A THESIS

entitled

STEREOCHEMISTRY OF SPERMIDINE SYNTHASE

by

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ABBREVIATIONS

A	ATP	adenosyl triphosphate
	aq.	aqueous
B	b.p.	boiling point
C	cm	centimeter
	^{o}C	degree
	C	charcoal
	c.i.	chemical ionisation
	conc.	concentration
D	D	deuterium
	dm	decameter
	DMSO	dimethylsulphoxide
	DSAM	decarboxylated S-adenosylmethionine
	DNA	deoxy ribonucleic acid
	DCCD	N,N'-dicyclohexylcarbodiimide
E	EDTA	ethylenediaminetetraacetic acid
	Eqn.	equation
	e.i.	electron impact
	e.g.	for example
F	Fig.	figure
G	g.l.c.	gas-liquid chromatography
	g	grams

H	h	hours
	h.p.l.c.	high pressure liquid chromatography
	H-ax	axial proton
	H-eq	equatorial proton
	HMPA	hexamethylphosphoramide
I	i.r.	infra-red
	i.d.	internal diameter
M	mol	mole(s)
	min.	minutes
	m.p.	melting point
	mmol	millimole(s)
	MHz	Megahertz
	m/z	mass/charge
	mm	millimeter
N	n.m.r.	nuclear magnetic resonance
	nm	nanometer
O	ODC	ornithine decarboxylase
P	Ph	phenyl
	p.p.m.	parts per million
	PhNCS	isothiocyanatobenzene
	pmol	picamole(s)
	p.l.c.	preparative layer chromatography
	PATC	phenylaminothiocarbonyl

R	r.t.	room temperature
	R_t	retention time
	rac.	racemic
	RNA	ribonucleic acid
	R_f	relative flow
S	SAM	S-adenosylmethionine
	SAMD	S-adenosylmethionine decarboxylase
T	TSS	3-(trimethylsilyl)-tetradeutero-propionic acid sodium salt
	Ts	tosyl
	TMS	tetramethyl silane
	THF	tetrahydrofuran
	TCA	trichloroacetic acid
	t.l.c.	thin-layer chromatography
U	u.v.	ultra violet
V	v/v	volume to volume
W	$w_{\frac{1}{2}}$	half width

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DECLARATION

The work described herein was performed at the Department of Chemistry and Molecular Sciences, University of Warwick, England, during the period January 1979-January 1982, and was funded by the Science and Engineering Research Council. The work described is thought to be original except where due and proper acknowledgement is given for ideas and work previously published, or performed by others. The work described has not been submitted for any other degree previously.

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ABSTRACT

The biosynthesis of polyamines is a topic of current interest. A number of methods for the isolation and analysis of these polyamines have been described in the literature. However, the stereochemical course of the biosynthesis of these polyamines is still undefined.

After a general introduction to the importance of the polyamines in living cells (Chapter 1), Chapter 2 outlines the materials, methods and the instruments used in this project. In Chapter 3, isolation, separation and analysis of the polyamines *via* their phenylaminothiocarbonyl derivatives is described. The ^{1}H and ^{13}C n.m.r. spectroscopic analyses of these derivatives are also described. The synthesis of amino acids specifically or stereospecifically labelled with deuterium after some modifications of a literature method, are described in Chapter 4. The biosynthesis of spermidine from putrescine and ^{2}H or ^{13}C labelled methionine is described in Chapter 5. This Chapter demonstrates the incorporation of the 3-aminopropyl group of the labelled methionine as an intact unit into spermidine. The condensation reaction between ethanal and 1,3-diaminopropane is described in Chapter 6. The products of such condensations, namely hexahydropyrimidine compounds were acetylated. Full ^{1}H n.m.r. analysis for the hexahydropyrimidines and their acetyl derivatives are described. Decouplings in some cases are necessary to assure the assignment of the spectrum. The stereochemistry of spermidine synthase is studied in Chapter 7. In this Chapter, hexahydropyrimidine derivatives of stereospecifically labelled biosynthetic [1',2'-$^{2}H_2$]spermidines were used to define the relative configurations at the labelled methylenes (by 400 MHz ^{1}H n.m.r. spectroscopy). A definite judgement of the stereochemistry of spermidine synthase necessitated the synthesis of stereospecifically labelled [1',2'-$^{2}H_2$]spermidines. The hexahydropyrimidines of these spermidines are compared with those of the biosynthetic spermidines obtained from stereospecifically labelled methionines by the action of *E. coli* cells. The outcome of this work demonstrates the inversion of configuration at C-3 of the 3-aminopropyl group (originally C-4 of methionine) after its incorporation into spermidine.

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PUBLICATIONS

Part of the work described in this thesis has been published, and a further part is being prepared for publication as follows:

1. M. P. Atkins, D. C. Billington, B. T. Golding, M. D. Johnson, I. K. Nassereddin, and P. J. Sellars, "Mechanistic Studies Using ^{13}C Labels", paper presented at the (Prochem) Symposium on Stable Isotopes, 27 September 1979, at the University of Warwick

2. I. K. Nassereddin, B. T. Golding and D. C. Billington, "Biosynthesis of Spermidine from L-Methionine in *E. coli*", *J. C. S. Chem. Comm.*, 1980, 90

3. I. K. Nassereddin and B. T. Golding, "Isolation, Separation and Analysis of Polyamines *via* their N-phenylaminothiocarbonyl Derivatives", *J. Chem. Research (S)*, 1981, 342 (M 3931)

4. I. K. Nassereddin, D. C. Billington, B. T. Golding, M. J. Kebbell, and in part I. M. Lockart, "Synthesis of Methionines Specifically Labelled with ^{2}H or ^{13}C", *J. Labelled Compounds and Radiopharmaceuticals*, 1981, 18, 1773

5. I. K. Nassereddin and B. T. Golding, "Stereochemistry of Spermidine Synthase", *J. Am. Chem. Soc.*, in the press

6. I. K. Nassereddin and B. T. Golding, "Biosynthesis of Polyamines", in preparation, May 1982

7. I. K. Nassereddin and B. T. Golding, "Reactions of Amine Compounds with Carbonyl Functions", in preparation, May 1982

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This thesis is dedicated to my lovely parents, without whom none of this would have been possible.

Thanks Mum and Dad.

Ishaq

CHAPTER I

INTRODUCTION

CHAPTER 1

INTRODUCTION

1.A GENERAL

A great deal of the biochemistry of the naturally occurring polyamines, putrescine (1), spermidine (2) and spermine (3), has been elucidated since their discovery in 1678.

$$H_2N-(CH_2)_4-NH_2$$
(1)

$$H_2N-(CH_2)_3-NH-(CH_2)_4-NH_2$$
(2)

$$H_2N-(CH_2)_3-NH-(CH_2)_4-NH-(CH_2)_3-NH_2$$
(3)

However, some aspects of their biological functions are still under investigation. The polyamines were found in a wide spectrum of living cells, which indicates the necessity of these compounds for the growth of living cells.

Large efforts have been devoted during the last 25 years to the study of the biosynthesis of these polyamines. Most of these experiments were carried out with radioactive precursors, and the resultant polyamines were screened for radioactive labelling.

The main part of this thesis is concerned with the use of the stable isotopes ^{2}H and ^{13}C in the study of the mechanism of the biosynthesis of polyamines, and the stereochemistry of the

enzyme spermidine synthase.

In this Chapter the author will discuss the biosynthesis of polyamines and some of their biological functions in living cells. The current work on the biosynthesis of the pyrrolizidine alkaloids from polyamines has, briefly, been discussed.

As methionine is the precursor of the 3-aminopropyl group in spermidine and spermine, it is important to include a brief discussion, on some of its biosynthetic reactions, in this Chapter.

I.B THE BIOCHEMISTRY OF POLYAMINES

I.B.1 Historical

The polyamines, putrescine (1), spermidine (2), and spermine (3) are low molecular weight, aliphatic, nonprotein nitrogenous bases. Spermidine (2) and spermine (3) are aminopropyl derivatives of putrescine (1).

The history of these polyamines precedes that of nucleic acids by some 200 years. It began in 1678 when Antony von Leeuwenhoek observed the gradual deposition of crystalline spermine phosphate from human semen[1]. This observation was reported to the Royal Society in London as a description of the crystallisation of spermatozoa.

Independently, Nicolas Vauquelin also reported[2] the formation of these crystals in his single paper on human semen, in 1791. He was apparently unaware of the report of Leeuwenhoek. These crystals were later rediscovered by many workers, in aged semen samples. However, the nature of this crystalline compound remained ambiguous for more than a century. In 1895, these crystals were described by

Boettcher, as a substance similar to proteins.[2] In 1878, Schreiner showed that the crystals were a phosphate salt of an organic base, identical with similar crystals obtained from many tissues. Three years later, Fuerbringer proved that the prostate gland is the main source of the spermine of semen. The name "spermine" was given to the base by Ladenburg and Abel in 1888[2], but the determination of the structure of this base waited thirty six years before the important work of Otto Rosenheim[3]. From 1924 to 1927, Rosenheim and his colleagues successfully synthesised spermine and the related base spermidine which they had also discovered[4] during the isolation of spermine.

I.B.2 General

Although the presence of polyamines in biological materials was reported[1] for the first time some 300 years ago, they have been relatively unfamiliar to most investigators. Because these compounds were thought to have no pharmacological activity, and because the significance of their presence was not known, little effort was directed towards their area of research. Indeed,until recently,these polyamines were thought to be metabolic end-products. It is only during the past thirty years or so, that interest in the biochemistry and biological function of these compounds has been greatly increased.

Several observations[5,6,7] indicate that the polyamines have important functions in cellular metabolism and are associated with many aspects of the growth of cells. This was supported by analytical studies showing the occurrence of polyamines in bacteria and plants in addition to animal tissues. The exact mechanism of action of these polyamines is still not understood.

The biosynthesis of polyamines in *Escherichia coli* cells was studied in the late fifties by Tabors[8] and Green[9]. Independently, they demonstrated that putrescine and L-methionine are the precursors for the spermidine molecule in bacteria (see Chapter 5). The Tabors were also the first to demonstrate that minced rat prostate could synthesisespermidine *in vitro* from radioactive putrescine[10]. It soon became clear that the biosynthesis of polyamines in animal tissues occurs through the same general pathway as in prokaryotic cells.

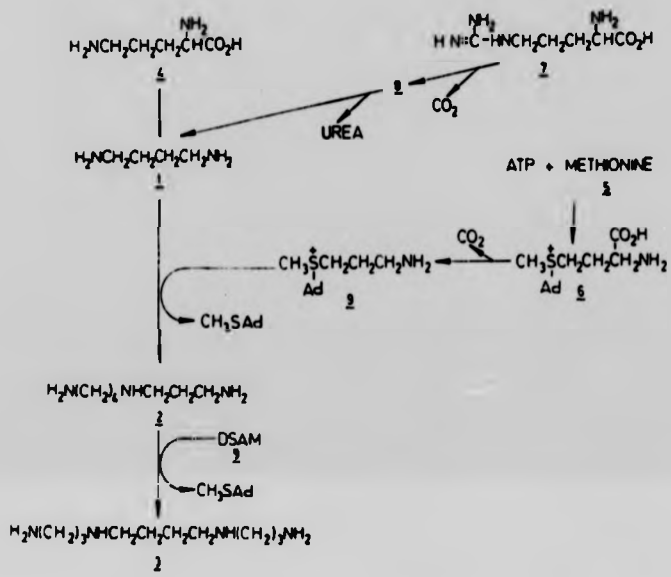
Convincing evidence has accumulated during the past 25 years that clearly indicates that these polyamines are very important physiologically. This includes the fact that several microorganisms have an absolute requirement for diamines or polyamines[10] for their growth. In addition, the concentrationof these amines and their biosynthetic enzymes is high in actively proliferating animal tissues and increases rapidly when growth or differentiation is induced in resting cells. It is also reported[11] that these changes precede increases in the DNA, RNA and the protein of the cell. *In vitro* experiments show that these polyamines, because of their polycationic nature, bind to nucleic acids and have a variety of effects on the biosynthesis and metabolism of these acids[11]. It is currently accepted that the biosynthesis of polyamines is very interrelated with the synthesis of nucleic acids and proteins. It has been postulated by some workers[12] that the polyamines specifically catalyse or control the biosynthesis of nucleic acids and that they are directly responsible for the increased macromolecular synthesis that occurs during growth. Even though there is a large accumulation of data about the relationship between polyamines and the nucleic acids there is still no conclusive evidence to support any of the specific proposals.

Large interest in the naturally occurring polyamines was triggered, after Russell *et al.*[13] reported that they had observed elevated diamine levels in hydrolysed urine samples of patients with malignant diseases. Since then numerous publications dealing with the polyamines have appeared. It is obvious that the analysis of these compounds in biological samples should take most of the effort in this area. However, the early suggestion that these polyamines might be useful in the early detection of occult cancer seems highly improbable at this time, mainly due to the time-consuming methodology which had been developed for the analysis of these compounds.

I.B.3 Biosynthesis of Polyamines

There are four enzymes involved in polyamine biosynthesis, two decarboxylases and two synthases. In animal tissues, putrescine (1) is formed from ornithine (4) by the action (Scheme I.B.I) of ornithine decarboxylase (ODC). In bacteria, however, this diamine is either formed from ornithine as above, or from arginine (7) *via* agmatine (8), with the enzymes involved being arginine decarboxylase and agmatinase. In most eukaryotic cells, the enzyme ODC is present only in low concentrations, and is rate limiting in the biosynthesis of spermidine (2).

Putrescine (1) is incorporated[8] (Scheme I.B.I) directly into spermidine, and the propylamine moiety is derived from methionine[9] (5). The sequence involves the synthesis of S-adenosylmethionine (SAM) (6) from methionine (5) and ATP, followed by decarboxylation of SAM to give decarboxylated SAM (DSAM) (9). This is then followed by the transfer of the propylamine moiety to putrescine by the



Scheme I.B.1 The biosynthesis of spermidine (2) and spermine (3) from putrescine (1) and methionine, *via* SAM (6) and DSAM (9).

action of spermidine synthase to give spermidine. Another synthase, spermine synthase, adds another propylamine group to spermidine to yield spermine (3).

Like most of the decarboxylases, mammalian ODC is a pyridoxal phosphate-requiring enzyme. It has been purified from a number of different sources[14-16]. The molecular weight of mammalian ODC is estimated to be *ca.* 70,000. It appears that only ODC obtained from livers of rats, which have previously been treated with thioacetamide, have shown a homogeneous preparation[12]. Mammalian ODC shows a specific requirement for thiols, and in the absence of these thiol compounds, the enzyme appears to undergo polymerisation leading to molecular species devoid of any enzymic activity[17]. One of the unique properties of ODC among other mammalian enzymes is

its extremely short half-life which is reported[18] to be as short as 10-20 minutes.

There are several lines of evidence indicating that the concentration of putrescine, as well as that of spermidine, plays an important role in the regulation of the activity of ODC in eukaryotic organisms. Putrescine appears to regulate the activity of ODC in cultured mouse fibroblasts[19]. Similarly, putrescine and spermidine at micromolar concentrations abolished the increase in the activity of ODC in cultured lymphocytes[20]. It has also been shown that an injection of a relatively large dose of putrescine into the rat could prevent the stimulation of ODC activity by partial hepatectomy[21]. Some evidence suggests that the changes in ODC activity may involve changes in its half-life, and in some instances the stimulation of the enzyme activity is associated with a lengthening of its half-life[22].

The production of the enzyme S-adenosyl-L-methionine decarboxylase (SAMD) in higher organisms is stimulated by putrescine and similar diamines[23]. This appears to be a universal characteristic of eukaryotic cells; however, some higher plants are possible exceptions to this rule[24]. This is in sharp contrast to the corresponding enzyme from bacterial sources (e.g. *E. coli*) which shows a stringent requirement for magnesium ions, and the enzyme from lower eukaryotes, such as protozoa, which is not influenced by either diamines or magnesium ions[25,26]. SAMD has been purified from many sources and that obtained from rat liver was found to have a molecular weight[12] of *ca.* 68,000. SAMD is found to be the only mammalian decarboxylase requiring pyruvate as a cofactor, rather than the more common pyridoxal phosphate[27]. The separation and purification of SAMD to homogenity has been achieved[12] in contrast to an earlier

report[28], which suggested that the formation of a functional complex between SAMD and spermidine synthase was contributing to the difficulty in purification of the two enzymes. Another difference between SAMDs from prokaryotic and eukaryotic organisms appears to be the sensitivity towards decarboxylated adenosylmethionine, (DSAM), the product of the reaction. DSAM (9) has been reported[17] to be a much more powerful inhibitor for eukaryotic (e.g. rat prostate and bakers' yeast) rather than for prokaryotic (e.g. *E. coli*) SAMD.

In all eukaryotic cells, the total activity of SAMD is much lower than that of spermidine synthase[14,29]. It thus appears that in most cases the rate of spermidine synthesis is determined by the activity of SAMD and hence the synthesis of spermidine is mainly, if not entirely, regulated by the concentration of cellular putrescine[30].

Mammalian SAMD also has a very short half-life of only 20-60 minutes[31,32]. Thus it ranks close to ODC in the list of the half-lives of mammalian enzymes.

The enzyme spermidine synthase catalyses the transfer of the propylamine moiety of DSAM to putrescine to yield spermidine, thiomethyladenosine, and one proton. This enzyme has been purified to homogenity from *E. coli* and has no known cofactor[33]. The corresponding enzyme from mammalian sources has been partially purified[29]. Spermidine synthase from rat ventral prostate has a high affinity for DSAM. The high activity of the enzyme for this substrate, and the fact that its total activity greatly exceeds that of SAMD[34], is probably the reason for the earlier suggestion[35] that only one enzyme was responsible for the decarboxylation of SAM and the transfer of the propylamine group to putrescine.

In contrast to ODC and SAMD, spermidine synthase from rat liver appears to have a rather long biological half-life since its

activity requires several hours for decay[36].

The enzyme spermine synthase utilises DSAM as the donor of the propylamine group and spermidine as the acceptor. Spermine synthase has been purified *ca.* 100-fold from rat brain. The slow progress in its purification and characterisation is attributed to its low activity[37]. No cofactor or coenzyme appears to be needed in the synthesis of spermine by the enzyme, which showed a high affinity[37] for DSAM. Like spermidine synthase, spermine synthase seems to be a rather stable enzyme with a long biological half-life[36].

I.B.4 Functions of polyamines

Many roles of the polyamines in both prokaryotic and eukaryotic cells are still undefined, but in the last 20 years numerous publications have appeared showing the importance of these polyamines in the cell proliferation processes. One of the first suggestions that polyamines might be involved in the proliferative response of cells and tissues came from the pioneering work of Herbst *et al.*[38] when they demonstrated increases in tissue levels of spermidine early in the regenerative process. Since this observation, the correlation between stimulation of cell growth and the large increases in the rate of polyamine biosynthesis, has been extended to a wide variety of systems[39]. These correlations were merely suggestive at the beginning, and it was only with the isolation of a well-defined putrescine auxotroph of *Escherichia coli*, that the growth requirement for polyamines was proved[40].

Closely related to the microbiological requirements for polyamines is the finding of Mager[41] that these substances have a marked stabilising effect on certain bacteria that require a high osmotic

environment for survival. This stabilising effect may explain some of the growth-promoting activity of polyamines.

A great number of researchers have taken the binding of polyamines to nucleic acids *in vitro* as the object of their researches[39,40]. Much of the information provided little evidence about the nature or the magnitude of this association, but definitely indicated the strong affinity between polyamines and nucleic acids. Complexing of polyamines and nucleic acids was suggested by such observations as the formation of an insoluble polyamine-nucleic acid complex when dilute solutions of spermidine or spermine were added to nucleic acid solutions[42].

The high affinity between polyamines and nucleic acids was demonstrated when the interaction between DNA and polyamines has resulted in the enhanced stability of the double helical structure against thermal denaturation[43]. X-ray analysis of the crystal structure of polyamine salts suggests that on interaction with DNA molecules, spermidine or spermine may form a bridge between the two strands of the DNA, *via* the protonated amino and imino groups. This model is supported by experimental evidence[44], which indicates the high specificity of this interaction.

In 1978, Cohen[11] suggested that one of the important roles of polyamines is to organise the structure and activity of t-RNA. t-RNA can be converted from its inactive configuration to a more compact active form by the addition of either magnesium ions or polyamines. One or two mols of polyamines per mol of t-RNA have been found in both eukaryotic and prokaryotic cells. Therefore, it was not surprising to find that a mixture of magnesium ions with spermidine would facilitate ordered crystallisation of t-RNA[45].

It was found that both spermidine and its analogue

ethidium, [Fig. 1.B.1(A)] which possesses similar distribution of free and substituted amino groups, bind similarly to tight and less tight binding-sites in t-RNA of *E. coli* or yeast. Spermidine and ethidium compete for the binding sites and at the same time they can displace each other, depending on the ionic strength of each one[11]. Numerous studies of the effect of cations on t-RNA indicated that one binding site for spermidine was in the vicinity of the relatively unstable stem of the dihydrouridine (D) arm [Fig. 1.B.1(B)*]. Both spermidine, and magnesium ions, appeared to act sequentially in this region in stabilising and inducing folding of the t-RNA[46].

The structural positions of spermine and magnesium ions in crystals of yeast t-RNA have recently been determined[47] [Fig. 1.B.1(B)]. Two spermine molecules and four magnesium ions have been located in the structure.

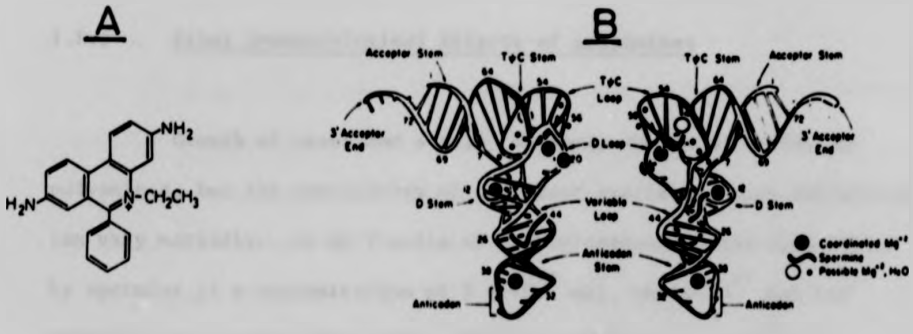


Fig. 1.B.1 (A) Ethidium structure
(B) Binding of spermine to t-RNA

Polyamines have been found in ribosomes isolated from both bacterial and animal cells[2]. On this basis, it has been widely accepted that ribosomes *in vivo* also contain polyamines, and that polyamines are essential for ribosomal structure and function.

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*This figure was taken from Ref. 11.

However, it has been demonstrated[48] that ribosomes can take up amines from the cytoplasm after the disruption of the cell. The binding of polyamines to ribosomes is reversible, and it was found[2] that, consequently, the amine content of the ribosomes changed during isolation procedures. Thus, it is impossible to be certain that ribosomes and polyamines bind to each other *in vivo*, or to the magnitude of this binding.

Several papers suggest that the polyamines play a role in peptide initiation, possibly by altering the ribosomal conformation. The amines decrease the requirement for magnesium ions for the formation of an initiation complex between aminoacyl-t-RNA, m-RNA, and ribosomes[49]. Spermine may also affect peptide elongation, since at low magnesium ion concentration, it stimulates the peptidyl transferase activity of ribosomes[50].

I.B.5 Other pharmacological effects of polyamines

Growth of yeast and several bacteria can be inhibited by polyamines, but the sensitivity of different species to such inhibition can vary markedly. At pH 7 cells of *Staphylococcus aureus* were killed by spermine at a concentration of 5×10^{-4} mol, while 10^{-3} mol was enough to kill cells of *E. coli*. The bactericidal potency[10] was increased 10-fold when the pH of the two media were raised from 7 to 8.

The antibacterial effect of polyamines could be due to their interference with the permeability mechanism of the cell membrane, or due to the diamines preventing uptake of essential amino acids[10].

In 1953, Hirsch and Dubos were the first to observe that

concentrations of spermidine that do not damage the cells or organisms, may become very toxic when mixed with beef plasma amine oxidase. They presumed that the products of the enzymatic oxidation of spermine are the responsible toxic factors[51].

In animals, the polyamines have a marked renal toxicity[10]. The administration of 0.075-0.15 mmol/kg of spermine to a variety of animals[51], produces only relatively mild acute effects. However, the resultant proteinuria and serum non-protein nitrogen retention which gradually develops are followed by death from renal failure. Spermidine is much less toxic than spermine and 1,4-diaminobutane is not toxic at comparable doses.

In man[10], vomiting, albuminuria, acetonuria, and hyperglycemia are observed after intramuscular injection of spermine (0.033 mmol/kg).

I.B.6 Cancer and polyamines

Russell[13] reported in 1971 that cancer patients had elevated levels of polyamines in their urine. Since that time numerous publications have supported this report (full account of this subject is discussed in the book "Polyamines in Biomedical Research", Ed. J. M. Gaugas, A. Wiley - Interscience, 1980). In a later report[52], Russell showed that changes in polyamine levels, in the plasma or urine of diagnosed cancer patients, were associated with alterations in tumour growth kinetics. Since these compounds are associated with many aspects of the growth of normal cells, it is not surprising to see them playing an important function in the cellular metabolism of cancer cells.

Since Russell's report[13] in 1971, the hope that the polyamines and some of their metabolites would be useful as a tumour marker

has motivated a considerable amount of research on these compounds. This includes the extension of polyamine analyses to other physiological fluids[53], such as whole blood, blood plasma, serum, and cerebrospinal fluid, along with variations in sample preparation and sophistication in the methodology of these analyses.

It is apparent from the literature in the last 10 years, that measuring polyamine levels in physiological fluids is a valuable tool in evaluating the pathogenesis of haematological diseases, as well as monitoring the course of the disease, with or without chemotherapeutic treatment. However, the early suggestion[13] that they might be useful in early detection of cancer seems highly improbable at this time. Firstly, the methodology is still time consuming and has not been applicable to mass-screening procedures, which would be necessary to ascertain their possible usefulness in early diagnosis. Secondly, other pathological conditions in which there is high cell turnover and high growth fractions also appear to result in increased urinary polyamines, e.g. patients with cystic fibrosis[54].

The use of polyamine analyses, as a general tumour marker and as an indication for either the state of health or disease stages, can be greatly improved if a specific antibody can be developed against putrescine, spermidine, spermine and the naturally occurring conjugated polyamines. This would establish the hoped-for immunoassay for quick, specific, reproducible and reliable analysis of the polyamines in biological fluids.

I.B.7 Polyamines as precursors of alkaloids

Recent studies have implicated the polyamines as

precursors of a number of alkaloids. In 1973, spermidine was demonstrated[55] to be the precursor of maytenin (10), in which two *trans*-cinnamoyl amide groups were added to the terminal amino groups of spermidine (Fig. 1.B.2).

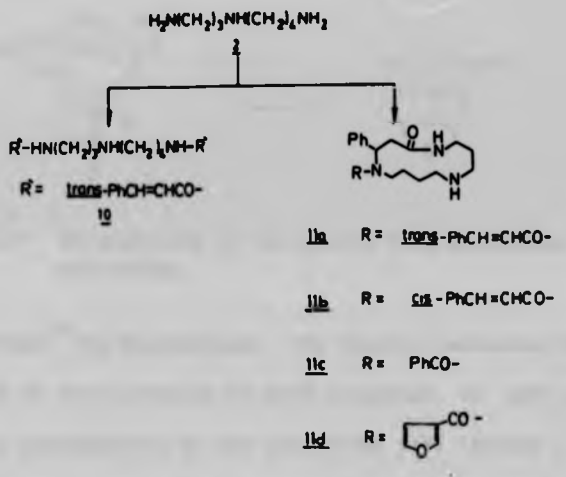
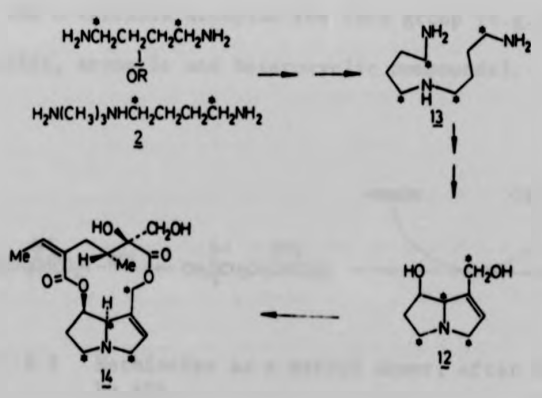


Fig. 1.B.2 Alkaloids which are believed to be derived from spermidine and cinnamoyl units.

In 1974, maytenin was reported to be the precursor of celacinnine (11a), in which the 13-membered ring is constructed from spermidine and one cinnamoyl unit. The 13-membered ring of celacinnine was also found in other alkaloid rings such as cellallocinnine (11b), celabenzene (11c), and celafurine[56] (11d).

Recently[57], evidence has been presented for the involvement of putrescine, spermidine and spermine in the biosynthesis of the pyrolizidine alkaloid, retrorsine. The most common base portion found in the pyrrolizidine alkaloids is retronecine (12), which was demonstrated, by the use of ^{14}C-labelled precursors, to be biosynthesised from arginine, ornithine and putrescine. The position of the ^{14}C-labelling



Scheme 1.B.2 Biosynthesis of retrorsine from spermidine or putrescine.

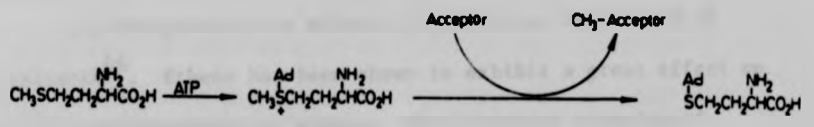
was determined[58] by degradation. The results indicated the utilisation of two molecules of each precursor, to give a symmetrical intermediate of the type shown (13) (Scheme 1.B.2). This is followed by the conversion of (13) into retrorsine (14) *via* retronecine (12).

1.C SOME BIOCHEMISTRY OF METHIONINE

In 1922 methionine was isolated and identified as a sulphur containing compound.[59] Six years later, the structure of this compound was demonstrated[60] to be $CH_3SCH_2CH_2CHNH_2COOH$. Methionine has been shown to be necessary for growth in a number of organisms[61] and an essential amino acid in man[62]. The biosynthesis of methionine in humans is closely linked to other sulphur-containing amino acids such as cysteine and homocysteine[61].

The importance of methionine can be demonstrated by the large number of methylation reactions[63] involving the activated

form of methionine, SAM (Scheme I.B.3), as a donor of the methyl group, and a suitable acceptor for this group (e.g. alcohols, amino acids, aromatic and heterocyclic compounds).



Scheme I.B.3 Methionine as a methyl donor, after its activation by ATP.

The activated form of methionine (SAM) may donate its methyl group, the alkyl chain or an adenosyl residue to a wide spectrum of acceptors. Many macromolecules, are now known, which after primary biosynthesis, are methylated in reactions[63] involving SAM. The methylation of nucleic acids, proteins and polysaccharides has been demonstrated[63].

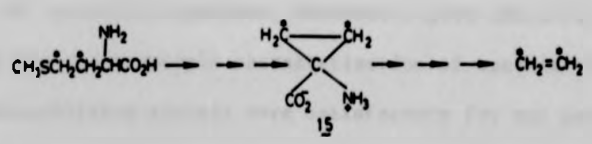
The methylation of the nucleic acid DNA plays an important role in the defence mechanism of the bacterial cell[64]. When foreign DNA enters a bacterial cell it may be degraded or it may survive and replicate. The specific enzyme endonuclease, which degrades foreign DNA, differentiates it from the native DNA by the degree of methylation in both acids. The foreign DNA is thus cleaved rather than the native methylated DNA. The methylation of t-RNA by SAM was first reported by Borek[65]; since then, these methylation reactions have been extensively studied[63]. It has been shown that the degree, and the pattern of this methylation, is different in t-RNA obtained from different organisms. It has also been shown that under different physiological conditions, different t-RNAs can be obtained from the same organism. The significance of t-RNA methylation,

with regard to its structure or function, has not yet been determined.

Another important aspect of methionine biochemistry is shown in the biosynthesis of spermidine and spermine. This topic is discussed in Chapter 5 of this thesis.

The biosynthesis of ethene in plants was the object of much research[66]. Ethene has been shown to exhibit a great effect on the growth and maturation of plants. The premature ripening of fruit by gases from unburned paraffin was identified[67] as the result of the ethene in these gases. The year 1934 brought the proof that ethene is a natural plant hormone, after it was demonstrated that apples produced ethene[68].

Many attempts for the elucidation of the biosynthetic route of ethene production have been carried out, by plant physiologists. Thus, since 1950 many precursors of the ethene produced by plants have been suggested. Most of the work on these precursors and their proposed pathways to ethene have been reviewed[66], the most preferred precursor of ethene was suggested to be methionine[66]. The most recent evidence being described by Adams and Yang[69] (Scheme 1.B.4).



15

Scheme 1.B.4

They infused ^{13}C-labelled methionine into apple tissue, and isolated labelled 1-aminocyclopropane-1-carboxylic acid (15). They then infused labelled 1-aminocyclopropane-1-carboxylic acid and obtained labelled ethene.

1.D OUTLINE OF THE PROJECT

1. General

A major problem facing the biochemist wishing to investigate the metabolic pathways, and stereochemistry of enzymic reactions, is the synthesis of labelled precursors and of labelled products for comparison with the biosynthetic molecules. The main aim of this project is to study the biosynthesis of spermidine and the stereochemistry of its synthase. This required the establishment of an efficient method for the isolation and analysis of spermidine from *E. coli* cells. When this had been achieved, labelled methionines were used to study the biosynthesis of spermidine. The problem of identifying the relative configurations of deuterium labelled spermidines was solved by the chemical synthesis of samples of labelled spermidines as standards which were compared with biosynthetic samples of spermidines.

2. Phenylaminothiocarbonyl (PATC) derivatives of polyamines

The eventual aim of this project, to study the stereochemistry of spermidine synthase, demanded a good and efficient method for the spectroscopic characterisation of spermidine. None of the published methods were satisfactory for our purposes. We studied various derivatives and eventually found that the PATC-derivatives of the polyamines were adequate for our purposes. Free amines can be recovered from these derivatives by acid hydrolysis. This method is now suitable for obtaining free spermidine, suitably labelled with ^{2}H or ^{13}C, which can be used to study its binding with nucleic acids.

3. Biosynthesis of spermidine from labelled methionines

Methionines labelled with ^{2}H or ^{13}C at C-2 and C-3 or at C-3 and C-4 with either ^{2}H or ^{13}C were used in studying the biosynthesis of spermidine from methionine and putrescine. The outcome of this work demonstrated the incorporation of the 3-aminopropyl group of methionine into spermidine as an intact unit.

4. Stereochemical studies

Studies on the stereochemical course of the transfer of the 3-aminopropyl group of methionine to putrescine were carried out with the aid of stereospecifically deuterium-labelled methionines. The establishment of the stereochemical outcome of this reaction proved to be difficult to analyse. Thus, the synthesis of deuterium labelled spermidine was carried out to assist in solving the cryptic stereochemistry of this reaction.

The thesis also describes synthesis of a number of model compounds, which were used to help understand some of the ^{1}H n.m.r. spectral features of the hexahydropyrimidine derivatives of the spermidines. These derivatives were then used as intermediates in the study of the relative configurations of the deuterium atoms in the labelled spermidines.

5. Synthesis of deuterium-labelled amino acids

Methionines specifically labelled with deuterium(s) were prepared by an exchange reaction method[70]. The utilisation of this method was extended as we have demonstrated to the preparation of *rac.*[2,3-$^{2}H_2$]valine, *rac.*[2-$^{2}H_1$]alanine, *rac.*[2,3-$^{2}H_2$]isoleucine and *rac.*[2,3,3-$^{2}H_3$]leucine.

Preparation of stereospecifically deuterium-labelled methionines were carried out, starting from [1,2-$^{2}H_{2}$]acetylene. The same route used in this synthesis was adapted in our laboratory for the synthesis of *rac.*[3,4-$^{13}C_{2}$]methionine from the commercially available [$^{13}C_{2}$]acetylene.

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CHAPTER 2

MATERIALS, METHODS, AND INSTRUMENTAL

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2.A MATERIALS

1. Solvents

All solvents used in the isolation and analysis of polyamines from *E. coli* cells were of analytical grade, which were distilled and stored over 3A or -4A molecular sieve. Solvents used in the h.p.l.c. analysis were h.p.l.c. grade and were purchased from "Rathburn Chemicals Ltd., Walkerburn, Scotland". Other solvents were of laboratory grade which were distilled before use. Anhydrous solvents were purified and dried according to a standard method. Chloroform was obtained ethanol free by chromatography on basic alumina. Absolute, anhydrous and deuterated solvents were stored in tightly stoppered bottles with parafilmed seals and were used in a dry box.

2. Chemicals

All chemicals were of the highest purity commercially available, and in some cases were purified before use.

2.B METHODS

1. Solutions in organic solvents were dried using $MgSO_4$, which had been stored at 110°C for at least 2 days. Evaporation of solvents under reduced pressure refers to the removal of bulk solvents by

using a Büchi rotary evaporator at 12-15 mmHg at room temperature. Otherwise, conditions of evaporation would be stated.

2. All glassware used in the synthesis of deuterated compounds, exchange experiments and in a moisture sensitive reaction were dried at 110°C overnight, flamed and then allowed to cool to room temperature under a blanket of dry nitrogen.

3. Exchange experiments were carried out in the dry box under a blanket of nitrogen.

4. Solutions of sodium methoxide in methanol were prepared in a dry box from anhydrous methanol and freshly cut clean sodium. Sodium was cleaned by dropping in a small amount of methanol before being added to the solvent. Titration of sodium methoxide solutions was done against standard hydrochloric acid solutions using phenolphthalein indication.

5. Preparation of hypochlorous acid: Chlorine(I) oxide was prepared[1] by passing Cl_2 (gas) through carbon tetrachloride. To the resultant solution was added, with stirring, the calculated amount of mercury(II) oxide. Filtration and the addition of water* was followed by the separation of the aqueous layer. The hypochlorous acid was titrated against 0.1 mol dm^{-3} sodium thiosulphate before use (N.B. for full details of this preparation see Ref. 1).

6. T.l.c. was carried out on home made plates of Kieselgel MN 60 F_{254} (Merck, Cat. No. 5554) which were activated at 110°C overnight before use. The analytical plates were of 0.25 mm thickness while the

*The amount of water was calculated according to the required strength of the acid.

preparative plates were of 0.5 mm thickness. The solvents were made up freshly and are quoted as volume to volume.

7. Gases were handled on an all glass vacuum line system. Standard techniques[2] were explored in handling and purification of all gas samples.

2.C INSTRUMENTAL

1. N.m.r.

^{1}H n.m.r. spectra were recorded using the following instruments.

(a) Perkin-Elmer (model R-34) 220 MHz ^{1}H n.m.r. spectrometer

(b) Bruker (model WH-400) 400 MHz ^{1}H n.m.r. spectrometer

Peaks are designated by their chemical shift (δ) in parts per million, followed in brackets by their relative integral value (e.g. 1H) in hydrogens, their multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, p = pentuplet, m = multiplet), and the spin-spin coupling constant (J) in Hertz, where appropriate. Spectra were recorded in deuterated solvents using TMS or TSS as a reference (zero δ).

^{13}C n.m.r. spectra were recorded on a Bruker (model WH-90) 22.63 MHz ^{13}C n.m.r. spectrometer, equipped with a variable temperature accessory. Peaks are designated as above. All spectra were run with broad band ^{1}H decoupling, and consists of singlets unless otherwise indicated.

^{2}H n.m.r. spectra were recorded on a Bruker (model WH-400) 61.424 MHz ^{2}H n.m.r. spectrometer. Peaks are designated as in the ^{1}H n.m.r. spectra.

All n.m.r. spectra were assigned by comparison to the n.m.r. spectra of model compounds, or of authentic materials.

2. Infra-red spectra

Infra-red (i.r.) spectra were recorded on a Perkin-Elmer (model 580B) grating infra-red spectrophotometer. Samples were either mulls (nujol), thin films, solutions or gases (10 cm gas cells) and were run using NaCl plates. Peaks are designated by their wave number (cm^{-1}) as strong (s), medium (m), weak (w), shoulder (sh) and as broad (br).

3. Optical rotations

Optical rotations were measured with a Bendix NPL automatic polarimeter (model 143D) using a 1.00 cm x 0.708 cm^{2} cell. The instrument was calibrated against a standard sucrose solution before each measurement. Values are expressed as specific rotations ([α]) .

4. Gas liquid chromatography

G.l.c. analyses were carried out using Perkin-Elmer (model F-11) flame ionisation gas chromatograph, using N_2 as carrier gas. Samples were compared to authentic materials.

5. Ultra violet spectra

U.v. spectra were recorded with a Pye-Unicam (model SP-1800) ultra violet spectrophotometer.

6. Melting points

Melting points (m.p.) were recorded using a Gallenkamp Instrument and are uncorrected.

7. Combustion analysis

C.H.N. combustion analysis were carried out by C.H.N. Laboratories, Leicester.

8. H.P.L.C.

High pressure liquid chromatography (h.p.l.c.) analyses were carried out on a Waters Associates (model 204) liquid chromatograph equipped with a U6K septumless injector, 6000A solvent delivery system, and a 400 single channel absorbance detector (254 nm).

9. Mass spectra

Mass spectra were recorded on a Kratos MS80 spectrometer. Peaks are quoted as m/z followed by their percentage where relevant. The molecular ion is designated as $M^{\bullet+}$.

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CHAPTER 3

ISOLATION, SEPARATION AND ANALYSES OF POLYAMINES

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3.A INTRODUCTION

Interest in the determination and analysis of the polyamines spermidine and spermine, and their precursor putrescine, has been greatly stimulated by the reports of Russell[1,2], which described elevated levels of these compounds in the urine of patients with metastatic cancer. Following these findings, numerous papers dealing with the estimation of these polyamines have been published. Polyamines have been successively described as the foul-smelling indicators of the male reproductive function, then as the promoters and regulators of cell growth, and most recently, as potentially useful indicators for the state of human health or disease. Parallel with these observations, polyamine analyses were developed that were qualitative at the beginning, but later quantitative measurements began to be used. Finally, highly sensitive measurements were needed if any useful clinical results were to be derived from these analyses.

The most favoured of the early methods for characterising polyamines was crystallisation of either their phosphate salts[3], or their picrates[4]. The latter were often identified by their melting points. Later on, ion-exchange chromatography[5] was used for the separation of polyamines. This method has successfully achieved separations in the nanomol range with fair specificity. Other methods of similar sensitivity followed, e.g. paper electrophoresis, paper chromatography and an enzymic assay using spermidine oxidase.

The final stage in the analysis of polyamines began when dansyl derivatives[6] of the polyamines were assayed fluorimetrically, after being separated by t.l.c. on silica gel plates. This technique permitted the assay of 20-200 pmol of spermidine. The use of g.l.c. was introduced in the analysis of polyamines as their trifluoroacetyl derivatives[7] and showed a reasonable sensitivity to 200 pmol . This method was later improved by the use of g.c./m.s., utilising analogues of polyamines as internal standards. In the mid-seventies numerous publications appeared describing the use of h.p.l.c. as a sensitive method for the quantitative analysis of polyamines. The most important of these was the separation of the dansyl derivatives[8] of the polyamines by h.p.l.c. This method exhibits a fairly good sensitivity (25-50 pmol), and allows a good separation between the diamines. The innovation of using antibodies against polyamines showed that immunoassay[9] was the ultimate analytical approach for routine estimation of the polyamines. Although this technique has its own drawbacks, mainly the cross-reaction between the antibodies, it is extremely sensitive ($\sim$ 0.05 pmol). Another drawback is the low specificity of the assay, but this may be compensated for by the high sensitivity of the method. The clinician observing abnormal values of a polyamine can then use a more specific method to identify the polyamine responsible.

Although many analytical methods of high sensitivity have been developed, this by no means suggests that the analysis has reached maturity. Many years of work are needed to bring the currently developing methods, especially immunoassay, into full flower.

Future developments in polyamine analysis should also

allow for the analysis of metabolites and conjugates of polyamines. The work of M. M. Abdel-Monem[10] on the dansyl derivatives of the acetyl conjugates of polyamines appears to have started this hoped-for new era in the field of polyamine analysis.

One step in the biosynthesis of spermidine is the reaction between decarboxylated S-adenosylmethionine and 1,4-diaminobutane (putrescine), which is catalysed by spermidine synthase (3-aminopropyl transferase)[11]. In order to study the biosynthesis and the stereochemistry of this reaction, we enlisted the aid of precursors labelled with stable isotopes. Thus, we required a convenient method for the isolation and separation of polyamines from cells of *Escheriohia coli*. Furthermore we required a derivative of spermidine whose n.m.r. spectra (^{1}H, ^{2}H and ^{13}C) would show, ideally, separate and easily assignable resonances for each carbon or methylene group [N.B. the 220 MHz ^{1}H n.m.r. spectrum of spermidine in $CDCl_3$ with TMS as internal standard, shows signals at δ 1.3 (5 H, brs), 1.49 (4 H, m), 1.64 (2 H, p) and 2.5-2.9 (8 H, m) p.p.m.]. No properly characterised derivatives of the polyamines, suitable for isolation, separation and spectroscopic analysis, have been described. After trying benzoyl[12], dansyl[13], 4-methoxybenzoyl[14] and p-toluenesulphonyl[15] derivatives and finding various shortcomings (especially incomplete derivatisation), we examined the phenylaminothiocarbonyl derivatives (PATC-derivatives) obtained by reacting polyamines with isothiocyanatobenzene (PhNCS). This classical procedure for derivatising amines[16] had not been applied to polyamines with the exception of 1,4-diaminobutane. We found these derivatives to be very suitable for our purposes. The fully blocked PATC-derivatives of polyamines were easy to prepare and separate by chromatography, using silica gel and

non-aqueous solvents. They were fully characterised by ^{1}H and ^{13}C n.m.r. spectroscopy and by combustion analysis.

In order to assign the spectroscopic data of phenylaminothiocarbonyl derivatives of polyamines, we needed simple amino compounds as models which would give PATC-derivatives whose ^{1}H and $\{^{1}H\}^{13}C$ n.m.r. spectra were straightforward to analyse.

We isolated the polyamines spermidine and putrescine from cells of *E. coli* by making the PATC-derivatives. The separation and purification was done on p.l.c. Preliminary analysis by h.p.l.c. was achieved with a mixture of authentic PATC-derivatives and was compared with PATC-derivatives isolated from *E. coli* cells. Finally, the separated PATC-spermidine was hydrolysed to give free spermidine, which later on was used for further studies, e.g. reactions with carbonyl compounds.

3.B PHENYLAMINOTHIOCARBONYL DERIVATIVES OF POLYAMINES

The natural polyamines do not possess structural features which enable their direct analysis and detection to be readily achieved. A potential method for detection and analysis of polyamines is through derivatisation. This method has the advantages of permitting, in principle, sensitive detection and good physical separation. It also has the advantage of allowing the accumulation of the polyamines by solvent extraction. None of the numerous derivatives of polyamines described in the literature fulfilled the needs of our objectives.

Most of the derivatives we examined (e.g. benzoyl[12], 2,4-dimethylbenzoyl, dansyl[13], 4-methoxybenzoyl[14], and p-toluenesulphonyl[15] have showed various shortcomings, such as incomplete

derivatisation and poor spectroscopic properties. Considerable effort was invested in the 4-methoxybenzoylamide derivatives of polyamines.

More than one procedure for the preparation of these derivatives was tried. The reaction between p-anisoylchloride and the polyamines was carried out in various organic solvents (DMSO, dichloromethane, pyridine) with pyridine as a base. Stirring for 12 h at room temperature gave mixtures of compounds containing partially reacted polyamines (n.m.r. and t.l.c. analysis) and some acid anhydride (i.r., ν_{max}, 1800 cm^{-1}). Attempts to purify such mixtures were unsuccessful.

Finally, preparation of these derivatives in a pure state was achieved, but in rather low yield, when the reactions were carried out in 2 mol dm^{-3} aqueous sodium hydroxide[14]. The 220 MHz ^{1}H n.m.r. spectra of these derivatives were recorded and showed them to be reasonably pure, but their combustion analyses showed unacceptable results, especially for the derivatives of spermidine and spermine. The n.m.r. spectra of these derivatives showed mainly broad, signals for which assignment was impossible due to overlapping of the resonances of the alkyl chain.

Analysis of the 4-methoxybenzoylamide derivatives of putrescine, spermidine and spermine by h.p.l.c. was attempted on columns of μ-C_{18}, μ-Porasil and Partisil. On the μ-C_{18} column, various combinations of water and methanol were used to elute the three derivatives, but with no success in achieving separation. When chloroform/methanol (99/1,v/v) was used as eluent on the silica gel columns, the Partisil column showed a good separation for the three derivatives, but the quality of the peaks was very poor (high $w_{\frac{1}{2}}$). The μ-Porasil gave the best results when this

solvent system was used, but the irreproducibility of the chromatograms made this system unreliable for accurate analyses.

We finally examined the phenylaminothiocarbonyl (PATC)-derivative of polyamines and found them to possess ideal chemical, chromatographic and spectroscopic properties in comparison to other derivatives in use. The PATC-derivatives have been fully characterised by CHN combustion analyses, m.p., t.l.c., h.p.l.c., i.r., u.v., ^{1}H n.m.r. and $\{^{1}H\}^{13}C$ n.m.r.

We found that the PATC-derivatives of putrescine, spermidine and spermine were difficult to prepare under similar conditions to those used for simple amines. Under these conditions (ethanolic solution), oiling out of products and failure of crystallisation of these oils, made it difficult to get analytically pure derivatives. Finally, the PATC of polyamines were obtained in crystalline form from solutions of aqueous ethanol. We have observed, by t.l.c. and ^{1}H n.m.r. spectroscopy, the full blockage of all amino groups of the polyamines, irrespective of whether the derivatisation was carried out in ethanol or in aqueous ethanol.

We did some preliminary kinetic experiments on the formation of the PATC-derivatives. This showed that blocking of all amino groups of the polyamines was complete within 5 minutes.

Isothiocyanatobenzene (PhNCS) is specific for amino functions. If used in excess during the reaction, it does not interfere with subsequent h.p.l.c. or t.l.c. analyses, because it can be removed by pumping at 10^{-5} mmHg/r.t. There is no need to remove the PhNCS before the chromatogram when it is present in relatively small quantity.

The chromatograph we used in the h.p.l.c. analysis was fitted with a single absorbance detector (254 nm). In applying

FIG. 3-B-1

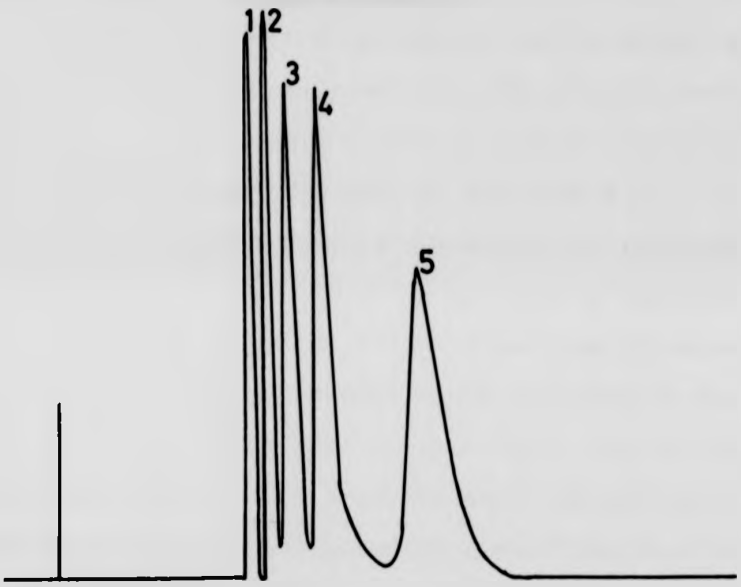

Fig. 3.B.1 Chromatogram for the h.p.l.c. analysis of authentic phenylaminothiocarbonyl derivatives of:

(1) ethylamine

(2) 1,3-diaminopropane

(3) putrescine

(4) spermidine

(5) spermine

h.p.l.c. analysis it was important for us to have a derivative which gives an appreciable absorption at this wavelength. We found by the u.v. analysis of PATC-derivatives that they possess a very high extinction coefficient at λ_{max} 252 nm. These characteristics of PATC-derivatives contributed to the high sensitivity of this method, which showed a detection limit of *ca.* 0.2 pmol at 10% of full-scale deflection.

Mixtures of the PATC-derivatives of ethylamine (16), 1,3-diaminopropane (18), 1,4-diaminobutane (19), spermidine (20) and spermine (21) could be analysed by isocratic h.p.l.c. [30 cm x 4.6 mm i.d. (μ-Porasil), 0.5 cm^3 min^{-1}]. Elution with 1% methanol in chloroform gave five nicely separated peaks (Fig. 3.B.1). The only setback of this h.p.l.c. analysis is the slight tailing of the peak corresponding to compound (21).

Mixtures of the PATC-derivatives can also be separated very easily by preparative layer chromatography. The PATC-derivatives behaved well on silica gel plates (Kieselgel MN) and showed no tailing after elution with dichloromethane/acetonitrile (87/13, v/v), and detection with iodine.

Another advantage in favour of the PATC-derivatives is the ease of recovery of the polyamines from the PATC-derivatives by hydrolysis in conc. HCl (overnight/115°C). For PATC-spermidine, evaporation and crystallisation from ethanol gave spermidine trihydrochloride, which could be passed through an ion exchange column to give hydrochloride-free spermidine.

The only disadvantage we faced while utilising the PATC-derivatives was their unsuitability for mass spectrometric analyses. Both c.i./e.i./m.s. gave no molecular ion or useful fragments for any of the PATC-derivatives. The advantages

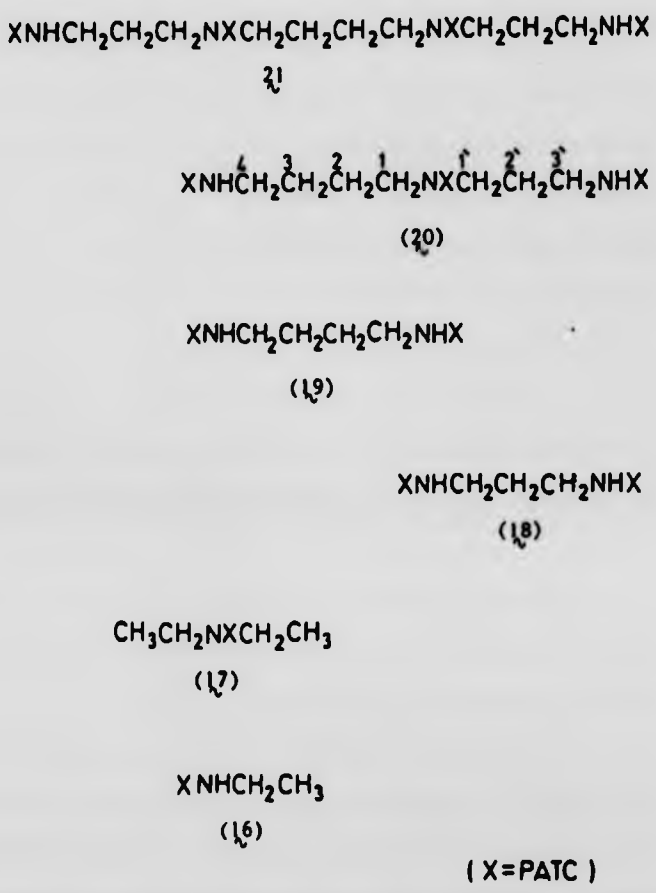

Fig. 3.C.1 Phenylaminothiocarbonyl derivatives of ethylamine (16), diethylamine (17), 1,3-diaminopropane (18), putrescine (19), spermidine (20) and spermine (21).

TABLE 3.C.I ^{1}H n.m.r. chemical shift assignments for PATC-derivatives (16), (17), (18), (19), (20) and (21)

Chemical shift of PATC-derivatives	H-1	H-2	H-3	H-4	H-1'	H-2'	H-3'
(16)	3.50	1.12					
(17)	3.75	1.15					
(18)	3.50	1.8	3.50				
(19)	3.50	1.57	1.57	3.50			
(20)	3.52	1.62	1.62	3.76	3.76	1.92	3.52
(21)*	3.78	1.68	1.68	3.78	3.78	1.92	3.52

*The chemical shifts of H-1", H-2" and H-3" are similar to H-1', H-2' and H-3' respectively.

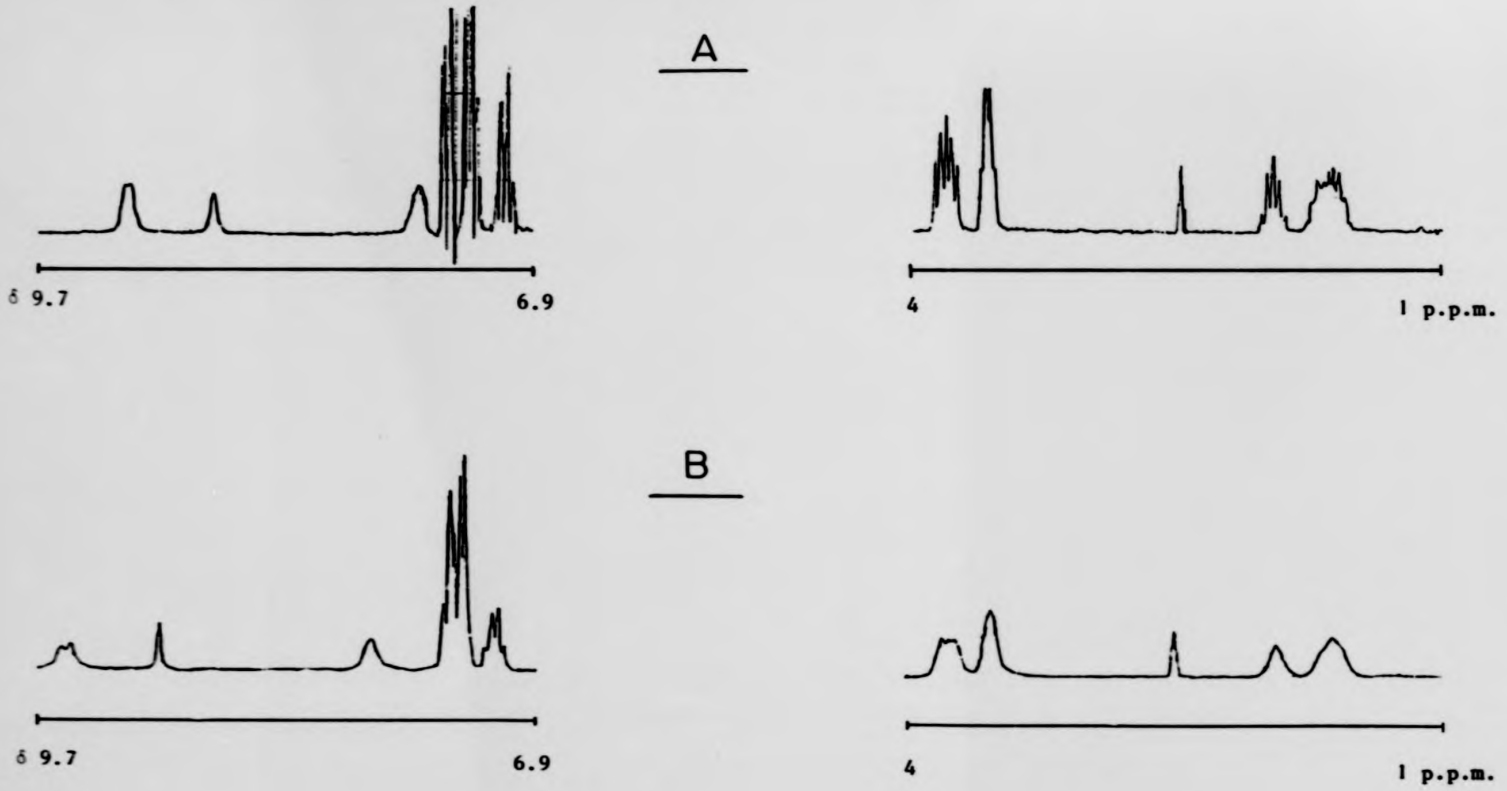

Fig. 3.C.2 The 220 MHz ^{1}H n.m.r. spectra of the phenylaminothiocarbonyl derivative of spermidine ($[^{2}H_{6}]$-DMSO, TMS)

(A) measured at 100°C

(B) measured at 30°C

mentioned above more than compensated for this disadvantage and put these derivatives among the most important pre-chromatographic derivatives known for polyamines.

3.C ASSIGNMENTS OF ^1H AND $\{^1H\}^{13}C$ n.m.r. SPECTRA

The assignment of resonances in the ^1H and $\{^1H\}^{13}C$ n.m.r. spectra for the phenylaminothiocarbonyl derivative of polyamines was undertaken with the aid of the spectra of model compounds. The assignment of signals in the ^1H n.m.r. spectra for the model compounds, N-(phenylaminothiocarbonyl)-ethylamine, [PATC-ethylamine (16)], N-(phenylaminothiocarbonyl)-diethylamine, [PATC-diethylamine (17)] and N,N'-*bis*(phenylaminothiocarbonyl)-1,3-diaminopropane, [PATC-1,3-diaminopropane (18)] presented no problem. The same can be said about N,N'-*bis*(phenylaminothiocarbonyl)-1,4-diaminobutane, [PATC-putrescine (19)] as can be seen from the relevant data in Table 3.C.1 and the structures in Fig. 3.C.1. It remains to justify the assignments of resonances in the ^1H n.m.r. spectra of the compounds N,N',N"-*tris*(phenylaminothiocarbonyl)-N-(3-aminopropyl)-1,4-diaminobutane, [PATC-spermidine (20)] and N,N',N",N"'-*tetrakis*-(phenylaminothiocarbonyl)-*bis*-N,N'-(3-aminopropyl)-1,4-diaminobutane, [PATC-spermine (21)]. For the ^1H n.m.r. spectra, assignments followed directly by comparison with the derivatives (16), (17), (18) and (19) (*cf.* Table 3.C.1).

Recording the ^1H n.m.r. spectra at 30°C gave poor resolution for the resonances from the alkyl chain. Resolution was greatly improved when the spectrum was measured at higher temperatures (e.g. 100°C) (*cf.* compound (20) Fig. 3.C.2). This could be due to an increase in the rate of equilibration of the many rotational

Fig. 3.C.2 The 220 MHz ^1H n.m.r. spectra of the phenylaminothiocarbonyl derivative of spermidine ($[^2H_6]$-DMSO, TMS)

(A) measured at 100°C

(B) measured at 30°C

FIG. 3-C-3

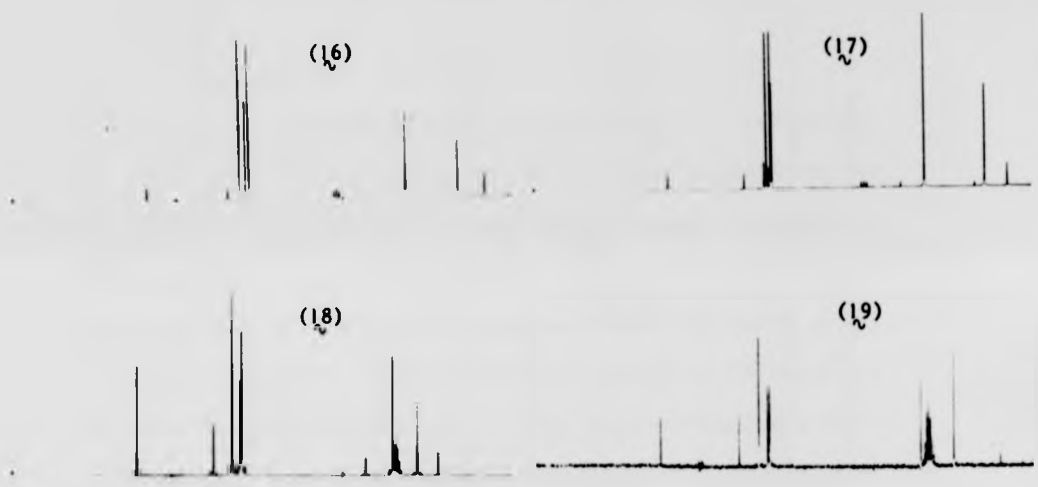

Fig. 3.C.3 The 22.6 MHz $\{^1H\}^{13}C$ n.m.r. spectra of phenylaminothiocarbonyl derivatives (16), (17), (18) and (19). Derivatives (16) and (17) were measured in ($CDCl_3$, TMS) while (18) and (19) were measured in ($[^2H_6]$-DMSO, TMS).

FIG. 3-C-4

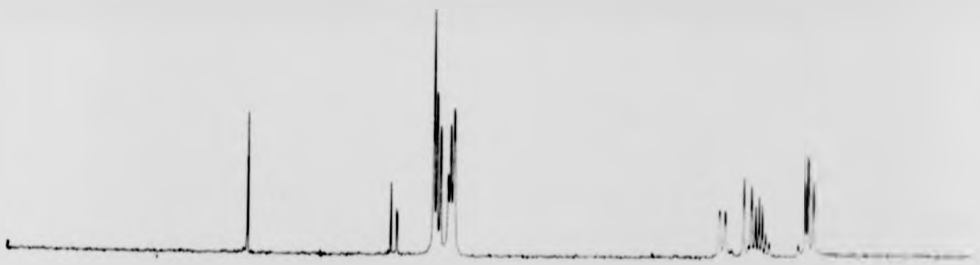

Fig. 3.C.4 $\{^1H\}^{13}C$ n.m.r. spectrum of phenylaminothiocarbonyl derivative of spermidine (5), in $[^2H_6]$-DMSO

TABLE 3.C.2 The $\{^{1}H\}^{13}C$ n.m.r. chemical shift assignments for PATC-derivatives (16), (17), (18), (19) and (20)

Chemical shift of PATC-derivatives	C-1	C-2	C-3	C-4	C-1'	C-2'	C-3'
(16)	40.17	14.30					
(17)	45.63	12.67					
(18)	41.48	28.49	41.48				
(19)	43.68	26.19	26.19	43.68			
(20)	50.19	24.72	26.15	43.69	48.63	26.86	41.74

$H_2NCH_2CH_2CH_2NH_2$

a

$H_2NCH_2CH_2CH_2CH_2NH_2$

b

$CH_3CH_2CH_2CH_2CH_2NH_2$

c

$CH_3CH_2CH_2CH_2CH_2NHCH_2CH_2CH_2CH_2CH_3$

d

FIG. 3-C-5

isomers of the derivatives.

The 22.63 MHz $\{^1H\}^{13}C$ n.m.r. spectra for the model compounds (16), (17), (18) and the PATC-putrescine (19) (Fig. 3.C.3) were easily assigned and need no further comment. It remains to justify the assignments made for compound (20). The $\{^1H\}^{13}C$ n.m.r. spectrum of compound (20) (Fig. 3.C.4 shows three distinct groups of signals) (*cf.* Table 3.C.2). The signals at 41.74 and 43.69 are assigned to C-3' and C-4, respectively by comparison with data for compounds (18) and (19). The signals at 48.63 and 50.19 [*cf.* C-1 for compounds (16) and (17)] are assigned to C-1' and C-1, respectively, by considering the C-1 of compounds (18) and (19). We assign the signal at 26.86 to C-2' [*cf.* C-2 positions for compounds (18) and (19)] and the signals at 24.72 and 26.15 to C-2 and C-3 respectively [*cf.* C-2 for compound (16) and C-2 for compound (17)]. The deshielding of C-1 for compound (17) compared to C-1 for compound (16) and the shielding of C-2 of compound (17) compared to C-2 of compound (16), parallel effects seen with, e.g. aminoalkanes, dialkylamines and trialkylamines[17] (Fig. 3.C.5). Data on the shielding and deshielding of these amino compounds can be seen in Table 3.C.3.

TABLE 3.C.3* ^{13}C n.m.r. chemical shift data for mono- and dialkylamines. For structures see Fig. 3.C.5.

Amine	C-1	C-2	C-3	C-4	C-5
a	39.5	39.2	39.5		
b	42.7	32.1	32.1	42.7	
c	42.5	34.0	29.7	23.0	14.3
d	50.8	30.7	30.3	23.3	14.6

*Data in this table was extracted from Ref. 17.

FIG. 3-D-1

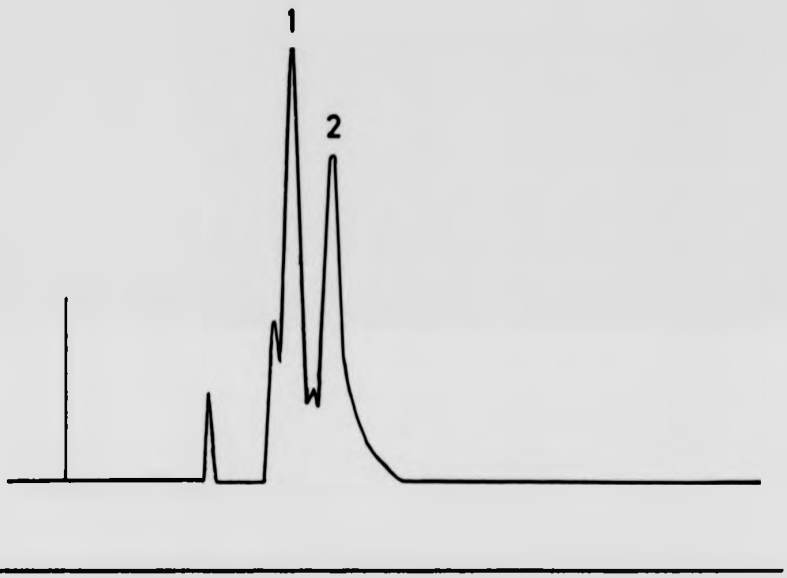

Fig. 3.D.1 Chromatogram for the hplc analysis of PATC-derivative of biosynthetic:

(1) putrescine (19)

(2) spermidine (20)

3.D ISOLATION, SEPARATION AND ANALYSIS OF PUTRESCINE AND SPERMIDINE FROM *E. COLI* CELLS

The inoculation of a standard salt medium, containing L-methionine, with the *E. coli* strain K_{12}, 630 Hf_{r1} (a methionine auxotroph) was carried out in small volumes to allow for maximum aeration of the cells. The polyamines putrescine and spermidine were obtained after extraction with trichloroacetic acid[18] and conversion into their PATC-derivatives. H.p.l.c. of the mixture of derivatives (Fig. 3.D.1) showed mainly two peaks corresponding to PATC-putrescine (19) and PATC-spermidine (20). T.l.c. and ^{1}H n.m.r. analyses were consistent with their identity as the PATC-derivatives (19) and (20).

The separation of the compounds (19) and (20) was done by p.l.c. and gave pure samples of the components (19) and (20) (^{1}H n.m.r. analysis).

This method of isolation and derivatisation of polyamines from *E. coli* has proved to be very useful for studying the biosynthesis of spermidine. The stability of the derivatives to air was checked using an authentic sample of the derivative (20). No change was observed after standing for 2 months in an aerated solution of chloroform.

The ease of separation, and the relatively high amount of isolated derivatives obtained by this method, has made it very easy to study the biosynthesis of several other polyamines in many different living species. Khan and Robins[19] have used our method to isolate homospermidine from the plant *Senecio isatideus*. This eventually provided some evidence for homospermidine being an intermediate in retronecine biosynthesis.

We noticed that there was no difference in the yield of

E. coli cells if the culture was inoculated with DL-methionine (0.1 g dm^{-3}) rather than L-methionine (0.05 g dm^{-3}). It is possible that the *E. coli* cells do not allow the D-isomer to pass the cell membrane, but leave it in the medium. Alternatively, by the action of an isomerase, the D-isomer is isomerised to the L-isomer. The latter possibility makes possible the utilisation of all the methionine.

3.E EXPERIMENTAL

3.E.1 Preparation of PATC-derivatives

1. Preparation of N-(phenylaminothiocarbonyl)-ethylamine [PATC-ethylamine (16)]

Ethylamine (1.8 g, 4 x 10^{-2} mol) was dissolved in ethanol (10 cm^3). To this mixture was added dropwise a solution of isothiocyanatobenzene (10.8 g, 8 x 10^{-2} mol) in ethanol (40 cm^3). After 1 h of stirring at room temperature a crystalline precipitate was formed, which was filtered off and washed with water (10 cm^3) and ethanol (10 cm^3). Recrystallisation of crude compound (16) was achieved by dissolving it in an excess of boiling ethanol. To the ethanolic solution was added decolourising charcoal and the mixture was stirred with warming for 30 minutes. The warm mixture was filtered through Celite and the volume of the filtrate was reduced to *ca.* 150 cm^3. After cooling at -20°C overnight the crystalline precipitate was filtered off and washed with cold ethanol (20 cm^3). The crystals were dried *in vacuo* (10^{-4} mmHg) to give pure (16) as white crystals (6.7 g, 94%).

M.p. 102-104°C (lit.[20] m.p. 106°C).

T.l.c. (neutral alumina/ethyl acetate/u.v. detection) R_f 0.64.

I.r. ν_{max} (cm^{-1}): 3240 (w, sh), 3210 (w, sh), 3110 (w, br), 1592 (s) and 1200 (m).

220 MHz ^1H n.m.r. ([$^2H_6$]-DMSO) at 30°C, δ 1.11 (3 H, t, J 7 Hz, H-2), 3.49 (2 H, p, J 7 Hz, H-1), 7.7 (1 H, brs, $CH_2\underline{NH}$), 9.4 (1 H, brs, Ph$\underline{NH}$) and 7.0-7.4 (phenyl protons) p.p.m.

220 MHz ^1H n.m.r. ([$^2H_6$]-DMSO) at 100°C, δ 1.12 (3 H, t, J 7 Hz, H-2), 3.5 (2 H, q, J 7 Hz, H-1) p.p.m., 7.5 (1 H, brs, $CH_2\underline{NH}$), 9.2 (1 H, brs, Ph$\underline{NH}$) and 7.0-7.4 (phenyl protons) p.p.m.

22.6 MHz {^1H}^{13}C n.m.r. ($CDCl_3$), δ 14.30 (C-2) and 40.17 (C-1) p.p.m. (*cf.* Fig. 3.C.3).

2. <u>Preparation of N-(phenylaminothiocarbonyl)-diethylamine [PATC-diethylamine (47)]</u>

Diethylamine (2.93 g, 4 x 10^{-2} mol) was dissolved in ethanol (10 cm^3). To this mixture was added dropwise a solution of PhNCS (10.8 g, 8 x 10^{-2} mol) in ethanol (40 cm^3). After 2 h of stirring at room temperature, compound (47) separated as an oil. All attempts to crystallise the oily product (47) have failed. Purification of the crude product (47) was achieved by decanting the solvent, adding ethanol (25 cm^3) and decanting again (two times). The oil was then dissolved in ethanol (500 cm^3). To the ethanolic solution was added decolourising charcoal and the mixture was stirred with warming for 2 h. The warm mixture was filtered through Celite. Ethanol was evaporated from the filtrate under reduced pressure to leave behind a colourless oil which was dried *in vacuo* (10^{-4} mmHg) to give pure (47) (8.94 g, 98%).

Oil, (lit.[20] m.p. 34°C)

T.l.c. (neutral alumina/ethyl acetate/u.v. detection) R_f 0.74

I.r. ν_{max} (cm^{-1}): 3242 (w, sh), 3213 (w, sh), 3110 (w), 1590 (s) and 1205 (m).

220 MHz ^1H n.m.r. ([$^2H_6$]-DMSO) at 30°C, δ1.15 (6 H, t, J 7 Hz,

2 x H-2), 3.75 (4 H, q, J 7 Hz, 2 x H-1), 8.9 (1 H, s, PhNH) and 7.4-7.8 (phenyl protons) p.p.m.

(N.B. broad signals at 30°C were sharpened when the spectrum was measured at 100°C.)

22.6 MHz $\{^1H\}^{13}C$ n.m.r. ($CDCl_3$) δ 12.67 (2 x C-2) and 45.63 (2 x C-1) p.p.m. (Fig. 3.C.3)

3. Preparation of N,N'-*bis*(phenylaminothiocarbonyl)-1,3-diaminopropane [PATC-1,3-diaminopropane (18)]

1,3-Diaminopropane (2.96 g, 4 x 10^{-2} mol) was dissolved in ethanol (10 cm^3). To this mixture was added dropwise a solution of PhNCS (16.2 g, 12 x 10^{-2} mol) in ethanol (40 cm^3). After 1 h of stirring at room temperature a crystalline precipitate appeared, which then was filtered off and washed with water (10 cm^3) and ethanol (10 cm^3). Recrystallisation of compound (18) was achieved by dissolving it in excess of boiling ethanol. To the ethanolic solution was added decolourising charcoal and the mixture was stirred with warming for 30 minutes. The warm mixture was filtered through Celite and the volume of the filtrate was reduced to *ca.* 200 cm^3. After cooling at -20°C overnight, the crystalline precipitate was collected and washed with cold ethanol (20 cm^3). The crystals were dried *in vacuo* (10^{-4} mmHg) to give compound (18) as white crystals (12.8 g, 94%).

M.p. 115-119°C (lit.[20] m.p. 116°C)

T.l.c. (neutral alumina/ethyl acetate/u.v. detection) R_f 0.46

I.r. ν_{max} (cm^{-1}) 3450 (w, sh), 3285 (w, sh), 3135 (w, br), 1595 (s) and 1170 (m)

220 MHz ^1H n.m.r. ([$^2H_6$]-DMSO) at 30°C, δ 1.8 (2 H, p, J 7 Hz, H-2), 3.5 (4 H, m, H-1 and H-3), 7.8 (2 H, br s, $2XCH_2NH$), 9.5 (2 H, br s, 2 x PhNH) and 7.0-7.4 (phenyl protons) p.p.m.

(N.B. broad signals at 30°C were sharpened when the spectrum was measured at 100°C.)

22.6 MHz $\{^1H\}^{13}C$ n.m.r. ($[^2H_6]$-DMSO) δ 28.49 (C-2) and 41.48 (C-1 and C-3) p.p.m. (*cf.* Fig. 3.C.3)

4. Preparation of N,N'-*bis*(phenylaminothiocarbonyl)-1,4-diaminobutane [PATC-putrescine (19)]

1,4-Diaminobutane (1.76 g, 2×10^{-2} mol) was dissolved in water (40 cm^3) and a solution of PhNCS (8.1 g, 6×10^{-2} mol) in ethanol (50 cm^3) was added. After 1 h of stirring at room temperature, the mixture was cooled to -20°C with stirring for 2 h to give a more easily filtered crystalline precipitate. The slight yellow crystalline product was filtered off and washed with water (50 cm^3). The precipitate was dissolved in excess of boiling ethanol. To the ethanolic solution was added decolourising charcoal and the mixture was stirred with heating for 1 h. The hot mixture was filtered through Celite and the volume of filtrate was reduced until the solution was turbid. The solution was left at -20°C overnight and the crystalline precipitate was filtered off and washed with cold ethanol (20 cm^3). The white crystals were dried *in vacuo* (10^{-4} mmHg) to give compound (19) (6.8 g, 95%).

M.p. 178-180°C (lit.[21] m.p. 177-179°C)

T.l.c. (neutral alumina/ethyl acetate/u.v. detection) R_f 0.4

U.v. λ_{max} 252 nm, ε 2.6×10^4 (in methanol).

C.H.N. combustion analysis - found: C, 60.25; H, 6.30; N, 15.60%.

$C_{18}H_{22}N_4S_2$ requires: C, 60.30; H, 6.20; N, 15.65%.

I.r. ν_{max} (cm^{-1}): 3250 (w), 3150 (m, br), 3030 (w), 1590 (s) and 1180 (s).

220 MHz ^1H n.m.r. ($[^2H_6]$-DMSO) at 30°C, δ 3.5 (4 H, br s, H-1 and H-4), 1.57 (4 H, br s, H-2 and H-3), 7.76 (2 H, br s, 2 x $CH_2\underline{NH}$),

9.43 (2 H, br s, 2 x PhNH) and 7.0-7.4 (phenyl protons) p.p.m. (N.B. raising the temperature to 100°C resulted in the sharpening of the signals.)

22.6 MHz $\{^1H\}^{13}C$ n.m.r. ($[^2H_6]$-DMSO) δ 26.19 (C-2 and C-3) and 42.68 (C-1 and C-4) p.p.m. (Fig. 3.C.3).

5. Preparation of N,N',N''-*tris*(phenylaminothiocarbonyl)-N-(3-aminopropyl)-1,4-diaminobutane [PATC-spermidine (20)]

Spermidine (2.9 g, 2 x 10^{-2} mol) was dissolved in water (40 cm^3). To this solution was added a solution of PhNCS (10.8 g, 8 x 10^{-2} mol) in ethanol (50 cm^3). After 1 h of stirring at room temperature the mixture was cooled at -20°C with stirring for 2 h to give a coagulated precipitate. The solvent was decanted off and the solid residue was dissolved in excess of boiling ethanol. To the ethanolic solution was added decolourising charcoal and the mixture was stirred with heating for 1 h. The hot mixture was filtered through Celite and the volume of the filtrate was reduced by boiling, with the addition of some water. The boiling and the addition of water was continued until the solution was turbid. The solution was left at -20°C overnight and the crystalline precipitate was filtered off and washed with cold ethanol (20 cm^3). The white crystals were dried *in vacuo* at 10^{-4} mmHg to give compound (20) (11 g, 97%).

M.p. 141-143°C.

T.l.c. (neutral alumina/ethyl acetate/u.v. detection) R_f 0.31.

U.v. λ_{max} 252 nm, ε 4.2 x 10^4 (in methanol).

C.H.N. combustion analysis - found: C, 61.20; H, 6.25; N, 15.10%.

$C_{28}H_{34}N_6S_3$ requires: C, 61.10; H, 6.20; N, 15.25%.

I.r. ν_{max} (cm^{-1}): 3280 (w, sh), 3260 (w), 3150 (w, sh), 1595 (s)

and 1172 (s).

220 MHz ^{1}H n.m.r. ([$^{2}H_6$]-DMSO) at 30°C, δ 1.62 (4 H, m, H-2 and H-3), 1.92 (2 H, br p, H-2'), 3.52 (4 H, m, H-3' and H-4), 3.76 (4 H, m, H-1 and H-1'), 7.77 (2 H, br s, 2 x $CH_2\underline{NH}$), 8.95, 9.42 and 9.48 (each is 1 H, br s, Ph$\underline{NH}$) and 7.0-7.4 (phenyl protons) p.p.m. [see Fig. 3.C.2(B)].

220 MHz ^{1}H n.m.r. ([$^{2}H_6$]-DMSO) at 100°C, δ 1.65 (4 H, m, H-2 and H-3), 1.95 (2 H, p, J 7 Hz, H-2'), 3.55 (4 H, q, J 7 Hz, H-3' and H-4), 3.8 (4 H, p, J 7 Hz, H-1 and H-1'), 7.62 (2 H, br s, 2 x $CH_2\underline{NH}$), 8.79, 9.26 and 9.48 (each is 1 H, br s, Ph$\underline{NH}$) and 7.0-7.4 (phenyl protons) p.p.m. [see Fig. 3.C.2(A)].

22.6 MHz {^{1}H}^{13}C n.m.r. ([$^{2}H_6$]-DMSO) δ 24.72 (C-2), 26.15 (C-3), 26.86 (C-2'), 43.69 (C-4), 41.74 (C-3'), 48.0 (C-1') and 50.19 (C-1) p.p.m. (see Fig. 3.C.4).

6. Preparation of N,N',N'',N'''-*tetrakis*(phenylaminothiocarbonyl)-bis-N,N'-(3-aminopropyl)-1,4-diaminobutane [PATC-spermine (21)]

Spermine (4 g, 2 x 10^{-2} mol) was dissolved in water (40 cm^3) and a solution of PhNCS (13.5 g, 10 x 10^{-2} mol) in ethanol (50 cm^3) was added with stirring. After 1 h at room temperature the mixture was cooled to -20°C with stirring for 2 h. An oil of slightly yellow colour separated. The mixture was cooled further to -78°C. Gradual warming with scratching gave a yellow crystalline precipitate. The precipitate was dissolved in excess of boiling ethanol. To the ethanolic solution was added decolourising charcoal and the mixture was stirred with heating for 1 h. The hot mixture was filtered through Celite and the volume of the filtrate was reduced under pressure until the solution was turbid. The solution was left at -20°C overnight and the crystalline precipitate was filtered off and washed with cold ethanol (20 cm^3). Drying *in vacuo*

at 10^{-4} mmHg gave pure product (21) (13.9 g, 92%).

M.p. 173-175°C.

T.l.c. (neutral alumina/ethyl acetate/u.v. detection) R_f 0.19.

U.v. λ_{max} 252 nm, ϵ 6.5×10^4 (in methanol).

C.H.N. combustion analysis found: C, 61.40; H, 6.10; N, 15.20%.

$C_{38}H_{46}N_8S_4$ requires: C, 61.40; H, 6.25; N, 15.10%.

I.r. ν_{max} (cm^{-1}): 3370 (m), 3200 (w, br), 1595 (m), and 1182 (m, br).

220 MHz ^1H n.m.r. ([$^2H_6$]-DMSO) at 30°C, δ 1.68 (4 H, br s, H-2 and H-3), 1.92 (4 H, br p, H-2' and H-2"), 3.52 (4 H, m, H-3' and H-3"), 3.78 (8 H, m, H-1, H-1', H-1'● and H-4), 7.8 (2 H, br s, 2 x $CH_2\underline{NH}$), 8.96 (2 H, s, 2 x Ph$\underline{NH}$), 9.5 (2 H, br s, 2 x Ph$\underline{NH}$), and 7.0-7.4 (phenyl protons) p.p.m.

(N.B. raising the temperature to 100°C resulted only in the sharpening of the signals.)

3.E.2 Analyses of PATC-derivatives by t.l.c. and h.p.l.c.

1. T.l.c: For analytical t.l.c. of PATC-derivatives, 0.25 mm thick layers of Kieselgel 60 HR reinst were used. Elution was carried out with dichloromethane/acetonitrile (87/13, v/v) followed by iodine detection.

A mixture of the PATC-polyamines was spotted on the plates (5 x 20 x 0.25 mm). After elution and developing, the following R_f's were recorded: PATC-putrescine (19) R_f 0.34, PATC-spermidine (20) R_f 0.25 and PATC-spermine (21) R_f 0.18.

The spots were of a nicely rounded shape, and were well separated from each other. No tailing, which is characteristic of other polyamine derivatives (e.g. dansyl), was observed in our analyses.

2. H.p.l.c: The analysis of PATC-derivatives was attempted on three different columns.

(a) μC_{18}: A reverse-phase column, which failed to separate a mixture of the PATC-derivatives. Elution was tried with different mixtures of water/methanol. No separation was achieved and all PATC-derivatives gave one wide peak of the same R_t.

(b) Partisil: A silica gel column which showed an appreciable separation for the PATC-derivatives (16), (17), and (19). Elution was carried out with chloroform/methanol (99/1, v/v). Compounds (20) and (21) gave overlapping peaks in this system.

(c) μ-Porasil: A silica gel column which provided a good separation for the compounds (16), (18), (19), (20) and (21). Compound (17) was overlapping with compound (18), so it was eventually excluded from the analysis. The chromatograms were developed using isocratic elution with chloroform/methanol (99/1, v/v), with a flow rate of 0.5 $cm^3 min^{-1}$ and a pressure reading of 500 psi. The pure PATC-derivatives (16), (18), (19), (20), and (21) each gave a single peak when tested separately by this system. A mixture of the derivatives (16), (18), (19), (20), and (21) were analysed by this system to give five nicely separated peaks with the following retention times [relative to (16)]: R_t (16), 1.00; R_t (18), 1.12; R_t (19), 1.21; R_t (20), 1.36 and R_t (20), 1.87 (*cf.* Fig. 3.B.1).

The μ-Porasil column was kept in n-hexane when not in use. After *ca.* 100 experiments the column was deactivated and reactivated. This was necessary to clean the column of any polar compounds or impurities, which would give unsatisfactory results in a later analysis. The column was washed with ethyl acetate, methanol, acetone and water (100 cm^3) and was reac-

tivated by reversing the order of the washing, i.e. acetone, methanol, ethyl acetate and chloroform (N.B. we have noticed that this washing is important for the reproducibility of the analyses).

3.E.3 Percentage recovery of polyamines *via* PATC-derivatives

The recovery of polyamines from their solution in aq. trichloroacetic acid (TCA) was measured by dissolving each of the polyamines, putrescine, spermidine and spermine (10^{-2} mol) in 0.4 mol dm^{-3} aq. TCA (7 cm^3). The solutions were extracted with ether (3 x 10 cm^3) rejecting the ethereal layers. The aqueous layers were adjusted to pH 9 by addition of 1 mol dm^{-3} aq. sodium carbonate. Isothiocyanatobenzene (1 mol equiv. + 1 mol equiv. per amino group) in ethanol (5 cm^3) was added to each reaction mixture and the resulting solutions were stirred for 1 h at room temperature. The solutions were extracted with dichloromethane (3 x 50 cm^3) and the combined extracts were washed with saturated aq. sodium bicarbonate (2 x 50 cm^3), 1 mol dm^{-3} sulphuric acid (2 x 50 cm^3) and water (2 x 50 cm^3). The dichloromethane layer of each reaction mixture was dried and evaporated, followed by pumping at 10^{-4} mmHg/40°C to leave white residues of the PATC-derivatives. The recovered derivatives were weighed and gave yields of 95, 92 and 90% for PATC-putrescine, PATC-spermidine and PATC-spermine respectively.

3.E.4 Rate of formation and hydrolysis of PATC-spermidine (20)

1. Rate of formation of compound (20)

Solutions containing spermidine and PhNCS were prepared in aqueous ethanol (8 or 10 cm^3, 1/1,v/v) and were incubated with

stirring at room temperature:

(a) spermidine (10^{-2} mol) and PhNCS (3×10^{-1} mol) in 10 cm^3 ethanol,

(b) spermidine (10^{-2} mol) and PhNCS (4×10^{-1} mol) in 10 cm^3 ethanol,

(c) spermidine (15×10^{-3} mol) and PhNCS (2×10^{-1} mol) in 8 cm^3 ethanol,

(d) spermidine (15×10^{-4} mol) and PhNCS (2×10^{-2} mol) in 8 cm^3 ethanol.

Mixtures (a) and (b) were analysed after 5 minutes by t.l.c. for PATC-spermidine and for free spermidine, using two different systems. [For PATC-spermidine see above. For t.l.c. analysis of free spermidine, silica gel plates (0.25 mm thick layers of MN Kieselgel plates) were used. Elution was done with methanol-formic acid (8/2, v/v) followed by ninhydrin detection.] The analysis showed the existence of PATC-spermidine, but free spermidine was undetectable. Mixture (b) showed residual PhNCS, whereas mixture (a) did not. For mixtures (c) and (d), t.l.c. showed both PATC- and free spermidine after 1 minute, but after 5 minutes no free spermidine could be detected.

2. Hydrolysis of compound (20)

Compound (20) (0.3 g, 55×10^{-2} mmol) was dissolved in concentrated hydrochloric acid (5 cm^3) and the mixture was boiled under reflux overnight. Evaporation to dryness under reduced pressure gave a white precipitate that was dissolved in water (15 cm^3) and the resulting solution was evaporated to dryness under reduced pressure. The last step (i.e. addition of water and evaporation) was repeated twice and gave a white residue.

Pre-cooled ethanol (15 cm^3) was added to the residue and the mixture was filtered off. The collected white crystals were washed with cold ethanol and dried *in vacuo* to give spermidine trihydrochloride (0.1 g, 74%). The 220 MHz ^1H n.m.r. spectrum (D_2O) of this trihydrochloride was completely identical to that of authentic spermidine trihydrochloride, δ 1.79 (6 H, m), 2.10 (2 H, p, J 7 Hz) and 3.0-3.3 (8 H, m) p.p.m. The filtrate contained no spermidine trihydrochloride and was mainly aniline hydrochloride (analysis by ^1H n.m.r. after evaporation). Free spermidine can be obtained by passing the hydrochloride through an ion exchange column [Amberlite IRA-400 ^-OH form, (1 x 10 cm)], using water as eluent. Evaporation of the first (100 cm^3) fraction under reduced pressure left behind a pure hydrochloride-free spermidine.

3.E.5 Isolation and separation of putrescine and spermidine from *E. coli* cells

1. *E. coli* strain and growth media

The *E. coli* strain K_{12}, 630 Hf_{r1} (a methionine auxotroph) used in this study was purchased from "The National Collections of Industrial and Marine Bacteria, Aberdeen, Scotland". The purity of the strain was maintained by a monthly transfer of pure colonies to new plates of media.

Growth media were prepared from the following solutions.

SOLUTION A: Contains K_2HPO_4 (140 g), KH_2PO_4 (36 g) per dm^3 H_2O.

SOLUTION B: Contains $MgSO_4.7H_2O$ (25 g), $CaCl_2.2H_2O$ (2 g), Fe-EDTA (1.5 g) and NaCl (20 g) per dm^3 H_2O.

SOLUTION C: Contains $ZnSO_4.7H_2O$ (3 mg), $NaMoO_4.2H_2O$ (4 mg), H_3BO_3 (50 mg), $MnSO_4.4H_2O$ (40 mg),

Pre-cooled ethanol (15 cm^3) was added to the residue and the mixture was filtered off. The collected white crystals were washed with cold ethanol and dried *in vacuo* to give spermidine trihydrochloride (0.1 g, 74%). The 220 MHz ^1H n.m.r. spectrum (D_2O) of this trihydrochloride was completely identical to that of authentic spermidine trihydrochloride, δ 1.79 (6 H, m), 2.10 (2 H, p, J 7 Hz) and 3.0-3.3 (8 H, m) p.p.m. The filtrate contained no spermidine trihydrochloride and was mainly aniline hydrochloride (analysis by ^1H n.m.r. after evaporation). Free spermidine can be obtained by passing the hydrochloride through an ion exchange column [Amberlite IRA-400 ^-OH form, (1 x 10 cm)], using water as eluent. Evaporation of the first (100 cm^3) fraction under reduced pressure left behind a pure hydrochloride-free spermidine.

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SOLUTION C: Contains $ZnSO_4.7H_2O$ (3 mg), $NaMoO_4.2H_2O$ (4 mg), H_3BO_3 (50 mg), $MnSO_4.4H_2O$ (40 mg),

$CuSO_4.5H_2O$ (4 mg) and 1 cm^3 of aq. $CoCl_2.6H_2O$ (0.2 g dm^{-3}) per 200 cm^3 H_2O.

SOLUTION D: Contains thiamine.HCl (50 mg), calcium pantothenate (100 mg) and 1 cm^3 of biotin (50 μg dm^{-3}) per 50 cm^3 H_2O.

SOLUTION H: Ammonium acetate (27.5 g) per dm^3 H_2O.

The final growth medium was made up from 10 cm^3 of solution B, 10 cm^3 of solution H, 1 cm^3 of solution D and 1 cm^3 of solution C, made up to 1 dm^3 with water. This solution was sterilised in an autoclave and then supplied with L-methionine solution (50 mg/10 cm^3), solution A (10 cm^3) and glucose (10 cm^3) (20%, w/v), which were sterilised separately.

2. Isolation of polyamines from *E. coli* cells[18]

The standard medium (10 x 1 dm^3) in 10 flasks of 2 dm^3 capacity was inoculated with *E. coli* cells K_{12}, $630Hf_{r1}$. The culture was incubated at 37°C for 30 h with aeration. The cells were harvested by centrifugation (6 x 10^3 r.p.m.) and were washed with aqueous NaCl and KCl solutions (200 cm^3, 0.25 w/v). The wet cells (30 g) were extracted with 0.4 mol dm^{-3} trichloroacetic acid (3 x 50 cm^3). The combined extracts were filtered through Celite and extracted with ether (3 x 300 cm^3), rejecting the ethereal layers (N.B. the procedure up to this stage was modified from Ref. 18). The aqueous layer was reduced to 10 cm^3 by evaporation under reduced pressure and the pH was adjusted to 9 by the addition of 1 mol dm^{-3} aqueous sodium carbonate. Isothiocyanatobenzene (1 cm^3) in ethanol (8 cm^3) was added and the resulting solution was stirred for 1 h at room temperature. The reaction mixture was then extracted with dichloromethane (3 x 100 cm^3), and the combined extracts were washed

with saturated aqueous sodium bicarbonate (2 x 100 cm^3), 1 mol dm^{-3} sulphuric acid (2 x 100 cm^3) and water (2 x 100 cm^3). The dichloromethane layer was dried and evaporated under reduced pressure to give a white residue of the phenylaminothiocarbonyl derivatives of putrescine and spermidine (by ^1H n.m.r. analysis) containing residual isothiocyanatobenzene which was removed by pumping at 10^{-5} mmHg/room temperature. The removal of residual PhNCS was necessary before the h.p.l.c. analysis of the isolated mixture, whereas it was not important for t.l.c. analysis. The residue was checked by t.l.c. on a silica gel plate (Kieselgel 60 HR reinst, 0.25 mm thick). Elution with dichloromethane/acetonitrile (87/13, v/v) gave two spots with R_f's 0.34 and 0.25 corresponding to PATC-putrescine and PATC-spermidine, respectively. Small amounts of polar impurities were also detected. The h.p.l.c. analysis was carried out as described for the authentic PATC-derivatives and showed mainly PATC-putrescine and PATC-spermidine, in addition to traces of residual PhNCS, and small peaks of unknown impurities before the peaks of the derivatives (19) and (20) (see Fig. 3.D.1).

3. Separation and purification of compounds (19) and (20) by p.l.c.

The mixture of compounds (19) and (20) was separated and purified by preparative layer chromatography. The mixture was dissolved in a minimum volume of dichloromethane. The solution was applied onto a silica gel plate [Kieselgel 60 HR reinst (0.5 mm x 20 x 100 cm)] . Double elution with dichloromethane/acetonitrile (9/1, v/v) gave two clear bands (detection with iodine was not necessary). Each band was scraped off and extracted with acetone (2 x 200 cm^3). Evaporation of each extract gave white crystalline residues.

H.p.l.c. analysis of each residue showed one single peak corresponding to either PATC-putrescine (70 mg) or PATC-spermidine (55 mg). ^{1}H n.m.r. spectra of the fractions were consistent with their identity as compounds (19) and (20) [comparison with spectra of authentic derivatives (19) and (20)].

3.E.6 Preparation of 4-methoxybenzoylamides of polyamines

To a solutions of polyamines (10 mmol) in 2 mol dm^{-3} aqueous sodium hydroxide (15 cm^3) was added a solution of p-anisoyl chloride (1 mol equiv. + 1 mol equiv. per amino group). The mixtures were stirred overnight at room temperature when a white sticky precipitate appeared in each reaction mixture. The supernatant of each solution was decanted off and the solid residue was dissolved in dichloromethane (50 cm^3). The organic solutions were each washed with 1 mol dm^{-3} sulphuric acid (3 x 50 cm^3), saturated aqueous sodium bicarbonate (3 x 50 cm^3) and water (2 x 50 cm^3). The organic solutions were separated, dried and evaporated under reduced pressure to leave behind an oily residue containing a small amount of acid anhydride. The oily residue was applied to a short column of silica gel. Elution with dichloromethane/methanol (94/6, v/v) gave fractions containing the 4-methoxybenzoylamide of a polyamine and these were combined and evaporated under reduced pressure to give slightly yellow crystals. The crude product was dissolved in ethanol (150 cm^3), decolourising charcoal was added and the solution stirred for 2 h at room temperature. The mixture was filtered through Celite, and the filtrate was reduced to 50 cm^3 under reduced pressure. The resulting solution was left at -20°C overnight to give white crystals of the 4-methoxybenzoylamide

of putrescine (68%), spermidine (63%) and spermine (60%), respectively.

The CHN combustion analyses gave:

4-Methoxybenzoylamide of putrescine, Found: C, 67.58; H, 6.70; N, 7.82%

$C_{20}H_{24}N_2O_4$ Requires: C, 67.39; H, 6.79; N, 7.86%

4-Methoxybenzoylamide of spermidine, Found: C, 67.58; H, 6.98; N, 7.44%

$C_{31}H_{37}N_3O_6$ Requires: C, 67.99; H, 6.81; N, 7.67%

4-Methoxybenzoylamide of spermine, Found: C, 64.91; H, 6.83; N, 7.17%

$C_{42}H_{50}N_4O_8$ Requires: C, 68.27; H, 6.82; N, 7.58%

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CHAPTER 4

SYNTHESES OF DEUTERIUM LABELLED AMINO ACIDS

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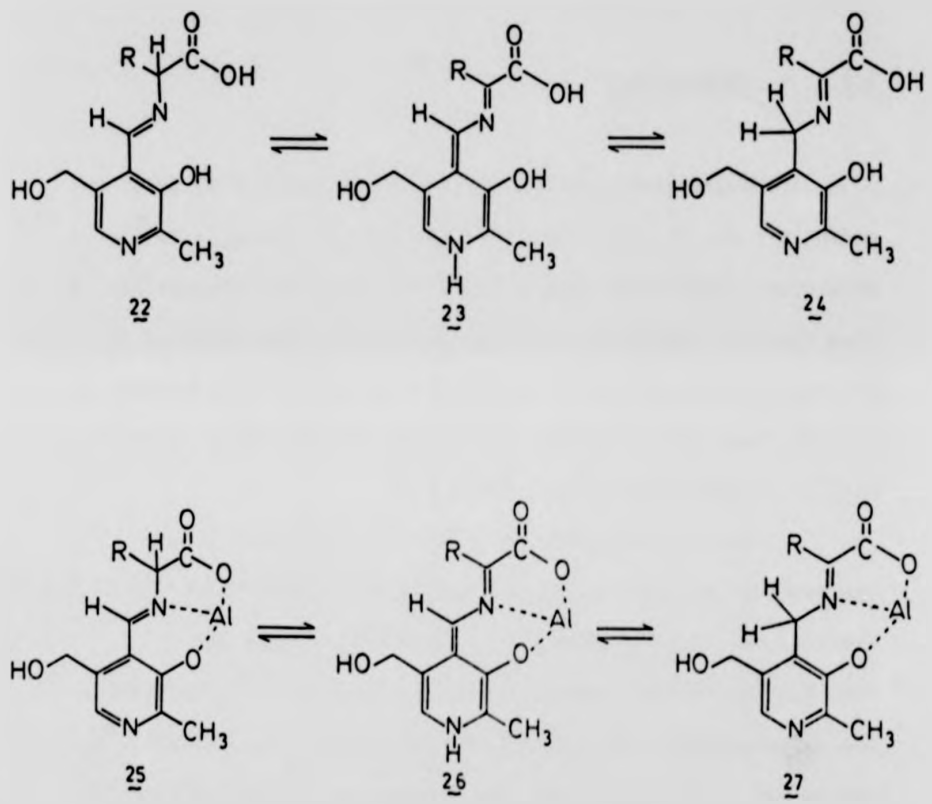
4.A INTRODUCTION

Methionines labelled at either C-2 and/or C-3, or at C-3 and/or C-4 are of great value for studying the catabolic fate of methionine, especially that of its alkyl chain (see Chapter 5). In this Chapter, syntheses of methionines specifically labelled at C-3 and C-4 with deuterium and of known relative configuration, will be discussed in detail. The routes of these syntheses were originally developed by D. C. Billington.

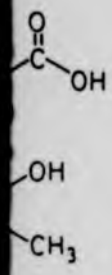
We have also modified a literature method[1] for the syntheses of *rac.*[2,3,3-$^2H_3$] and *rac.*[3,3-$^2H_2$]methionine, *rac.*[2,3,3-$^2H_3$]-leucine, *rac.*[2,3-$^2H_2$]isoleucine, *rac.*[2-$^2H_1$]alanine and *rac.*[2,3-$^2H_2$]valine. Resolution of the *rac.*[2,3,3-$^2H_3$]methionine was achieved following a literature procedure[2]. The L-[2,3,3-$^2H_3$]-methionine obtained was used for studying the biosynthesis of spermidine in *E. coli* cells (*cf.* Chapter 5).

4.B *Rac.* AMINO ACIDS

In 1974, a method for the selective deuteration of amino acids at the α- and/or β-position(s) was published[1]. This method made use of the observation that the relative exchange of α- and β-protons in an amino acid/aluminium(III)/pyridoxal (vitamin B-6) complex is a pH-dependent process. Vitamin B-6 is an essential cofactor for enzymes which carry out a multitude of amino acid reactions. In



Scheme 4.B.1 Postulated mechanism for the transamination and racemisation reactions of amino acids and vitamin B-6 in presence or absence of Al(III).



1954, Snell[3] showed that the mechanism for two of the vitamin B-6 reactions, which involve cleavage of the α-hydrogen of amino acids, i.e. racemisation and transamination, could be represented by interconversions of the amino acid - vitamin B-6 complexes (Schiff's bases) of (22), (23) and (24) (see Scheme 4.B.1) or their metal-ion complexes (25), (26) and (27). Snell[4] has also shown that with some metal ions the ratio of (22) to (24) at equilibrium is highly pH dependent. This could reflect some difference in stability between (23) and (24), although it was suggested to be due to different pH maxima for the transamination and racemisation reaction. An amino acid may react with pyridoxal to form the aldimine (22). Racemisation may occur *via* (22) → (23) → enantiomer of (22), while transamination to the keto acid is the result of (22) → (23) → (24) followed by hydrolysis of (24). Abbott *et al.*[1] have demonstrated that selectivity between these two reactions can occur through modification of coenzyme (pyridoxal) in the absence of enzyme. In the presence of excess of amino acid the Schiff's base (22) is most likely to be formed. This aldimine might tautomerise to (24) and back to (22) *via* (23), or to (23) and back to enantiomer of (22) without going to (24). After returning to (22) this aldimine is at equilibrium with the free amino acid and after many passes through this sequence of reactions, all the amino acid will have been acted upon. From the above analysis it would be expected that if the reactions were done in the presence of ^2H_2O, the α-position of the amino acid would become deuterated. Actually, it was found[5] that both the α- and β-positions were deuterated. This was explained[6,7] as arising from imine-enamine tautomerisation of (24).

Abbott and his coworkers[1] have carried out pH-dependent studies on these exchange reactions in the presence of Al(III), which

is supposed to give complexes (25), (26) and (27) (Scheme 4.B.1). This metal serves as a model for the enzymes involved in transamination and racemisation. They concluded that when these exchanges were carried out at pH *ca.* 5 the α- and β-protons of an amino acid are exchanged at comparable rates, whereas at pH *ca.* 10 the rate of α-exchange greatly exceeds that of β-exchange. The absence of Al(III) from the reaction mixture gave no significant deuteration. It was concluded that Al(III) is important for the transamination, which is considered to go from (25) → (26) → (27) and in reverse, and also for the racemisation, (25) → (26) → enantiomer of (25). The key intermediate in the deuteration of amino acids in the presence of Al(III) is complex (27). This should have two tautomeric forms (the imine shown and a derived enamine) at pH 5, which lead to the deuteration of α- and β-positions. At pH 10 the imine is presumably not converted to the enamine (because this requires N-protonation), and so exchange only occurs at the α-position.[8] We have applied the method of Ref. 1 (N.B. this reference describes only the preparation of deuterated alanine, valine and α-amino butyric acid) to the preparation of *rac.*[2,3,3-$^2H_3$]methionine, *rac.*[3,3-$^2H_2$]methionine, *rac.*[2,3,3-$^2H_3$]leucine, *rac.*[2,3-$^2H_2$]isoleucine and we repeated the syntheses of *rac.*[2,3-$^2H_2$]valine and *rac.*[2-$^2H_1$]alanine. The reaction conditions for each amino acid were optimised by monitoring the exchange by 220 MHz ^1H n.m.r. spectroscopy. The labelled amino acids were directly crystallised from the reaction mixture in a good yield (N.B. no yields were reported in Ref. 1).

The synthesis of *rac.*[3,3-$^2H_2$]methionine was first tried in our laboratory by D. C. Billington[9], who claimed a 97% yield of pure product. In trying to synthesise more of this compound,

following this procedure, the author obtained a yield of 105%! Checking the product for metal ions by carrying out a flame test indicated the presence of a large amount of sodium chloride. This could only arise from the sodium hydroxide added to the reaction mixture to adjust the pH to 10.2. To avoid this problem the author used lithium hydroxide* to adjust the pH of the reaction mixture. The isolated *rac.*[3,3-$^2H_2$]methionine (yield 87%), when tested for lithium ions, gave a negative result (flame test).

The time allowed for each amino acid to exchange was variable, depending on the solubility of the amino acid, besides structural factors. Methionine was the fastest amino acid to exchange at both its α- and β-positions. Monitoring the reaction by ^1H n.m.r. spectroscopy showed that *ca.* 98% of the α-position had been exchanged after 24 h., whilst *ca.* 80% of the β-position was deuterated. The 220 MHz ^1H n.m.r. of unlabelled methionine shows resonances at (^2H_2O, TSS) δ 2.12 (3 H, s), 2.2 (2 H, m), 2.7 (2 H, t) and 3.82 (1 H, t) p.p.m. The disappearance of the signals at δ 3.82 and 2.2 p.p.m. (corresponding to α- and β-protons, respectively) were used to follow the production of *rac.*[2,3,3-$^2H_3$]methionine. It should be mentioned that part of the resonance of the β-protons is covered by the resonance of the methyl group of methionine. Hence, accurate judgement (by ^1H n.m.r. analysis) about the extent of deuteration at this position is rather difficult. Because of this, *rac.*[2,3,3-$^2H_3$]-methionine was analysed by e.i.m.s. to quantify the total amount of deuterium present. The N-trifluoroacetyl butyl ester[10] derivative was made and examined by m.s. and showed an overall deuterium content of ≥ 86%. From the ^1H n.m.r. spectrum of *rac.*[2,3,3-$^2H_3$]methionine we

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*Lithium chloride is much more soluble in ethanol than sodium chloride.

estimated that most of the α-position had been exchanged (≥ 98%) leaving ≥ 80% of deuterium on the β-position.

The exchange reaction of leucine took 15 days for complete deuteration. In the ^{1}H n.m.r. spectrum of unlabelled leucine, signals at δ 3.72 and 1.68 p.p.m. correspond to α- and β/γ-positions, respectively. The disappearance of the α-resonance (δ 3.72 p.p.m.) and the simplification of the signal at δ 1.68 p.p.m. from a multiplet to pentuplet (1 H), corresponding to the γ-position, is a good indication for the completion of the exchange process. The integrals in the 220 MHz ^{1}H n.m.r. spectrum gave a [^{2}H] content of ≥ 95%.

The exchange reaction of isoleucine took 10 days for the production of *rac.*[2,3-$^{2}H_{2}$]isoleucine. In the 220 MHz ^{1}H n.m.r. spectrum of the produced *rac.*[2,3-$^{2}H_{2}$]isoleucine, no signals at δ 3.67 and 1.96 p.p.m. (originally belonging to α- and β-protons in the unlabelled isoleucine, respectively) were present. Integrals in the 220 MHz ^{1}H n.m.r. of labelled isoleucine gave a [^{2}H] content of ≥ 93%.

In the preparation of *rac.*[2-$^{2}H_{1}$]alanine, the exchange reaction was monitored for two days when deuteration of the α-proton was judged to be complete. The reaction was stopped after the disappearance of the resonance at δ 3.75 p.p.m. corresponding to the α-proton of unlabelled alanine. The integration of the ^{1}H n.m.r. spectrum of *rac.*[2-$^{2}H_{1}$]alanine suggested a [^{2}H] content of ≥ 97% at the α-position.

The exchange reaction of valine to prepare *rac.*[2,3-$^{2}H_{2}$]valine took 10 days before showing complete exchange of the α- and β-protons. This was checked by the disappearance of signals at δ 3.62 and 2.27 p.p.m. corresponding to α- and β-protons, respectively. The integration in the ^{1}H n.m.r. spectrum of *rac.*[2,3-$^{2}H_{2}$]valine suggested a [^{2}H] content

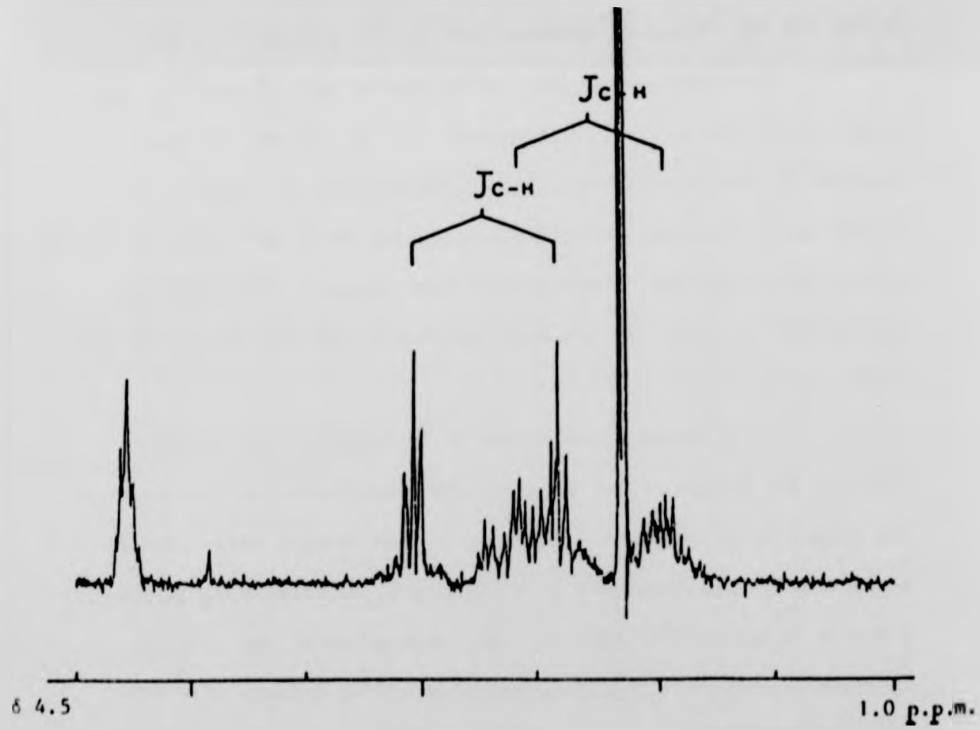

Fig. 4.C.1 The 220 MHz ^{1}H n.m.r. ($^{2}HCl/^{2}H_{2}O$, TSS) spectrum of *rac.*[3,4-$^{13}C_2$]methionine, showing the $J^{13}C-^{1}H$ coupling.

of ≥ 94%.

4.C RESOLUTION* OF *Rac.*[3,4-$^{13}C_2$]METHIONINE

Rac.[3,4-$^{13}C_2$]methionine** was used in studying the biosynthesis of spermidine from methionine in *E. coli* cells (*cf.* Chapter 5). It is well established[11] that this microorganism utilises only the L-isomer of methionine in this biosynthesis. We therefore resolved the *rac.*[3,4-$^{13}C_2$]methionine, following a literature procedure[2], in order to make use of the L-isomer in the biosynthesis of spermidine. The recovered D-isomer was then racemised and so could be used to obtain more of the L-isomer after another resolution. The 220 MHz ^1H n.m.r. analysis of L-[3,4-$^{13}C_2$]methionine gave a spectrum (Fig. 4.C.1) identical to the published[9] spectrum of *rac.*[3,4-$^{13}C_2$]methionine.

4.D STEREOSPECIFICALLY LABELLED *Rac.*(3R,4R)-[3,4-$^2H_2$]METHIONINE***

In the course of our studies on the biosynthesis of spermidine from L-methionine in *E. coli* cells, aimed specifically at the mechanisms of spermidine synthase, we needed a stereospecifically labelled methionine. The *rac.*(3R,4R) and (3R,4R)-[3,4-$^2H_2$]methionine used in part of this work were originally synthesised by D.C.B.[9]. The author

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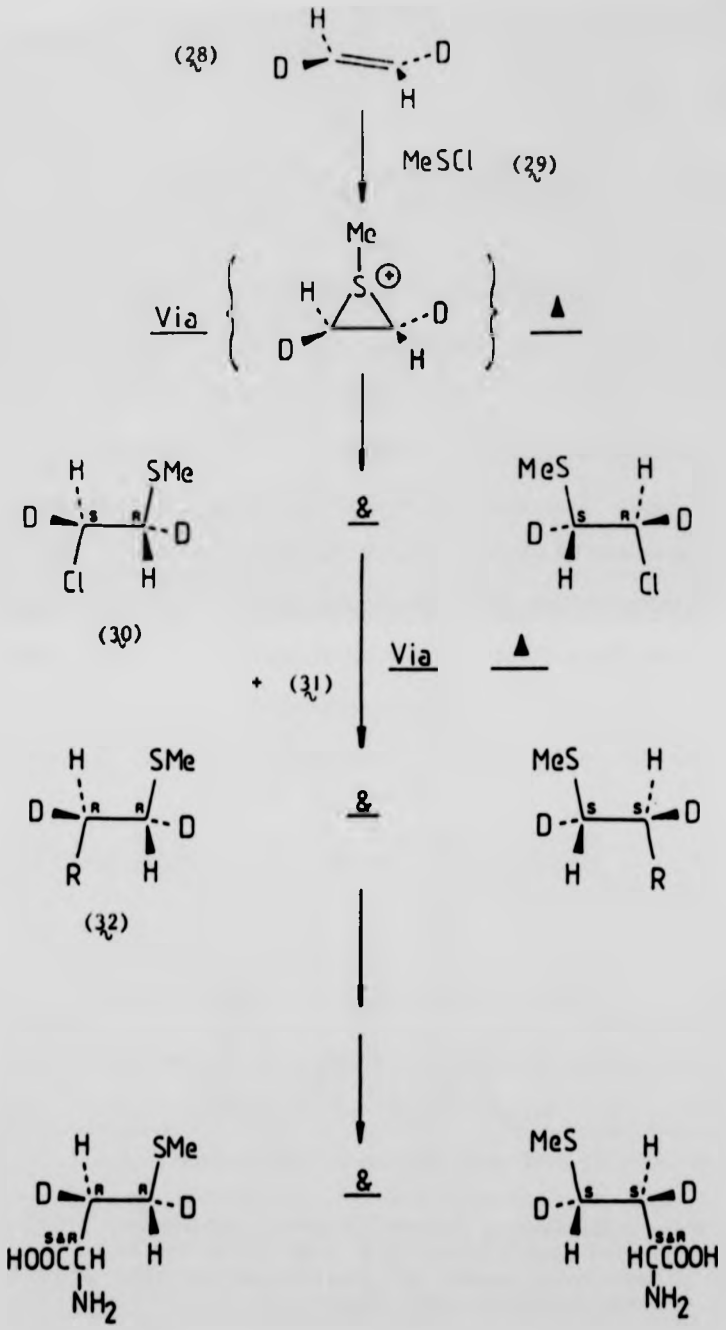
*The resolution of DL-methionines was carried out before we observed that cultures of *E. coli* can be fed with DL-methionine rather than L-methionine and still yield a similar weight of cells (Chapter 3).

**This methionine was synthesised by D.C.B.[9] from [1,2-$^{13}C_2$]ethene containing 81% [$^{13}C_2$], 18% [^{12}C-^{13}C] and 1% [$^{12}C_2$] giving overall 90% atom [^{13}C]. The route used was similar to that described for the synthesis of *rac.*(3R,4R)-[3,4-$^2H_2$]methionine in this Chapter (Section 4.E.3).

***Rac.*(3R,4R)-[3,4-$^2H_2$]methionine is an abbreviation for a mixture of equal amounts of (2R,3R,4R), (2S,3R,4R), (2R,3S,4S), and (2S,3S,4S)-[3,4-$^2H_2$]methionines. *Rac.*(3S,4R)-[3,4-$^2H_2$]methionine is an abbreviation for a mixture of equal amounts of (2R,3R,4S), (2S,3R,4S), (2R,3S,4R) and (2S,3S,4R)-[3,4-$^2H_2$]methionines.



Scheme 4.D.1 The synthesis of *rac.* (3R,4R)-[3,4-$^2H_2$]methionine

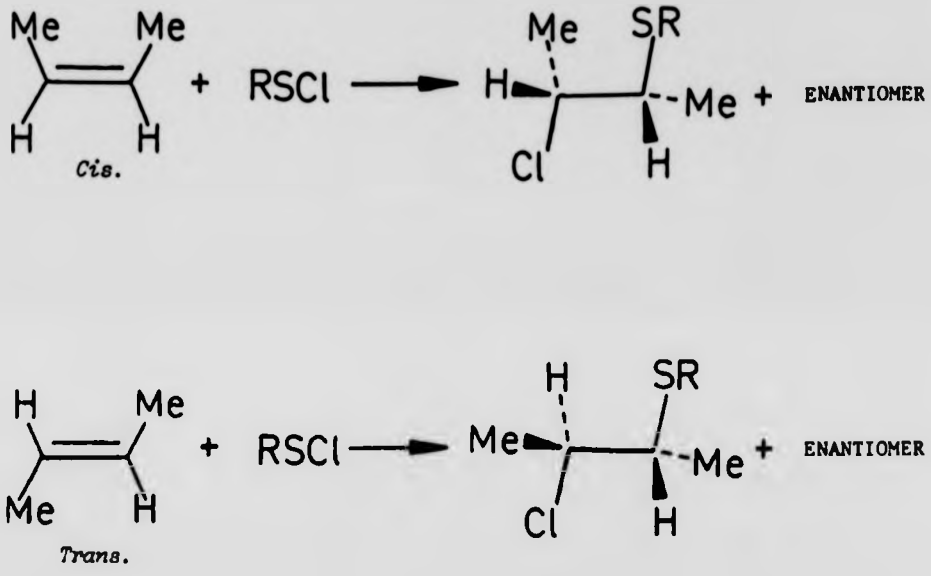
R = $AcNH(EtO_2C)_2C$-

(31) = Sodium diethyl acetamidomalonate

The Fundamental Bands (cm^{-1}) of Deuterated Ethylenes

Band	Type	C_2H_4	C_2D_4	C_2H_3D	CH_2CD_2	*cis*-$(CHD)_2$	*trans*-$(CHD)_2$	C_2HD_3
ν_1	symmetrical C—H(D) stretching	—	—	3002	3019	2300	—	2280
ν_2	C=C stretching	—	—	1605	1585	1567	—	1546
ν_3	$CH_2(D_2)$ symmetrical bending	—	—	1290	1032	1215	—	1047
ν_4	out-of-plane C—H(D) bending	—	—	1001	—	—	988	765
ν_5	C—H(D) stretching	—	—	3062	2335	3055	—	2232
ν_6	in-plane CH(D) bending	—	—	1128	1150	—	—	995
ν_7	out-of-plane C—H(D) bending	949	720 (*us*)	807	752	842	727	724
ν_8	out-of-plane C—H(D) bending	—	—	943	944	—	—	919
ν_9	C—H(D) stretching	3106	2345 (*ts*)	3103	3095	3058	3065	3045
ν_{10}	C—H(D) bending	810	584	715	—	646	678	610
ν_{11}	C—H(D) stretching	2990	2200 (*m*)	2272	2231	2251	2270	2222
ν_{12}	C—H(D) bending	1444	1078 (*s*)	1403	1384	1342	1300	1290

Table 4.D.1 The fundamental bands in the i.r. spectrum of deuterated ethenes. This table was copied from Ref. 14.



Scheme 4.D.2 The scheme shows the stereospecificity of the addition reaction of p-chlorobenzenesulphenyl chloride to *cis*- or *trans*-2-butene.

repeated the route to synthesise *rac.* (3R,4R)-[3,4-$^2H_2$]methionine and obtained spectroscopic data agreeing well with those published[9].

The first step in the synthesis of these methionines is the addition of methanesulphenyl chloride (29) to (E)-[1,2-$^2H_2$]ethene (28) to give 1-chloro-2-(methylthio)ethane[12] (30) (Scheme 4.D.1). The [$^2H_2$]ethene (28) was prepared by the stereospecific reduction[13] of [$^2H_2$]acetylene with chromium(II) solution. The purity of (28) was checked by i.r. analysis[14]. It was found to be free from any (Z)-[1,2-$^2H_2$]ethene. The i.r. spectrum of (28) was characterised by the presence of a band at 987 cm^{-1}. No band at 843 cm^{-1} (characteristic of (Z)-[1,2-$^2H_2$]ethene) was present. Complete i.r. data for (E)- and (Z)-[1,2-$^2H_2$]ethene is presented in Table 4.D.1[14].

The mechanistic description of the addition reaction between methanesulphenyl chloride (29) and (E)-[1,2-$^2H_2$]ethene (28) suggests a rate determining formation of a thiiranium ion[15], which undergoes a nucleophilic opening by chloride as the product-forming step. Numerous studies of the reactions between alkyl or aryl-sulphenyl halides and alkenes provide convincing evidence in favour of this interpretation. It was found that such reactions proceed completely in a *trans* stereospecific manner[16]. The *trans* addition is favoured because of steric hindrance generated by the sulphur and its substituent. The ring opening step can be assumed to follow the classical S_N2 pathway (i.e. inversion). Stereochemical studies[17] on the addition of p-chlorobenzenesulphenyl chloride to *cis* or *trans*-2-butene at various temperatures in 1,1,2,2-tetrachloroethane, proved that these additions proceed stereospecifically (Scheme 4.D.2). This shows that if leakage to an open-chain carbenium ion were involved in these additions, two isomers should have been detected, rather than one, from each addition.

FIG.4-D-1

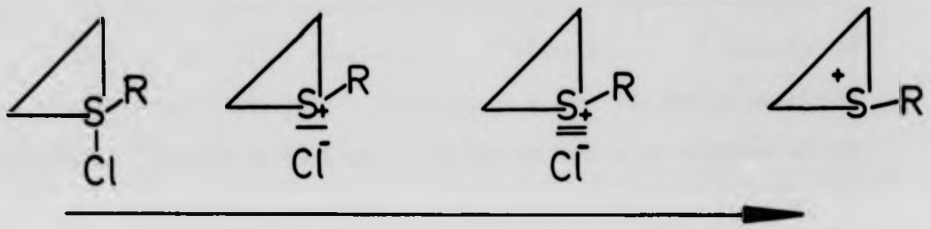


Fig. 4.D.1 Spectrum of structures for the proposed thiiranium intermediate . The direction of the arrow indicates the change in the structure upon increasing the polarity of the solvent.

Generally, the formation of cationoid intermediates in an organic reaction (including addition processes) produces a mixture of products due to the ability of such intermediates to rearrange. This can lead to stereoconversion and reaction with other nucleophiles present in the reaction mixture. None of these characteristics were observed in the addition reactions of sulphenyl chlorides to alkenes. Recently, Smit *et al.*[18] have suggested the involvement of other bridged species, in the addition of sulphenyl halides to alkenes, such as the covalent σ-sulphurane (Fig. 4.D.1). They pointed out various abnormalities in the behaviour of so-called thiiranium intermediates, that could be explained by proposing a spectrum of structures[18] (Fig. 4.D.1). The precise structure of the "thiiranium ion" depends on its environment (e.g. solvent polarity). However, whatever the structure of the intermediate involved in the reaction of halide (29) with ethene (28) in dichloromethane, the stereochemical outcome can be assumed to be rigorously *trans* addition. Rearrangements and loss of stereochemical integrity have only been observed in systems well known to rearrange *via* cationic species (e.g. norbornyl)[19]. The intermediacy of the primary carbenium ion 2-(methylthio)ethyl carbocation can be excluded both on theoretical grounds (primary carbocations have never been observed in non-polar solvents[20]), and experimental evidence (Ref. 9) that (E)-[1,2-$^2H_2$]ethene leads to a different set of [3,4-$^2H_2$]methionines than (Z)-[1,2-$^2H_2$]ethene.

The reaction of ethene (28) with the halide (29) will result in a racemic mixture of chloride (30). [N.B. In Scheme 4.D.1 we show this reaction to proceed *via* a 1-methylthiiranium ion only for simplification; it should be kept in mind that other less polar intermediates may be involved under these conditions[18] (*cf.* Fig. 4.D.1).]. Chloride (30) was immediately used in the next

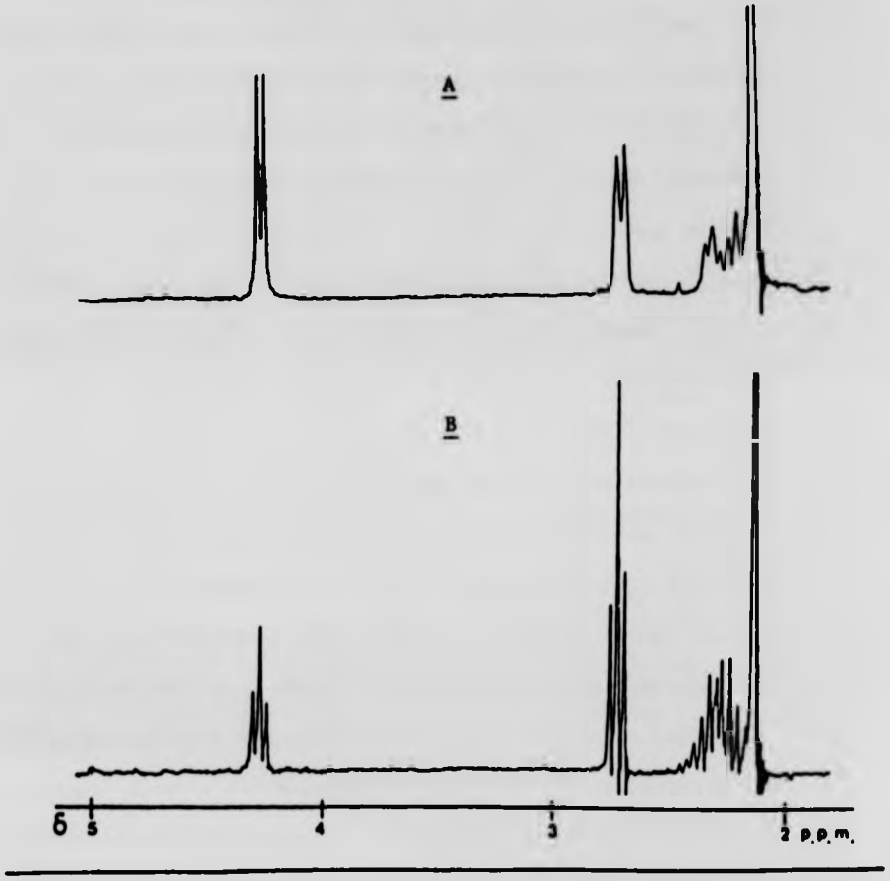


Fig. 4.D.2 The 220 MHz ^{1}H n.m.r. ($^{2}H_2O/^{2}HCl$, TSS) of:

A - (2R,3R,4R), (2S,3R,4R), (2R,3S,4S), and (2S,3S,4S)-[3,4-$^{2}H_2$]methionines.

B - Standard unlabelled methionine

step of the synthesis, because of its toxicity and in order to reduce the risk of racemisation by S_N2 attack of chloride ions on (30), [which might be formed reversibly from (30)].

Condensation of chloride (30) with the sodium salt of diethyl acetamidomalonate (31) is also expected to proceed *via* a thiiranium ion under the conditions used (in ethanol, a relatively polar solvent). It was necessary to show that this reaction does not proceed *via* a S_N2 pathway, which would lead to products of inverted configuration at C-3 of methionine. Under good S_N2 conditions, (KI/acetone), chloride (30) reacted 1.5 times faster than n-butyl chloride[21]. The chloride (30) can react with salt (31) *via* a thiiranium ion or *via* an S_N2 pathway, whereas n-butyl chloride can only react *via* an S_N2 pathway. A competitive reaction between chloride (30) and n-butyl chloride and a limited amount of salt (31), was conducted[9] under the same conditions as was used with the labelled *rac.* chloride (30) [1-chloro-2-(methylthio)-[1,2-$^2H_2$]ethane]. The outcome of this experiment showed a product derived only from the reaction of chloride (30) with salt (31) and no product from the reaction of salt (31) with n-butyl chloride. This proved that the rate of reaction of chloride (30) is much faster than n-butyl chloride, and indicated that chloride (30) reacts with salt (31) *via* a thiiranium ion rather than by direct S_N2 displacement.

The stereochemistry of the ethyl 2-acetamido-2-ethoxycarbonyl-4-(methylthio)butanoate [derivative (32)] derived from (E)-[1,2-$^2H_2$]-ethene is shown in Scheme 4.D.1. Acidic hydrolysis of derivative (32) resulted directly in a pure mixture of (2R,3R,4R), (2S,3R,4R), (2R,3S,4S), and (2S,3S,4S)-[3,4-$^2H_2$]methionines. This will be abbreviated as *rac.* (3R,4R)-[3,4-$^2H_2$]methionine. The 220 MHz ^1H n.m.r. (Fig. 4.D.2) analysis agreed with data published in Ref. 9.

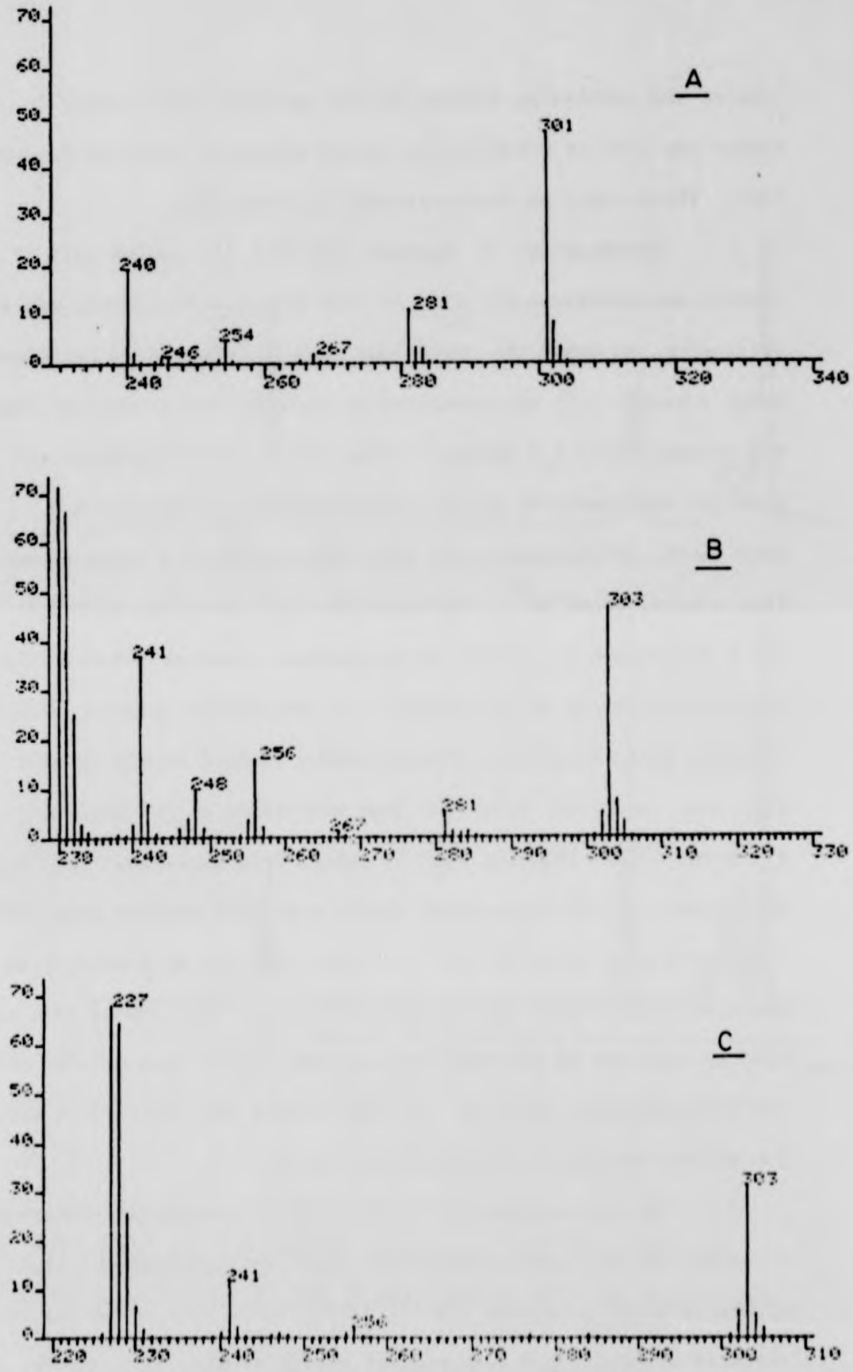


Fig. 4.D.3 The e.i.m.s. analysis on the N-trifluoroacetyl butyl ester derivative of:

A - Unlabelled methionine

B - (3R,4R)-[3,4-$^2H_2$]methionine

C - (3R,4S)-[3,4-$^2H_2$]methionine

The m.s. analysis of the ^2H content in *rac.*(3R,4R)-[3,4-$^2H_2$]-methionine was carried out on the N-trifluoroacetyl butyl ester derivative[10] as described previously for L-[2,3,3-$^2H_3$]methionine (*cf.* Section 4.E.1). The m.s. analysis showed the presence of 10% [$^2H_1$] species and 90% [$^2H_2$] species. The spectrum was compared with spectra for derivatives from unlabelled methionine and (3R,4S)-[3,4-$^2H_2$]methionine (obtained from D. C. Billington[9]) as shown in Fig. 4.D.3.

4.E EXPERIMENTAL

4.E.1 Syntheses of deuterium-labelled (C-2 and/or C-3) *rac.* amino acids

1. Preparation, resolution and m.s. analysis of *rac.*[2,3,3-$^2H_3$]methionine

(a) Preparation of *rac.*[2,3,3-$^2H_3$]methionine

*Rac.*methionine (4.36 g, 3 x 10^{-2} mol) was suspended in ^2H_2O (5 cm^3) and heated with stirring for 30 minutes. The mixture was pumped to dryness and the residue was dissolved with warming in ^2H_2O (30 cm^3). To this solution was added Al(III)* in ^2H_2O (3 cm^3, 7.5 x 10^{-4} mol) and pyridoxal hydrochloride (0.6 g, 3 x 10^{-3} mol). The pH of the resulting solution was adjusted to 5.0 (p^2H 5.4) by addition of 40% NaO^2H in ^2H_2O. The reaction mixture was heated at reflux with stirring under nitrogen for 24 h. After cooling the reaction mixture to 0°C addition of pre-cooled ethanol (100 cm^3) gave a

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*A solution of Al(III) in ^2H_2O was prepared[1] by heating hydrated aluminium sulphate [$Al_2(SO_4)_3.16H_2O$, 0.775 g, 1.23 x 10^{-3} mol] at 250°C to a constant weight, and then cooling and adding ^2H_2O (2 cm^3). The resulting solution was dried to a constant weight and the residue was dissolved in ^2H_2O (5 cm^3) to give a 0.25 mol dm^{-3} Al(III) solution.

precipitate of slightly yellow crystals. These were filtered off, washed with cold ethanol and were dissolved in water (40 cm^3). To the aqueous solution was added decolourising charcoal and the mixture was stirred with warming for 1 h. The mixture was filtered through Celite and the volume of the filtrate was reduced to *ca.* 8 cm^3 before the addition of boiling ethanol (40 cm^3). After cooling at -20°C overnight the crystals which formed were collected, washed with ethanol (20 cm^3) and ether (20 cm^3). Air drying gave *rac.*[2,3,3-$^2H_3$]methionine (3.1 g, 71%) as a white crystalline solid, m.p. 275-277°C, pure by t.l.c. [Kieselgel F_{254}, 88O, ammonia/ethanol (23/77,v/v), ninhydrin spray, R_f 0.45], 220 MHz ^1H n.m.r., (^2H_2O, TSS) δ 2.13 (3 H, s) and 2.63 (2 H, s) p.p.m.

(b) Resolution of *rac.*[2,3,3-$^2H_3$]methionine[2]

Rac.[2,3,3-$^2H_3$]methionine (1.49 g, 10 x 10^{-3} mol) and ammonium-l-α-bromocamphor-π-sulphonate (3.28 g, 10 x 10^{-3} mol) were dissolved in pre-warmed 1 mol dm^{-3} hydrochloric acid (10 cm^3). The solution was allowed to cool to room temperature and soon deposited crystals of the bromocamphorsulphonate of L-[2,3,3-$^2H_3$]-methionine (*ca.* 1.9 g). These crystals were filtered off and were then dissolved in hot water (4 cm^3). L-[2,3,3-$^2H_3$]Methionine was precipitated by addition of conc. ammonia to pH 5.9 followed by addition of hot methanol (40 cm^3). The methionine was collected (472 mg, 63% of available L-isomer) and recrystallised by dissolving in hot water (10 cm^3), reducing the volume to a minimum, and adding hot methanol (40 cm^3). After standing the resulting mixture overnight at 0°C, the crystals of L-[2,3,3-$^2H_3$]methionine were filtered off, washed with cold methanol (10 cm^3) and dried *in vacuo* to give a yield of 445 mg (60%), $[\alpha]_D^{20}$ + 22 (c 0.037 in 1 mol dm^{-3} HCl). The

220 MHz ^{1}H n.m.r. spectrum was identical with that of the starting *rac.*[2,3,3-$^2H_3$]methionine (*cf.* data in Section 4.B.1).

(c) Preparation of N-trifluoroacetyl-L-[2,3,3-$^2H_3$]methionine butyl ester[10] for mass spectrometric analysis

The derivatisation of L-[2,3,3-$^2H_3$]methionine (0.15 g, 10^{-3} mol) was carried out by adding HCl/BuOH* solution (10 cm^3, 1.25×10^{-2} mol). The resulting suspension was heated on a steam bath for 2 h, with the exclusion of atmospheric moisture. The solvent was removed from the cooled solution by evaporation under reduced pressure (50°C, 20 mmHg, the last traces being removed at 35°C, 0.1 mmHg) to provide a pale yellow oil. This oil was taken up in dichloromethane (10 cm^3) and trifluoroacetic anhydride (1 cm^3, 1.45 g, 7×10^{-3} mol) was added. The yellow reaction mixture was sealed, and stored at 25°C for 90 minutes, during which time it became colourless. The solvent, and excess trifluoroacetic anhydride were removed under reduced pressure (30°C, 20 min. then 30°C, 1 mmHg) to give a pale yellow oil (*ca.* 0.25 g). T.l.c. (silica gel, CH_2Cl_2, H_2SO_4 spray and char) showed a major spot at R_f 0.38 and some impurities at the origin. Purification was done by flash column chromatography[22] [(2.5 mm dia. x 10 cm) column of Merck Kieselgel 60 (200-400 mesh)], eluting with CH_2Cl_2. The progress of the column was monitored by t.l.c. Evaporation of the relevant fractions gave pure product (0.22 g, 73%) as a pale yellow oil pure by t.l.c. (R_f 0.38).

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*A solution of dry hydrogen chloride in absolute butan-1-ol (HCl/BuOH) was prepared by the dropwise addition of acetyl chloride (17.6 cm^3, 19.5 g, 25×10^{-2} mol) to ice-cold, stirred, absolute butanol (200 cm^3), under an atmosphere of dry nitrogen.

The mass spectrometric analysis showed the following peaks and relative intensities for the molecular ion cluster: m/z 301 (0), 302 (19), 303 (21) and 304 (100), denoting 14% [$^2H_1$], 14% [$^2H_2$], 72% [$^2H_3$] and an overall deuterium content of ≥ 86%.

2. Preparation of *rac*.[3,3-$^2H_2$]methionine

Rac.[2,3,3-$^2H_3$]methionine (1.0 g, 6.6 x 10^{-3} mol) was dissolved in water (30 cm^3). To this solution aluminium sulphate solution (0.66 cm^3, 1.7 x 10^{-4} mol) and pyridoxal hydrochloride (0.13 g, 66 x 10^{-4} mol) were added. The pH of the mixture was adjusted to 10.0 by the addition of lithium hydroxide solution (5 mol dm^{-3}). The rate of exchange at the α-position was monitored by 220 MHz ^1H n.m.r. analysis. After standing for 18 h at 37°C the reaction mixture was evaporated under reduced pressure to *ca*. 10 cm^3. Hydrochloric acid (5 mol dm^{-3}) was added until the pH fell to 5.2, and the precipitated solid was redissolved by heating. Boiling ethanol (50 cm^3) was added, and the solution was stored for 24 h at -20°C. The precipitated crystals were filtered off and washed with cold ethanol (20 cm^3) and ether (20 cm^3). Air drying gave *rac*.[3,3-$^2H_2$]methionine (0.86 g, 86%) as a white crystalline solid, m.p. 275-278°C. The 220 MHz ^1H n.m.r. analysis showed resonances at (^2H_2O, TSS) δ 2.13 (3 H, s), 2.63 (2 H, s) and 4.03 (1 H, s) p.p.m. The product was pure by t.l.c. (*cf*. Section 4.E.1) and showed the absence of lithium ions (flame test). The integrals in the 220 MHz ^1H n.m.r. spectrum gave a [^2H] content of ≥ 82%.

3. Preparation of *rac*.[2,3,3-$^2H_3$]leucine

Rac.leucine (3.93 g, 3 x 10^{-2} mol) was suspended

in ^2H_2O (5 cm^3) and heated with stirring for 30 minutes. The mixture was pumped to dryness and the residue was dissolved with warming in ^2H_2O (90 cm^3). To this solution was added Al(III) in ^2H_2O (3 cm^3, 7.5 x 10^{-4} mol) and pyridoxal hydrochloride (0.6 g, 3 x 10^{-3} mol). The pH of the resulting solution was adjusted to 5.0 (p^2H 5.4) by addition of 40% NaO^2H in ^2H_2O. The reaction mixture was heated at reflux with stirring under nitrogen and monitored by ^1H n.m.r. analysis for 15 days. After cooling the reaction mixture to 0°C addition of pre-cooled ethanol (200 cm^3) gave a precipitate of yellow crystals. These were filtered off, washed with ethanol and were dissolved in water (200 cm^3). To the aqueous solution was added decolourising charcoal and the mixture was stirred with warming for 3 h. The warm mixture was filtered through Celite and the volume of the filtrate was reduced to *ca.* 15 cm^3 before addition of hot ethanol (100 cm^3). After cooling at -20°C for 12 h, the crystalline precipitate was collected, washed with cold ethanol (20 cm^3), and ether (20 cm^3). Air drying gave *rac.*[2,3,3-$^2H_3$]leucine (3.0 g, 79%) as a white crystalline solid, m.p. 292-295°C. The 220 MHz ^1H n.m.r. analysis showed signals at (^2H_2O, TSS) δ0.94 (6 H, d) and 1.68 (1 H, p) p.p.m. The signal at δ 3.72 p.p.m. in the starting unlabelled leucine disappeared and the complicated multiplet at δ 1.68 p.p.m. from the protons at C-3 and C-4 was simplified to a pentuplet (1 H) corresponding to H-4 only. The integrals in the 220 MHz ^1H n.m.r. gave a [^2H] content of ≥ 95%.

4. Preparation of *rac.*[2,3-$^2H_2$]isoleucine

Rac. isoleucine (2.62 g, 2 x 10^{-2} mol) was suspended in ^2H_2O (5 cm^3) and heated with stirring for 30 minutes. The mixture was pumped to dryness and the residue was dissolved with warming in

^2H_2O (50 cm^3). To this solution was added Al(III) in ^2H_2O (2 cm^3, 5 x 10^{-4} mol) and pyridoxal hydrochloride (0.4 g, 2 x 10^{-3} mol). The pH of the resulting solution was adjusted to 5.0 (p^2H 5.4) by addition of 40% NaO^2H in ^2H_2O. The reaction mixture was heated at reflux with stirring under nitrogen and monitored by ^1H n.m.r. analysis for 10 days. After cooling the reaction mixture to 0°C addition of pre-cooled ethanol (100 cm^3) gave a precipitate of yellow crystals. These were filtered off, washed with ethanol, and were dissolved in water (100 cm^3). To the resulting aqueous solution was added decolourising charcoal and the mixture was stirred with warming for 3 h. The warm mixture was filtered through Celite and the volume of the filtrate was reduced to *ca.* 10 cm^3 before addition of boiling ethanol (70 cm^3). After cooling to -20°C for 2 h, the crystalline precipitate was collected, washed with cold ethanol (20 cm^3), and ether (20 cm^3). Air drying gave *rac.*[2,3-$^2H_2$]isoleucine (1.74 g, 70%) as a white crystalline solid. The 220 MHz ^1H n.m.r. analysis showed signals at (^2H_2O, TSS) δ 0.98 (3 H, m), and 1.4 (2 H, m) p.p.m. The removal of the signals corresponding to α- and β-protons of the unlabelled isoleucine [at δ 3.67 (1 H, d) and 1.96 (1 H, m) p.p.m., respectively] was complete. The [^2H] content by the 220 MHz ^1H n.m.r. analysis was ≥ 93%.

5. Preparation of *rac.*[2-$^2H_1$]alanine

*Rac.*alanine (3.12 g, 3.5 x 10^{-2} mol) was dissolved in ^2H_2O (30 cm^3). To this solution was added Al(III) in ^2H_2O (3.5 cm^3, 8.8 x 10^{-4} mol) and pyridoxal hydrochloride (0.7 g, 3.5 x 10^{-3}). The pH of the resulting solution was adjusted to 10.2 (p^2H 10.6) by addition of 40% NaO^2H in ^2H_2O. The rate of exchange at the α-position was monitored by 220 MHz ^1H n.m.r. analysis.

After 2 days of incubation at 30°C, the reaction mixture was evaporated under reduced pressure to *ca.* 10 cm^3. Boiling ethanol (70 cm^3) was added and the solution was stored for 24 h at -20°C. The crystals which formed were filtered off and were washed with cold ethanol (20 cm^3) and ether (20 cm^3). Air drying gave *rac.*[2-$^2H_1$]alanine (2.2 g, 70%) as a white crystalline solid, m.p. 290-292. The 220 MHz ^1H n.m.r. analysis showed a signal at (^2H_2O, TSS) δ 1.46 (3 H, s) p.p.m. No signal corresponding to the α-proton in the starting unlabelled alanine, [δ 3.75 (1 H, q) p.p.m.] was detected. The integrals in the 220 MHz ^1H n.m.r. spectrum gave a [^2H] content of ≥ 97%.

6. Preparation of *rac.*[2,3-$^2H_2$]valine

*Rac.*valine (1.17 g, 1 x 10^{-2} mol) was suspended in ^2H_2O (5 cm^3) and heated with stirring for 30 minutes. The mixture was pumped to dryness and the residue was dissolved with warming in ^2H_2O (20 cm^3). To this solution was added Al(III) in ^2H_2O (1 cm^3, 2.5 x 10^{-4} mol) and pyridoxal hydrochloride (0.2 g, 1 x 10^{-3} mol). The pH of the resulting solution was adjusted to 5.0 (p^2H 5.4) by the addition of 40% NaO^2H in ^2H_2O. The reaction mixture was heated at reflux with stirring under nitrogen. The rate of exchange at the α- and β-positions was monitored by 220 MHz ^1H n.m.r. analysis. After 10 days, cooling the reaction mixture to 0°C and addition of pre-cooled ethanol (80 cm^3) gave a precipitate of yellow crystals. These were filtered off, washed with ethanol and were dissolved in water (50 cm^3). To the aqueous solution was added decolourising charcoal and the mixture was filtered through Celite. The volume of the filtrate was reduced to *ca.* 8 cm^3 before addition of boiling ethanol (50 cm^3). After cooling at -20°C for 12 h the crystalline

precipitate was collected, washed with cold ethanol (10 cm^3) and ether (10 cm^3). Air drying gave *rac.*[2,3-$^2H_2$]valine (1.01 g, 86%) as a white crystalline solid, m.p. 297-299°C. The 220 MHz ^1H n.m.r. analysis showed a resonance at (^2H_2O, TSS) δ 1.0 (6 H, s) p.p.m. The disappearance of the signals corresponding to the α- and β-protons of the unlabelled valine [at δ 3.62 (1 H, d) and 2.27 (1 H, m) p.p.m., respectively] was complete. The integrals in the 220 MHz ^1H n.m.r. spectrum gave a [^2H] content of ≥ 94%.

4.E.2 Resolution of *rac.*[3,4-$^{13}C_2$]methionine

Rac.[3,4-$^{13}C_2$]methionine (0.91 g, 6 x 10^{-3} mol) and ammonium-l-α-bromocamphor-π-sulphate (1.98 g, 6 x 10^{-3} mol) were dissolved in pre-warmed 1 mol dm^{-3} hydrochloric acid (6 cm^3). The solution was allowed to cool to room temperature and soon deposited crystals of the bromocamphor sulphonate of L-[3,4-$^{13}C_2$]methionine (*ca.* 1.2 g). These were filtered off and were dissolved in water (3 cm^3). The amino acid was precipitated by addition of conc. ammonia to pH 5.9, followed by addition of hot methanol (25 cm^3). The methionine was collected (0.26 g, 58% of available L-isomer) and was recrystallised by dissolving in hot water (10 cm^3), reducing the volume to a minimum and adding hot methanol (30 cm^3). After standing the resulting mixture overnight at 0°C, the crystals of L-[3,4-$^{13}C_2$]methionine were filtered off, washed with cold methanol and dried *in vacuo* (0.24 g, 54% of available L-isomer), $[\alpha]_D^{20}$ + 21.5 (c 0.009 in 1 mol dm^{-3} HCl). The 220 MHz ^1H n.m.r. spectrum (^2H_2O/ ^{2}HCl, TSS) showed resonances at δ 2.15 (3 H, d), 2.28 (0.9 x 2 H, 2 x m, $J^{13}C-^1H$ 135 Hz, and 0.1 x 2 H, m), 2.78 (0.9 x 2 H, 2 x t, $J^{13}C-^1H$ 135 Hz and 0.1 x 2 H, t) and 4.29 (1 H, m) p.p.m. (see

Fig. 4.C.1). The compound was pure by t.l.c. (see Section 4.E.1), R_f 0.45.

The mother liquor from the first filtration containing mainly the bromocamphorsulphonate of D-methionine, was combined with the mother liquor from the above crystallisation. The pH of the mixture was adjusted to 5.9 by addition of conc. ammonia. Hot methanol (50 cm^3) was added and the precipitated methionine was filtered off and recrystallised (as described for the L-isomer) to give (0.64 g, 89%) of methionine which was mainly the D-isomer (optical rotation measurement). Racemisation of this methionine (0.64 g, 4.2×10^{-3} mol) was achieved by dissolving it in water (10 cm^3) and adding aluminium sulphate (0.038 g, 1.1×10^{-4} mol) and pyridoxal hydrochloride (0.08 g, 4.2×10^{-4} mol); the pH of the solution was adjusted to 5.2 by addition of 40% NaOH and the mixture was left standing for 2 h/r.t. The reaction mixture was cooled to 0°C followed by addition of pre-cooled ethanol (200 cm^3). The mixture was left at -20°C overnight and the precipitated crystalline solid was collected and washed with ethanol (20 cm^3) and ether (20 cm^3) to give *rac.*[3,4-$^{13}C_2$]methionine (0.58 g, 90%). The 220 MHz ^1H n.m.r. spectrum was identical with the spectrum of the L-isomer (*cf.* Fig. 4.C.1).

4.E.3 Synthesis of *rac.*(3R,4R)-[3,4-$^2H_2$]methionine

1. Preparation of (E)-[1,2-$^2H_2$]ethene[13]

Using dry apparatus, ^2H_2O (20 cm^3, 20 g, 1 mol), 99.8% atom,was added dropwise to calcium carbide (20 g, 0.3 mol). The generated [$^2H_2$]acetylene was collected by displacement of water from 2 x 2.3 dm^3 conical flasks. The generation of the gas was stopped when 2 x 1.9 dm^3 of water had been displaced by the generated [$^2H_2$]-

acetylene. The remaining water was removed from the flask using an aspirator and replaced by chromium(II) solution (2 x 250 cm^3)*. The flask was sealed and shaken at room temperature in an orbital shaker. The progress of the reduction was monitored by g.l.c. (Chromosorb 101, N_2 at 20 psi, 25°C). The reaction was stopped when no acetylene could be detected (usually 36 h). The resulting (E)-[1,2-$^2H_2$]ethene in the two flasks was transferred to a vacuum line and dried (trap to trap distillation) to give 3.6 dm^3 of (E)-[1,2-$^2H_2$]ethene, free from any traces of (Z)-[1,2-$^2H_2$]ethene by i.r. analysis [*cf.* Table 4.D.1[14]]: (gas cell, 50 mmHg) 3070 (m), 2270 (w, sh), 1300 (m, sh), 988 (s, sh), 727 (s, sh) and 675 (m, sh) cm^{-1}. The absence of peaks at 1342 and 842 cm^{-1} is characteristic of (E)-[1,2-$^2H_2$]ethene free from (Z)-isomer. Two small peaks appearing at 1001 and 807 cm^{-1} were characteristic of monodeuterated ethene[14].

2. Preparation of methanesulphenyl chloride (29)[23]

Sulphuryl chloride (21.45, 0.15 mol) was added dropwise over 15 minutes to stirred dimethyldisulphide (14.1 g, 0.15 mol) at -15°C. The reaction was allowed to warm up to room temperature with stirring over 2 h and the product was then fractionally distilled. The fraction boiling between 30 and 32°C (110 mmHg) was collected at -78°C to give methanesulphenyl chloride as a deep orange liquid (18.8 g, 72%), 220 MHz ^1H n.m.r. ($CDCl_3$, TMS) δ 2.9 (3 H, s) p.p.m. CH_3SCl [N.B. absence of peak at δ 2.4 (s) p.p.m. due to $(CH_3S)_2$]. The compound has a very

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*Potassium chromium(III) sulphate dodecahydrate (100 g, 0.2 mol) was dissolved in hydrochloric acid (250 cm^3, 3 mol dm^{-3}) and 2% zinc amalgam (50 cm^3) was added. The mixture was shaken under an atmosphere of nitrogen until the colour changed from dark green to translucent blue. The amalgam was run off and the chromium(II) solution was used at once.

strong smell and was stored in a sealed container at -20°C. The material was directly used after preparation and any residues were destroyed with sodium hypochlorite solution.

3. Preparation of (1S,2R)/(1R,2S)-1-chloro-2-(methylthio)-[1,2-$^2H_2$]ethane (30)

A solution of methanesulphenyl chloride (6 g, 7.2 x 10^{-2} mol) in dichloromethane (50 cm^3) was placed in a 250 cm^3 flask, cooled to -25°C and evacuated to *ca.* 20 mmHg on a vacuum line. Dry (E)-[1,2-$^2H_2$]ethene (28) was admitted to the solution with swirling. The admission of ethene (28) was continued until no further absorption occurred, and the solution was colourless (40-50 minutes), whilst the temperature of the solution was kept below -25°C. Dichloromethane was distilled off at 20°C/50 mmHg, to give 1-chloro-2-(methylthio)-[1,2-$^2H_2$]ethane (30). The 220 MHz ^1H n.m.r. ($CDCl_3$, TMS) showed resonances at δ 2.15 (3 H, s), 2.8 (1 H, br d, J 9 Hz) and 3.6 (1 H, br d, J 9 Hz) p.p.m. The compound was taken directly to the next stage (N.B. this compound is a potent vesicant and should be handled with care).

4. Reaction between 1-chloro-2-(methylthio)-[1,2-$^2H_2$]ethane (30) and sodium diethyl acetamidomalonate (31)

Using dry apparatus, sodium (1.25 g, 5 x 10^{-2} mol) was dissolved in anhydrous ethanol (35 cm^3) by boiling the mixture under reflux with the exclusion of atmospheric moisture. Diethyl acetamidomalonate (11.25 g, 5 x 10^{-2} mol) was added and the mixture was boiled under reflux until a clear solution resulted. The chloride (30) (consisting of *ca.* 7.2 x 10^{-2} mol + traces of dichloromethane) was added and the reaction was boiled under reflux, with the exclusion of atmospheric moisture, for 5 h. After cooling to 0°C, the precipitated sodium chloride was filtered off, under suction, and washed with ice-cold ethanol (30 cm^3). The combined filtrates were evaporated to dryness

under reduced pressure to provide an orange oil. This oil was extracted into dichloromethane (3 x 50 cm^3), the solution was filtered through Celite and was evaporated under reduced pressure (20 mmHg, then 1 mmHg for 1 h) to give ethyl 2-acetamido-2-ethoxycarbonyl-4-(methylthio)--[3,4-$^2H_2$]butanoate (32), (16 g, 93%) as an orange crystalline mass. The 220 MHz ^1H n.m.r. ($CDCl_3$, TMS) showed resonances at δ 1.25 (6 H, t), 2.04 (3 H, s), 2.08 (3 H, s), 3.34 (1 H, d, J 9 Hz), 3.65 (1 H, d, J 9 Hz), 4.3 (4 H, q) and 6.9 (1 H, br s) p.p.m. This material was directly hydrolysed without any further purification.

5. Hydrolysis of (3R,4R)/(3S,4S)ethyl 2-acetamido-2-ethoxycarbonyl-4-(methylthio)-[3,4-$^2H_2$]butanoate to *rac.*(3R,4R)-[3,4-$^2H_2$]methionine

The above product (32) (16 g, 5.5 x 10^{-2} mol) was dissolved in 2 mol dm^{-3} hydrochloric acid (61 cm^3) and the mixture was boiled under reflux for 6 h with stirring. A further amount of 2 mol dm^{-3} hydrochloric acid (61 cm^3) was added and the reaction was boiled under reflux for a further 3 h. The orange solution which resulted was evaporated to dryness under reduced pressure (70°C, 10 mmHg), to give an orange oil. This oil was taken up in water (30 cm^3) and the pH of the solution was adjusted to 7 by the addition of saturated lithium hydroxide. To the resulting solution was added decolourising charcoal and the mixture was stirred with warming for 1 h. The mixture was filtered through Celite. The filtrate was evaporated to dryness under reduced pressure and the solid residue was dissolved in boiling water (10 cm^3). After addition of boiling ethanol (50 cm^3), the mixture was stored at -20°C for 24 h. The precipitated solid was filtered off under suction and washed with pre-cooled ethanol (20 cm^3) and ether (20 cm^3). Air drying gave *rac.*(3R,4R)-[3,4-$^2H_2$]methionine (4.7 g, 3.0 x 10^{-2} mol) as a white crystalline solid, m.p. 175-178°C, pure by t.l.c. (*cf.* Section 4.E.1). The 220 MHz ^1H n.m.r. analysis

(DCl/^2H_2O/TSS) showed signals at δ 2.12 (3 H, s), 2.25 (1 H, 2 x t, J 7 Hz), 2.7 (1 H, br d, J 7 Hz), and 4.28 (1 H, d, J 7 Hz) p.p.m. (Fig. 4.D.2). This product was checked by a flame test and was found to be free of any lithium ions. The overall yield of *rac.*(3R,4R)-[3,4-$^2H_2$]methionine based on the ethene (28) used was 42%. The reported yield[9] for the synthesis of *rac.*(3R,4R)-[3,4-$^2H_2$]methionine in the above manner was 35%.

Mass spectrometric analysis on pure (3R,4R)-[3,4-$^2H_2$]methionine was carried out *via* the N-trifluoroacetyl-[3,4-$^2H_2$]methionine butyl ester[10] derivative following the procedure described for the derivatisation of L-[2,3,3-$^2H_3$]methionine in Section 4.E.1. This analysis (Fig. 4.D.3) showed the following peaks and relative intensities for the molecular ion cluster: m/z 301 (0), 302 (10), 303 (100), denoting 10% [$^2H_1$], 90% [$^2H_2$] and an overall deuterium content of ≥ 95%. Figure 4.D.3 shows the m.s. of the above derivative in comparison with spectra for derivatives of unlabelled methionine, and (3S,4R)-[3,4-$^2H_2$]methionine supplied by D.C.B.[9].

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CHAPTER 5

BIOSYNTHESIS OF SPERMIDINE FROM L-[3,4-$^{13}C_2$] AND L-[2.3,3-$^{2}H_3$]METHIONINES

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5.A INTRODUCTION

The polyamines putrescine, spermidine, and spermine are naturally occurring non-protein nitrogeneous bases. These polyamines are known to be widely distributed in almost all living cells[1].

The first experiments on the biosynthesis of spermidine were carried out with growing cultures of *E. coli*, using either labelled ornithine or $^{14}C^{15}N$-labelled 1,4-diaminobutane[2] (putrescine). These experiments demonstrated the expected conversion,by the action of a decarboxylase,of ornithine into 1,4-diaminobutane, which was then incorporated into spermidine. Green[3] has shown that the 3-aminopropyl unit of spermidine is derived from methionine. When *Neurospora crassa* was grown on [2-^{14}C]methionine, the label was incorporated into spermidine. In the biosynthesis of spermidine from methionine, it was postulated that the first step is the formation of S-adenosyl methionine (SAM) from methionine and ATP. This reaction was described in yeast[4] as the first step in the methylation reactions. The methyl moiety of SAM is transferred to a variety of substrates (e.g. histamine). For the biosynthesis of spermidine, however, SAM is first decarboxylated and then the aminopropyl group is transferred to 1,4-diaminobutane. The latter reaction, catalysed by spermidine synthase is analogous to transmethylation except that the aminopropyl unit rather than the methyl group is transferred from a sulphonium centre. Early experiments by Tabor *et al.*[2] showed, as expected, that

no ^{14}C was incorporated into spermidine when cultures of *E. coli* were fed with [1-^{14}C]methionine. This confirms the decarboxylation of SAM before the incorporation of the aminopropyl group into spermidine, by the catalytic action of spermidine synthase[5].

This Chapter describes our study of the biosynthesis of spermidine from L-methionine with the aid of methionines labelled with stable isotopes (^{2}H and ^{13}C). The metabolite spermidine was isolated, derivatised and subjected to ^{13}C, ^{1}H and ^{2}H n.m.r. analyses. These n.m.r. spectra were compared with ^{1}H and ^{13}C (natural abundance) n.m.r. spectra of authentic derivatives which have been previously assigned (*cf.* Chapter 3).

The methionines used in this study were L-[2,3,3-$^{2}H_3$] and L-[3,4-$^{13}C_2$]methionine. The importance of the ^{13}C-labelled methionine is to confirm that C-2 and C-3 of the aminopropyl unit are both incorporated into spermidine without cleavage of the C-2/C-3 bond. This complements the work of Green[3], in which he describes the incorporation of C-1 of the aminopropyl unit, derived from [2-^{14}C]-methionine, into spermidine. Taken together, these results would confirm the transfer of the aminopropyl group as an intact unit from methionine into spermidine.

One reason for using L-[2,3,3-$^{2}H_3$]methionine as precursor of spermidine was to confirm the result obtained with [3,4-$^{13}C_2$]methionine. However, the main purpose of using this methionine was to observe the fate of the deuteriums during the biosynthetic process leading to spermidine. The loss of deuterium at C-2 or C-3 might provide additional information about the mechanistic action of S-adenosylmethionine decarboxylase and spermidine synthase. Part of the research project described in this thesis is the study of the stereochemistry of the reaction between 1,4-diaminobutane and decarboxylated adenosylmethionine

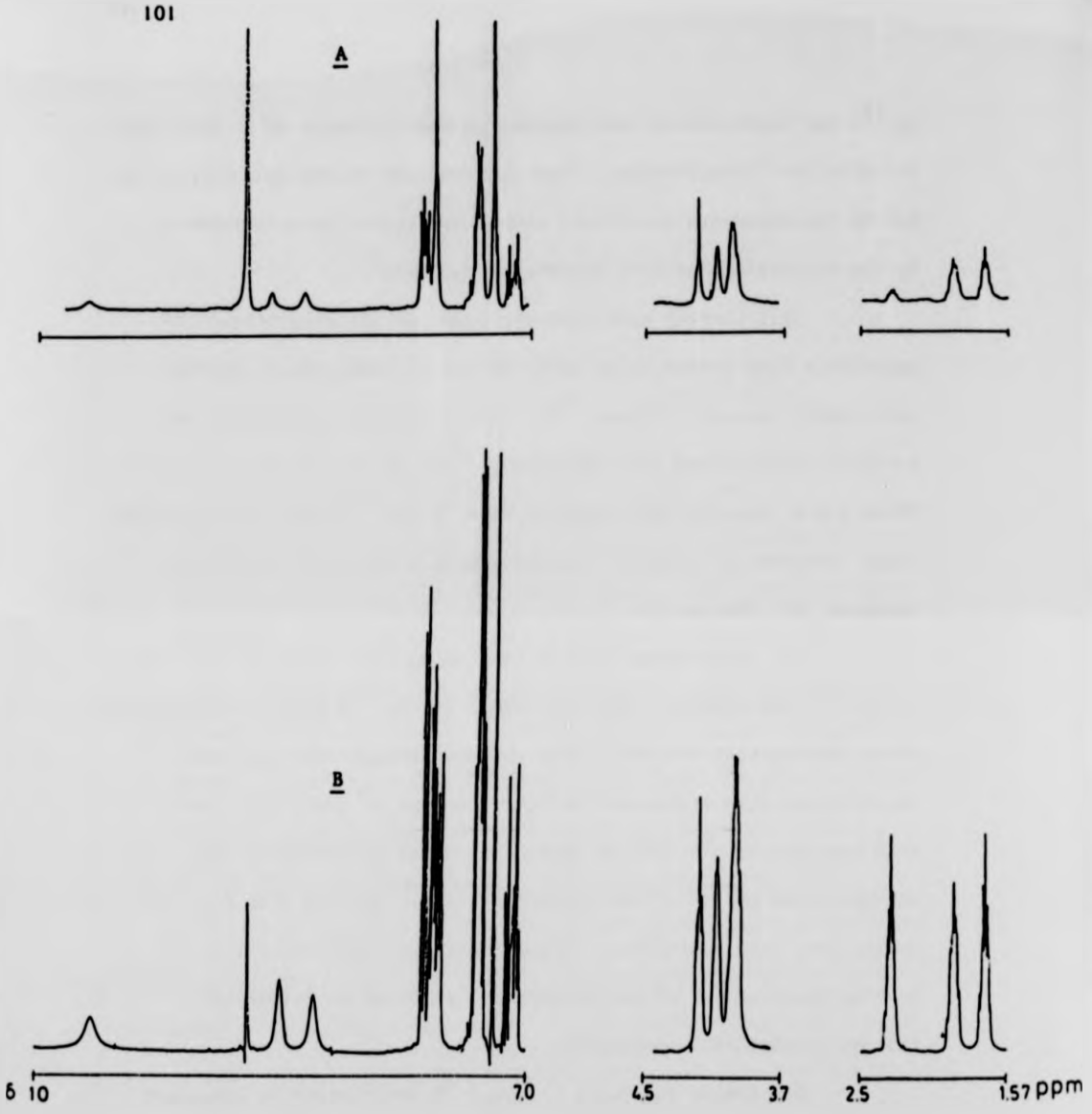

Fig. 5.B.1 The 400 MHz ^{1}H n.m.r. ($[^{2}H_{5}]$pyridine, TMS) spectra of:

A - PATC-[2'-$^{2}H_{2}$]spermidine (20a)

B - PATC-spermidine (20) (authentic unlabelled)
where PATC = phenylaminothiocarbonyl group.

in *E. coli* using methionine stereospecifically labelled with deuterium at C-3 and C-4. It was important for the success of this project to confirm that there is no loss of deuterium or its stereochemical integrity during the biosynthesis of spermidine from methionine.

5.B BIOSYNTHESIS OF SPERMIDINE FROM L-[2,3,3-$^2H_3$]METHIONINE

Cultures of *E. coli* were grown on a salt medium which was supplied with L-[2,3,3-$^2H_3$]methionine*. The biosynthesised putrescine and spermidine were isolated and purified *via* their phenylaminothiocarbonyl (PATC) derivatives as described for unlabelled compounds (*cf.* Chapter 3).

The isolated PATC-putrescine (19) was subjected to 400 MHz ^1H n.m.r. spectral analysis and was found to be devoid of deuterium [spectrum very similar to that of the authentic (19)].

The isolated PATC-spermidine (20a) was examined by 400 MHz ^1H n.m.r. spectral analysis [Fig. 5.B.1(A)]. The spectrum showed some similar features to a spectrum of authentic unlabelled (20) [Fig. 5.B.1(B)], the only significant differences being consistent with the presence of deuterium atoms at the positions indicated in labelled (20a) [Fig. 5.B.1(A)]. Thus the spectrum of (20a) showed complete retention of deuterium at C-2' of spermidine (originally C-3 in the labelled methionine). [N.B. The resonance for H-1' in the unlabelled (20) was a triplet; this signal appeared as a singlet in the spectrum of the labelled PATC-spermidine (20a), due to absence of coupling between this position and the adjacent

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*L-[2,3,3-$^2H_3$]methionine has a [^2H] content of ≥ 86% (m.s. analysis). By ^1H n.m.r. spectral analysis most (*ca.* 98%) of the C-2 position was labelled with deuterium, leaving *ca.* 80% deuterium at C-3.

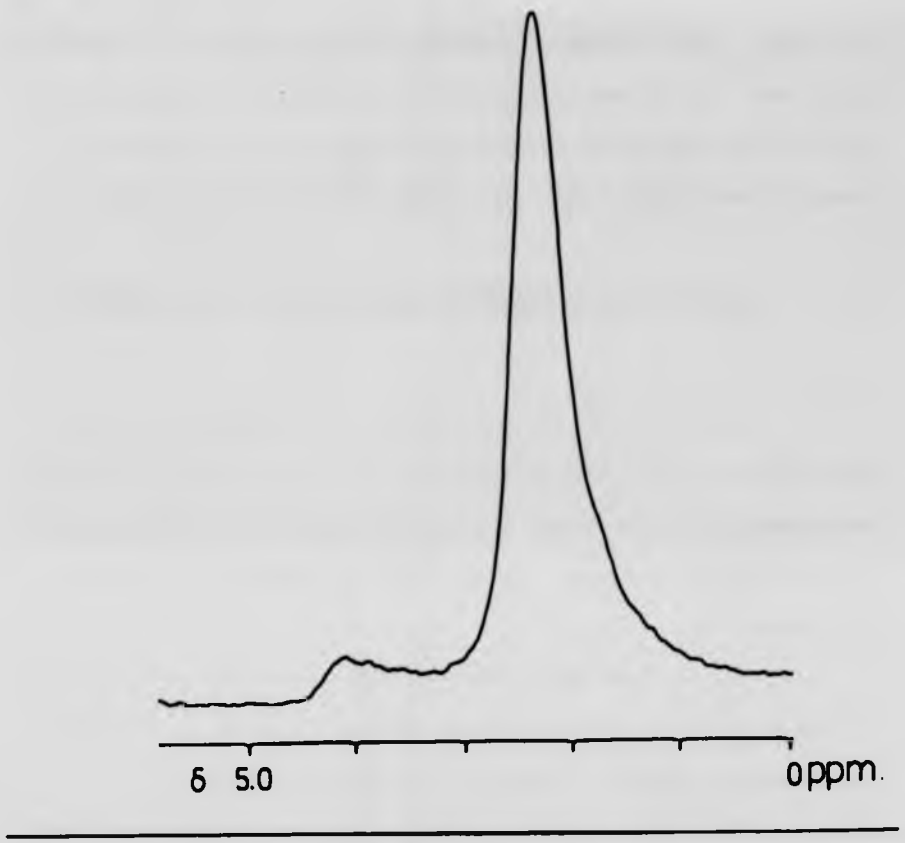
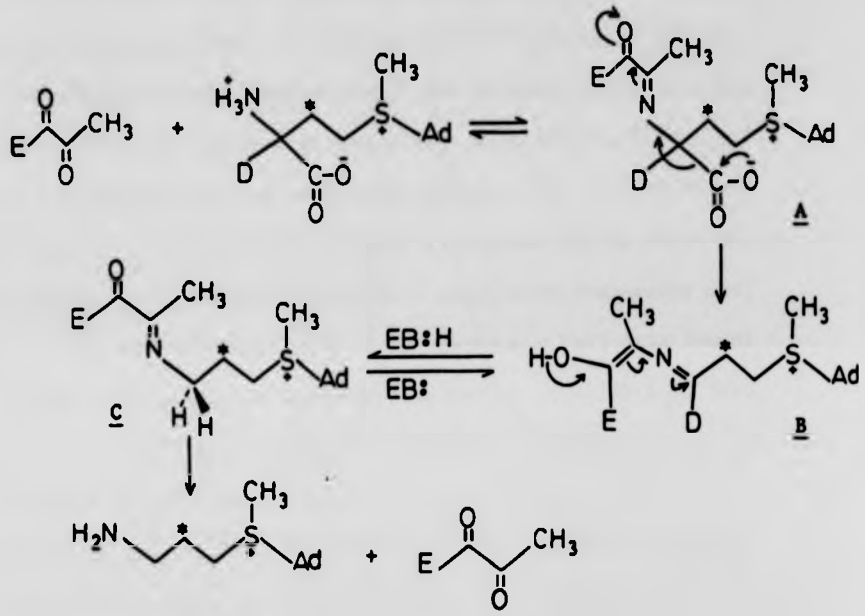


Fig. 5.B.2 The 61.6 MHz ^{2}H n.m.r. spectrum of PATC-spermidine (20a) (in pyridine). Spermidine was isolated from *E. coli* cells which were fed with L-[2,3,3-$^{2}H_3$]methionine.

H-2' (largely deuterated)]. The residual resonance at δ 2.33 (0.2 x 2 H, q, H-2') of the isolated labelled PATC-spermidine (20a) is the result of proton impurities at C-3 of the labelled methionine, estimated to be 20% (see the footnote). The intensity of this signal relative to the unlabelled methylene in the spectrum of compound (20a) was 1:5, which supports the estimate made above for protons at C-3 of the labelled methionine. This result supports the contention that C-1' → C-3' of the isolated spermidine has been biosynthesised from C-2 → C-4 of the methionine supplied to the culture. No dilution from endogenous methionine took place. The ^{1}H n.m.r. spectrum of (20a) showed also that a substantial loss of deuterium took place from C-3' [δ 3.95 (4 H, m, H-3' and H-4) p.p.m.], originally deuterium at C-2 in the labelled methionine [*cf.* Fig. 5.B.1(B)].

The 61.6 MHz ^{2}H n.m.r. spectrum of labelled PATC-spermidine (20a) was examined. This spectrum (Fig. 5.B.2) confirmed the loss of deuterium from C-3'. The integration of the resonances for deuterium at C-2' and C-3' in the 61.6 MHz ^{2}H n.m.r. spectrum of the isolated labelled PATC-spermidine (20a) indicate that ≥ 90% of deuterium at C-3' had been lost in the process of spermidine formation. Thus, the main product was [2',2'-$^{2}H_2$]spermidine rather than the expected [2',2',3'-$^{2}H_3$]spermidine.

In the biosynthesis of spermidine, the enzyme SAM-decarboxylase catalyses[6] the decarboxylation of SAM to give S-(5'-deoxy-5'-adenosyl)-1-(methylthio)propylamine (decarboxylated-SAM)[7]. The enzyme spermidine synthase catalyses the transfer of the aminopropyl moiety from the decarboxylated-SAM to 1,4-diaminobutane (putrescine) to give spermidine[4]. Recently, the enzyme SAM-decarboxylase has been purified to homogeneity from mammalian tissues[8,9], yeast[10], and bacteria[6]. Many amino-acid decarboxylases utilise either pyridoxal phosphate or an enzyme-bound



Scheme 5.B.1 Proposed mode of binding between SAM-decarboxylase and SAM which might lead to the exchange of deuterium at C-2 in L-[2,3,3-$^2H_3$]methionine during the decarboxylation process.

α-ketoacid as prosthetic group. The decarboxylation requires the formation of an intermediate Schiff's base from the α-amino nitrogen of the amino-acid and the carbonyl function of the prosthetic group. The SAM-decarboxylase of *E. coli* contains 1-2 mols of pyruvate covalently bound to each mol of enzyme[6]. This pyruvate was identified as the prosthetic group essential for the catalytic activity of the enzyme[6]. The most common prosthetic group in amino-acid decarboxylases is pyridoxal-5'-phosphate. The pyruvyl residue in SAM-decarboxylase is a relatively uncommon feature.

Abdel-Monem[11] has proposed a binding mode between S-adenosyl-L-methionine decarboxylase and SAM. An azomethine bond was reported to be formed between the substrate and the enzyme (Scheme 5.B.1). Incubation of the enzyme with substrate analogues resulted in a time-dependent irreversible inactivation of the enzyme[11].

We can extend the proposed[11] mode of binding between analogues of substrate and the enzyme, to explain the substantial loss of deuterium, originally present at C-2 of the labelled methionine. The first step in the decarboxylation of the substrate SAM is the formation of an azomethine bond between the amino group of SAM and the ketonic carbonyl of pyruvate to give enzyme-bound adenosylmethionine (A) (Scheme 5.B.1). This is followed by decarboxylation of the adduct (A) to give an enzyme-bound imine (B). The cavity of the active site of the enzyme could have a functional group, which equilibrates the adduct (B) with (C), faster than (C) is hydrolysed to decarboxylated SAM. This functional group acts as a base (e.g. $-NH_2$) in the conversion of (B) into (C). Another possibility is that decarboxylated SAM is released, but undergoes a slower exchange *via* re-formation of (B).

The fact that deuterium originally at C-2 of methionine was

$$RNHCH_2^{*}CH_2^{*}CH_2NRCH_2CH_2CH_2CH_2NHR$$

(20b)

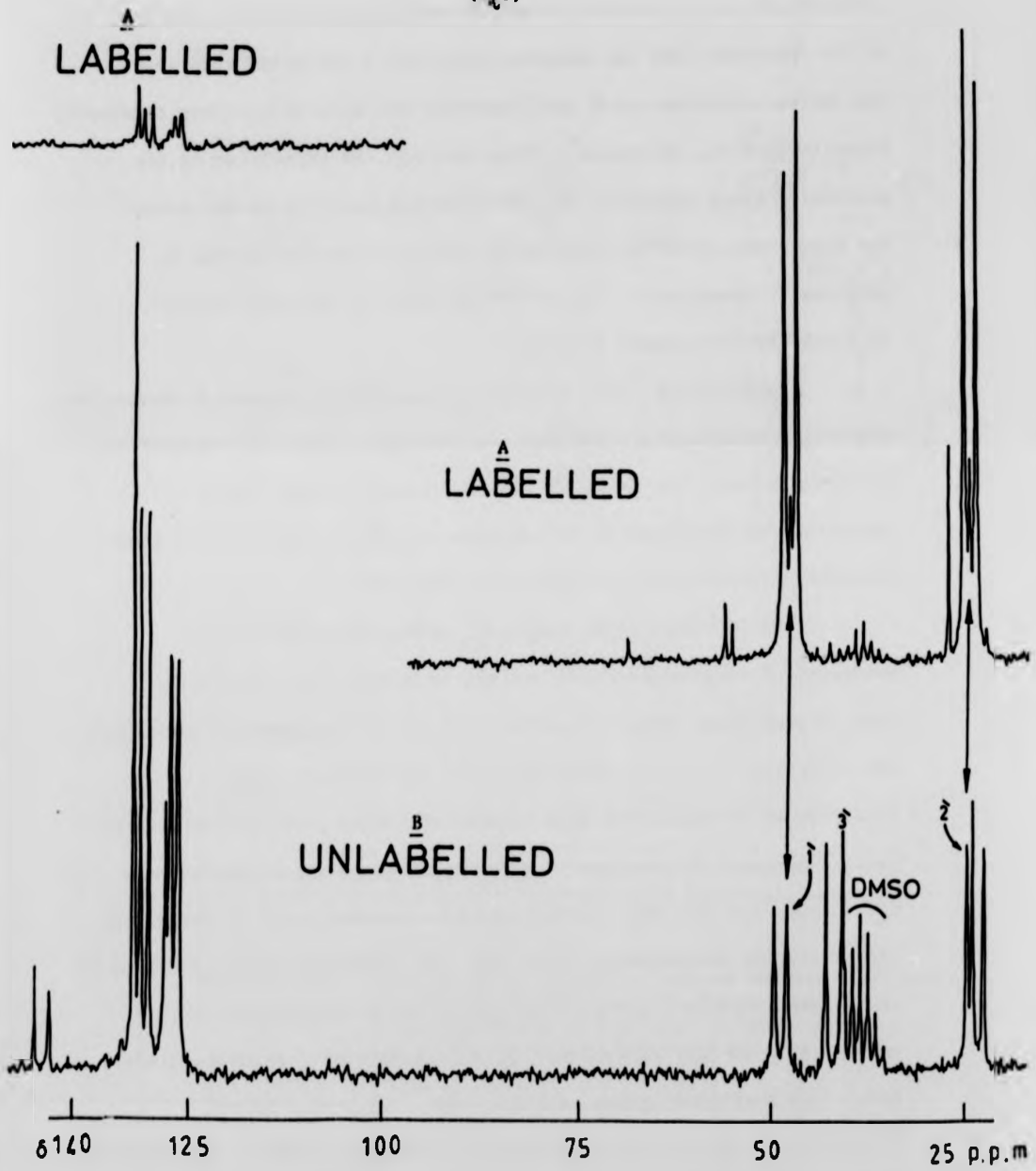


Fig. 5.C.1 The 22.6 MHz $\{^1H\}^{13}C$ n.m.r. ($[^2H_6]$-DMSO, TMS) spectra of:

A - PATC-[1',2'-$^{13}C_2$]spermidine (20b)

B - PATC-spermidine (20) (authentic unlabelled)

where R = phenylaminothiocarbonyl group

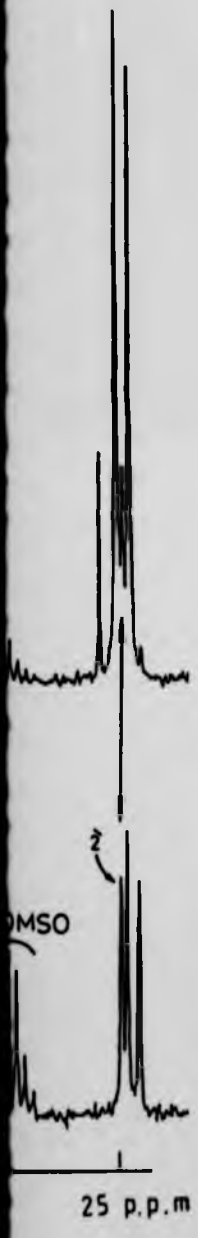
* = ^{13}C

lost, whereas that at C-3 was retained, has provided some more information about the mode of action of SAM-decarboxylase.

It was very important to make sure that deuterium originally at C-3 of methionine does not exchange in the formation of spermidine. We intended to study the stereochemistry of spermidine synthase using stereospecifically labelled [3,4-$^{2}H_{2}$]methionines. Any exchange of deuterium at C-3 of these methionines would result in the loss of stereochemical information (*cf.* Chapter 7).

5.C BIOSYNTHESIS OF SPERMIDINE FROM L-[3,4-$^{13}C_{2}$]METHIONINE

L-[3,4-$^{3}C_{2}$]Methionine (containing 81% $^{13}C_{2}$, 18% $^{12}C^{13}C$ and 1% ^{12}C) was added to a growing culture of *E. coli*. Putrescine and spermidine were isolated as described for unlabelled materials (*cf.* Chapter 3) *via* their phenylaminothiocarbonyl derivatives. The isolated derivatives (19) and (20b) were subjected to ^{1}H and ^{13}C n.m.r. spectral analyses. The ^{1}H and ^{13}C n.m.r. spectra of compounds (19) were consistent with spectra of authentic unlabelled (19) and proved, as expected, no incorporation of ^{13}C into putrescine. The isolated sample of compound (20b) showed in its 22.6 MHz(^{1}H)^{13}C n.m.r. spectrum an intense AX system [doublets $J^{13}C-^{13}C$ 35 Hz, astride singlets at δ 26.8 (C-2') and 48.5 (C-1') p.p.m.] in addition to signals at natural abundance [Fig. 5.C.1(A)]. The 22.6 MHz ^{13}C n.m.r. spectrum of unlabelled PATC-spermidine (20) was assigned with the aid of model compounds (*cf.* Chapter 3). The signals at δ 26.86 and 48.63 p.p.m. in the spectrum of the unlabelled PATC-spermidine (20) [Fig. 5.C.1(B)] were also assigned, with the aid of data for model compounds to C-2' and C-1', respectively (*cf.* Chapter 3, Table 3.C.2). The position of the doublets in the ^{13}C n.m.r. spectrum of labelled



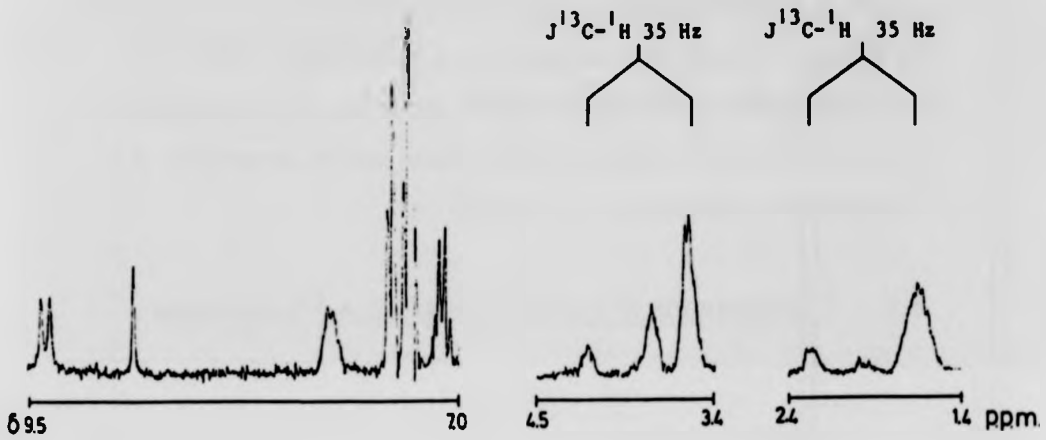


Fig. 5.C.2 The 220 MHz ^{1}H n.m.r. ($[^{2}H_{6}]$-DMSO, TMS) spectrum of PATC-[1',2'-$^{13}C_{2}$]spermidine (20b) showing ^{13}C-^{1}H couplings.

compound (20b) confirm that C-1' is as assigned previously in the unlabelled compound (at δ 48.63 p.p.m.). It shows also that the resonance for C-2' is as assigned in the unlabelled PATC-spermidine (20) at δ 26.86.

The intensities of the signals for C-1' and C-2', in comparison with the natural abundance signals, suggests that methionine had been fully incorporated into spermidine without any significant dilution from endogenous sources of methionine.

The 220 MHz ^{1}H n.m.r. spectrum of the ^{13}C-labelled (20b) was examined (Fig. 5.C.2) and was similar to the spectrum of unlabelled compound (20) [*cf.* Chapter 3 (Fig. 3.C.2)] with some modification due to the presence of two ^{13}C atoms at C-1' and C-2'. The resonance for H-1' appeared at δ 3.8 [double multiplet (0.9 x 2 H, $J^{13}C-^{1}H$ 135 Hz and 0.1 x 2 H, m)] p.p.m. Part of this signal was covered by the broad resonances from H-4 and H-3'at δ3.52 p.p.m. The resonance for H-2' appeared at δ 1.95 [double multiplet (0.9 x 2 H, $J^{13}C-^{1}H$ 135 Hz and 0.1 x 2 H, m)] p.p.m. Part of this signal was covered by resonances from H-3 and H-2 at δ 1.65 p.p.m. The residual signals for H-1' and H-2' at δ 3.8 and 1.95 p.p.m. derive from the *ca.* 10% of unlabelled methionine.

The amount of L-[3,4-$^{13}C_2$]methionine used in this batch of cultures was 0.035 gdm^{-3} rather than the usual amount (0.05 gdm^{-3}). This reduction in the amount of methionine did not affect the amount of cells produced or the spermidine produced by them. The amount of PATC-spermidine obtained (0.065 g) and the mass of wet cells (29 g) were consistent with yields from other batches.

The ^{13}C and ^{1}H n.m.r. spectral analyses described above have proved that C-3 and C-4 of methionine are precursors for C-2' and C-1', respectively, of spermidine. This is the first published[12] work to demonstrate the incorporation of more than one methylene group

from the C-2 → C-4 moiety of methionine into spermidine. It is also the first work to utilise stable isotopes in demonstrating this incorporation.

We considered the possible recovery of pure spermidine from a PATC-derivative, labelled at C-1' and/or C-2' with stable isotope(s). It is possible to hydrolyse PATC-spermidine to free spermidine (*cf.* Chapter 3). This provides specifically labelled spermidine which may be of value for studying the metabolism[13] of spermidine and its interaction with nucleic acids[14]. Recent studies have shown that spermidine is a precursor of a number of alkaloids[15-17]. Specifically labelled spermidine would be useful for studying details of the biosyntheses of these alkaloids.

5.D EXPERIMENTAL

5.D.1 Isolation of [2',2'-$^2H_2$]spermidine (20a)

The standard medium (10 x 1 dm^3) in 10 flasks of 2 dm^3 capacity was supplied with L-[2,3,3-$^2H_3$]methionine (0.05 g dm^{-3}) and inoculated with *E. coli* cells. The culture was incubated at 37°C for 30 h. The cells were harvested by centrifugation to give 32 g cells (wet). The polyamines putrescine and spermidine were extracted from the cells with TCA[2]. The extracted polyamines were converted into PATC-derivatives as described for the unlabelled compounds in Chapter 3.

The isolated mixture of PATC-putrescine (19) and PATC-spermidine (20a) was purified by p.l.c. on a silica gel plate [Kieselgel 60 HR reinst 2 x (0.5 mm x 20 x 100 cm)]. Double elution with dichloromethane/acetonitrile (9/1, v/v) gave two bands. Each

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band was scraped off and extracted with acetone (2 x 200 cm^3). Evaporation of each fraction gave white residues. T.l.c. analysis for each fraction, on a silica gel plate (Kieselgel 60 HR reinst, 0.25 mm thick) and elution with dichloromethane/acetonitrile (87/13, v/v) gave R_f's 0.34 and 0.25 corresponding to compounds (19) and (20a) respectively. The purity of each fraction was checked by h.p.l.c. and each gave a single peak. The yield of compounds (19) and (20a) was (50 mg) and (65 mg), respectively.

The 400 MHz ^1H n.m.r. spectrum ([$^2H_5$]-pyridine, TMS) of compound (19) showed resonances at δ 2.0 (4 H, m) and 4.0 (4 H, m) p.p.m. As expected, no deuterium was observed to be incorporated into compound (19). The 400 MHz ^1H n.m.r. spectrum ([$^2H_5$]-pyridine, TMS) of isolated compound (20a), showed resonances at δ 1.74 (2 H, p, J 7 Hz, H-3), 1.94 (2 H, p, J 7 Hz, H-2), 2.33 (0.2 x 2 H, q, J 7 Hz, H-2'), 3.95 (4 H, m, H-3' and H-4), 4.07 (2 H, br s, H-1), 4.18 (2 H, s, H-1') 8.31, 8.54 and 9.67 (1 H, br s, PhNH) and 7.0-7.7 (phenyl protons) p.p.m. The 61.6 MHz ^2H n.m.r. (pyridine) of compound (20a) was examined (Fig. 5.B.2) and showed resonances at δ 2.35 (2 ^2H, br s, ^2H-2') and 4.0 (0.1 x ^2H, br s, ^2H-3') p.p.m. The n.m.r. indicates that ≥ 90% of the deuterium at C-3' has been lost during the formation of spermidine.

5.D.2 Isolation of [1',2'-$^{13}C_2$]spermidine (20b)

The standard medium (10 x 1 dm^3) in 10 flasks of 2 dm^3 capacity was supplied with [3,4-$^{13}C_2$]methionine (0.035 g dm^{-3}) and inoculated with *E. coli* cells. The culture was incubated at 37°C for 30 h. The cells were harvested by centrifugation to give 29 g cells (wet). The polyamines were isolated and purified *via* their PATC-derivatives, as described for the unlabelled compounds (*cf.*

Chapter 3). The pure compound (19) isolated from this culture was subjected to ^{13}C n.m.r. spectral analysis and gave no indication for the presence of ^{13}C above natural abundance. Its ^{1}H n.m.r. spectrum was very similar to that of authentic (19). The 22.6 MHz $\{^{1}H\}^{13}C$ n.m.r. ($[^{2}H_6]$-DMSO) analysis of isolated compound (20b) showed an intense AX system [doublets (J 35 Hz) astride singlets at δ 26.8 (C-2') and 48.5 (C-1') p.p.m.] in addition to signals at natural abundance (Fig. 5.C.1). The 220 MHz ^{1}H n.m.r. ($[^{2}H_6]$-DMSO, TMS) of isolated derivative (20b) was examined (Fig. 5.C.2) and showed signals at δ 1.65 (4 H, m, H-2 and H-3), 1.95 (0.9 x 2 H, 2 x m, J ^{13}C-^{1}H 135 Hz, and 0.1 x 2 H, m, H-2'), 3.52 (4 H, m, H-3' and H-4), 3.75 (2 H, m, H-1), 3.8 (0.9 x 2 H, 2 x m, J ^{13}C-^{1}H 135 Hz and 0.1 x 2 H, m, H-1'), 7.75 (2 H, br m, 2 x $CH_2\underline{NH}$), 8.88, 9.45, 9.52 (each 1 H, s, Ph$\underline{NH}$) and 7.0-7.4 (phenyl protons) p.p.m.

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CHAPTER 6

HEXAHYDROPYRIMIDINES FROM SPERMIDINE AND RELATED COMPOUNDS

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6.A INTRODUCTION

Recent studies on the physiological significance of amine-carbonyl interactions have centred on:

(1) the design of polyfunctional agents for sequestering metabolically generated ethanal[1] and

(2) reactions between catecholamines and aldehydes[2].

The possible physiological significance of polyamine-aldehyde interactions has usually been discussed in terms of imines or poly-imines as the supposed products of condensation.

In this Chapter the author describes the reaction of some polyamines with ethanal in neutral solvents (e.g. water and dichloromethane). The reaction of 1,3-diaminopropane, spermidine, and 1,2-diaminoethane always resulted in the formation of a cyclic product, whereas 1,4-diaminobutane afforded a bis-imine. Some chemical aspects of these reactions and the products are discussed.

Our interest in the reaction of ethanal with polyamines started when we faced the problem of solving the stereochemistry of spermidine synthase. We could not achieve direct analysis for the stereochemistry of deuterium-labelled spermidines prepared synthetically or obtained biosynthetically from *E. coli* cells. It was decided to approach this problem by conversion of these spermidines into hexahydropyrimidines. We found that spermidine reacts with ≥ 2 mol equiv. ethanal to give first a hexahydropyrimidine* (13) and then an

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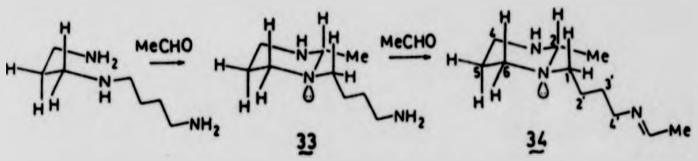
*The name hexahydropyrimidine of spermidine is an abbreviation for 1-(4'-aminobutyl)-2-methylhexahydropyrimidine, and the name imine-hexahydropyrimidine is an abbreviation for 1-[4'-(N-ethylidene)-aminobutyl]-2-methylhexahydropyrimidine.



(Eqn. 6.1)

imine-hexahydropyrimidine (34) (Eqn. 6.1). The assignment of the ^{1}H n.m.r. spectrum of such a derivative was rather difficult by direct analysis. We found that the preparation and the ^{1}H n.m.r. analysis of model hexahydropyrimidines was essential for understanding the reactions between ethanal and polyamines and to achieve a correct analysis and understanding of the hexahydropyrimidines from spermidine. In this Chapter, full analysis of the ^{1}H n.m.r. spectra of some model compounds are discussed in detail.

6.B SOME ASPECTS OF THE CHEMISTRY OF HEXAHYDROPYRIMIDINES

The condensation of aldehydes or ketones with an amino function is the most common way for the formation of an azomethine bond. The product of the condensation of a primary amine with a carbonyl group is usually called an aldimine (or Schiff's base). The condensation process is usually accompanied by the elimination of 1 molecule of water. Usually, in the preparation of an aldimine, potassium hydroxide is added to assist dehydration[1,2].

The importance of the amine/carbonyl condensation was demonstrated in the formation of an azomethine[3] bond in many enzyme-mediated processes[4] (e.g. transmethylation and decarboxylation).

Hexahydropyrimidines are mostly synthesised by the direct condensation of 1,3-diaminopropane or its N-alkyl or N-aryl derivative with an aldehyde or ketone. Hexahydropyrimidines frequently exist in equilibrium with an open-chain amino-imine[5,6], with the position of the

equilibrium dependent on factors such as the degree of substitution of the ring and the reaction solvent. The ring-chain tautomerism in hexahydropyrimidines becomes evident when the cyclic structure is partially destabilised by the introduction of two alkyl groups at C-2[7]. Thus, hexahydropyrimidine itself exists completely as the cyclic form and has no absorption in the double bond region of the infra-red spectrum. However, its 2,2-dimethyl derivative must be a tautomeric mixture in which the open-chain form gives rise to the C=N stretching vibration at 1670 cm^{-1} in the infra-red spectrum. By replacement of one hydrogen atom of an NH group of a hexahydropyrimidine with t-butyl, the tautomeric equilibrium shifts completely in favour of the open-chain form.

Hexahydropyrimidines show the normal behaviour of aliphatic bases, but some are unstable in the sense that C-2, being directly linked to two nitrogen atoms, is very readily eliminated by acidic hydrolysis yielding a 1,3-diaminopropane and an aldehyde or ketone.

Preparation of hexahydropyrimidines by the catalytic hydrogenation of pyrimidines is rather unlikely to succeed, because under normal hydrogenation conditions, the hexahydropyrimidines undergo reductive ring cleavage. Those cases where steric factors cause strain in the cyclic structure and give rise to a dynamic equilibrium with an open-chain tautomer containing an azomethine bond[8] are more readily hydrogenated.

The oxidation of hexahydropyrimidine derivatives has only been recorded for the N,N'-dimethyl compound, which gave N,N'-dimethyl-1,3-diaminopropane[7]. 2-Methylhexahydropyrimidine (35) is unique among

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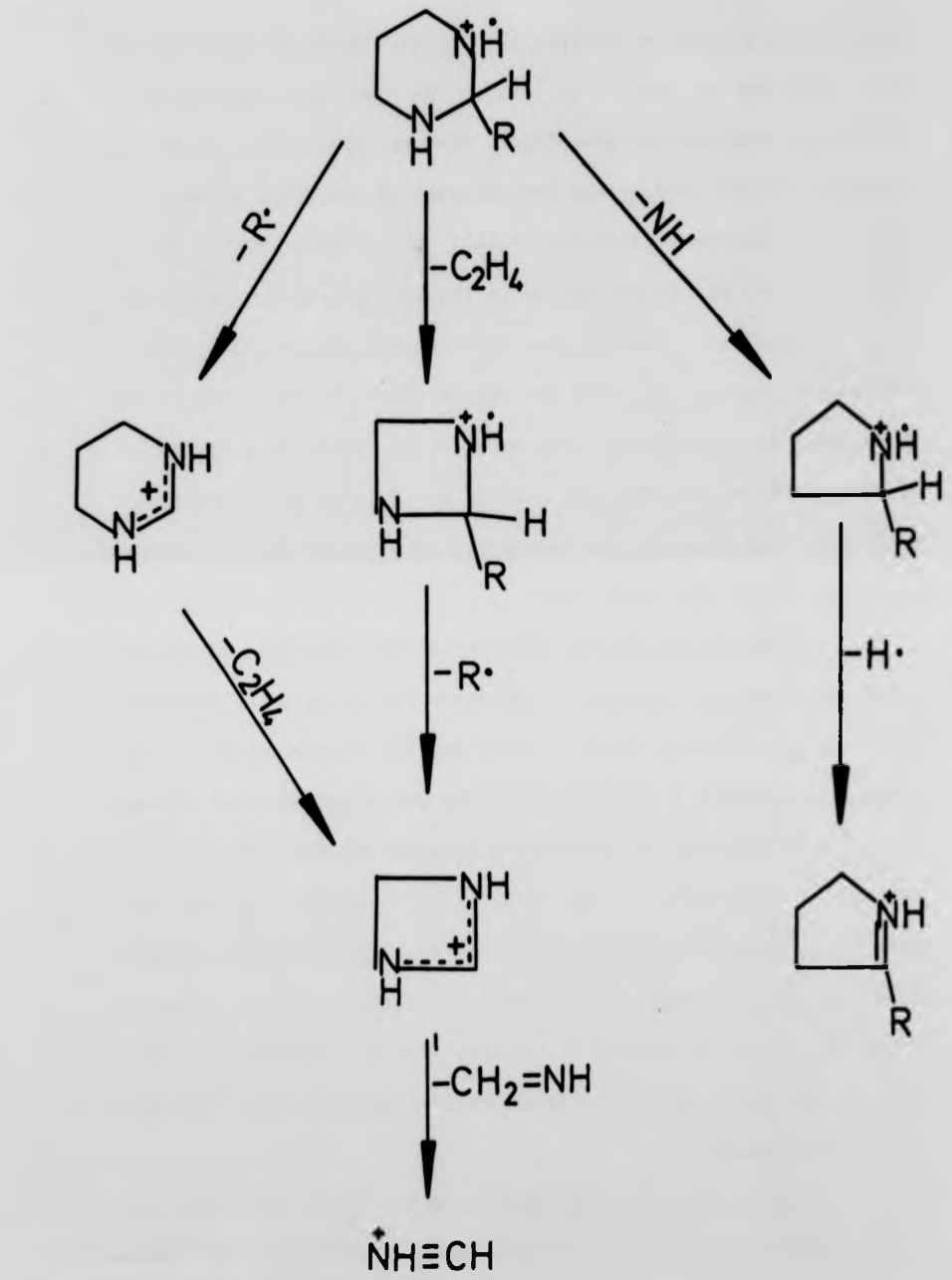


Scheme 6.B.1 Fragmentation patterns of hexahydropyrimidines (abstracted from Ref. 9a).

the hexahydropyrimidines examined in that, when this compound was shaken with platinum and hydrogen under vigorous conditions, hydrogen was evolved from the reaction and 1,4,5,6-tetrahydropyrimidine was isolated. Thus, under apparent reducing conditions, an oxidation occurred[8]! It could be assumed that under these conditions, a platinum-catalysed equilibrium between the hexahydro and the tetrahydro derivatives was set up which favoured the tetrahydro species in the case of the 2-methyl derivative.

Much mass spectral data is available for hexahydropyrimidines. The favoured pathway for the fragmentation of a hexahydropyrimidine is shown in Scheme 6.B.1[9]. Of the three patterns indicated in the Scheme, the left hand side one is of interest because by loss of the R group the molecular ion gives a resonance-stabilised cyclic amidinium ion, already noted as responsible for the highly basic nature of 1,4,5,6-tetrahydropyrimidine.[10] The stability of the amidinium ion is indicated by its intensity being greater than that of the parent molecular ion.

Other alkylated and N-substituted hexahydropyrimidines exhibit this outstanding feature in their mass spectra, whereby a group is ejected from C-2 to give a resonance-stabilised entity of greater abundance than the molecular ion. When there is a choice of ejection of one of two radicals from C-2 to give two different amidinium ions of comparable stability, the cracking pattern involving the more stable and hence less energetic free radical predominates. Thus, with the molecular ion of 2-methylhexahydropyrimidine (35) the preferred pathway by a factor of over 3 is ejection of a methyl radical to give the 1,4,5,6-tetrahydropyrimidinium ion, rather than loss of a hydrogen atom to give the 2-methyl analogue. The ratio of peak heights is reported[7] to be $(M-15)^+/(M-1)^+/M^+ = 18:5:1$. The presence of a cyclic

amidinium ion in the mass spectrum of a hexahydropyrimidine is a useful means of differentiating between the cyclic and the open-chain tautomers. The mass spectrum of the latter is not expected to show any peaks corresponding to this ion.

The cyclic tautomers of hexahydropyrimidines can be identified by infra-red spectroscopy: presence of a strong peak at 3270 cm^{-1}, which can be assigned to the NH stretching vibration of a secondary amine[11]. There is no sharply defined absorption in the 1600-1700 cm^{-1} region which would be assigned to the -C=N- stretching vibration of the open-chain tautomer.

The δ values for certain absorption peaks in the ^{1}H n.m.r. spectra of hexahydropyrimidine and some of its derivatives are reported in Ref. 7.

The spectrum of 1,3-diaminopropane consists of two peaks: a triplet in the 2.5-3.25 δ region assigned to the two protons of each methylene next to the nitrogen atoms, and a multiplet in the 1.3-2.3 δ region due to the central methylene[12]. The ^{1}H n.m.r. spectra of hexahydropyrimidine and its derivatives, when examined at room temperature (which is well above the temperature where ring inversion between two equivalent chair forms is slow) do not differentiate[13] between the axial and equatorial protons at C-2. Full ^{1}H n.m.r. data for some model compounds is given in the next section.

The conformational preference of saturated heterocyclic six-membered rings is believed, like cyclohexane derivatives, to be for chair conformations with equatorial substituents. Hydrogen atoms on the nitrogens are axial leaving the lone pairs equatorial. This has been demonstrated for a wide variety of compounds such as piperidine[14] and piperazine[15]. These have chair conformations of

geometry closely analogous to that of cyclohexane. In substituted cyclic compounds, the non-bonded interactions between an axial alkyl substituent and the β-axial hydrogens, largely determine the conformational preference of that substituent. Large groups have a greater preference for the equatorial position than small groups. In an N-alkyl substituted heterocyclic compound the substituents may occupy either the equatorial or axial position. Interconversion of these two conformations by nitrogen inversion is a much easier process ($\Delta G^{\ddagger} \leq 40$ kJ mol^{-1}) than ring inversion ($\Delta G^{\ddagger} > 40$ kJ mol^{-1})[16], which enables the N-alkyl group to change its orientation without the intervention of ring inversion. The position of the equilibrium between the two chair conformations is determined by the size of the N-substituent and the interaction of the lone-pair(s) with adjacent substituents.

In hexahydropyrimidines, the presence of another nitrogen atom in the ring increases the rate of nitrogen inversion (relative to cyclic compounds containing one hetero atom, e.g. piperidine). It was no surprise to find that the barrier to ring inversion[13,17] is 45 kJ mol^{-1}, whereas the barrier to nitrogen inversion is very much less than this[18]. The most important question concerning the conformations of hexahydropyrimidines is the position of the conformational equilibrium on nitrogen. This question was answered by ^{1}H n.m.r.[19], ^{13}C n.m.r.[20] and dipole moment[21] measurements. The N-methyl derivative of hexahydropyrimidine was found from measurements of N-H to C-2 methylene coupling constants to have an equatorial methyl but an axial N-H group. The N,N'-dimethyl derivative of hexahydropyrimidine was studied by comparing its spectral characteristics with models (e.g. 1,3,5,5-tetramethylhexahydropyrimidine)[19b]. The diequatorial conformation is *ca.* 2 kJ mol^{-1} more stable than either of the enantiomeric

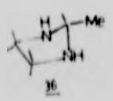
axial-equatorial conformations.

6.C MODEL HEXAHYDROPYRIMIDINES

6.C.1 Introduction

The work on hexahydropyrimidine derivatives was started when we faced the problem of assigning the stereochemistry of spermidine synthase. The assignment of the relative configuration of the deuterium atoms in spermidine, introduced at C-1' and C-2', was considered to be impossible from examination of spectra of the free [1',2'-$^2H_2$]spermidine because of signals overlapping. Although the PATC-derivative of spermidine gives separated signals for the protons at C-1' and C-2', it is impossible to assign the relative configuration of the deuterium atoms at C-1' and C-2' in such a derivative because of the lack of knowledge about the conformations of this compound. It was therefore necessary to find a derivative of spermidine having a well-defined conformation and a ^1H n.m.r. spectrum easy to analyse. We considered the possibility of forming a diamagnetic metal complex of spermidine, which would have a 6-membered ring including the metal ion and C-1' → C-3' of spermidine. However, we found that polyamines in general and spermidine in particular react with ethanal to give hexahydropyrimidine derivatives which would have the desired properties.

We found that 1,2-diaminoethane reacted with one mol equiv. of ethanal to give 2-methylimidazolidine (36). The ^1H n.m.r. spectrum of this compound showed resonances at ($CDCl_3$, TMS) δ 1.28 (3 H, $-CHC\underline{H}_3$),



36

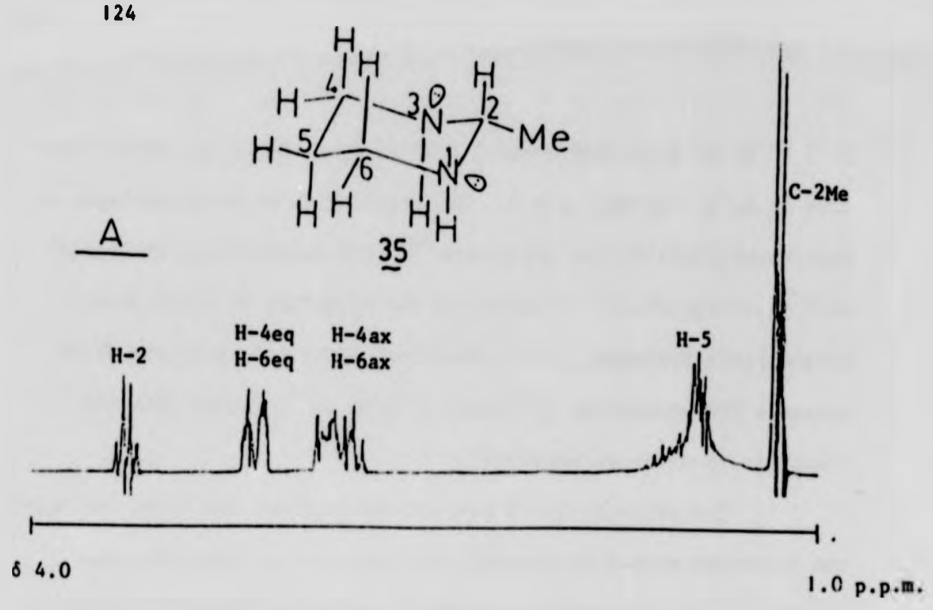
2.78 (2 H, m, H-4ax and H-5ax), 3.01 (2 H, m, H-4eq and H-5eq) and 3.24 (1 H, q, -CH-CH_3) p.p.m. The preparation of such compounds is well established in the literature[22]. The condensation of ethanal with 1,4-diaminobutane resulted in the formation of 1,4-diamino-butane-*bis*(ethylimine) [N.B. characterised by i.r. analysis which showed a C=N stretching vibration at 1670 cm^{-1}] rather than the 2-methyl-1,3-diazacycloheptane.

The preparation of hexahydropyrimidines was first attempted using the classical method of Branch[5], who condensed a monoprotonated 1,3-diamine with a carbonyl compound to give the hexahydropyrimidine ring. Following this procedure, the maximum yield achieved by the author for the preparation of hexahydropyrimidine derivatives was 20-25%. This low yield could be due to acid-catalysed polymerisation of the hexahydropyrimidine produced. However, we found that carrying out the condensation of the 1,3-diamine with a carbonyl compound in a neutral organic solvent ($CDCl_3$ or CH_2Cl_2) or in water (^2H_2O) (i.e. without the protonation of the starting diamine) gave much higher yields (60-70%).

6.C.2 ^1H n.m.r. analysis of model compounds

1. Preparation and acetylation of 2-methylhexahydropyrimidine (35)

The condensation of 1,3-diaminopropane with 1.1 mol equiv. of ethanal in water gave rise to the rapid formation of 2-methylhexahydropyrimidine (35) (65% yield), which was purified by distillation. The ^1H n.m.r. spectrum of compound (35) [Fig. 6.C.1(A)] showed resonances at ($CDCl_3$, TMS) δ 2.85 p.p.m., corresponding to the axial protons at C-4 and C-6, and resonances at δ 3.15 p.p.m. (Jgem 14.0 Hz), corresponding to the equatorial protons at C-4 and C-6.



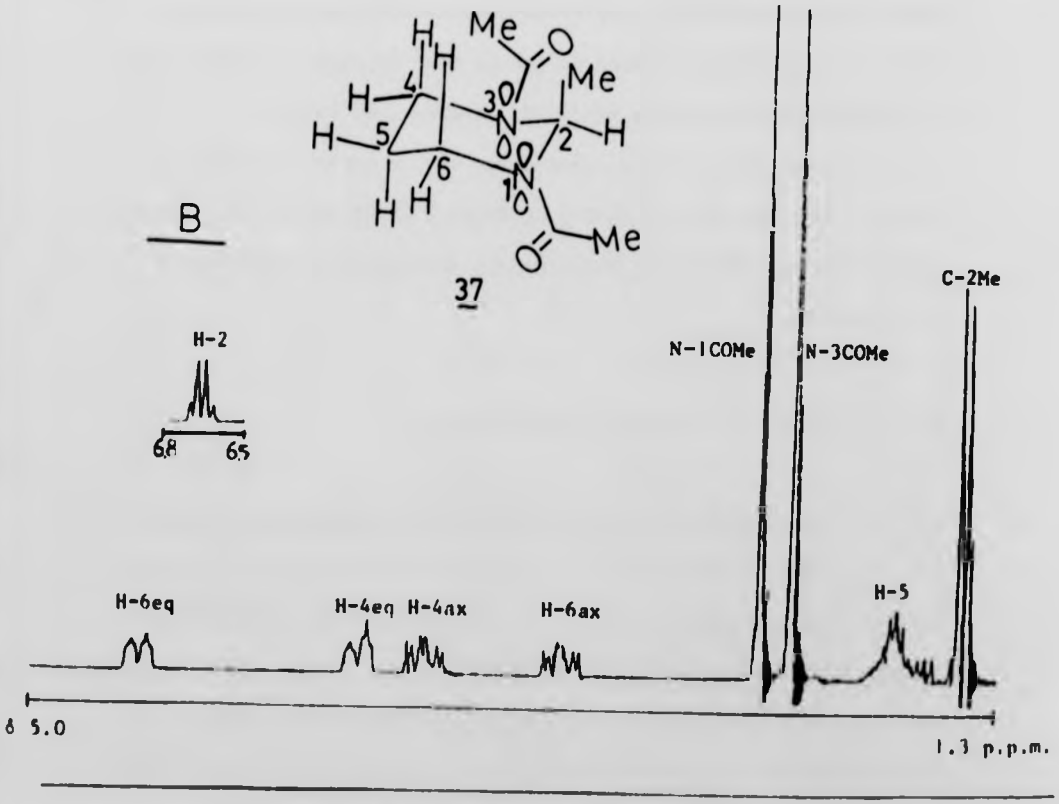
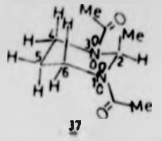


Fig. 6.C.1 The 220 MHz ^{1}H n.m.r. ($CDCl_3$, TMS) spectra of
A - 2-Methylhexahydropyrimidine (35)
B - 1,3-Diacetyl-2-methylhexahydropyrimidine (37)

The difference in the chemical shifts between the axial and equatorial protons in compound (35) is due to the shielding effect of the lone-pairs of the nitrogen atoms on the adjacent axial protons[16]. The spectrum also shows an AX_3 system for the proton at C-2 [δ 3.64 (1 H, q) p.p.m.], and the methyl group [δ 1.16 (3 H, d) p.p.m.] attached to that position. The protons at C-5 appeared at δ 1.48 p.p.m. as a broad multiplet.

Acetylation[23] of compound (35), using the reagent acetic anhydride in aqueous sodium hydroxide, gave 1,3-diacetyl-2-methyl-hexahydropyrimidine (37). The purity of this compound was confirmed by its combustion analysis. The e.i.m.s. analysis showed two



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small peaks for M^+ (0.05%) and $(M-1)^+$ (0.007%) with a large peak for $(M-15)^+$ (100%). The amidinium ion $(M-15)^+$ resulting from the ejection of the methyl radical at C-2, is preferred to either the ion generated by the ejection of a hydrogen atom or the parent ion M^+ [relative intensities = 100:5:1, respectively]. This ratio is rather different from the ratio observed with hexahydropyrimidine (35), in which the $(M-15)^+$ ion is not much preferred to $(M-1)^+$ or M^+ [relative intensities 18:5:1, respectively].

The i.r. spectral analysis of compound (37) showed a very strong and broad peak at ν_{max} 1630cm^{-1}. This peak was assigned to the two acetyl groups at N-1 and N-3.

The 220 MHz ^{1}H n.m.r. spectrum of compound (37) [Fig. 6.C.1(B)] showed signals at ($CDCl_3$, TMS) δ 1.45 and 6.65 p.p.m. These were assigned to the AX_3 system of the methyl group and the proton attached to C-2. [N.B. The C-2 methyl became axial because the carbonyl function of the acetyl group is directed towards the equatorial position.] The signals at δ 2.08 and 2.21 p.p.m. were asisgned to the methyls

of the acetyl groups at N-3 and N-1, respectively. The difference in the chemical shifts of these two methyls suggested a difference in their environments [see structure in Fig. 6.C.1(B)]. It is necessary to postulate that one of the acetyl groups (say that at N-3) is positioned in such a way that its carbonyl group is directed away from the C-2 methyl, whereas the carbonyl of the acetyl group at N-1 is pointing in the opposite direction (i.e. towards the C-2 methyl). Further evidence for this conclusion follows from examining the signals for protons at C-4 and C-6. The protons at C-4 showed resonances at δ 3.49 (1 H, t x d, Jgem 15 Hz, Jvic 3 and 15 Hz, H-4ax) and δ 3.74 (1 H, d x t, Jgem 15 Hz, Jvic 2.5 and 2.5 Hz, H-4eq) p.p.m. The change in the chemical shifts of the H-4 signals as a result of adding an electron withdrawing acetyl at N-3 was found to be 0.64 and 0.6 p.p.m. for H-4ax and H-4eq, respectively [from comparison with data for the precursor (35)]. The protons at C-6 showed resonances at δ 2.97 (1 H, t x d, Jgem 15 Hz, Jvic 3 and 15 Hz, H-6ax) and δ 4.59 (1 H, d x t, Jgem 15 Hz, Jvic 2.5 and 2.5 Hz, H-6eq) p.p.m. The shifts in the signals of the C-6 protons of compound (37), as a result of acetylating N-1 and N-3, would be expected to be similar to the shifts of the H-4 signals (see above). By comparison with data for compound (35) [the precursor of (37)], H-6eq was found to have shifted downfield by 1.44 p.p.m., while H-6ax was shifted by only 0.12 p.p.m. These shifts resulting from acetylation at N-1 are different from the values obtained from acetylation at N-3.

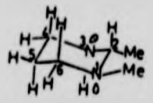
The observed differences for the chemical shifts of protons at C-4 and C-6 can be explained by the proposed conformation in Fig. 6.C.1(B). The proton H-6eq lies in the deshielding region of the adjacent carbonyl group and therefore appears at a very different

chemical shift from H-4eq.

Confirmation for the assignment of the C-4 and C-6 protons of compound (37) was achieved by decoupling experiments. Irradiation at δ 4.59 (H-6eq) p.p.m. resulted in the loss of the Jgem coupling of H-6ax (δ 2.97 p.p.m.). The signal for H-6ax changed from a triple doublet to a double doublet (Jvic 3 and 15 Hz). The multiplet for protons at C-5 also showed some modification. Irradiation at δ 2.97 p.p.m. resulted in loss of the Jgem coupling of H-6eq, which changed from a doublet of triplets to one triplet with both Jvic 2.5 Hz. The resonances for protons at C-5 were also modified as expected. Irradiation at δ 3.74 (H-4eq) and 3.49 (H-4ax) p.p.m. showed the expected modifications.

2. Preparation and attempted acetylation of 1,2-dimethyl-hexahydropyrimidine (38)

Preparation of 1,2-dimethylhexahydropyrimidine (38) was achieved by the condensation of N-methyl-1,3-diaminopropane with 1.1 equiv. of ethanal in water. The yield obtained in the preparation of this compound was 60%. The e.i.m.s. analysis of this compound



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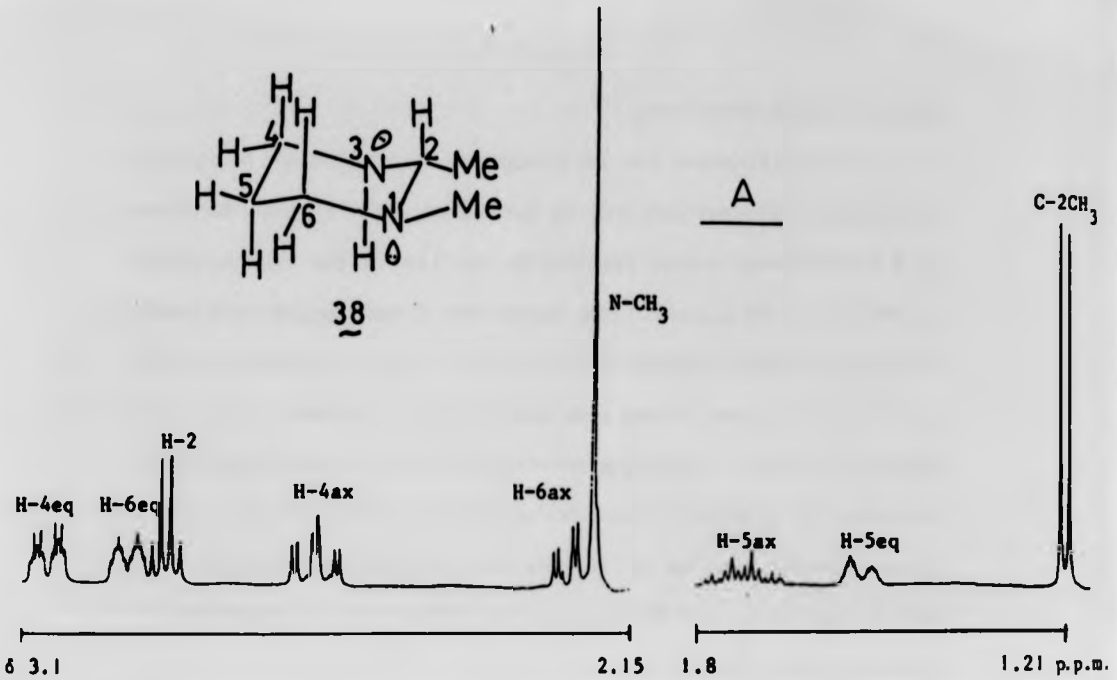
showed M^+, $(M-1)^+$, $(M-2)^+$ and $(M-15)^+$ ions in a ratio of 5:27:88:100%, respectively. It seems that the displacement of the amino hydrogen in hexahydropyrimidine (35) by a methyl group, increases the stability of the $(M-1)^+$ ion at the expense of the $(M-15)^+$ ion. The loss of two hydrogens from C-2 and the unsubstituted amino group gave rise to a new ion $(M-2)^+$, which showed a relatively high stability in comparison to the parent ion.

The 400 MHz ^1H n.m.r. spectrum of compound (38) [Fig. 6.C.2(A)] showed an AX_3 system at δ 1.21 (3 H, d) and 2.88 (1 H, q) p.p.m. for



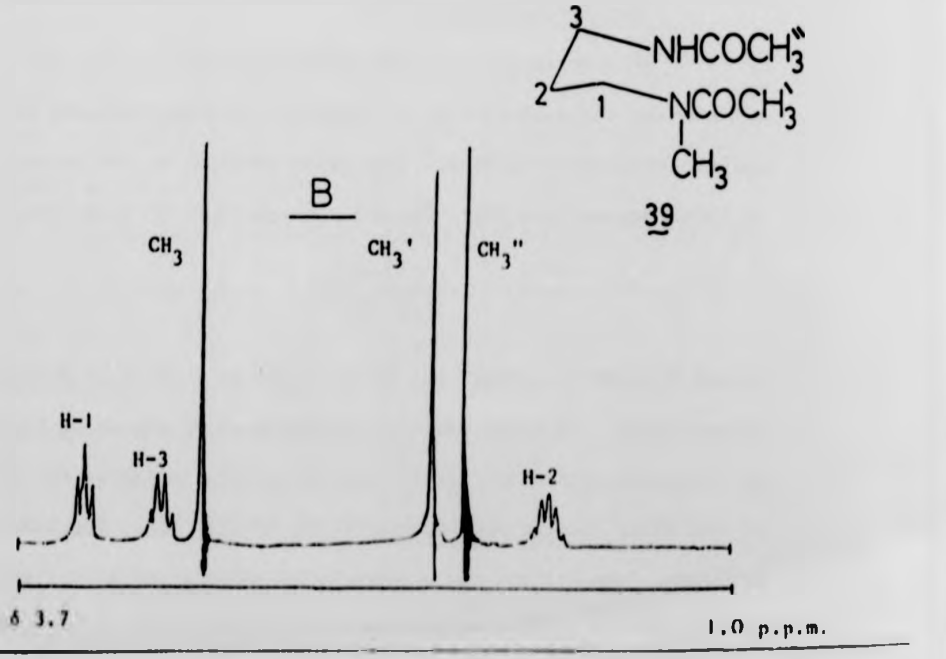


Fig. 6.C.2 ^{1}H n.m.r. spectra of:

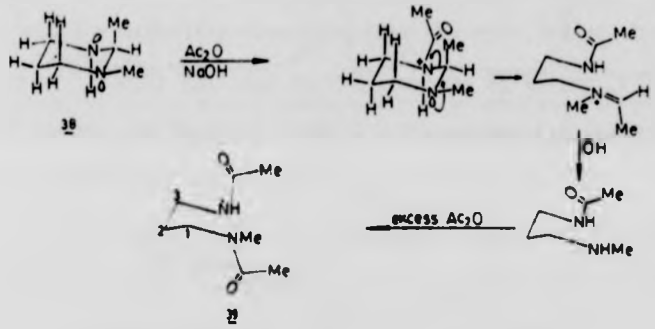
A - 1,2-Dimethylhexahydropyrimidine (38)

B - N,N'-Diacetyl-N-methyl-1,3-diaminopropane (39)

the proton and methyl group at C-2. The quartet of the C-2 proton at δ 2.88 p.p.m. was shielded by 0.76 p.p.m. compared to the quartet of the C-2 proton of compound (35). This shielding arises from the presence of the equatorial methyl group at N-1 [Fig. 6.C.2.(A)]. The signal for C-5 protons in the 2-methylhexahydropyrimidine (35) showed a broad resonance at δ 1.48 p.p.m. The C-5 protons of compound (38) showed a signal of double multiplets at δ 1.53 p.p.m. for H-5eq and a quartet of triplets at δ 1.74 p.p.m. for H-5ax. The presence of a methyl group (δ 2.21 p.p.m.) at N-1 of the ring causes the lone-pair of that nitrogen to take up an axial position. The 1,3-diaxial interaction between this lone-pair and H-5ax, compared to a 1,3-diaxial H-H interaction in (35), is probably responsible for the separation of H-5ax from H-5eq in (38). The single N-substituent in (38) leads to the appearance of resonances for H-4ax and H-6ax, as well as H-4eq and H-6eq at different chemical shifts. The signal for H-6ax at δ 2.25 p.p.m. (t x d, Jgem 13 Hz, Jvic 3 and 13 Hz) was shielded by 0.6 p.p.m. [in comparison to the signal of H-6ax (δ 2.85 p.p.m.) in compound (35)] due to the presence of the axial lone-pair at N-1. To a lesser extent, the lone-pair also shielded the H-6eq at δ 2.95 p.p.m. (d x m Jgem 13 Hz) by 0.2 p.p.m. [in comparison to the signal of H-6eq (δ 3.15 p.p.m.) in compound (35)]. The N-3 lone-pair probably prefers an equatorial orientation as in (35), and so there are only small chemical shift differences between H-4ax/H-4eq in (38) and the corresponding protons in (35). The assignments of the C-4 → C-6 protons were confirmed by a series of decoupling experiments by irradiating at each proton in turn.

The attempted synthesis of 1,2-dimethyl-3-acetyl-hexahydropyrimidine failed due to the opening of the hexahydropyrimidine ring under the conditions used for the acetylation. These conditions were

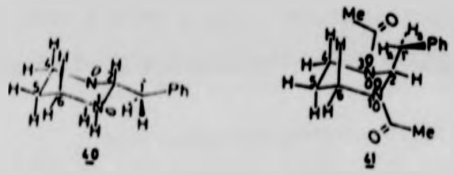
acetic acid and DCC in THF, or acetic anhydride and NaOH in water. Acetylation of compound (38) was also attempted with acetic anhydride and NaOH in methanol or dichloromethane. The product of all these reactions, when examined by ^{1}H n.m.r. spectroscopy showed the formation of N,N'-diacetyl-N-methyl-1,3-diaminopropane (39) [see Fig. 6.C.2(B)]. This was obtained *via* an imine intermediate, which resulted from the ring-opening of the hexahydropyrimidine as illustrated in Scheme 6.C.1.



SCHEME 6-C-11

3. Preparation and acetylation of 2-benzylhexahydropyrimidine (40)

Preparation of 2-benzylhexahydropyrimidine (40) was achieved by the condensation of 1.1 mol equiv. phenylacetaldehyde with 1 mol equiv. 1,3-diaminopropane in water, and gave the pure compound (40), on distillation (80°C at 2 x 10^{-2} mmHg), in 73% yield. The 220 MHz ^{1}H n.m.r. spectrum of compound (40) showed resonances [Fig. 6.C.3(A)] at δ 2.26 p.p.m. (d, CH-C$\underline{H}_2$-Ph) overlapping with resonances of H-4ax and H-6ax at δ2.27 p.p.m. The signals for H-6eq and H-4eq resonated at δ 3.15 (d, Jgem 13.6 Hz, with additional fine



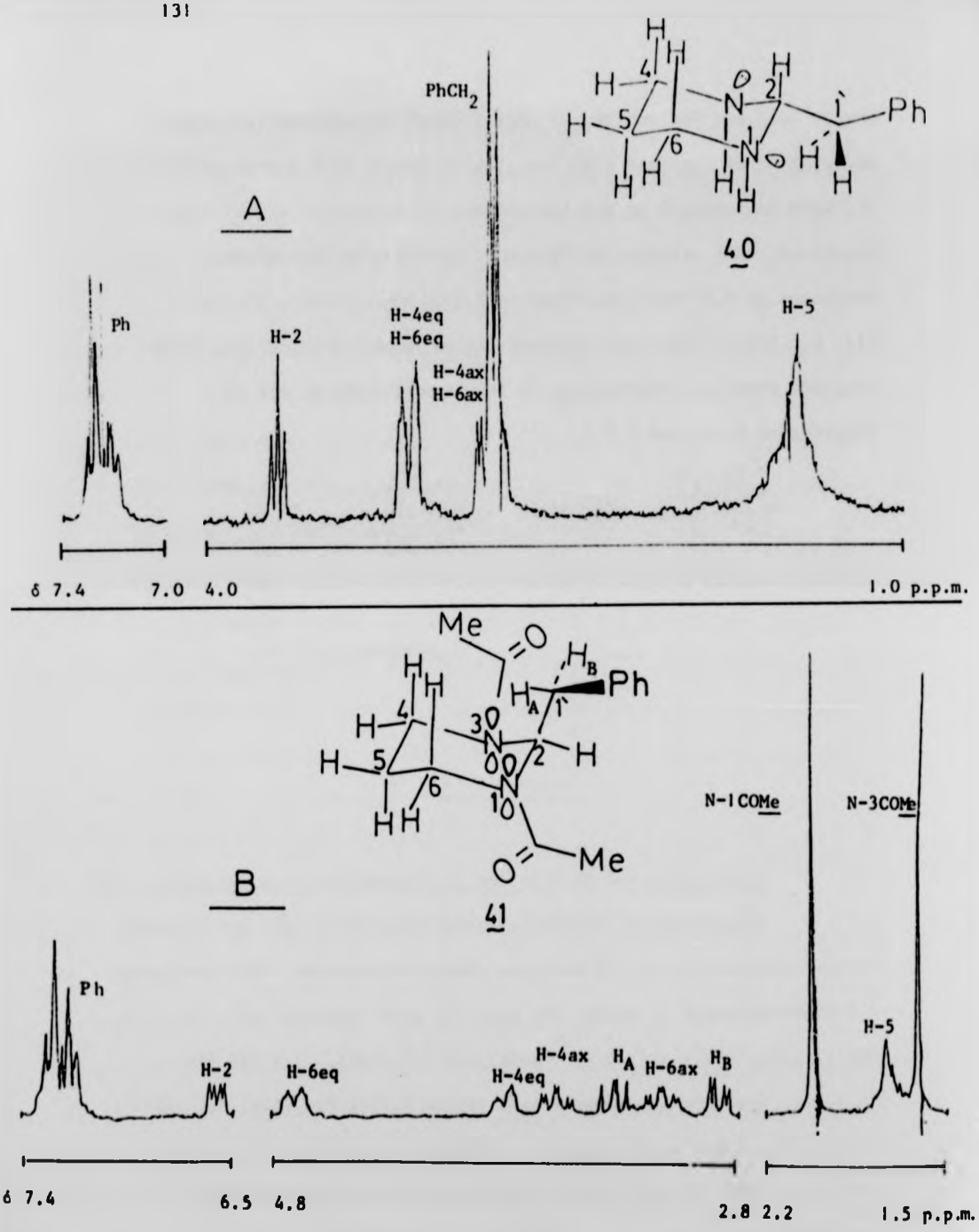


Fig. 6.C.3 The 220 MHz ^{1}H n.m.r. ($CDCl_3$, TMS) spectra of

A - 2-Benzylhexahydropyrimidine (40)

B - 1,3-Diacetyl-2-benzylhexahydropyrimidine (41)

splitting from the vicinal couplings) p.p.m. Signals for C-5 protons appeared as a broad multiplet at δ 1.46 and signals for H-2 appeared at δ 3.7 p.p.m. as a triplet.

Acetylation of compound (40) afforded a pure product of 1,3-diacetyl-2-benzylhexahydropyrimidine (41). The i.r. spectral analysis of compound (41) showed two strong peaks at ν_{max} 1630 and 1645 cm^{-1}. These peaks were assigned to the acetyl groups at N-1 and N-3.

The e.i.m.s. analysis of compound (41) showed amidinium ions of similar stability resulting from the ejection of the benzyl group $(M-91)^+$ (100%) followed by eliminations of ketene [ions at $(M-133)^+$ (100%) and $(M-175)^+$ (100%)]. The parent ion showed a very small percentage (*ca.* 4%) relative to the amidinium ions.

The 220 MHz ^1H n.m.r. analysis for compound (41) was consistent in every detail with the structure of the compound [Fig. 6.C.3(B)]. The effect of acetylation on compound (40) is similar to that seen with compound (35). The assignment of signals for C-4 and C-6 protons on compound (41) was done directly by comparison with the ^1H n.m.r. spectrum of compound (37) [Fig. 6.C.1(B)]. The only new feature in the ^1H n.m.r. spectrum of compound (41) is the appearance of an ABX system between the protons of C-1' ($-\underline{CH}_2-Ph$) and the adjacent H-2 proton. The two methyls of the acetyl groups are pointing in opposite directions as for compound (37). One of the protons (H_A) of C-1' is assumed to be pointing in the direction of the carbonyl group attached to N-1 and is therefore expected to be deshielded. The signal of the H_A proton appeared at δ 3.32 (d x d, Jgem 12 Hz, Jvic 14 Hz) p.p.m. The signal of the H_B proton appeared at δ 2.88 (d x d, Jgem 12 Hz, Jvic 3.6 Hz) p.p.m. The H_X proton (i.e. H-2) resonated at δ 6.57 (d x d, Jvic 3.6 and 14 Hz) p.p.m.

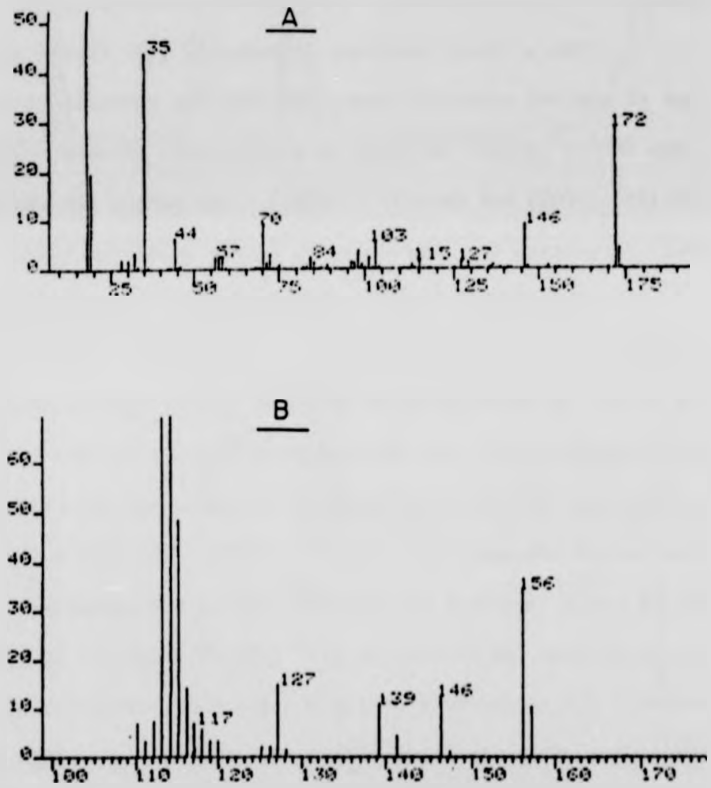


Fig. 6.D.1 The mass spectrometric analysis of compound (33):

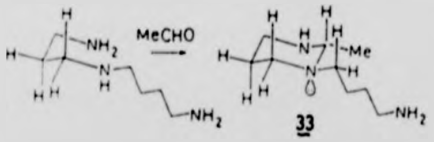
A - The c.i.m.s. showing the ion $(M+1)^+$ at 172

B - The e.i.m.s. showing the ion $(M-15)^+$ at 156

6.D HEXAHYDROPYRIMIDINES FROM SPERMIDINE

1. Preparation of 1-(4'-aminobutyl)-2-methylhexahydropyrimidine (33)

The hexahydropyrimidine derivative of spermidine (33) was prepared by dropwise addition of 1 mol equiv. of ethanal into a solution of spermidine in chloroform. This reaction was monitored by ^{1}H n.m.r. spectral analysis which showed after 5 minutes the disappearance of free spermidine and the complete formation of the cyclic derivative (33). [N.B. The ^{1}H n.m.r. spectrum of compound (33) is very similar to that of 1-[4'-(N-ethylidine)aminobutyl]-2-methyl-hexahydropyrimidine (34) (see below), the only differences being the absence of the resonance for the N=CH-CH_3 group, and the appearance of the C-4' protons partly overlapping with the resonances of the H-1'eq and H-4ax at δ *ca.* 2.67 p.p.m.] Compound (33) was analysed



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by c.i. and e.i. mass spectrometry. The c.i. mass spectrum [Fig. 6.D.1(A)] showed a major peak for the protonated molecular ion $(M+1)^+$ at m/z 172. The e.i. mass spectrum [Fig. 6.D.1(B)] showed a major peak at m/z 156 corresponding to the ion $(M-15)^+$. An exact mass measurement for this ion gave a value of 156.1494 ($C_8H_{18}N_3$, error -3.8 p.p.m.).

2. Preparation of 1-[4'-(N-ethylidene)aminobutyl]-2-methyl-hexahydropyrimidine (34)

The imine-hexahydropyrimidine derivative of spermidine (34) was prepared by dropwise addition of ethanal (≥ 2 mol equiv.) into a solution of spermidine in chloroform. For ^{1}H n.m.r. analysis

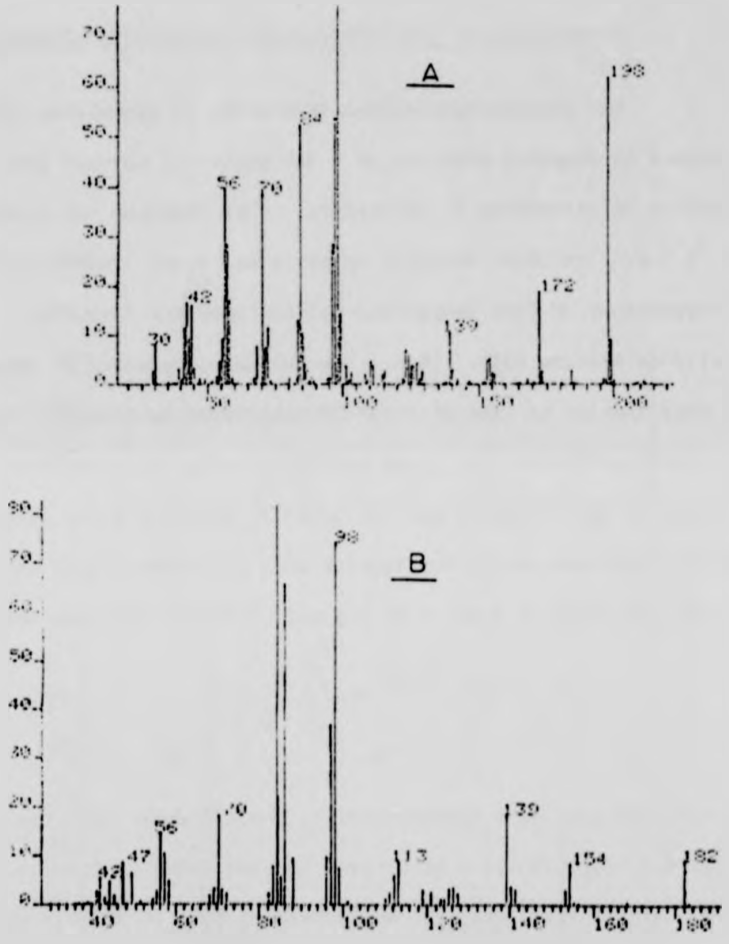

Fig. 6.D.2 The mass spectrometric analysis of compound (14)

A - The c.i.m.s. showing the ion $(M+1)^+$ at 198

B - The e.i.m.s. showing the ion $(M-15)^+$ at 182

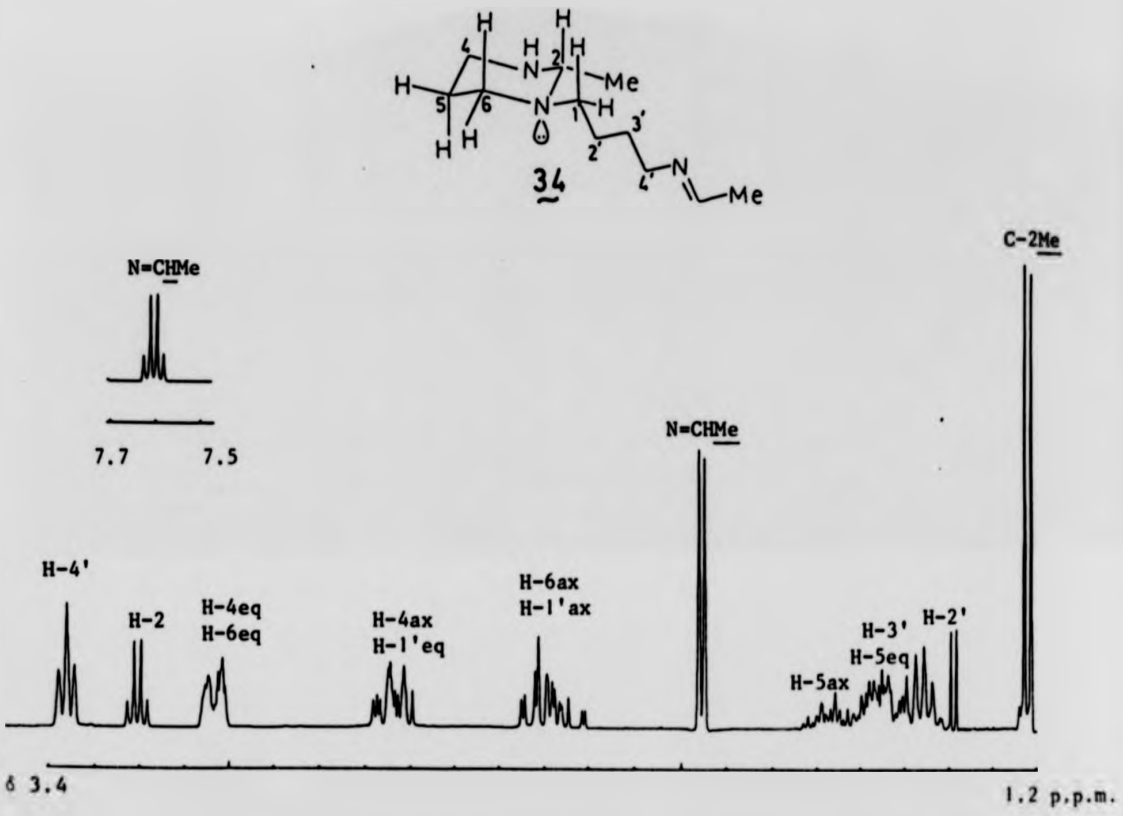

Fig. 6.D.3 The 400 MHz ^{1}H n.m.r. spectrum ($CDCl_3$, TMS) of 1-[4'-(N-ethylidene)aminobutyl]-2-methylhexahydropyrimidine (34).

this reaction was carried out, directly in the n.m.r. tube. The cyclisation and imine formation of compound (34) was complete within 5 minutes at room temperature (see Eqn. 6.1). Compound (34) was analysed by c.i. and e.i. mass spectrometry. The c.i. mass spectrum [Fig. 6.D.2(A)] showed a major peak for $(M+1)^+$ at m/z 198. The e.i. mass spectrum [Fig. 6.D.2(B)] showed a peak at 182 corresponding to the $(M-15)^+$ ion. An exact mass measurement for this ion gave a value of 182.1657 ($C_{18}H_{20}N_3$, error 0 p.p.m.).

The assignment of the 400 MHz ^1H n.m.r. ($CDCl_3$, TMS) spectrum of compound (34) (Fig. 6.D.3) was difficult. This assignment was achieved with the aid of data for model compounds (*cf.* 6.C). The 4'-(N-ethylidene)aminobutyl chain of compound (34) is expected to take up an equatorial position on the hexahydropyrimidine ring leaving an axial lone-pair at N-1 (*cf.* 6.B). It is expected on steric grounds that the butyl chain will take up a conformation which extends away from the ring.

The first order analysis for spectrum of compound (34) (Fig. 6.D.3) showed signals at δ 1.22 (3 H, d, J 6 Hz, C-2Me), 1.95 (3 H, d, J 4.5 Hz, N=CHMe), 3.20 (1 H, q, J 5.8 Hz, H-2), 3.36 (2 H, t, J 6.9 Hz, H-4'), and 7.60 (1 H, q, J 4.5 Hz N=CHMe) p.p.m. The signals for the protons H-5eq, H-2' and H-3' were all in the region δ 1.35-1.65 p.p.m., which appears as a complicated multiplet. Although the position of each signal for H-5eq, H-2' and H-3' can be roughly assigned, the exact shape and position of these signals is impossible to judge because of overlap. The H-5ax proton appeared at δ 1.67 (q x t, Jgem 12 Hz, Jvic 4,4,12 and 12 Hz) p.p.m. This assignment was confirmed by decoupling experiments. Irradiation at δ 3.04 p.p.m. (H-6eq and H-4eq), resulted in the appearance of the H-5ax signal as a quartet (Jgem 12 Hz, Jvic 12 and 12 Hz) due to

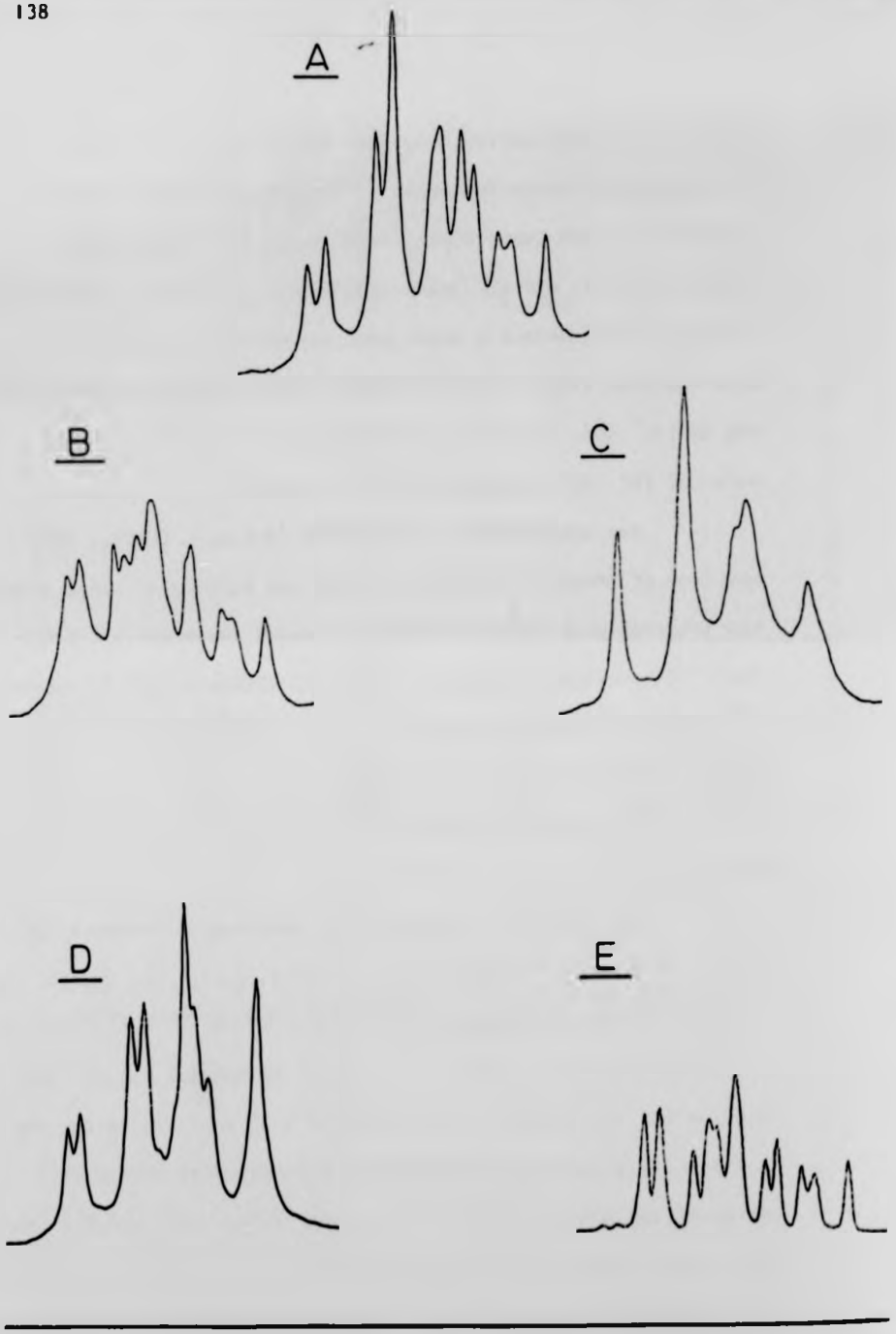

Fig. 6.D.4 The remaining resonances of the H-6ax and H-1'ax signals (A) after decoupling the protons of:
(B) H-5ax, (C) H-5eq, (D) H-2' and (E) H-6eq.

the loss of the small Jvic coupling with H-4eq and H-6eq. Irradiation at δ 2.33 p.p.m. (H-6ax) changed the signal from H-5ax into a triple triplet (Jgem 12 Hz, Jvic 4, 4 and 12 Hz) owing to the loss of the big Jvic coupling with H-6ax. A similar result (i.e. to the effect from H-6ax decoupling) was obtained when the H-4ax proton was decoupled. Signals at δ 3.04 p.p.m. were assigned to H-6eq and H-4eq (d, Jgem 12 Hz, with additional fine splitting). The assignment was confirmed by decoupling H-5ax at δ 1.67 p.p.m. This caused the signals from H-6eq and H-4eq to lose the fine splitting [due to coupling with H-5ax (Jvic ∿ 4 Hz)]. It remains to justify the assignments for H-6ax, H-1'ax, H-4ax and H-1'eq.

The signal from H-6ax appeared at δ 2.33 (t x d, Jgem 12 Hz, Jvic 3 and 12 Hz) p.p.m., and were partly overlapping with the signals of H-1'ax at δ 2.28 (d x d x d, Jgem 13 Hz, Jvic 6 and 8.5 Hz) p.p.m. [see Fig. 6.D.4(A)]. Confirmation of these assignments was derived from a series of decoupling experiments involving the protons of H-5ax, H-5eq, H-6eq and H-2' and spectral simulation with the aid of the instrument's computer. Irradiation at H-5ax (δ 1.67 p.p.m.) resulted in the formation of a double doublet in place of the original signal of H-6ax (Jgem 12 Hz and Jvic 3 Hz). The signal of H-1'ax was essentially unaffected [*cf.* Fig. 6.D.4(B)]. The decoupling of H-5eq (*ca.* δ 1.52 p.p.m.) from H-6ax resulted in the collapse of the signal of H-6ax into a triplet with Jgem = Jvic = 12 Hz. The power of the irradiation also affected the signal of H-1'ax [N.B. because the signals of H-5eq and H-2' are very near, the power of the irradiation also decoupled H-2' protons from H-1'ax], which collapsed into a broad doublet [*cf.* Fig. 6.D.4(C)]. This doublet was sharpened when the irradiation was centred at the protons of C-2' (*ca.* δ 1.47 p.p.m.). This irradiation did not affect the triple doublet of H-6ax which became easier to discern [*cf.* Fig. 6.D.4(D)].

Irradiation at δ 3.04 p.p.m. (H-6eq) resulted in the collapse of the triple doublet of H-6ax into a double doublet. The double double doublet of H-1'ax was essentially unaffected [*cf.* Fig. 6.D.4(E)].

The assignments for H-6ax and H-1'ax were further supported by simulation of their signals on the Bruker WH-400 computer ('Panic' Programme). The parameters measured from the spectrum were fed to the computer and were iterated. The simulated signals* for H-1'ax and H-6ax [Fig. 6.D.5(A)] were very similar to the observed signals (B)

Fig. 6.D.5 The 400 MHz ^{1}H n.m.r. signals for H-1'ax and H-6ax
(A) simulated (B) observed

(above Figure) and gave the following coupling constants:

H-6ax-H-6eq, J 12.0 Hz

H-6ax-H-5eq, J 3.6 Hz

H-6ax-H-5ax, J 11.4 Hz

H-1'ax-H-1'eq, J 12.1 Hz

H-1'ax-H-2'**, J 6.5 Hz

H-1'ax-H-2'**, J 8.2 Hz

δ (H-6ax) - δ (H-1'ax) = 15.36 Hz

The signals for H-4ax at δ 2.66 p.p.m. appeared as a triple doublet (Jgem *ca.* 13 Hz, Jvic 3 and *ca.* 12 Hz) and was partially overlapping with the signal of H-1'eq at δ 2.63 p.p.m.[(theoretically d x d x d) observed couplings Jgem 13 Hz, Jvic *ca.* 7 and 8. Hz) [Fig. 6.D.6(A)]. The assignments were confirmed by a sequence of

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*These signals have a line width of 2 Hz.
**The H-2' protons are not individually assigned.

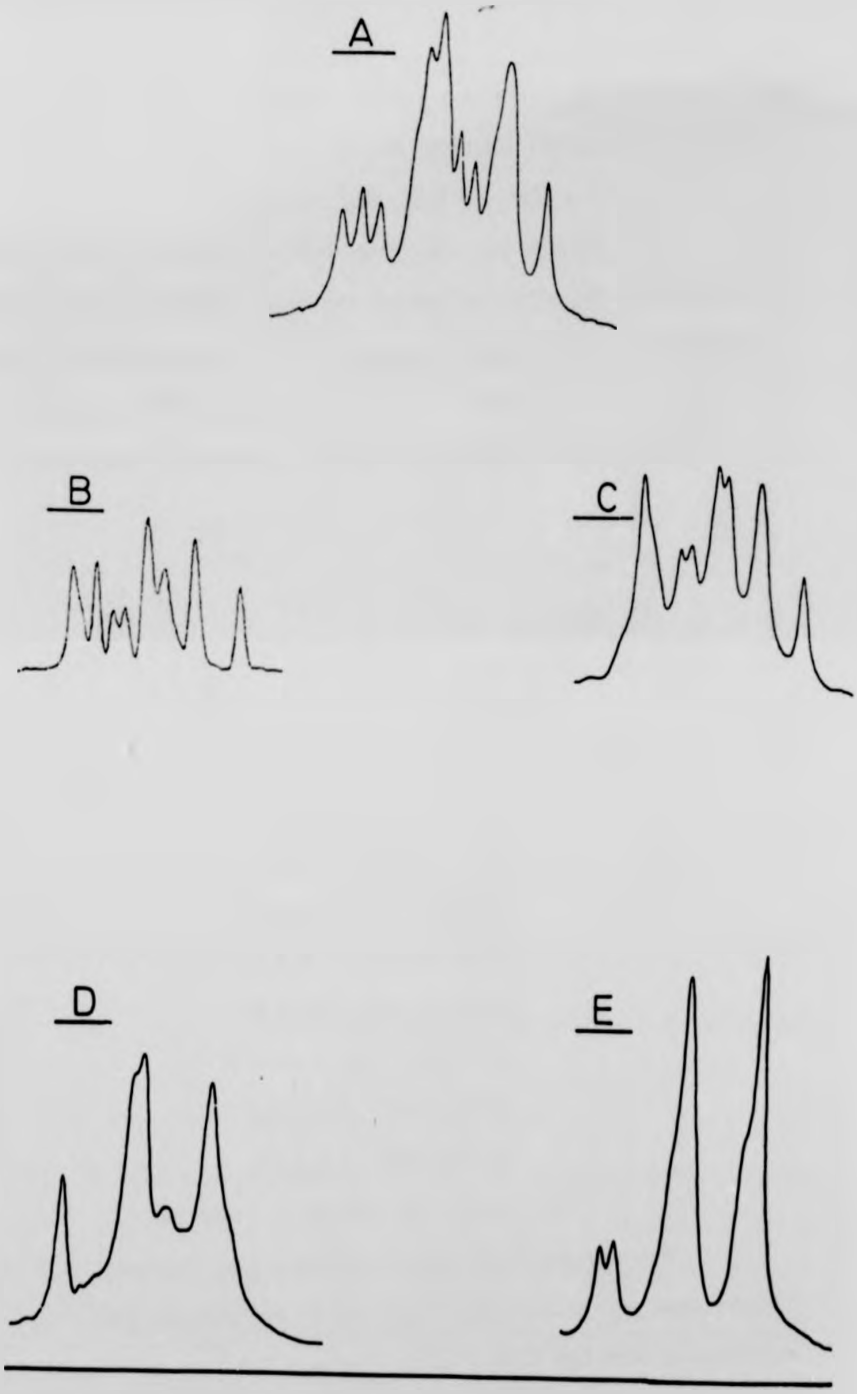

Fig. 6.D.6 The remaining resonances from H-4ax and H-1'eq signals (A) after decoupling the protons of: (B) H-4eq, (C) H-5ax, (D) H-5eq and (E) H-2'.

decoupling experiments at H-4eq, H-5ax, H-5eq, and H-2'. Irradiation at δ 3.04 p.p.m. (H-4eq) resolved the signal of H-4ax into a double doublet. The signal of H-1'eq was unaffected [Fig. 6.D.6(B)]. Irradiation at δ 1.67 p.p.m. (H-5ax) also resolved the signal of H-4ax into a broad doublet due to the removal of Jvic 12 Hz [Fig. 6.D.6(C)]. Decoupling the H-5eq from H-4ax by irradiation at δ 1.52 p.p.m. showed the collapse of the signal of H-4ax into a triplet, which was still overlapping with the remnant of H-1'eq [N.B. upon irradiation at H-5eq the protons at C-2' were also affected; this led to appreciable decoupling between H-2' and H-1'eq] [Fig. 6.D.6(D)]. Decoupling of H-2' from H-1'eq collapsed the signal from the proposed dxd xd into a doublet with a strong Jgem coupling (13 Hz). Each peak of this doublet was overlapping with two peaks from the triple doublet of H-4ax [Fig. 6.D.6(E)].

The above assignments were further supported by simulation of the overlapping H-4ax, H-1'eq signals using the Bruker WH-400 computer ('Panic' Programme). The parameters measured from the spectrum were fed to the computer and were iterated. The simulated signals* for H-4ax and H-1'eq [Fig. 6.D.7(A)] were mostly similar to the observed signals

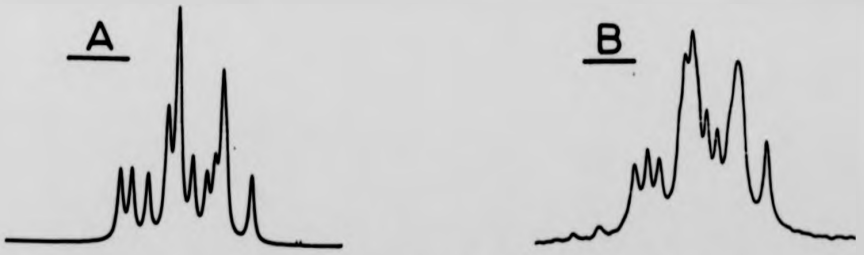

Fig. 6.D.7 The 400 MHz ^{1}H n.m.r. signals for H-4ax and H-1'eq (A) simulated (B) observed.

[Fig. 6.D.7(B)], and gave the following coupling constants:

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*These signals have a line width of 1.5 Hz.

H-1'eq-H-1'ax, J 12.1 Hz

H-1'eq-H-2'*, J 6.5 Hz

H-1'eq-H-2'*, J 8.2 Hz

H-4ax-H-4eq, J 12.0 Hz

H-4ax-H-5eq, J 3.6 Hz

H-4ax-H-5ax, J 11.4 Hz

δ (H-4ax) - δ (H-1'eq) = 7.5 Hz

Finally, it is worth mentioning that a number of polyamine conjugates play a key biochemical role and possess interesting pharmacological properties[24]. One of these conjugates is trimethylated spermidine[25]. This conjugate was synthesised recently from the parent spermidine *via* a hexahydropyrimidine derivative which served, in the synthesis, as a protecting group and as a latent N-methyl function. Hexahydropyrimidine derivatives of spermidine were also used in the syntheses of a variety of plant alkaloids[26]. The importance of the hexahydropyrimidine ring in these syntheses is that it brings about the differentiation of the two primary amino groups of spermidine by the reversible blocking of one amino group (usually the amino group of the aminopropyl chain). This leaves the other primary amino group of spermidine free for reactions, without any interference from the blocked amino group. [N.B. The two primary amino groups in free spermidine have similar basicity[27] and reactivity[28]. This feature is characteristic of the general problem facing researchers on the metabolically important polyamines[29].]

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*The two H-2' protons are not individually assigned.

6.E EXPERIMENTAL

6.E.1 Preparation of model compounds

1. 2-Methylhexahydropyrimidine (35)

A pre-cooled solution of ethanal (9.7 g, 2.2 x 10^{-1} mol) in water (30 cm^3) was reacted, by dropwise addition, with 1,3-diaminopropane (15.4 g, 2 x 10^{-1} mol) in water (50 cm^3), whilst the temperature was kept at 5-10°C (ice bath). The reaction mixture was sealed and magnetically stirred overnight. A slightly yellow product was brought out of the solution by the action of solid sodium hydroxide and cooling. The product was separated and dried. Distillation of the crude product gave a colourless oil, b.p. 139-142°C (65% yield). The ^1H n.m.r. spectrum [Fig. 6.C.1(A)] showed resonances at ($CDCl_3$, TMS) δ 1.16 (3 H, d, J 6 Hz, -CH-$\underline{CH_3}$), 1.48 (2 H, m, H-5), 2.85 (2 H, m, H-4ax and H-6ax), 3.15 (2 H, d , with additional fine structure, Jgem 14 Hz, H-4eq and H-6eq) and 3.64 (1 H, q, J 6 Hz, H-2) p.p.m.

2. Preparation of 1,3-diacetyl-2-methylhexahydropyrimidine (37)

2-Methylhexahydropyrimidine (1 g, 10^{-2} mol) was dissolved in water (5 cm^3). The aqueous solution was added at 10°C to a stirring solution of sodium hydroxide (2.4 g/12 cm^3). To the resulting solution, acetic anhydride (6.12 g, 6 x 10^{-2} mol) was added over 1 h. The reaction mixture was stirred for a further 2 h. Evaporation gave a viscous yellow residue which was taken into dichloromethane. Decolourising charcoal was added and the mixture was stirred at room temperature for 30 minutes. The charcoal was removed by filtration through Celite, followed by the removal of dichloromethane

to give crude product (37) as an oil. Crystallisation was achieved by dissolving the product in the minimum volume of dichloromethane (5 cm^3), followed by addition of ether (120 cm^3). Cooling at -20°C for 3 h precipitated clean white crystals. The precipitate was filtered off and washed with ether (20 cm^3). Drying at 40°C gave pure product (37) (1.35 g, 73%), m.p. 85°C.

C.H.N. combustion analysis, found: C, 58.81; H, 8.71; N, 15.31, $C_9H_{16}N_2O_2$ requires: C, 58.67; H, 8.75; N, 15.21.

The e.i.m.s. analysis showed peaks at m/z 284 $(M)^+$, (1%), 283 $(M-1)^+$, (5%), and 269 $(M-15)^+$, (100%).

The 220MHz ^1H n.m.r. spectrum [Fig. 6.C.1(B)] showed resonances at ($CDCl_3$, TMS) δ 1.45 (3 H, d, J 6 Hz, -CH-CH_3), 1.69 (2 H, m, H-5), 2.08 (3 H, s, N-3-$COCH_3$), 2.21 (3 H, s, N-1-$COCH_3$), 2.97 (1 H, t x d, Jgem 15 Hz, Jvic 3 and 15 Hz, H-6ax), 3.49 (1 H, t x d, Jgem 15 Hz, Jvic 3 and 15 Hz, H-4ax), 3.74 (1 H, d x t, Jgem 15 Hz, Jvic 2.5 and 2.5 Hz, H-4eq), 4.59 (1 H, dxt Jgem 15 Hz, Jvic 2.5 and 2.5 Hz, H-6eq) and 6.65 (1 H, q, J 6 Hz, H-2) p.p.m.

The i.r. spectral analysis showed the presence of a strong and broad peak which was assigned to the acetyl groups at ν_{max} 1630 cm^{-1}.

3. Preparation of 1,2-dimethylhexahydropyrimidine (38)

A pre-cooled solution of ethanal (4.85 g, 11 x 10^{-2} mol) in water (15 cm^3), was reacted by dropwise addition with N-methyl-1,3-diaminopropane (8.8 g, 10^{-2} mol) solution in water (20 cm^3), whilst the temperature was kept at 5-10°C (ice-bath). The reaction mixture was sealed and magnetically stirred overnight. The crude product was brought out of the solution by the action of an excess of

sodium hydroxide and cooling. The crude product (38) was separated and dried. Distillation of crude (38) gave a pure product, b.p. 35°C at 12 mmHg (6.85 g, 60%), as a colourless oil. The 400 MHz ^{1}H n.m.r. spectrum [Fig. 6.C.2(A)] showed resonances at ($CDCl_3$, TMS) δ 1.21 (3 H, d, J 6Hz, -CH-CH_3), 1.53 (1 H, d x m Jgem 13 Hz, H-5eq), 1.74 (1 H, q x t, Jgem 13 Hz, Jvic 3, 3, 13 and 13 Hz, H-5ax), 2.21 (3 H, s, N-1-CH_3), 2.25 (1 H, t x d, Jgem 13 Hz, Jvic 3 and 13 Hz, H-6ax), 2.65 (1 H, t x d, Jgem 13 Hz, Jvic 3 and 13 Hz, H-4ax), 2.88 (1 H, q, J 6 Hz, H-2), 2.95 (1 H, d x m, Jgem 13 Hz, H-6eq) and 3.06 (1 H, d x m, Jgem 13 Hz, H-4eq) p.p.m. The e.i.m.s. analysis of compound (36) showed peaks at m/z M^+ (5%), $(M-1)^+$ (27%), $(M-2)^1$ (88%) and $M-15)^+$ (100%).

4. Preparation of 2-benzylhexahydropyrimidine (40)

A pre-cooled solution of phenylacetaldehyde (12 g, 10^{-2} mol) in water (20 cm^3) was reacted with a solution of 1,3-diaminopropane (7.1 g, 9.6×10^{-2} mol) in water (30 cm^3) by dropwise addition to the latter whilst the temperature of the mixture was kept at 5-10°C (ice-bath). The reaction flask was sealed and magnetically stirred overnight. The crude product was brought out of the solution by the action of an excess of sodium hydroxide. Separation of the crude product was followed by drying and distillation at 80°C at 2×10^{-2} mmHg to give pure compound (40) (12 g, 73%). Its 220 MHz ^{1}H n.m.r. spectrum [Fig. 6.C.3(A)] showed resonances at ($CDCl_3$, TMS) δ 1.96 (2 H, m, H-5), 2.26 (2 H, d, J 6 Hz, -CH_2Ph), 2.27 (2 H, m, H-4ax and H-6ax), 3.15 (2 H, d, with additional fine splitting, Jgem 13.6 Hz, H-4eq and H-6eq), 3.7 (1 H, t, J 6 Hz, H-2) and 7.25 (5 H, m, phenyl protons) p.p.m.

5. Prpearation of 1,3-diacetyl-2-benzylhexahydropyrimidine (41)

2-Benzylhexahydropyrimidine (1.3 g, 7.4×10^{-3} mol) was dissolved in water (5 cm^3). To the aqueous solution was added a sodium hydroxide solution (3 g, 12 cm^3) with cooling at 10°C. Acetic anhydride (3.5 g, 3.5×10^{-2} mol) was added dropwise over 1 h. The reaction was brought to room temperature and left stirring overnight. Evaporation of the mixture gave a crude residue as an oil. The crude compound was extracted into dichloromethane (3 x 100 cm^3). Decolourising charcoal was stirred with the organic solution for 1 h at room temperature. The solution was filtered through Celite, dried and evaporated to leave a colourless oily residue. The compound was dissolved in dichloromethane (3 cm^3) and to the resulting solution ether (30 cm^3) was added. The mixture was kept at -20°C overnight. The precipitated crystals were collected, washed with ether (20 cm^3) and dried, to give pure compound (41) (1.01 g, 52%), m.p. 113-115°C. The e.i.m.s. analysis for compound (41) showed peaks at m/z M^+ (5%), $(M-1)^+$ (3%), $(M-91)^+$ (100%), $(M-133)^+$ (97%) and $(M-175)^+$ (92%). Its 220 MHz ^1H n.m.r. spectrum [Fig. 6.C.3(B)] showed resonances at ($CDCl_3$, TMS) δ 1.64 (3 H, s, N-3-$COCH_3$), 1.78 (2 H, m, C-5), 2.1 (3 H, s, N-1-$COCH_3$), 2.88 (1 H, d x d, Jgem 12 Hz, Jvic 3.6 Hz, H-1'B), 3.12 (1 H, t x d, Jgem 12 Hz, Jvic 3.4 and 12 Hz, H-6ax), 3.32 (1 H, d x d, Jgem 12 Hz, Jvic 14 Hz, H-1'A), 3.57 (1 H, t x d, Jgem 12 Hz, Jvic 3.4 and 12 Hz, H-4ax), 3.79 (1 H, d x t, Jgem/2 Hz, Jvic 3.4 and 3.4 Hz, H-4eq), 4.7 (1 H, d x t, Jgem 12 Hz, H-6eq), 6.57 (1 H, d x d, Jvic 3.6 and 14 Hz, H-2) and 7.25 (5 H, m, phenyl protons) p.p.m.

The CHN combustion analysis for compound (41):- found: C, 69.19; H, 7.74; N, 10.74. $C_{15}H_{20}N_2O_2$ requires: C, 69.20; H, 7.74; N, 10.76.

The i.r. spectral analysis for compound (41) showed the presence of two strong peaks corresponding to the acetyl groups at ν_{max} 1639 and 1645 cm^{-1}.

6.E.2 Preparation of hexahydropyrimidines from spermidine

1. Preparation of 1-(4'-aminobutyl)-2-methylhexahydropyrimidine [hexahydropyrimidine derivative of spermidine (33)]

The hexahydropyrimidine derivative of spermidine (33) was prepared by the dropwise addition of ethanal (0.1 g, 2.2×10^{-3} mol) into a stirring solution of spermidine (0.145 g, 1×10^{-3} mol) in chloroform (5 cm^3), with cooling at 5-10°C. The reaction was stirred for 5 minutes. The solvent was removed under reduced pressure to give oily compound (33). The product was checked by ^1H n.m.r. analysis and found to be pure. Its ^1H n.m.r. spectrum showed signals at ($CDCl_3$, TMS) δ 1.22 (3 H, d, -CH-CH_3), 1.35-165 (5 H, complex multiplets, H-5eq, H-2' and H-3'), 1.67 (1 H, q x t, H-5ax), 2.28 (1 H, d x d x d H-1'ax), 2.33 (1 H, t x d, H-6ax), 2.63 (1 H, m, H-1'eq), 2.66 (3 H, t x d, H-4ax), 2.67 (2 H, m, H-4'), 3.04 (2 H, d, with additional splitting, H-4eq and H-6eq) and 3.20 (1 H, q, H-2) p.p.m. The analysis of the signals in the spectrum of compound (33) is similar to the analysis of the signals for compound (34) (see below).

The c.i. mass spectrometric analysis of compound (33) showed a major peak for the ion $(M+1)^+$ at m/z 172. The e.i. mass spectrometric analysis showed a peak for the ion $(M-15)^+$ at m/z 156. An exact mass measurement for this ion gave a value of 156.1494 ($C_8H_{18}N_3$, error -3.8 p.p.m.) [*cf.* Fig. 6.D.1(A) and (B)].

2. Preparation of 1-[4'-(N-ethylidene)aminobutyl]-2-methyl-hexahydropyrimidine [Imine-hexahydropyrimidine derivative of spermidine (34)]

The imine-hexahydropyrimidine derivative of spermidine (34) was prepared by dropwise addition of ethanal (0.2 g, 4.5×10^{-3} mol) into a stirring solution of spermidine (0.29 g, 2×10^{-3} mol) in chloroform (6 cm^3), with cooling at 5-10°C. The reaction was magnetically stirred and monitored by ^1H n.m.r. analysis. The cyclisation of spermidine and imine formation at the terminal nitrogen was complete within 5 minutes. The solvent was evaporated to dryness leaving a colourless oily residue of (34) (0.38 g, 97%), pure by 400 MHz ^1H n.m.r. analysis, which showed (Fig. 6.D.3) resonances ($CDCl_3$, TMS) at δ1.22 (3 H, d, J 6 Hz, CH-CH_3), 1.35-1.65 (5 H, complex multiplets, H-5eq, H-2' and H-3'), 1.67 (1 H, q x t, Jgem 12 Hz, Jvic 4, 4, 12 and 12 Hz, H-5ax), 1.95 (3 H, d, J 4.5 Hz, N=CHCH_3), 2.28 (1 H, dxdxd, Jgem 13 Hz, Jvic 6 and 8.5 Hz, H-1'ax), 2.33 (1 H, t x d , Jgem 12 Hz, Jvic 3 and 12 Hz, H-6ax), 2.63 (1 H, m, Jgem 13 Hz, Jvic *ca.* 7 and 8 Hz, H-1'eq), 2.66 (1 H, t x d, Jgem *ca.* 13 Hz, Jvic 3 and *ca.* 12 Hz, H-4ax), 3.04 (2 H, d, Jgem 12 Hz with additional fine splitting, H-4eq and H-6eq), 3.20 (1 H, q, J 5.8 Hz, H-2), 3.36 (2 H, t, J 6.9 Hz, H-4') and 7.6 (1 H, q, J 4.5 Hz -N=CHMe) p.p.m.

The above assignments were derived by a sequence of decoupling experiments of the protons at C-4, C-5, C-6 and C-1'. The parameters presented above were either obtained directly from the spectrum, or from the spectra of the decoupling experiments (see above 6.D).

The assignment of signals for H-6ax, H-1'ax and H-4ax, H-1'eq were further supported by simulation of their signals on the Bruker WH-400 computer ('Panic' Programme). The observed parameters (measured above) were fed to the computer and were iterated. The simulated signals were similar to the observed ones [*cf.* Fig. 6.D.5 and Fig. 6.D.7].

The c.i. mass spectrometric analysis of compound (34) showed a major peak for the ion $(M+1)^+$ at m/z 198. The e.i. mass spectrometric analysis showed a peak for the ion $(M-15)^+$ at m/z 182. An exact mass measurement for this ion gave a value of 182.1657 ($C_{18}H_{20}N_3$, error 0 p.p.m.) [*cf.* Fig. 6.D.2(A) and (B)].

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CHAPTER 7

STEREOCHEMISTRY OF SPERMIDINE SYNTHASE

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7.A INTRODUCTION

The enzyme spermidine synthase catalyses the formation of the polyamine spermidine [N-(3-aminopropyl)-1,4-diaminobutane] from putrescine (1,4-diaminobutane) and decarboxylated adenosylmethionine. The primary function of spermidine synthase is to assist the transfer of the aminopropyl group of decarboxylated adenosylmethionine to one of the nitrogen atoms of 1,4-diaminobutane. In this Chapter we will study the cryptic stereochemistry of this reaction, at the reacting methylene group of the aminopropyl unit of the decarboxylated adenosylmethionine.

In principle, the stereochemistry of spermidine synthase could be elucidated by converting [4-^{2}H]methionine of known configuration at C-4 into [1'-^{2}H]spermidine by cells of *E. coli*. However, we could not conceive a simple method for establishing the absolute stereochemistry of such a labelled spermidine. Our approach has been to use *E. coli* to convert methionine labelled with deuterium at both C-3 and C-4 and of known relative configuration (see Chapter 4) into spermidine labelled at C-1' and C-2' of its aminopropyl group.

A culture of *E. coli* was fed (2R,3R,4R), (2S,3R,4R), (2R,3S,4S), and (2S,3S,4S)-[3,4-$^2H_2$]methionine (abbreviated as *rac.*(3R,4R)-[3,4-$^2H_2$]methionine). Another culture was fed (2R,3R,4S), (2S,3R,4S), (2R,3S,4R), and (2S,3S,4R)-[3,4-$^2H_2$]methionine (abbreviated as *rac.*(3R,4S)-[3,4-$^2H_2$]methionine). Dideuterated spermidines were isolated either *via* their PATC-derivative (*cf.* Chapter 3) or by direct

purification on a basic ion exchange column. The free dideuterated spermidines were reacted with ≥ 2 mol equiv. of ethanal to give imine-hexahydropyrimidines. These derivatives were subjected to 400 MHz ^{1}H n.m.r. analysis and decoupling experiments. The imine-hexahydropyrimidine from unlabelled spermidine showed a very complicated n.m.r. spectrum. Although all signals in the spectrum were assigned and the assignments were supported by a series of decoupling experiments (*cf.* Chapter 6), it was difficult to determine the relative configuration of deuteriums in the dideuterated imine-hexahydropyrimidine by comparison with the spectrum of the unlabelled derivative because of the problem of signal overlap.

These complexities necessitated the synthesis of a reference sample for one of the dideuterated imine-hexahydropyrimidines. Synthetic possibilities were explored with unlabelled materials. Initially, 4-acetamidobutylaziridine was prepared, but its aziridine ring could not be opened. We eventually achieved an efficient synthesis of spermidine *via* 2-trifluoromethyl-Δ^2-oxazoline. This method was then adapted to prepare a dideuterated spermidine from (E)-[1,2-$^{2}H_2$]ethene. The dideuterated samples of the imine-hexahydropyramidines originating from the spermidine of the *E. coli* cells were then compared with the sample of dideuterated imine-hexahydropyrimidine generated from the synthetic dideuterated spermidine.

7.B SOME CHEMICAL ASPECTS OF AZIRIDINES

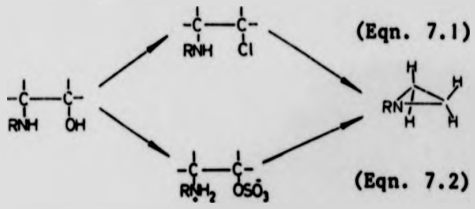
Aziridines have attracted considerable attention in recent years because of their fundamental importance as examples of highly-strained and reactive rings. As a result of this interest, the chemistry of aziridines have been the subject of many reviews[1,2,3].

Haloamines were used in the original preparation of aziridines by Gabriel[4] in 1888. He treated 2-bromoethylamine with potassium hydroxide and he obtained a product which he formulated as vinylamine. Later, Marckwald[5] pointed out the aziridine structure as being more likely for the product of this reaction. The subsequent observations of Gabriel were, thus, later reinterpreted[6] as ring-opening reactions characteristic of the strained three-membered ring.

The dimensions of the three-membered ring in aziridine, as determined by microwave spectroscopy[7,8], electron diffraction spectroscopy[9] and by x-ray diffraction[10], showed that the C-C bondlength equals the C-N bondlength (*ca.* 1.49 Å). The internal bond angles are expected to be close to 60° (compared with 111.3° for the C-N-C bond angle in dimethylamine). The resultant ring strain is reflected in the infra-red spectrum[11] as an increase in the C-H vibrational frequency and a decrease in the N-H vibrational frequency.

The fact that aziridine is a relatively weak base has been discussed in terms of pseudoaromaticity or electron delocalisation in the three-membered ring[12]. A variety of alkyl aziridines have pK_b in the range 7.93-9.47, whereas for ammonia it is 9.5 and for diethylamine 10.7[13].

The preparation of aziridine derivatives is most frequently accomplished in a two-step synthesis from a suitably substituted 2-aminoalcohol. The reaction may be carried out *via* the 2-haloamine, which is treated with alkali to give the aziridine (Gabriel synthesis Eqn. 7.1). Esterification of the 2-aminoalcohol by treatment with sulphuric acid, followed by base-induced cyclisation forms the basis of the Wenker route[14] (Eqn. 7.2). In both the Gabriel and Wenker



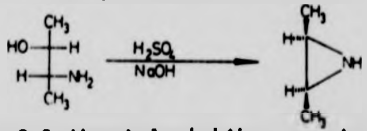
(Eqn. 7.1)

(Eqn. 7.2)

reactions, the formation of piperazine is an important side reaction[15].

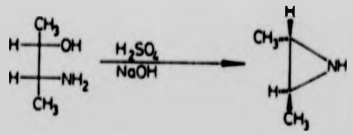
The Wenker reaction offers some advantages in ease of handling of the reagents. A major drawback of this reaction is dehydration, which occurs when the hydroxyl group is attached to a tertiary carbon[16].

It is well established[17] that both the Gabriel and Wenker ring closures occur with inversion of configuration at the substituted carbon atom. The nucleophilic displacement of the halogen or haloester group by nitrogen follows an S_N2 pathway and ring closure is therefore stereospecific. An optically active (2R,3R)-3-amino-2-butanol gave a *cis*-2,3-dimethylaziridine (Eqn. 7.3) whereas an



(Eqn. 7.3)

optically active *trans*-2,3-dimethylaziridine was obtained from an optically active (2S,3R)-3-amino-2-butanol (Eqn. 7.4). It is also

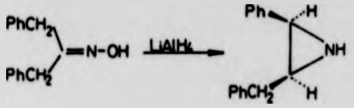


(Eqn. 7.4)

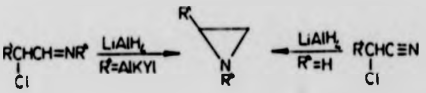
well established that the esterification of the hydroxyl group in the Wenker reaction occurs with retention of configuration[17].

Many methods for the preparation of aziridines are published in the literature[18]. One of these methods utilises the formation of a ß-carbanion[19] species. This method requires the presence of a good leaving group at the amino-function, to facilitate the intra-

molecular displacement leading to the cyclic aziridine. Another useful method for the preparation of aziridines is by the metal hydride reduction of some oximes[20] (Eqn. 7.5). The α-chlorination of nitriles[21] or imines[22,23] followed by metal reduction also gives a substituted aziridine ring (Eqn. 7.6).

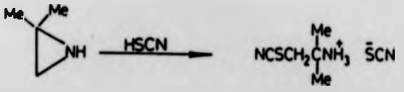


(Eqn. 7.5)



(Eqn. 7.6)

The strain present in the ring system of aziridines amounts to *ca.* 113 kJ mol^{-1}[24]. This results in a much easier ring-opening reaction, compared to, e.g. piperazines, by cleavage of C-N, and occasionally C-C bonds. Ring-opening in aziridines is an acid-catalysed process which proceeds *via* an S_N2 pathway. There may be an S_N1 contribution when alkyl or other suitable substituents are in a position to stabilise a developing carbenium ion. The hydrolysis of such carbenium ions gives the expected amino-tertiary alcohol[25]. However, the description of ring-opening in terms of extreme S_N1 or S_N2 paths does not adequately explain the proportions of the two possible products from ring-opening of unsymmetrically substituted aziridines. It is usually easy to decide which of the two ring-carbons is more S_N1-susceptible (e.g. more highly alkylated) and which is the more S_N2-susceptible (less substituted). Changes in solvent polarity or nucleophilicity of the attacking group may be expected to influence the degree of bond-breaking and bond-making at the transition state. Using a strong nucleophile (NCS^-), S_N2 substitution occurs at the primary carbon in the 2,2-dimethylaziridine (*cf.* equation 7.7). Using a relatively weaker nucleophile (Cl^-), the bond-breaking is



(Eqn. 7.7)

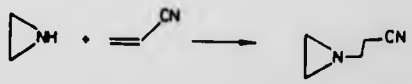
presumably ahead of bond-making and the most S_N1-susceptible carbon is attacked (*cf.* Eqn. 7.8).

(Eqn. 7.8)

Much evidence, provided by kinetic measurements, supports the view that the ring-opening proceeds usually *via* an S_N2-pathway with the aziridinium ion as an intermediate in this process[26]. The ease of rupture of the aziridine ring is really the ease of ring-opening in the postulated quaternary ammonium intermediate[27]. Aziridinium salts are usually reactive towards nucleophiles and they are only isolable using weakly nucleophilic solvents in the presence of a suitable counter-ion (e.g. perchlorate).

The ring-opening of aziridines with water or halogen acids is not of great preparative significance because the 2-aminoalcohols or 2-haloamines are often the starting materials for aziridine syntheses. Ring-opening with amines to give a 1,2-diamine, and with other nucleophiles, is of more value.

Reactions of aziridines involving ring-retention are well described[19]. N-Alkylation of secondary aziridines with alkyl halides requires the presence of a base, such as potassium carbonate, to remove quickly any acid generated, which would otherwise cause acid-catalysed ring-opening. Nucleophilic addition of aziridine to the carbonyl function of ketones and aldehydes and to α,β-unsaturated nitriles (Eqn. 7.9) or carbonyl compounds occurs smoothly in the absence of acid or base. Acylation of aziridines may be achieved with the appropriate acylating agents in the presence of a base.



(Eqn. 7.9)

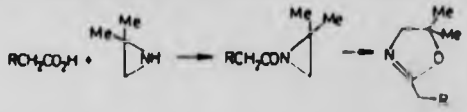
The aziridine ring itself is opened by nucleophiles. Such reactions are normally extremely slow. When the nitrogen bears an acyl group, which can bring about the stabilisation of the developing negative change, the reaction may proceed more readily.

Although many aziridines are quite stable and may be stored for a long time in the pure state or in the presence of alkali, contact with acid or alkylating agents frequently brings about rapid polymerisation of the aziridine. This acid-catalysed polymerisation may be explained as a nucleophilic attack on an aziridinium ion by a molecule of aziridine[28].

7.C SOME CHEMICAL ASPECTS OF Δ^2-OXAZOLINES

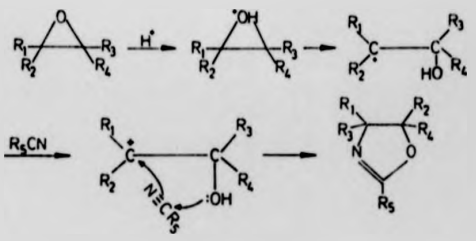
The first alkyloxazoline was obtained by Gabriel[29], who heated 2-bromoethylamine hydrobromide with acetic anhydride and sodium acetate and obtained a 3% yeield of 2-methyloxazoline. Usually oxazolines can be obtained from N-acyl derivatives of 2-aminoalcohols in many ways[30], e.g. the action of p-toluenesulphonyl chloride on N-aroyl derivatives of 2-amino-2-methyl-1-propanol in pyridine gives an oxazoline in good yield[31].

Carboxylic acids are readily converted into Δ^2-oxazolines by treatment with 2-aminoethanol or with 2,2-dimethylaziridine[32,33] (Eqn. 7.10). The acid-catalysed reaction of oxiranes with nitriles



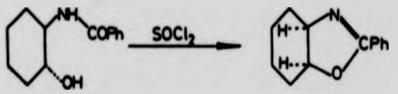
(Eqn. 7.10)

provides an excellent route for the synthesis of Δ^2-oxazolines[34,35] (Scheme 7.C.1).



(Scheme 7.C.1)

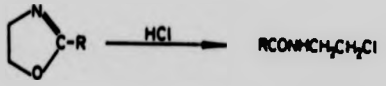
The significant observation has been made[36] that DL-N-benzoylallothreonine ethyl ester with thionyl chloride gives an oxazoline which can be hydrolysed by acid to DL-threonine. Inversion at one of the chiral centres is thus involved, and the fact that the O-toluenesulphonate of benzoylallothreonine ester gives exactly the same oxazoline on treatment with alcoholic potassium acetate, makes it probable that inversion at the β-carbon atom occurs when the ring is formed. The correctness of this general mechanism is indicated by the behaviour of N-benzoyl-*cis*- and *trans*-2-aminocyclohexanol with thionyl chloride. The *trans* isomer gave the *cis*-oxazoline (Eqn. 7.11).



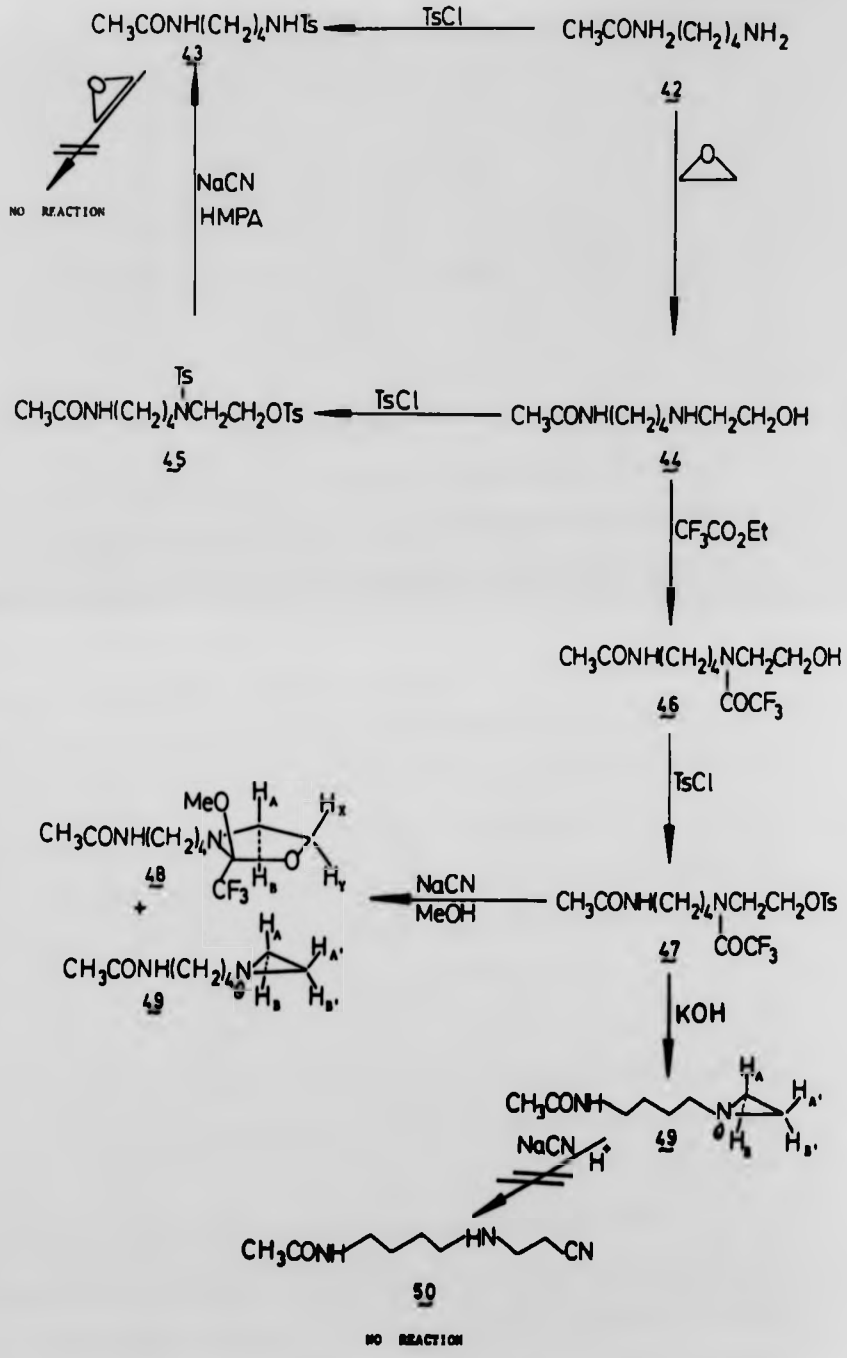
(Eqn. 7.11)

With the *cis*-isomer, where the inversion mechanism is not applicable, replacement of the hydroxide by the chloride was the only reaction observed[37].

Oxazolines are much more resistant to alkalis than to acids. Acid-catalysed ring-opening of oxazolines (e.g. Eqn. 7.12) occurs readily to give 2-chloroethyl amides[29]. The nucleophilic ring-opening of an oxazoline by an S_N2 attack at the C-5 position has not been



(Eqn. 7.12)



Scheme 7.D.1 Attempted synthesis of spermidine *via* N-(4-acetamidobutyl)aziridine.

detailed in the literature. Although, oxazolines are considered more stable to bases than acids, we found that the oxazoline ring of 2-trifluoromethyl-Δ^2-oxazoline is slowly opened at the C-5 carbon by nucleophilic attack with cyanide ion (in DMSO, at room temperature). The product of this ring-opening was N-trifluoroacetyl-3-aminopropionitrile.

7.D ATTEMPTED SYNTHESIS OF (1'S,2'S)/(1'R,2'R)-[1',2'-$^2H_2$]-SPERMIDINE

The synthesis of stereospecifically deuterium-labelled spermidine was attempted by examining what appeared to be an easy route (Scheme 7.D.1). The synthesis of N-acetamido-1,4-diaminobutane (42) was achieved by modification of a literature[38] method. Compound (42) was tosylated[39] to give N-(4-acetamidobutyl)-p-toluenesulphonamide (43). Attempted reaction between ethylene oxide and either compound (43) or its lithium salt [$MeCONH(CH_2)_4NLiTs$] failed. It was hoped that tosylation would enable clean N-hydroxyethylation to be achieved, avoiding the disubstitution expected if compound (42) was reacted with ethylene oxide.

Finally, we found that addition of one 2-hydroxyethyl group to the nitrogen atom of compound (42) can be achieved in acceptable yield by condensing 1 mol equiv. of ethylene oxide with 3 mol equiv. of compound (42) in propan-1-ol at 85°C. Distillation of the reaction mixture gave pure N-(4-acetamidobutyl)-2-aminoethanol (44) in 54% yield (based on ethylene oxide). Tosylation of compound (44) gave N-(4-acetamidobutyl)-2-aminoethanol N,O-*bis*-p-toluenesulphonate (45). Displacement of the O-tosyl group in (45) by a cyano-group was tried using sodium cyanide in HMPA. The product recovered from this reaction was N-(4-acetamidobutyl)-p-toluenesulphonamide (43),

rather than the expected nitrile. Presumably, formation of the nitrile took place, but under the reaction conditions this underwent a base-catalysed elimination to give (43) and acrylonitrile.

In another approach, compound (44) was reacted with ethyl trifluoroacetate in acetonitrile to give N-(4-acetamidobutyl)-N-trifluoroacetyl-2-aminoethanol (46). Direct displacement of the hydroxyl group in (46) by a cyano-function was tried using the system tri-n-butylphosphine, potassium cyanide, 18-crown-6 and carbon tetrachloride in acetonitrile[40]. However, the product isolated from this reaction was N-(4-acetamidobutyl)-N-trifluoroacetyl-1-amino-2-chloroethane, showing that chloride rather than cyano displaced the activated hydroxyl group of compound (46).

Compound (46) was converted into its tosylate: N-(4-acetamidobutyl)-N-trifluoroacetyl-2-aminoethanol O-p-toluenesulphonate (47). The attempted displacement of the O-tosyl by a cyano function using sodium cyanide in methanol, resulted in the formation of cyclic products (see below). Treating compound (47) with sodium hydroxide, rather than sodium cyanide, in methanol, gave a similar result, but the reaction was cleaner. The crude product was examined by ^{1}H n.m.r. spectral analysis and showed the formation of two cyclic systems, namely the oxazolidine (48) and aziridine (49) in a 1:1 ratio (Scheme 7.D.1). The 220 MHz ^{1}H n.m.r. spectrum (CD_3OD, TMS) showed resonances for the protons of the aziridine ring as an AA'BB' system at δ 1.27 (2 H, s with additional fine splittings, H_A and $H_{A'}$) and 1.73 (2 H, s with additional fine splittings, H_B and $H_{B'}$) p.p.m. The protons of the N-(4-acetamidobutyl) group showed resonances at δ 1.55 (4 H, m, central methylenes), 2.82 (2 H, t, $-CH_2N$) and 3.19 (2 H, q, $CH_3CONHCH_2-$) p.p.m. The methyl of the acetyl group showed a signal at δ 1.92 (3 H, s) p.p.m.

The signals in the ^1H n.m.r. spectrum corresponding to the oxazolidine adduct were mostly clear and separated from the signals of the aziridine adduct, except for partial overlapping between the signals of the protons of the alkyl chain in both adducts. Signals at δ 1.55 p.p.m. were assigned to the central methylenes in the chain of the N-(4-acetamidobutyl) group. These signals were overlapping with the corresponding methylenes from the aziridine. A signal at δ 3.19 p.p.m. was assigned to ($-CONHC\underline{H}_2-$) and ($-CH_2C\underline{H}_2N$) protons. These signals were overlapping with those of the methylene adjacent to the acetamido group in the aziridine. Signals of the methylenes in the oxazolidine ring showed an ABXY system at δ 2.25 (1 H, t, $-N-CH_A$), 2.41 (1 H, t, $-N-CH_B$), 4.03 (1 H, t, $-O-CH_X$) and 4.18 (1 H, t, $-O-CH_Y$) p.p.m. The methyl of the acetyl group in the oxazolidine showed a signal overlapping with the corresponding methyl in the aziridine adduct.

No attempts were made to separate the mixture of the aziridine and oxazolidine, and the structural assignment for the latter compound is tentative. Sole production of the aziridine (49) from compound (47) was achieved when the reaction was carried out using potassium hydroxide in benzene. The ^1H n.m.r. spectral analysis (Fig. 7.D.1) of the aziridine obtained from this preparation

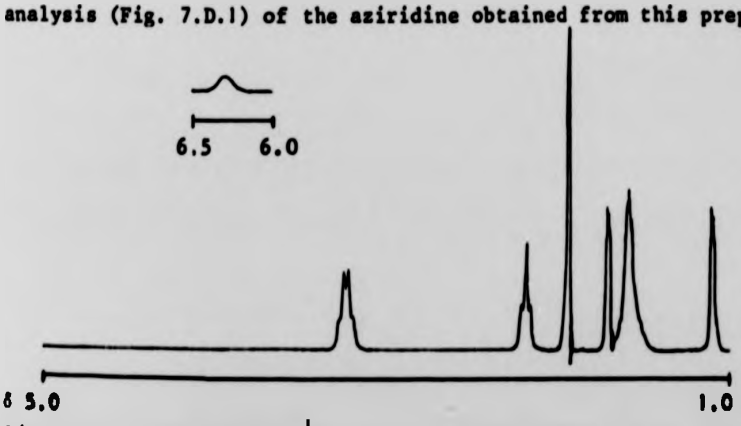


Fig. 7.D.1 The 220 MHz ^1H n.m.r. spectrum ($CDCl_3$, TMS) of pure aziridine (49).

showed results similar to the one obtained above with small changes in the chemical shifts due to the solvent effect (N.B. The ^1H n.m.r. spectrum of the aziridine and oxazolidine mixture was measured in CD_3OD.)

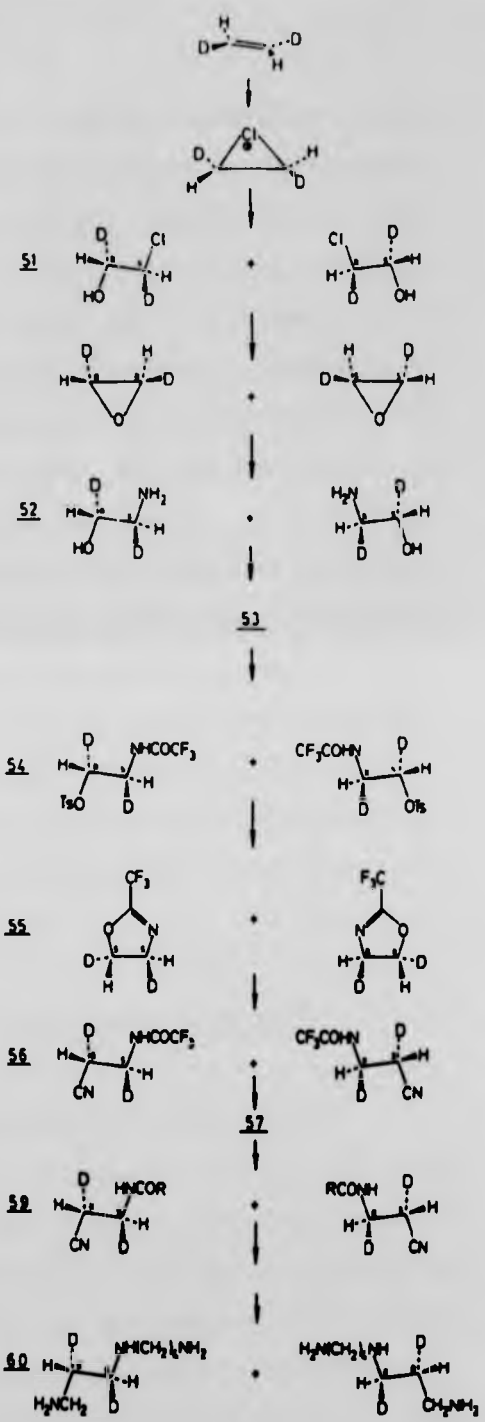
Nucleophilic (S_N2) attack by cyanide at one of the methylenes of the aziridine (49) would be expected to give the desired compound, N-(4-acetamidobutyl)-N-3-aminopropionitrile (50). However, treatment of the aziridine (49) with sodium cyanide, in dichloromethane or DMSO, in the presence of aqueous acid or Lewis acid failed to produce compound (50). Polymerisation of (49) appeared to be the dominant process under these reaction conditions.

Although the synthesis of spermidine *via* an aziridine was unsuccessful, the chemistry described provides a convenient method for the synthesis of aziridine derivatives. This was demonstrated by the synthesis of 1-methylaziridine, 1-propylaziridine, 2-methylaziridine and 1-azabicyclo[3,1,0]hexane from O-tosylated N-trifluoroacetylated precursors.

7.E SYNTHESIS OF (1'S,2'S)/(1'R,2'R)-[1',2'-$^2H_2$]SPERMIDINE

The synthesis of a stereospecifically labelled spermidine was more difficult than we anticipated (*cf.* previous section). This synthesis was finally achieved by coupling N-benzyloxycarbonyl-4-aminobutyric acid (58) with the amino-function of (2R,3S)/(2S,3R)-[2,3-$^2H_2$]3-aminopropionitrile (57), followed by hydrogenolysis and reduction to give the desired spermidine.

The synthesis of the labelled 3-aminopropionitrile (57) was achieved, after defining the synthetic route with unlabelled materials, by starting from (E)-[1,2-$^2H_2$]ethene. This was prepared



Scheme 7.E.1 Synthesis of (1'S,2'S)/(1'R,2'R)-[1',2'-$^{2}H_{2}$]spermidine

from the reduction of [$^2H_2$]acetylene (*cf.* Chapter 4). (E)-[1,2-$^2H_2$]-ethene was reacted[41] with hypochlorous acid [prepared from chlorine oxide[42] (Cl_2O) as described in Chapter 2] to give (1R,2S)/(1S,2R)-[1,2-$^2H_2$]2-chloroethanol (Scheme 7.E.1). The reaction of hypochlorous acid with an olefinic double bond is a typical electrophilic anti-addition[43,44]. The addition of the hypochlorous acid to (E)-[1,2-$^2H_2$]ethene would generate the cyclic chloronium intermediate. Nucleophilic attack by hydroxide from the least hindered direction of the chloronium intermediate (Eqn. 7.13) would generate a mixture

(Eqn. 7.13)

of (1S,2R) and (1R,2S) isomers of the [1,2-$^2H_2$]chlorohydrin (51)* in 1:1 ratio (Scheme 7.E.1). The ^1H n.m.r. spectrum of compound (51) showed resonances at δ 2.61, 3.63 and 3.83 p.p.m. (each is s, corresponding to 1H, for -OH, -CHDOH and -CHDCl, respectively).

Compound (51) was reacted with an excess of potassium hydroxide to generate by intramolecular S_N2 displacement, (1S,2S)/(1R,2R)-[1,2-$^2H_2$]ethylene oxide (N.B. full details for the reaction of 2-chloroethanol with potassium hydroxide and for the vacuum line technique used in this preparation is given in Ref. 41). The ethylene oxide was distilled (trap to trap) and reacted, in the vacuum line, with an excess of ammonia. This reaction is generally believed to proceed *via* an S_N2 pathway to give (1R,2S)/(1S,2R)-[1,2-$^2H_2$]2-amino-ethanol (52). Its 220 MHz ^1H n.m.r. spectrum showed signals at δ 1.82 (1 H, br s, OH), 2.82 (1 H, s, NH_2CHD-) and 3.58 (1 H, s, -CHOH) p.p.m.

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*The labelled compounds are differentiated from their corresponding unlabelled compounds by the letter (a) which follows the number of the latter.

The broadness in the signals of the ^1H n.m.r. spectrum resulted from small H-D couplings.

The reaction of compound (52) with ethyl trifluoroacetate in acetonitrile afforded pure (1R,2S)/(1S,2R)-N-trifluoroacetyl[1,2-$^2H_2$]-2-aminoethanol (53) in a 98% yield. The 220 MHz ^1H n.m.r. spectrum of compound (53) showed resonances at δ 2.5 (1 H, brs, OH), 3.5 (1 H, s, -CHDNH) and 3.78 (1 H, s, -CHDOH) p.p.m. Tosylation of the hydroxyl group of compound (53) afforded (1R,2S)/(1S,2R)-N-trifluoroacetyl-[1,2-$^2H_2$]2-aminoethanol O-p-toluenesulphonate (54) in 86% yield. Its ^1H n.m.r. spectrum showed signals at δ 3.65 and 4.15 p.p.m. (each 1 H, s, -NHCHD- and -CHDOTs, respectively). The remaining signals in the spectrum corresponded to resonances in the spectrum of N-trifluoroacetyl-2-aminoethanol O-p-toluenesulphonate (54a).

Preparation of (4S,5S)/(4R,5R)-2-trifluoromethyl[4,5-$^2H_2$]Δ^2-oxazoline (55) was carried out by reacting compound (54) with potassium hydroxide in a non-polar solvent (e.g. dichloromethane). The ^1H n.m.r. spectrum of compound (55) was compared with the spectrum of 2-trifluoromethyl-Δ^2-oxazoline (55a) (Fig. 7.E.1). The triplets of the methylenes

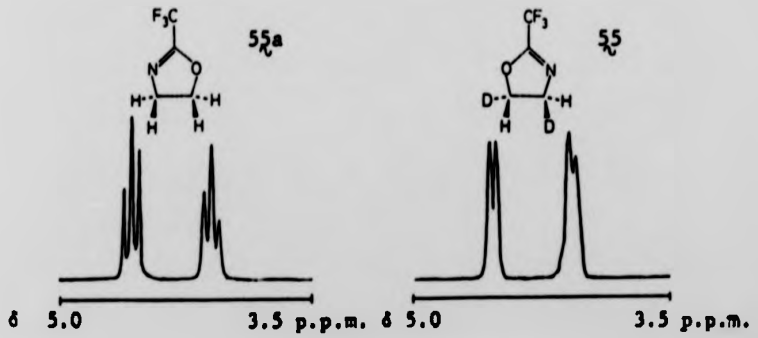

Fig. 7.E.1 The 220 MHz ^1H n.m.r. spectra ($CDCl_3$,TMS) of labelled oxazoline (55) and unlabelled oxazoline (55a).

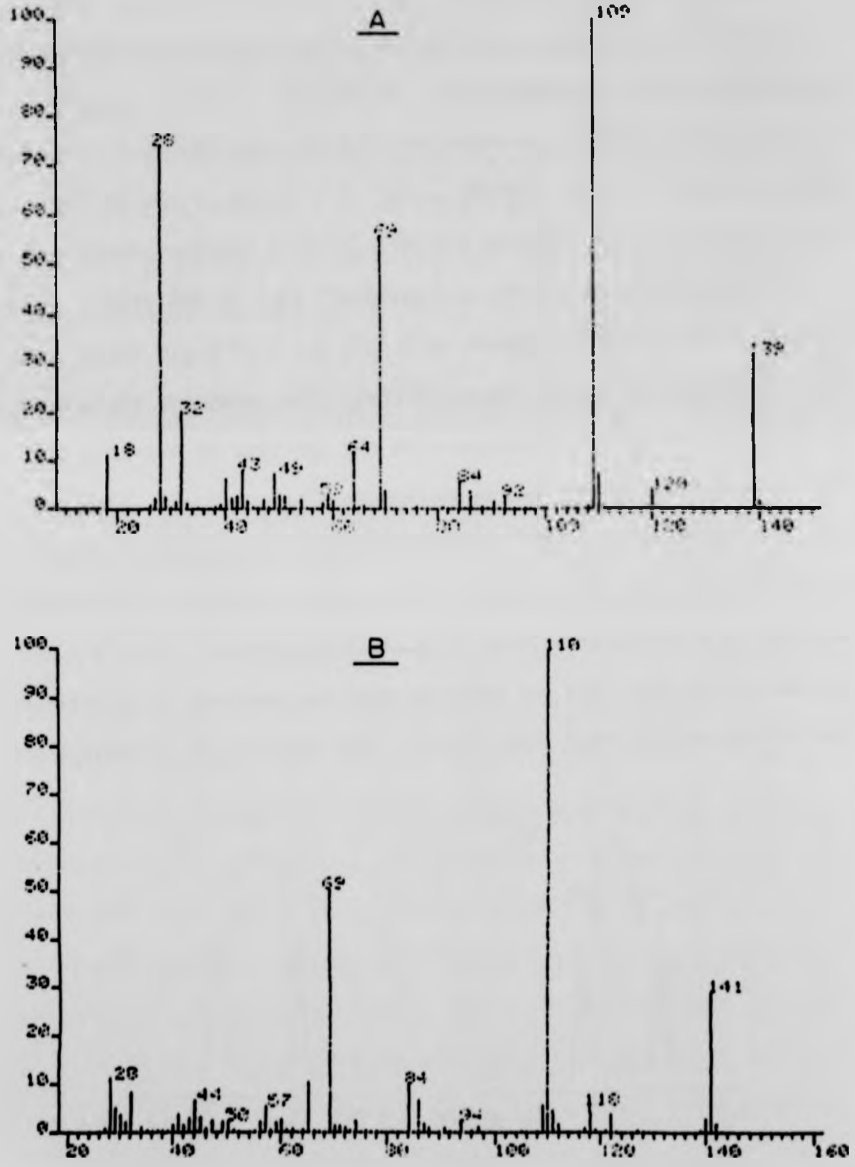

Fig. 7.E.2 The e.i.m.s. analysis of:

A - 2-Trifluoromethyl-Δ^2-oxazoline (55a)

B - (4R,5R)/(4S,5S)-2-trifluoromethyl[4,5-$^2H_2$]Δ^2-oxazoline (55).

in the 2-trifluoromethyl-Δ^2oxazoline have lost the strong geminal couplings and therefore appear in the labelled compound (55) as doublets at δ 4.09 and 4.56 p.p.m. (H-4 and H-5, respectively). The e.i.m.s. analysis of 2-trifluoromethyl-Δ^2-oxazoline showed a peak at m/z 139 for the parent ion M^+ [Fig. 7.E.2(A)]. Compound (55) showed two peaks for M^+ and $(M+1)^+$ at m/z 140 (10%) and 141 (90%) indicating the absence of any undeuterated species and the presence of *ca.* 10% of mono-deuterated species [Fig. 7.E.2(B)].

The ring-opening of the oxazoline (55) was carried out by nucleophilic attack of cyanide in DMSO at the C-5 position of the ring to give (2S,3R)/(2R,3S)-N-trifluoroacetyl-[2,3-$^2H_2$]3-aminopropionitrile (56). The oxazolines are considered to be more stable to bases than acids[43]. We found that the ring-opening of compound (55) with cyanide needs between 5-7 days for completion at room temperature. Obviously, the electron-withdrawing trifluoromethyl group activates C-5 of compound (55) to nucleophilic attack. The product of this ring-opening, compound (56), was difficult to separate from DMSO.

It was found to be more practical to proceed with the next stage of the synthesis without the purification of compound (56). The volume of the reaction mixture was reduced to a minimum by pumping (10^{-4} mmHg/50°C). The residue of the reaction was then dissolved in a minimum volume of water. Lithium hydroxide was added to hydrolyse the trifluoroacetyl group and affording (2R,3S)/(2S,3R)-[2,3-$^2H_2$]3-aminopropionitrile (57) which was isolated as its hydrochloride. The ^1H n.m.r. spectrum of the nitrile (57) was consistent with the presence of deuterium atoms at C-2 and C-3.

The coupling between the nitrile (57) and N-benzyloxy-carbonyl-4-aminobutyric acid (58) was carried out in dichloromethane in the presence of DCCD to give (2S,3R)/(2R,3S)-(N-benzyloxycarbonyl-

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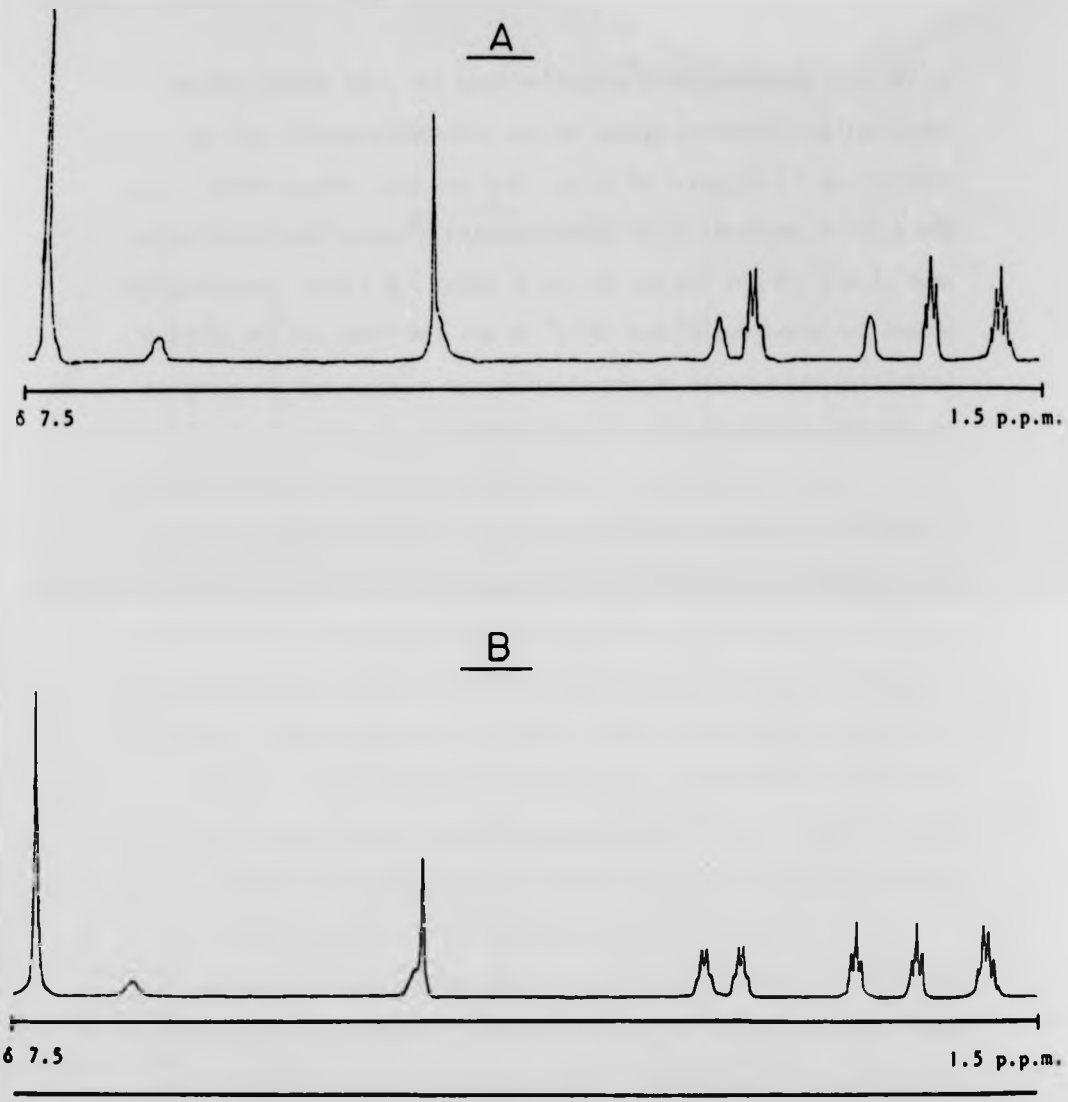


Fig. 7.E.3 The 220 MHz ^{1}H n.m.r. ($CDCl_3$, TMS) of:

A - (2S,3R)/(2R,3S)-(N-benzyloxycarbonylamino)-butyryl-[2,3-$^{2}H_2$]-3-aminopropionitrile (59)

B - (N-benzyloxycarbonylamino)butyryl-3-aminopropionitrile (59a)

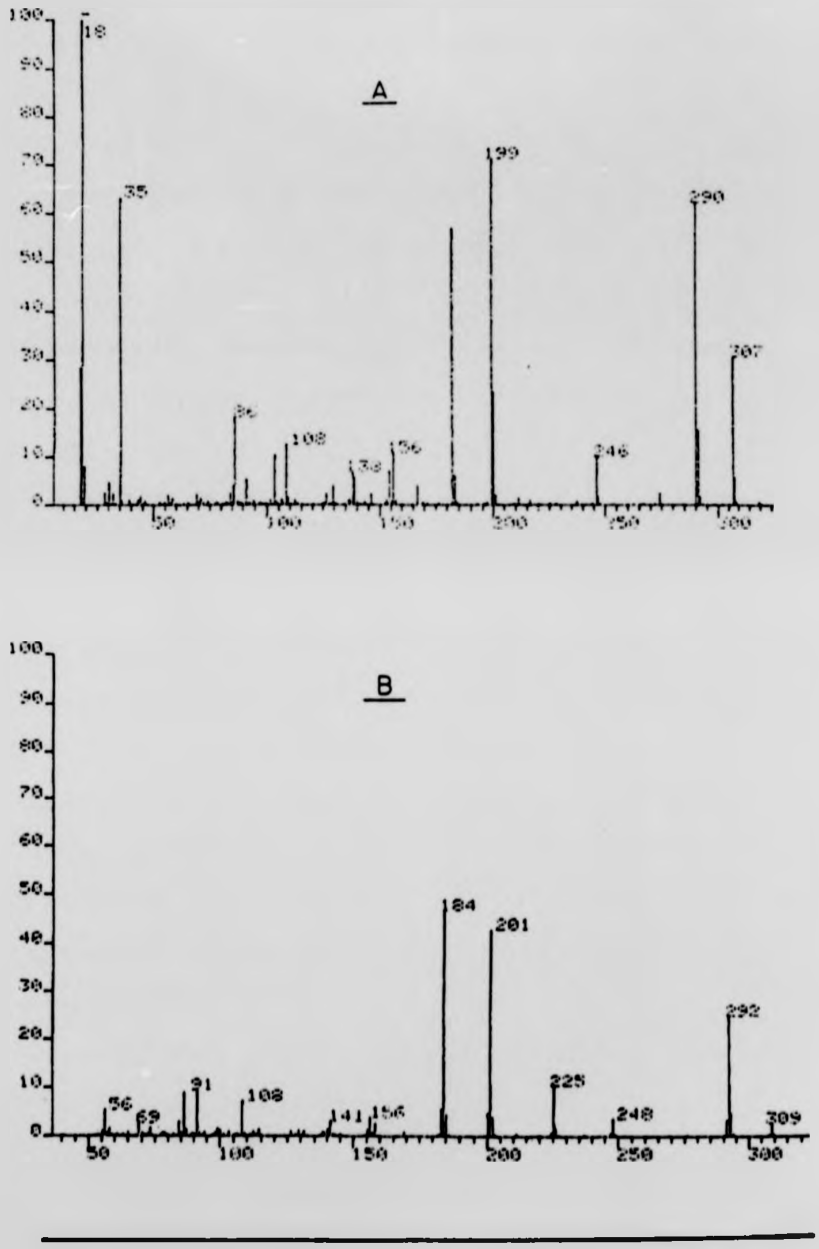


Fig. 7.E.4 The c.i.m.s. analysis of:

A - (N-benzyloxycarbonylamino)butyryl-3-amino-propionitrile (59a)

B - (2S,3'R)/(2R,3S)-[2,3-$^{2}H_{2}$]-(N-benzyloxycarbonyl-amino)butyryl-3-aminopropionitrile (59)

amino)butyryl-[2,3-$^2H_2$]3-aminopropionitrile (59). Its ^1H n.m.r. spectrum [Fig. 7.E.3(A)] showed resonances at δ 2.6 and 3.45 p.p.m., each corresponding to one proton at C-2 and C-3, respectively, of the propionitrile unit of compound (59) (-CONHCHDCHDCN). The remaining resonances were consistent with resonances for compound (59a) [Fig. 7.E.3(B)]. The c.i.m.s. analysis for a sample of the compound (59a) showed a peak at m/z 290 for the ion $(M+1)^+$ [Fig. 7.E.4(A)], whereas the compound (59) showed peaks at m/z 291 and 292 for the ion M^+ (10%) and $(M+1)^+$ (90%), respectively [Fig. 7.E.4(B)]. This analysis showed, as for the oxazoline (55), the presence of 10% total monodeuterated species, and an overall deuterium content of 95 atom %.

The reductive hydrogenation of compound (59) by the reagent $Pd/C/H_2$ followed by borane/THF afforded (1'R,2R)/(1'S,2'S)-[1',2'-$^2H_2$]-spermidine (60), isolated as its trihydrochloride, m.p. 254-257°C (lit.[45] m.p. 256-258°C). The procedure followed above for the conversion of (59) → (60) was provided by Dr. David J. Robins[46].

Free [1',2'-$^2H_2$]spermidine was obtained by running the trihydrochloride through a column of a basic ion exchange resin. The 220 MHz ^1H n.m.r. analysis of [1',2'-$^2H_2$]spermidine showed resonances at δ 1.5 (10 H, m, H-2', H-2, H-3, and 5xNH) and 2.7 (8 H, m, H-1, H-4, H-1' and H-3') p.p.m. The overall yield of labelled spermidine based on ethene was 15%.

7.F BIOSYNTHESIS OF (1'S,2'R)/(1'R,2'S) AND (1'S,2'S)/(1'R,2'R)-[1',2'-$^2H_2$]SPERMIDINE FROM STEREOSPECIFICALLY DEUTERIUM-LABELLED METHIONINES[47]

Two standard salt media each containing either (3R,4R)-[3,4-$^2H_2$]methionine or (3R,4S)-[3,4-$^2H_2$]methionine were inoculated

with *E. coli* cells (N.B. for the synthesis and the terminology of these methionines see Chapter 4). The cells were grown for 37 h. The polyamines, putrescine and [1',2'-$^2H_2$]spermidine, were isolated and purified, separately from each culture, *via* their PATC-derivatives, as described in Chapter 3. The PATC-spermidines isolated from the cultures were separately hydrolysed by conc. hydrochloric acid to give the trihydrochloride of either (1'S,2'S)/(1'R,2'R) or (1'R,2'S)/(1'S,2'R)-[1',2'-$^2H_2$]spermidine. The free spermidines were obtained by running the hydrochlorides through a basic ion exchange column. The ^1H n.m.r. spectra of the labelled spermidines were identical to the ^1H n.m.r. spectrum of the synthetic (1'S,2'S)/(1'R,2'R)-[1',2'-$^2H_2$]spermidine (see above). Finally, the dideuterated spermidines were reacted, separately, with ≥ 2 mol equiv. ethanal to give the corresponding imine-hexahydropyrimidines which were then analysed by ^1H n.m.r. spectroscopy (see coming section).

7.C STEREOCHEMISTRY OF SPERMIDINE SYNTHASE : ^1H N.M.R. SPECTROSCOPIC ANALYSIS OF SYNTHETIC AND BIOSYNTHETIC SAMPLES OF STEREOSPECIFICALLY LABELLED SPERMIDINES

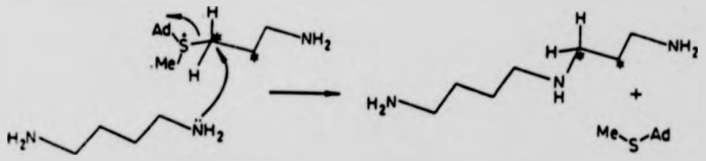
The enzyme spermidine synthase catalyses the formation of the polyamine spermidine from putrescine and decarboxylated adenosylmethionine[48]. This enzyme has no identified cofactor[49].

The stereochemistry of spermidine synthase can be studied, in principle, by converting a stereospecifically deuterium-labelled methionine at C-4 into spermidine deuterated at C-1'. However, as mentioned in the introduction (7.A) we failed to devise a synthesis of this labelled methionine and for establishing the absolute stereochemistry of the derived deuterated spermidine. We decided to study the

stereochemistry of spermidine synthase by converting deuterium-labelled methionines at C-3 and C-4 and of known relative configurations into spermidines labelled at C-1' and C-2'.

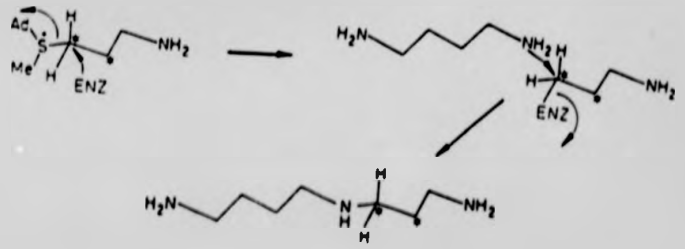
There are three plausible mechanisms for the formation of spermidine from 1,4-diaminobutane and decarboxylated adenosylmethionine:

(i) Enzyme-mediated S_N2 attack of a nitrogen atom of 1,4-diaminobutane at C-1 of the aminopropyl group of decarboxylated adenosylmethionine (Scheme 7.C.1). This mechanism would bring about an inversion of configuration at this carbon atom (i.e. at C-1' of spermidine).



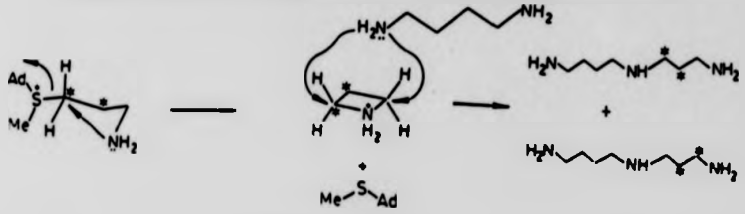
(Scheme 7.C.1)

(ii) S_N2 attack at C-1 of the aminopropyl group of decarboxylated adenosylmethionine by a nucleophilic group of spermidine synthase, giving an aminopropylated enzyme. This reacts by an S_N2 mechanism with 1,4-diaminobutane to give spermidine (Scheme 7.C.2). The stereochemical consequence of this mechanism would be retention of configuration at C-1 of the aminopropyl group of decarboxylated adenosylmethionine.



(Scheme 7.C.2)

(iii) Enzyme-induced intramolecular closure of decarboxylated adenosylmethionine to give azetidine, which then reacts with 1,4-diaminobutane by an S_N2 mechanism to give spermidine (Scheme 7.G.3). This mechanism would bring about retention of configuration at C-1 of the aminopropyl group of decarboxylated adenosylmethionine.

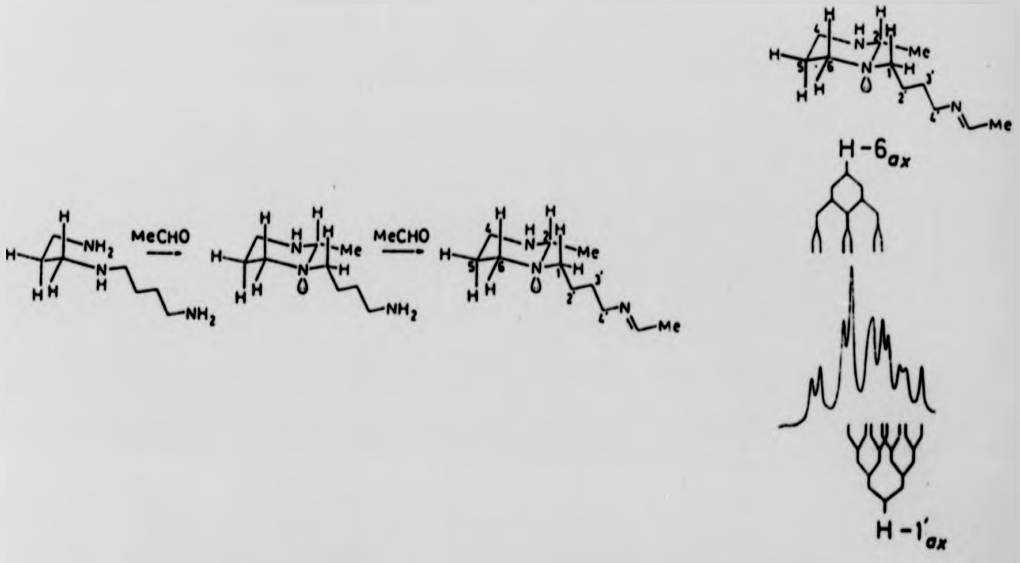


(Scheme 7.G.3)

Mechanism (iii) can be distinguished from (i) and (ii) by feeding [3,4-$^{13}C_2$]methionine to *E. coli* and determining the distribution of ^{13}C in the spermidine produced. The biosynthesis of spermidine from such a labelled methionine was studied[48] (for details see Chapter 3). The spermidine produced showed ^{13}C label only at C-1' and C-2'. In the proposed azetidine [mechanism (iii) above], C-1' and C-3' are homotopic, so if spermidine was derived from the [3,4-$^{13}C_2$]methionine *via* the azetidine (Scheme 7.G.3), this spermidine must be a mixture of two isomers. One isomer would be labelled at C-1' and C-2', while the other would be labelled at C-2' and C-3'. The spermidine produced showed label only at C-1' and C-2'. This result excluded mechanism (iii) as a possibility for the mechanism of spermidine synthase.

We demonstrated (*cf.* Chapter 6) that the reaction of spermidine with ethanal led to the rapid formation of an imine-hexahydropyrimidine derivative. This derivative exhibited a 400 MHz ^1H n.m.r. spectrum which by first order analysis yielded chemical shifts and coupling constants for all the protons in the molecule,

with the exception of H-5eq, 2xH-2' and 2xH-3'. These data and analyses of spectra for models (*cf.* Chapter 6) show that the preferred conformation of the imine-hexahydropyrimidine is a chair with equatorial substituents. That the N-substituent extends away from the chair is indicated by the identity of chemical shifts for all ring protons and by the chemical shifts of H-1'ax and H-1'eq in the imine-hexahydropyrimidine of spermidine. The proton H-6ax shows a triple doublet at δ 2.33 (Jgem 12 Hz, Jvic 3 and 12 Hz) superimposed on a double double doublet for H-1'ax (Fig. 7.G.1). Hence the relative configuration of deuterium atoms in a specimen of [1',2'-$^{2}H_2$]spermidine can be determined after its reaction with ≥ 2 mol equiv. ethanal, by analysing the resonance for H-6ax. This will show a vicinal H-H coupling constant of either 3 or 12 Hz depending on the relative configuration of the deuterium atoms at H-5 and H-6 (axial-equatorial or equatorial-equatorial, respectively).



(Fig. 7.G.1)

Using the methionine-requiring auxotroph of *E. coli* K_{12}; 630 Hf_{ri}, two cultures of cells were grown in media supplemented with either (3R,4R)*-[3,4-$^2H_2$]methionine or (3R,4S)*-[3,4-$^2H_2$]methionine. Deuterated spermidine was isolated[48] from each medium by the method described in Chapter 3.

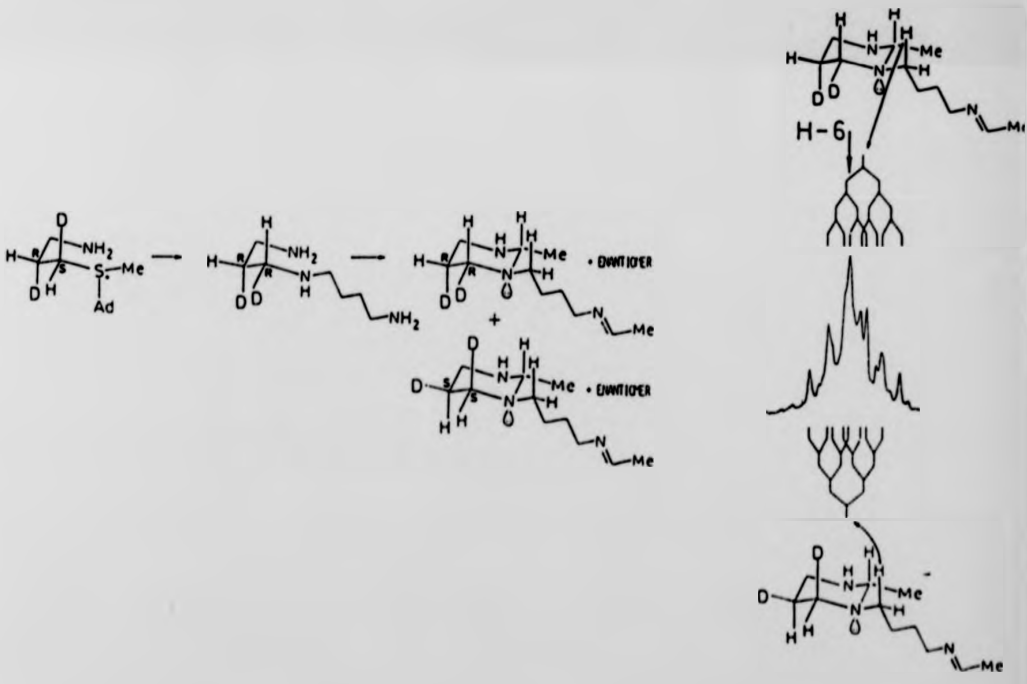
The dideuterated spermidines from the (3R,4R) and (3R,4S)-[3,4-$^2H_2$]methionines were reacted with ethanal in deuterochloroform. These reactions were monitored by 400 MHz ^1H n.m.r. spectroscopy, which showed sequential formation of hexahydropyrimidines and imine-hexahydropyrimidines. If the formation of spermidine proceeds *via* the enzyme-mediated S_N2 attack of a nitrogen atom of 1,4-diaminobutane on C-1 of decarboxylated adenosylmethionine [i.e. mechanism (i)], the imine-hexahydropyrimidine obtained from the (3R,4S)-[3,4-$^2H_2$]methionine will be a mixture of (2S,5R,6R)- and (2S,5S,6S)-[5,6-$^2H_2$]isomers and their enantiomers (*ca.* 25% of each isomer). The imine-hexahydropyrimidine obtained from the (3R,4R)-[3,4-$^2H_2$]methionine will be a mixture of (2S,5S,6R)- and (2S,5R,6S) isomers and their enantiomers (*ca.* 25% of each isomer).

The ^1H n.m.r. spectra of each enantiomer of a pair will be identical, whereas the spectra from the diastereoisomeric pairs will differ. In the ^1H n.m.r. spectrum of each dideuterated imine-hexahydropyrimidine H-5ax, H-5eq, H-6ax and H-6eq therefore corresponds to 0.5 H each, with H-5ax paired with either H-6ax or H-6eq, and H-5eq paired with either H-6eq or H-6ax, depending on the relative configuration of the deuterium atoms in each pair of enantiomers.

---

*(3R,4S) is an abbreviation for (2R,3R,4S),(2R,3S,4R), (2S,3R,4S) and (2S,3S,4R) isomers while (3R,4R) is an abbreviation for (2R,3R,4R),(2S,3R,4R), (2R,3S,4S) and (2S,3S,4S) isomers.

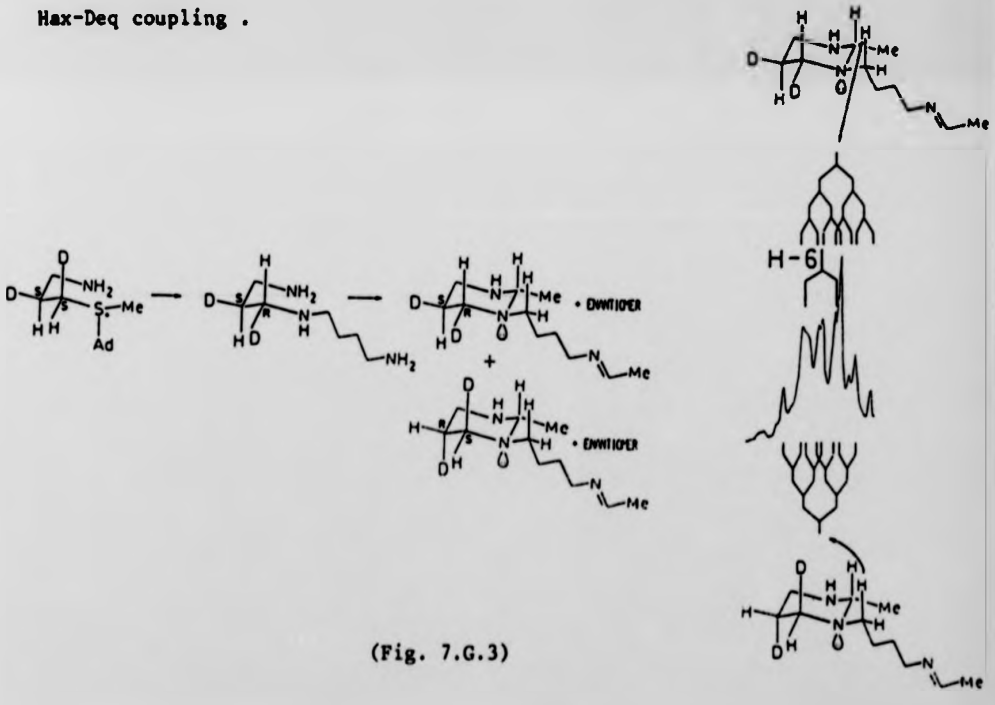
As expected, for the stereochemical course of spermidine synthase operating *via* mechanism (i), the resonance for H-6ax, from the imine hexahydropyrimidine of the (2S,5R,6R)- and (2S,5S,6S)-isomers and their enantiomers, was observed as a broad singlet (δ 2.30, $w_{\frac{1}{2}}$ ∿ 6 Hz) superimposed on resonances for H-1'ax (Fig. 7.G.2). [N.B. the H-D couplings are *ca.* 1/6 of corresponding H-H couplings[50] and so the 'theoretical' appearance of the H-6ax signal is 10 lines with maximum separation of 11 Hz and with the 4 intense central lines each separated by *ca.* 1 Hz.]



(Fig. 7.G.2)

The dideuterated spermidine [from (3R,4R)-[3,4-$^2H_2$]methionine], assuming the operation of mechanism (i), led to an imine-hexahydropyrimidine mixture of (2S,5S,6R)- and (2S,5R,6S)-isomers and their enantiomers (*ca.* 25% of each isomer). Again, the ^1H n.m.r.

spectrum of each enantiomer of a pair will be identical, whereas the spectra from the pairs will be different. [N.B. The explanation given above for the ^{1}H n.m.r. spectrum of the mixture of enantiomers of the imine-hexahydropyrimidine derived from (3R,4S)methionine, applies also to the mixture obtained from (3R,4R)methionine.] The 400 MHz ^{1}H n.m.r. spectrum of this mixture showed a doublet for H-6ax at 2.30 (J ∿ 12 Hz). This arises from the (2S,5S,6R)-[5,6-$^{2}H_{2}$]-imine-hexahydropyrimidine (*cf.* Fig. 7.G.3). This signal is expected to be a doublet (J 12 Hz) of 1:1:1 triplets (J 2 Hz) ignoring vicinal Hax-Deq coupling .



(Fig. 7.G.3)

The signals for H-6ax of dideuterated imine-hexahydropyrimidines (see Figures 7.G.2 and 3) derived from dideuterated spermidine were broadened and shifted upfield (*ca.* 12 Hz) compared to H-6ax in the unlabelled imine-hexahydropyrimidine, presumably due to a slightly different inductive effect of deuterium(s) compared to hydrogen. A further complication is that these signals overlap those

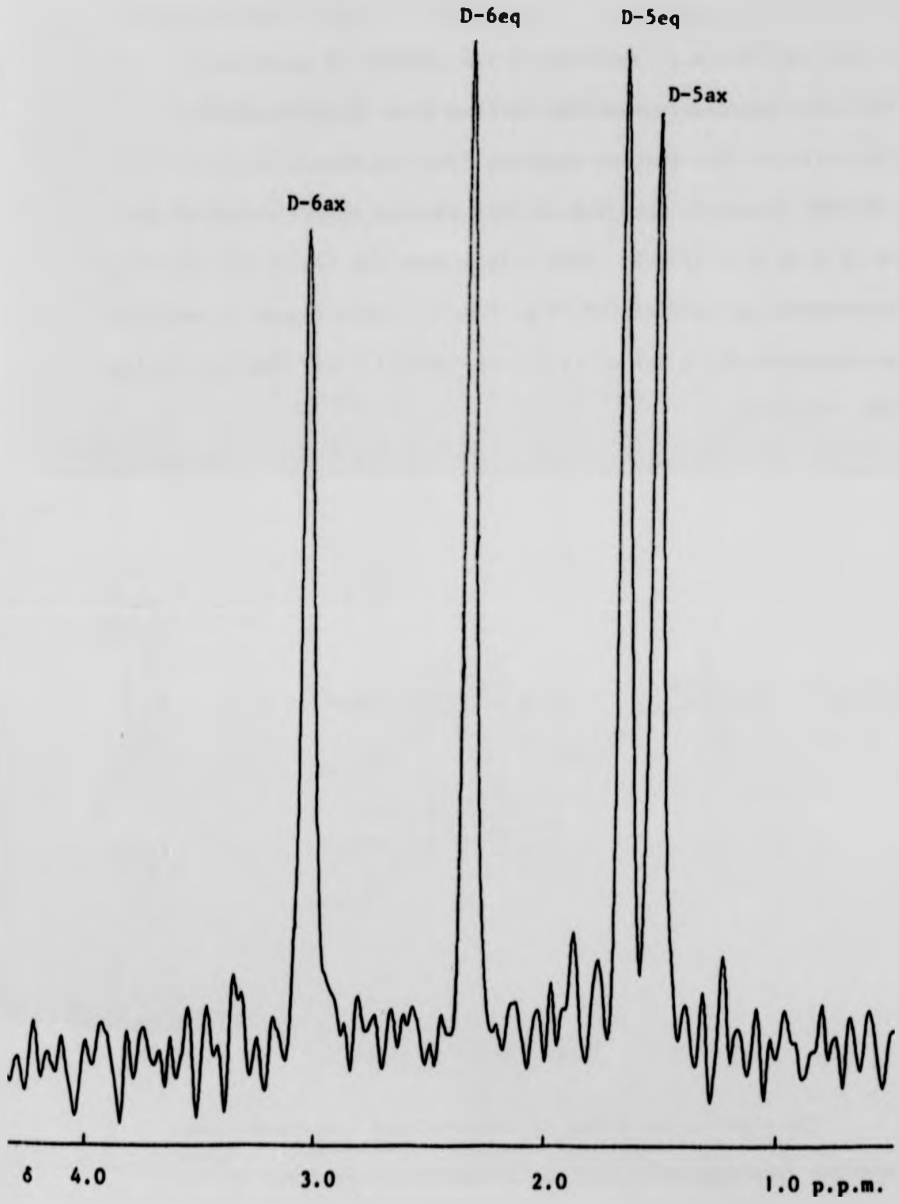
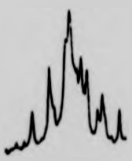


Fig. 7.G.4 The 61.4 MHz $\{^1H\}^2H$ n.m.r. spectrum of (2S,5R,6R)/(2S,5S,6S)-[5,6-$^2H_2$]imine-hexahydropyrimidine derivative of synthetic (1'R,2'R)/(1'S,2'S)-[1',2'-$^2H_2$]spermidine. The spectrum shows four peaks corresponding to the signals of the diastereoisomeric pairs in the mixture.

for H-1'ax, which appears as two double double doublets separated by 7 Hz in the spectrum of each dideuterated imine-hexahydropyrimidine. These two double double doublets (originally one double double doublet in the unlabelled imine-hexahydropyrimidine), arise from an isotope effect of deuterium *versus* hydrogen transmitted from axial H or D through the nitrogen lone-pair (axial) to H-2 (axial) and H-1'ax. [N.B. In the spectra of the dideuterated imine-hexahydropyrimidines the signal of H-2 appears as two quartets of similar intensities.]

The above complexities necessitated the synthesis of a reference sample of one of the dideuterated imine-hexahydropyrimidine. This was achieved from (E)-[1,2-$^{2}H_{2}$]ethene *via* (2S,3R)/(2R,3S)-[2,3-$^{2}H_{2}$]3-aminopropionitrile (*cf.* Section 7.E). The synthetic (1'R,2'R)/(1'S,2'S)-[1',2'-$^{2}H_{2}$]spermidine was reacted with ≥ 2 mol equiv. ethanal in deuterochloroform to give the (2S,5R,6R) and the (2S,5S,6S)-[5,6-$^{2}H_{2}$]imine-hexahydropyrimidines and their enantiomers. The 61.4 MHz $\{^{1}H\}^{2}H$ n.m.r. spectrum of this mixture showed four singlets of equal intensities (Fig. 7.G.4) at δ 1.66 and 3.03 p.p.m. corresponding to the D-5eq and D-6ax, respectively, in the (2S,5S,6S)-isomer, and at δ 1.52 and 2.33 p.p.m. corresponding to the D-5ax and D-6eq, respectively, in the (2S,5R,6R)-isomer. The 400 MHz ^{1}H n.m.r. spectrum of the synthetic (2S,5R,6R) and (2S,5S,6S) isomers of imine-hexahydropyrimidine was similar (peak for peak matching) to the dideuteroimine-hexahydropyrimidine derived from the (3R,4S)-[3,4-$^{2}H_{2}$]methionine. In particular the resonance for H-6ax (Fig. 7.G.5) was, as expected, a broad singlet at



(Fig. 7.G.5)

δ 2.30 p.p.m., superimposed on resonances from H-1'ax.

The similarity between the signals for H-6ax in both the synthetic (2S,5R,6R)/(2S,5S,6S)-[5,6-$^2H_2$]imine-hexahydropyrimidine and the [5,6-$^2H_2$]imine-hexahydropyrimidines derived originally from (3R,4S)-[3,4-$^2H_2$]methionine, confirms that enzyme-catalysed S_N2 attack of the nitrogen atom of putrescine on the C-1 of the aminopropyl group of decarboxylated adenosylmethionine, causing inversion of configuration at this carbon, is the route by which spermidine is biosynthesised.

A recent kinetic study[51] of spermidine synthase from *E. coli* concluded that a ping pong Bi Bi mechanism[52] operates, *via* an intermediate aminopropylated enzyme. It was suggested[53] that this conclusion should be confirmed by a stereochemical investigation. However, if this conclusion is correct the stereochemical course of spermidine synthase should be an overall retention *via* two inversion steps. The contrasting conclusion from the present study is that spermidine synthase operates by a sequential Bi Bi mechanism exhibiting the stereochemistry of a classical S_N2 reaction, i.e. inversion [mechanism (i)]. It is therefore analogous to enzymic transmethylation with S-adenosylmethionine, for which inversion at the sulphonium methyl has been conclusively demonstrated[54] and also analogous to enzymic transadenosylation with methionine for which inversion at the C-5' has been recently demonstrated[55].

7.H EXPERIMENTAL

7.H.1 Attempted synthesis of [1',2'-$^2H_2$]spermidine

1. Preparation of N-acetamido-1,4-diaminobutane (42)

1,4-Diaminobutane (14 g, 1.6×10^{-1} mol) was added dropwise

to a stirring solution of glacial acetic acid (92 cm^3). The resultant solution was kept in a water bath at 50-60°C during the dropwise addition, over 1 h, of acetic anhydride (1.26 g, 1.34×10^{-1} mol). The resultant mixture was stored overnight at room temperature and was then evaporated to dryness under reduced pressure. The oily residue was dissolved in a mixture of hot water (50 cm^3) and 6 mol dm^{-3} hydrochloric acid (33 cm^3). The acidic mixture was pumped to dryness (10^{-3} mmHg/40°C), to give unreacted 1,4-diaminobutane-2HCl and N-acetyl-1,4-diaminobutane-HCl. Extraction of the mixture with propan-2-ol (5 x 100 cm^3) dissolved the acetylated fraction, which was separated by filtration from 1,4-diaminobutane-2HCl. The volume of the organic solution was reduced to 200 cm^3 and kept at -20°C overnight to give a white precipitate. Filtration and drying gave the hydrochloride of the product (42), 13.7 g (51% yield), m.p. 138-140°C (lit.[38] m.p. 136-139°C). The hydrochloride-free N-acetyl-1,4-diaminobutane was obtained by dissolving in methanol (200 cm^3) and adding sodium methoxide in methanol (2.7 mol dm^{-3}, 36 cm^3). The mixture soon deposited most of the available sodium chloride. The salt was filtered off and the filtrate was reduced to a minimum volume. Ether (300 cm^3) was added. The precipitated salt was filtered off and the ethereal layer was evaporated under reduced pressure to give crude compound (42). Distillation (b.p. 126°C/0.1 mmHg) gave pure (42), 9.4 g (88% yield from its hydrochloride). Its 220 MHz ^1H n.m.r. spectrum showed resonances at ($CDCl_3$, TMS) δ 1.27 (2 H, s, $\underline{NH}_2$), 1.53 (4 H, m, central methylenes), 1.97 (3 H, s, $-COC\underline{H}_3$), 2.72 (2 H, t, $-C\underline{H}_2NH_2$) and 3.24 (2 H, q, $-CONHC\underline{H}_2-$) p.p.m.

2. <u>Preparation of N-acetamido-1,4-diaminobutane-p-toluenesulphonamide</u> (43)

p-Toluenesulphonyl chloride (7.0 g, 3.7×10^{-2} mol) was

added in portions during 30 minutes to a mechanically stirred solution of mono-acetyl-1,4-diaminobutane (6.0 g, 3.6 x 10^{-2} mol) and sodium carbonate (7.7 g, 7.3 x 10^{-2} mol) in water (85 cm^3) at 60°C. Stirring at 60°C was continued for 2 h. The mixture was allowed to cool to 0°C, and stirring was continued until the product precipitated. The crystals were collected and recrystallised from ethyl acetate at -20°C to give the pure product (43) (8.0 g, 80%), m.p. 124-127°C (lit.[39] m.p. 125-126). Its 220 MHz ^{1}H n.m.r. spectrum showed resonances at ($CDCl_3$, TMS) δ 1.52 (4 H, m, central methylenes), 1.95 (3 H, s, $-COCH_3$), 2.43 (3 H, s, Ar-CH_3), 2.95 (2 H, q, $-CH_2NH-SO_2-$) 3.22 (2 H, q, $-CONHCH_2-$), 5.15 (1 H, t, $-SO_2NH-$), 5.82 (1 H, br s, -CONH-) and 7.31 and 7.75 [each 2 H, d, phenyl protons] p.p.m.

3. Preparation of N-(4-acetamidobutyl)-2-aminoethanol (44)

Mono-acetyl-1,4-diaminobutane (145 g, 1.1 mol) was dissolved in propan-1-ol (1 dm^3) in a 3-necked flask which was fitted with an acetone-dry ice condenser. Ethylene oxide (14 g, 3.2 x 10^{-1} mol) was added to the above solution. The resulting mixture was incubated for 8 h at 85°C. The organic solvent was removed under reduced pressure to leave an oily residue which contained the product (44) and some unreacted starting amide. The product was purified by distillation (140°C at 0.3 mmHg) to give pure compound (44) as a colourless oil (3.0 g, 54%). The e.i.m.s. analysis showed a large peak for the ion $(M-31)^+$ at m/z 143, probably for the loss of $-CH_2OH$. The CHN combustion analysis found: C, 55.20; H, 10.49; N, 16.13, $C_8H_{18}N_2O_2$ requires, C, 55.4; H, 10.41; N,16.08. The 220 MHz ^{1}H n.m.r. spectrum of compound (44) showed resonances at ($CDCl_3$, TMS) δ 1.54 (4 H, m, $-CH_2CH_2CH_2CH_2-$), 1.96 (3 H, s, $-COCH_3$), 2.64 (2 H, t, $-NHCH_2CH_2OH$), 2.75 (2 H, m, $-CH_2NHCH_2-$), 3.24 (2 H, q,

-CONHC$\underline{H}_2$-), 3.66 (2 H, t, -C$\underline{H}_2$OH) and 6.58 (1 H, br s, CONH-) p.p.m.

4. Preparation of N-(4-acetamidobutyl)-2-aminoethanol N,O-bis-p-toluenesulphonate (45)

Alcohol (44) (6.6 g, 3.8×10^{-2} mol) was dissolved in pre-cooled pyridine (70 cm^3). Tosyl chloride (15.6 g, 8.2×10^{-2} mol) was added portionwise with stirring. The resulting mixture was stored at 0°C overnight. Addition of water (50 cm^3) gave an aqueous solution which was extracted with dichloromethane (4 x 60 cm^3). The organic layers were combined and washed with cold 2.5 mol dm^{-3} hydrochloric acid (3 x 120 cm^3) and water (2 x 120 cm^3). Decolourising charcoal was added to the organic solution and the mixture stirred for 30 minutes at room temperature. The solution was filtered through Celite, dried and the solvent removed under reduced pressure to give an oily residue of compound (45). The attempts to crystallise this oil failed. The oil (12.6 g, 86% yield) was pure by ^{1}H n.m.r. spectral analysis which showed resonances at ($CDCl_3$, TMS) δ 1.52 (4 H, m, central methylenes), 1.95 (3 H, s, COC$\underline{H}_3$), 2.43 and 2.48 (each 3 H, s, Ar-C$\underline{H}_3$), 3.0 [2 H, t, -$(CH_2)_3$C$\underline{H}_2$N], 3.25 (4 H, m, -NTs-CH_2C$\underline{H}_2$OTs and -CONHC$\underline{H}_2$-), 4.15 (2 H, t, -NTsC$\underline{H}_2$$CH_2$OTs) and 7.3-7.8 (8 H, m, phenyl protons) p.p.m.

5. Preparation of N-(4-acetamidobutyl)-N-trifluoroacetyl-2-aminoethanol (46)

The alcohol (44) (13.2 g, 7.7×10^{-2} mol) was dissolved in acetonitrile (100 cm^3) and ethyl trifluoroacetate (22 g, 1.54×10^{-1} mol) was added. The resulting mixture was heated under reflux for 2 h. Removal of the solvent under reduced pressure gave a residue containing mainly the product (46), 99%, and traces of solvent. The 220 MHz ^{1}H n.m.r. spectrum of compound (46) showed resonances at ($CDCl_3$, TMS) δ 1.6 (4 H, m, -CH_2C$\underline{H}_2$C$\underline{H}_2$$CH_2$-), 1.96 (3 H, s, -COC$\underline{H}_3$), 3.25 (2 H, q, -CONHC$\underline{H}_2$-), 3.55 (4 H, m, -C$\underline{H}_2NCOCF_3C\underline{H}_2$-) and 3.8 (2 H, q, -C$\underline{H}_2$OH) p.p.m.

6. Preparation of N-(4-acetamidobutyl)-N-trifluoroacetyl-2-aminoethanol O-p-toluenesulphonate (47)

N-trifluoroacatylaminoethanol (44) (2.7 g, 10 x 10^{-3} mol) was dissolved in dry pyridine (50 cm^3). Tosyl chloride (3.8 g, 20 x 10^{-3} mol) was added to the above solution with stirring. The resulting mixture was stored at 0°C overnight. Addition of water (50 cm^3) gave an aqueous solution which was extracted with dichloromethane (4 x 50 cm^3). The organic layers were combined and washed with cold 2.5 mol dm^{-3} hydrochloric acid (3 x 100 cm^3) and water (2 x 100 cm^3). Decolourising charcoal was added to the organic solution. The mixture was stirred for 30 minutes and then filtered through Celite. The filtrate was dried and removal of the solvent under reduced pressure gave an oily residue of product (47). All attempts to crystallise this oil failed. The oil of compound (47) (3.1 g, 70% yield), was checked by ^1H n.m.r. analysis and found to be pure. The 220 MHz ^1H n.m.r. spectrum showed resonances at ($CDCl_3$, TMS) δ 1.6 (4 H, m, $-CH_2\underline{CH_2}\underline{CH_2}CH_2-$), 1.97 (3 H, s, $COC\underline{H_3}$), 2.45 (3 H, s, $Ar-C\underline{H_3}$), 3.27 (2 H, q, $CH_3CONHC\underline{H_2}-$), 3.44 [2 H, t, $-(CH_2)_3\underline{CH_2}N(COCF_3)CH_2-$], 3.65 [2 H, t, $-CH_2N(COCF_3)\underline{CH_2}CH_2-$], 4.2 (2 H, t, $-CH_2\underline{CH_2}OSO_2-$), 5.9 (1 H, br s, $-CO\underline{NH}-$) and 7.35 and 7.75 [each (2 H, d, phenyl protons)] p.p.m.

7. Preparation of N-(4-acetamidobutyl)aziridine (49)

Powdered potassium hydroxide (5.9 g, 10.4 x 10^{-2} mol) was added to a stirring solution of compound (47) (22 g, 5.2 x 10^{-2} mol) in benzene (150 cm^3). The mixture was sealed and left stirring overnight at room temperature. The precipitated salt was removed by filtration. The solvent was evaporated from the filtrate to leave oily compound (49) (5.8 g, 72%). This product was reasonably pure by ^1H n.m.r. analysis. The 220 MHz ^1H n.m.r. spectrum (Fig. 7.D.1) of compound (49) showed resonances at ($CDCl_3$, TMS) δ 1.1 (2 H, s, with additional fine

splitting, H_A and H_A'), 1.6 [4 H, m, $-CH_2(\underline{CH_2})_2\underline{CH_2}-$], 1.81 (2 H, s, with additional fine splitting, H_B and H_B'), 1.95 (3 H, s, $-COC\underline{H_3}$), 2.2 (2 H, t, $-\underline{CH_2}N-$), 3.25 (2 H, q, $CH_3CONHC\underline{H_2}-$) and 6.3 (1 H, br s, $CH_3CON\underline{H}$) p.p.m. (Fig. 7.D.1).

7.H.2 Synthesis of (1'R,2'R)/(1'S,2'S)-[1',2'-$^2H_2$]spermidine

1. Preparation of (1R,2S)/(1S,2R)-[1,2-$^2H_2$]2-chloroethanol (51)

Hypochlorous acid [2.9 mol dm^{-3} (42 cm^3)] was placed in a 2 dm^3 round-bottom flask which was supplied with a two-way tap. The acid was frozen by application of a dry-ice bath. The frozen acid was pumped *in vacuo* to remove any traces of chlorine. The flask was attached to a vacuum line in which (E)-[1,2-$^2H_2$]ethene (2.5 dm^3, 1.1 x 10^{-1} mol) was trapped. The flask containing the acid was placed in a liquid-nitrogen bath. The trapped ethene was allowed to condense over the acid. The flask containing the reaction mixture was isolated from the rest of the vacuum line. [N.B. A safety device (rubber balloon) was attached to the flask and the tap was opened to allow for excess of pressure to escape.] The flask was allowed gradually to warm up to room temperature. The reaction mixture was then left stirring (orbital shaker) in the dark overnight. Saturated sodium chloride (100 cm^3) was added, and the reaction mixture was then extracted with dichloromethane (3 x 100 cm^3). The combined organic layers were separated, dried and evaporated to leave behind an oily residue of compound (51) (8 g, 89% yield). The residue was checked by 220 MHz ^1H n.m.r. spectroscopy ($CDCl_3$, TMS) and showed resonances at δ 2.61 (1 H, s, $O\underline{H}$), 3.63 (1 H, s, $-C\underline{H}DOH$) and 3.83 (1 H, s, $-C\underline{H}DCl$) p.p.m.

2. Preparation of (1R,2R)/(1S,2S)-[1,2-$^{2}H_{2}$]2-aminoethanol (52)

Compound (51) (4.1 g, 5 x 10^{-2} mol) was dissolved in water (60 cm^{3}) in a 500 cm^{3} flask containing a magnetic stirrer bar. The solution was deep-frozen in liquid nitrogen. After addition of potassium hydroxide (pellets) (56 g, 1 mol), the flask was attached to the vacuum line and the whole apparatus was evacuated. The frozen solid in the flask was slowly allowed to melt, which resulted in a vigorous evolution of ethylene oxide. The ethylene oxide produced was trapped by the action of a dry ice-acetone bath. The flask which originally contained the reaction mixture was replaced by a 200 cm^{3} flask containing concentrated ammonia solution (100 cm^{3}). The flask containing the ammonia solution was deep-frozen and evacuated, before the tap joining it to the main line was opened. Ethylene oxide was gradually released from its trap by warming and cooling*. The flask containing the ammonia was magnetically stirred and was cooled by liquid nitrogen to trap some of the released ethylene oxide. After 30 minutes of stirring, the flask containing the ammonia was kept in liquid nitrogen for 15 minutes to trap all the remaining ethylene oxide (traces). The flask was isolated from the line by switching the relevant tap. After warming to 0°C stirring was continued for a further 5 minutes. The flask containing the reaction mixture was removed from the line and was evaporated under reduced pressure (12 mmHg at 35°C) to leave behind an oily residue (2.2 g, 70%) of compound (52). Its 220 MHz ^{1}H n.m.r. ($CDCl_{3}$, TMS) spectrum showed resonances at δ 1.82 (1 H, br s, -OH), 2.82 (1 H, s, $NH_{2}CHD$-)

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*The release of ethylene oxide into the line was controlled by warming and cooling the trap containing it by removing or replacing the dry ice-acetone bath. The manometer should always be observed, so as not to release too much gas.

and 3.58 (1 H, s, -CHDOH) p.p.m.

3. Preparation of (1R,2S)/(1S,2R)-N-trifluoroacetyl [1,2-$^2H_2$]-2-aminoethanol (53)

Compound (52) (2.2 g, 3.5 x 10^{-2} mol) was dissolved in acetonitrile (30 cm^3). Ethyl trifluoroacetate (6.0 g, 4.5 x 10^{-2} mol) was added to the solution. The resulting mixture was sealed and magnetically stirred for 20 minutes at room temperature. The solvent was removed under reduced pressure to leave behind an oily residue of compound (53), (5.4 g, *ca.* 98% yield + 2% acetonitrile). Its 220 MHz ^1H n.m.r. ($CDCl_3$, TMS), showed resonances at δ 2.5 (1 H, br s, OH), 3.5 (1 H, s, -CHDNH-) and 3.78 (1 H, s, -CHDOH) p.p.m.

4. Preparation of (1R,2S)/(1S,2R)-N-trifluoroacetyl[1,2-$^2H_2$]-2-aminoethanol O-p-toluenesulphonate (54)

The N-trifluoroacetylaminoalcohol (53) (5.3 g, 3.3 x 10^{-2} mol) was dissolved in pre-cooled pyridine (40 cm^3). Tosyl chloride (8.5 g, 4.4 x 10^{-2} mol) was added portionwise to the above solution. The resulting mixture was kept at 0°C overnight. Water (20 cm^3) was added to the organic solution, which was then extracted with dichloromethane (3 x 100 cm^3). The combined organic layers were washed with cold 2.5 mol dm^{-3} hydrochloric acid (3 x 100 cm^3) and water (2 x 100 cm^3). The organic layer was separated, dried and evaporated to leave behind an oily residue of compound (54). The oil was dissolved in ether (10 cm^3). Petroleum ether (50 cm^3) was added gradually with stirring to the ethereal solution which caused immediate precipitation of white crystals of compound (54). Filtration and air-drying afforded pure compound (54) as white crystals m.p. 58-60°C (8.8 g, 86% yield). Its 220 MHz ^1H n.m.r. spectrum ($CDCl_3$, TMS) showed resonances at δ 2.46 (3 H, s, $-CH_3$), 3.65 (1 H, s, -NHCHD-), 4.15 (1 H, s, -CHDOTs), 6.95

(1 H, br s, -NH-) and 7.37 and 7.79 (each 2 H, d, phenyl protons) p.p.m. E.i.m.s. analysis for compound (54) gave peaks at m/z 312 (10%) and 313 (90%) corresponding to the ions $(M-1)^+$ and M^+ respectively, and denoting an overall deuterium content of *ca.* 95 atom %. An unlabelled sample of the compound (54a) showed a peak at m/z 311 corresponding to the M^+ ion.

5. Preparation of (4R,5R)/(4S,5S)-2-trifluoromethyl [4,5-$^2H_2$]Δ^2-oxazoline (55)

Compound (54) (8.8 g, 2.8 x 10^{-2} mol) was dissolved in dichloromethane (150 cm^3). To the organic solution was added powdered potassium hydroxide (2.8 g, 5 x 10^{-2} mol). The mixture was sealed and magnetically stirred at room temperature. The reaction soon started the precipitation of potassium tosylate. The mixture was kept stirring for 4 h. The precipitate was discarded by filtration through Celite. The filtrate was evaporated under reduced pressure to leave behind a colourless residue of compound (55), (3.2 g, 81% yield). The purity of product (55) was checked by ^1H n.m.r. analysis and found to be ≥ 98%. Its 220 MHz ^1H n.m.r. ($CDCl_3$, TMS) spectrum showed peaks (Fig. 7.E.1) at δ 4.05 (1 H, d, J *ca.* 9.5 Hz, H-4) and 4.52 (1 H, d, J *ca.* 9.5 Hz, H-5) p.p.m. The ^1H n.m.r. spectrum for sample of 2-trifluoromethyl-Δ^2-oxazoline[56] (55a) showed resonances (Fig. 7.E.1) at δ 4.09 (2 H, t, J *ca.* 9.5 Hz, H-4) and 4.56 (2 H, t, J *ca.* 9.5 Hz) p.p.m. The e.i.m.s. analysis for compound (55a) showed a peak [Fig. 7.E.2(A)] at m/z 139, corresponding to the ion M^+. The e.i.m.s. analysis for compound (55) showed peaks [Fig. 7.E.2(B)] at m/z 140 (10%) and 141 (90%). The overall deuterium content was *ca.* 95 atom %.

6. Preparation of (2R,3S)/(2S,3R)-[2,3-$^{2}H_{2}$]3-aminopropionitrile (57) *via* (2R,3S)/(2S,3R)-N-trifluoroacetyl-[2,3-$^{2}H_{2}$]-3-aminopropionitrile (56)

Dry sodium cyanide (1.5 g, 3 x 10^{-2} mol) was added to a stirring solution of oxazoline (55), (2.3 g, 1.6 x 10^{-2} mol) in dry DMSO (30 cm^{3}). The mixture was sealed and magnetically stirred at room temperature for 6 days, the reaction being monitored by ^{1}H n.m.r. analysis. The volume of the reaction was reduced (10^{-4} mmHg/40°C) to 5 cm^{3}. Lithium hydroxide (1.23 g, 3 x 10^{-2} mol) solution in water (10 cm^{3}) was added to the residue and the resulting mixture was magnetically stirred for 5 minutes. To it was added dropwise 5 mol dm^{-3} hydrochloric acid (10 cm^{3}). The mixture was evaporated to dryness (10^{-4} mmHg/40°C), to leave behind a viscous residue. A mixture of water and ethanol (1/4) (30 cm^{3}) was added to this residue. The resulting solution was stirred over decolourising charcoal for 30 minutes. The charcoal was removed by filtration through Celite. The filtrate was kept at -20°C overnight to precipitate a white crystalline product (57). The precipitate was filtered off and dried under reduced pressure to give pure compound (57) as a mono-hydrochloride (0.95 g, 55% yield) m.p. 162-164°C (lit.[57] m.p. 165°C). Its 220 MHz ^{1}H n.m.r. analysis showed resonances at ($^{2}H_{2}O$, TSS) δ 2.93 (1 H, d, J *ca.* 6 Hz, -CHDCN) and 3.32 (1 H, d, J *ca.* 6 Hz, -CHDN$\overset{+}{H}_{3}\bar{C}$l)p.p.m. The 220 MHz ^{1}H n.m.r. spectrum for sample of 3-aminopropionitrile hydrochloride (57a) showed resonances at δ 2.92 (2 H, t, -CH_{2}CN) and 3.32 (2 H, t, -$CH_{2}N\overset{+}{H}_{3}\bar{C}$l) p.p.m. The i.r. analysis of compound (57) gave peaks at ν_{max} 1708 and 2245 cm^{-1}.

7. Preparation of N-benzyloxycarbonyl-4-aminobutyric acid (58)

4-Aminobutyric acid (20.6 g, 2 x 10^{-1} mol) was dissolved in 4 mol dm^{-3} sodium hydroxide (50 cm^{3}). The aqueous solution was

cooled at 0°C (ice-bath) with stirring, before the dropwise addition of benzyl chloroformate (37.4 g, 2.2×10^{-1} mol) in 4 mol dm^{-3} sodium hydroxide (60 cm^3). The reaction mixture was magnetically stirred at room temperature for 4 h, and then extracted with ether (2×100 cm^3). The pH of the aqueous layer was adjusted to 3. The acidified solution soon precipitated white crystals when placed in a dry-ice/methanol bath. The collected crystals were recrystallised by dissolving in chloroform (30 cm^3) and adding petroleum ether (50 cm^3). The resulting mixture was cooled with stirring in a dry-ice/methanol bath and soon precipitated transparent crystals of compound (58). The crystalline precipitate was collected and dried *in vacuo* (0.5 mmHg, overnight) to afford pure compound (58) (38 g, 80% yield), m.p. 65-68°C (lit.[58] m.p. 65-66). The 220 MHz ^1H n.m.r. ($CDCl_3$, TMS) spectrum showed resonances at δ 1.81 (2 H, p, $-CH_2\underline{CH}_2CH_2-$), 2.38 (2 H, t, $-\underline{CH}_2CO_2H$), 3.25 (2 H, q, $-CONHCH_2-$) 5.09 (2 H, s, benzyl methylene) and 7.33 (5 H, s, phenyl protons) p.p.m.

8. Preparation of (2S,3R)/(2R,3S)-(N-benzyloxycarbonylamino)-butyryl[2,3-$^2H_2$]3-aminopropionitrile (59)

N-Benzyloxycarbonyl-4-aminobutyric acid (58) (1.54 g, 6.5×10^{-3} mol) and 3-aminopropionitrile hydrochloride (0.71 g, 6.5×10^{-3} mol) were dissolved in dichloromethane (10 cm^3). The resulting mixture was cooled to -5°C (salt/ice-bath), and triethylamine (0.9 cm^3, 6.5×10^{-3} mol) was added. DCCD (1.36 g, 6.6×10^{-3} mol) was added to the reaction mixture, which then was left stirring overnight. The resulting mixture was filtered and the filtrate was reduced to *ca.* 5 cm^3. A white precipitate came out on cooling at -20°C (4 h). The precipitate was filtered off and dried under reduced pressure to give white crystals of compound (59). Recrystallisation from dichloromethane gave pure (59) 1.7 g, 90% yield, m.p. 114-115°C. The purity of the compound (59)

was checked by ^{1}H n.m.r. spectroscopic analysis [Fig. 7.E.3(A)]. Its 220 MHz ^{1}H n.m.r. spectrum ($CDCl_3$, TMS) showed resonances at δ 1.84 (2 H, p, $-CH_2\underline{CH_2}CH_2CONH-$), 2.25 (2 H, t, $-(CH_2)_2\underline{CH_2}CONH-$), 2.6 (1 H, br s, $-CONHCHDC\underline{H}DCN$), 3.26 (2 H, q, $ph-CH_2OCONHC\underline{H_2}CH_2-$), 3.45 (1 H, br s, $-CONHC\underline{H}DCHDCN$), 5.1 (2 H, s, $ph\underline{CH_2}OCONH-$), 5.11 (1 H, br s, $CH_2CON\underline{H}CH_2$), 6.71 (1 H, br s, $ph-CH_2OCON\underline{H}-$) and 7.36 (5 H, s, phenyl protons) p.p.m. The ^{1}H n.m.r. spectrum of unlabelled sample (59a) showed resonances similar to those of the labelled compound (59), except for the signals of the methylenes in the aminopropyl unit. In the unlabelled sample (59a) these appeared [Fig. 7.E.3(B)] at δ 2.6 (2 H, t, $-CONHCH_2\underline{CH_2}CN$) and 3.46 (2 H, q, $-CONHC\underline{H_2}CH_2CN$) p.p.m. The e.i.m.s. analyis (NH_4^+) for the unlabelled compound (59a) showed a peak [Fig. 7.E.4(A)] at m/z 290 $(M+1)^+$. The labelled compound (59) showed peaks [Fig. 7.E.4(B)] at m/z 291 (10%) and 292 (90%) corresponding to the ions M^+ and $(M+1)^+$, respectively, indicating an overall deuterium content of ≥ 95 atom %.

9. Hydrogenation and reduction of compound[45] (59)* to give (1'R,2'R)/(1'S,2'S)-[1',2'-$^2H_2$]spermidine (60)

The nitrile (59) (0.3 mg, 1 x 10^{-3} mol) in dry methanol (10 cm^3) was hydrogenolysed for 2 h at room temperature in the presence of 10% Pd/C (0.035 g). The product of this reaction, obtained by filtration and removal of the solvent, was used directly in the next step. A solution of 1 mol dm^{-3} borane in tetrahydrofuran (19 cm^3) was added to the product of the previous reaction in dry tetrahydrofuran (30 cm^3) and the solution was heated under reflux for 18 h. The solvent of the reaction was evaporated under reduced pressure.

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*The detailed procedure for the hydrogenolysis and reduction of compound (59) was kindly supplied by Dr. D. J. Robins (Chemistry Department, University of Glasgow).

This was followed by addition of pre-cooled dry ethanol (30 cm^3). Dry hydrogen chloride was passed through the resulting solution. The solid trihydrochloride of (1'R,2'R)/(1'S,2'S)-[1',2'-$^2H_2$]spermidine was filtered off and recrystallised from ethanol to give the pure trihydrochloride of compound (60) as white crystals (0.16 g, 62% yield), m.p. 254-257°C (lit.[45] m.p. 256-258°C). Its 220 MHz ^1H n.m.r. spectrum showed resonances at (^2H_2O, TSS) δ 1.8 (4 H, m, 2 x H-2 and 2 x H-3), 2.1 (1 H, m, H-2') and 3.12 (7 H, m, H-1', 2xH-3', 2xH-1 and 2xH-4) p.p.m. Free spermidine was obtained by running the compound through a column of a basic ion exchange resin (Amberlite IR 400, $\bar{O}H$). The first 100 cm^3 of the eluent (water) were collected and evaporated to dryness to leave behind the hydrochloride-free (1'R,2'R)/(1S',2'S)-[1',2'-$^2H_2$]spermidine (60). The 220 MHz ^1H n.m.r. spectrum ($CDCl_3$, TMS) for the free base showed resonances at δ 1.5 (10 H, m, H-2', 2xH-2, 2xH-3 and 5xN-H), and 2.7 (7 H, m, 2xH-1, 2xH-4, H-1' and 2xH-3') p.p.m. The overall yield of (1'R,2'R)/(1'S,2'S)-[1',2'-$^2H_2$]spermidine based on the (E)-[1,2-$^2H_2$]ethene used was 15%.

7.H.3 Biosynthesis of stereospecifically labelled [1',2'-$^2H_2$]-spermidine from (3R,4S)- and (3R,4R)-[3,4-$^2H_2$]methionines

Two standard media, each (10 x 1 dm^3) in 10 flasks of 2 dm^3 capacity, were prepared as described in Chapter 3. Each medium was supplied with one of the following: (3R,4R) or (3R,4S)-[3,4-$^2H_2$]methionine (0.05 g dm^{-3}), and inoculated with *E. coli* cells. The cultures were incubated at 37°C for 30 h. The cells of each culture were harvested separately, by centrifugation to give *ca.* 30-33 g cells (wet). The polyamine spermidine was extracted from each batch of cells with TCA. The extracted spermidines were converted into PATC-derivative as

described in Chapter 3.

The PATC-derivative of the dideuterated spermidine from each run was separately purified by p.l.c. on a silica gel plate [Kieselgel 60 HR reinst 2 x (0.5 x 20 x 100 cm)] (*cf.* Chapter 3).

The PATC-derivatives of the two samples of the dideuterated spermidine were hydrolysed by dissolving each in concentrated hydrochloric acid (7 cm^3) and boiling under reflux overnight. The trihydrochloride of each sample of the dideuterated spermidine was isolated as described in Chapter 3 (*cf.* Section 3.E.4). The hydrochloride-free dideuterated spermidines were obtained by running each sample through an ion exchange column [Amberlite IR 400, $\bar{O}H$ form (1 x 10 cm)], using water as eluent. Evaporation of the first fraction (100 cm^3) under reduced pressure left behind a pure hydrochloride-free (1'S,2'S)/(1'R,2'R)-[1',2'-$^2H_2$]spermidine from one batch and (1'R,2'S)/(1'S,2'R)-[1',2'-$^2H_2$]spermidine from the other culture.

The relative configuration of deuterium atoms in these spermidines are discussed and compared to synthetic dideuterated spermidine in the text of this Chapter (see Section 7.G).

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