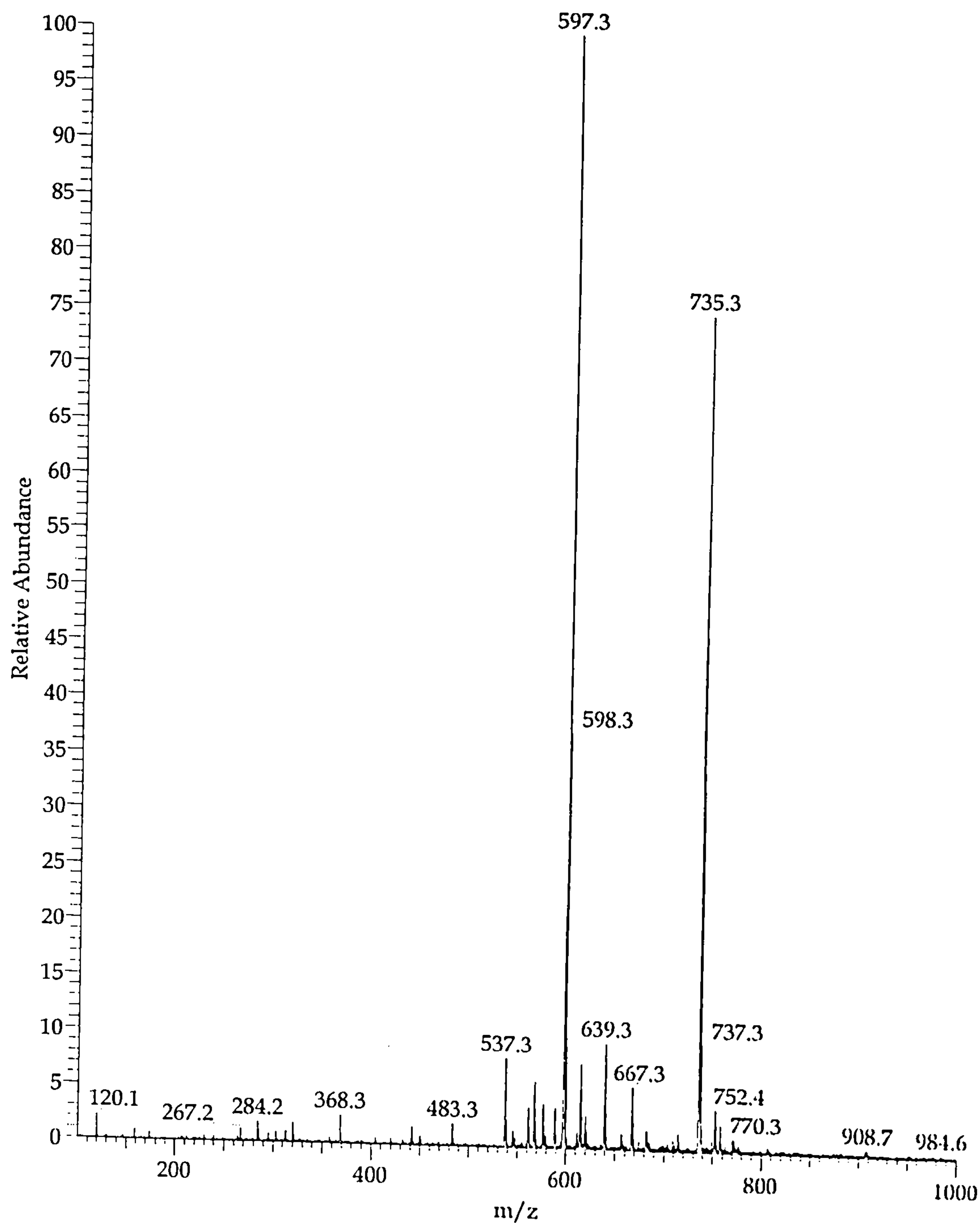
*m/z* (electrospray)

*m/z* (electrospray) msms



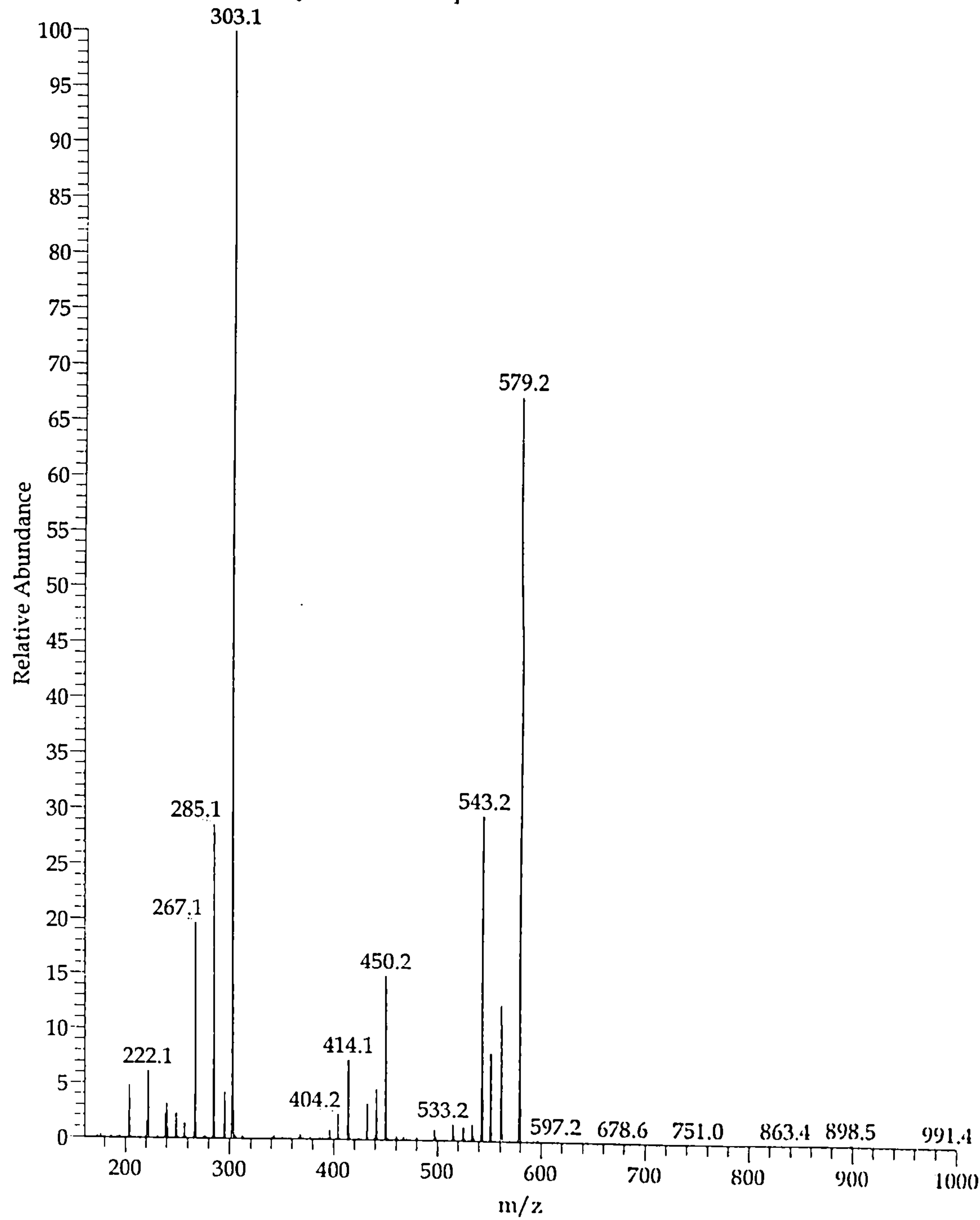


LN226 #1-30 RT: 0.02-0.49 AV: 30 NL: 1.82E7  
T: + p Full ms [ 100.00-1000.00]



LN226msms #1-34 RT: 0.00-0.61 AV: 34 NL: 8.33E6

T: + p Full ms2 597.30@34.00 [ 160.00-1000.00]

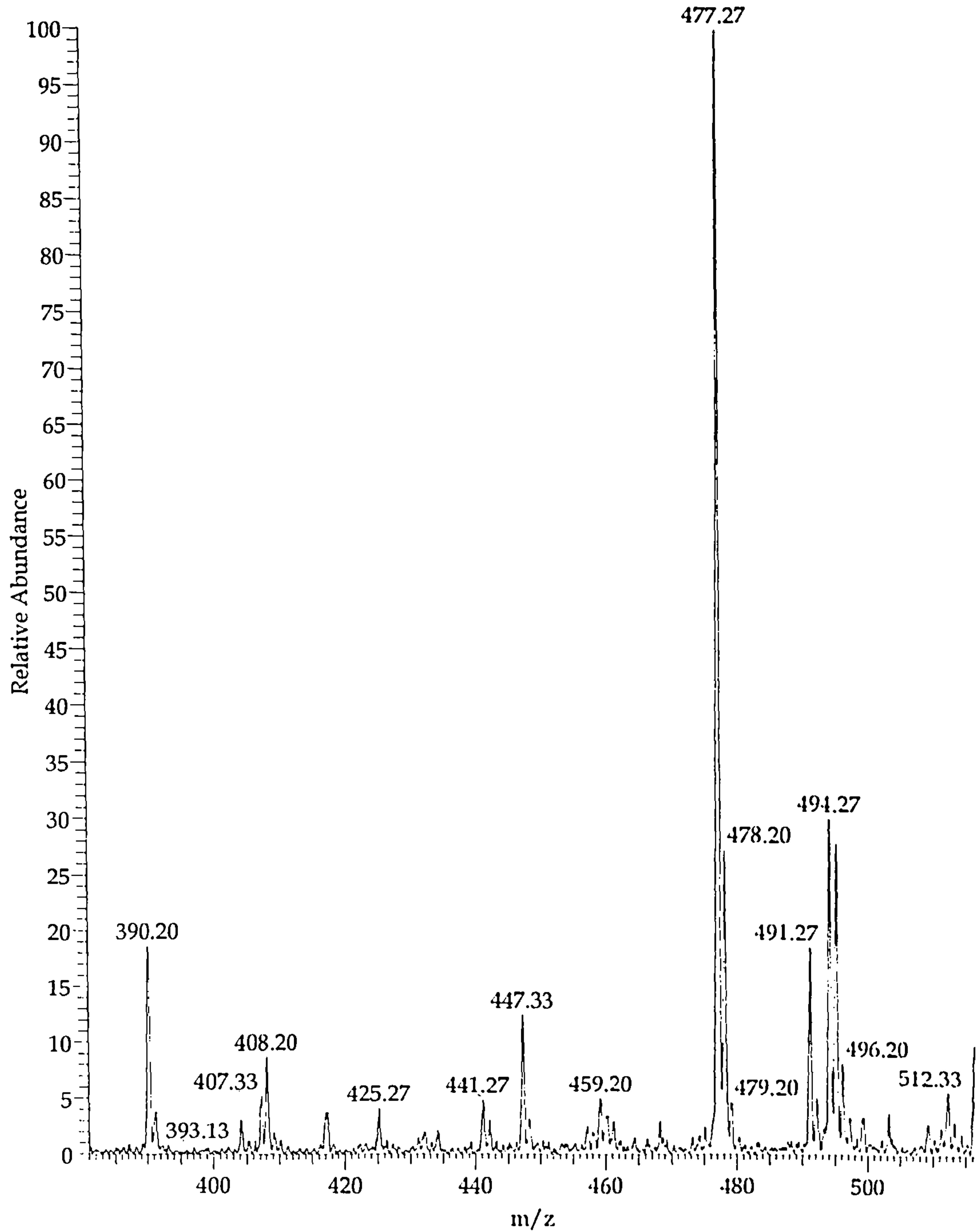






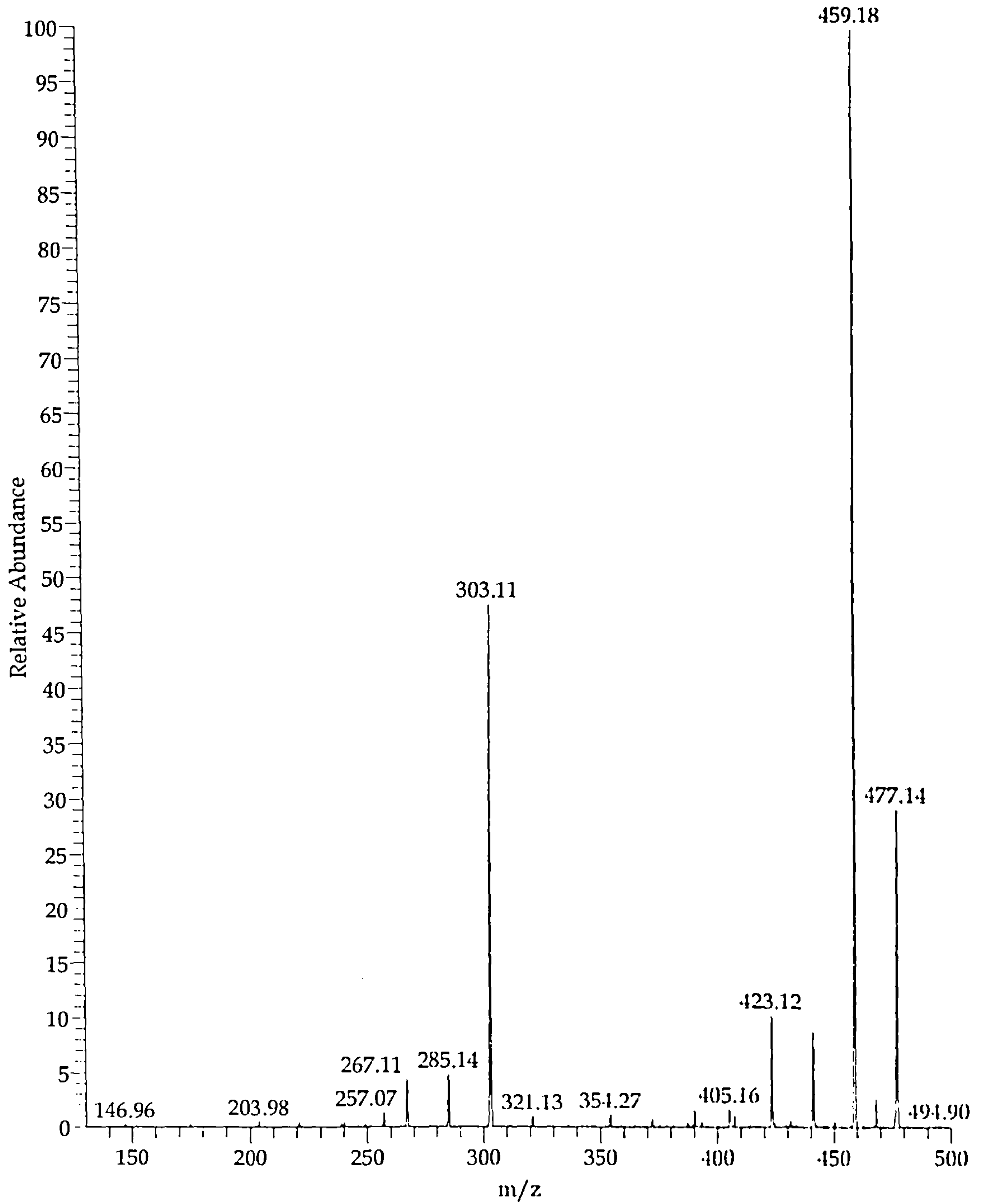


LN227 #1-42 RT: 0.01-0.66 AV: 42 NL: 2.67E6  
T: + p Full ms [ 100.00-1000.00]

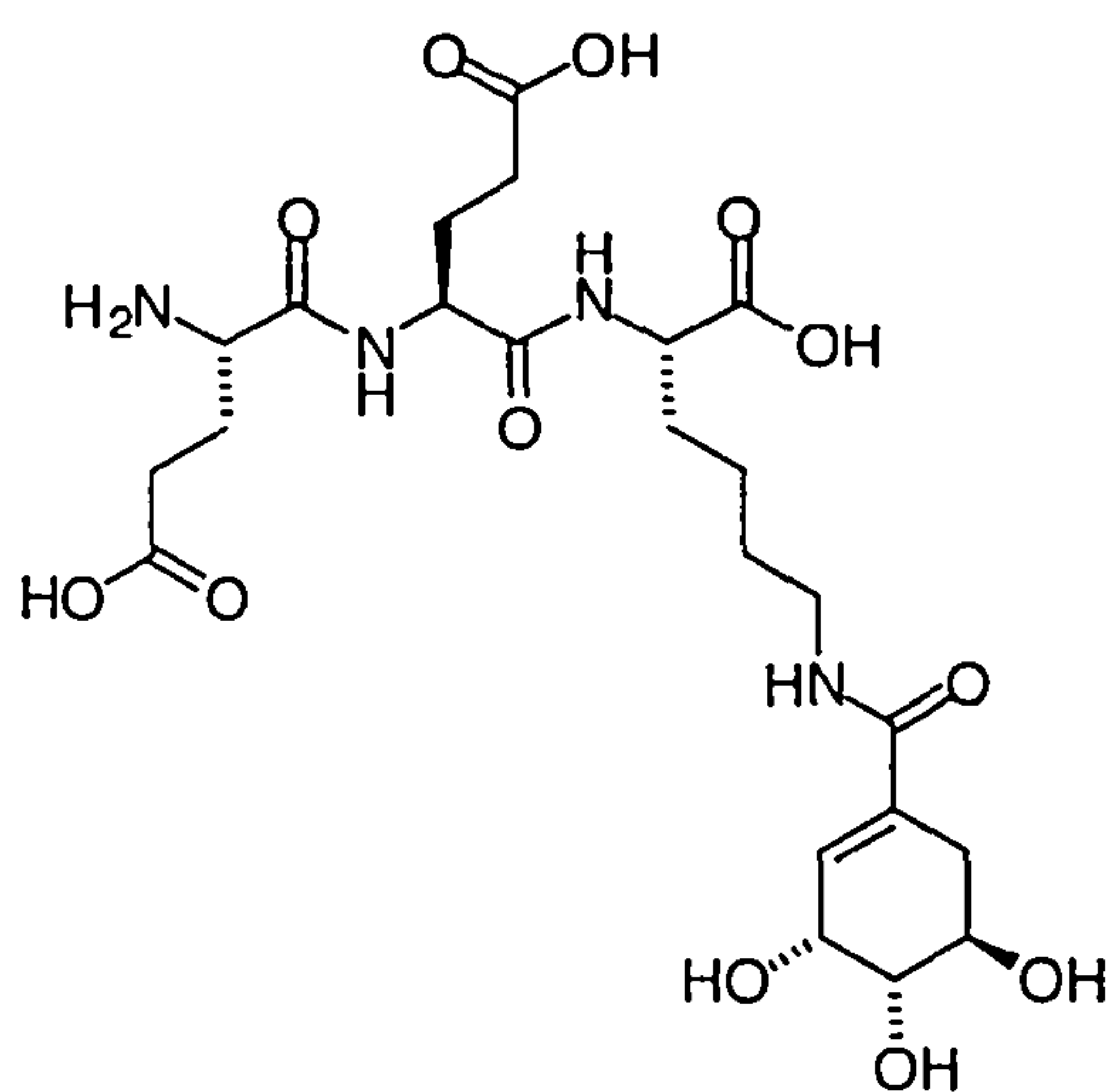


LN227msms #2-33 RT: 0.01-0.45 AV: 32 NL: 4.42E5

T: + p Full ms2 477.00@23.00 [ 130.00-500.00]



**L-glutamoyl-L-glutamoyl-N-ε-shikimoyl-L-lysine 88e**



<sup>1</sup>H-NMR of crude product from resin (CD<sub>3</sub>OD, 400 MHz)

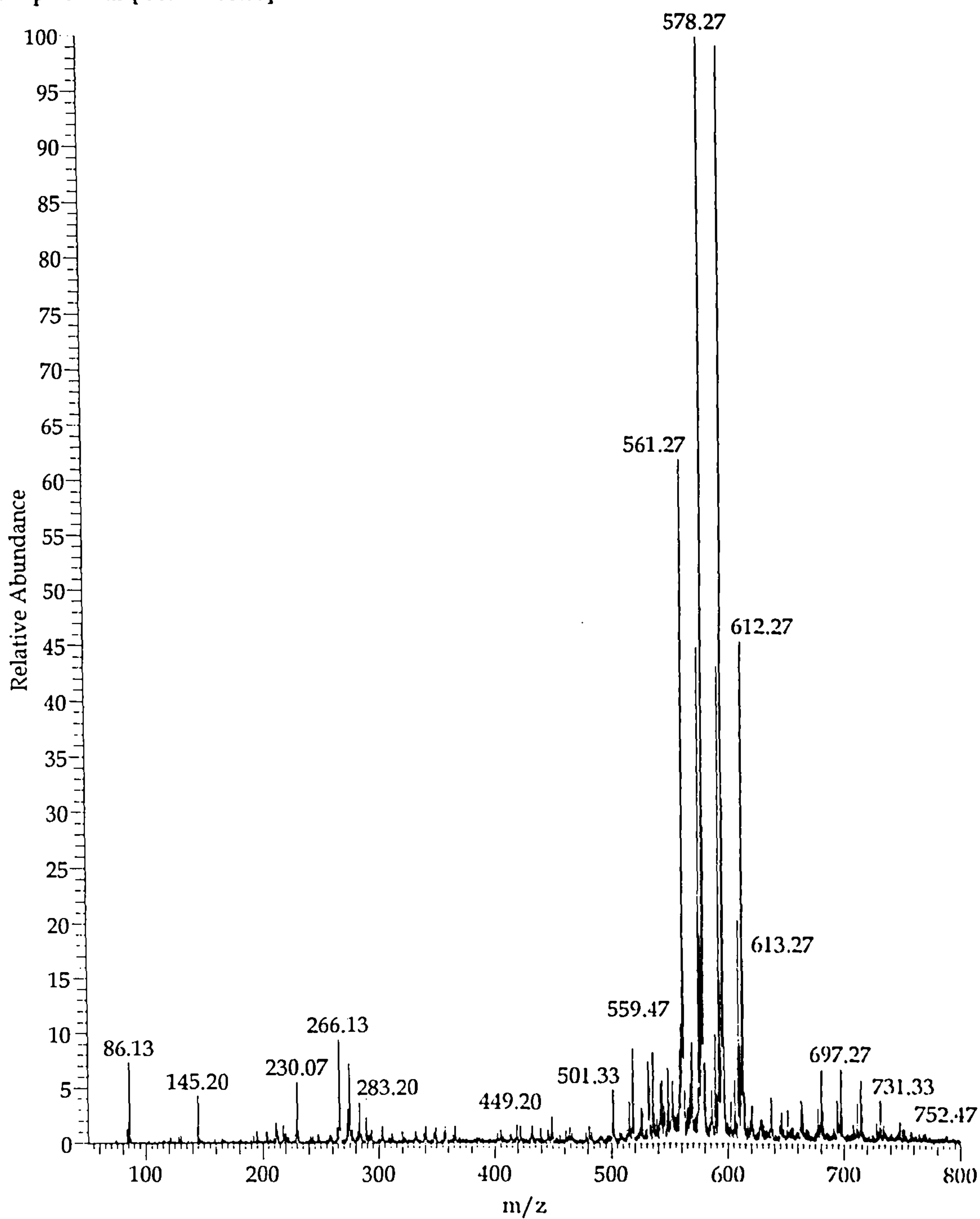
*m/z* (electrospray)

*m/z* (electrospray) msms



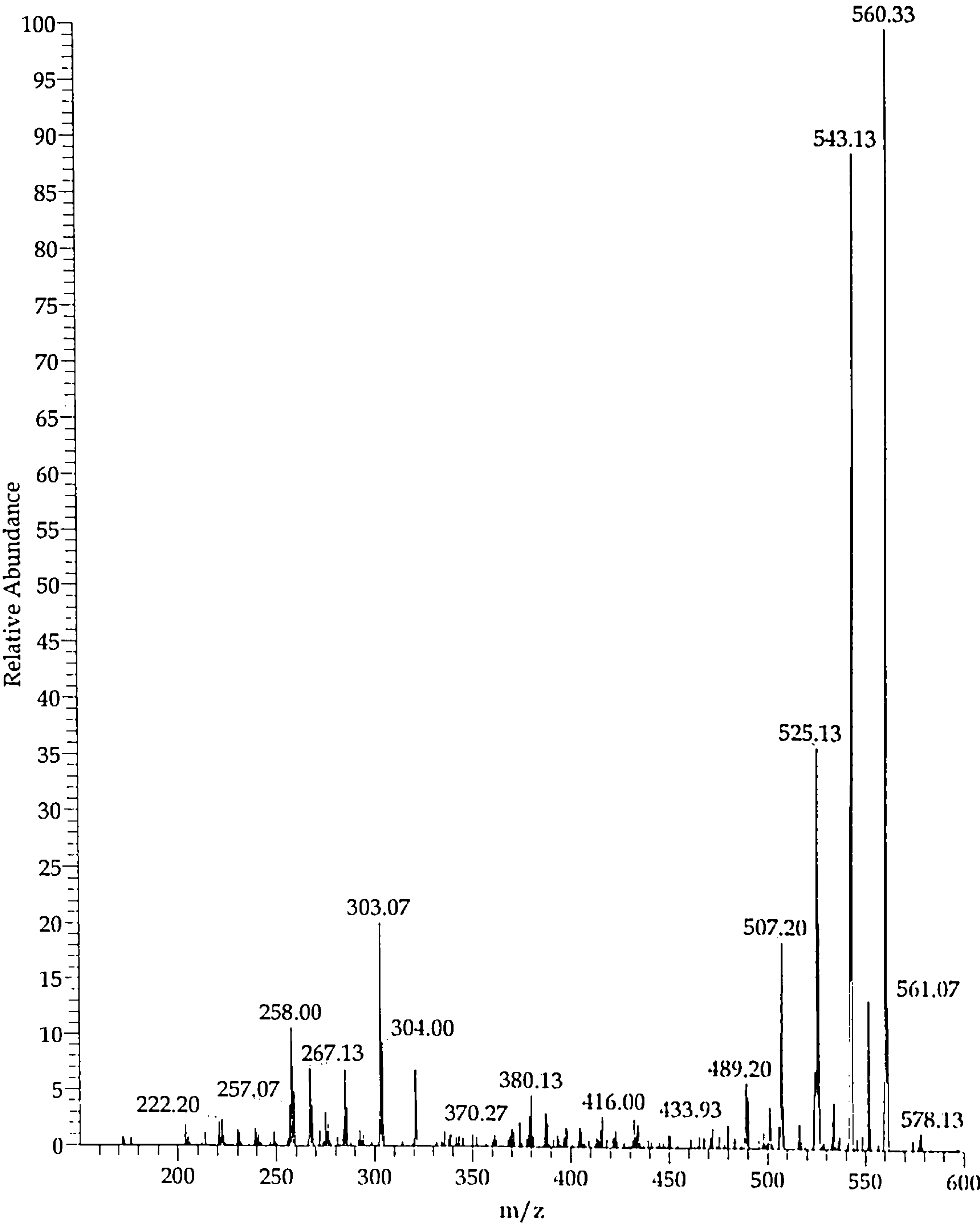
LN224RAW #1-51 RT: 0.01-0.72 AV: 51 NL: 5.67E6

T: + p Full ms [ 50.00-800.00]



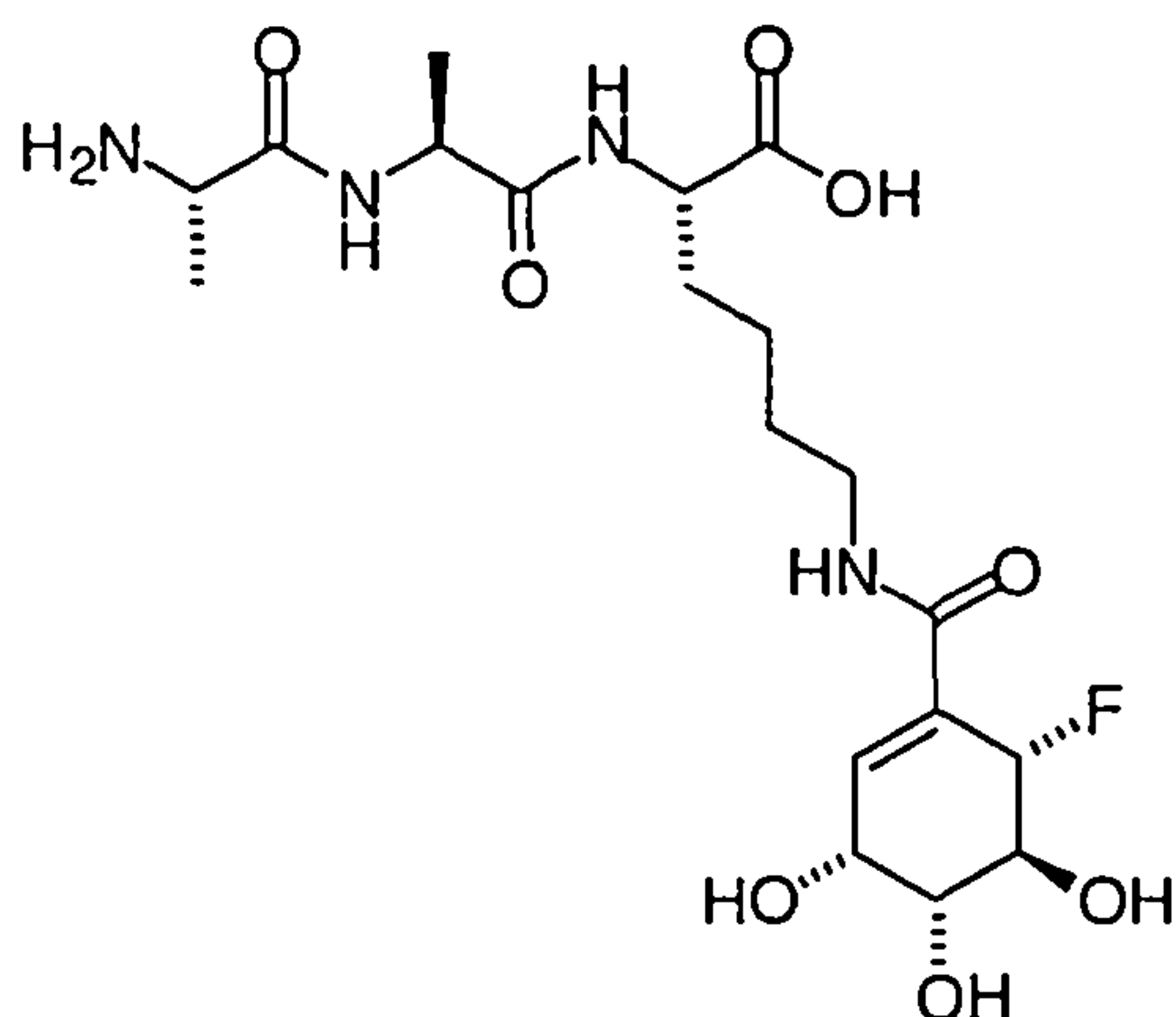


LN224msms1 #1-24 RT: 0.00-0.39 AV: 24 NL: 3.07E4  
T: + p Full ms2 561.10@27.00 [ 150.00-600.00]



### Appendix 3: Peptide - (6S)-6-Fluoroshikimate conjugates (4 - 8)

#### L-alanyl-L-alanyl-N-ε-(6S)-6-fluoroshikimoyl-L-lysine 4



<sup>1</sup>H-NMR of crude product from resin (CD<sub>3</sub>OD, 400 MHz)

*m/z* (electrospray)

*m/z* (electrospray) msms

HPLC/ms output (RP max column, 3 - 50 % 50 mM formic acid in MeCN)

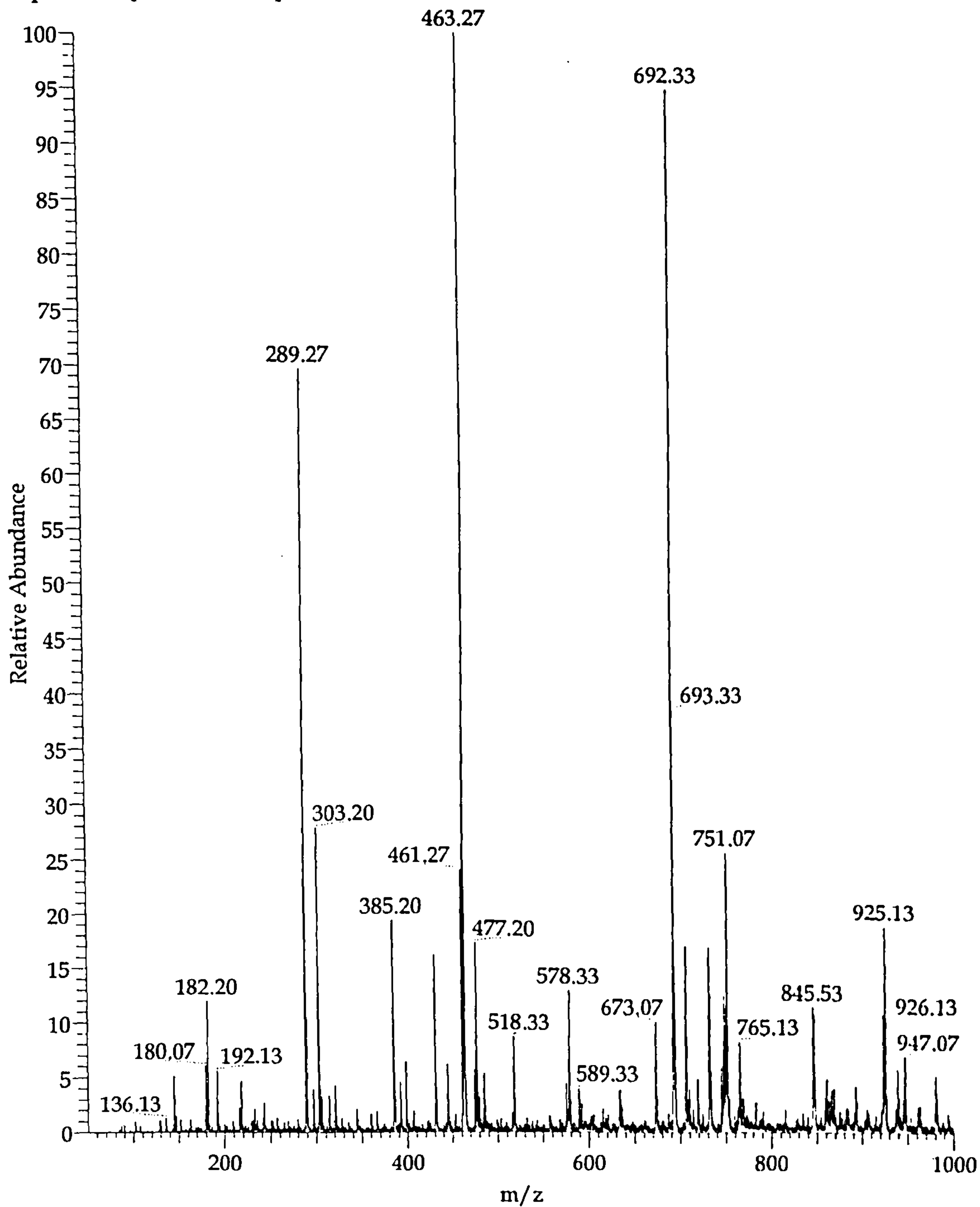






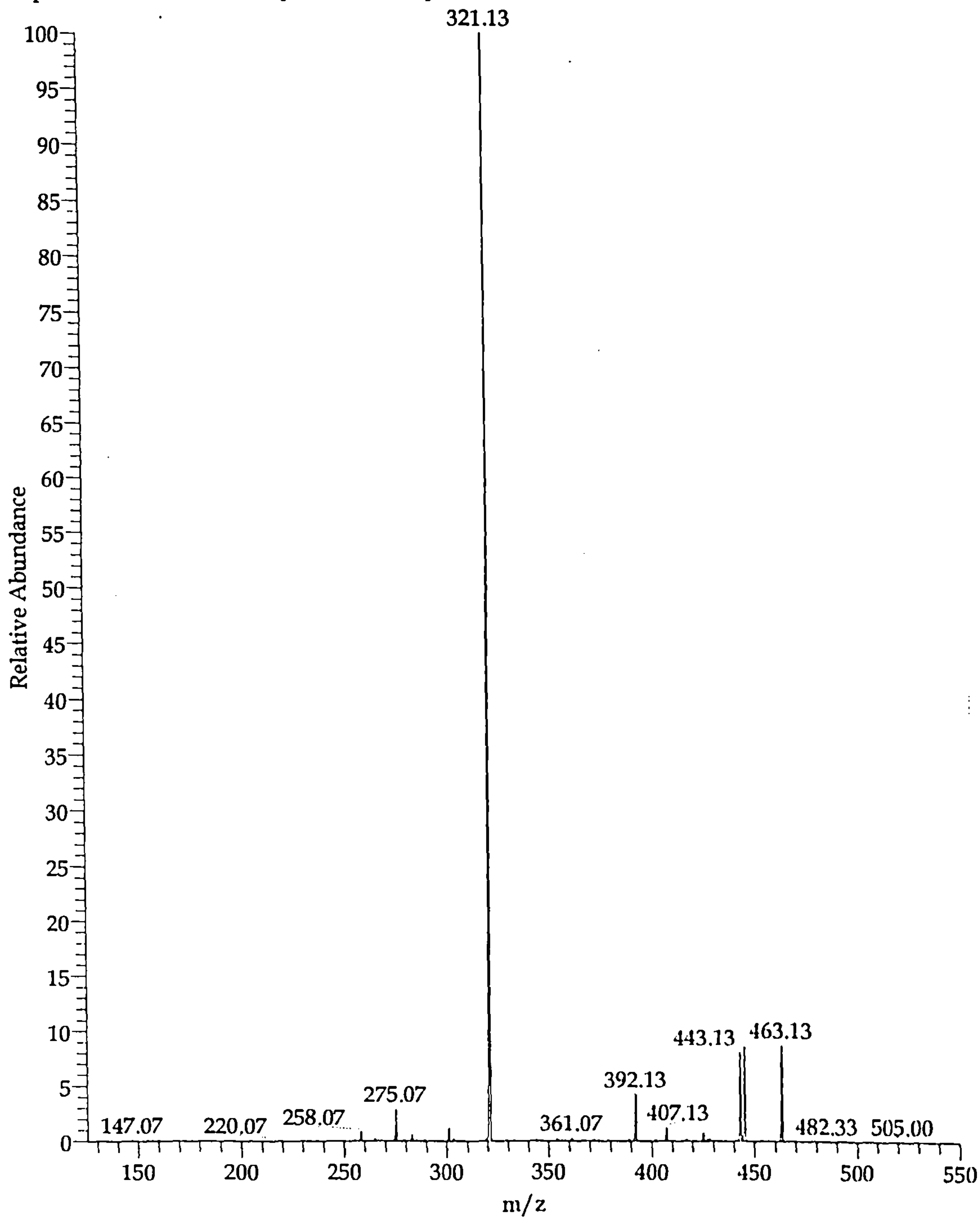
LN272\_021014100415 #4-68 RT: 0.07-1.14 AV: 65 NL: 3.26E6

T: + p Full ms [ 50.00-1000.00]



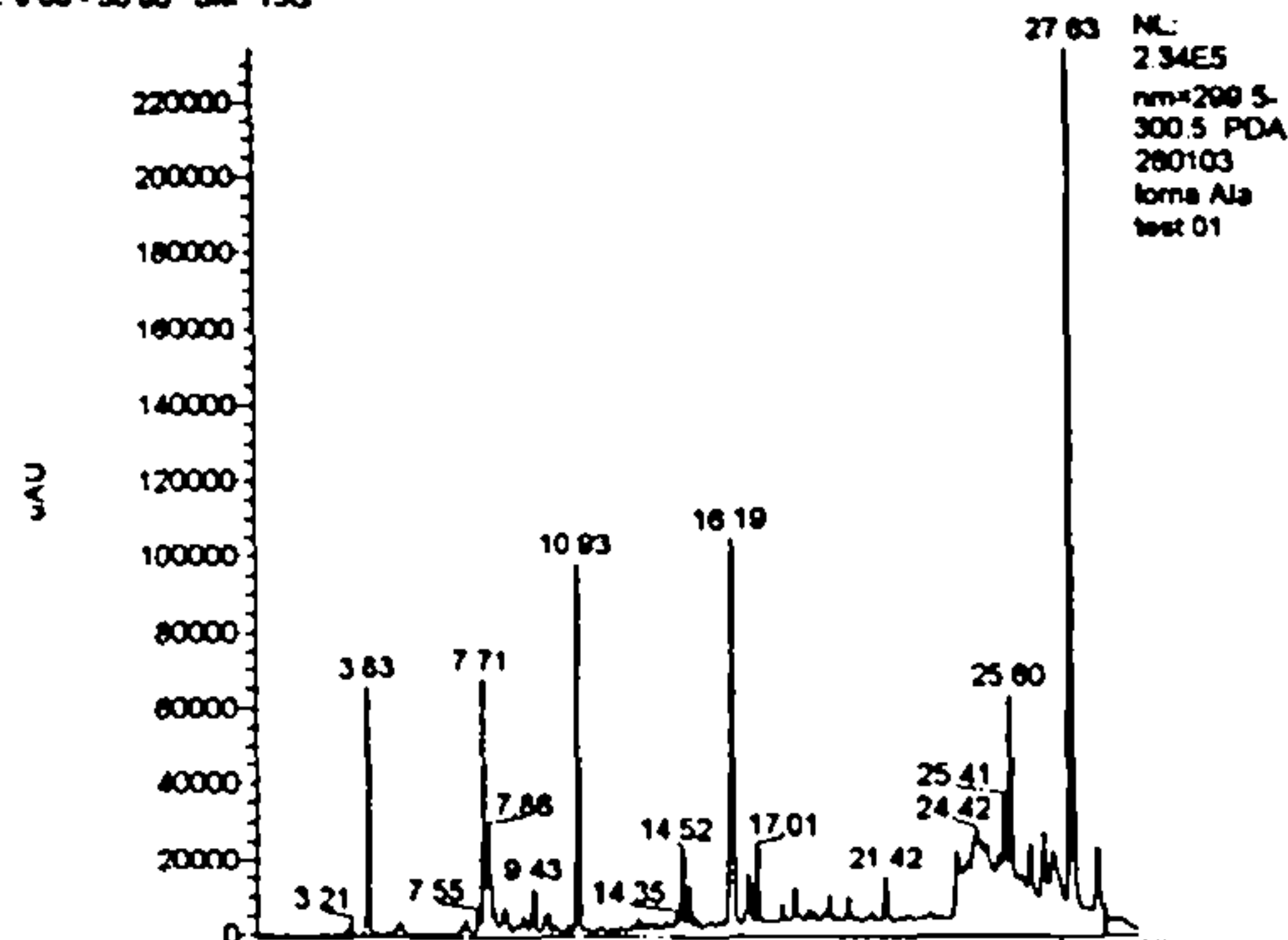
LN272msms #2-66 RT: 0.02-0.96 AV: 65 NL: 2.88E6

T: + p Full ms2 463.30@24.00 [ 125.00-550.00]



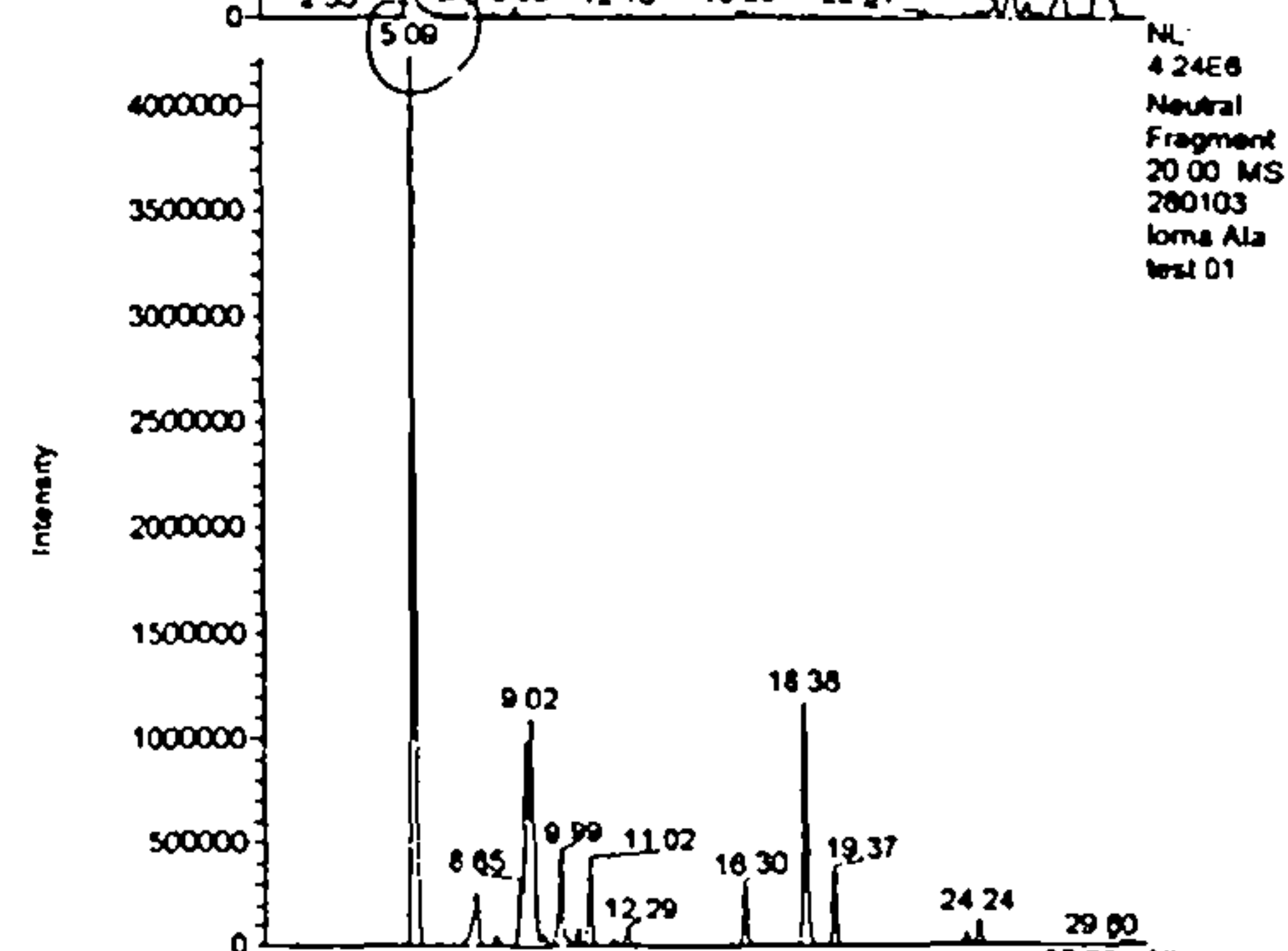
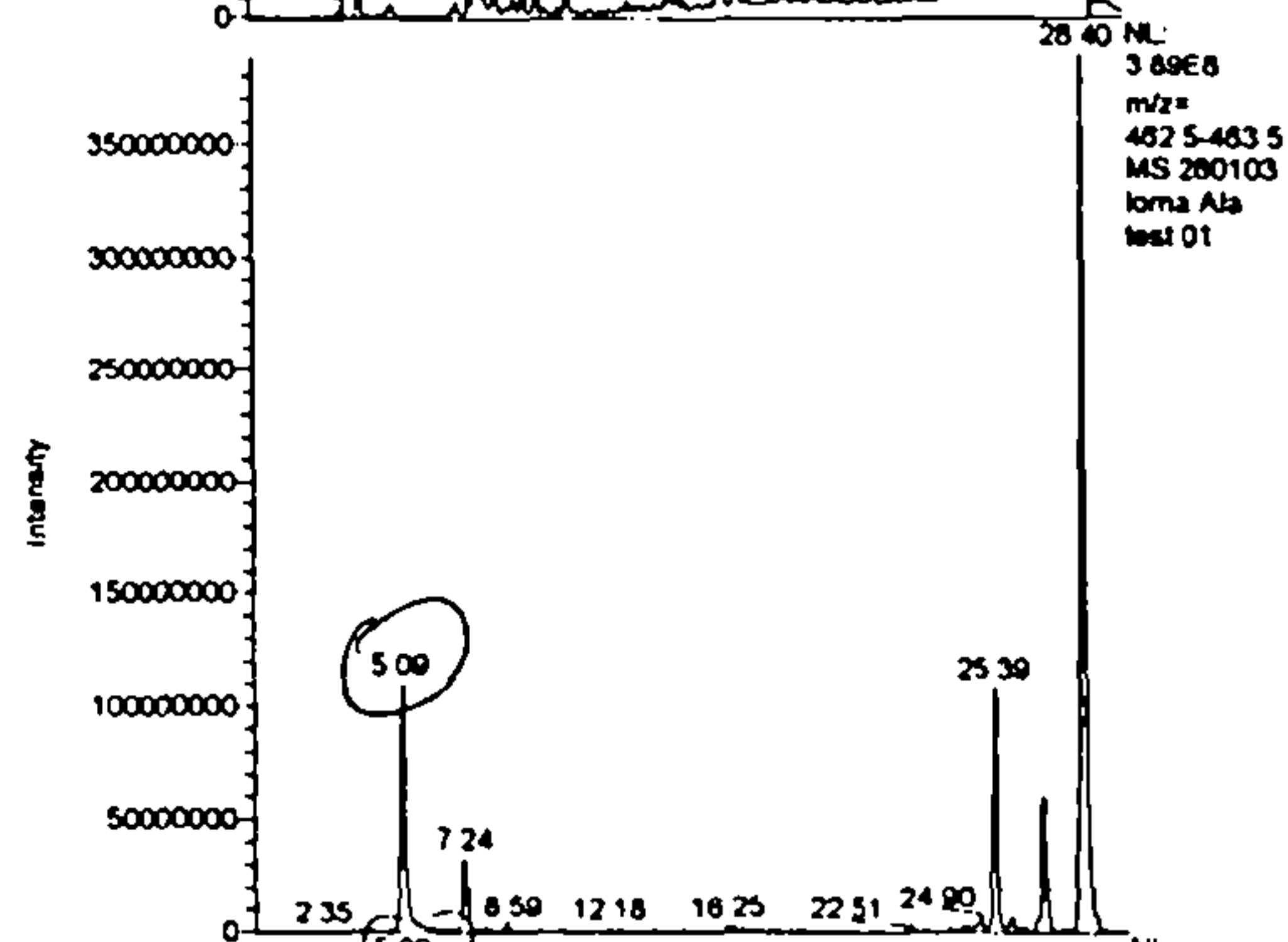


RT: 0.00 - 30.00 SM 153

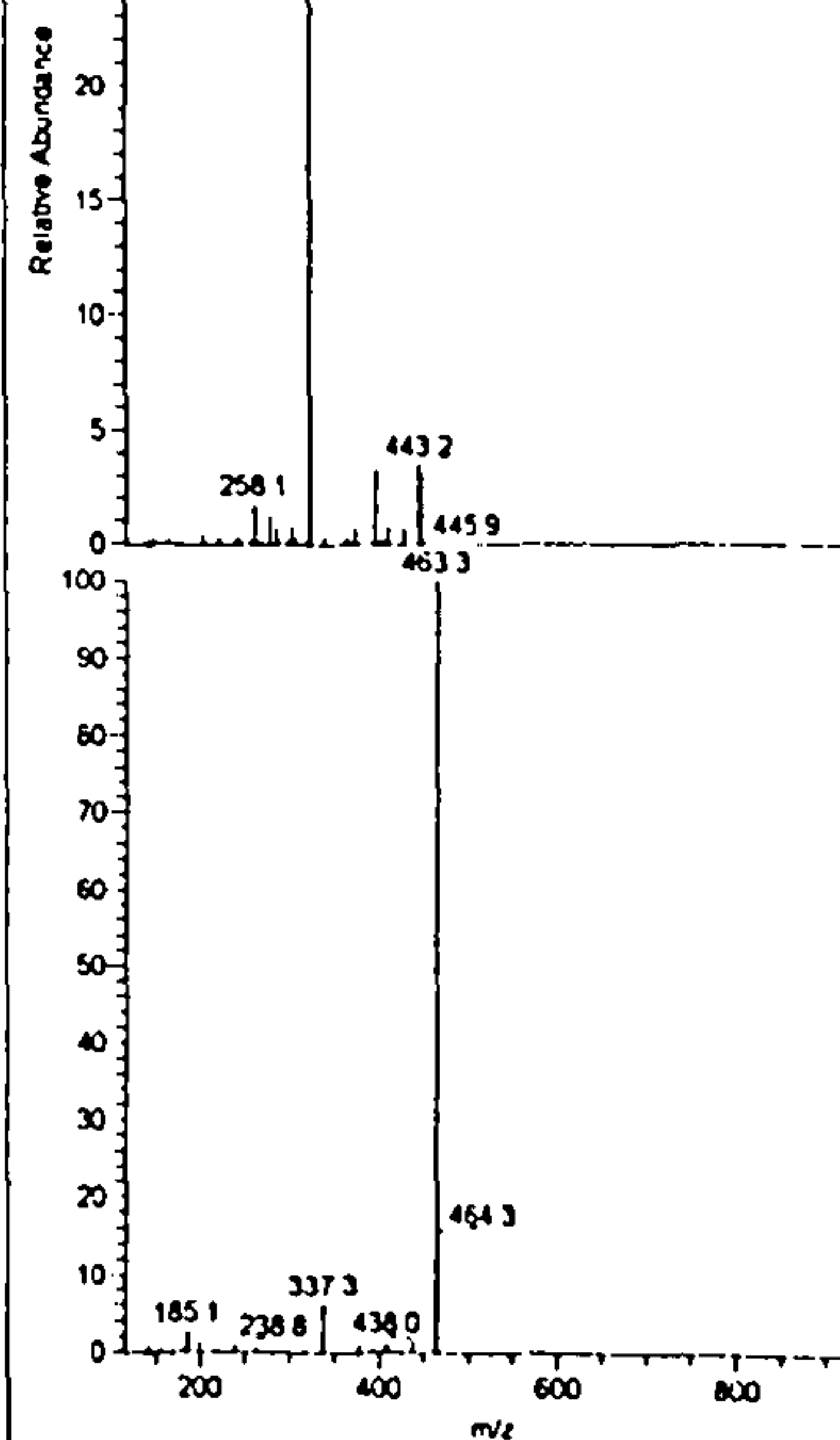
280103 lorna Ala test 01  
Type: Unknown ID: C:25 Row: 18

Sample Name:  
Study:  
Client:  
Laboratory:  
Company:  
Phone:  
Instrument Method: C:\Xcalibur\methods\lorna\020902 20 min  
grad 3-50% C n D pos.meth  
Processing Method:  
Vial: D:21  
Injection Volume (µl): 10.00  
Sample Weight: 0.00  
Sample Volume (µl): 0.00  
ISTD Amount: 0.00  
Dil Factor: 1.00

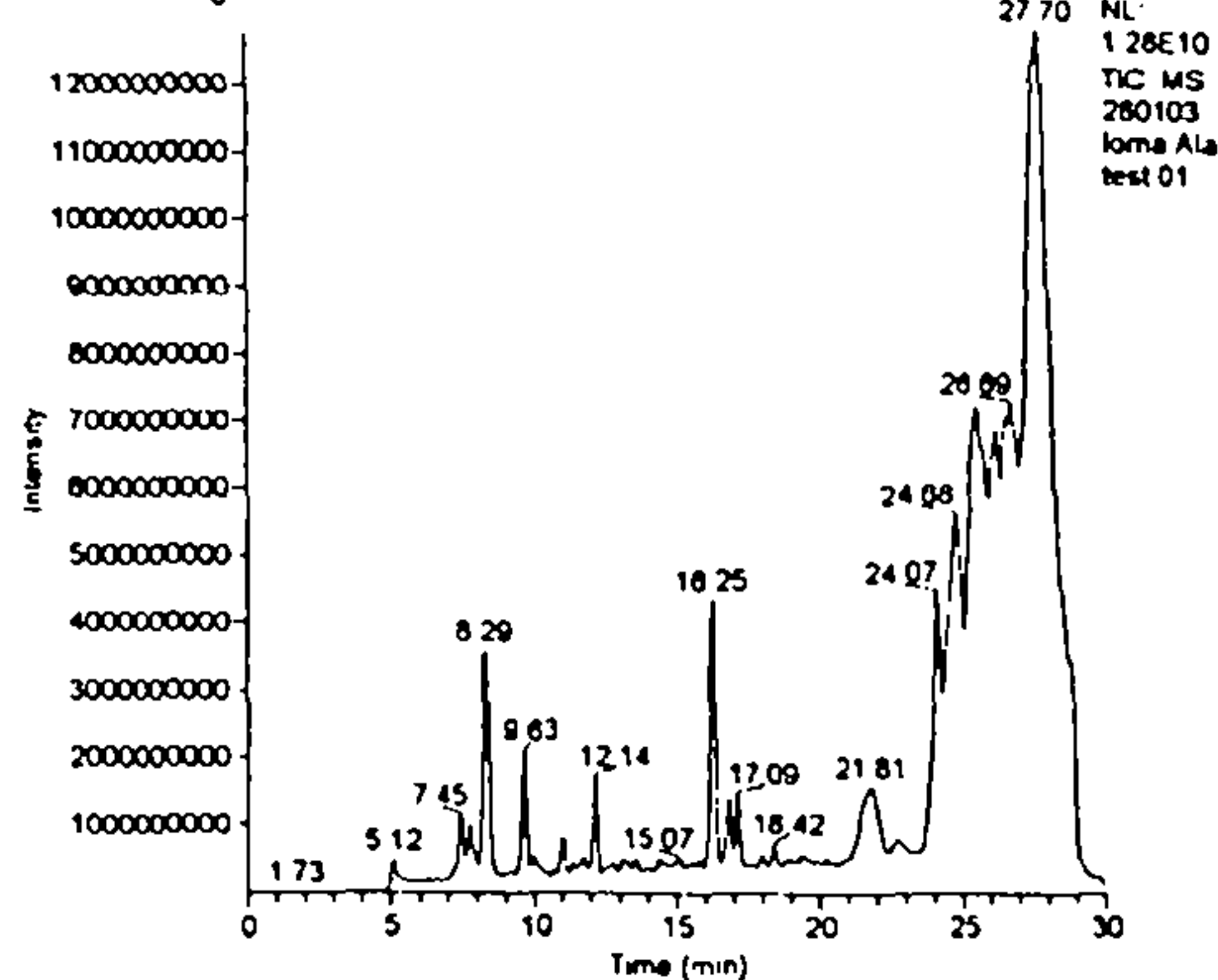
RP MAX  
I HAVE PREP COLUMN  
LIKE THIS!



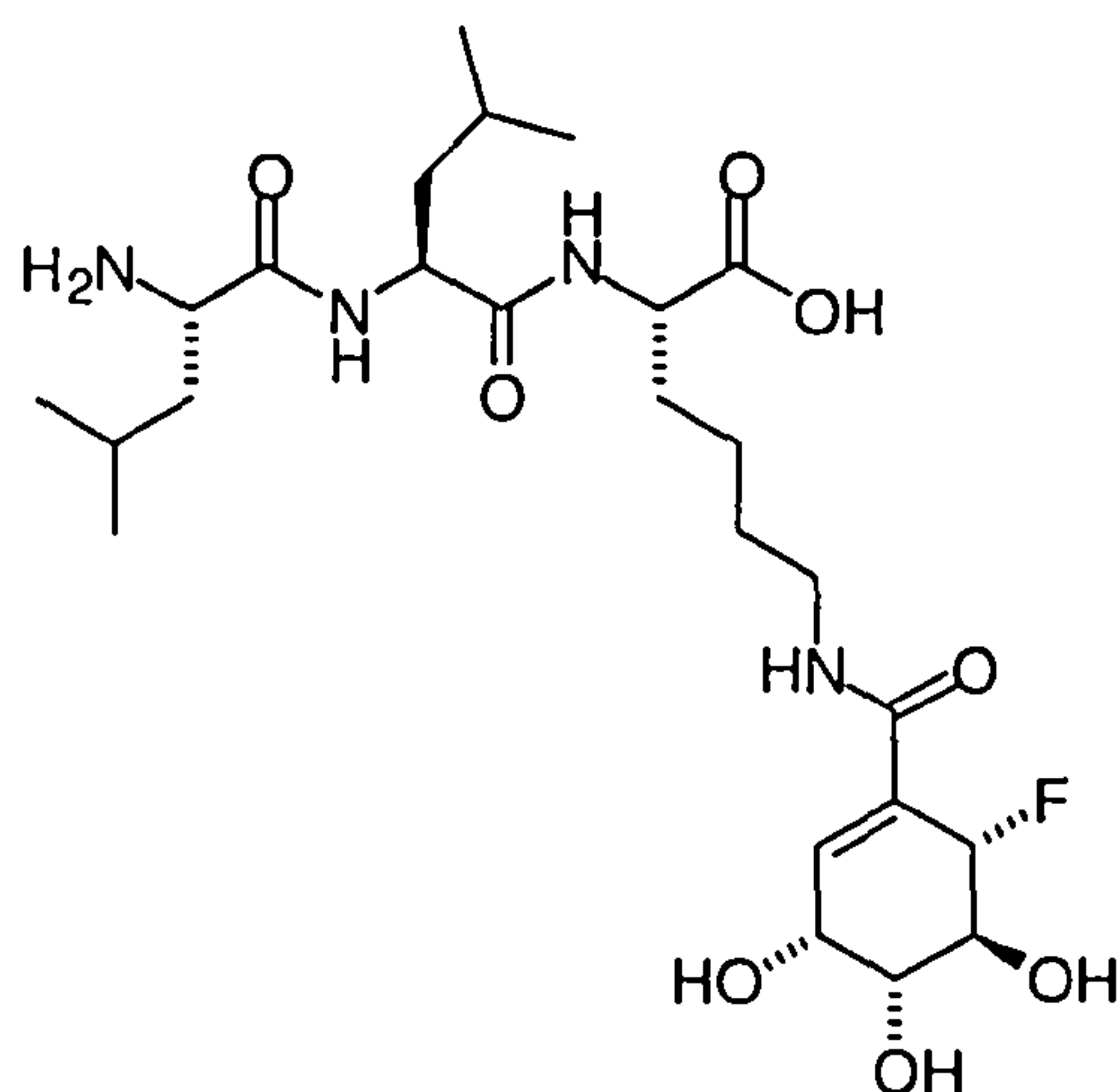
280103 lorna Ala test  
010174 RT 5.08 AV 1 NL  
21168 Y = c d Full ms2  
463.17 @ 35.00 |  
115.00-940.00 |



280103 lorna Ala test  
010174 RT 29.37 AV 1  
NL 61828 Y = c d Full  
ms2 463.17 @ 35.00 |  
115.00-940.00 |



**L-leucyl-L-leucyl-N- $\epsilon$ -(6*S*)-6-fluoroshikimoyl-L-lysine 5**



$^1\text{H}$ -NMR of crude product from resin ( $\text{CD}_3\text{OD}$ , 400 MHz)

$m/z$  (electrospray)

$m/z$  (electrospray) msms

HPLC/ms output (RP max column, 3 - 50 % 50 mM formic acid in MeCN)

```

Current Data Parameters
NAME LN27A-021001 3.8
PROG 10 00
PROCNO 1

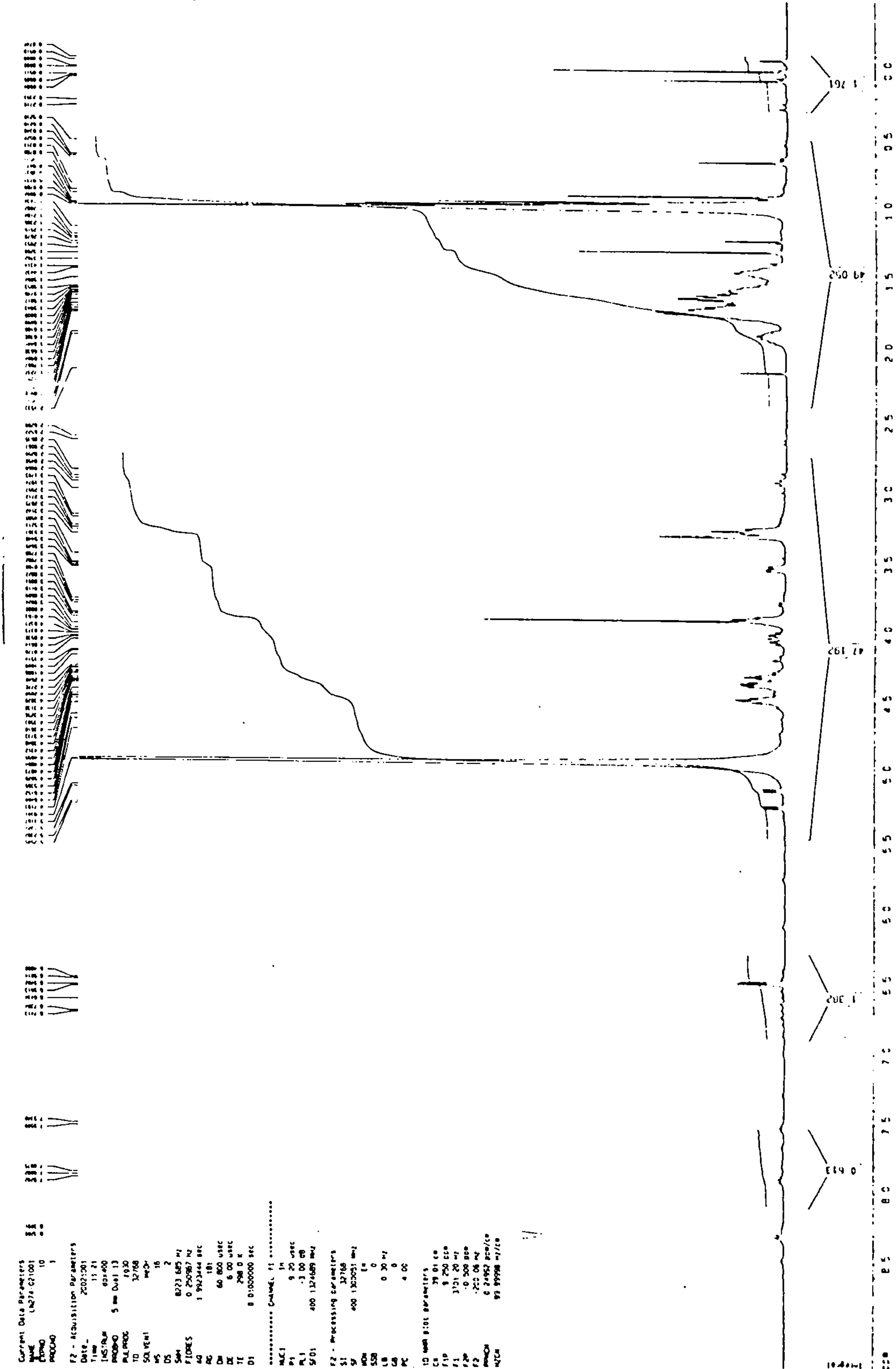
F2 - Acquisition Parameters
Date_ 20021001
Time 15:21
INSTRUM 602400
PROBHD 5 mm Dual 13
PULPROG 1930
TO 32768
SOLVENT me2o
MS 16
DS 2
SMA 8223.685 Hz
FIDRES 0.250967 Hz
AQ 1.99823444 sec
RG 181
DE 60.800 usec
ZE 6.00 usec
TE 298.0 K
D1 8.01000000 sec

***** CHANNEL F1 *****
NUC1 1H
P1 9.20 usec
PL1 -3.00 dB
SF01 400.1324609 MHz

F2 - Processing parameters
SI 32768
SF 400.1305051 MHz
WDW EM
SSB 0
LB 0.30 Hz
GB 0
PC 4.00

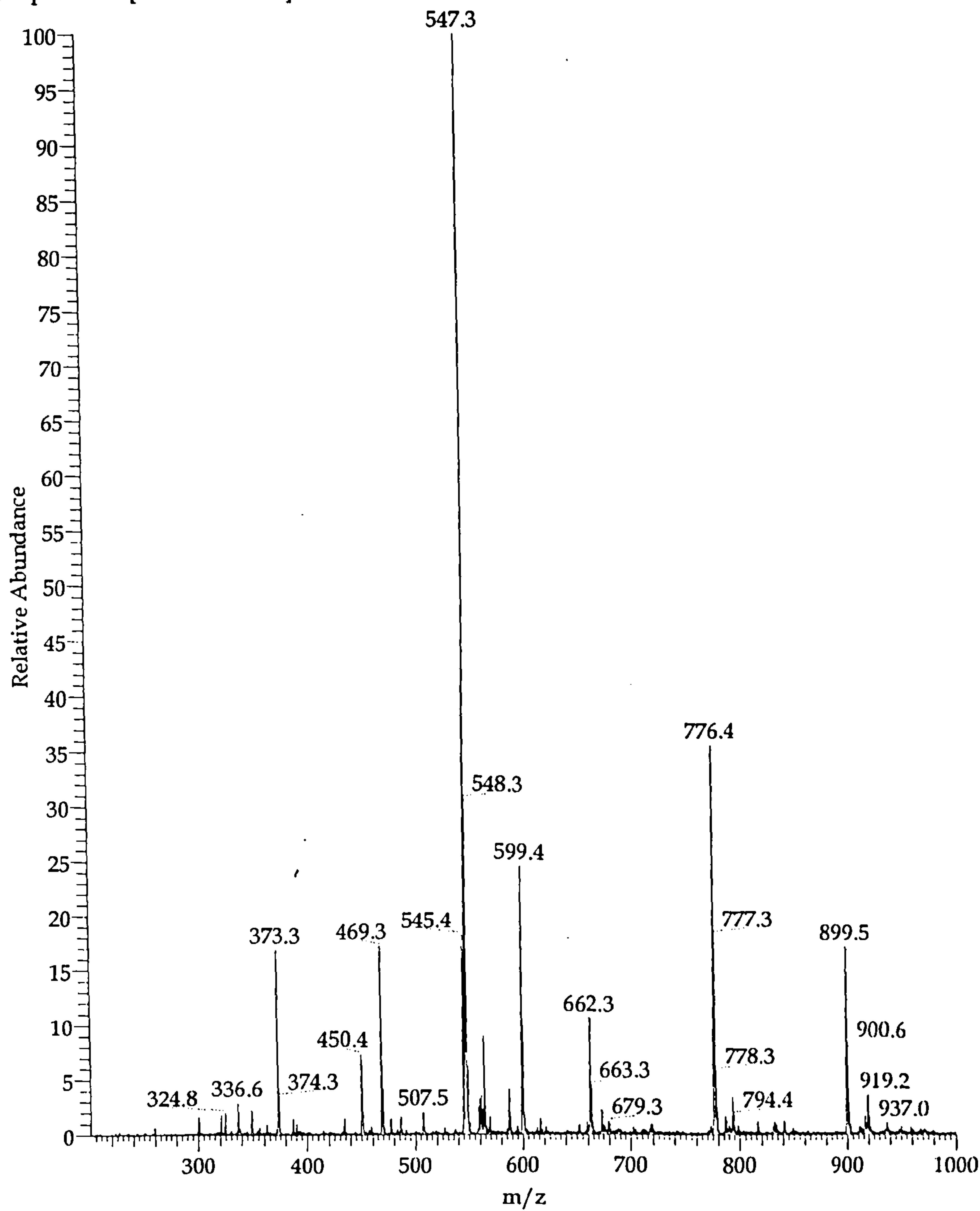
10 user plot parameters
CL 29.01 cm
F10 8.250 cm
F1 3101.20 Hz
F20 -0.500 cm
F2 -200.06 Hz
PMDCH 6.24952 sec/cg
MTCn 93.99998 m/sec

```



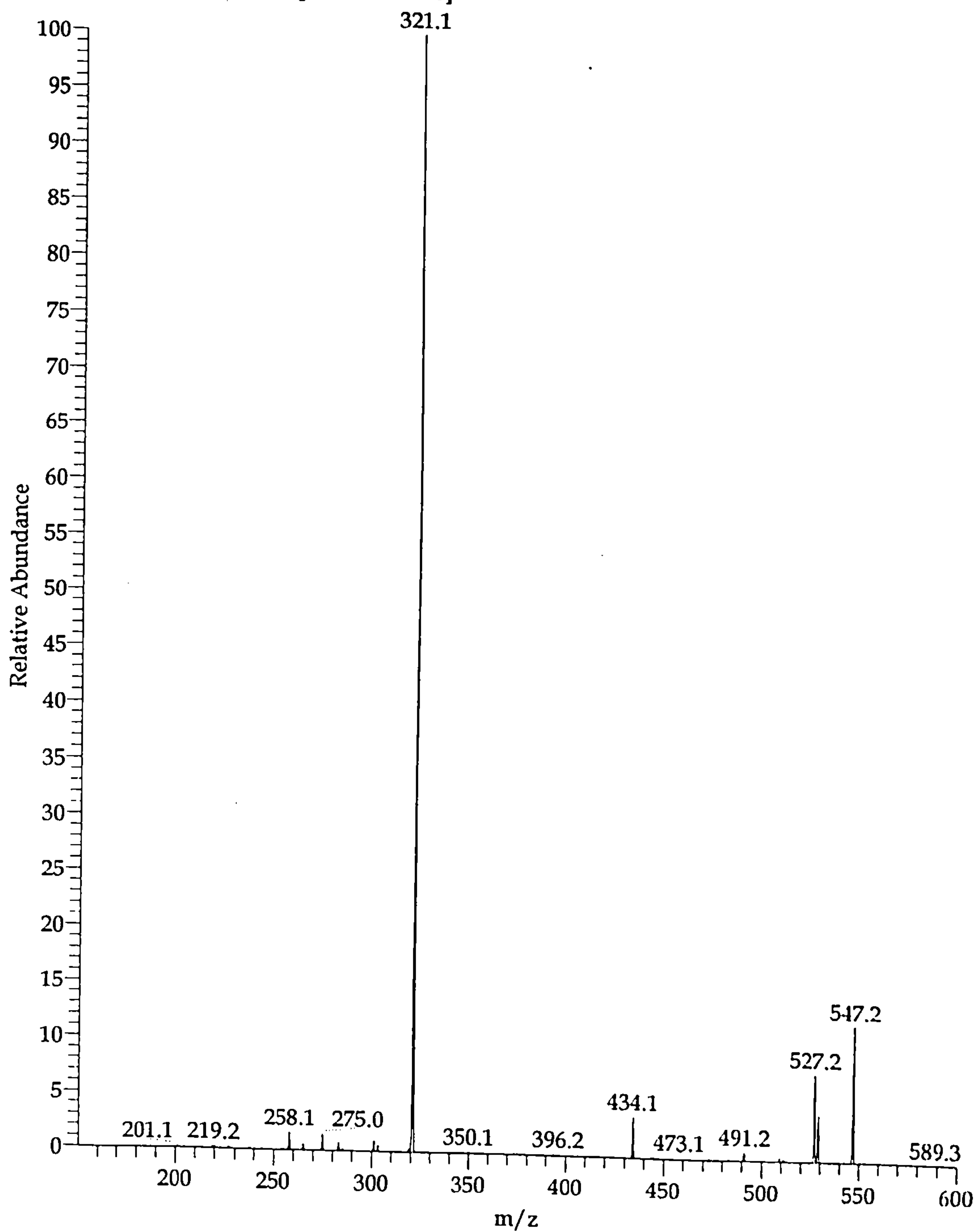
Lorna1 #3-57 RT: 0.03-0.82 AV: 55 NL: 2.98E7

T: + p Full ms [ 200.00-1000.00]



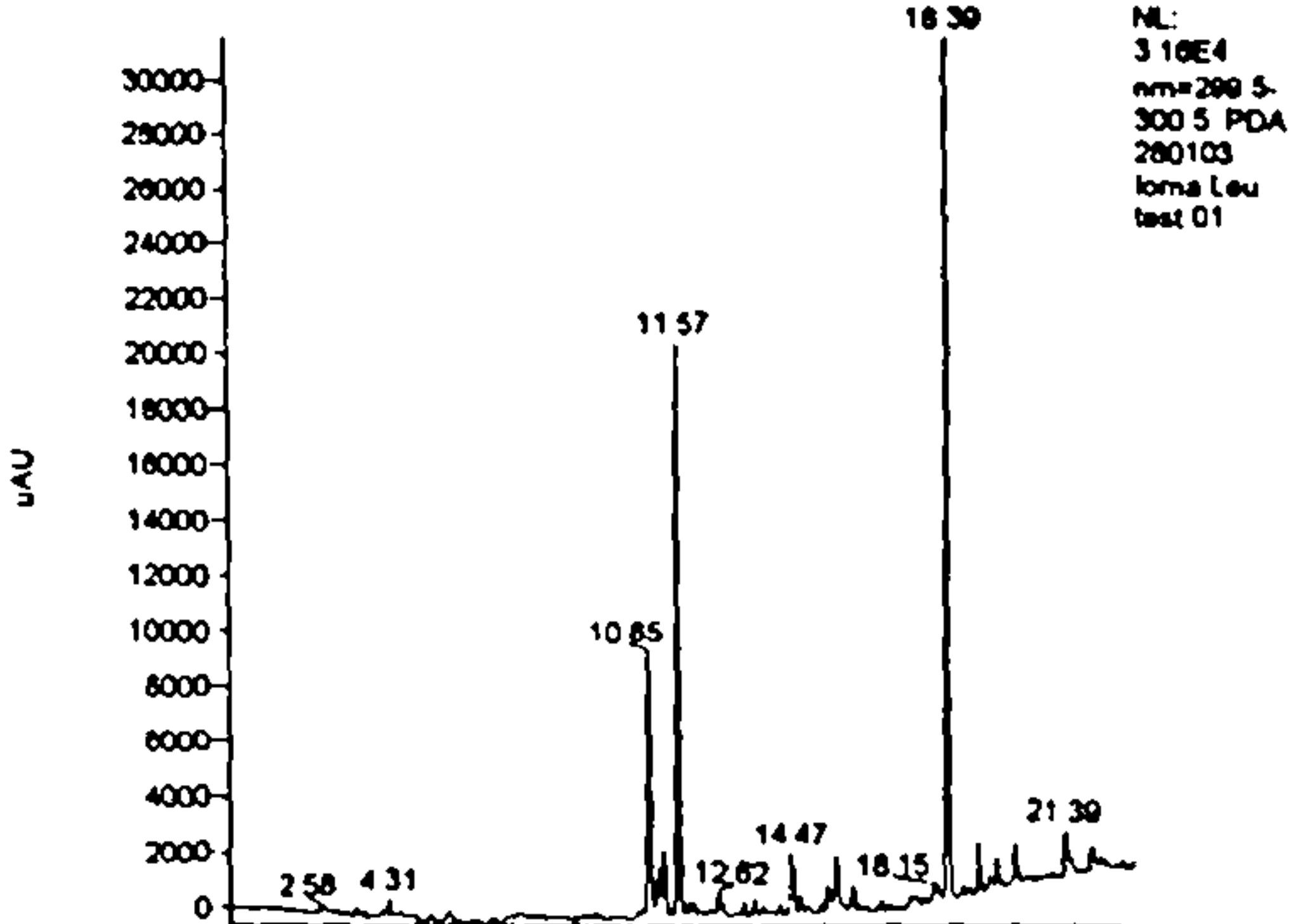
Lorna1msms #1-24 RT: 0.01-0.33 AV: 24 NL: 3.60E7

T: + p Full ms2 547.30025.00 [150.00-600.00]





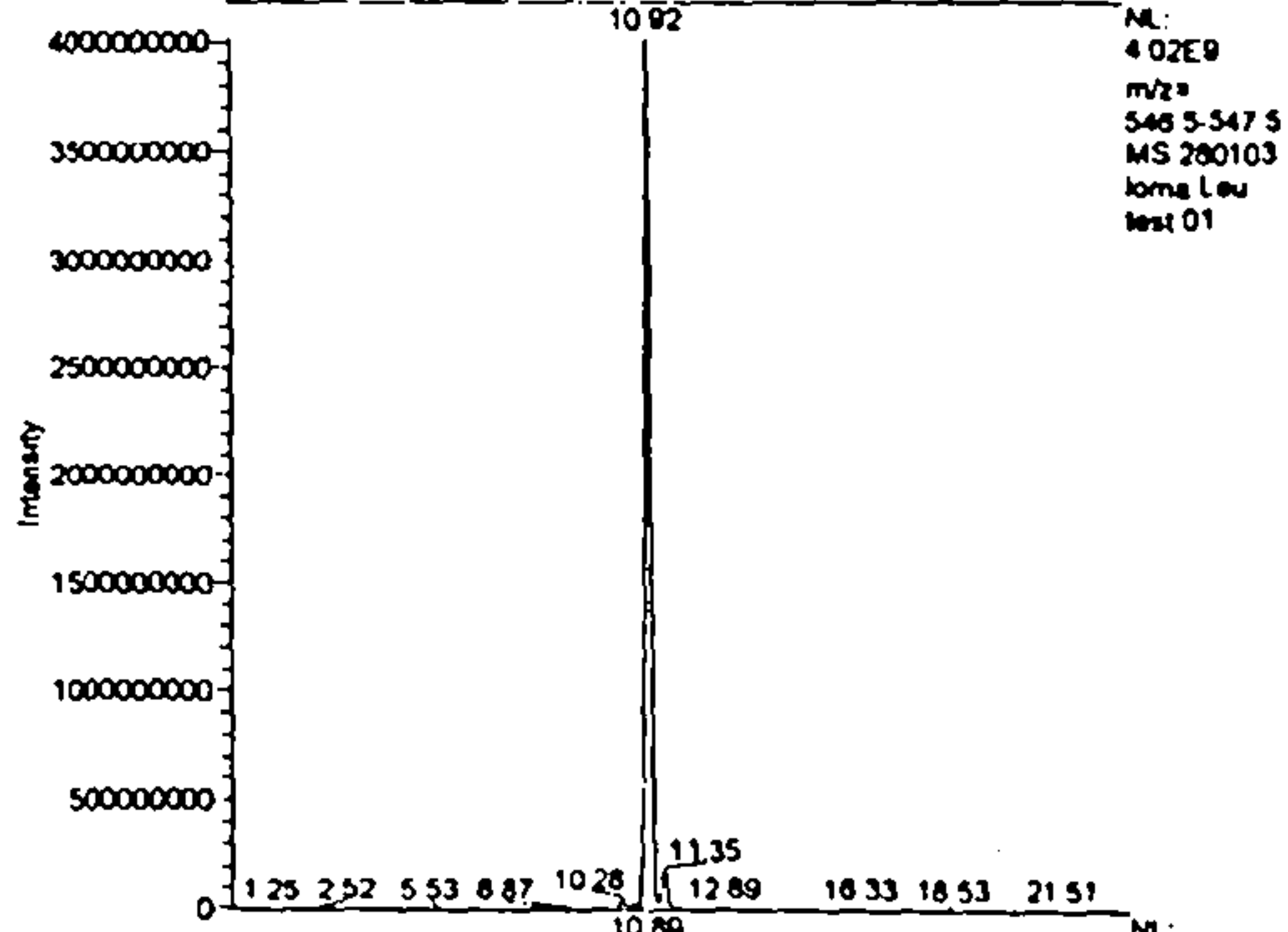
RT: 0.25 - 23.22 SM: 150



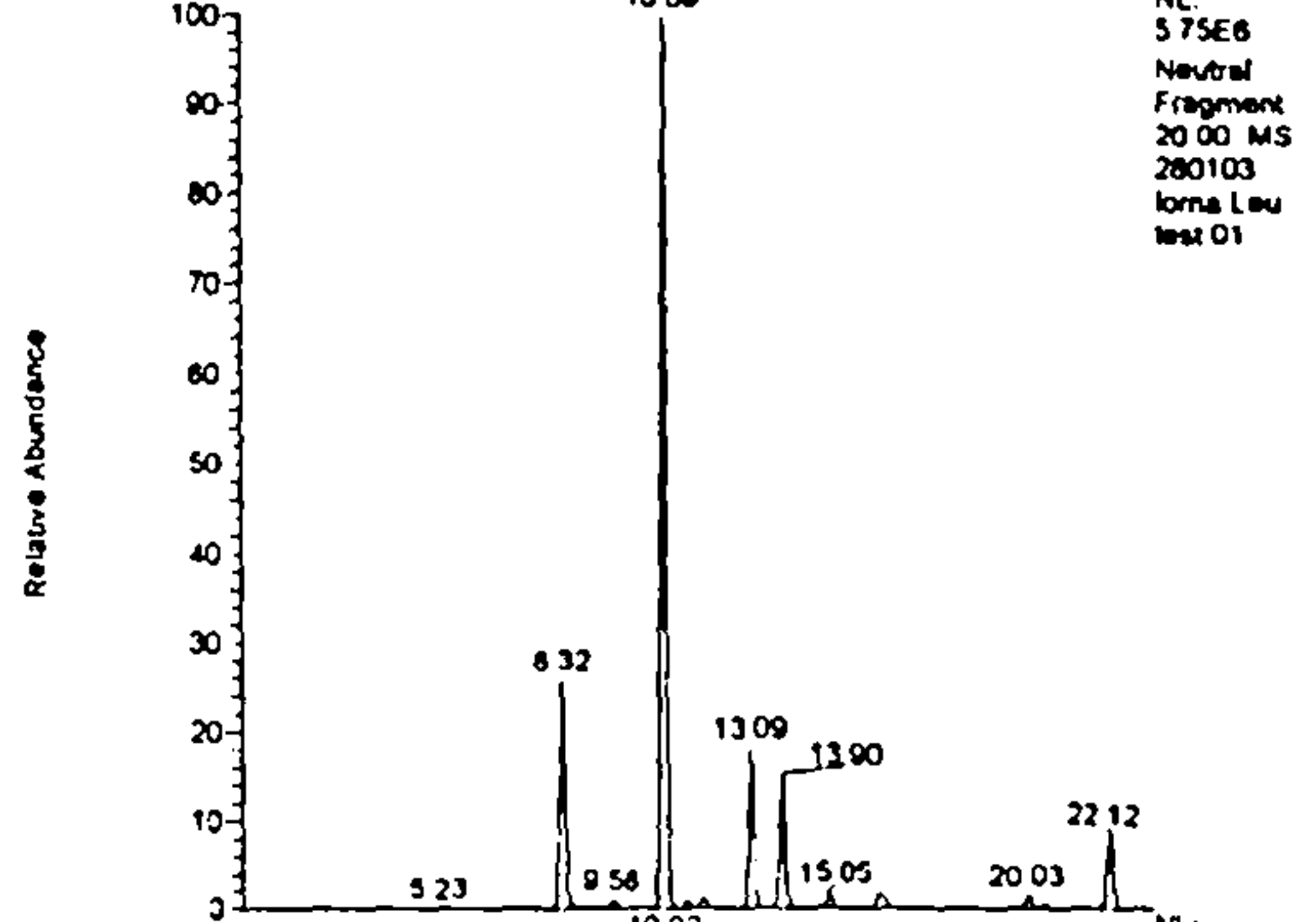
NL: 310E4  
nm=280.5-  
300.5 PDA  
280103  
lorna Leu  
test 01

280103 lorna Leu test 01  
Type: Unknown ID: C:25 Row: 18  
Sample Name:  
Study:  
Client:  
Laboratory:  
Company:  
Phone:  
Instrument Method: C:\Xcalibur\methods\lorna\020902 20 min  
grad 3-50% C n D pos.meth  
Processing Method:  
Vial: D:23  
Injection Volume (µl): 10.00  
Sample Weight: 0.00  
Sample Volume (µl): 0.00  
ISTD Amount: 0.00  
Dil Factor: 1.00

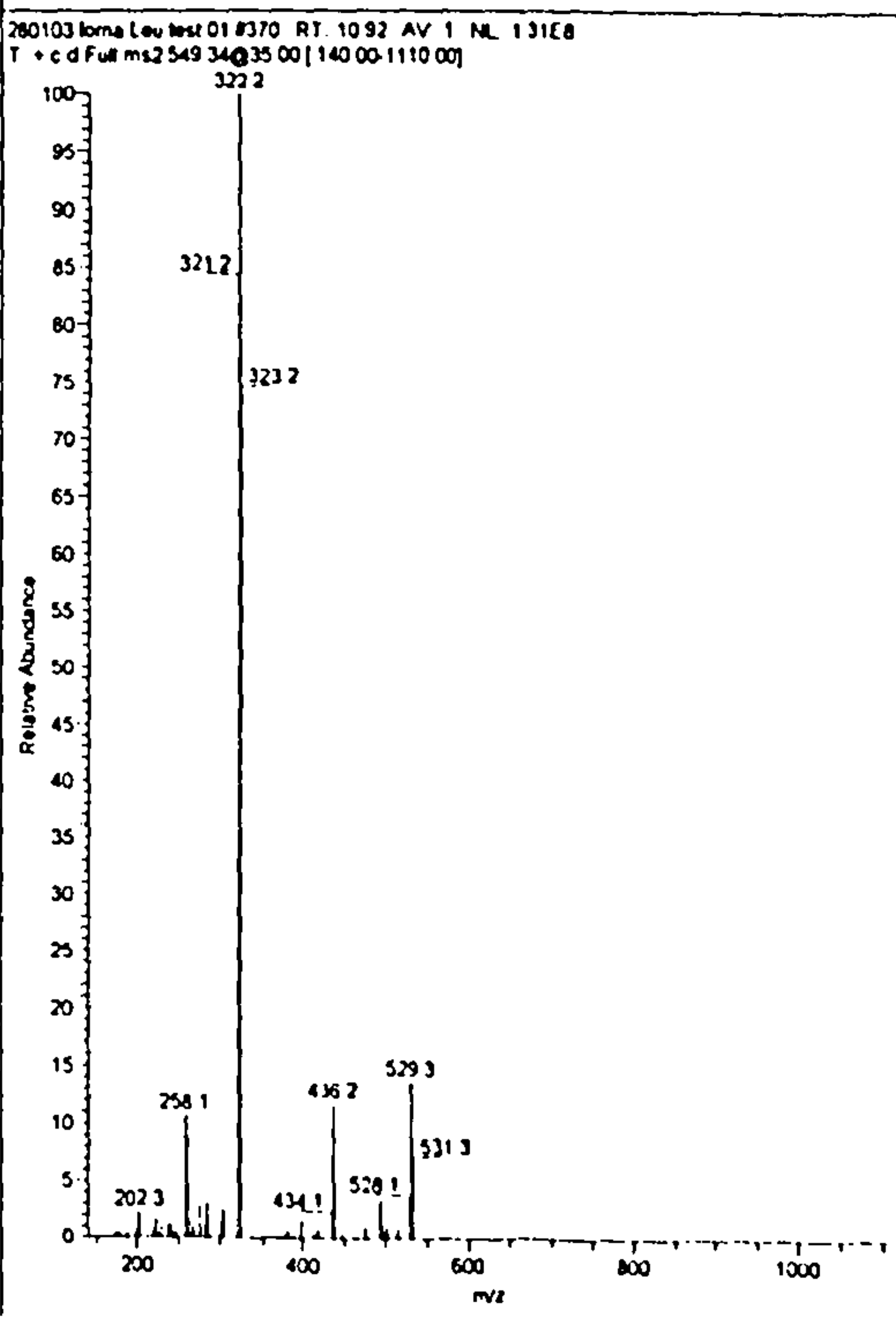
RP MAX COLUMN.  
I HAVE PREP UER.



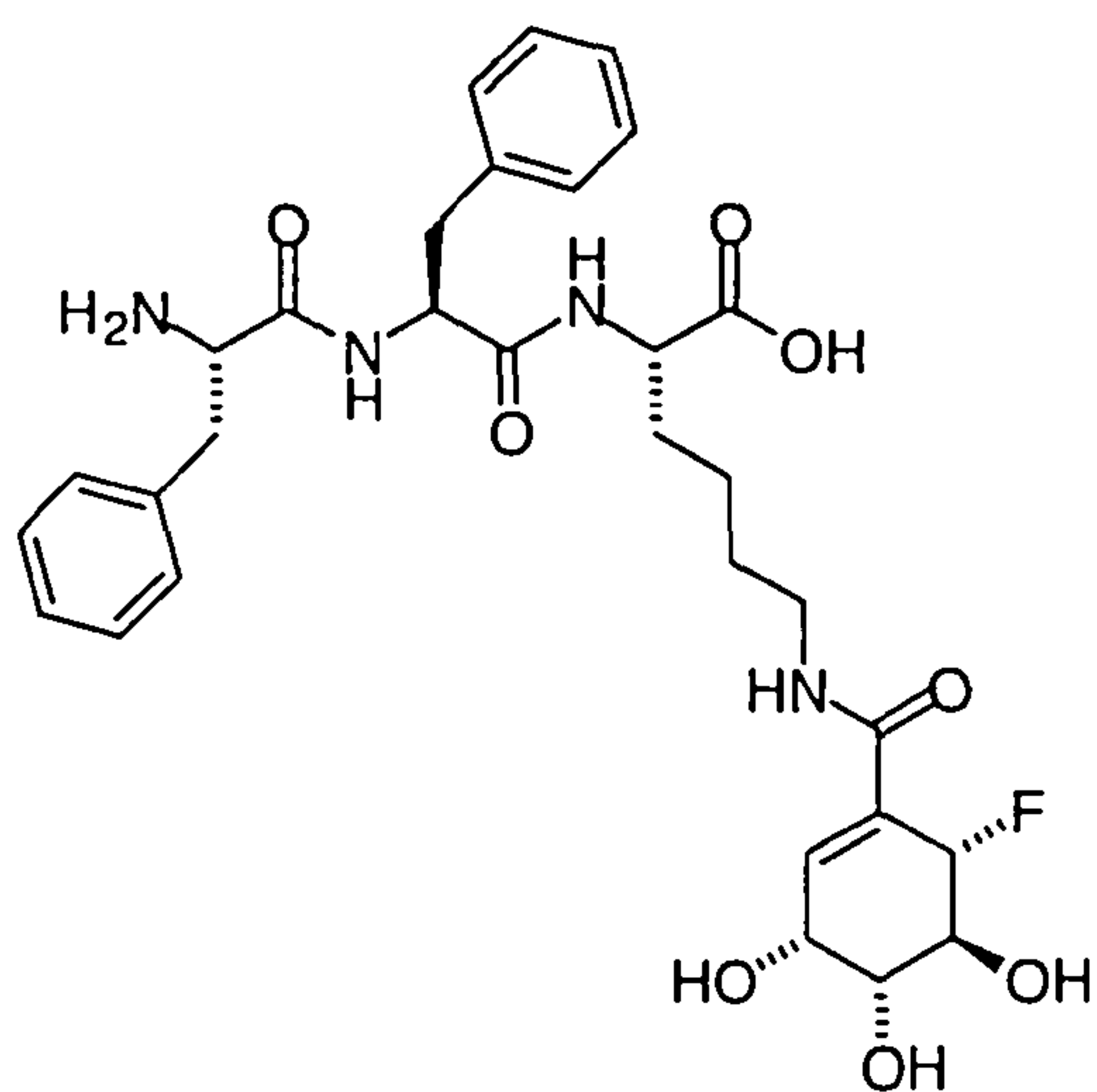
NL: 402E9  
m/z=546.5-547.5  
MS 280103  
lorna Leu  
test 01



NL: 575E6  
Neutral  
Fragment  
20.00 MS  
280103  
lorna Leu  
test 01



**L-phenylalanyl-L-phenylalanyl-N-ε-(6*S*)-6-fluoroshikimoyl-L-lysine 6**

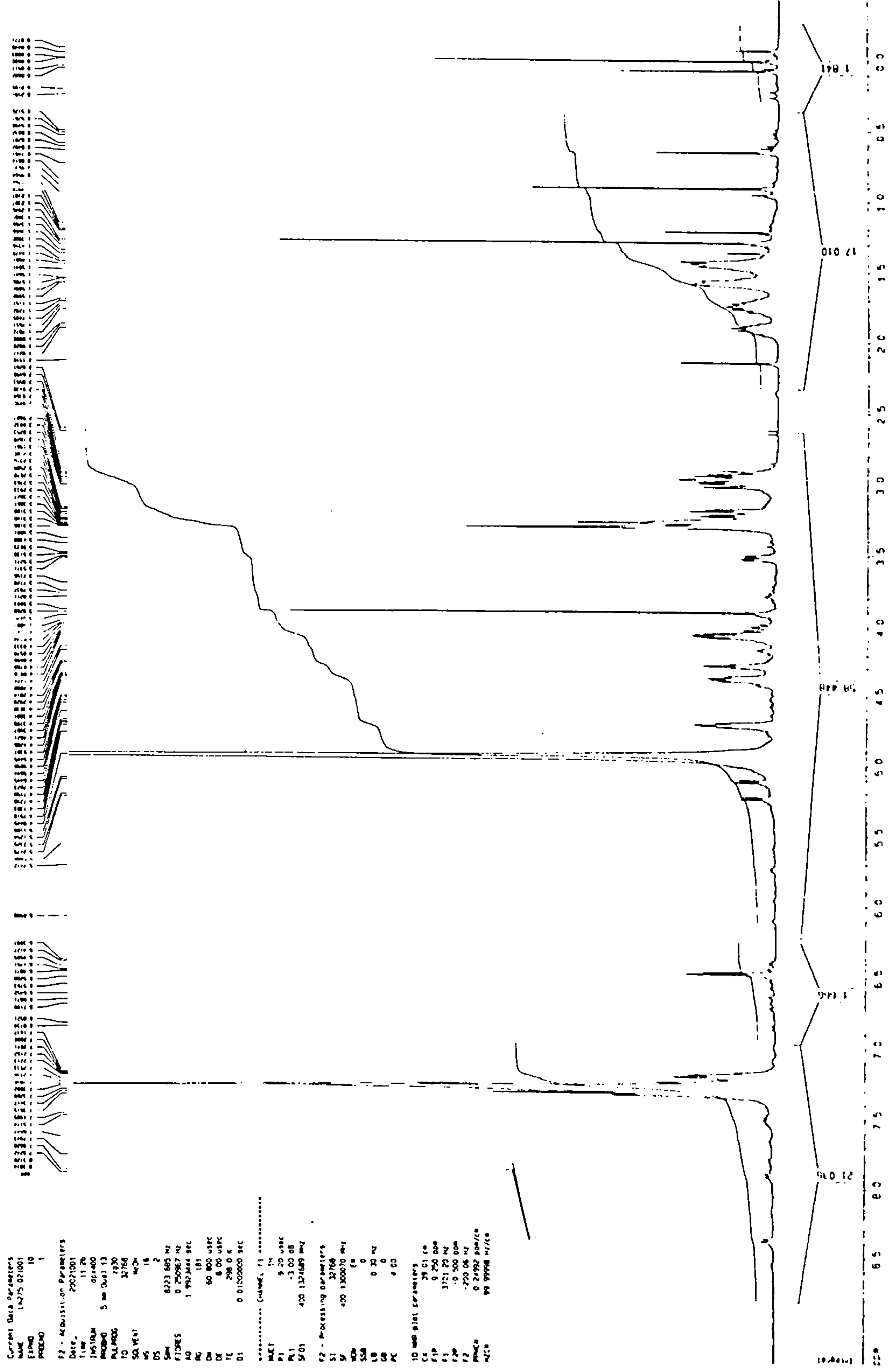


<sup>1</sup>H-NMR of crude product from resin (CD<sub>3</sub>OD, 400 MHz)

*m/z* (electrospray)

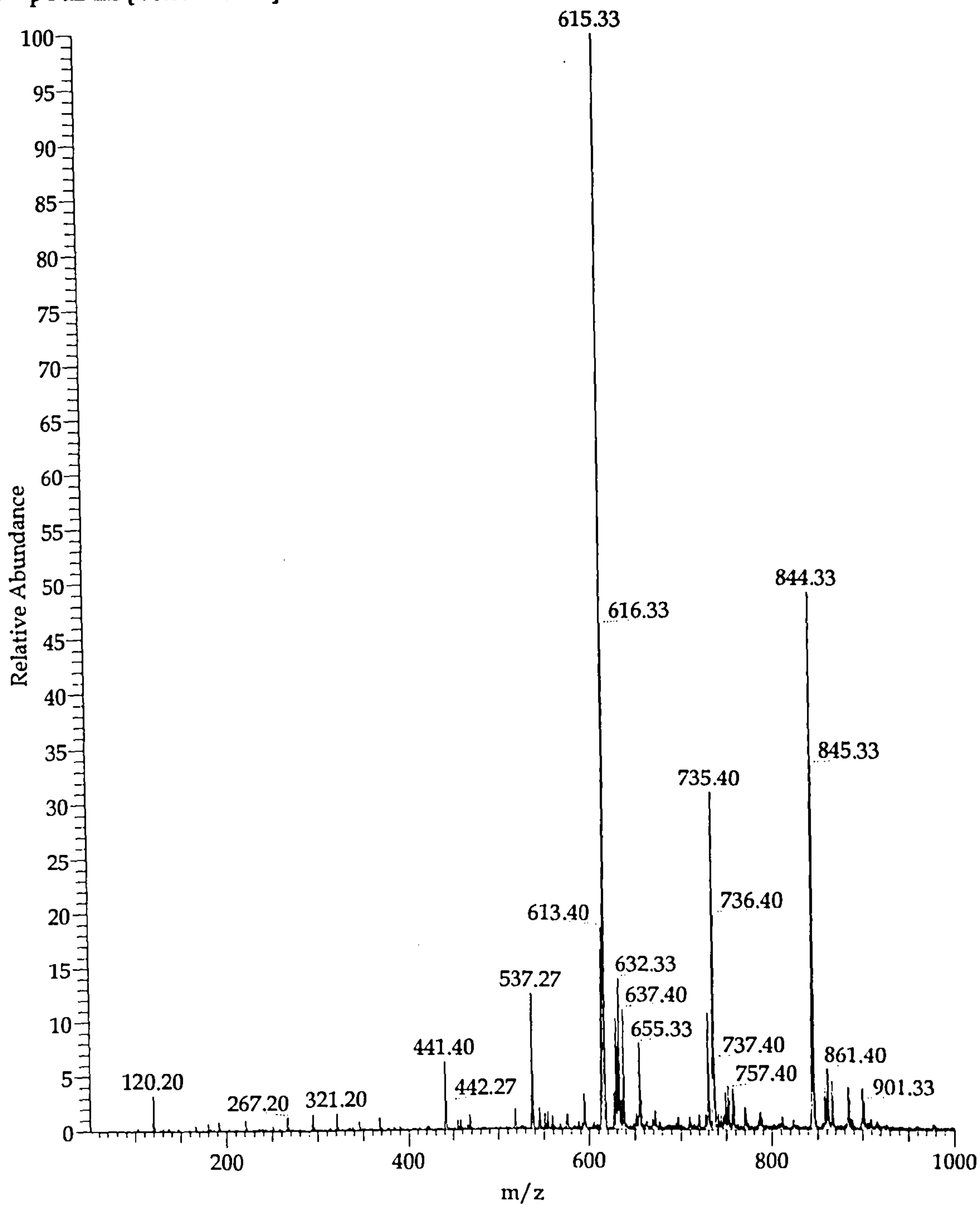
*m/z* (electrospray) msms

HPLC/ms output (RP max column, 3 - 50 % 50 mM formic acid in MeCN)



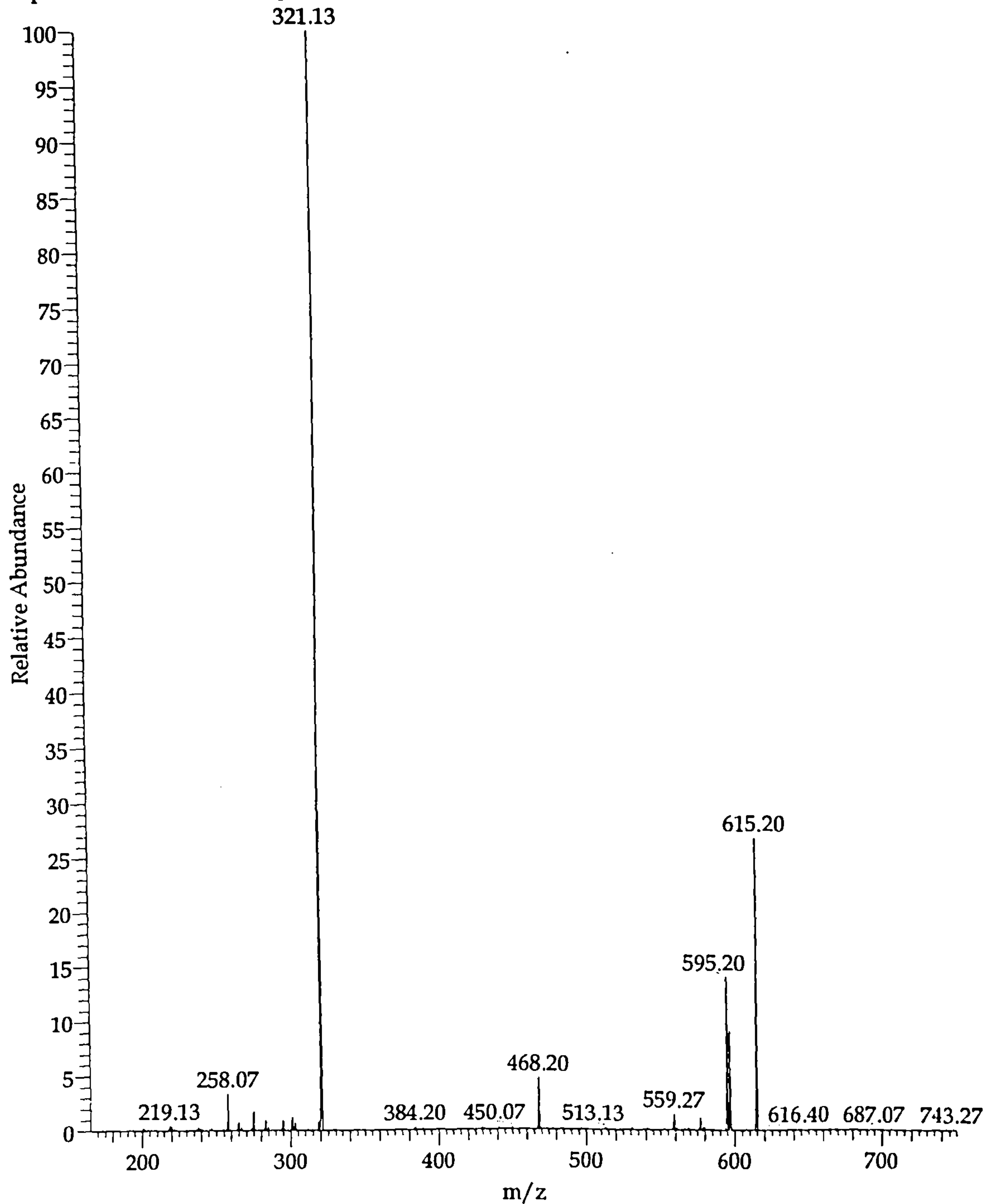
LN275 #2-89 RT: 0.02-1.45 AV: 88 NL: 1.14E7

T: + p Full ms [ 50.00-1000.00]



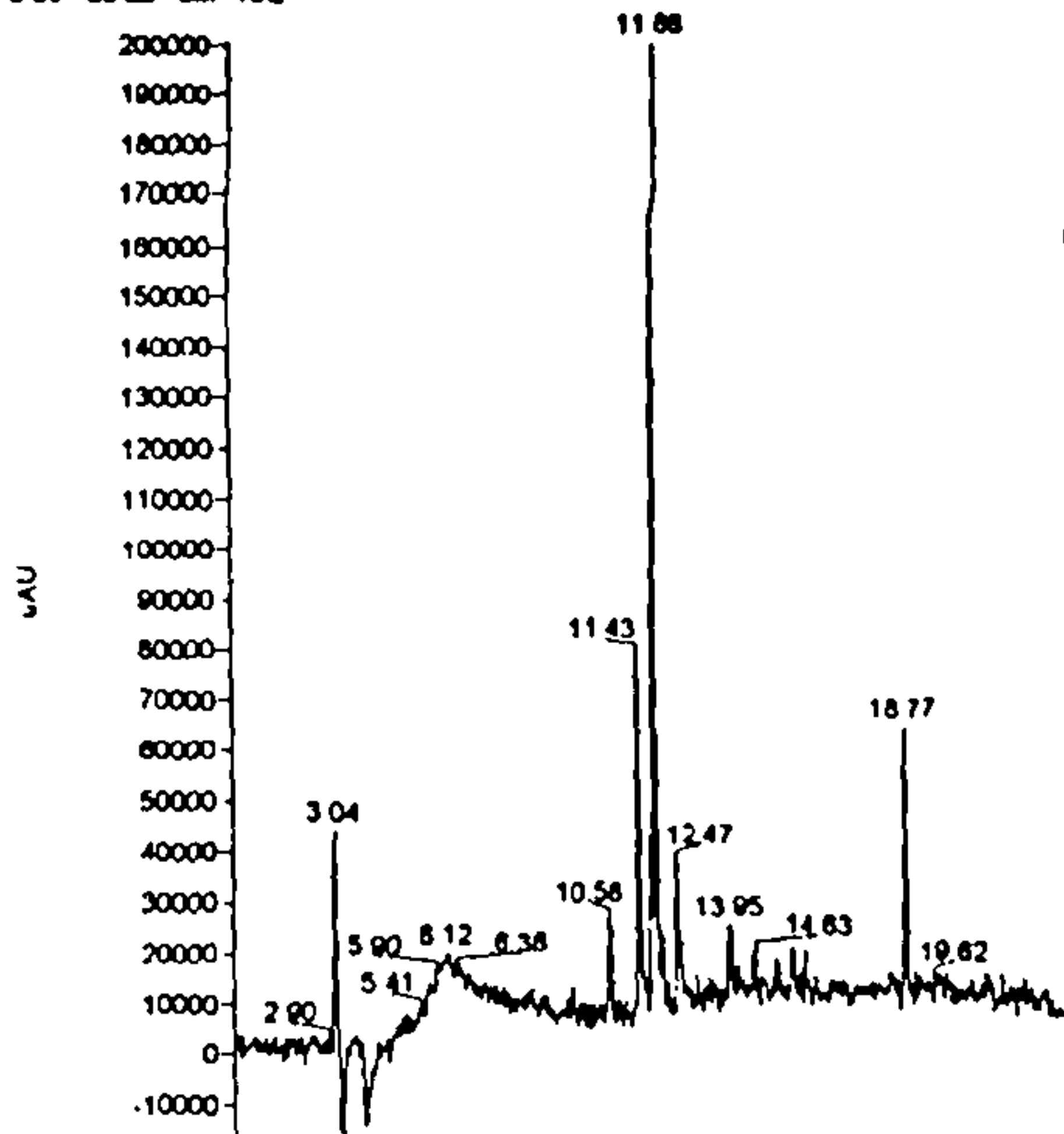
LN275msms #1-42 RT: 0.01-0.65 AV: 42 NL: 2.70E6

T: + p Full ms2 615.40@25.00 [165.00-750.00]





RT: 0.25 - 23.22 SM 150



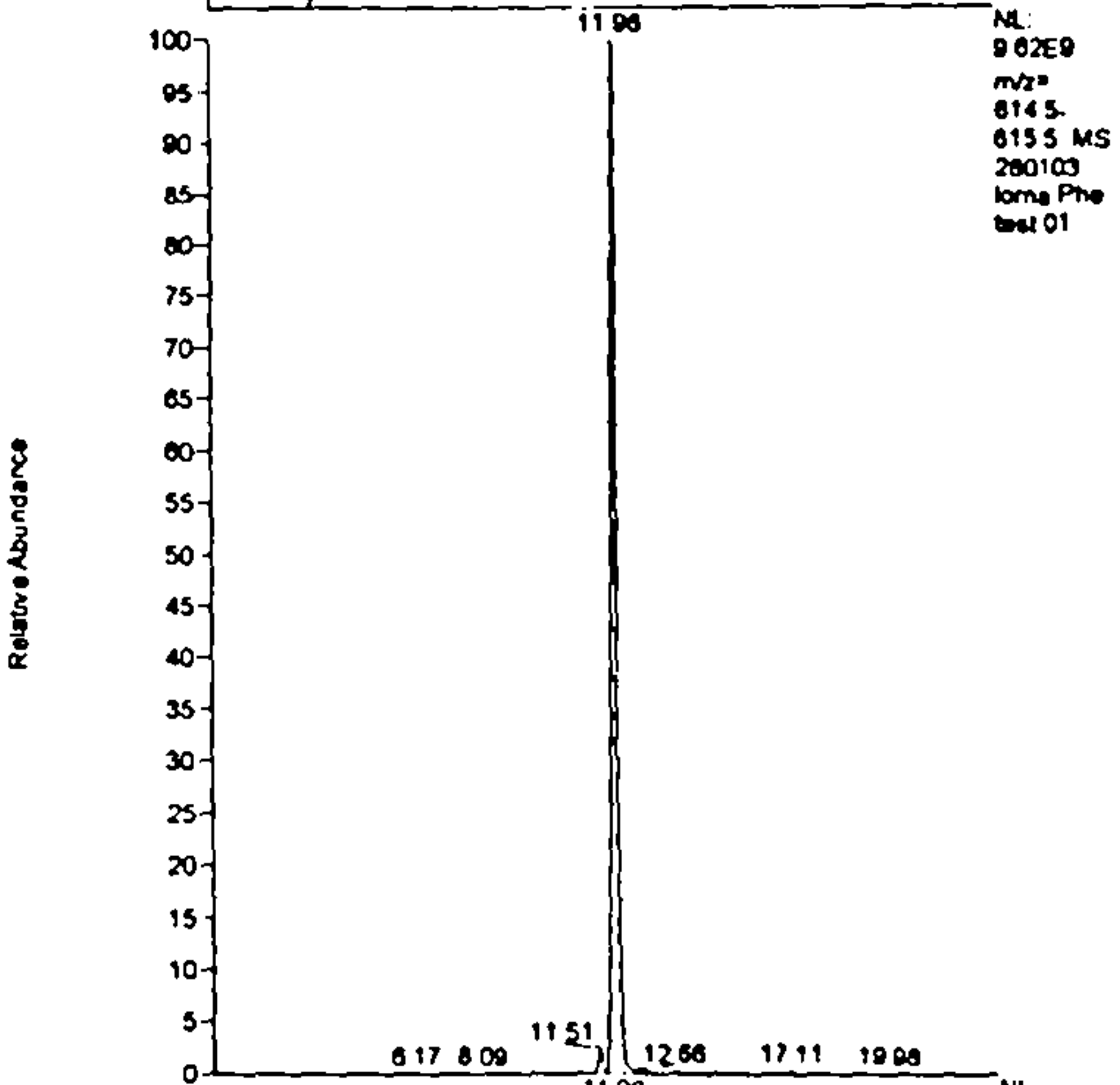
ML:  
200E5  
nm=214.5  
215.5  
PDA  
280103  
lorna Phe  
test 01

280103 lorna Phe test 01  
Type: Unknown ID: C:25 Row: 18  
Sample Name:  
Study:  
Client:  
Laboratory:  
Company:  
Phone:

Instrument Method: C:\Xcalibur\methods\lorna\020902 20 min  
grad J-50% C n D pos.meth

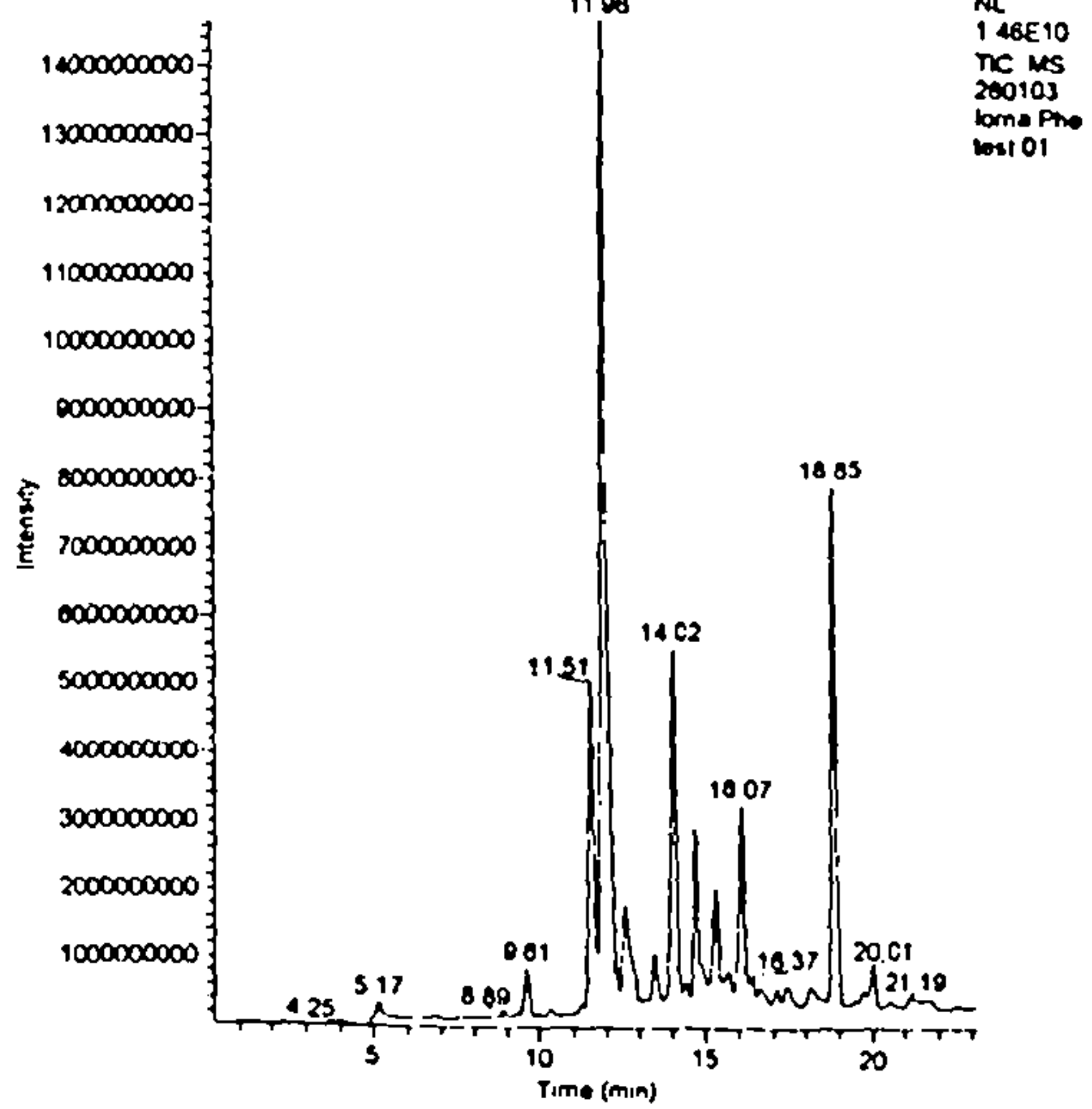
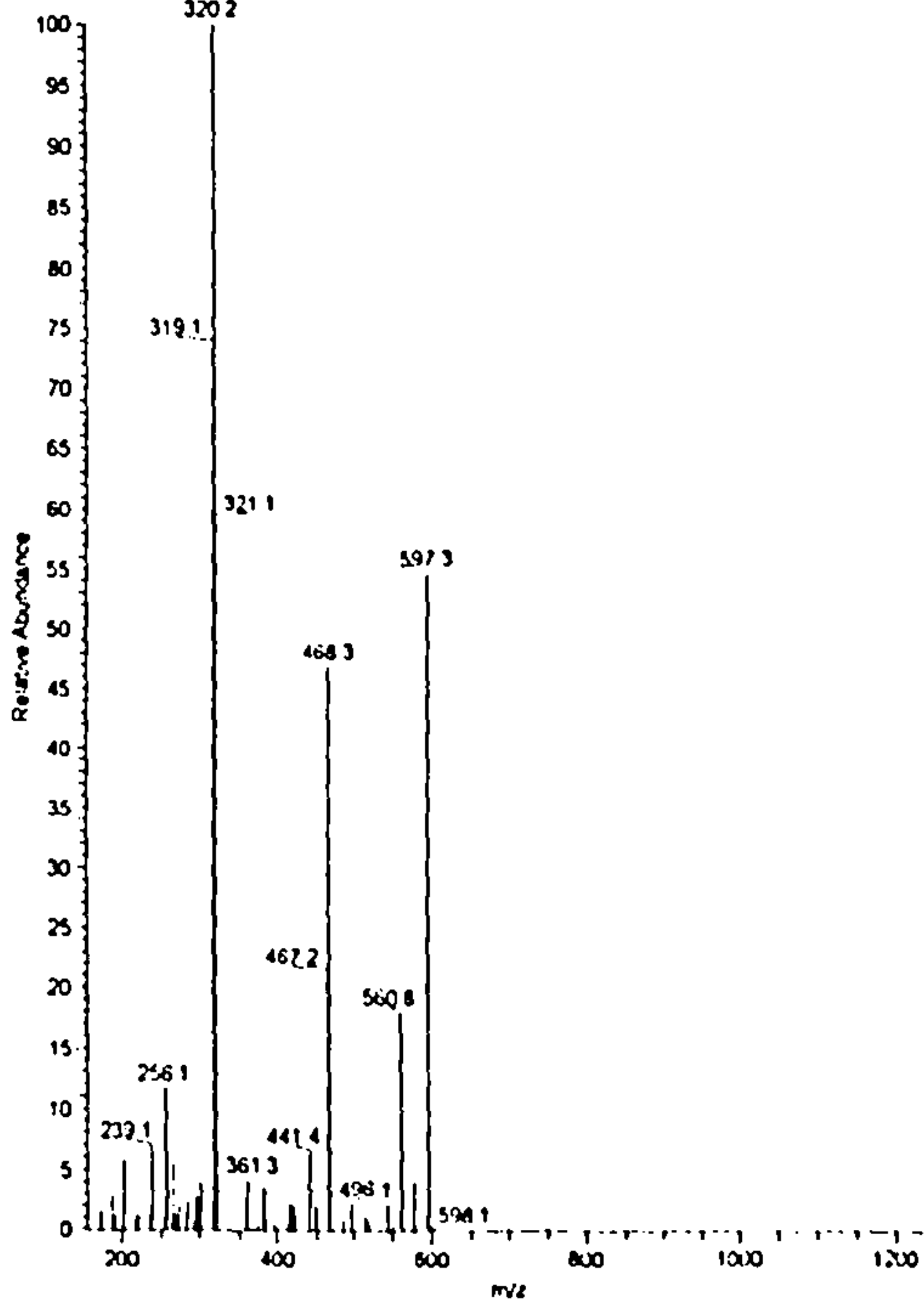
Processing Method:  
Vial: D:25  
Injection Volume (µl): 10.00  
Sample Weight: 0.00  
Sample Volume (µl): 0.00  
ISTD Amount: 0.00  
Dil Factor: 1.00

RP MAX COLUMN.  
I HAVE PREP VERSION!



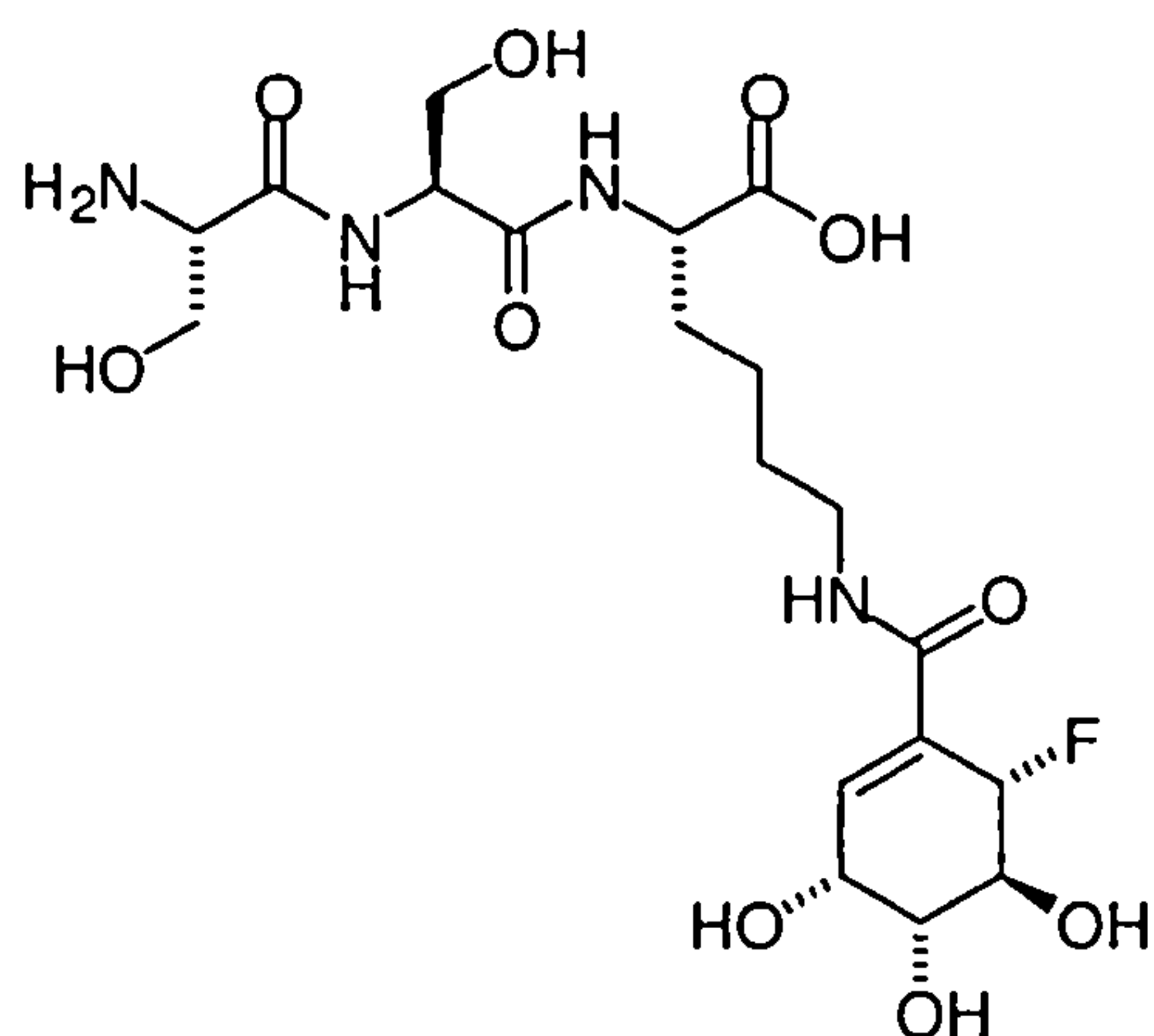
ML:  
9.02E9  
m/z= 614.5  
615.5 MS  
280103  
lorna Phe  
test 01

280103 lorna Phe test 01 8389 RT: 11.50 AV: 1 NL: 1.56E7  
T: c d Full ms2 615.31@35.00 [155.00-1245.00]



ML:  
1.46E10  
TIC MS  
280103  
lorna Phe  
test 01

**L-seryl-L-seryl-N- $\epsilon$ -(6*S*)-6-fluoroshikimoyl-L-lysine 7**

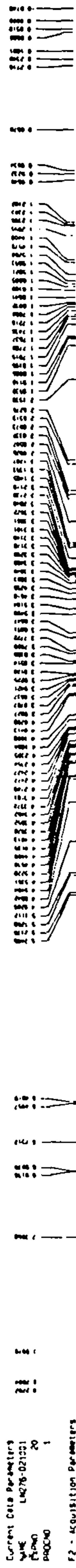


$^1\text{H-NMR}$  of crude product from resin ( $\text{CD}_3\text{OD}$ , 400 MHz)

$m/z$  (electrospray)

$m/z$  (electrospray) msms

HPLC/ms output (RP max column, 3 - 50 % 50 mM formic acid in MeCN)



Current Data Parameters  
Name: LQ276-021011  
Pch: 20  
Proc: 1

F2 - Acquisition Parameters  
Date: 20021001  
Time: 13:28  
F1: 100.000  
F2: 100.000  
PUL: 5.000  
T0: 10.000  
SOL: 10.000  
NS: 10  
S: 2  
Sum: 0.000000 Hz  
F1: 0.000000 Hz  
F2: 1.992344 sec  
F3: 20.000  
F4: 60.000 usec  
F5: 6.00 usec  
F6: 200.0 Hz  
F7: 0.0100000 sec

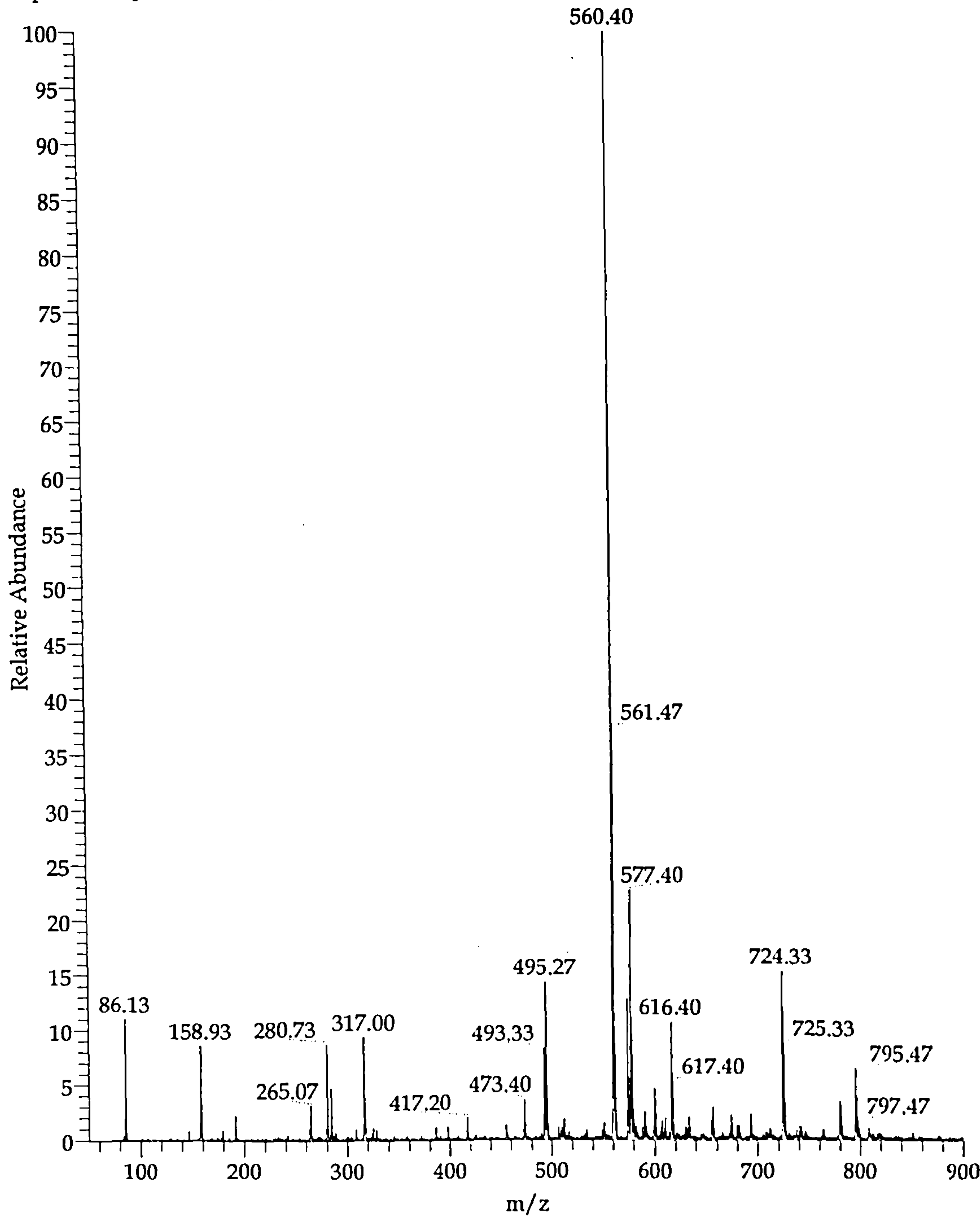
..... DUMPL 11 .....

MACI: 11  
P1: 9.20 usec  
P2: -3.00 dB  
P3: 400.132/1000 Hz

F2 - Processing parameters  
S1: 32768  
S2: 400.130000 Hz  
S3: 10  
S4: 0.00 Hz  
S5: 0.00 Hz  
S6: 0.00 Hz  
S7: 4.00

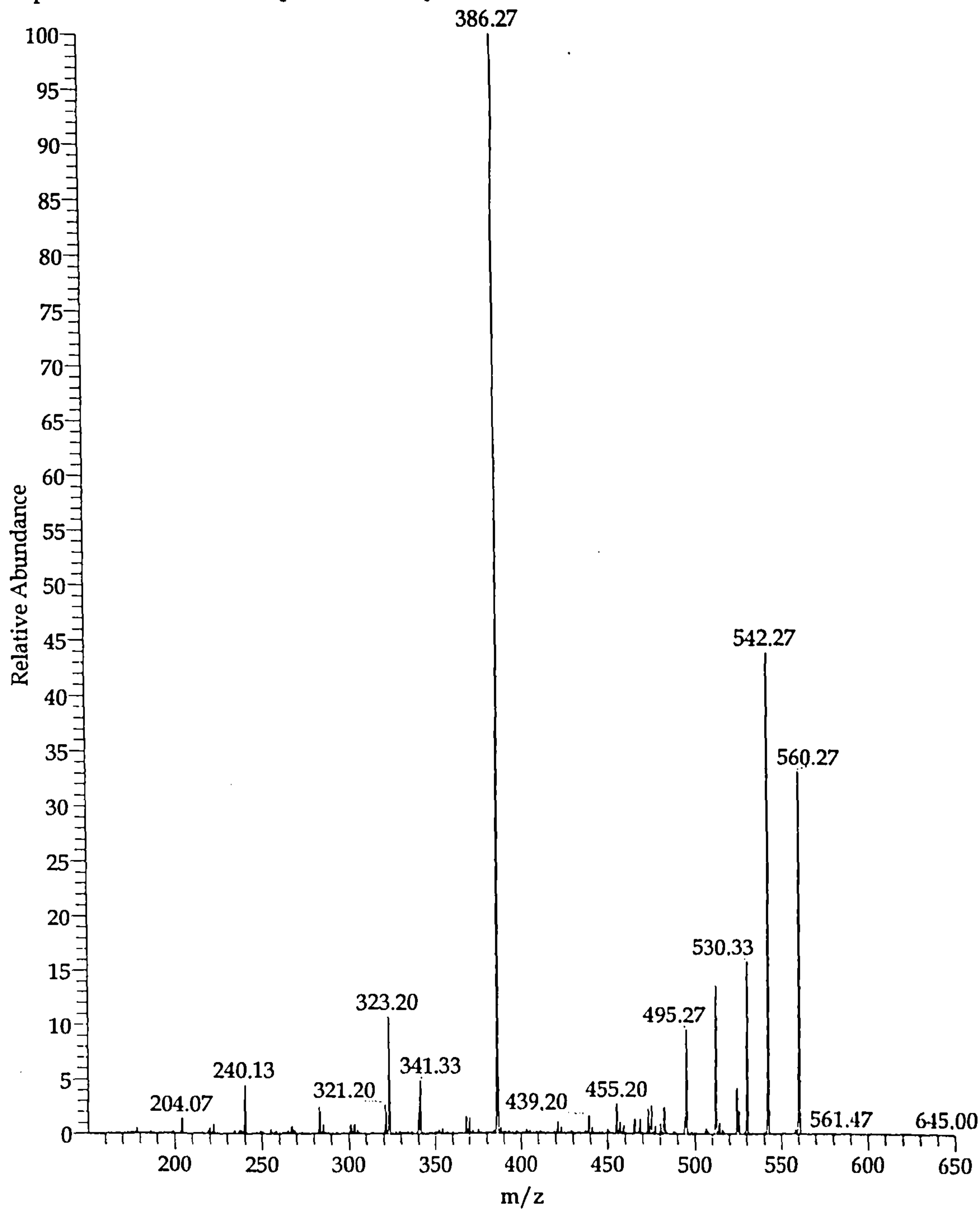
12 band plot parameters  
C1: 20.000 Hz  
C2: 20.000 Hz  
C3: 20.000 Hz  
C4: 20.000 Hz  
C5: 20.000 Hz  
C6: 20.000 Hz  
C7: 20.000 Hz  
C8: 20.000 Hz  
C9: 20.000 Hz  
C10: 20.000 Hz  
C11: 20.000 Hz  
C12: 20.000 Hz

LN276 #2-54 RT: 0.02-0.83 AV: 53 NL: 8.86E6  
T: + p Full ms [ 50.00-900.00]



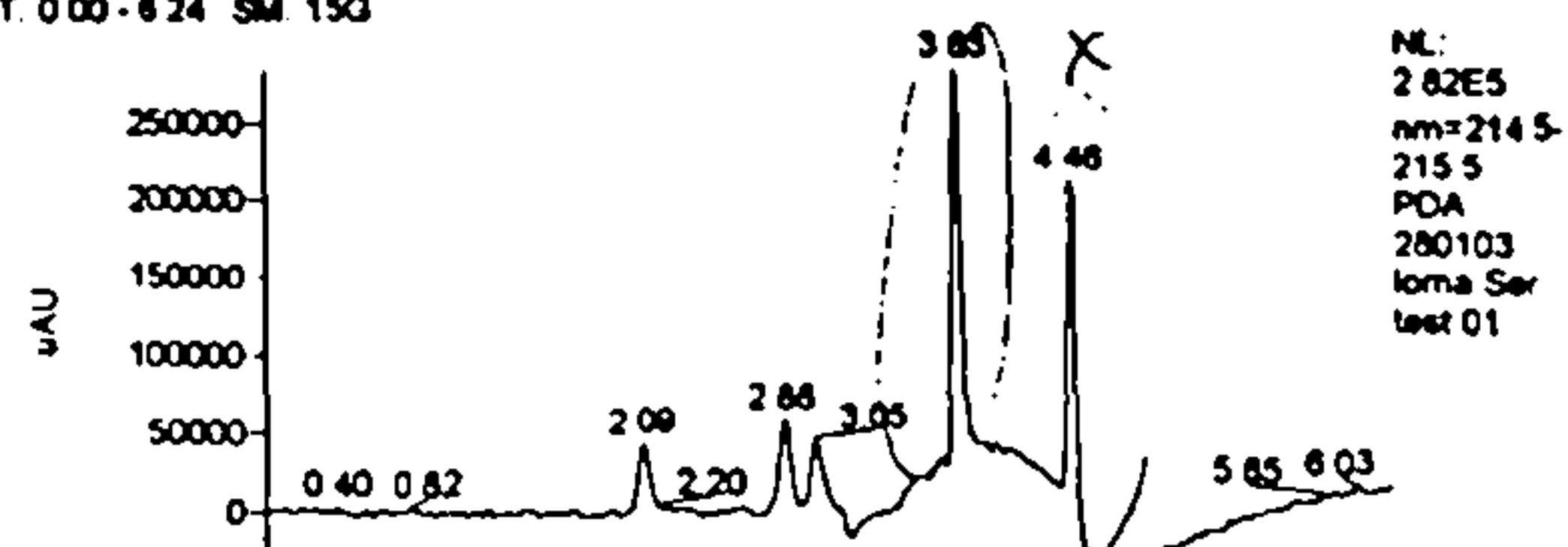
LN276\_021014094124 #2-41 RT: 0.02-0.58 AV: 40 NL: 3.98E6

T: + p Full ms2 560.30@31.00 [150.00-650.00]





RT: 0.00 - 6.24 SM 150



NL:  
282E5  
nm=214.5  
215.5  
PDA  
280103  
lorna Ser  
test 01

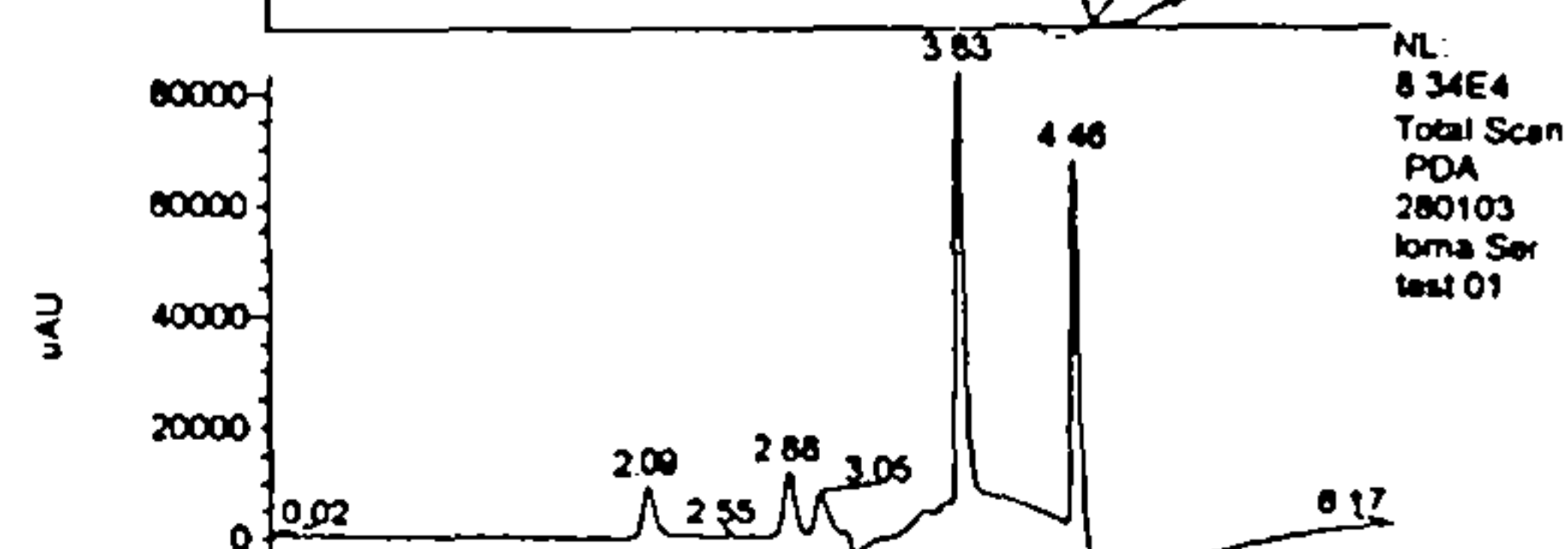
280103 lorna Ser test 01  
Type: Unknown ID: C:25 Row: 18

Sample Name:  
Study:  
Client:  
Laboratory:  
Company:  
Phone:

Instrument Method: C:\Xcalibur\methods\lorna\020902 20 min  
grad 3-50% C n D pos.meth

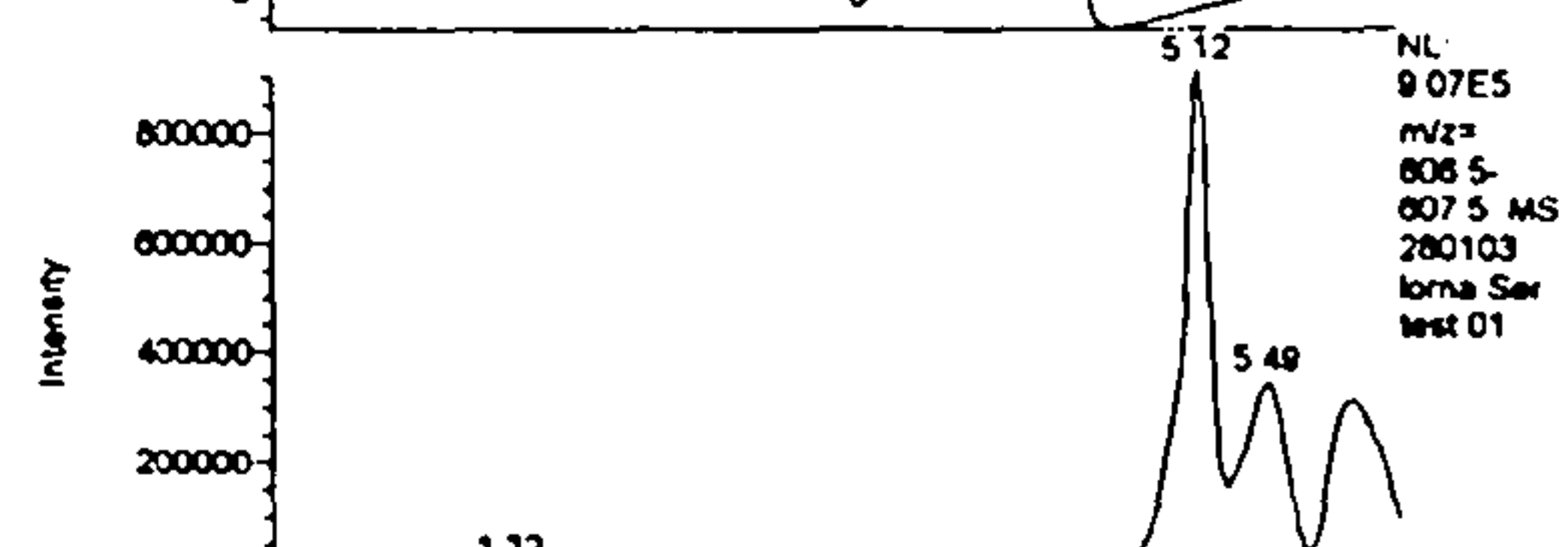
Processing Method:

Vial: D:24  
Injection Volume (µl): 10.00  
Sample Weight: 0.00  
Sample Volume (µl): 0.00  
ISTD Amount: 0.00  
Dil Factor: 1.00

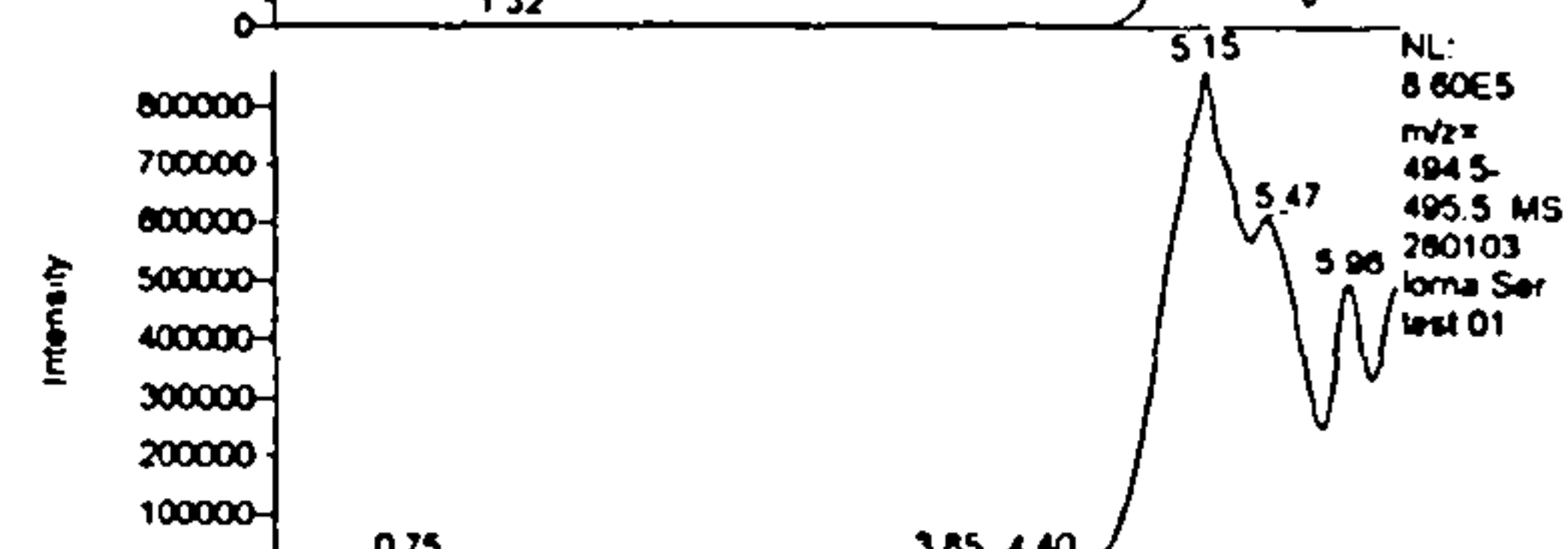


NL:  
834E4  
Total Scan  
PDA  
280103  
lorna Ser  
test 01

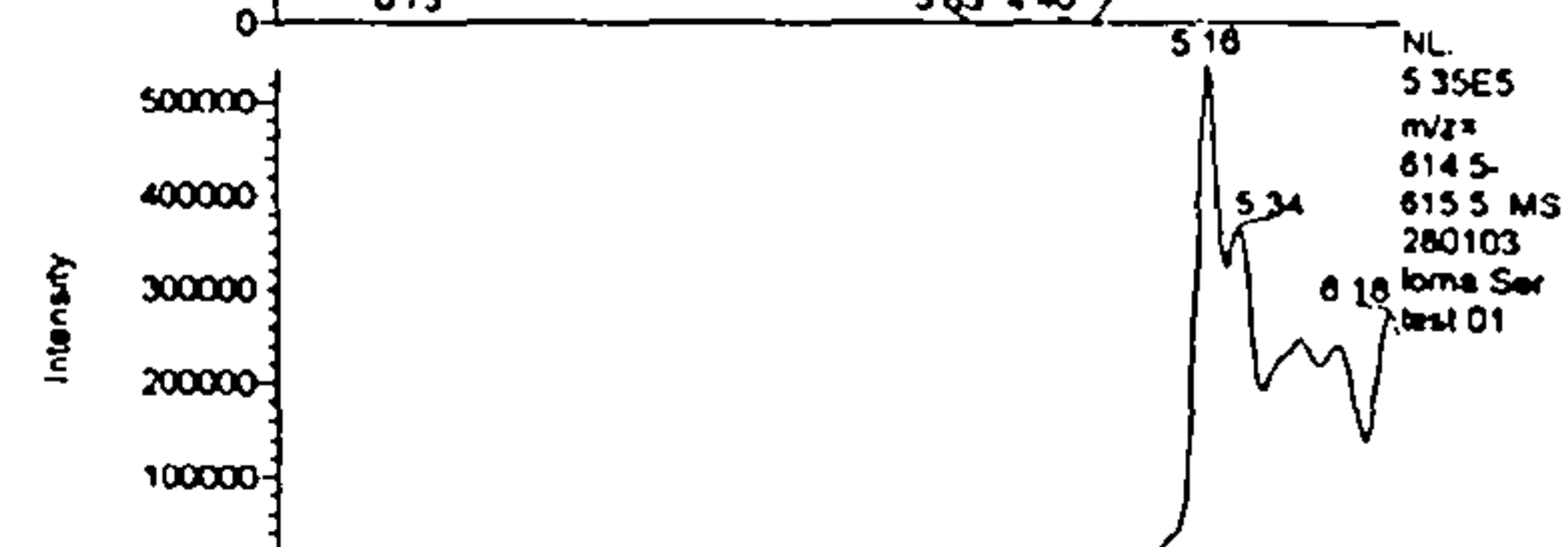
RP MAY



NL:  
907E5  
m/z= 806.5  
807.5 MS  
280103  
lorna Ser  
test 01



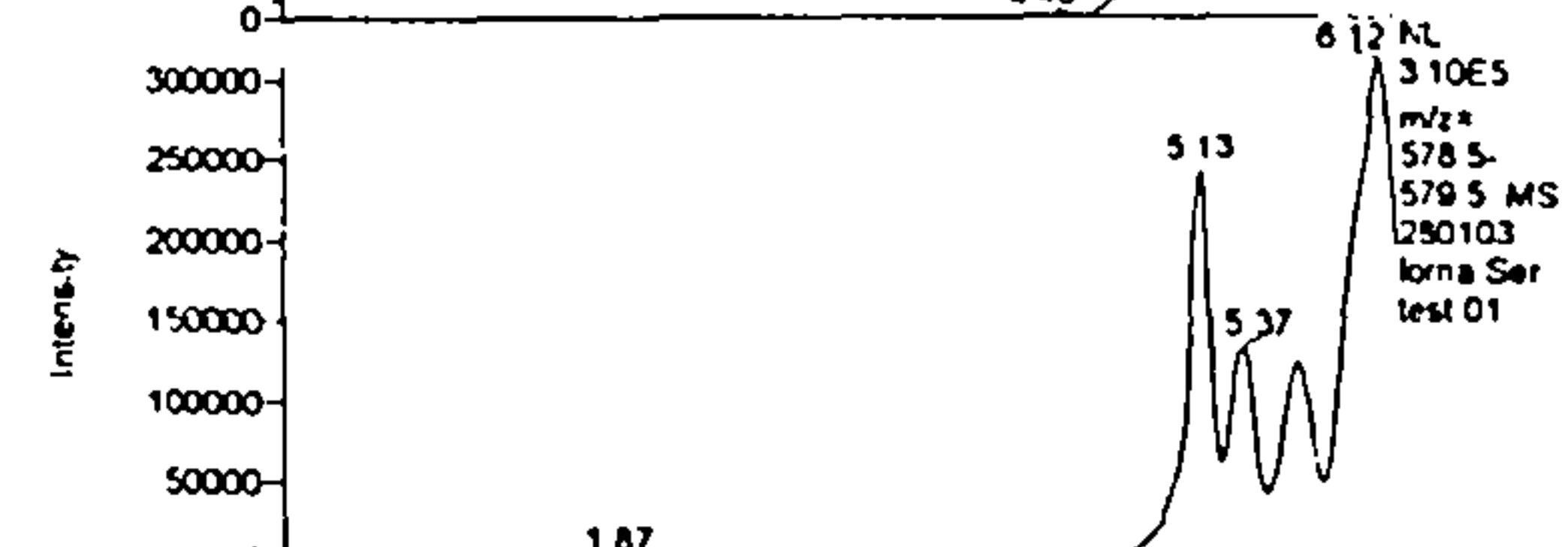
NL:  
880E5  
m/z= 494.5  
495.5 MS  
280103  
lorna Ser  
test 01



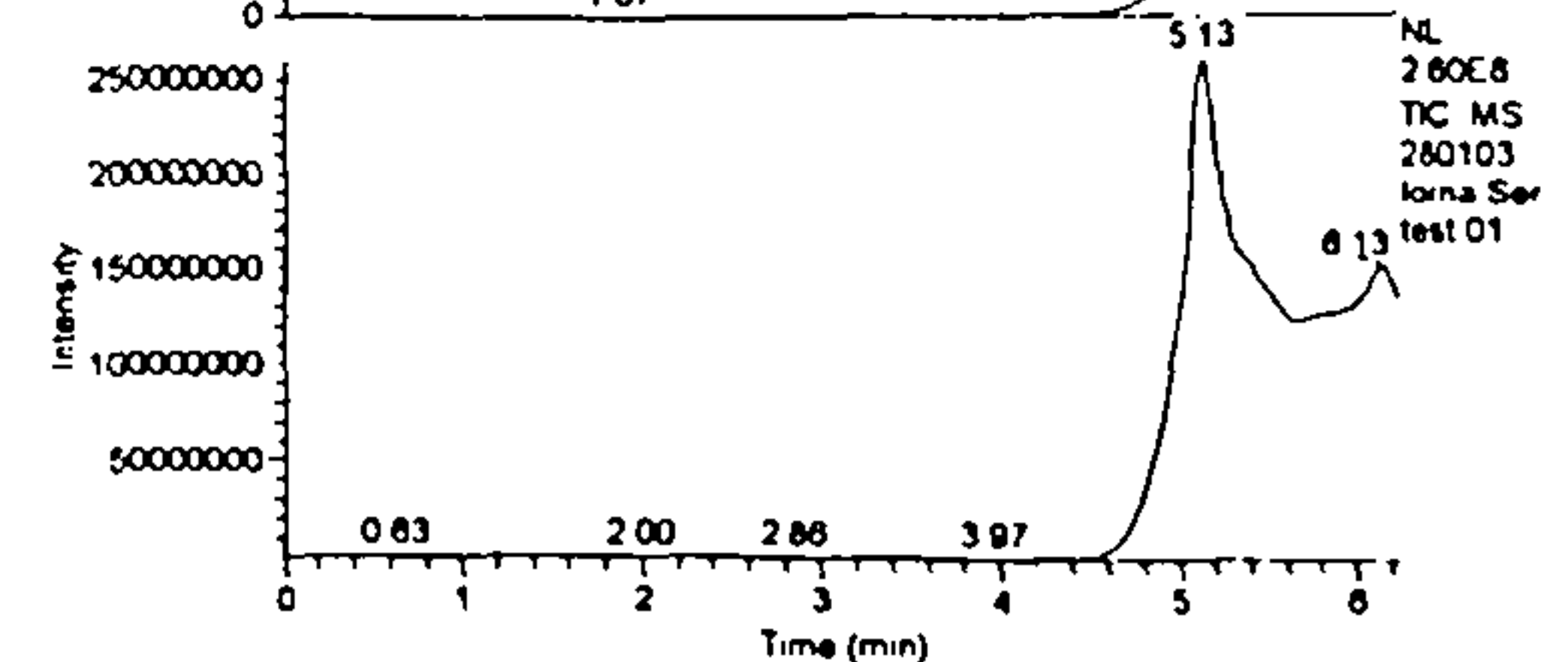
NL:  
535E5  
m/z= 614.5  
615.5 MS  
280103  
lorna Ser  
test 01



NL:  
477E5  
m/z= 462.5  
463.5 MS  
280103  
lorna Ser  
test 01

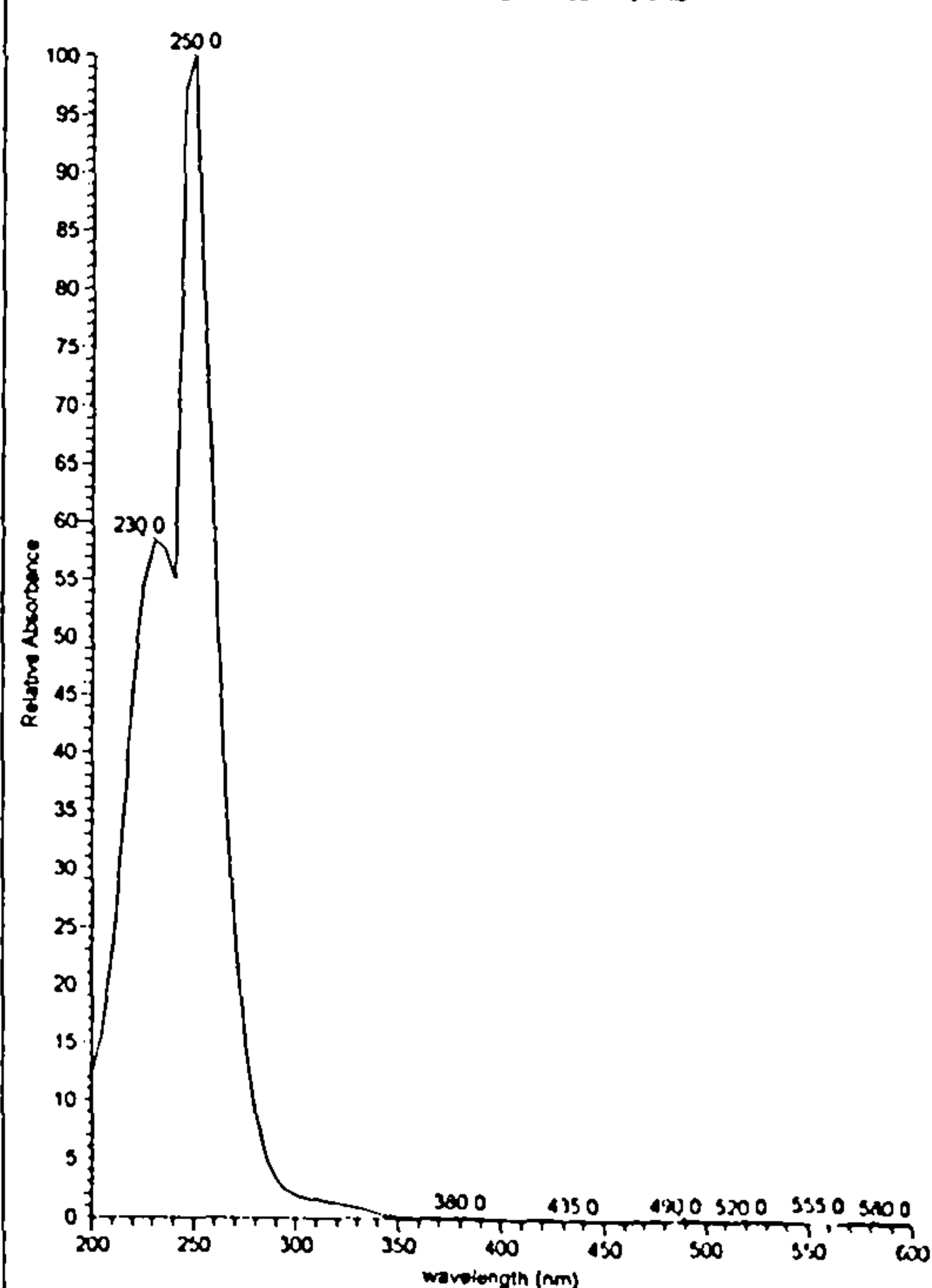


NL:  
310E5  
m/z= 578.5  
579.5 MS  
280103  
lorna Ser  
test 01

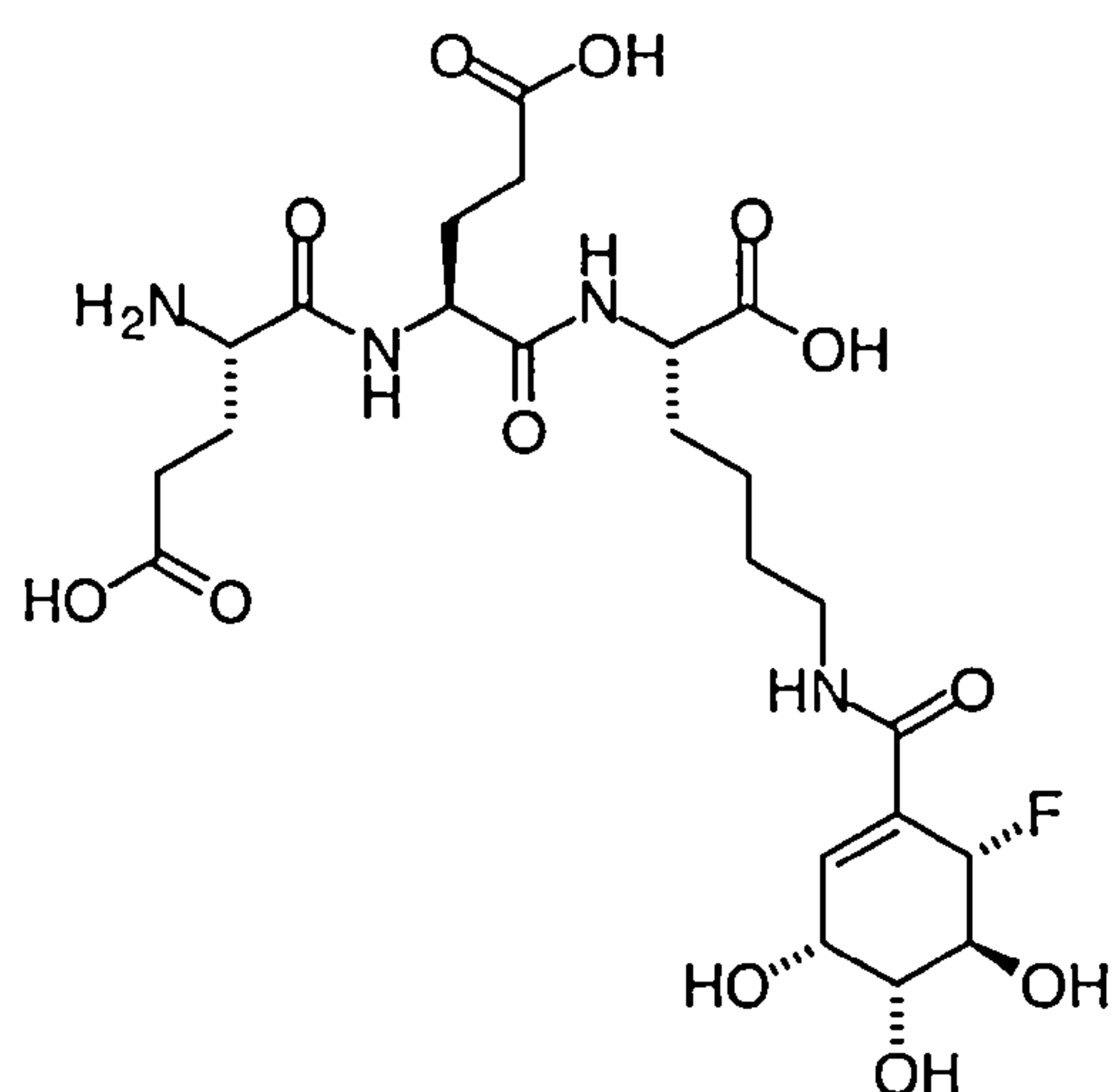


NL:  
280E8  
TIC MS  
280103  
lorna Ser  
test 01

280103 lorna Ser test 01 #1145 RT 3.81 AV 1 NL 666E5 microAU



**L-glutamoyl-L-glutamoyl-N-ε-(6S)-6-fluoroshikimoyl-L-lysine 8**



<sup>1</sup>H-NMR of crude product from resin (CD<sub>3</sub>OD, 400 MHz)

*m/z* (electrospray)

*m/z* (electrospray) msms

HPLC/ms output (RP max column, 3 - 50 % 50 mM formic acid in MeCN)



D:\Andy

LN273 #1 0.01-0.80 AU: 4 NL: 42456  
T: p Au [50.00-100.00]

10/14/2002 09:32:49 AM

Relative Abundance

9500 0  
8000 0  
6500 0  
5000 0  
3500 0  
2000 0  
500 0  
0

1000 0  
800 0  
600 0  
400 0  
200 0  
0

1.000 0  
0.800 0  
0.600 0  
0.400 0  
0.200 0  
0.000 0

1.000 0  
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Current Data Parameters  
NAME LN273-021001  
PROCNO 40  
PROCNO 1

F2 - Acquisition Parameters  
Date 20021001  
Time 11:14  
INSTRUM dx400  
PROBHD 5 mm Dual 13  
PULPROG zg30  
TO 32768  
SOLVENT MECH  
AS 15  
DS 2  
SWH 8423.865 Hz  
FIDRES 0.250967 Hz  
AQ 1.9923444 sec  
RG 181  
RG 60.800 usec  
DE 6.00 usec  
TE 298.0 K  
D1 0.01000000 sec

\*\*\*\*\* CHANNEL f1 \*\*\*\*\*  
NUC1 1H  
P1 9.20 usec  
PL1 -3.00 dB  
SF01 400.1324685 MHz

F2 - Processing parameters  
SI 32768  
SF 400.1300075 MHz  
WDW EM  
SSB 0  
LB 0.30 Hz  
GB 0  
PC 4.00

10 NMR plot parameters  
CX 39.01 cm  
FIP 9.250 ppm  
F1 3701.20 Hz  
F2 -0.500 ppm  
F2 -200.06 Hz  
RPMON 0.24992 ppm/cm  
H2ON 99.99996 Hz/cm

2.061

2.533

7.050

49.582

2.069

1.062

0.613

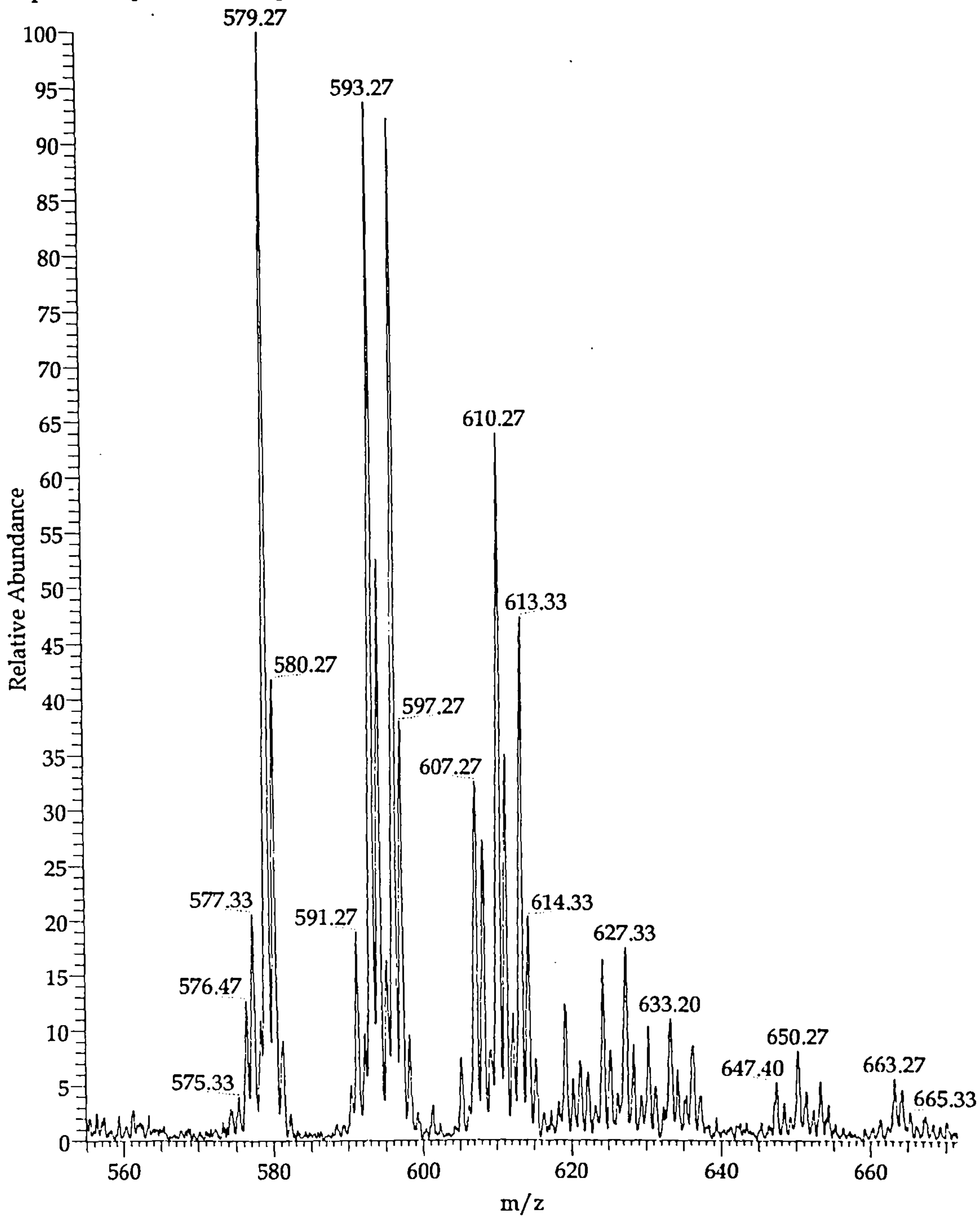
Integrals

0.0 0.5 1.0 1.5 2.0 2.5 3.0 3.5 4.0 4.5 5.0 5.5 6.0 6.5 7.0 7.5 8.0 8.5 9.0



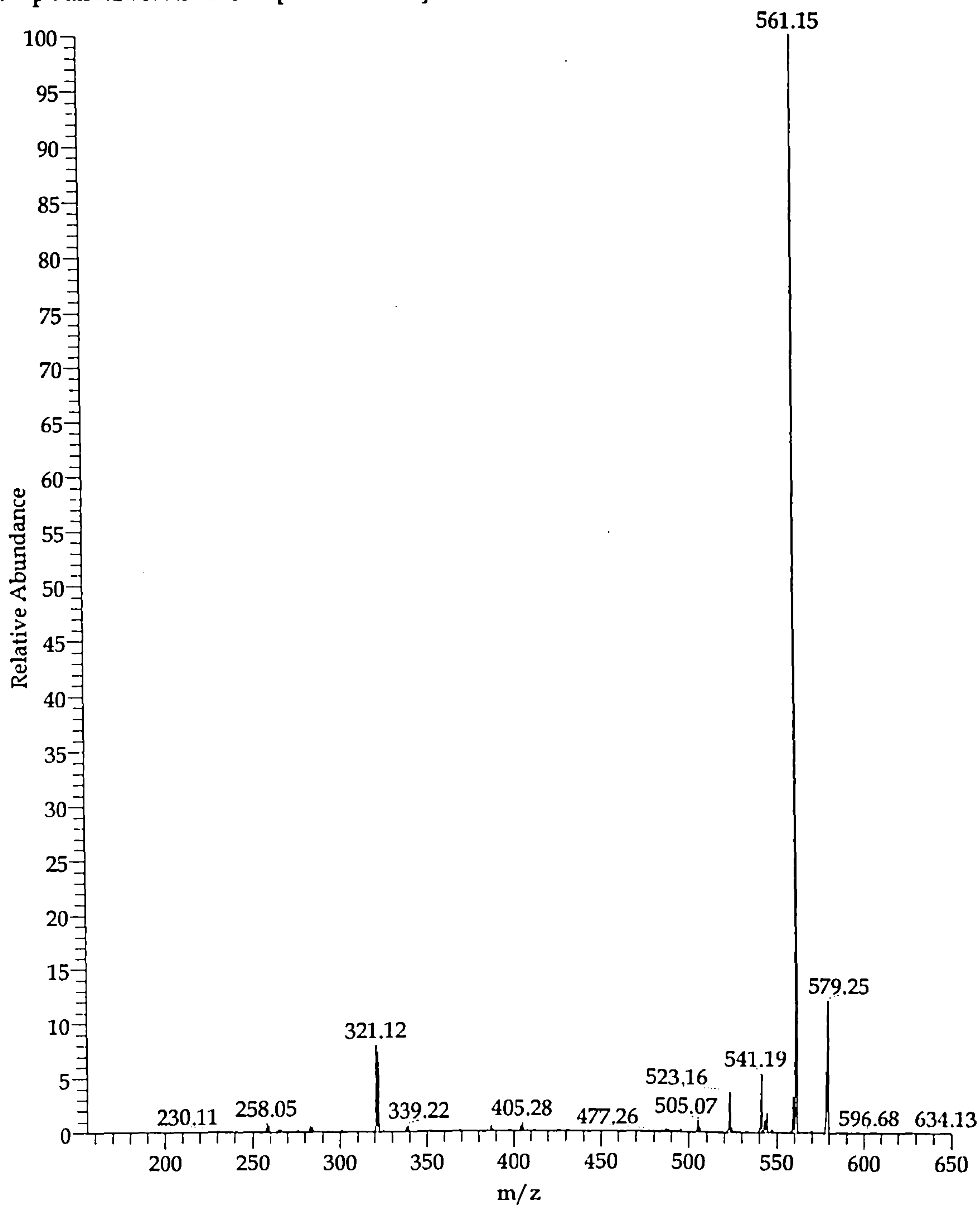
LN273 #1-49 RT: 0.01-0.80 AV: 49 NL: 6.26E6

T: + p Full ms [ 50.00-1000.00]



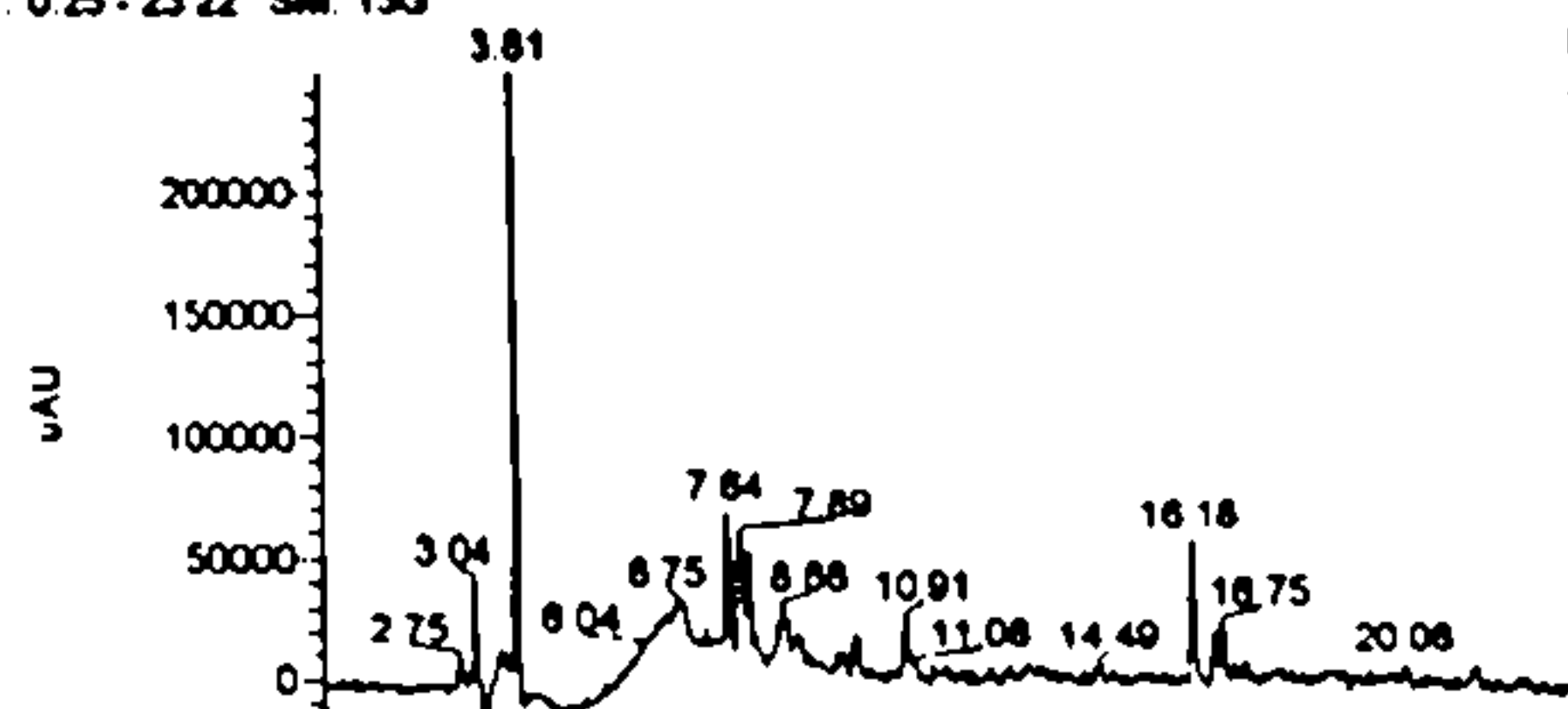
LN273msms1 #1-34 RT: 0.02-0.53 AV: 34 NL: 1.04E6

T: + p Full ms2 579.30@23.00 [155.00-650.00]





RT: 0.25 - 23.22 SM: 150



NL:  
2.49E5  
nm=214.5  
215.5  
PDA  
280103  
lorna Glu  
test 01

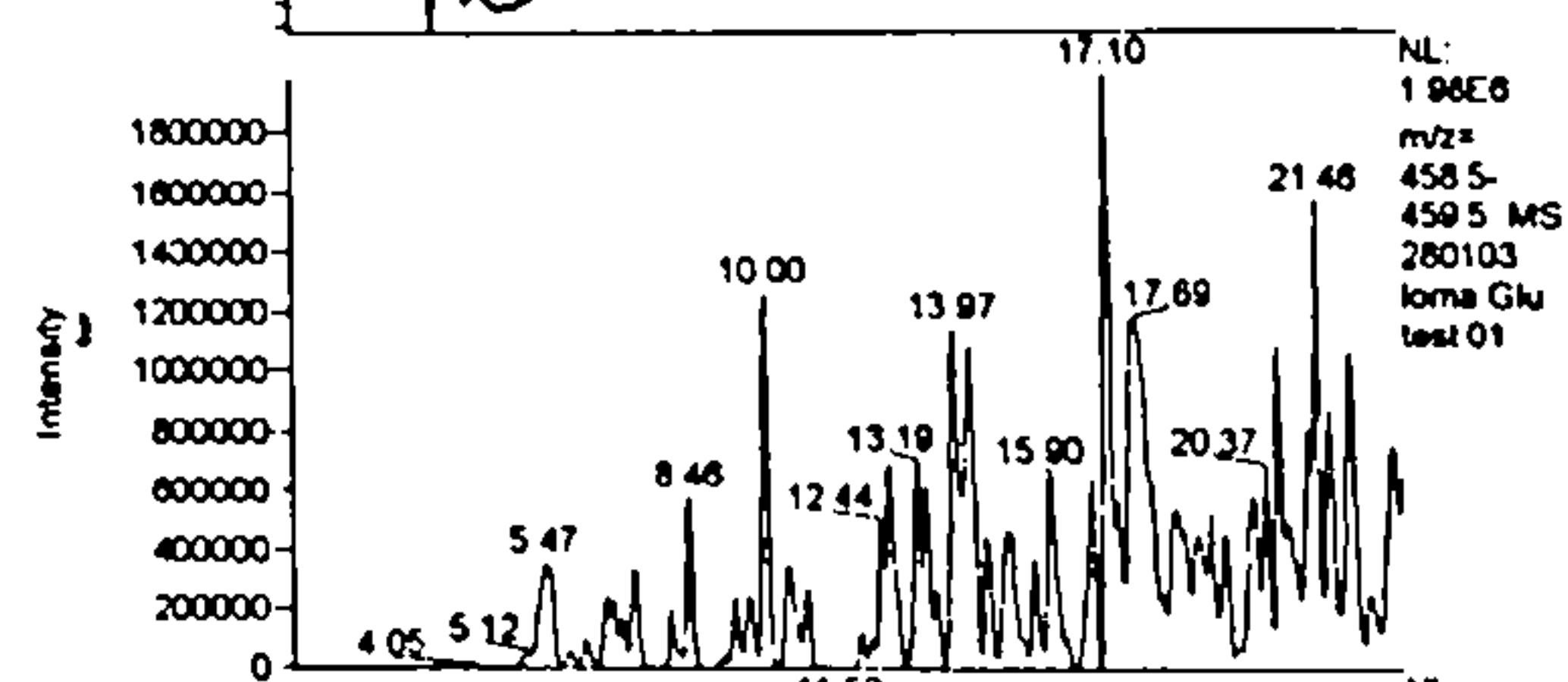
280103 lorna Glu test 01  
Type: Unknown ID: C:25 Row: 18

Sample Name:  
Study:  
Client:  
Laboratory:  
Company:  
Phone:

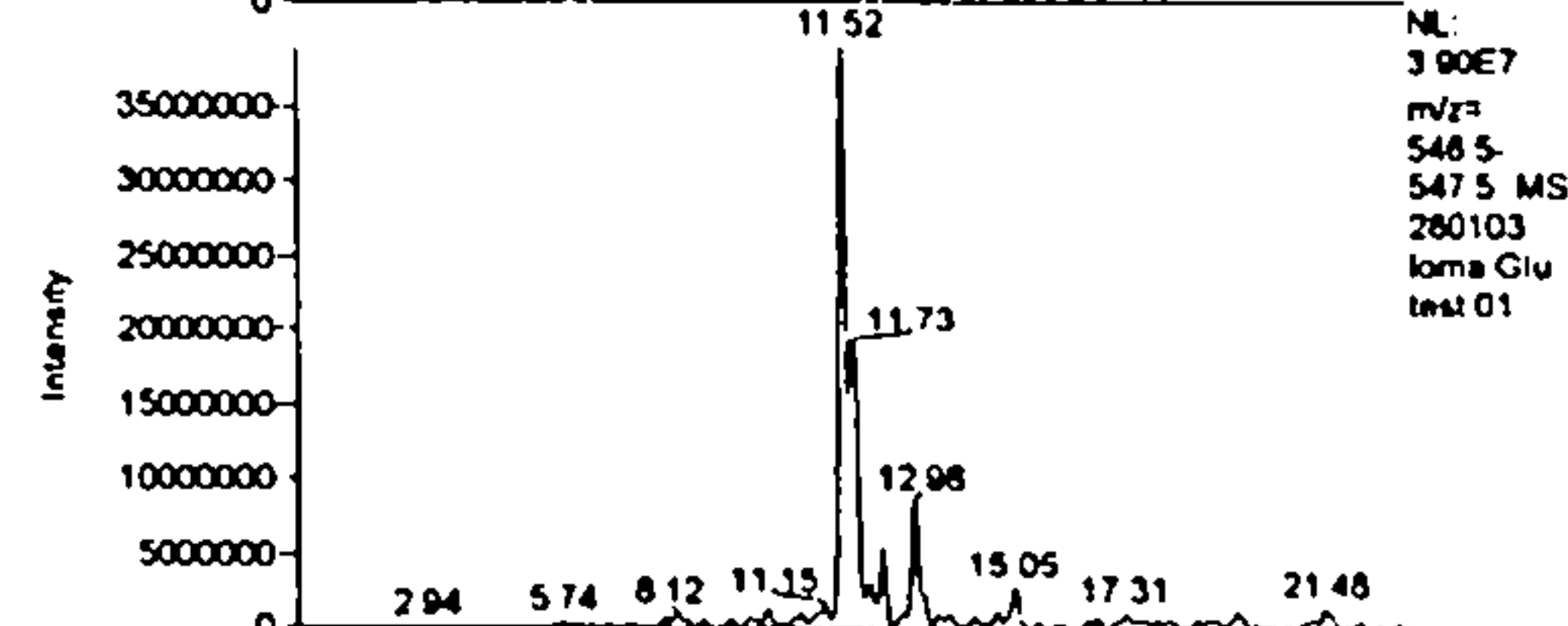
Instrument Method: C:\Xcalibur\methods\lorna\020902 20 min  
grad 3-50% C n D pos.meth

Processing Method:

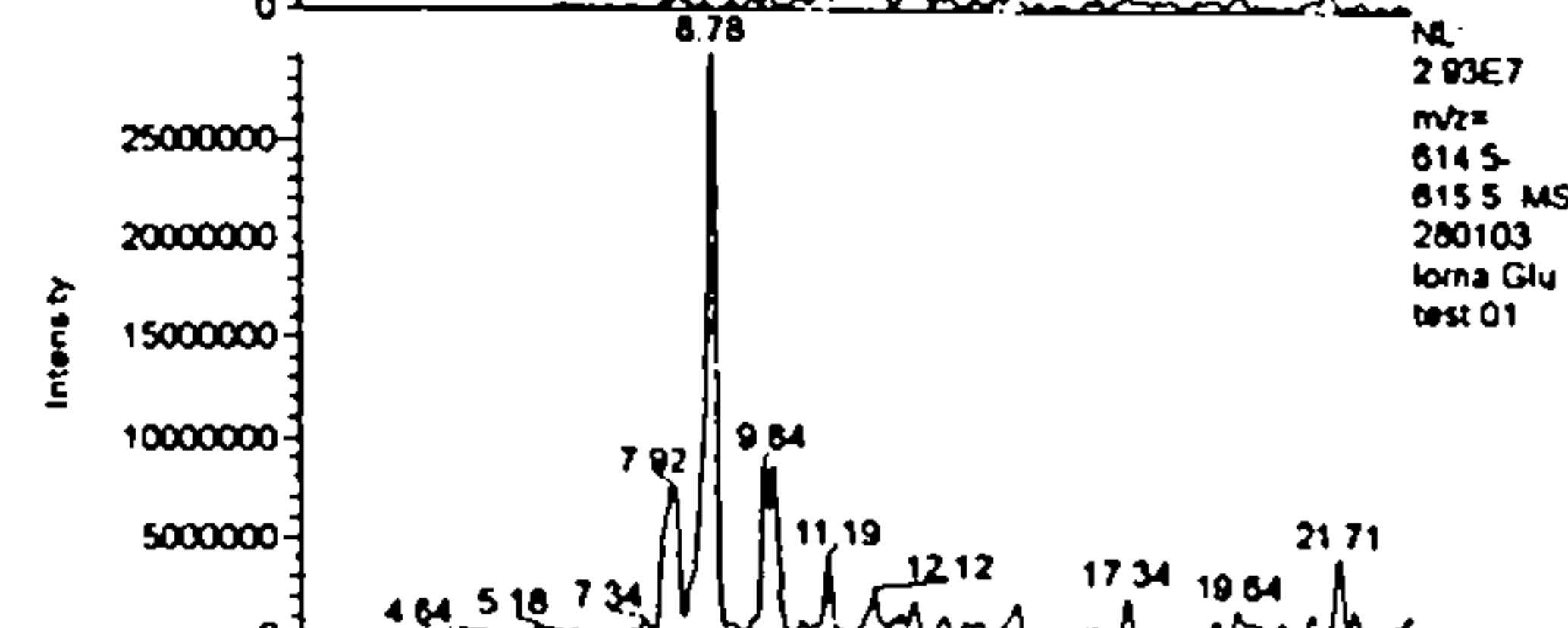
Vial: D:22  
Injection Volume (µl): 10.00  
Sample Weight: 0.00  
Sample Volume (µl): 0.00  
ISTD Amount: 0.00  
Dil Factor: 1.00



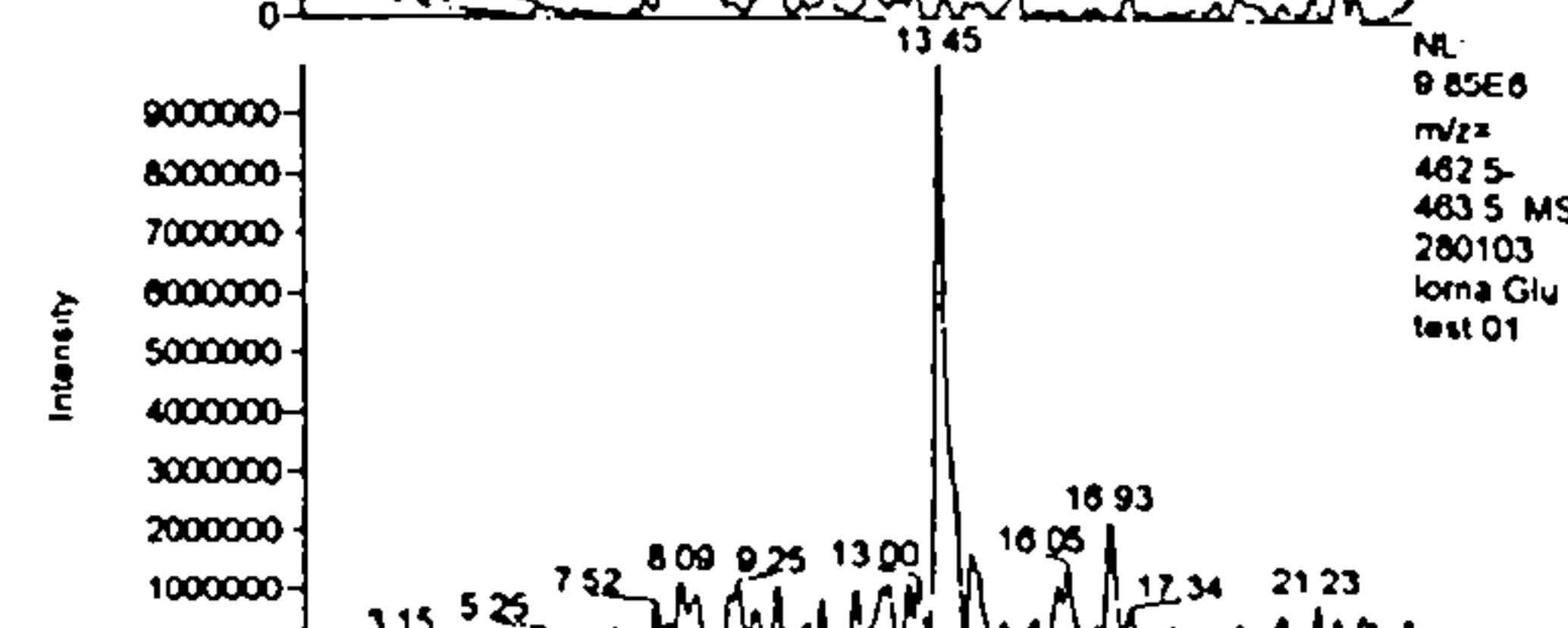
NL:  
1.98E8  
m/z= 458.5  
459.5 MS  
280103  
lorna Glu  
test 01



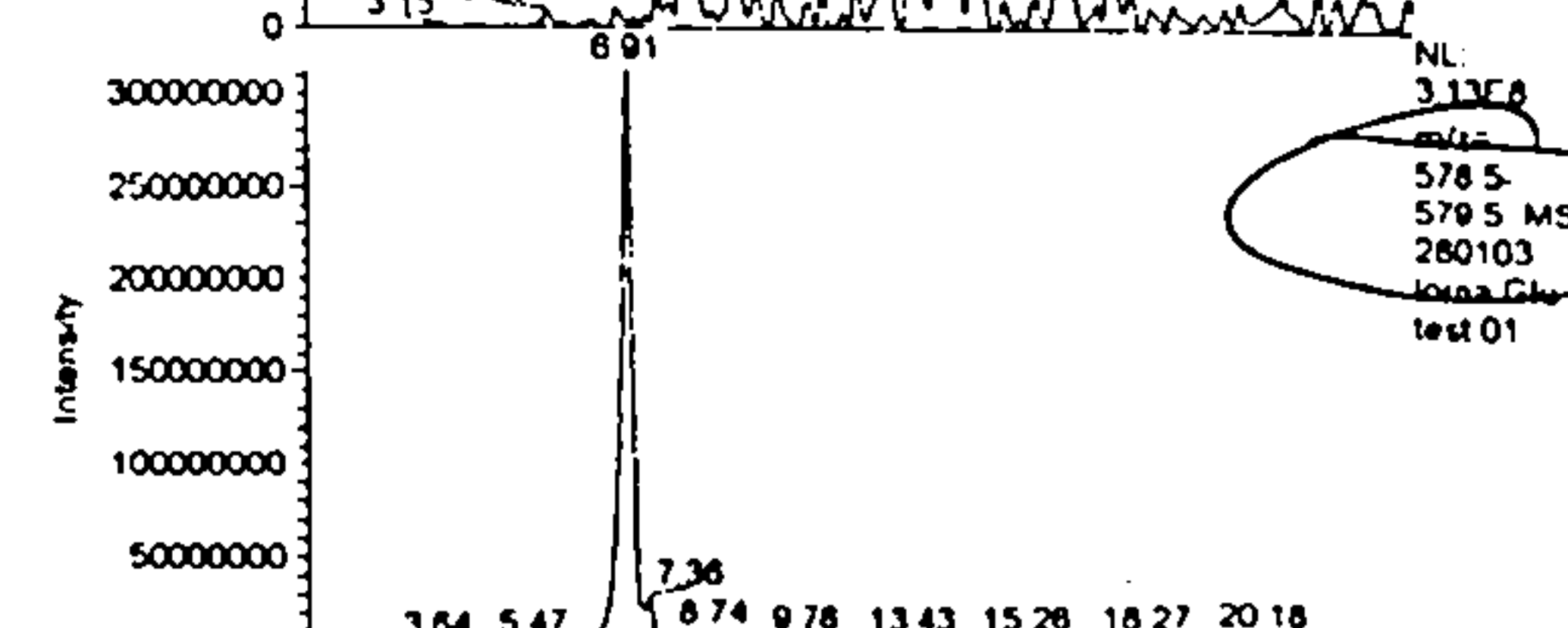
NL:  
3.90E7  
m/z= 548.5  
547.5 MS  
280103  
lorna Glu  
test 01



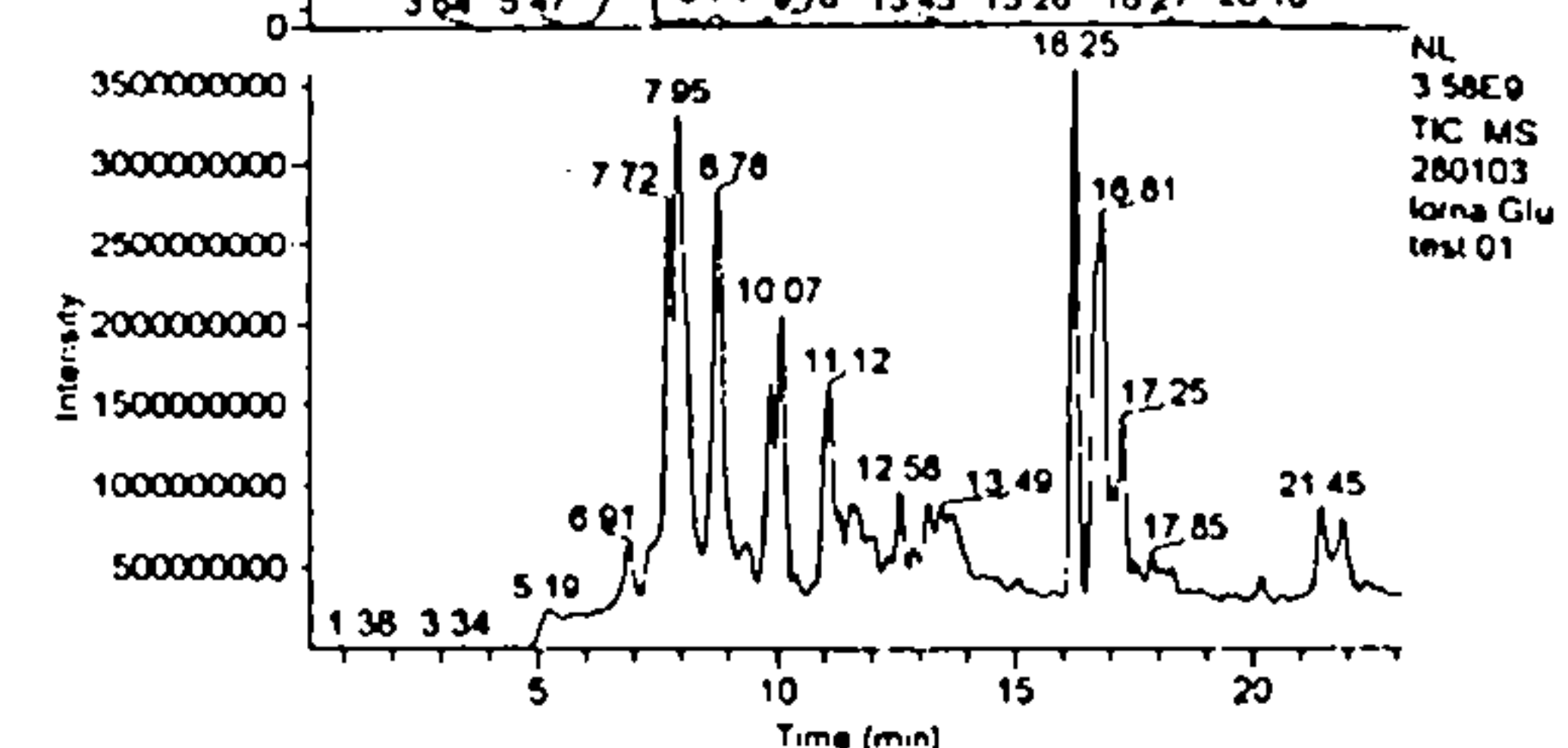
NL:  
2.93E7  
m/z= 614.5  
615.5 MS  
280103  
lorna Glu  
test 01



NL:  
9.85E6  
m/z= 482.5  
483.5 MS  
280103  
lorna Glu  
test 01

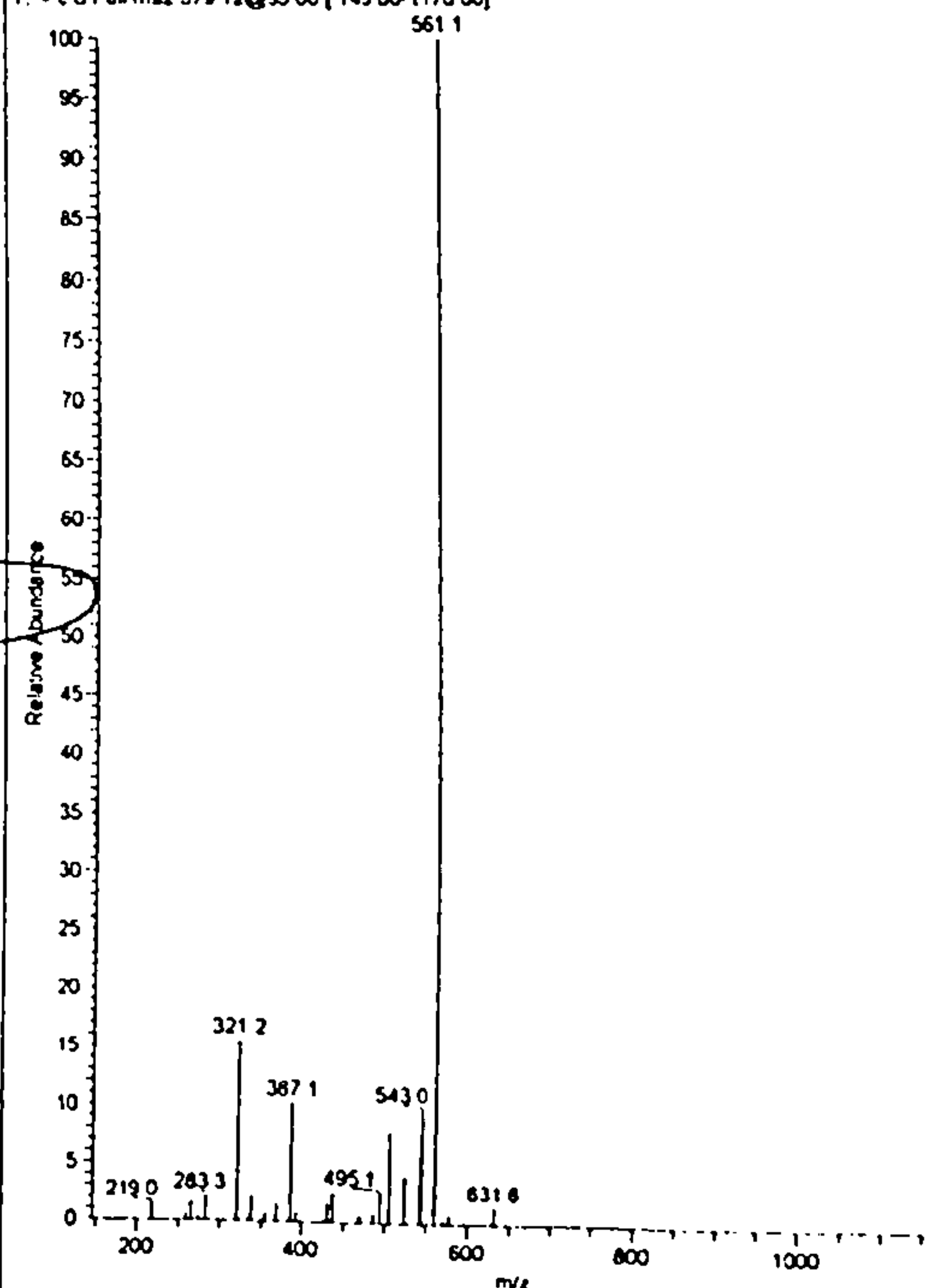


NL:  
3.13E6  
m/z= 578.5  
579.5 MS  
280103  
lorna Glu  
test 01



NL:  
3.58E9  
TIC MS  
280103  
lorna Glu  
test 01

280103 lorna Glu test 01 #160 RT: 6.25 AV: 1 NL: 207E6  
T: + c d Full ms 2 579 12@35 00 [ 145 00-1170 00]



R.P. MAX