The Synthesis and Pharmacology of Ephedrine Analogues

A Thesis Submitted for

the Degree of Master of Science in Chemistry

By

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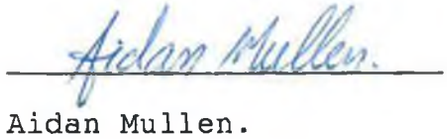
DECLARATION

This is to certify that the research presented in this thesis is entirely the work of Aidan Mullen , except where duly acknowledged.



Aidan Mullen.

To Rita and Stephanie Mullen

THE SYNTHESIS AND PHARMACOLOGY OF EPHEDRINE ANALOGUES

By Aidan J. Mullen

ABSTRACT

In this report we set out to extend recent studies on substituted PAC analogues produced by the fermentation of aromatic aldehydes with Saccharomyces cerevisiae. The medium selected contained glucose as a carbon source for the biotransformation, and sodium pyruvate as an inhibitor for alcohol dehydrogenase, the enzyme responsible for the conversion of the carbinol to a benzyl alcohol derivatives. An investigation of sodium cyanoborohydride for a selective reductive amination was carried out on these carbinols to produce the corresponding ephedrine analogues. Poor selectivity was apparent for this reagent but when the reduction products were converted to their hydrochloride derivatives the optically pure compounds separated readily by fractional crystallisation. Their pharmacological activity was investigated using commercial ephedrine as a reference compound. The synthesised (-) ephedrine had an identical activity to the commercial compound, while the substituted analogues all had a slightly lower activity, with the exception of (-)-4-chloro ephedrine which showed a stronger antispasmodic effect. The stereoselectivity of the synthesis and the enantiomeric purity of the resolved compounds were investigated by high field nuclear magnetic resonance spectrometry employing chiral praseodymium shift reagents .

LIST OF ABBREVIATIONS

PAC	Phenylacetylcarbinol
THF	Tetrahydrofuran
Ac	Acetyl
Pd/c	Palladium on charcoal
UV	Ultra Violet
HPLC	High Performance Liquid Chromatography
NMR ^{1} H	Proton Magnetic Resonance
NMR ^{13} C	Carbon 13 Magnetic Resonance
hfc	Tris(3-heptafluoropropylhydroxymethylene)-d-camphorato
tfc	Tris(3-(trifluoromethylhydroxymethylene)-d-camphorato
fod	Tris(6,6,7,7,8,8,8,-heptafluoro-2,2-dimethyl-3,5,octanedionato)
ATP	Adenosine Tri Phosphate
cAMP	3',5'-cyclic adenosine Mono Phosphate
CNS	Central Nervous System
TLC	Thin Layer Chromatography
ppm	Parts Per Million
$[\alpha]_D$	Specific Optical Rotation (sodium D line)
Rf	Retention Value on TLC plate

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INTRODUCTION

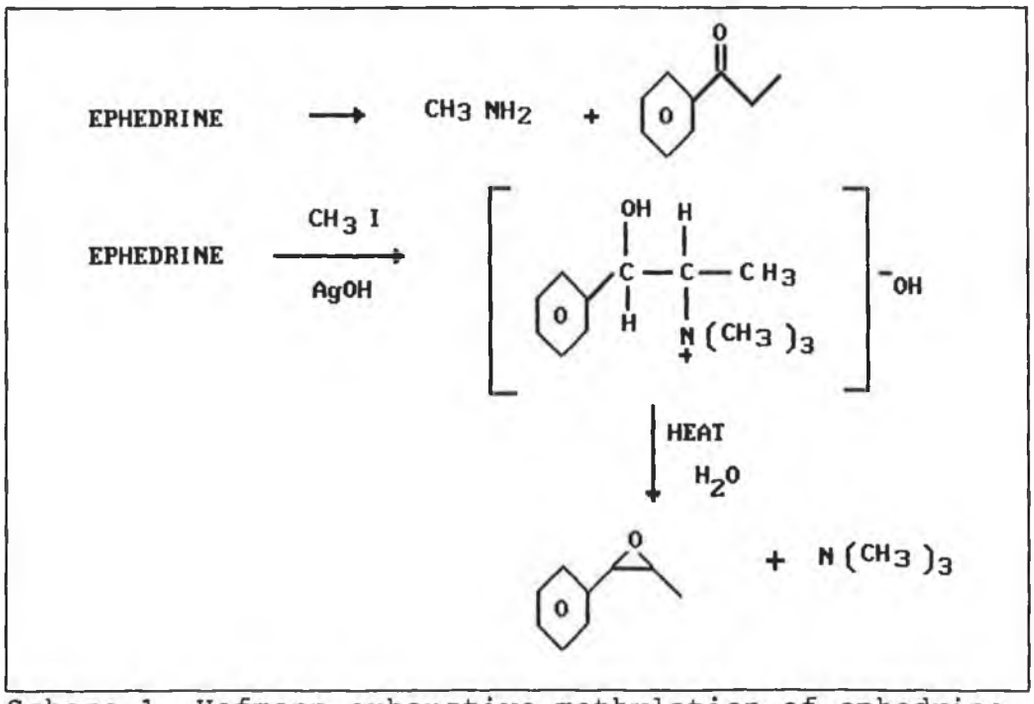
1.1 HISTORY The first reported use of ephedrine was in China over 5000 years ago as a drug called Ma-huang (meaning astringent yellow). It was mentioned in herbal books as early as 2000 BC. A Chinese medical plant publication in 1596, recommended the drug for improving bad circulation, reducing fever, and treating respiratory ailments. The main ingredient in the Ma-huang was dried green leaves and shoots from Ephedra sinicra.[1]

The drug did not appear in the west until Yamanashi isolated an impure alkaloid in 1885 and two years later Nagai and Hari[2] isolated a pure compound which they named ephedrine. Merck of Darmstadt, a German firm, began to investigate a European plant Ephedra helvetica and in 1888 isolated ephedrine and ψ-ephedrine.

Chen, a Chinese American, and his colleague Schmidt studied Ma-huang in Peking, and on returning to America in 1923 began pharmacological trials on native American Ephedra species. It is now known that of the 45 species in the Ephedra genus only 25 contain the alkaloid, which can be present in leaves at concentrations of 1-2% . Today Ephedrine is prepared by synthesis via the reductive amination of Phenyl acetyl carbinol (PAC) , which is produced by Saccharomyces cerevisiae during the fermentation of sugar medium containing benzaldehyde.

1.2 STRUCTURE AND PROPERTIES

The structure of ephedrine was elucidated using the following reasoning, and was first synthesised by Späth.[3] The molecular formula was known to be $C_{10}H_{15}NO$ and on oxidation benzoic acid was formed thus suggesting it contains a benzene ring with only one side chain. When treated with nitrous acid ephedrine yielded a nitroso-compound and must therefore be a secondary amine, and when heated with hydrochloric acid ephedrine forms methylamine and propiophenone (1). Scheme 1



Scheme 1 Hofmann exhaustive methylation of ephedrine

This still did not fully assign the structure as two possible geometric isomers still existed ,(Figure 1.). When it was observed that Hofmann exhaustive methylation of ephedrine produced sym-methylphenylethylene oxide (2) structure A seemed more probable as this had been known to undergo hydramine fission. The proof of the final structure was gained by the fact that removal of a hydroxyl group still gave an optically active product. This was not possible with the alternative structures proposed ie. structure B. (Figure 1.)

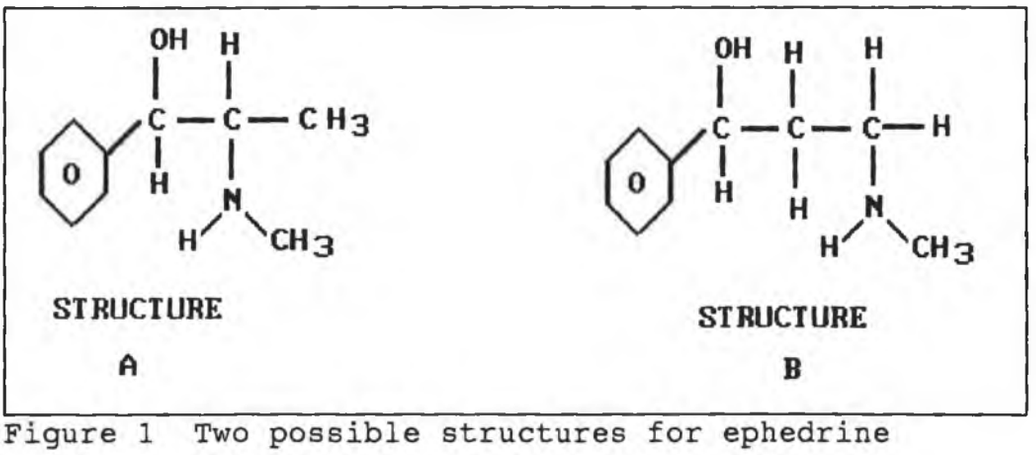


Figure 1 Two possible structures for ephedrine

Ephedrine or 1-phenyl-1-hydroxy-2-methylamino-propane contains two asymmetric carbon centres and consequently there are four possible isomers. These are divided into two classes according to their symmetry . The erythro or ephedrine series contains the enantiomers (±)-ephedrine and the threo or pseudoephedrine series contains the (±)-ψ-ephedrine. The configuration of the pairs of enantiomers was first proposed by Freudenberg [4] .(Figure 2.)

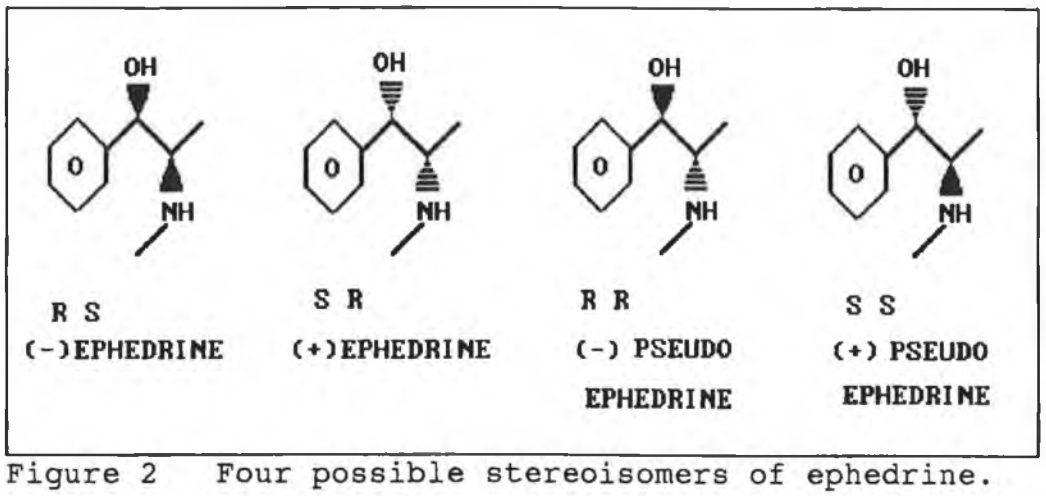


Figure 2 Four possible stereoisomers of ephedrine.

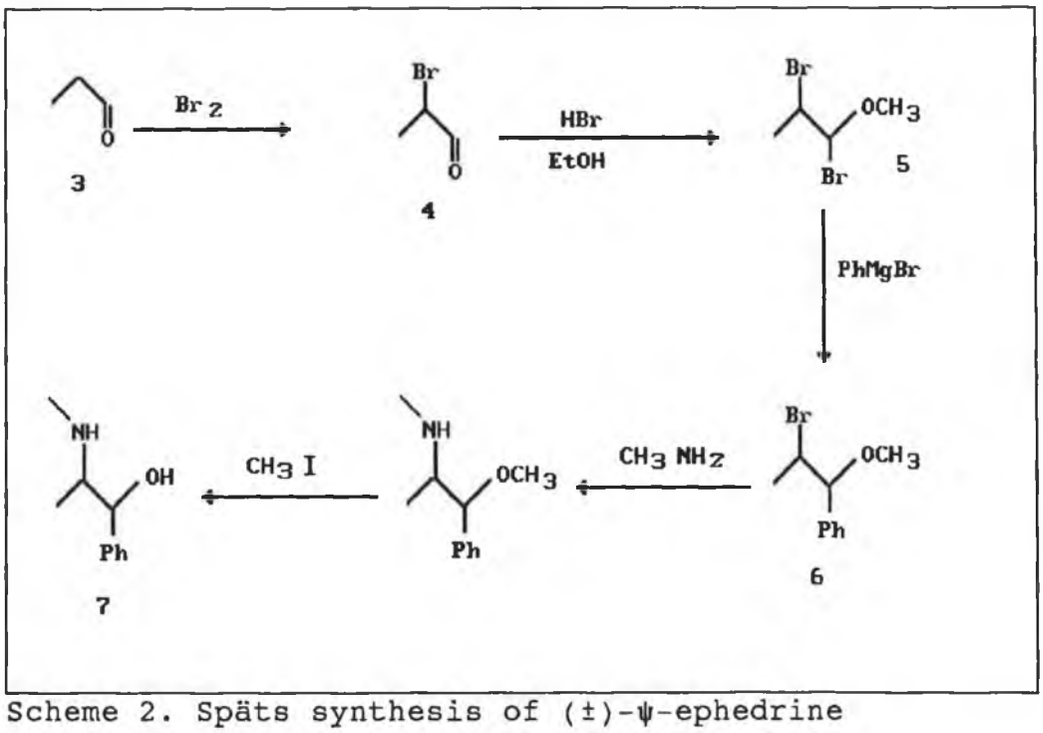
Ephedrine is a colourless, odourless, waxy compound which is deliquescent and partly crystalline. It decomposes gradually in the presence of light and atmospheric carbon dioxide. The anhydrous material has a melting point of 34°C but rapidly absorbs water to form a hemihydrate with a slightly higher melting point (40°C). Ephedrine is volatile at room temperature, is laevorotary and is a slightly weaker base (pKa 9.4) than ψ-ephedrine. It is soluble in water (one part in 36 @ 20°C), alcohol, organic solvents and oils. The free base forms derivatives such as hydrobromides, hydrochlorides, phosphates, oxalates, and sulphates.[5] The individual ephedrine and ψ-ephedrine isomers are often characterised as their hydrochloride derivative. This data is summarised in Table 1.

	(+)ephedrine	(-)ephedrine	(+) ψ-ephedrine	(-) ψ-ephedrine
Melting point	37°-39°C	37°-39°C	118°-119°C	118°-119°C
R S config.	1S 2R	1R 2S	1S 2S	1R 2R
$[\alpha]_D$		-40° C=5, 1N HCl		-49° C=0.6,EtOH
HCl derivative MP	218°-220°C	218°-220°C	185°-188°C	185°-188°C
HCl derivative $[\alpha]_D$	+34.3° C=5,WATER		+68.0° C=0.6, EtOH	

Table 1. Properties of the isomers of ephedrine and their hydrochloride derivatives

1.3 CHEMICAL SYNTHESIS

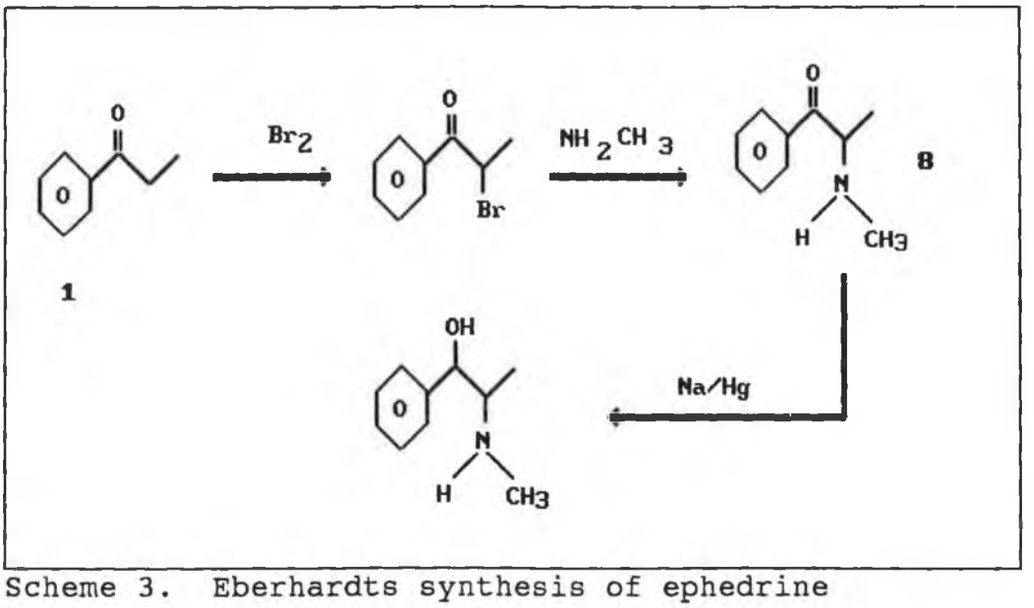
The synthesis by Späts [3] was initiated by the bromination of ethanal (3) to give α-bromo ethanal (4) which on refluxing in ethanol acidified with hydrogenbromide gave (5). The intermediate (6) was produced by the Grignard reaction of (5) with phenyl magnesium bromide followed by de-bromination using methylamine. Hydrogen iodide removed the methoxy group to give (±)-ψ-ephedrine, which was resolved using tartaric acid (Scheme 2).



Scheme 2. Späts synthesis of (±)-ψ-ephedrine

(-)-ephedrine was synthesised by Mansake[6] via the catalytic reduction of 1-phenylpropane-1,2-dione in a methanolic solution of methylamine followed by resolving the racemic (±)-ephedrine with mandelic acid.

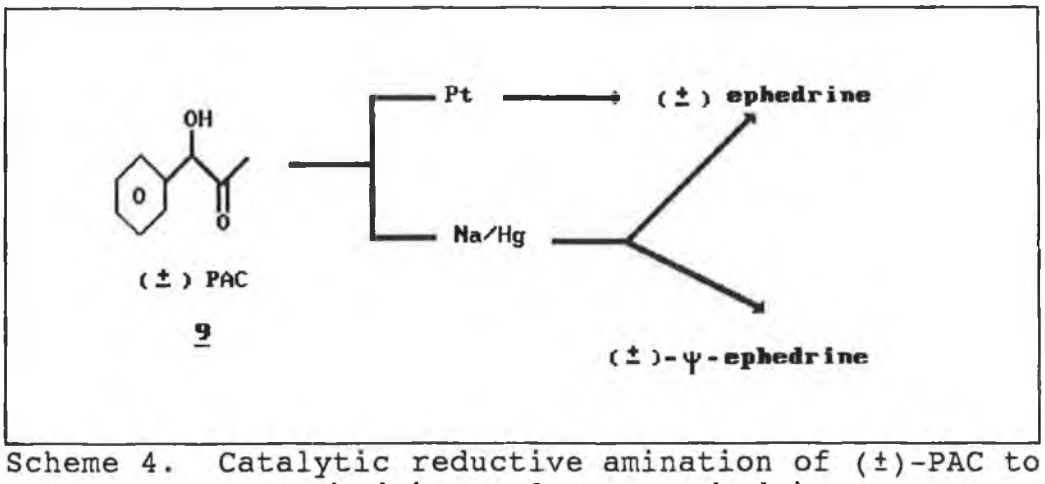
Another synthetic route to (ψ)-ephedrine used (±)-α-methylaminopropiophenone (8) as a precursor. This is readily obtained from propiophenone (1) by bromination followed by reaction with methylamine. Reduction of the ketone with sodium amalgam gives a mixture of (±)-ephedrine and (±)-ψ- ephedrine, with the latter in slightly higher proportion.[7] (Scheme 3)



Scheme 3. Eberhardts synthesis of ephedrine

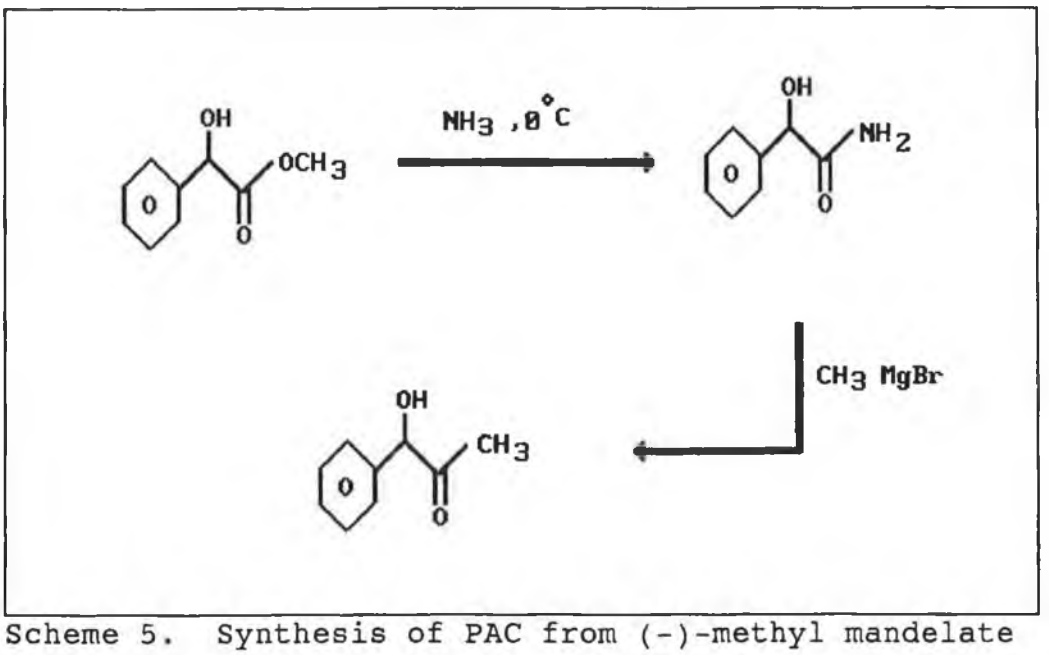
ψ-ephedrine was converted into ephedrine by heating with aqueous hydrochloride solution (25%) in a sealed tube to give an equilibrium ratio of 60:40.[8] This was an important discovery as the (-)-ephedrine isomer has a higher pharmacological activity than the pseudo isomer

Catalytic reduction of α-methyl aminopropiophenone hydrochloride over platinum gives almost exclusively (±)-ephedrine in yields of over 90%.[9] In this particular case the stereoselectivity is based on "kinetic control" as ephedrine is formed faster than the pseudo isomer.



Scheme 4. Catalytic reductive amination of (±)-PAC to (±)-ephedrine and (±)-ψ-ephedrine.

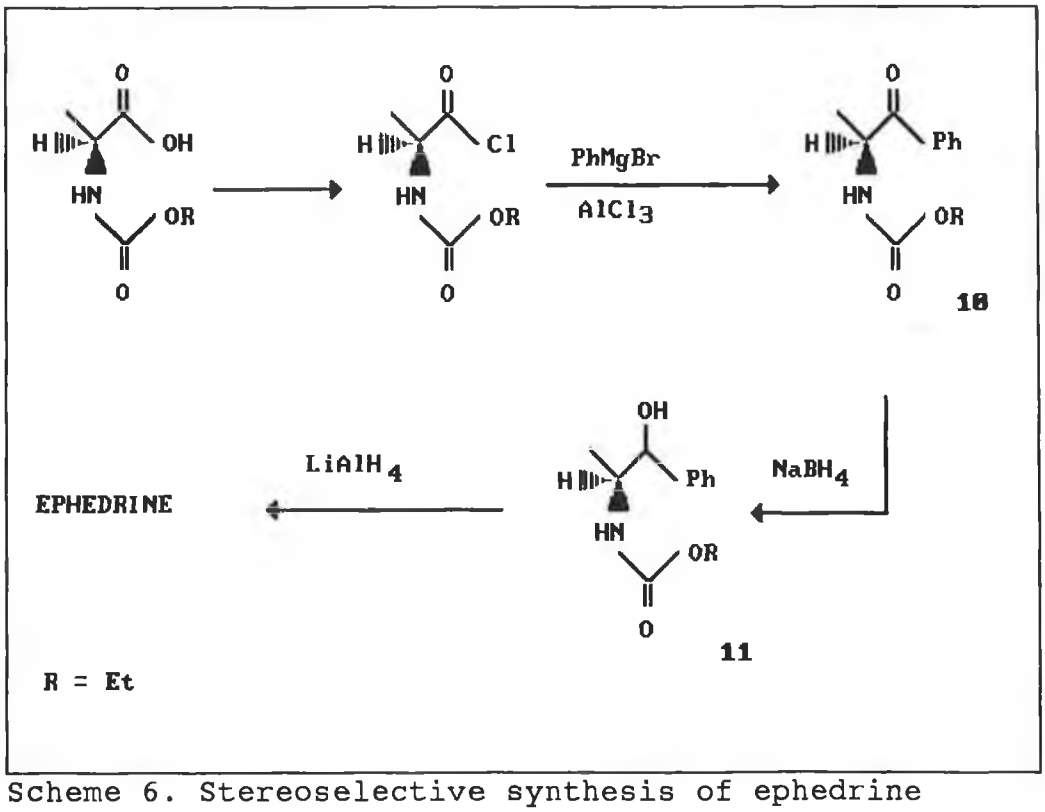
Of the four stereoisomers only (+)-ψ-ephedrine and (-)-ephedrine are pharmacologically active, with the latter being of more commercial value. A synthetic route producing a predominance of the (-)-isomer was desired, and this was achieved by the reductive amination of phenylacetylcarbinol (9) with methylamine and hydrogen over a platinum catalyst [10] , Scheme 4 . (-)Phenylacetylcarbinol can be synthesised from (-)-methyl mandelate (Scheme 5) by the method of Freudenberg [4] , or produced by fermentative mixed acetoin condensation between benzaldehyde and acetaldehyde in the presence of the pyruvate decarboxylase enzyme found in Saccharomyces cerevisiae.



Scheme 5. Synthesis of PAC from (-)-methyl mandelate

A number of authors have published work relating to stereospecific synthesis of ephedrine and ψ-ephrdrine. Buckley [11] used chiral N-ethoxycarbonyl and N-benzene sulphonyl derivatives of alanine as the bases for the production of various oxygenated aromatic α-amido ketones via two routes.

The first route involved converting the α-amino acid derivative to the corresponding acid chloride, followed by a Friedel-Crafts acylation carried out with phenyl magnesium bromide to give the ketone (10) (Scheme 6.)

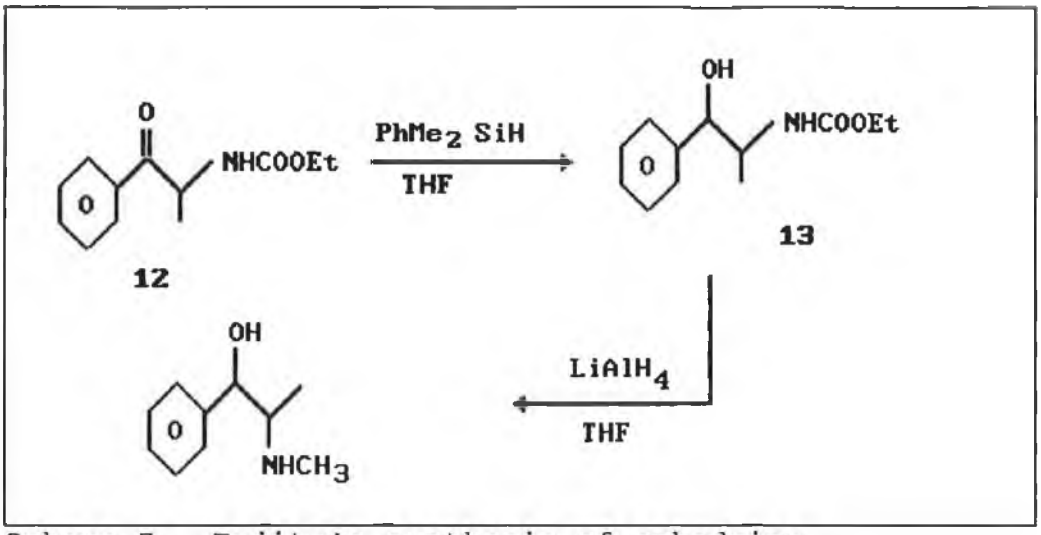


Scheme 6. Stereoselective synthesis of ephedrine

A second method was via an arylmetallo route using phenyl lithium and this also produced the ketone (10). The products were optically active in each of the above cases.

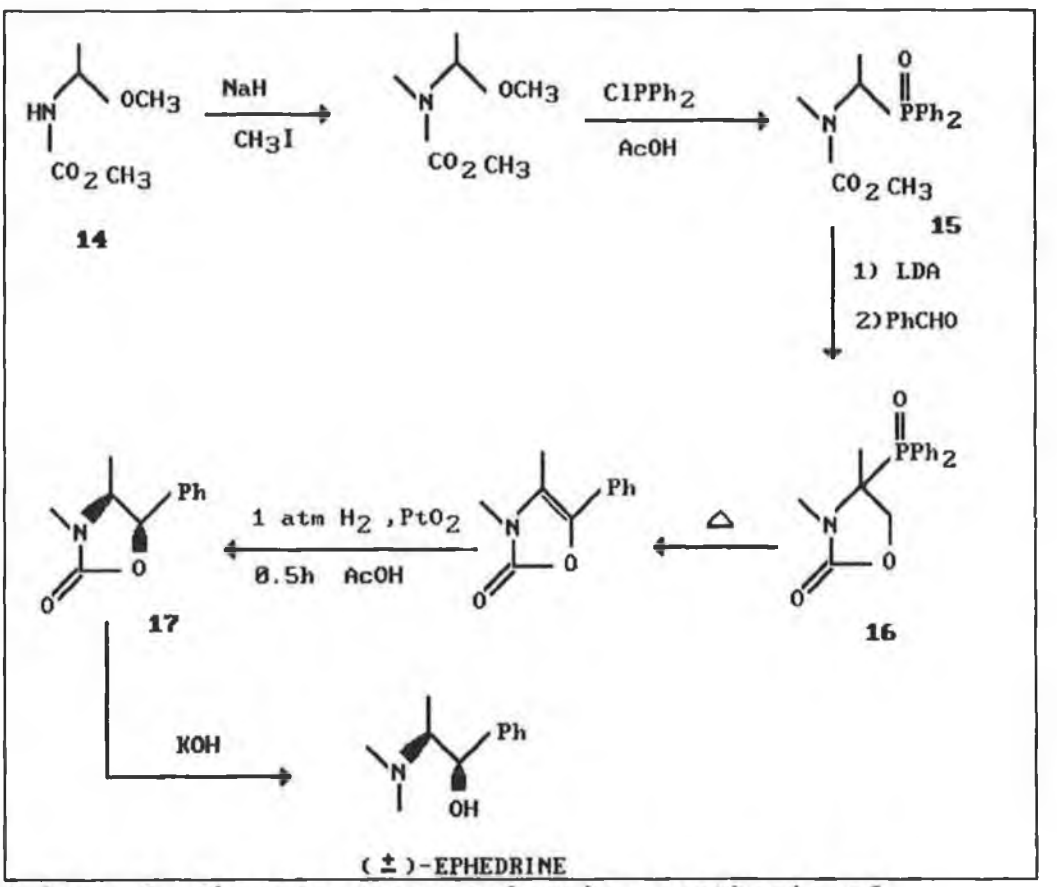
The stepwise reduction of (S)-2-((ethoxycarbonyl)amino)-1-phenyl-propanone (10) afforded variable ratios of ephedrine and ψ-ephedrine. In each case the ketone carbonyl was first converted to the alcohol (11) using $NaBH_4$ followed by reduction with lithium aluminum hydride to give the desired secondary amino alcohol. A 4:1 ratio of ephedrine to ψ-ephedrine was achieved in a yield of 90 % by this method. The ortho, meta methoxyphenyl,and the 2,4 dimethoxyphenyl analogue of ketone (10) were also prepared by the Friedel-craft method, but these were not reduced to the corresponding ephedrine compounds.

Fujita [12] produced optically active 2-amino alcohols by a diastereocontrolled reduction using hydrosilanes (Scheme 7). In this method dimethylphenyl silane in THF was used to reduce the ketone (12) yielding 2-ethoxycarboxyl amino-1-phenylpropan-1-ol (13) in a 1:9 threo to erythro ratio. Reduction of (13) with lithium aluminium hydride in THF at 60°C gave (1)-ephedrine with a yield of 80% . The stereochemical outcome is attributed to the bulky reduction species which attacks the ketone carbonyl carbon according to the Felkin [13] transition state model.



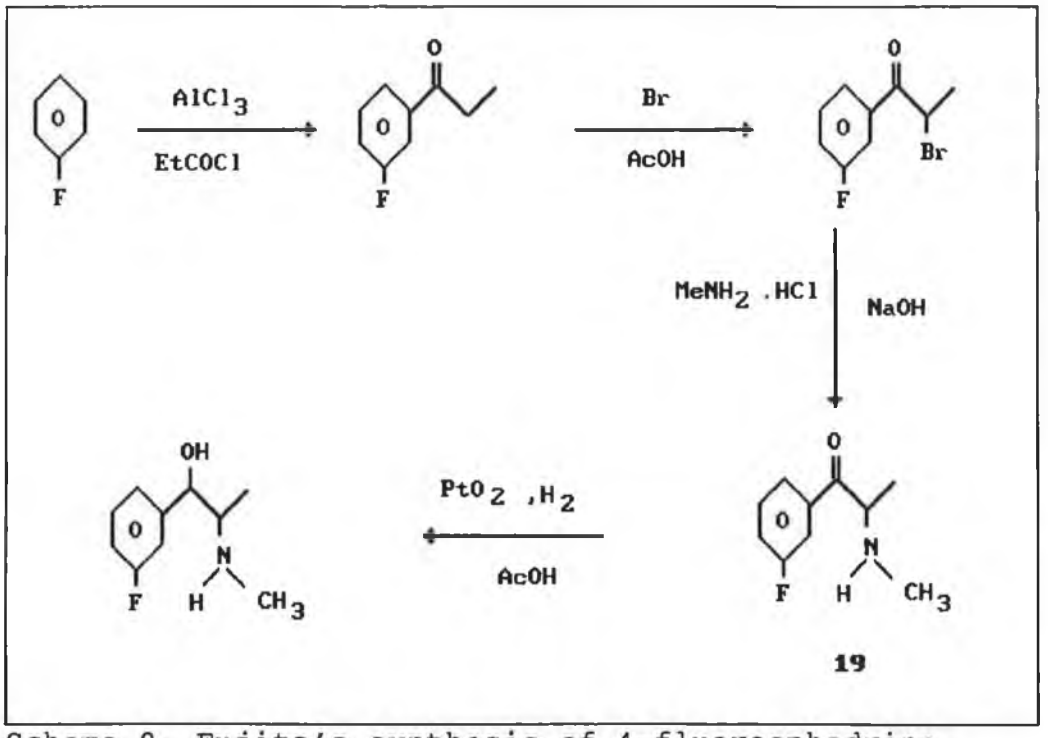
Scheme 7. Fujita's synthesis of ephedrine

Shono [14] reported a stereoselective synthesis of vicinal erythro amino alcoholic moieties utilising carbanions in which the negative charge is located at a position α to the nitrogen atom of the N-acylamine, Scheme 8. The synthesis was started by preparing α-methoxy carbamate (14) by the anodic oxidation of α-carbomethoxyethylamine in methanol. The N-methylation derivative of (14) was followed by treatment with chlorodiphenylphosphene to give (15), Scheme 8. On reaction with benzaldehyde the 2-oxazolidone derivative (17) was formed. Careful hydrogenation in the presence of PtO_2 at atmospheric pressure, for a short period gave the highest yields of (18) which on alkaline hydrolysis afforded (±)-ephedrine selectively



Scheme 8. Shono's stereoselective synthesis of ephedrine

A number of researchers have published work relating to the synthesis of substituted ephedrine derivatives. Kraft[15] prepared 4-fluoroephedrine hydrochloride as a racemic mixture by the platinum oxide catalysed reductive amination of N-methylamino 4-fluoropropiophenone (18). The reaction sequence is shown below (Scheme 9).



Scheme 9. Fujita's synthesis of 4-fluoroephedrine.

Pfanz [16] reported the synthesis of p-nitro and p-amino -ψ-ephedrine hydrochloride in a research paper outlining various methods of converting ephedrine from the erythro to

the threo isomer via the OAc derivative. The O,N diacetyl-ψ-ephedrine derivative was nitrated by reacting nitric acid (d 1.52) and a small quantity of para aminobenzosulphonic acid, at 0°C for one hour. Refluxing in 2 M HCl produced the hydrochloride derivative, and this was converted to the 4-amino analogue by the catalytic reduction of the nitro group with platinum oxide.

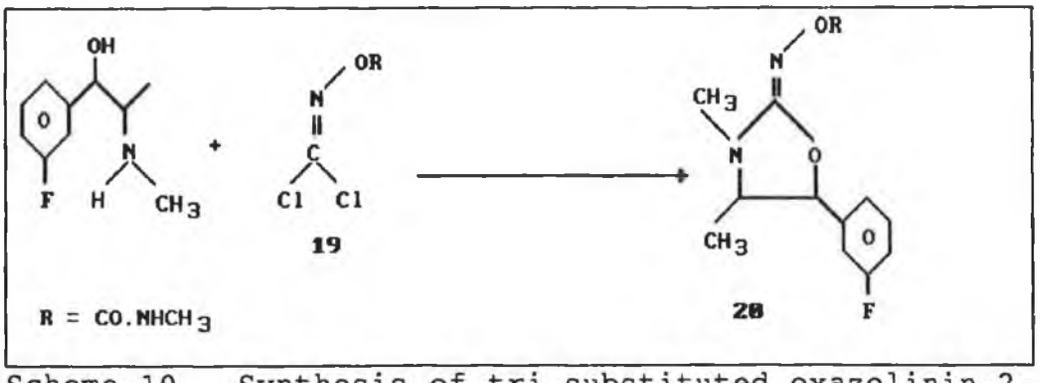
Oamu [17] patented the synthesis and use of 4-(trifluoromethyl)-ephedrine as a cardiovascular β blocking agent. The synthesis involves the halogenation of the substituted ethyl phenyl ketone, followed by reaction with methylamine. The amino ketone was reduced to the corresponding amino alcohol using a Pd/C catalyst.

Weichet [18] synthesised 4-hydroxyphenylacetyl carbinol from the intermediate ethynylcarbinol produced by reacting acetylene with 4-hydroxybenzaldehyde in liquid ammonia. The hydroxy carbinol was then reduced in the presence of methyl amine using sodium borohydride to give the corresponding ephedrine analogue.

Ose and Takamatsu [19] patented a method of synthesising racemic erythro and threo 2-chloroephedrine hydrochloride by reducing 2'-chloro-2-methylamino propiophenone with lithium aluminium hydride in the presence of methylamine. They later patented another method for the production of

2-chloro, 3,4 and 2,5 dichloroephedrine hydrochloride which used an acidic solution of $KClO_4$ in the presence of UV light to induce aryl chlorination of ephedrine.

Ziman [20] studied the synthesis of a range of di- and tri-substituted oxazolinin-2-one oximes, which were patented as antidepressants. The 3,5 dimethyl,-5-(4-fluorophenyl) derivative (20) was prepared by reacting Phosgene oxime O-(N-methyl)-carbamate (19) with the corresponding substituted ephedrine (Scheme 10). This in turn was synthesised by reacting a substituted benzaldehyde with lithium acetylide , and the resulting yne-ol was hydrated with mercuric oxide/methanol/water. Reductive amination using methylamine and sodiumborohydride/methanol gave the ephedrine analogue as a mixture of erythro and threo isomers. The 4-chloro, and the 4-fluoro analogue were prepared by this method and converted to the substituted oxazolinin-2-one oxime.



Scheme 10. Synthesis of tri-substituted oxazolinin-2-one oximes

1.4 PRODUCTION OF PHENYL ACETYL CARBINOL

Neuberg[21] studied the transformation of benzaldehyde to benzyl alcohol using broths of fermenting yeast and sucrose. After 3-5 days no sugar or benzaldehyde remained but the quantity of benzyl alcohol produced was not proportional to the original amount of benzaldehyde used. They searched for a side product in the fermentation broth and found a compound which exhibited leavo-rotation. All the tests carried out indicated an optically active α β ketone alcohol.[22] This compound was phenylacetylcarbinol.

Further studies showed that the addition of carboxy-acetaldehyde improved the yield, and they fermented other aldehydes such as cinamaldehyde which led them to the conclusion that carboligase was an active enzyme component in forming these compounds. Smith[23] proposed that acetylation of benzaldehyde proceeded via a coenzyme dependent system and Hanc[24] showed that pyruvic acid produced during the fermentation of sucrose is decarboxylated to an active acetaldehyde which reacts with benzaldehyde in a benzoin condensation reaction mediated by an enzyme but requiring a coenzyme thiamine pyrophosphate. The enzyme was identified as pyruvate decarboxylase and not carboligase as suggested by Neuberg. Magnesium ions (Mg^{2+})

also activated the biotransformation which indicated that the enzyme molecule has four coenzyme binding sites, three of which are occupied by thiamine pyrophosphate and one by magnesium[25]

Many research workers have studied the bioconversion of benzaldehyde to PAC in order to maximise the yield. Smith[23] showed that when pyruvic acid was added in concentrations exceeding that of benzaldehyde the latter inhibits the production of benzyl alcohol. Groger[26] found the maximum production of PAC was 5g/L using 20% cane sugar in the medium ,but the addition of beer wort increased the yield to 8g/L as a result of the high levels of thiamine it contains.

Voets[27] demonstrated that agitation and aeration of the medium during fermentation was important if yields were to be maximised, and that in the first 10 hours intensive bud formation occurred in the yeast corresponding to the period when the highest levels of PAC were produced. The possibility of other strains of yeast being used in this fermentation was investigated by Netrval[28] and he found that Saccharmoyces calsbergensis could produce up to 12.4g/L of PAC. These values only represented analytical and not extracted yields. Agarwal[29] studied the material balance of the medium and suggested that the optimum level of benzaldehyde was 16mM. At levels of 4mM or below the

production of benzyl alcohol was a maximum while levels above 20mM inhibited the production of PAC completely.

The acyloin condensation reaction has also been reported to occur with substituted benzaldehydes. Most of the early work was carried out by Neuberg[22,30] while Groger[31] demonstrated that 1-naphthaldehyde and 2-furaldehyde were also substrates for this bioconversion.

Long[32] studied the production of substituted PAC's. The yield of these compounds, and the corresponding benzyl alcohol derivative, were measured colorimetrically and by high performance liquid chromatography (HPLC) respectively. They demonstrated that the aromatic aldehydes substituted in the ortho position were poor substrates, while those with $-CH_3$, $-CF_3$, and -Cl substituent located at para positions produced high carbinol yields. The opposite was observed with methoxy substituent, which gave highest yields when meta substituted rather than in either of the other positions. The measured yields of carbinol compound and substituted aromatic alcohol are given in Table 2.

Substrate aromatic aldehyde	L-acetyl aromatic carbinol	yield (mg/mL)	aromatic alcohol	yield (mg/mL)
Benzaldehyde	L-phenylacetyl carbinol	10.1-10.2	benzyl alcohol	0.4
o-Tolualdehyde	L-2-methylphenyl acetyl carbinol	2.0-2.5	2-methylbenzyl alcohol	1.0
m-Tolualdehyde	L-3-methylphenyl acetyl carbinol	5.6-6.2	3-methylbenzyl alcohol	0.4
p-Tolualdehyde	L-4-methylphenyl acetyl carbinol	5.4-6.4	4-methylbenzyl alcohol	0.3
2-Chlorobenz-aldehyde	L-2-chloroacetyl carbinol	0.6-0.7	2-chlorobenzyl alcohol	0.8
3-Chlorobenz-aldehyde	L-3-chloroacetyl carbinol	2.1-3.2	3-chlorobenzyl alcohol	0.6
4-Chlorobenz-aldehyde	L-4-chloroacetyl carbinol	6.5-8.0	4-chlorobenzyl alcohol	0.3
o-Anisaldehyde	L-2-methoxyphenyl acetyl carbinol	0.8-0.9	2-methoxybenzyl alcohol	0.9
m-Anisaldehyde	L-3-methoxyphenyl acetyl carbinol	4.5-5.7	3-methoxybenzyl alcohol	0.7
p-Anisaldehyde	L-4-methoxyphenyl acetyl carbinol	1.2-3.4	4-methoxybenzyl alcohol	0.6

Table 2. Yields of carbinol and aromatic alcohol produced by fermentation with Saccharomyces cerevisiae [32]

In the above study gas chromatography was used to determine the level of aromatic alcohols produced, and to validate the colorimetric method of Groger[34] used in the determination of L-acetyl aromatic carbinol. Acetylbenzoyl (1-phenyl-1,2 propanedione) was used as a standard in both of the above methods.

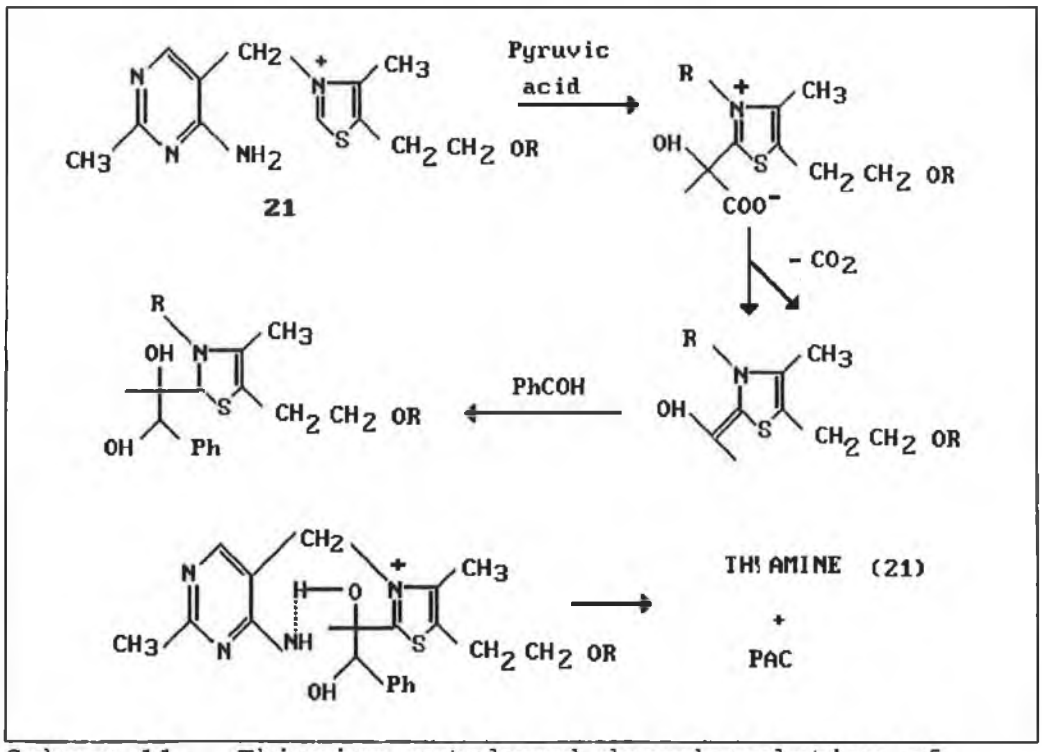
The highest levels of PAC were obtained at benzaldehyde concentrations of 6g/L (56mM) and a medium pH of 4.5, while yeast dose rates of 60g/L also gave a maximum yield of carbinol. In the case of all the aromatic aldehydes studied, the production of the corresponding alcohol was suppressed when the medium containing sodium pyruvate was used. Consequently in these cases the yield of carbinol was highest. It was noted in this report that aldehydes having substituents in the ortho position of the ring were poor substrates for L-acetyl aromatic carbinol production.

In subsequent studies the reaction rates of a number of aromatic aldehydes as substrate for Saccharomyces cerevisiae were investigated.[33] Parameters such as aldehyde concentration, medium pH, and yeast dose rate were varied and the effect on the level of PAC produced monitored. These fermentations were for short periods
(1 hour) and two mediums were tried. The first contained sucrose as the sole carbon source while the second only contained sodium pyruvate.

The benzoin condensation is also a reaction which involves an active aldehyde intermediate.[35] [36] The product is a carbinol and is similar to PAC. Thiamine (21) will act as a catalyst for the benzoin condensation in an analogous way to cyanide [37]. The thiazolium zwitterion stabilises the active aldehyde intermediate allowing it to react with another molecule of benzaldehyde. The reaction has been studied extensively as a model for important biochemical carbon-carbon bond formation reactions in which the catalytic entity is the thiazolium function of thiamine pyrophosphate. Some of these pathways are the decarboxylation of pyruvic acid to acetoin and the transketolase reaction.

During the enzymatic decarboxylation of pyruvic acid the thiazolium zwitterion also acts as a catalyst stabilising the active aldehyde intermediate, and this intermediate can then react further with a molecule of benzaldehyde to produce carbinol compound, Scheme 11. In this case the active aldehyde is acetaldehyde and the resultant carbinol will have the hydroxyl group α to the aromatic ring. The enzyme structure is hydrogen bonded to the thiamine complex

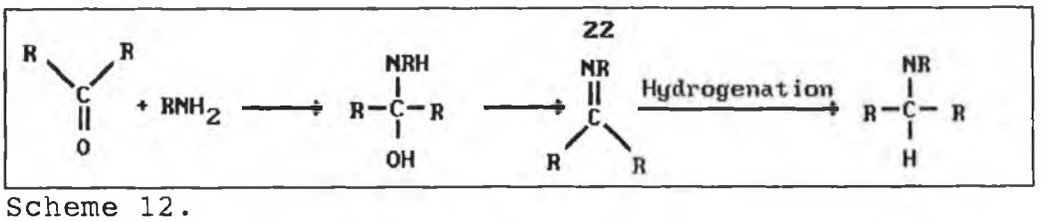
increasing the basicity of the nitrogen at position 4 on the pyrimidine ring. This allows interaction with the hydroxyl proton of the α-hydroxyethyl group in the active aldehyde which labilises the bond at C-2 and releases the product (PAC) from the enzyme complex.



Scheme 11. Thiamine catalysed decarboxylation of pyruvic acid, and the synthesis of PAC from the active aldehyde

1.5 REDUCTIVE AMINATION

When aldehydes or ketones are treated with a primary or secondary amine in the presence of hydrogen and a hydrogenating catalyst, reductive amination of the carbonyl compound takes place. The reaction is thought to occur through the imine intermediate (22).[38]



Scheme 12.

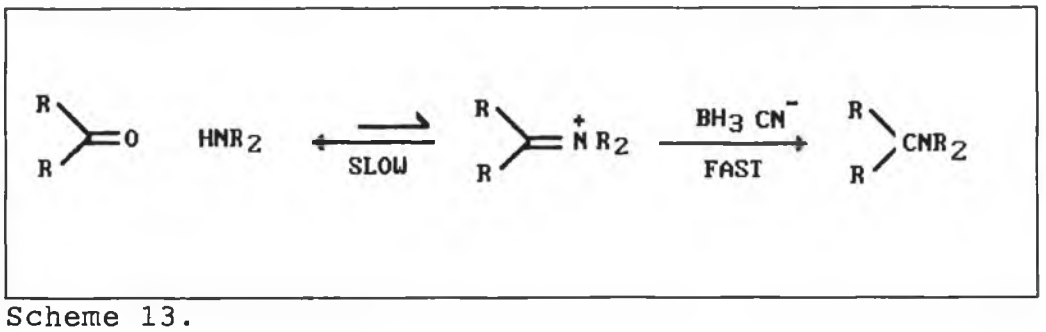
Many reagents have been proposed for such a reduction , with hydrogen and a metallic catalyst being applied commercially. Sodium borohydride, sodium cyanoborohydride, iron pentacarbonyl and alcoholic KOH, and selenophenol (PhSeH) have all been successfully applied to this reduction.

In a one step reaction from the carbonyl compound the reagent selected must be sufficiently inert not to reduce the original carbonyl compound before the imine is formed, and to reduce the imine in preference to the carbonyl. As

mentioned above sodium cyanoborohydride fits such a description. The strong electron withdrawing cyano group increases the lewis acidity of the corresponding cyano borane and the resulting cyanoborohydride anion is more reluctant to deliver a hydride. The result is a toned down reducing capacity and increased stability which allows more discriminative selection among the functional groups.[39]

The selectivity of the reagent for the reduction of imines under acidic conditions was first demonstrated by Kreevoy [40], who showed that the BH_3CN^- anion was stable in acid down to pH 3. Further work was carried out by Borch [41], who extensively studied the selectivity of this compound as a reducing agent in a number of reactions. This selectivity was induced solely by altering the pH of the medium. Aldehydes and ketones showed optimum reduction using either lithium or sodium cyanoborohydride at pH 3-4 in methanol, while oximes were reduced to the corresponding N-alkylhydroxylamine at pH 4. When it was established that the imminium moieties ($>C=N^+R_2$ and $>C=N^+RH$) were reduced with BH_3CN^- at pH 6 to 7, and that the reduction of ketones was negligible in this range, Borch realised the potential of the method to trap the imminium group by reduction in the presence of aldehydes and ketones.

The formation of the imminium moiety is reversible but has an optimum at pH 6 (Scheme 13), yet Borch successfully exploited the reaction at pH as low as 4 and as high as 10. The only requirement was that there was enough proton source to generate a positively charged >C=N$^+$< species.



Scheme 13.

The reductive amination of phenylacetyl carbinol has been extensively studied by many researchers. This reaction is an important step in the synthesis of ephedrine. If the reductive amination can be performed stereoselectively using (-)-phenylacetyl carbinol as a substrate, then the only product formed is the pharmacologically active (-)-ephedrine. Some methods of synthesising ephedrine have already been discussed in Section 1.3.

Weichet [18] produced ephedrine by reductive amination of (-)-PAC using sodium borohydride and methylamine. He found a 4:1 ratio of erythro to threo isomers in the isolated product. The method of Hennion and Fleck [42] was applied to the synthesis of racemic 3-hydroxy PAC and 4-flouro PAC which were used in the synthesis of the corresponding ephedrine analogues by the method described above. A Czechoslovak patent [43] was later issued to the same authors in relation to the synthesis of these compounds by this method.

Ose [44] used lithium aluminium hydride as the reductive agent in the reductive amination of aryl chlorinated phenylacetyl carbinols to produce racemic mixtures of the erythro and threo 2-chloro and the 2,4-dichloro ephedrine analogues. This work was also the subject of two patents [19,45] and included the synthesis of 3,4-dichloroephedrine hydrochloride by the action of an acidic solution of $KClO_4$ on ephedrine in the presence of UV light.

1.6 DETERMINATION OF ENATIOMERIC PURITY

Only two of the four possible stereoisomers of ephedrine have pharmacological activity. These are (1R,2S)(-) ephedrine , and (1S,2R) (+)-ψ-ephedrine. When ephedrine is prepared from 1-Phenylacetyl carbinol by reductive amination, the configuration of carbon-1 is predetermined by the stereoselective biotransformation. The reduction amination of the carbinol then produces a pair of diastereoisomers by introducing another asymetric centre at carbon-2, and their ratio can be determined by quantitative high field nuclear magnetic resonance (^{1}H nmr). Alteration of the configuration about carbon-1 still remains a possibility during this reaction, and this would lead to two pairs of enantiomers being formed. The enantiomeric excess must now be determined, and by far the most succesful method reported was ^{1}H nmr employing chiral lanthanide shift reagents.

The study of the structure of ephedrine using ^{1}H nmr was first reported by Hyne.[46] He prepared oxazolidine, spirodecane, and oxazolidone derivatives of (-) ephedrine and (+)-ψ-ephedrine by ring closure across the hydroxyl and amino function. He then used ^{1}H nmr to study the influence of the phenyl ring on the terminal methyl group as a method of detecting the relative spatial distribution of the various groups in the molecule.

Hofer [47] applied chiral relaxation reagents in the ^{13}C nmr spectroscopy of ephedrine in order to develop an analytical method for determining the enantiomeric purity. He reported the formation of diastereomeric complexes from enantiomeric ephedrine and the gadolinum relaxation reagent, tris[d,d-dicampholymethanate] gadolinum (III) . The difference in relaxation time ($\Delta\Delta t1$) was dramatic, and showed a dependence on the concentration of the lanthanide reagent used. This decreased rapidly at low concentrations of reagent and decreased slowly at higher values. The maximum difference (0.754 sec) occurred at a concentration of 2.5 mg of $Gd(dcm)_3$ for the substituted aromatic carbon.

A similar method was applied by Dewar [48], using ^{1} H nmr on a 60 MHz machine. The susceptibility of various groups showing sharp lines such as $N-CH_3$ and $C-CH_3$ were investigated. Four lanthanide shift reagents were employed, the two non chiral reagents were $Eu(fod)_3$ and $Pr(fod)_3$, while the two chiral reagents were $Eu(tfc)_3$ and $Pr(tfc)_3$. The latter gave relatively large differential values (0.33) for the $C-CH_3$ doublet in deuterated benzene even though line broadening was observed. By using constant shift reagent to sample molar ratios, constant instrument conditions and a mathematical base line technique, a calibration curve giving optical purity was constructed.

A further study[49] also used chiral shift ^1H nmr techniques to determine the optical purity of (±)-ephedrine and (±)-ψ-ephedrine. They prepared ephedrine solutions containing the europium and prasodynmun derivatives $Eu(hfc)_3$ and $Pr(tfc)_3$, and observed the shift of the CH_3 doublet. A plot of the induced chiral shift against the molecular proportion of the lanthanide reagent to substrate was linear at low values of the latter. A calibration curve was developed by preparing a series of enantiomeric mixtures and plotting the theoretical optical purity, determined by polarimetary, against the ratio of peak heights of the doublets produced by the enantiomers present. Statistical interpretation of the results by methods of least squares and analysis of variance was applied to the data generated. The agreement with the polarimetric method was within ± 1% .

1.7 GENERAL PHARMACOLOGY

The autonomic nervous system is divided in two separate systems i.e. the Sympathetic and the Parasympathetic nervous systems. In the case of the former the pregangalonic neuron synapse transmitter is acetylcholine and the postgangalonic transmitter is noradrenaline ,but acetylcholine is the synapse transmitter in both the pre- and postgangalonic neurons in the parasympathetic nervous system. As a result of these differences, stimulation of either type of nervous system results in opposite biological functions.

Activation of the sympathetic system is characterised by the release of noradrenaline and the symptoms observed are those resulting from the effects of this compound. The heart beat rate and blood pressure are raised, the pupils and bronchi are dilated, and the liver is stimulated to convert glycogen to glucose. It causes the blood to shunt from the skin to the muscles, brain, and heart, while peristalsis in the intestine and contraction of the bladder is inhibited. Overall the effect is one of stimulating the body into a highly active state, similar to the effect caused by the release of adrenaline into the blood stream from the adrenal medulla. Stimulation of the parasympathetic nervous system has the opposite effect and tends to return the body back to the original deactivated

state by suppressing the effect of noradrenaline.

Ephedrine is a sympathomimetic amine and falls in the category of non-catecholamine drugs as it does not have a hydroxyl substituent on the aromatic ring. Ahlquist [50] suggested that the drugs acted through two cellular receptors, α and β, while Furchgust [51] subdivided these into $\beta 1$, $\beta 2$, $\alpha 1$,and $\alpha 2$ andrenergic receptors. The $\beta 1$ receptor has a direct effect on the heart and the $\beta 2$ effects the smooth muscle tissue of the bronchi, uterus, and blood vessels. These receptors are believed to be on the cell surface and stimulation causes the release of the enzyme adenyl cyclase which catalyses the conversion of adenosine tri phosphate (ATP) to 3',5'-cyclic adenosine monophosphsate (cAMP). It is this secondary cellular messenger which is believed to cause the effects observed.

Ephedrine stimulates both the α and β andrenergic receptors, and the pharmacological effect is described as both direct and indirect. The direct effect results from stimulation of the above receptors causing increased production of cAMP, while the indirect effect is due to

stimulation of the sympathetic nervous system to release noradrenaline, which in turn activates the andrenergic receptors.

Ephedrine is used as a nasal decongestant and a vasoconstrictor, but unlike adrenaline it is effective by the oral route. It is less potent as a bronchodilator but has a longer duration of activity than adrenaline, and is not metabolised by catechol-o-methyl transferase in the blood or monoamine oxidase in the liver. It does however undergo deamination and conjugation. The side effects during clinical use are tachycardia, hypertension, urinary retention, and evidence of CNS stimulation such as nausea , vomiting, sweating, vertigo, tremor, and insomnia. As a result ephedrine has been largely been replaced by more specific β2 stimulators.[52]

RESULTS AND DISCUSSION

2.1 FERMENTATION OF AROMATIC ALDEHYDES

The initial experiments were designed to find a method, and fermentation medium which gave an appreciable yield of PAC or substituted PAC. The mediums used are described in Appendix 1 and were initially tried in small batches (200 cm^3) . The extracted PAC was purified using sodium bisulphate method (Section 3.4)

2.1.1 SELECTION OF MEDIUM FOR FERMENTATION

The first medium tried (medium A) was that described by Long [32]. The extraction and purification of the PAC produced was carried out according to their method. The yield was very low and is given in Table 3. below. This medium contained only pyruvic acid as a carbon source for the biotransformation.

Because of the low yield the pyruvic acid was replaced with sucrose (medium B) and the experiment repeated. The yield did increase , but was still too low for the later reductive amination stage. The medium was further adjusted by the addition of a range of minerals. Magnesium ions were added and are known to be a necessary co-factor for the enzyme involved, and ammonium sulphate was added as a source of nitrogen. This corresponded to the medium used by

Voets [27] and was denoted medium C. Again another increase in yield was observed. The final modification was to include the minerals from medium C and to add both sucrose and pyruvic acid from medium A and B. This medium (medium D) gave the highest yields.

MEDIUM	YIELD	CONCENTRATION mg/L
Medium A	0.206g	1.03
Medium B	0.301g	1.50
Medium C	0.523g	2.61
Medium D	0.716g	3.58

Table 3. PAC yields from the various mediums tried

2.1.2 SCALE UP OF FERMENTATION

To produce sufficient PAC for the reductive amination stage the scale of the fermentation had to be increased. Medium D was selected as the most suitable and a batch size of 800 cm^3 was the maximum which could be handled. The first large scale fermentation yielded 1.74g of PAC (2.175 g/L) after purification. This yield was not as good as that produced under the same conditions using 200 cm3 of medium. To improve the air incorporation in the medium, during the large scale fermentation, a small pump was used to blow a continuous stream of air into the bottom of the vessel. The air was dispersed through a narrow nozzle to give a small bubble size, and the resulting yield increased to 3.3g (4.12g/L).

2.1.3 FERMENTATION OF 4-CHLOROBENZALDEHYDE

The production of 4-chloro PAC used the same medium, fermentation scale, and extraction method as described above in 2.1.2 . The first attempt yielded 0.230g (0.25 g/L) and 1.5g of the solid aldehyde, which had not dissolved, was recovered from the medium. To overcome the low solubility of 4-Chlorobenzaldehyde the fermentation was repeated but the aldehyde was first dissolved in acetone ($10cm^3$) and the appropriate aliquot added. The yield increased to 0.6g (0.75 g/L).

When the purification was performed in the usual manner 0.73g of 4-chloro PAC was recovered, however the ether washings were retained and when evaporated yielded a further 1.8g of the crude compound, which was identified by TLC and ^{1}H nuclear magnetic resonance (section 2.1.10) This brought the overall yield to approximatly 2.5g

2.1.4 FERMENTATION OF 4-METHOXYBENZALDEHYDE

The 4-methoxybenzaldehyde was fermented using medium D (800 cm^3). The cells were washed with ethanol and the washings were returned to the medium before extraction with ether, as described in the modified extraction method (section 3.3). The aldehyde was added over the seven hour period to maintain a concentration of
11 mM . The yield after purification with sodium bisulphate was 1.057g (1.32g/L). The fermentation was repeated , but the purification step was omitted. The ether extract was washed with sodiumcarbonate and evaporated to give a crude fraction of 4-methoxy PAC. This crude fraction totalled 8.13g, which was higher than the reported yield.

2.1.5 FERMENTATION OF 2-CHLOROBENZALDEHYDE

The fermentation was carried out using medium D and a batch size of 800 cm3. The recovered yeast was in poor condition and very dark in colour, which suggests an intolerance of the added aldehyde. The modified extraction technique was used, and the purification step was omitted. The aldehyde was added in aliquots of 1.4 cm^3 to give a medium concentration of (15 mM). The extraction yielded 3.8g of viscous yellow liquid, and the ^1H nmr results indicated that this liquid contained approximately a 50% mixture of the parent aldehyde (2-chlorobenzaldehyde) and the corresponding substituted PAC.

2.1.6 FERMENTATION OF 4-METHYLBENZALDEHYDE

4-Methylbenzaldehyde was fermented using medium D (800 cm^3). Six aliquots of 1.3 cm^3 of the aldehyde were added over the time period and the medium was extracted with the revised extraction method (section 3.3). The purification step was omitted and 5.134g (6.41mg/L) of a thick yellow liquid was recovered. This was identified as 4-methyl PAC by ^1H nmr (section 2.1.10), and TLC showed it to be sufficiently pure for the reductive amination step.

2.1.7 FERMENTATION OF 4-FLUOROBENZALDEHYDE

4-Fluorobenzaldehyde was fermented and extracted in the same manner as described above (Section 3.1.6) . Six additions of 1.3 cm^3 of the aldehyde were made over the fermentation period, and 6.7g (8.73g/L) of crude 4-Fluro PAC was produced. This was recovered as a yellow oil

2.1.8 FERMENTATION OF 3-PYRIDINECARBOXALDEHYDE

The fermentation was preformed as described in section 3.2, and the recovered yeast was in good condition. The extraction of the medium was carried out as described in section 3.3, but only a small quantity (0.3g) of semi solid material was recovered. TLC analysis showed this to contain a number of different compounds. The ^1H nmr data was not consistent with that of a carbinol, but showed some aldehyde to be present.

2.1.9 THIN LAYER CHROMATOGRAPHY

The presence of the carbinol compound in the crude fermentation extract was confirmerd by TLC, as described in section 3.4 . In all cases chloroform was used as the solvent and the plates were developed with iodine. A good resolution of the carbinol was achieved under these conditions with the parent aldehyde just below the solvent front and the carbinol midway along the plate. The Rf values of the compounds mentioned above
are given in Table 4.

CARBINOL COMPOUND	Rf VALUE	PARENT ALDEHYDE Rf VALUE
(-)PHENYLACETYL CARBINOL (PAC)	0.47	0.83
(-)-4-CHLORO PAC	0.48	0.83
(-)-4-METHOXY PAC	0.41	0.79
(-)-4-METHYL PAC	0.45	0.73
(-)-4-FLUORO PAC	0.43	0.79
(-)-2-CHLORO PAC	0.45	0.85

Table 4. Rf vales of aromatic substituted PAC and parent aldehyde

During the fermentation of 4-methoxybenzaldehyde, the TLC plate of the crude extract was compared to that of the purified extract. The latter showed a higher concentration of parent aldehyde than the crude extract. This seems to suggest that during the bisulphate purification step the parent aldehyde is extracted as efficiently as the carbinol compound. This step does not have any significant benefit in preparing the carbinol for the reductive amination reaction.

Column chromatography was performed on the crude extract from the fermentation of benzaldehyde and 4-chlorobenzaldehyde, by the method described in section 3.6 , using chloroform as the eluant. In each case the carbinol was identified as the largest band present and followed a small yellow band. It was well resolved from all the other compounds present, and was recovered as a thick yellow oil in the case of PAC and as a yellow semi-solid in the case of 4-chloro PAC.

2.1.10 ^{1}H NMR OF THE FERMENTATION EXTRACTS

The nmr data were recorded on the Perkin Elmer R-12B 60 MHz machine as described in section 3.10. The data was recorded on the neat compound in some cases.

^{1}H NMR OF PHENYLACETYL CARBINOL

^{1}H NMR (ppm, $CDCl_3$)

The sample had been passed through a chromatography column prior to recording the data.

2.02 ppm (3H,s, CH-C<u>H</u>$_3$) 4.40 ppm (1H,s,C<u>H</u>-OH)

5.05 ppm (1H,s,C-OH) 7.30 ppm (5H,s, ArH)

see appendix II

^{1}H NMR OF 4-CHLORO PHENYLACETYL CARBINOL

^{1}H NMR (ppm, $CDCl_3$)

The sample had been passed through a chromatography column prior to recording the data.

2.02 ppm (3H,s, CH-C<u>H</u>$_3$) 4.3 ppm (1H,s,C<u>H</u>-OH)

5.10 ppm (1H,s,C-OH) 7.30 ppm (5H,s, ArH)

^{1}H NMR OF 4-METHOXY PHENYLACETYL CARBINOL

^{1}H NMR (ppm, $CDCl_3$)

The sample was extracted from the fermentation medium and purified using sodium bisulphate(section 3.4)

2.05 ppm (3H,s, CH-C<u>H</u>$_3$) 3.75 ppm (3H,s, ArOC<u>H</u>$_3$)

4.5 ppm (1H,s,C<u>H</u>-OH) 5.10 ppm (1H,s,C-OH)

6.7-7.4 ppm (4H,M, ArH)

^{1}H NMR OF 4-METHYL PHENYLACETYL CARBINOL

^{1}H NMR (ppm, neat)

The sample was extracted from the fermentation medium.

1.4 ppm (3H,s, CH-C<u>H</u>$_3$) 1.65 ppm (3H,s,Ar-C<u>H</u>$_3$)

4.05 ppm (1H,s,C<u>H</u>-OH) 4.6 ppm (1H,s,C-OH)

6.3-6.9 ppm (4H,m, ArH)

The peak at 4.05 ppm showed a broad shoulder on the downfield side.

^{1}H NMR OF 4-FLUORO PHENYLACETYL CARBINOL

^{1}H NMR (ppm, neat)

The sample was extracted from the fermentation medium.

1.5 ppm (3H,s, CH-C<u>H</u>$_3$) 4.05 ppm (1H,s,C<u>H</u>-OH)

4.06 ppm (1H,s,C-OH) 6.2-7.0 ppm (5H,s, ArH)

9.3 ppm (trace of parent aldehyde)

2.1.11 APPRAISAL OF RESULTS

The results indicate the viability of a fermentation method for the production of substituted PAC compounds with the exception of 2-chlorobenzaldehyde and 3-pyridinecarboxaldehyde, which are not good substrates for this biotransformation. The purification step described by Long[32] has been shown not to be a necessary step prior to the reductive amination and was omitted during further work.

2.2 REDUCTIVE AMINATION AND PREPARATION OF EPHEDRINE ANALOGUES

2.2.1 REDUCTIVE AMINATION OF ACETOPHENONE

Borch[41] carried out a reductive amination on acetophenone using sodium cyanoborohydride and methylamine in absolute methanol at pH 6 - 8. The reported yields of N-methylphenethylamine were 78% and the melting point of the hydrochloride derivative was 178°-178°C. In this researchthe reaction was performed replacing methylamine free base by the hydrochloride derivative (section 3.7). In both runs of this reaction 20mM of acetophenpone were used and the yields of crude products were 1.35g and 1.43g respectively. The product was recovered as a thick viscous oil which was slightly yellow in colour.

^{1}H NMR (neat)

1.4ppm (3H,s,C-CH_3)

1.7ppm (3H,s,N-H)

2.3ppm (3H,s, N-CH_3)

3.7ppm (1H,m,C<u>H</u>-OH)

7.4ppm (5H,S,ArH)

The hydrochloride derivative was prepared by the method described in section 3.8, to give sticky white solid. It was recrystallised from petroleum ether/methanol to give white waxy solid with a melting point range 159.1°-165.3°C. Two subsequent recrystallisations from

carbon tetrachloride / chloroform yielded a similar white solid with a melting point of 172.8°-173.4°C.

These results indicate that the reductive amination of crowded ketones can be prepared using methylamine hydrochloride as a replacement for the methanolic solution of methylamine used by Borch[38]. The yield were lower, and in the order of 25% .

2.2.2 SYNTHESIS OF EPHEDRINE

The reductive amination of PAC was carried out as described in section 3.7. In two sequential reactions 0.4g and 1.86g of PAC yielded 0.34g and 0.91g of crude ephedrine respectively. This product was a viscous yellow oil which contained a waxy white solid. These yields represent a mixture of the two isomers, the oil being the ψ-ephedrine and the solid being ephedrine which has a higher melting point. The presence of the ephedrine isomers were confirmed by ^1H nmr.

^1H NMR of crude ephedrine free base (ppm $CDCl_3$)

0.9ppm (3H,s,CH-$C\underline{H}_3$) 2.4ppm (3H,s, N-CH_3)

2.5-3.0 (m,not assigned) 4.7ppm (1H,d, C$\underline{H}$-OH)

7.3ppm (5H,s,ArH)

This recorded spectrum was identical to that available in the reference literature [51]

PREPARATION OF THE HYDROCHLORIDE DERIVATIVE

The hydrochloride was initially prepared by dissolving the crude ephedrine mixture in methanol and adding concentrated hydrochloric acid. The yield from this method was as low as 10%, and the acidity may have caused the ephedrine to decompose back to the original PAC and methylamine. A modified method using hydrogenchloride gas was developed and used in all subsequent preparations (section 3.8).

FRACTIONAL CRYSTALLISATION OF EPHEDRINE HYDROCHLORIDE MIXTURE

The isomers were crudely separated from the oily matrix in which it was recovered, using cold acetone. The ephedrine fraction is insoluble in this solvent and separates as a waxy white solid. The ephedrine isomer is only slightly soluble in hot chloroform, while the ψ-isomer is fully soluble . This property was exploited to crudely separate the two isomers, the ephedrine fraction being recovered with a higher purity.

RECRYSTALLISATION OF THE EPHEDRINE FRACTION

The hydrochloride fraction was recrystallised twice from acetone/methanol (15:1) to give white needles with a melting point range of 215.5°-216.3°C

MICROANALYSIS OF EPHEDRINE HYDROCHLORIDE

	THEORY	MEASURED
CARBON	59.55%	59.07%
HYDROGEN	7.94%	8.02%
NITROGEN	6.72%	6.94%

OPTICAL POLARIMETRY OF EPHEDRINE HYDROCHLORIDE

The optical rotation of the compound was measured using deionised water as a solvent (section 3.10). The small quantity of pure compound available made it necessary for concentrated solutions to be used, and a short path length cell was employed. The optical rotation is concentration dependent and as no literature values were available at the concentrations used the commercial (-)ephedrine hydrochloride was employed as a standard.

SYNTHETIC EPHEDRINE HYDROCHLORIDE.

$[\alpha]_D$ - 24.28° H_2O C = 28.5

COMMERCIAL (-) EPHEDRINE HYDROCHLORIDE

$[\alpha]_D$ - 25.9° H_2O C = 27.0

RECRYSTALLISATION OF THE ψ-EPHEDRINE FRACTION

This fraction was recovered by evaporating the chloroform used to separate the ephedrine hydrochloride. It was recrystallised twice from carbon tetrachloroide / chloroform mixture (10:2) to give a white crystalline solid with a melting point range of 160.3° - 162.6°C .

^{1}H NMR of ψ-EPHEDRINE HYDROCHLORIDE

^{1}H NMR (ppm D_2O)

1.1ppm (3H,d,CH-C$\underline{H}_3$)

2.8ppm (3H,s,N-CH_3)

3.3-3.8ppm (not assigned)

4.5ppm (H_2O in D_2O)

7.5ppm (5H,s,ArH)

MICROANALYSIS OF ψ-EPHEDRINE HYDROCHLORIDE

	THEORY	MEASURED
CARBON	59.55%	59.36%
HYDROGEN	7.94%	7.84%
NITROGEN	6.72%	6.64%

OPTICAL POLARIMETRY OF ψ- EPHEDRINE HYDROCHLORIDE

The optical rotation of the ψ-ephedrine hydrochloride was measured using the described method (Section 3.10).

$[\alpha]_D$ - 33.77° H_2O C = 26.4

APPRAISAL OF RESULTS

The analytical results on the (-)-ephedrine hydrochloride isolated suggest that the sample was optically pure and that it had been successfully separated from the pseudo isomer. The (-)-ψ-ephedrine hydrochloride had a lower melting point than that quoted in the literature suggesting that some of the erythro isomer still remained despite the repeated recrystallisations. Evidence to suggest this was also observed during the pharmacological experiments (Section 2.4).

2.2.3 SYNTHESIS OF 4-CHLOROEPHEDRINE

The reductive amination of 4-chloro PAC was carried out as described in section 3.7. The product was recovered as a semi solid yellow oil which solidified on cooling below room temperature. The hydrochloride derivative was prepared and recovered as a waxy cream coloured solid, with an overall yield in the order of 10 to 15%.

SEPARATION OF ISOMER MIXTURE

The 4-chloroephedrine hydrochloride isomers were washed with cold acetone to remove the oily matrix in which they were recovered. The erythro isomer was crudely separated from the pseudo isomer using hot chloroform, the later being very soluble in this solvent. The former was recovered as a gritty white solid with a melting point of 243.6° - 244.1°C .

RECRYSTALLISATION OF 4-CHLOROEPHEDRINE HYDROCHLORIDE

The crude 4-chloroephedrine described above was recrystallised twice from a mixture of acetone and methanol (17:3) to give large clear crystal needles which had a sharp melting point 244.5°- 245.0°C .

^{1}H NMR OF 4-CHLOROEPHEDRINE HYDROCHLORIDE

^{1}H NMR (ppm, D_2O)

1.25 ppm (3H,s, CH-$C\underline{H}_3$) 2.9 ppm (3H,s, N-CH_3)

5.25 ppm (1H,m, not assigned) 7.55 ppm (4H,s, ArH)

MICROANALYSIS OF 4-CHLOROEPHEDRINE HYDROCHLORIDE

	THEORY	**MEASURED**
CARBON	50.85%	50.83%
HYDROGEN	6.36%	6.57%
NITROGEN	5.93%	5.82%

OPTICAL POLARIMETRY OF 4-CHLOROEPHEDRINE HYDROCHLORIDE

The optical polarimetry of 4-chloroephedrine hydrochloride was carried out as described in section 3.10. The solubility of this compound in water was much less than that of ephedrine, consequently the observed rotation using the 0.1 mL cell was very low. The measurement was repeated using a larger cell and the results are given below.

0.1 mL cell	$[\alpha]_D$ -33.22°	H_2O C = 6.0
1.0 mL cell	$[\alpha]_D$ -31.11°	H_2O C = 4.5

RECRYSTALLISATION OF ψ-4-CHLOROEPHEDRINE HYDROCHLORIDE

The pseudo isomer was recrystallised from a carbon tetrachloride / chloroform mixture (4:1) to give white solid (47 mg) with a melting point of 144.2°- 146°C. The crystals differed to that observed for the 4-chloroephedrine isomer.

^{1}H NMR OF ψ-4-CHLOROEPHEDRINE HYDROCHLORIDE

^{1}H NMR (ppm, D_2O)

1.20 ppm (3H,d, $CH-CH_3$) 2.85 ppm (3H,s, $N-CH_3$)

4.3 ppm (1H,M,not assigned) 7.45 ppm (4H,s, ArH)

OPTICAL POLARIMETRY OF ψ-4-CHLOROEPHEDRINE HYDROCHLORIDE

Because the 4-chloroephedrine hydrochloride was not very soluble in water , dilute HCl (5M) was selected as a solvent for the pseudo isomer. The compound was however very soluble in this solvent, and the optical rotation was measured at a low concentration using the 0.1 mL cell.

$[\alpha]_D$ -31.8° HCl 5M C = 6.0

APPRAISAL OF RESULTS

The 4-chloroephedrine hydrochloride was easily recovered in the form of large pure crystals which were produced in sufficient quantity for further investigation. The melting point recorded was in the same range as the values published[44] , 245°-6°C. The ^{1}H nmr data was in agreement with that expected for the structure, and the microanalysis data agreed with the theoretical values. Although no literature values were available for the optical rotation of this isomer, the observed values were of the correct direction.

The ψ-4-chloroephedrine hydrochloride was more difficult to recrystallised than the isomer described above, and the yields were consequently much lower. No literature melting point, or optical rotation values were available for this compound.

2.2.4 SYNTHESIS OF 4-METHOXYEPHEDRINE

The reductive amination of 4-methoxy PAC was carried out as described in section 3.7. The isomeric mixture was recovered as a yellow oil. The hydrochloride derivative was prepared by passing hydrogen chloride gas through a solution of the compound (section 3.8) and the crude product was recovered as a waxy solid which had a slight yellow coloured.

SEPARATION OF ISOMER MIXTURE

The crude mixture of isomers were separated from the oily material by washing with cold acetone. The resulting white solid was washed with hot chloroform to remove the (1R,2R)-ψ-isomer, and the insoluble crude material had a melting point of 199°-204°C. The chloroform washings were evaporated but did not yield the ψ-isomer,and consequently the acetone washings of the original crude material were retained and examined. This yielded a small quantity of white solid on evaporation of some of the solvent. This crude compound had a melting point range of 160°-166°C

RECRYSTALLISATION OF 4-METHOXYEPHEDRINE HYDROCHLORIDE

The 4-methoxyephedrine hydrochloride was recrystallised from a chloroform / methanol (20:1) mixture to give a fine white crystalline solid. This was further recrystallised from acetone / methanol (20:1) mixture until a constant melting point was obtained(236°-239°C)

^{1}H NMR OF 4-METHOXYEPHEDRINE HYDROCHLORIDE

^{1}H NMR (ppm, D_2O)

1.1 - 1.25ppm (3H,d, CH-C$\underline{H}_3$) 2.8 ppm (3H,s, N-CH_3)

3.85ppm (3H,s, OCH_3) 5.1 ppm (1H,M,not assigned)

6.9 - 7.6 ppm(4H,s, ArH) see appendix II

MICROANALYSIS OF 4-METHOXYEPHEDRINE HYDROCHLORIDE

	THEORY	MEASURED
CARBON	57.02%	56.86%
HYDROGEN	7.78%	7.64%
NITROGEN	6.05%	5.97%

OPTICAL POLARIMETRY OF 4-METHOXYEPHEDRINE HYDROCHLORIDE

The optical rotation of this compound was measured as described in section 3.10. The compound was only slightly soluble in water and the measurement was recorded using a 1.0 ml cell

$[\alpha]_D$ -29.3° H_2O C = 4.75

EXAMINATION OF " ψ-4-METHOXYEPHEDRINE " HYDROCHLORIDE

The hydrochloride fraction recovered from the acetone washings above, was recrystallised from ethanol to give clear cubic crystals with a melting point of 167°-168°C. This compound had no optical rotation, and the ^1H nmr data was not consistent with that expected from ψ-4-methoxyephedrine.

^{1}H NMR (ppm, D_2O)

2.4 ppm (3H,s, N-CH_3) 3.51 ppm (3H,s, OCH_3)

3.85 ppm (2H,s, Ar-CH_2-N) 6.6 - 7.3 ppm(4H,m, ArH)

The characteristic doublet between 1-2 ppm resulting from the methyl group is not present, and the methine multiplet at 4.8 - 5.5 ppm resulting from the proton α to the aromatic ring is also missing. These have been replaced by a new peak at 3.85 ppm which integrates for two protons. This suggests that the compound is the product of the reductive amination of 4-methoxy PAC . This was confirmed by synthesis.

REDUCTIVE AMINATION OF 4-METHOXYBENZALDEHYDE

A reductive amination was preformed on 4-methoxybenzaldehyde as described in section 3.7. The hydrochloride derivative of the resulting yellow oil was prepared as according to Section 3.8, and the white solid produced was washed with acetone prior to recrystallisation from ethanol. The product was recovered as white cubic crystals similar to those described above. The melting point was recorded as 167°-168°C and the ^{1}H nmr data for this compound was idenyical to the product described from the attempt to synthesise ψ-4-methoxyephedrine.

APPRAISAL OF RESULTS

No literature data was found on any of the possible isomers of 4-methoxyephedrine. The microanalysis data and the ^1H nmr results suggest that the synthesised compound has the correct structure, and the sharp melting point suggests that it is quite pure. The optical rotation is of the correct direction but this cannot confirm the optical purity of the compound. The ψ-4-methoxyephedrine analog was not found during the synthesis, but the reductive amination product of 4-methoxybenzaldehyde was detected.

2.2.5 SYNTHESIS OF 4-METHYLEPHEDRINE

The 4-methyl PAC produced by fermentation was reduced in the presence of methylamine hydrochloride to give the methyl substituted ephedrine analogue (section 3.7). This was recovered as a thick yellow oil, and the hydrochloride derivative was prepared. It was recovered as a white solid in a yellow oily matrix.

SEPARATION OF ISOMER MIXTURE

The hydrochloride mixture was washed with acetone to give a white solid and yellow acetone washing. These were concentrated by evaporation of the solvent and a further yield of the white solid was recovered by the addition of diethyl ether. The two fractions were combined, but the isomers could not be separated using hot chloroform as described in previous sections, as the compound was very soluble in this solvent. Hot acetone was used and the insoluble fraction recovered as a white solid with a melting point 212°-225°C. The acetone soluble fraction was recovered by evaporation to give a white solid with a melting point of 131.2°-138.7°C .

RECRYSTALLISATION OF 4-METHYLEPHEDRINE HYDROCHLORIDE

The crude 4-methylephedrine hydrochloride was dissolved in a 50/50 acetone/chloroform mixture by heating and adding methanol dropwise. The compound recrystallised from this solvent mixture to give 134mg of white powder with a melting point of 220°-222°C .

MICROANALYSIS OF 4-METHYLEPHEDRINE HYDROCHLORIDE

	THEORY	MEASURED
CARBON	61.52%	61.56%
HYDROGEN	8.35%	8.57%
NITROGEN	6.50%	6.38%

OPTICAL POLARIMETRY OF 4-METHYLEPHEDRINE HYDROCHLORIDE

The optical rotation was measured according to section 3.10, and as the compound has a good solubility in water only a small quantity of concentrated solution was prepared which gave an easily detectable rotation in the 0.1mL cell.

$[\alpha]_D$ -30.70° H_2O C = 13.0

2.2.6 SYNTHESIS OF 4-FLUOROEPHEDRINE

The reductive amination of 4-fluoro PAC was carried out as described in section 3.7, to yield a thick yellow oil. This oil was dissolved in diethyl ether and when the volume of solvent was reduced a white solid precipitate was formed leaving a yellow solution. This solid was recrystallised from the same solvent to give large cubic crystals of ψ-4-fluoroephedrine (0.34g), with a melting point of 133.5°-135°C. The yellow solution described above was evaporated to give a thick oil, and the hydrochloride derivative of this compound was prepared as described in section 3.8, producing a white solid.

SEPARATION OF ISOMER MIXTURE

The isomers were readily separated at this stage as the threo isomer was only sparingly soluble in diethyl ether and was precipitated as the free base .

RECRYSTALLISATION OF 4-FLUOROEPHEDRINE HYDROCHLORIDE

The hydrochloride was insoluble in hot chloroform but was recrystallised from a 50/50 acetone chloroform mixture to give a white powder (58mg), with a melting point 195°-197°C

MICROANALYSIS OF 4-FLUOROEPHEDRINE HYDROCHLORIDE

	THEORY	**MEASURED**
CARBON	54.67%	54.84%
HYDROGEN	6.83%	7.06%
NITROGEN	6.38%	6.22%

OPTICAL POLARIMETRY OF 4-FLUOROEPHEDRINE HYDROCHLORIDE

The optical rotation of the erythro isomer was measured as described in section 3.10, using a 0.1mL cell.

0.1 mL cell $[\alpha]_D$ -25.06° H_2O C = 20.0

PREPARATION OF THE HYDROCHLORIDE DERIVATIVE OF ψ-4-FLUOROEPHEDRINE

A solution of the ψ-4-fluoroephedrine free base in diethyl ether / chloroform (60:40) was treated with hydrochloride gas. The resulting hydrochloride was fully soluble in this solvent, and the compound was recovered by rotary evaporation to yield a sticky white solid. This was washed with cold acetone to give the crude hydrochloride derivative.

RECRYSTALLISATION OF ψ-4-FLUOROEPHEDRINE HYDROCHLORIDE

The hydrochloride derivative of ψ-4-fluoroephedrine was recrystallised from chloroform to give a white powder (140 mg) with a melting point of 169.8°-170.4°C .

^{1}H NMR OF ψ-4-FLUOROEPHEDRINE FREE BASE

^{1}H NMR (ppm, $CDCl_3$)

0.95-1.0 ppm (3H,d, CH-$C\underline{H}_3$) 2.45 ppm (3H,s, N-CH_3)

4.1-4.25 (1H,d,not assigned) 6.9-7.5 ppm (4H,m, ArH)

The peak at 2.45 ppm had a small shoulder extending downfield to 2.9 ppm .

^{1}H NMR OF ψ-4-FLUOROEPHEDRINE HYDROCHLORIDE

^{1}H NMR (ppm, D_2O?)

0.95-1.1 ppm (3H,d, CH-$C\underline{H}_3$) 2.7 ppm (3H,s, N-CH_3)

5.05 ppm (1H,m,not assigned) 6.9-7.5 ppm (4H,s, ArH)

OPTICAL POLARIMETRY OF ψ-4-FLUOROEPHEDRINE HYDROCHLORIDE

The optical rotation was measured as described in section 3.10 , using a 0.1mL cell.

$[\alpha]_D$ -47.15° H_2O C = 21.2

APPRAISAL OF RESULTS

The synthesis of para fluoroephedrine differed from the other synthesis in that the (-)-ψ-4-fluoroephedrine free base was easily separated from the erythro isomer after the

reductive amination step. The melting point recorded for this free base is higher than that reported in the literature [18], where the published value was 114°C for a racemic mixture. However the melting point for the hydrochloride derivative of this isomer did agree with the reported value of 170°C by the same author.

The ^{1}H nmr data for the two hydrochloride derivatives, and the ψ-free base, are in agreement with that expected for their structures, and the microanalysis results also agreed well with the theoretical values. The melting point recorded for the (-)-4-fluoroephedrine was lower than that reported by Weichet [18], and also lower than that reported earlier by Kraft and Dengel,(222°C)[15]. This suggests that the erythro compound may still contain a trace of the ψ-isomer even after repeated recrystallisations. The optical rotation value cannot be used to resolve this matter as the are no literature values for comparison.

2.3 HIGH FIELD ^1H NMR STUDIES

The high field nmr data was collected using a Bruker AC 400, 400 MHz instrument as described in section 3.10.4 , and the chiral shift studies were preformed using $Pr(tfc)_3$ in deuterated chloroform.

High field nmr study of PAC

The crude PAC extract was passed through a chromatography column as described in section 3.6 , and a sample (20 mg) was dissolved in deuterated chloroform (0.5 ml) and placed in an nmr tube (5mm). The singlet due to the $C-CH_3$ and $N-CH_3$ was observed at 2.09 ppm and 4.7ppm respectively. $Pr(tfc)_3$ (1mg) was added and these bands were shifted upfield to 1.95ppm and 4.48ppm , no splitting of the bands were observed. Addition of another 1mg of shift reagent caused a complete collapse of the signal due to line broadening. This suggests that the biotransformation is indeed stereoselective at carbon 1 and that the molar ratio of compound to shift reagent for optimum resolution lies between 1 : 0.0042 and 1 : 0.0128

High field nmr of racemic synthetic ephedrine

The nmr spectrum of the mixture produced by the reductive amination of PAC suggested the presence of a pair of diastereoisomers, (-) ephedrine and (-)-ψ-ephedrine. It showed a doublet at 0.75-0.83ppm due to $C-CH_3$ and on close inspection each band was further split into two bands.

The characteristic $N-CH_3$ band at 2.35ppm could also be seen to be split into a doublet, and the relative hight of these bands indicated that the (-) ephedrine/ (-)-ψ-ephedrine ratio was approximately 65/35, (Figure 6 ,appendix II) . As these compounds were collected after the reductive amination step the results seem to suggest only slight degree of stereoselectivity in this reaction.

High field nmr of (-)ephedrine

The (-) ephedrine fraction of the racemate described above was separated by fractional crystallisation of the hydrochloride derivative as described in section 3.9 . This hydrochloride was converted back to the free base prior to the nmr studies. The spectrum observed was identical to that observed with the commercial (1R,2S)-ephedrine purchased from Aldrich chemicals,(Figure 7 and 8 Appendix II). Chiral $Pr(tfc)_3$ (1mg) was added to a 2M solution of synthetic (-) ephedrine in $CDCl_3$ the $N-CH_3$ band at 2.37ppm collapsed completely into a broad hump. The $C-CH_3$ doublet at 0.76ppm was shifted upfield to 0.74ppm but retained the same JJ coupling (6Hz). Two further additions of 1mg of shift reagent moved the band upfield to 0.7ppm, but they remained clearly defined as a doublet. The presence of anything but minute quantities of the (+) ephedrine isomer can thus be ruled out, and suggests that the configuration about carbon 1 does not alter during the synthesis.

High field nmr of enantiomeric mixture of ephedrine

During the studies described above the use of chiral $Pr(tfc)_3$ under the conditions stated did not indicate the presence of any enantiomeric mixtures. For this to be acceptable as evidence of their absence racemic mixtures of commercial (-) and (+) ephedrine were prepared. This was dissolved in deuterated chloroform to give a 2M solution. The nmr tube (5mm) was loaded with this solution (0.5ml) and the spectrum recorded as described in section 3.10.4 . The chiral shift reagent (1mg) was added and the data collected again. The $N-CH_3$ peak collapsed and the $C-CH_3$ doublet at 0.76ppm was shifted upfield to 0.7ppm . Two distinct doublets were apparent with the two enantiomers separating by about 6.2Hz . Two more additions of the chiral shift reagent (0.5mg) caused the peaks to shift further upfield and increased the resolution of the enantiomers, before the bands became excessively broad, (Figure 9. Appendix II). This indicates the effectiveness of this reagent and conditions for resolving ephedrine enantiomers and the absence of band splitting indicates enantiomeric purity in the compounds described.

2.4 PHARMACOLOGICAL STUDY OF THE EPHEDRINE ANALOGUES

The measurement of antispasmodic activity in the synthesised ephedrine analogues was attempted on tissue section which were stored overnight in thyroid solution at 5°C . The spasms observed from this tissue were very variable , and a constant base line could not be obtained for comparison. The recovery of the tissue after addition of ephedrine was prolonged and no reasonable measurements were obtained.

The experiments were repeated using fresh tissue . The observed spasms were stronger and faster, with a constant base line, and recovered faster after washing. The experimental conditions and procedures are described in Section 3.11 .

The observed reduction in the length of the spasm trace was expressed as a percentage of the original trace length and the results are given in Table 5.

COMPOUND NAME	R S CONFIG.	SOLUTION MOLARITY	% SUPRESSION	% SUPRESSION	% SUPRESSION
Commercial (-)ephedrine HCl	1R2S	0.0003M	77%	79%	87%
Synthetic (-)ephedrine HCl	1R2S	0.0003M	80%	80%	-
Commercial (-)-ψ-ephedrine HCl	1R2R	0.0003M	0%	0%	0%
Synthetic (-)-ψ-ephedrine HCl	1R2S	0.0003M	14%	-	-
Commercial (+)-ψ-ephedrine HCl	1S2S	0.0003M	35%	43%	28%
Synthetic (-)-4-fluoroephedrine HCl	1R2S	0.0003M	92%	94%	96%
Synthetis (-)-ψ-4-fluoroephedrine HCl	1R2R	0.0003M	0%	0%	-
Synthetic(-)-4-methylephedrine HCL	1R2S	0.0003M	83%	81%	83%
Synthetic(-)-4-methoxyephedrine HCl	1R2S	0.0003M	17%	18%	-
Synthetic(-)-4-chloroephedrine HCl	1R2S	0.0003M	>100%	>100%	>100%
Commercial (-)ephedrine HCl	1R2S	0.0001M	40%	-	-
Synthetic (-)-4-chloroephedrine HCl	1R2S	0.0001M	80%	-	-
Synthetic (-)-4-fluoroephedrine HCL	1R2S	0.0001M	29%	-	-

Table 5. Observed antispasmodic activity of the synthesised compounds and commercial references. See appendix III

In most cases the compounds were tried on three pieces of tissue. Commercial ephedrine hydrochloride isomers were used as control compounds , and as expected only the (-) ephedrine and the (+)-ψ-ephedrine hydrochloride had any activity. The synthesised (-) ephedrine hydrochloride showed very similar activity to the corresponding commercial control, but the synthesised (-)-ψ-ephedrine hydrochloride showed a small level

of activity which was not expected. This suggests that the compound may still contain a small trace of the erythro isomer.

The (-)-4-fluoro and the (-)-4-methylephedrine hydrochloride analogues also showed similar activity to the control (-)-ephedrine compound, but the synthesised (-)-4-chloroephedrine hydrochloride showed a greater suppression of the tissue spasms. The (-)-4-methoxy analogue showed very little activity and the (-)-ψ-4-fluoroephedrine isomer showed no activity.

A comparison of the commercial (-)-ephedrine and the corresponding 4-chloro and 4-fluoro analogues at lower dosage also showed the (-)-4-chloroephedrine hydrochloride to have a higher activity than the control compound.

2.5 DISCUSSION

The production of phenyl acetyl carbinol by fermentative techniques has been extensively applied to the commercial synthesis of ephedrine. The complete stereospecificity of the enzyme catalysed reaction being the major attribute. Much work has already been undertaken to maximise the yield of phenyl acetyl carbinol produced, and during this research small changes were made to the methods already published which resulted in sufficiently high yields for the second step of the synthesis pathway to be possible. The fermentation method used here is not commercially viable due to the cost of the medium.

While some publications mentioned the possibility of novel carbinol compounds being produced by employing substituted benzaldehyde as a substrate for the fermentation, very little work seems to have been published on this matter. Long[32,33] demonstrated the usefulness of this technique on a range of substituted aldehydes and indicated the potential yields based on quantitative analytical methods. In this report some of the aldehydes previously used were fermented and again the extracted carbinols were produced in sufficiently high yields to allow subsequent reductive amination. No attempt was made to maximise the yields in these cases, but adjustment of the medium aldehyde concentrations during the fermentation and comparing this

to the subsequent carbinol yield, would give information of the correct dosage for each of the substituted aldehydes. 4-chlorobenzaldehyde was the only solid aldehyde used, and it was found to be only sparingly soluble in the aqueous medium. Dissolving the compound in acetone prior to addition to the fermentation medium was found to be far more productive, giving extracted yield at the upper range of that measured by Long[32].

3-Pyridinecarboxaldehyde had not previously been fermented by other research workers, and yet it seemed an ideal substrate due to its high solubility in water. When this biotransformation was attempted no parent aldehyde or pyridal carbinol were recovered in the medium extract. One possible explanation is that the carbinol would also be highly soluble in water and would not be removed during the ether extraction.

The fermentation of 2-chlorobenzaldehyde gave a crude extract which contained a high quantity of parent aldehyde mixed with the substituted PAC. the reductive amination of this compound was a not attempted for this reason. Long also observed a low biotransformation for this aldehyde. The fermentation of 4-methoxybenzaldehyde however gave a higher crude yield than that reported by Long.

The purification of the carbinol by extracting with sodium bisulphate was used in the initial stages of the research. The yields of 'purified' compound were only in the order of 10% to 20% of the original crude extract, and were found to contain a higher proportion of parent aldehyde than the extract. For this reason the purification step was omitted in further work.

Column chromatography was carried out on the crude PAC extract as a means of purification but the yields were so small that its use became questionable. This was further amplified when the reductive amination step of the purified compound yielded a product which still required a number of recrystallisation steps before a pure compound could be reached. It was then decided to use the crude extract as a starting point for the reductive amination and to purify the compounds at a later stage.

The reductive amination of the substituted carbinols was based on the method of Borch[41], using sodium cyanoborohydride as a selective reducing agent by controlling the solution pH. Methylamine was replaced with the hydrochloride derivative and acetophenone was used as a trial compound. The results compared well with the data published by Borch indicating a successful synthesis.

When this method was applied to PAC, ephedrine and ψ-ephedrine were produced. An accurate yield can not be calculated because the starting compound may contain some benzyl alcohol, and the number of recrystallisation steps resulted in considerable loss of compound. Consequently any method of estimating the stereoselectivity of the reaction based on the weight of the diastereoisomers produced cannot be applied. This may however be possible using high field nmr or chiral high performance liquid chromatography.

In the case of the substituted PAC the reductive amination produced the corresponding ephedrine analogue as a mixture of isomers. It was very difficult to obtain the ψ-4-methylephedrine hydrochloride in a pure form, due to the similar solubility of the erythro isomer. Consequently very little data was collected on this compound. The reductive amination of 4-methoxy PAC did not seem to yield any threo isomer. Its presence may have been missed due to the production of side products, or if it was highly soluble in acetone it may have been removed completely by the initial washing.

The hydrochloride derivative was prepared for each compound synthesised. In all cases the compounds were recovered as a sticky solid in a oily matrix. This even occurred when pure commercial ψ-ehhedrine free base was used and may

suggest that the ephedrine decomposes back to PAC on contact with the hydrochloride gas. A more sophisticated system which has the capacity to measure the quantity of gas added may overcome this problem, and increase the purity and yield of hydrochloride derivative produced.

Many authors have published work relating to the synthesis of ephedrine analogues, but in all these cases the product was formed as a racemic mixture. As a result of this no optical rotation measurements are available. While all the erythro and threo compounds synthesised in this research were observed to be laevorotary, no figures for the compounds purity can be drawn from this data.

The melting point data was used as an indication of the compound purity and in the case of ephedrine and the 4-chloroephedrine isomers it agreed completely with the published figures. The other analogues agreed with the published melting point data to a varying degree, yet they all gave very acceptable microanalysis results. This is consistent with compounds which may be chemically pure but not fully optically pure.

High field ^{1}H nmr was used to give more detailed information on the purity of the compounds. The (-) ephedrine produced by the method of synthesis described in this research was indistinguishable from the commercial ephedrine using this

nmr technique. The application of chiral shift reagents caused the $C-CH_3$ band to shift upfield but the band multiplicity remained unchanged. Inspection of this data suggests that the fractional crystallisation step successfully separated the diastereoisomer pairs and the resulting fractions were present as single enantiomers. The orientation of the hydroxyl group on carbon 1 must therefore remain stable through out the reductive amination reaction. The high field nmr data for the unresolved synthetic ephedrine mixture indicated only slight stereoselectivity during the reduction at carbon 2.

The pharmacological testing of the ephedrine analogues gave some information on the potential antispasmodic activity of these compounds. The initial attempt did not yield any results and this was explained by the age of the rabbit tissue used. The subsequent trials were more successful and gave results which allow a tentative comparison of activity of the analogues with that of commercial (1R,2S)-(-)-ephedrine hydrochloride.

These experiments suggest that (-)-4-chloroephedrine hydrochloride may be a stronger antispasmodic than (-)-ephedrine hydrochloride, and that (-)-4-fluoro and (-)-4-methylephedrine hydrochloride have a similar activity to the commercially prepared control. Of these two synthesised compounds the former has a stronger activity.

While synthesis of these substituted ephedrine compounds may find useful application as antispasmodics, it is more likely that they may be used as chiral precursors for the synthesis of other molecules. One such application would be in the preparation of the antidepressant compounds (tri-substituted oxazolidin-2-one oximes) described by Ziman[20]. The activity of these compounds could possibly be enhanced if they were prepared stereoselectively.By using the method of synthesising (1R,2S) ephedrines analogues described in this research, this type of preparation is possible.

EXPERIMENTAL

3.1 MATERIALS

The aromatic aldehydes used in the fermentations were obtained from Aldrich chemicals, Dorset, with the exception of benzaldehyde and 4-methylbenzaldehyde which were supplied by Riedel de Haen and Merck respectively. The materials used to prepare the fermentation medium were sodium pyruvate and Peptone from Sigma Chemicals, St. Louis,USA., citric acid from Riedel de Haen, and glucose monohydrate (Food Grade) from Roquette Chemicals. The yeast used was "Proofex" compressed bakers yeast supplied by Irish Yeast Products, Glasnevin.

The sodium cyanoborohydride and methylamine hydrochloride were both supplied by Aldrich Chemicals, Dorset, and the four commercial ephedrine standards were also supplied by Aldrich Chemicals ie (1R,2S)-(-)-ephedrine, (1S,2R)-(+)-ephedrine hydrochloride, (1R,2R)-(-)-ψ-ephedrine, and (1S,2R)-(+)-ψ-ephedrine hydrochloride.

The Kieselgel S (.032-.063 mm) Silica gel, and the pre-coated TLC plated were obtained from Riedel de Haen, while the deuterated solvents used in the nmr study were supplied by Aldrich Chemicals.

The chiral lanthanide shift reagents used in the high field nmr study were supplied by Aldrich Chemicals,and all other reagents and solvents used were general purpose reagents.

3.2 FERMENTATION OF AROMATIC ALDEHYDES

The selected medium was prepared as described in appendix I . The ingredients were dissolved in tap water and the pH adjusted to within the range 4.4 to 4.6 by the dropwise addition of sodium hydroxide soln. (40% w/v). The temperature of the medium was adjusted to 29°C and placed in the fermentation vessel figure in the water bath (previously heated to 29°C). Fresh bakers yeast (Saccharomyces Cerevisiae) (30g) was added and the aeration and stirring mechanism were started. After an initial period of one hour fermentation the aromatic aldehyde substrate was added to give a concentration of 16mM. In the case of some of the less soluble substituted benzylaldehydes eg. 4-Chlorobenzylaldehyde it was necessary to dissolve them initially in acetone and add a fixed aliquot to give the desired concentration. Hourly additions of the substrate were continued until a total fermentation time of 4 hours was reached.The medium was then inoculated with extra yeast (20g) and the process of substrate additions continued until a total of 7 additions were made. Fermentation was continued for an extra hour before extraction of the medium.

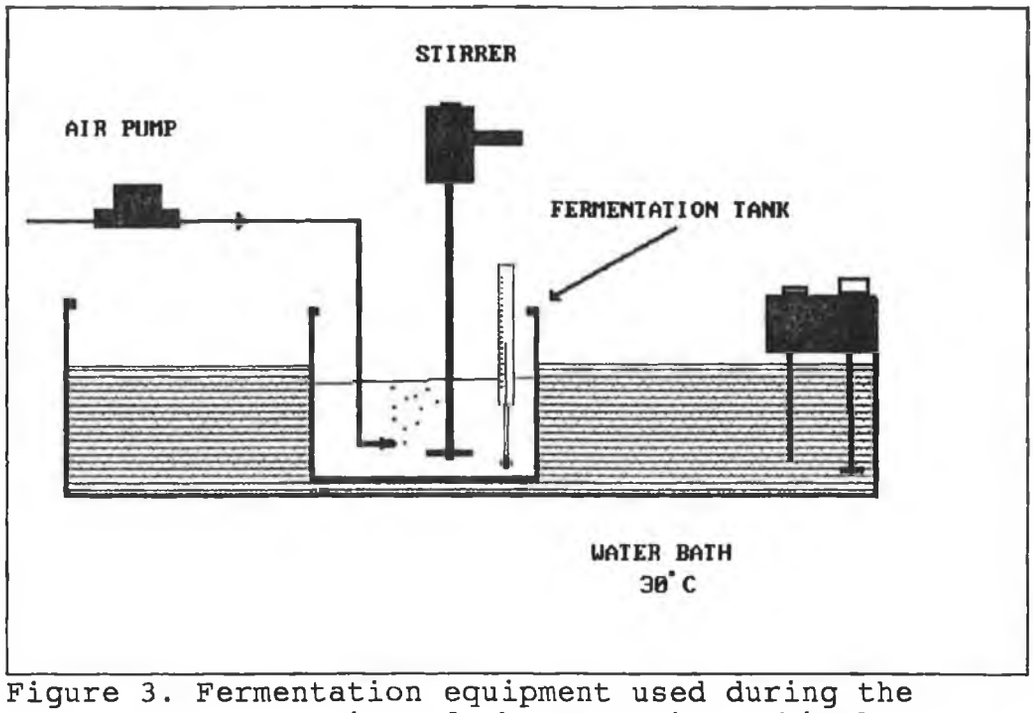


Figure 3. Fermentation equipment used during the preparation of the aromatic carbinols

3.3 EXTRACTION OF CABINOL FROM MEDIUM

The medium was centrifuged at 6000g for five minutes to remove the yeast cells from suspension. The supernatant liquid was removed by suction taking care not to disturb the semi solid layer of cells at the bottom, which were then extracted twice with ethanol (40 cm^3) and filtered. The ethanol extract was added to the aqueous layer and transferred to a large separation funnel where it was extracted twice with equal volumes of diethyl ether. This was reduced to 50 cm^3 by rotary evaporation to give a yellow liquid, which was washed twice with equal volumes of 10% Na_2CO_3. The ether layer was dried over magnesium sulphate and evaporated under reduced pressure to give a thick opaque liquid. This was maintained at reduced pressure for one hour to remove all traces of solvent prior to ^1H nmr spectroscopy.

3.4 PURIFICATION OF CRUDE PAC

The crude material was dissolved in ether to give complete solution. This was extracted twice with equal volumes of a 10% solution of sodium metabisulphate. The aqueous layer was removed and washed with ether to extract any remaining alcohol. Solid sodium hydrogen carbonate was added to decompose the organic-bisulphate complex resulting in the release of carbon dioxide. When the effervescence had ceased the carbinol was re-extracted into ether. The phenyl acetylcarbinol was recovered by rotary evaporation of the solvent.

3.5 THIN LAYER CHROMATOGRAPHY

The pre-coated chromatography plates were cut to size and marked to show a thin pencil line at a hight just above the solvent height. Solid samples were dissolved in a suitable solvent prior to loading on the plates, otherwise neat liquid samples were used. The plate was dried in air and the location and identification of each spot was marked. The plate was then placed vertically in the chromatography chamber. When the solvent front had travelled about 7cm the plate was removed and dried in air. The dried plates were developed with iodine and the Rf values calculated.

3.6 COLUMN CHROMATOGRAPHY

A clean dry glass separating funnel was packed with cotton wool at the neck above the tap. Silica gel (Kieselgel S 0.032-0.063 mm) approximately 50g was weighed and mixed with the elution solvent to give a thick paste. This was slowly poured down the side of the funnel and the side tapped gently to remove any air bubbles in the gel. The solvent level was increased and the tap opened to allow a small trickle to elute. When necessary the solvent level was increased to insure that the column did not dry out. This was achieved using a Pasteur pipette and letting the solvent flow down the side without disturbing to top layer of the gel. The column had compacted to about half the original volume on standing for three hours, and a layer of sand was placed on the top of the gel.

The level of elutant in the column was dropped to just above the top of the sand layer, and the sample (dissolved in a suitable solvent) was loaded onto the top of the column using a pipette. Extra elutant was immediately added to prevent the column from drying, and when the sample had entered the silica gel the eluent reservoir was filled . This reservoir level was maintained

while the eluting solvent was collected in marked test tubes. The solvent in these was removed by rotary evaporation and thin layer chromatography was applied to each fraction to find the sections which contained the desired compound. These sections were combined and the compound recovered.

3.7 REDUCTIVE AMINATION

Methylamine hydrochloride (60mM) was dissolved in dry methanol (60cm^3) and added to a solution of the selected carbinol (10mM) in the same solvent. Sodium cyanoborohydride (7mM) was added to the above mixture and extra solvent introduced to insure complete solution of the compound. The flask was capped and stirred for 72 hours at room temperature, and then acidified with conc. HCl (3 cm^3) in a fume cupboard. When the effervescence had ceased the solvent was removed under reduced pressure at 40°C to give a sticky white solid. This was dissolved in water (20 cm^3) and washed twice with equal quantities of ether. It was basified with KOH pellets and saturated by the addition of sodium chloride, the liberated free base removed by extraction into ether (100cm^3) , and the volume reduced by rotary evaporation. The free base was recovered as a yellow oil in all cases.

3.8 PREPARATION OF THE HYDROCHLORIDE DERIVATIVE

The carbinol compound was dissolved in methanol ($20cm^3$) and hydrochloric acid was added ($3\ cm^3$). The mixture was refluxed for 30 minutes and the solvent and excess acid removed by rotary evaporation, leaving the solid hydrochloride derivative behind.

MODIFIED METHOD

Due to decomposition the yields experienced with the method described above were very low. It was modified by dissolving the ephedrine compound in diethyl ether and bubbling hydrogen chloride gas through the solution for 10 minutes. The hydrochloride derivative was insoluble in this solvent in all but one case, and fell out of solution as a white solid. The solvent was removed by rotary evaporation and the product was recovered and purified.

3.9 SEPARATION OF ISOMERS BY FRACTIONAL CRYSTALLISATION

The selected solvent, or solvent mixture, was heated to boiling point and the compound was placed in a pre-heated beaker (10mL or 20mL depending on size of sample). The solvent was added dropwise to the compound and any dissolution was observed. More solvent was added as necessary until the compound had just dissolved. The solution was quickly filtered through a warm funnel to remove the insoluble compound, and the filtrate was collected. When the filtrate had cooled the resulting crystals were removed by filtration and washed. The original filtrate was let stand to produce a second yield of crystals which were treated in the same manner as the first yield. Each of the separated compounds were recrystallised from suitable solvents and their purity assessed by determining their melting points (section 3.10.1). When necessary the compounds were recrystallised a number of times untill no further change was observed.

3.10 INSTRUMENTATION METHODS

3.10.1 Determination of melting point

The melting point of all the solid compounds were determined using a Griffet Melting Point Apparatus. The temperature was recorded from a mercury thermometer, which had previously been calibrated.

3.10.2 Determination of optical rotation

The optical rotation of the samples were measured using an Optical Activity, digital, electrically balancing instrument. A sample of the compound was weighed and dissolved in a known quantity of a suitable solvent. The machine as set to read zero using the solvent, and the cell was loaded with the sample solution. The observed rotation was noted and if this was less than the measurement accuracy (.05) a different cell or a more concentrated solution was tried. The machine was calibrated using L-cysteine.

3.10.3 Determination of ^1H nmr data

The low field ^1H nmr spectrum was recorded on a Perkin Elmer R-12B 60 Hz instrument.The samples were prepared by dissolving in an appropriate solvent and loading the solution in a nmr tube (5 mm). This was warmed to operating temperature and set spinning in the magnetic field. Collecting and intergrating the nmr data was performed according to the manufacturers instructions for the instrument.

3.10.4 **Determination of high field ^1H nmr data**

The high field ^1H nmr was run on a Buker Ac-400 at 400MHz. The solutions were made up in deuterated chloroform and the residual hydrogen peak was used as a reference and assigned 7.24ppm. The chiral shift reagent was tris(3-(trifluoromethylhydroxymethylene)-d-camphorato) Paresodymium (III) which was purchased from Aldrich chemicals. This was added to the deuterated chloroform solution in known quantities (0.5-1mg) and the chemical shift observed. All the operating ,shimming, and plotting operations were under software control.

The hydrochloride derivatives of ephedrine do not dissolve to any appreciable extent in this deuterated solvent and must be converted back to the free base before the nmr data can be collected. This is achieved by basifying an aqueous solution of the compound with sodium hydroxide and extracting with diethyl ether. The ether is dried over magnesium sulphate before evaporating the solvent.

3.11 PHARMACOLOGICAL ACTIVITY

Fresh Thyroid solution was prepared and the bath reservoirs filled. A large rabbit was killed by cervical dislocation extrangulation and dissected. The jejunum was removed and placed in the thyroid solution while the three tissue baths ($10cm^3$) reached the operation temperature (36°C). The Poligraph was switched on and the electrodes calibrated using a 2g weight, setting the full scale deflection to 8g. Lengths of tissue (1 cm) were removed from the stomach end of the jejunum and were suspended from the electrode by threads at either ends.

The bath was filled with pre-heated thyroid solution from the reservoir and the tissue adjusted to 2g tension by observing the position of the pen on the graph and adjusting the vertical height of the electrode. The solution in the bath was aerated with a mixture of oxygen /carbon dioxide, and the tissue spasms recorded on the graph. The gain control was used to maintain the contractions within the calibrated range, and when these spasms had stabilised the test sample was added ($0.3\ cm_3$ of 10^{-3}M aqueous soln.) . The effect that this had

on the contractions was observed by the change in the graph. After 10 minutes the bath was washed out with thyroid solution, and when the tissue stabilised again a different quantity of the test compound was added to ascertain the dose related response. This procedure was repeated with fresh tissue sections for each of the test compounds. Each of the compounds were tested on the tissue in the three baths.

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APPENDIX I

APPENDIX I

FERMENTATION MEDIUM

MEDIUM A

For 800 cm^3 of medium the following ingredients were weighed and diluted with tap water.

* Peptone 4.8g
* Sodium pyruvate 49.3g
* Citric acid 8.4 g

The pH was adjusted to 4.5 by the addition of 40% sodium hydroxide solution.

MEDIUM B

For 800 cm^3 of medium the following ingredients were weighed and diluted with tap water.

* Peptone 4.8g
* Sucrose 80g
* Citric acid 8.4 g

The pH was adjusted to 4.5 by the addition of 40% sodium hydroxide solution.

MEDIUM C

For 800 cm^3 of medium the following ingredients were weighed and diluted with tap water.

*	Yeast extract	4.8g
*	Sucrose	80g
*	Ammonium sulphate	7.32g
*	Magnesium Sulphate	0.4g
*	Potassium dihydrogen phosphate	0.8g

The pH was adjusted to 5.5 by the addition of 40% sodium hydroxide solution.

MEDIUM D

For 800 cm^3 of medium the following ingredients were weighed and diluted with tap water.

*	Peptone	4.8g
*	Sucrose	80g
*	Sodium pyruvate	20g
*	Citric acid	8.4g
*	Ammonium sulphate	7.3g
*	Magnesium Sulphate	0.4g
*	Potassium dihydrogen phosphate	0.8g

The pH was adjusted to 4.5 by the addition of 40% sodium hydroxide solution.

APPENDIX II

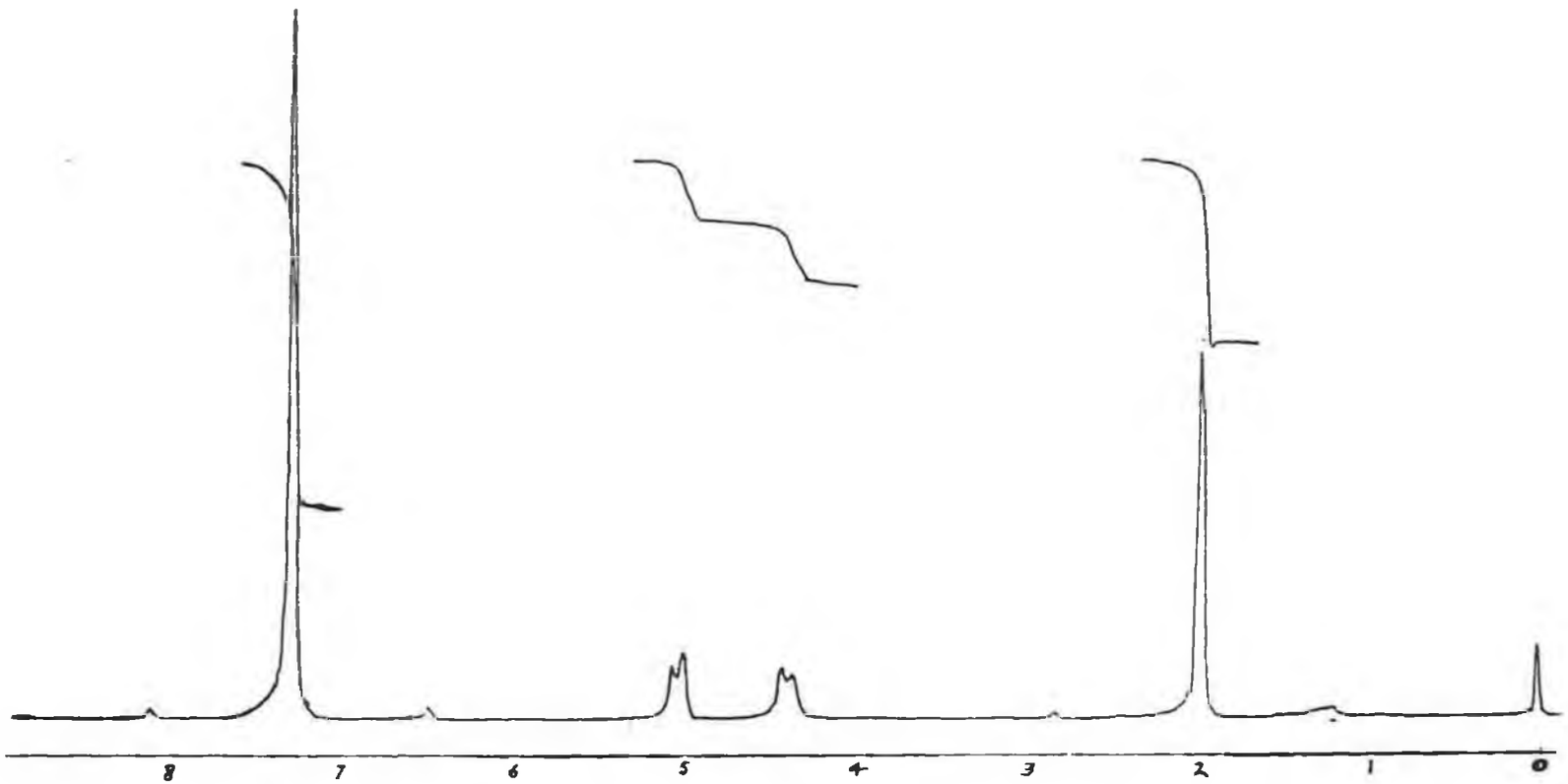

Figure 4

60 MHZ nmr of phenyl acetyl carbinol in CDCl3, compound purified by column chromatography. Instrument : Perkin Elmer R 12B

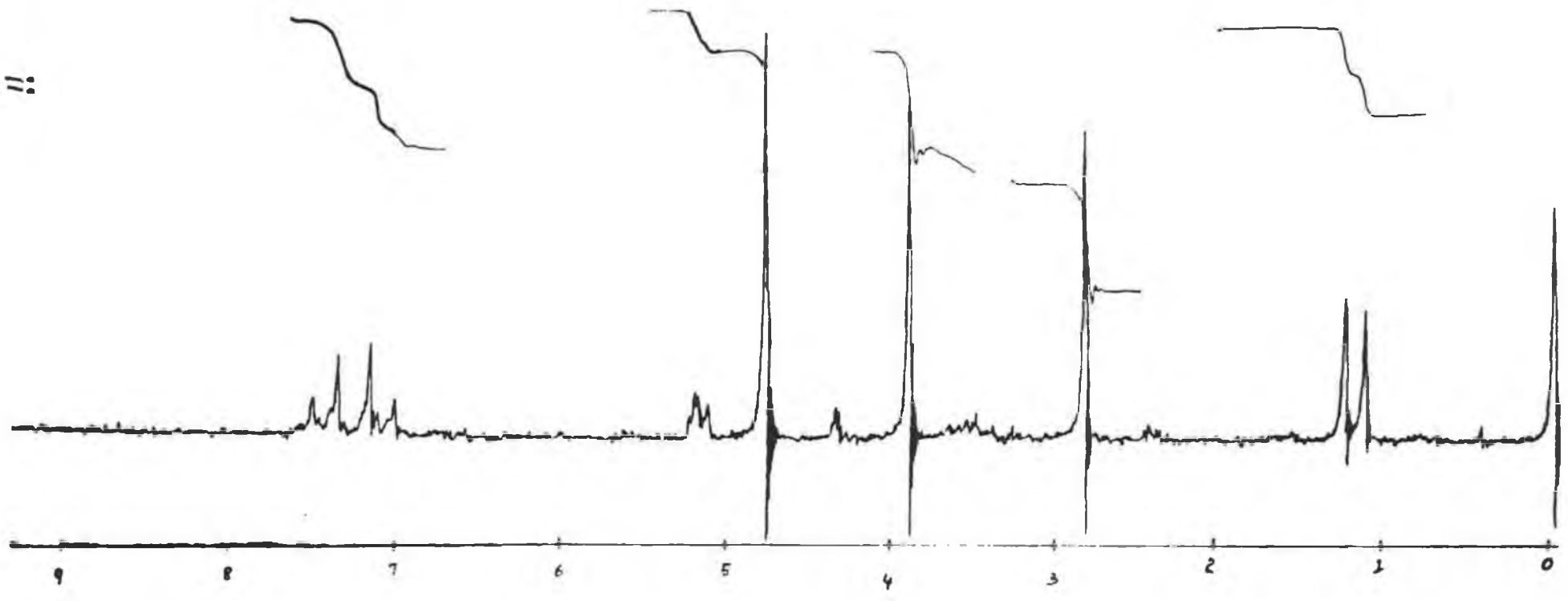

Figure 5

60 MHz nmr of 4 methoxy ephedrine hydrochloride in deuterated water.

Instrument : Perkin Elmer R 12B

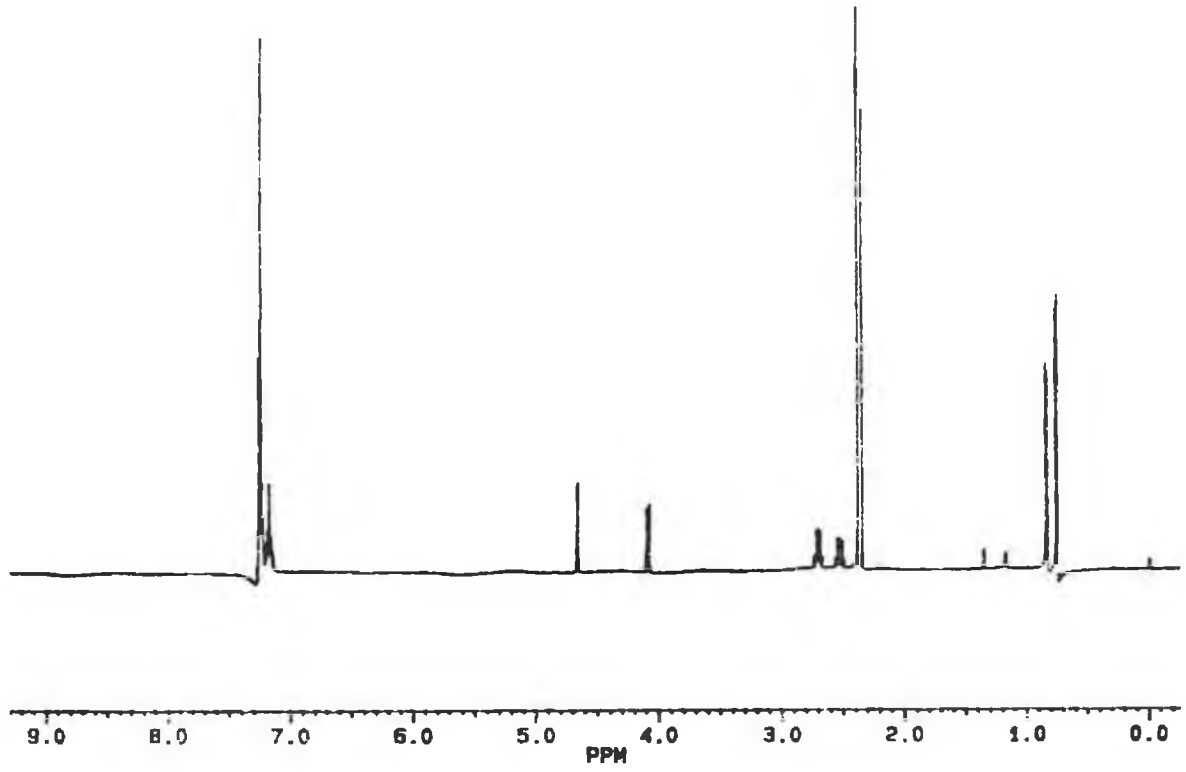

Figure 6.
400 MHz nmr of the product from the reductive amination of PAC.

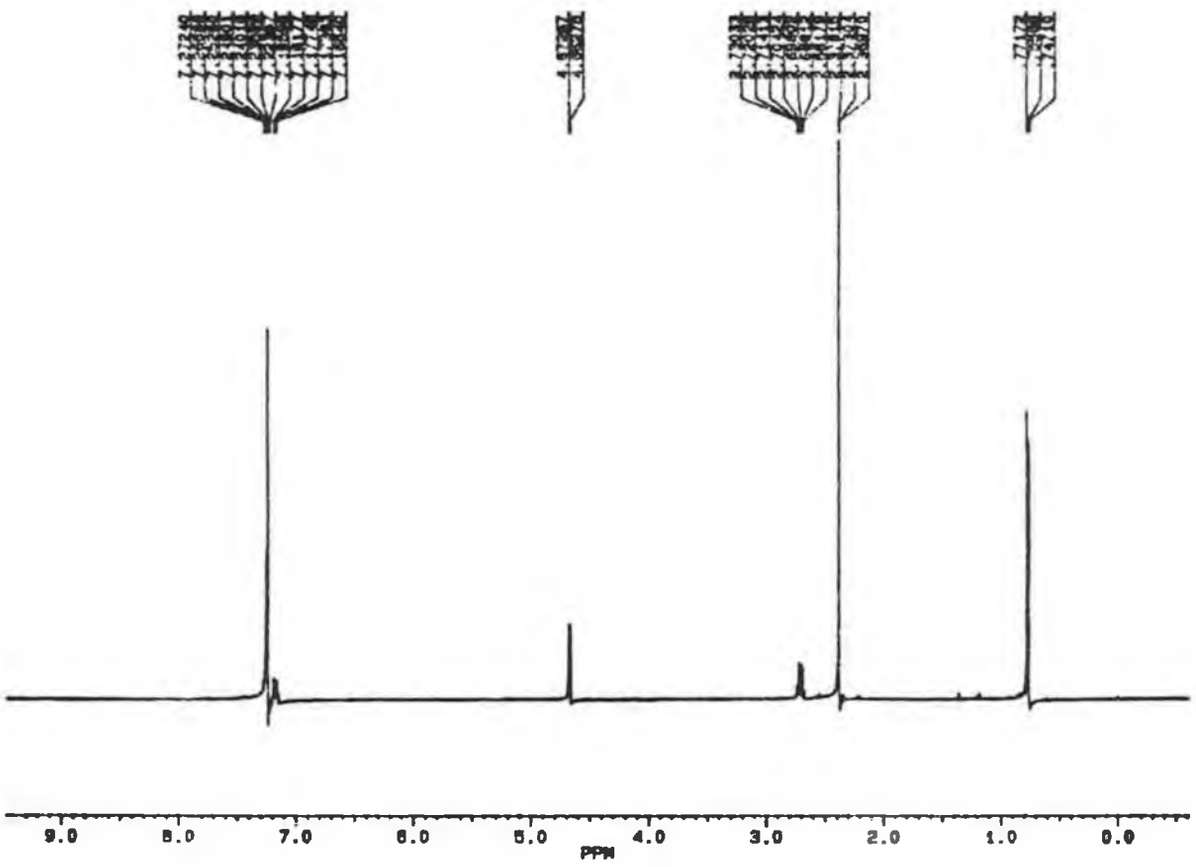

Figure 7

400MHZ nmr of (−) ephedrine synthesised by the reductive amination of PAC, in CDC13,

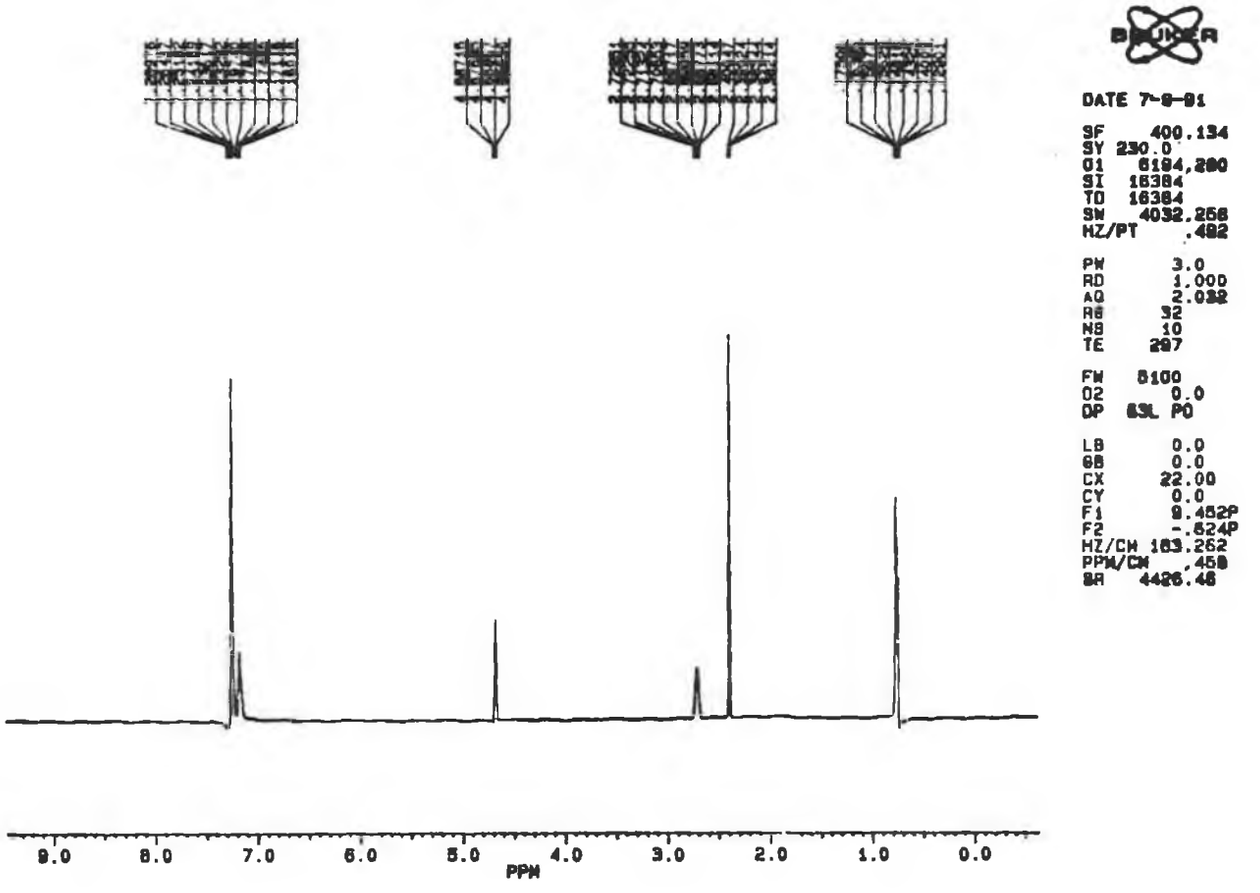

Figure 8

400 MHz nmr of commercial ephedrine (Aldrich Chemicals), in CDCl3 ,

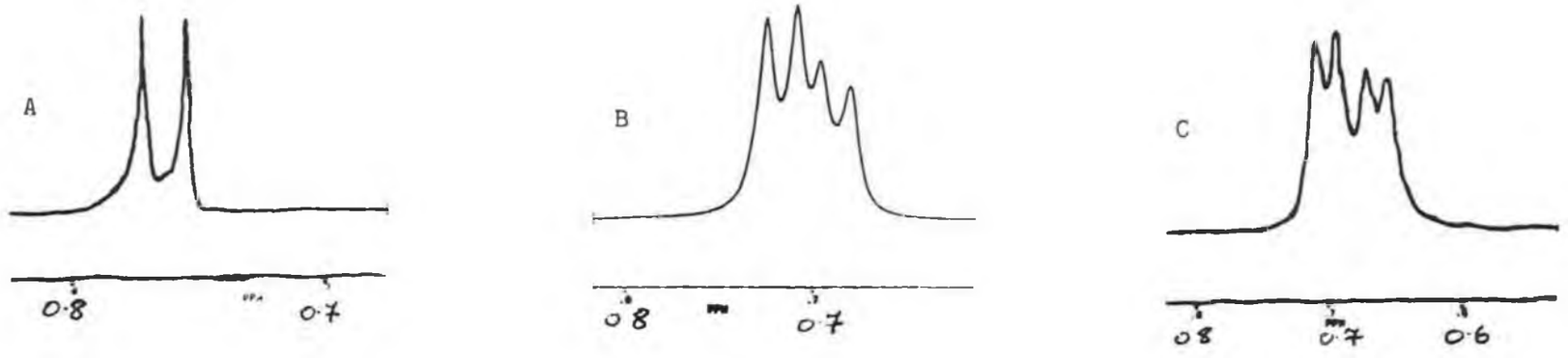

Figure 9

400MHz nmr of racemic ephedrine in CDCl3, residual hydrogen as reference

B) sample + 1mg Pr(tfc)3

C) sample + 2mg Pr(tfc)3

APPENDIX III

APPENDIX III

FIGURE 10 Trace of tissue spasm for (1R 2S)-ephedrine hydrochloride at a concentration of $3x10^{-3}$ M

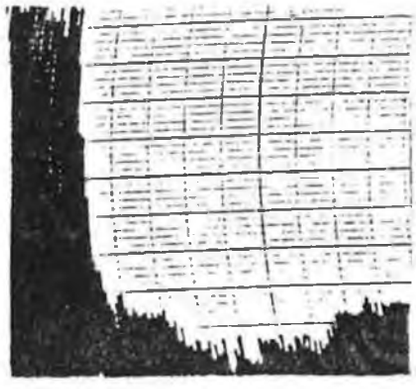

FIGURE 11 Trace of tissue spasm for commercial (1R 2S)-ephedrine hydrochloride at a concentration of $3x10^{-3}$ M

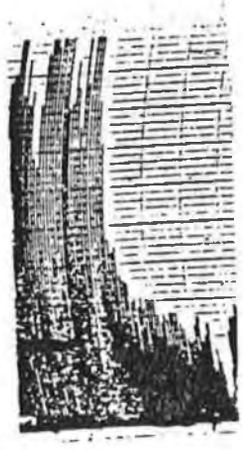

FIGURE 12 Trace of tissue spasm for (1R 2S)-4-fluoroephedrine hydrochloride at a concentration of $3x10^{-3}$ M

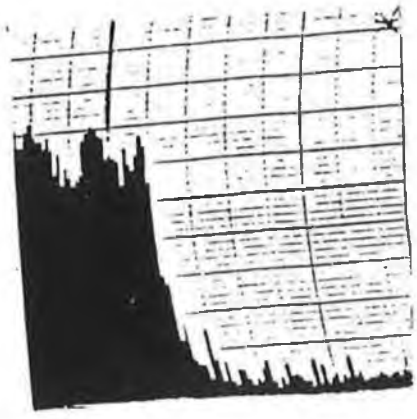

FIGURE 13 Trace of tissue spasm for (1R 2S)-4-chloroephedrine hydrochloride at a concentration of $3x10^{-3}$ M

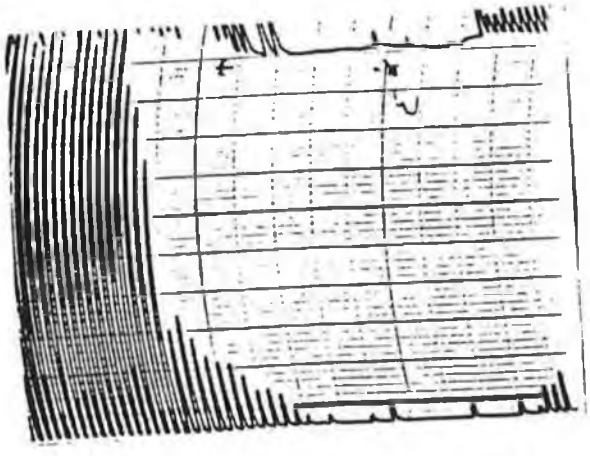

FIGURE 14 Trace of tissue spasm for (1R 2S)-4-methylephedrine hydrochloride at a concentration of $3x10^{-3}$ M