The Biosynthesis of Spermidine. Part 3:† The Stereochemistry of the Formation of the N-CH_2 Group in the Biosynthesis of Spermidine

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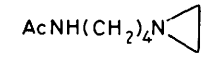
The stereochemistry of the formation of spermidine from butane-1,4-diamine and decarboxylated adenosylmethionine in *Escherichia coli* is shown to be inversion. That is to say, spermidine synthase mediates an S_N2 displacement in which the nucleophile is an amino group of butane-1,4-diamine, the carbon centre undergoing substitution with inversion is C-1 of the 3-aminopropyl group of decarboxylated adenosylmethionine and the leaving group is 5-methylthioadenosine. This conclusion is based on the analysis, with the help of high-field ^1H n.m.r. spectroscopy, of the relative stereochemistries of derivatives of the [1′,2′-$^2H_2$]spermidines derived from [3,4-$^2H_2$]methionines of known relative configuration.

The enzyme spermidine synthase catalyses the formation of the polyamine spermidine [*N*-(3-aminopropyl)butane-1,4-diamine] from putrescine (butane-1,4-diamine) and decarboxylated adenosylmethionine (see Part 1 of this series of papers[1]). 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 butane-1,4-diamine. Determination of the cryptic stereochemistry of this reaction,[2] at the reacting methylene group of the aminopropyl unit of the decarboxylated adenosylmethionine, would distinguish between two plausible, alternative mechanisms for this reaction.[1]

In principle, the stereochemistry of the reaction catalysed by spermidine synthase could be elucidated by having cells of *Escherichia coli* convert [4-^{2}H]methionine of known configuration at C-4 into [1′-^{2}H]spermidine.§ We attempted to prepare such a labelled methionine from dehydromethionine.[3] However, exchange induced by MeONa–MeOD could only be achieved at the methyl group of dehydromethionine. We then prepared methionines labelled with deuterium at both C-3 and C-4 and of known relative configuration[4] and used *E. coli* to convert these methionines into spermidines labelled at C-1′ and C-2′ of their aminopropyl groups. A method for determining the relative configurations of deuterium atoms in such spermidines was developed in research described in Part 2 of this series of papers.[5]

A culture of *E. coli*[6] was fed (2*R*,3*R*,4*R*)-, (2*S*,3*R*,4*R*)-, (2*R*,3*S*,4*S*)-, and (2*S*,3*S*,4*S*)-[3,4-$^2H_2$]methionine {abbreviated as *rac.* (3*R*,4*R*)-[3,4-$^2H_2$]methionine}. Another culture was fed (2*R*,3*R*,4*S*)-, (2*S*,3*R*,4*S*)-, (2*R*,3*S*,4*R*)-, and (2*S*,3*S*,4*R*)-[3,4-$^2H_2$]methionine {abbreviated as *rac.* (3*R*,4*S*)-[3,4-$^2H_2$]methionine}. Dideuteriated spermidines were isolated either *via* their PATC-derivative[1,6] or by direct purification on a basic ion exchange column. The free dideuteriated spermidines were treated with ⩾2 mol equiv. of ethanal to give iminohexahydropyrimidines.[5] These derivatives were subjected to 400 MHz ^1H n.m.r. analysis and decoupling experiments. The iminohexahydropyrimidine 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 (see Scheme 2a, Figure 2a, and ref. 5) it was difficult to determine the relative configuration of deuterium atoms in the dideuteriated iminohexahydropyrimidine 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 dideuteriated iminohexahydropyrimidines. Several synthetic possibilities were explored with unlabelled



(1)

materials. Initially, *N*-(acetamidobutyl)aziridine (**1**) was prepared,[7] but its aziridine ring could not be opened. We eventually achieved an efficient synthesis of spermidine *via* 2-trifluoromethyl-4,5-dihydro-oxazole. This method was then adapted to prepare a dideuteriated spermidine from (*E*)-[1,2-$^2H_2$]ethene. The dideuteriated samples of the iminohexahydropyrimidines originating from the spermidine of the *E. coli* cells were then compared with the sample of dideuteriated iminohexahydropyrimidine generated from the synthetic dideuteriated spermidine.

Synthesis of (1′S,2′S)/(1′R,2′R)-[1′,2′-$^2H_2$]*Spermidine* (**12**) *from* (E)-[1,2-$^2H_2$]*Ethene* (**2**).—The synthesis of a stereospecifically labelled spermidine was eventually achieved by coupling 4-benzyloxycarbonylaminobutyric acid with the amino function of (2*R*,3*S*)/(2*S*,3*R*)-[2,3-$^2H_2$]aminopropiononitrile (**3**), followed by hydrogenolysis and reduction to give the desired spermidine.

The synthesis of the labelled 3-aminopropiononitrile (**3**) was achieved, after defining the route with unlabelled materials, by starting from (*E*)-[1,2-$^2H_2$]ethene (Scheme 1), which was prepared by the reduction of [$^2H_2$]ethyne with Cr^{II} ion.[8] (*E*)-[1,2-$^2H_2$]Ethene was treated[9] with hypochlorous acid to give the (1*S*,2*R*) and (1*R*,2*S*) isomers of the [1,2-$^2H_2$]chlorohydrin (**4**) in 1:1 ratio, assuming that this reaction of hypochlorous acid with an alkene is a typical electrophilic *anti*-addition.[10] The ^1H n.m.r. spectrum of compound (**4**) showed resonances at δ 2.61, 3.63, and 3.83 (each s, corresponding to 1 H, OH, C*H*DOH, and CHDCl, respectively). Compound (**4**) was then treated with an excess of potassium hydroxide to generate by, it is presumed, intramolecular S_N2 displacement, (1*S*,2*S*)/(1*R*,2*R*)-[1,2-$^2H_2$]oxirane (**5**). The oxirane was treated with an excess of ammonia. This reaction should proceed by an S_N2 pathway to give (1*R*,2*S*)/(1*S*,2*R*)-2-amino[1,2-$^2H_2$]ethanol (**6**). 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, C*H*DOH).

The reaction of compound (**6**) with ethyl trifluoroacetate in acetonitrile afforded (1*R*,2*S*)/(1*S*,2*R*)-2-trifluoroacetylamino[1,2-$^2H_2$]ethanol (**7**) in a 98% yield. The 220 MHz ^1H n.m.r. spectrum of this compound showed resonances at δ 2.5 (1 H, br s, OH), 3.5 (1 H, s, –C*H*DNH), and 3.78 (1 H, s,

† Part 2, preceding paper.

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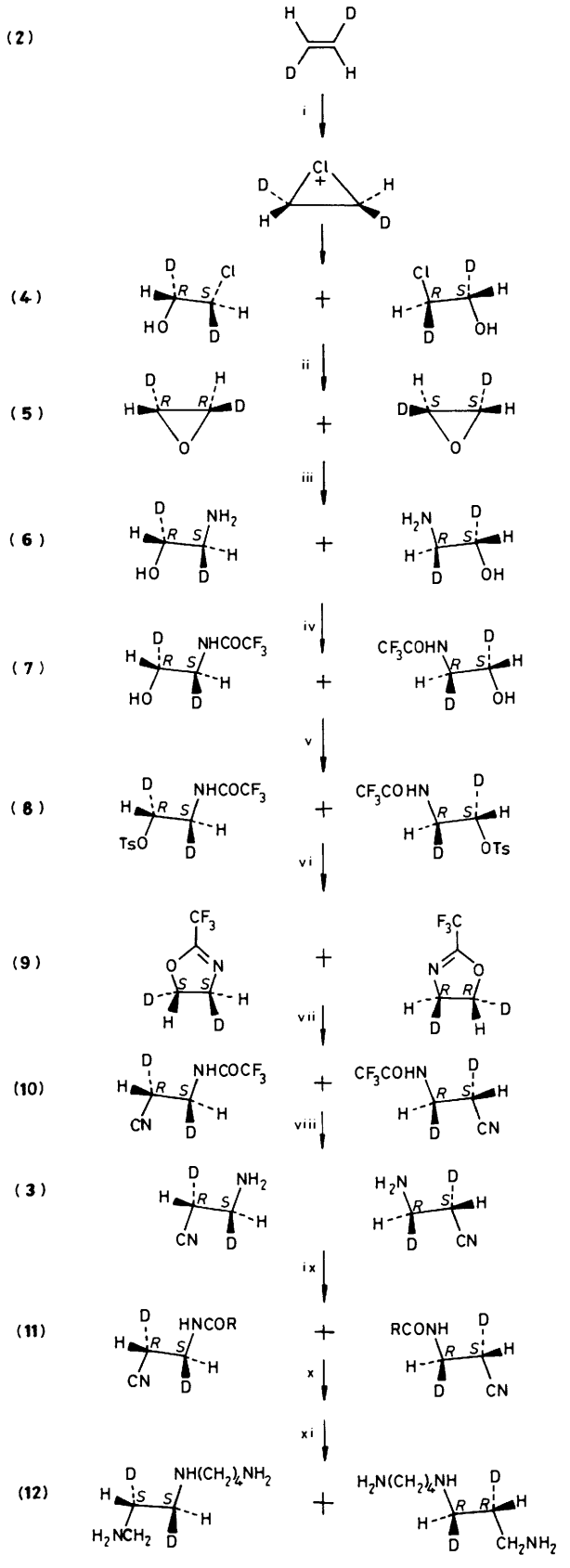
§ Primed numbers refer to the propyl group.



Scheme 1. Synthetic route to (1′*R*,2′*R*)/(1′*S*,2′*S*)-[1′,2′-$^2H_2$]spermidine: i, HOCl; ii, KOH; iii, NH_3; iv, $(CF_3CO)_2O$; v, TsCl–pyridine; vi, KOH–CH_2Cl_2; vii, NaCN–DMSO; viii, aq. LiOH; ix, $ZNH(CH_2)_3CO_2H$–DCC–CH_2Cl_2; x, Pd–C–CH_2; xi, B_2H_6–THF.

–C*H*DOH). Tosylation of the hydroxyl group of compound (**7**) afforded (1*R*,2*S*)/(1*S*,2*R*)-2-trifluoroacetylamino[1,2-$^2H_2$]-ethanol *O*-toluene-*p*-sulphonate (**8**) in 86% yield. Its ^1H n.m.r. spectrum showed signals at δ 3.65 and 4.15 (each 1 H, s, NHC*H*D and C*H*DOTs, respectively).

Preparation of (4*S*,5*S*)/(4*R*,5*R*)-2-trifluoromethyl[4,5-$^2H_2$]-4,5-dihydro-oxazole (**9**) was achieved by treating compound (**8**) with potassium hydroxide in a non-polar solvent (*e.g.* dichloromethane). 2-*N*-Trifluoroacetamidoglycosyl halides have been converted into 2-trifluoromethyl-4,5-dihydro-oxazoles.[11] The ^1H n.m.r. spectrum of compound (**9**) was compared with the spectrum of 2-trifluoromethyl-4,5-dihydro-oxazoline. The triplets of the methylene groups in the 2-trifluoromethyl-4,5-dihydro-oxazol are simplified in the spectrum of the labelled compound (**9**) to doublets at δ 4.09 and 4.56 (4-H and 5-H, respectively). The e.i.m.s analysis of 2-trifluoromethyl-4,5-dihydro-oxazole showed M^+ at *m*/*z* 139. Compound (**9**) showed peaks at *m*/*z* 140 (10%) and 141 (90%) indicating the absence of any undeuteriated species and the presence of *ca.* 10% of mono-deuteriated species.

The ring-opening of the dihydro-oxazole (**9**) was carried out by nucleophilic attack of cyanide in DMSO at the C-5 position of the ring to give (2*S*,3*R*)/(2*R*,3*S*)-3-trifluoroacetylamino[2,3-$^2H_2$]propiononitrile (**10**). This reaction needed 5—7 days for completion at room temperature. Obviously, the electron-withdrawing trifluoromethyl group activates C-5 of compound (**9**) toward nucleophilic attack. It was possible to convert (**8**) into (**10**) without isolation of the dihydro-oxazole (**9**), by treatment of (**8**) with sodium cyanide in dimethyl sulphoxide. Presumably, cyanide ion in dimethyl sulphoxide is a sufficiently strong base to deprotonate the trifluoroacetamido group of (**8**). The resulting ambident ion effects intramolecular displacement of toluene-*p*-sulphonate *via* its more reactive heteroatom.

The product of the ring-opening of the dihydro-oxazole (**9**), compound (**10**) was difficult to separate from DMSO and it was found to be more practical to proceed with the next stage of the synthesis without purification. Aqueous lithium hydroxide hydrolysed the trifluoroacetyl group of compound (**10**) and afforded (2*R*,3*S*)/(2*S*,3*R*)-3-amino[2,3-$^2H_2$]propiononitrile (**3**) which was isolated as its hydrochloride. The ^1H n.m.r. spectrum of the nitrile (**3**) was consistent with the presence of a single deuterium atom at both C-2 and C-3.

The coupling of the nitrile (**3**) with 4-benzyloxycarbonyl-aminobutyric acid was carried out in dichloromethane in the presence of dicyclohexylcarbodi-imide to give (2*S*,3*R*)/(2*R*,3*S*)-3-(4-benzyloxycarbonylamino)butyryl[2,3-$^2H_2$]propiono-nitrile (**11**). Its ^1H n.m.r. spectrum showed resonances at δ 2.6 and 3.45, each corresponding to one proton at C-2 and C-3 respectively, of the propiononitrile unit of compound (**11**). The remaining resonances matched those observed for the corresponding undeuteriated compound. The c.i.m.s. analysis of an undeuteriated sample showed a peak at *m*/*z* 290 for the ion $(M + 1)^+$ whereas compound (**11**) showed peaks at *m*/*z* 291 (10%) and 292 (90%). This analysis showed the presence of 10% total monodeuteriated species, and an overall deuterium content of 95 atom%. We had considered the possibility that during the cyanide-induced conversion of the dihydro-oxazole (**9**) into the nitrile (**10**), exchange and/or epimerisation at C-4 of (**9**) or at C-2 of (**10**) might occur. None of these processes actually occurred, the deuterium content of (**9**) being found to be similar to that of (**11**).

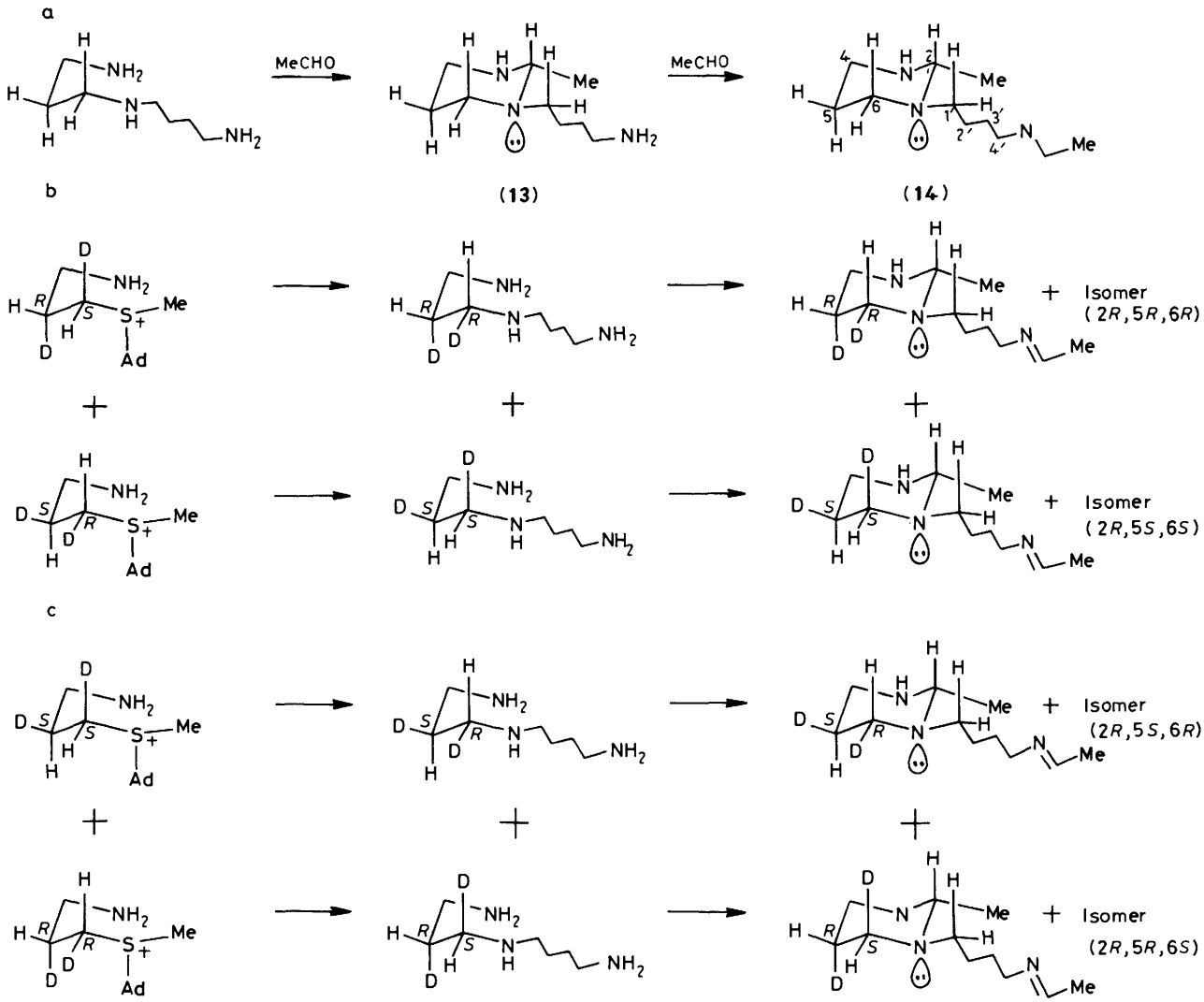
Hydrogenolysis of compound (**11**) by the reagents Pd–C–H_2 followed by reduction of the nitrile with borane–THF afforded (1′*R*,2′*R*)/(1′*S*,2′*S*)-[1′,2′-$^2H_2$]spermidine (**12**), isolated as its trihydrochloride.*

* The procedure for the conversion of (**11**) into (**12**) was kindly provided by Dr. David J. Robins, University of Glasgow.

Free [1′,2′-$^2H_2$]spermidine was obtained by passing 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 1.15 (10 H, m, 2′-H, 2-H, 3-H, and 5 × N*H*) and 2.7 (7 H, m, 1-H, 4-H, 1′-H, and 3′-H). The overall yield of labelled spermidine based on ethene was 15%.

Biosynthesis of Dideuteriated Spermidines and Their Stereochemical Analysis.—Two standard salt media each containing either (3*R*,4*R*)-[3,4-$^2H_2$]methionine or (3*R*,4*S*)-[3,4-$^2H_2$]-methionine were inoculated with *E. coli* cells.[6] After allowing cell growth to proceed, the polyamines putrescine and [1′,2′-$^2H_2$]spermidine were isolated and purified from each culture, *via* their PATC-derivatives.[6] The PATC-spermidines isolated from the cultures were hydrolysed by concentrated 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. Free spermidines were obtained by passing the hydrochlorides through a basic ion exchange column. The ^1H n.m.r. spectra at 220 MHz of the labelled spermidines were similar to the ^1H n.m.r. spectrum of the synthetic (1′*S*,2′*S*)/(1′*R*,2′*R*)-[1′,2′-$^2H_2$]spermidine. Free dideuteriated spermidines were treated with ethanal in deuteriochloroform.[5]

The dideuteriated spermidines from the (3*R*,4*R*)- and (3*R*,4*S*)-[3,4-$^2H_2$]methionines (*cf.* Introduction) were each treated with ethanal in deuteriochloroform. These reactions were monitored by 400 MHz ^1H n.m.r. spectroscopy, which showed sequential formation of a hexahydropyrimidine and then an iminohexahydropyrimidine.[5] If the formation of spermidine proceeds *via* the enzyme-mediated S_N2 attack of a nitrogen atom of butane-1,4-diamine on C-1 of decarboxylated adenosylmethionine [*i.e.* mechanism (i) of ref. 1], the iminohexahydropyrimidine obtained from the (3*R*,4*S*)-[3,4-$^2H_2$]methionine will be a mixture of (2*S*,5*R*,6*R*)- and (2*S*,5*S*,6*S*)-[5,6-$^2H_2$]isomers and their enantiomers (*ca.* 25% of each isomer) [*cf.* Scheme 2b]. The iminohexahydropyrimidine obtained from the (3*R*,4*R*)-[3,4-$^2H_2$]methionine will be a mixture of (2*S*,5*S*,6*R*)- and (2*S*,5*R*,6*S*) isomers and their enantiomers (*ca.* 25% of each isomer) [*cf.* Scheme 2c]. 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 dideuteriated iminohexahydropyrimidine, 5-H_{ax}, 5-H_{eq}, 6-H_{ax}, and 6-H_{eq} therefore correspond to 0.5 H each, with 5-H_{ax} paired with either 6-H_{ax} or 6-H_{eq}, and 5-H_{eq} paired with either 6-H_{eq} or 6-H_{ax} depending on the relative configuration of the deuterium atoms in each pair of enantiomers.



Scheme 2. (a) Reaction between spermidine and ethanal leading to hexahydropyrimidine (**13**) and the iminohexahydropyrimidine (**14**); (b) the dideuteriated iminohexahydropyrimidine derived from (2*R*,3*R*,4*S*)/(2*R*,3*S*,4*R*)/(2*S*,3*R*,4*S*)/(2*S*,3*S*,4*R*)-[3,4-$^2H_2$]methionine; and (c) the dideuteriated iminohexahydropyrimidine derived from (2*R*,3*R*,4*R*)/(2*R*,3*S*,4*S*)/(2*S*, 3*R*,4*R*)/(2*S*,3*S*,4*S*)-[3,4-$^2H_2$]methionine.

As expected, for the stereochemical course of spermidine synthase operating *via* mechanism (i),[1] the resonance for 6-H_{ax}, from the iminohexahydropyrimidine of the (2*S*,5*R*,6*R*)- and (2*S*,5*S*,6*S*)-isomers and their enantiomers, was observed as a broad singlet (δ 2.30, $w_{\frac{1}{2}}$ *ca.* 6 Hz) superimposed on resonances for 1′-H_{ax} (*cf.* Figure 1b). H–D couplings are *ca.* a sixth of the corresponding H–H couplings[12] and so the 'theoretical' appearance of the 6-H_{ax} signal is a doublet (*J* 3 Hz) of 1:2:3:2:1 pentuplets (*J ca.* 2 Hz).

The dideuteriated spermidine from (3*R*,4*R*)-[3,4-$^2H_2$]methionine, assuming the operation of mechanism (i)[1] leads to an iminohexahydropyrimidine mixture of (2*S*,5*S*,6*R*)- and (2*S*,5*R*,6*S*)-isomers and their enantiomers (*ca.* 25% of each isomer, *cf.* Scheme 2c). 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. The 400 MHz ^1H n.m.r. spectrum of this mixture showed a broad doublet of 6-H_{ax} at 2.30 (*J ca.* 12 Hz), (Figure 1c). This arises from the (2*S*,5*S*,6*R*)-[5,6-

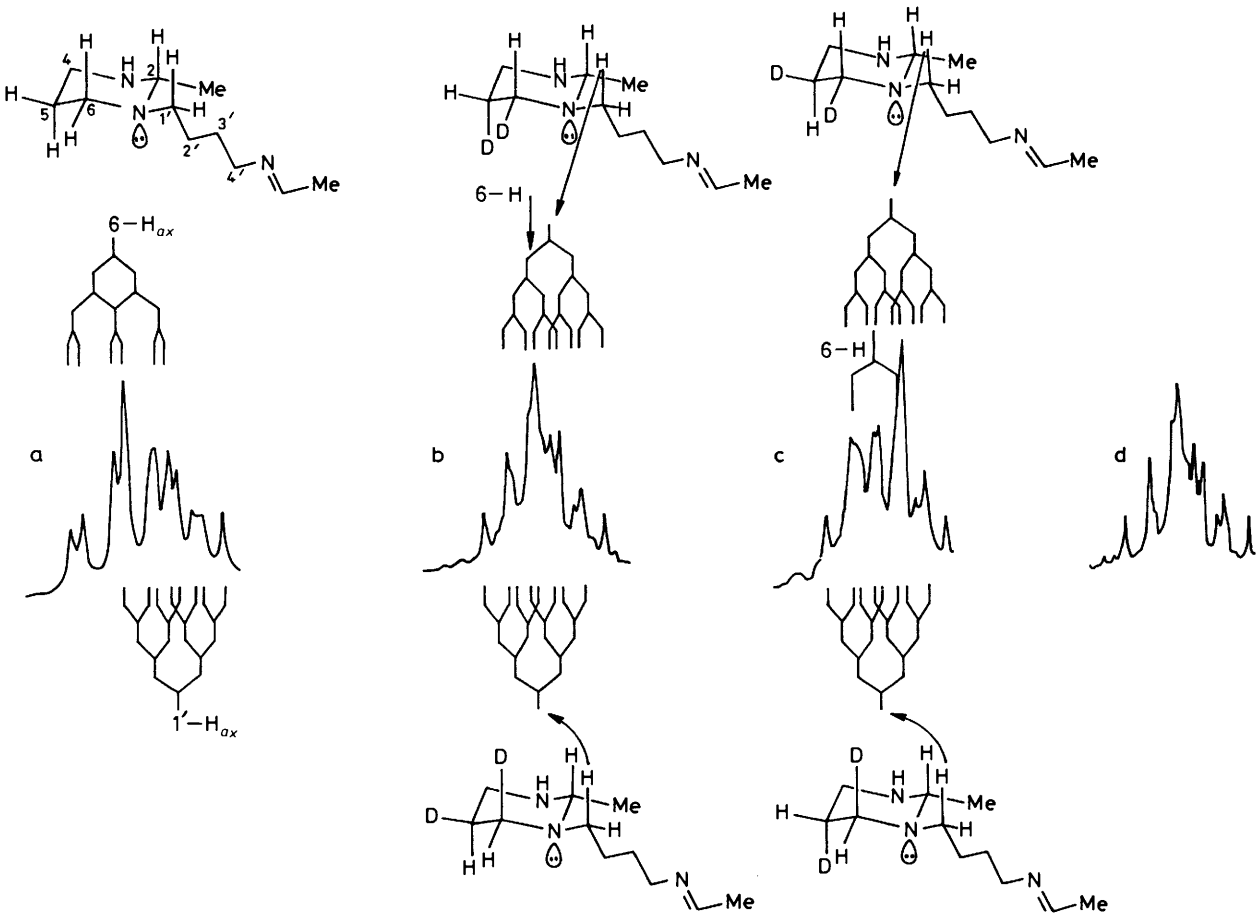


Figure 1. Portions of (*ca.* δ 2.3) of the 400 MHz n.m.r. spectra (scale: 1 mm = 4.5 Hz) of (a) unlabelled iminohexahydropyrimidine (**14**); (b) dideuteriated iminohexahydropyrimidine (*cf.* Scheme 2b) from (2*R*,3*R*,4*S*)/(2*R*,3*S*,4*R*)/(2*S*,3*R*,4*S*)/(2*S*,3*S*,4*R*)-[3,4-$^2H_2$]methionine; (c) dideuteriated iminohexahydropyrimidine (*cf.* Scheme 2c) from (2*R*,3*R*,4*R*)/(2*R*.3*S*,4*S*)/(2*S*,3*R*,4*R*)/(2*S*,3*S*,4*S*)-[3,4-$^2H_2$] methionine; (d) synthetic dideuteriated iminohexahydropyrimidine (*cf.* Scheme 1).

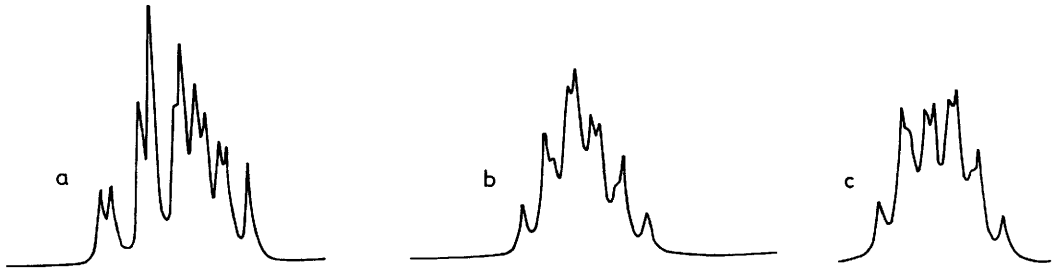


Figure 2. Spectra simulated with a SIMEQ-II program (Varian). (a) This spectrum was obtained by using the following parameters for 6-H_{ax} and 1′-H_{ax} in compound (**14**): 6-H_{ax}, δ 495.0 Hz (arbitrary setting), *J* values 3.28, 11.75, and 11.75 Hz; 1′-H_{ax}, δ 480.0 Hz, *J* values 6.0, 8.19, and 12.0 Hz (line width 2.0 Hz). (b) This spectrum was obtained by summation of two octets for 1′-H_{ax} at δ 480.0 and 486.0 Hz (*J* values as in (a), line width 2.0 Hz) with a component for 6-H_{ax} obtained by using δ 485.0 Hz and J_{vic} = 3.28 Hz; the effect of deuterium was approximated (the program used could not cope with spin ^{1} nuclei) by superimposing a line width of 8 Hz on the doublet for 6-H_{ax}, which was converted into a broad singlet. (c) This spectrum was obtained in a manner similar to (b) by using identical parameters for the octets and δ 485.0 Hz J_{vic} = 11.75 Hz, and line width 5 Hz for 6-H_{ax}.

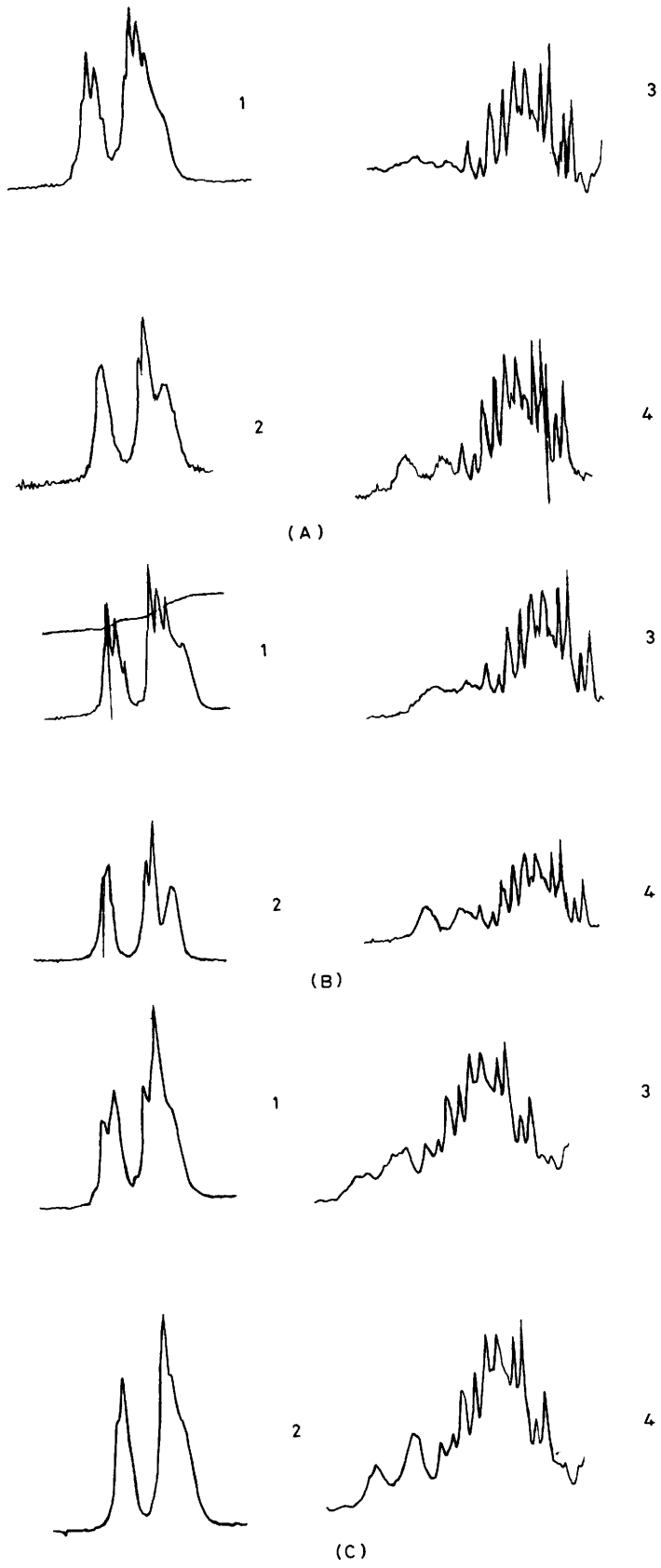


Figure 3. Undecoupled (1 and 3) and decoupled (2 and 4) signals in the ^{1}H n.m.r. spectra of dideuteriated iminohexahydropyrimidines (for description see text).

$^2H_2$]iminohexahydropyrimidine and its enantiomer. This signal is expected to be a doublet (J 12 Hz) of 1 : 1 : 1 triplets (J 2 Hz) ignoring vicinal H_{ax}–D_{eq} coupling.

The signals for 6-H_{ax} of dideuteriated iminohexahydropyrimidines (see Figures 1b and 1c) derived from dideuteriated spermidines were broadened and exhibit an upfield isotope shift[13] (*ca.* 12 Hz) compared to 6-H_{ax} in the unlabelled iminohexahydropyrimidine (*cf.* Figure 1a). A further complication is that these signals overlap those for 1′-H_{ax}, which appears as two double double doublets ('octets') separated by 7 Hz in the spectrum of each dideuteriated iminohexahydropyrimidine. These two octets (originally one octet in the unlabelled iminohexahydropyrimidine) are suggested to arise from an isotope effect of deuterium *versus* hydrogen transmitted from an axial H or D at C-6 of the hexahydropyrimidine through the nitrogen lone-pair (axial) to 1′-H. For similar reasons, in the spectra of the dideuteriated iminohexahydropyrimidine *but not the unlabelled iminohexahydropyrimidine*, the signal of 2-H appears as two quartets of similar intensities separated by 7 Hz.

The complexities described necessitated the synthesis of a reference sample of one of the dideuteriated iminohexahydropyrimidines. This was achieved from (*E*)-[1,2-$^2H_2$]ethene *via* (2*S*,3*R*)/(2*R*,3*S*)-[2,3-$^2H_2$]-3-aminopropiononitrile (see above). The synthetic (1′*R*,2′*R*)/(1′*S*,2′*S*)-[1′,2′-$^2H_2$]spermidine was treated with >2 mol equiv. of ethanal in deuteriochloroform to give the (2*S*,5*R*,6*R*)- and the (2*S*,5*S*,6*S*)-[5,6-$^2H_2$]iminohexahydropyrimidines and their enantiomers. The 61.4 MHz {^{1}H}^{2}H n.m.r. spectrum of this mixture showed four singlets of equal intensities at δ 1.66 and 3.03 corresponding to the 5-D_{eq} and 6-D_{ax}, respectively, in the (2*S*,5*S*,6*S*)-isomer, and at δ 1.52 and 2.33 corresponding to the 5-D_{ax} and 6-D_{eq}, respectively in the (2*S*,5*R*,6*R*)-isomer. The 400 MHz ^{1}H n.m.r. spectrum of the synthetic (2*S*,5*R*,6*R*)- and (2*S*,5*S*,6*S*)-isomers of iminohexahydropyrimidine was similar (peak for peak matching) to the dideuterioiminohexahydropyrimidine derived from the (3*R*,4*S*)-[3,4-$^2H_2$]methionine. In particular, the resonances for 6-H_{ax} (Figure 1d) was, as expected, a broad singlet at δ 2.30, superimposed on resonances from 1′-H_{ax}.

The analyses of the resonances in Figure 1a—c were supported by simulations carried out with a SIMEQ-II program (Varian) (cf. Figure 2a—c).

The above conclusions were reinforced by a series of double resonance experiments, the most pertinent results of which are shown in Figure 3. The iminohexahydropyrimidines from (3*R*,4*S*)-[3,4-$^2H_2$]methionine should have 6-H_{ax} paired with 5-H_{eq} [in the (2*S*,5*R*,6*R*)-isomer and its enantiomer] and 6-H_{eq} paired with 5-H_{ax} [in the (2*S*,5*S*,6*S*)-isomer and its enantiomer]. As expected, irradiation of 6-H_{ax} showed no effect on observed 5-H_{ax}. However, whilst irradiation of 5-H_{ax} hardly affected the 6-H_{ax}/1′-H_{ax} multiplet, 6-H_{eq} was observed as a less broad singlet (see Figure 3A, *cf.* 1 and 2). Irradiation of 6-H_{eq} had no effect on 6-H_{ax} but the broad doublet for 5-H_{ax} was sharpened (see Figure 3A, *cf.* 3 and 4). The identical series of double resonance experiments applied to the iminohexahydropyrimidine from synthetic (1′*R*,2′*R*)/(1′*S*,2′*S*)-spermidine gave a similar set of results (see Figure 3B). However, the iminohexahydropyrimidines from (3*R*,4*R*)-[3,4-$^2H_2$]-methionine gave a different set of results because 6-H_{ax} is paired with 5-H_{ax} and 6-H_{eq} is paired with 5-H_{eq}. Thus, irradiation of 5-H_{ax} had no effect on 6-H_{eq} (see Figure 3C, *cf.* 1 and 2). Irradiation of 6-H_{eq}, converted 5-H_{ax} from a broad triplet into a sharper triplet (the right-hand portion of each was obscured; see Figure 3C, *cf.* 3 and 4), because the removal of 6-H_{eq} to 5-D_{ax} coupling.

Conclusions

The conclusion from the study described is that cells of *Escherichia coli* manufacture spermidine by butane-1,4-diamine *directly displacing* 5-methylthioadenosine from decarboxylated adenosylmethionine. It is presumed that this reaction is mediated by spermidine synthase, an enzyme that has been isolated in pure form from *E. coli* and shown to catalyse the formation of spermidine from the substrates cited.[14] The principal function of the enzyme is to align the substrates in a manner to permit an S_N2 displacement at C-1 of the 3-aminopropyl group of decarboxylated adenosylmethionine. This reaction is analogous to enzymic transmethylation from adenosylmethionine for which an S_N2 pathway has been established for several examples.[15] Adenosylmethionine is biosynthesised by S_N2 attack of methionine on the 5′-adenosine triphosphate, catalysed by methionine adenosyltransferase.[16] S_N2 Displacements at methylene centres and more highly substituted carbon atoms are relatively uncommon in biological systems. Numerous studies of displacements in chemical systems have shown that compared to substitution at methyl centres, there is a steric impediment with methylene that is accentuated with methine.[17] Alkylating agents (*e.g.* alkyl halides, epoxides) are toxic to biological systems because they react with nucleophilic sites in enzymes and nucleic acids, usually by an S_N2 mechanism.[18] Glutathione may scavenge alkylating agents in cells and it has recently been shown that the reactions between this thiol and phenethyl halides catalysed by a glutathionine *S*-transferase are probably S_N2 displacements.[19]

Experimental

Synthesis of (1′R,2′R)/(1′S,2′S)-[1′,2′-$^2H_2$]*Spermidine.*

(1R,2S)/(1S,2R)-2-*Chloro*-[1,2-$^2H_2$]*ethanol* (**4**).—Hypochlorous acid (2.9M; 42 cm^3), prepared and assayed as described in ref. 20 was placed in a 2 dm^3 flask fitted with a two-way tap. The acid was frozen by immersing the flask in a solid CO_2 bath. The frozen acid was pumped *in vacuo* to remove any traces of chlorine. The flask was then attached to a vacuum line in which (*E*)-[1,2-$^2H_2$]ethene[8] (2.5 dm^3, 0.11 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 to room temperature. The reaction mixture was then stirred (orbital shaker) in the dark overnight. Saturated aqueous sodium chloride (100 cm^3) was added, and the reaction mixture was then extracted with dichloromethane (3 × 100 cm^3). The combined organic layers were separated, dried, and evaporated to leave an oily residue of compound (**4**) (8 g, 89% yield), δ (220 MHz; $CDCl_3$, TMS) 2.61 (1 H, s, OH), 3.63 (1 H, s, CHDOH), and 3.83 (1 H, s, CHClD).

(1R,2R)/(1S,2S)-2-*Amino*-[1,2-$^2H_2$]*ethanol* (**6**).—Compound (**4**) (4.1 g, 0.05 mol) was dissolved in water (60 cm^3) in a 500 cm^3 flask containing a magnetic stirrer bar and 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 (modelled on that of ref. 9) was evacuated. The frozen solid in the flask was slowly allowed to melt, which resulted in a vigorous evolution of dideuteriated oxirane (**5**). The oxirane was trapped in a tube cooled by a solid CO_2–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 to the main line was opened, and oxirane 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 oxirane. After 30 min, the flask containing the ammonia was kept in liquid nitrogen for 15 min to trap all the remaining oxirane (traces). The flask was isolated from the line, warmed to 0 °C, and stirring was continued for a further 5 min. The reaction mixture was removed from the line and was evaporated under reduced pressure (12 mmHg at 35 °C) to leave an oily residue (2.2 g, 70%) of compound (**6**), δ (220 MHz; $CDCl_3$, TMS) 1.82 (1 H, br s, OH), 2.82 (1 H, s, NH_2CHD) and 3.58 (1 H, s, $CHDOH$).

(1R,2S)/(1S,2R)-2-*Trifluoroacetylamino*-[1,2-$^2H_2$]*ethanol* (**7**).—Compound (**6**) (2.2 g, 35 mmol) was dissolved in acetonitrile (30 cm^3). Ethyl trifluoroacetate (6.0 g, 45 mmol) was added to the solution. The resulting mixture was sealed and stirred for 20 min at room temperature. The solvent was removed under reduced pressure to leave an oily residue of compound (**7**), (5.4 g, *ca.* 98% yield + 2% acetonitrile), δ (220 MHz; $CDCl_3$, TMS), 2.5 (1 H, br s, OH), 3.5 (1 H, s, $CHDNH$), and 3.78 (1 H, s, $CHDOH$).

(1R,2S)/(1S,2R)-2-*Trifluoroacetylamino*-[1,2-$^2H_2$]*ethanol* O-*Toluene*-p-*sulphonate* (**8**).—The *N*-trifluoroacetylaminoalcohol (**7**) was tosylated[24] to afford compound (**8**) as white crystals, m.p. 58—60 °C (8.8 g, 86% yield), δ_H 2.46 (3 H, s, Me), 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, ArH); *m/z* (e.i.) 312 ($M^+ - 1$, 10%) and 313 (M^+, 90). An unlabelled sample of the compound (**8**) showed a peak at *m/z* 311 (M^+).

(4R,5R)/(4S,5S)-2-*Trifluoromethyl*[4,5-$^2H_2$]-4,5-*dihydro-oxazole* (**9**).—To compound (**8**) (8.8 g, 28 mmol) in dichloromethane (150 cm^3) was added powdered potassium hydroxide (2.8 g, 5 mmol). The mixture was sealed and stirred at room temperature for 4 h. The precipitated potassium toluene-*p*-sulphonate was removed by filtration through Celite. The filtrate was evaporated under reduced pressure to give a colourless residue of compound (**9**) (3.2 g, 81%). The purity of product (**9**) was found to be ≥98% (by ^1H n.m.r.); δ 4.05 (1 H, d, *J ca.* 9.5 Hz, 4-H) and 4.52 (1 H, d, *J ca.* 9.5 Hz, 5-H); *m/z* (e.i.) 140 (10%) and 141 (90). The ^1H n.m.r. spectrum for a sample of 2-trifluoromethyl-4,5-dihydro-oxazole prepared similarly from unlabelled precursors (*cf.* ref. 21) showed resonances at δ 4.09 (2 H, t, *J ca.* 9.5 Hz, 4-H) and 4.56 (2 H, t, *J ca.* 9.5 Hz); *m/z* (e.i.) 139 (M^+).

(2R,3S)/(2S,3R)-3-*Amino*[2,3-$^2H_2$]*propiononitrile* (**3**) via (2R,3S)/(2S,3R)-3-*Trifluoroacetylamino*[2,3-$^2H_2$]*propiono-nitrile* (**10**).—Dry sodium cyanide (1.5 g, 30 mmol) was added to a stirred solution of the dihydro-oxazole (**9**), (2.3 g, 16 mmol) in dry dimethyl sulphoxide (30 cm^3). The mixture was sealed and stirred at room temperature for 6 days and the reaction was monitored by ^1H n.m.r. spectroscopy. The volume of the reaction was reduced (10^{-4} mmHg/40 °C) to 5 cm^3. Lithium hydroxide (1.23 g, 30 mmol) solution in water (10 cm^3) was added to the residue and the resulting mixture was stirred for 5 min before the dropwise addition of 5M-hydrochloric acid (10 cm^3). The mixture was evaporated to dryness (10^{-4} mmHg/40 °C), to leave 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 min. The charcoal was removed by filtration through Celite and the filtrate was kept at −20 °C overnight to precipitate a white crystalline product. The precipitate was filtered off and dried *in vacuo* to give pure compound (**3**) as its monohydrochloride (0.95 g, 55% yield), m.p. 162—164 °C), δ (^2H_2O, TSS) 2.93 (1 H, d, *J ca.* 6 Hz, CHDCN) and 3.32 (1 H, d, *J ca.* 6 Hz, $CH\overset{+}{N}H_3$). The 220 MHz ^1H n.m.r. spectrum for a sample of 3-aminopropiononitrile hydrochloride (*cf.* ref. 22) showed resonances at δ 2.92 (2 H, t, CH_2CN) and 3.32 (2 H, t, $CH_2\overset{+}{N}H_3$).

4-*Benzyloxycarbonylaminobutyric acid.*—This was prepared in the standard manner[23] from 4-aminobutyric acid m.p. 65—68 °C (lit.,[24] 65—66 °C), δ 1.81 (2 H, pent., $CH_2CH_2CH_2$), 2.38 (2 H, t, CH_2CO_2H), 3.25 (2 H, q, $CONHCH_2$), 5.09 (2 H, s, $PhCH_2$), and 7.33 (5 H, s, ArH).

(2S,3R)/(2R,3S)-3-(4-*Benzyloxycarbonylaminobutyryl-amino*)[2,3-$^2H_2$]*propiononitrile* (**11**).—4-Benzyloxycarbonyl-aminobutyric acid (1.54 g, 0.65 mol) and the hydrochloride of (2*R*,3*S*)/(2*S*,3*R*)-3-amino-[2,3-$^2H_3$]propiononitrile (**3**) (0.71 g, 6.5 mmol) 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 mmol) was added. Dicyclohexyl-carbodi-imide (1.36 g, 6.6 mmol) was added and the reaction mixture stirred overnight. The mixture was filtered and the filtrate was reduced to *ca.* 5 cm^3. A white precipitate came out on cooling to −20 °C (4 h), which was filtered off and dried *in vacuo* to give white crystals of compound (**11**). Recrystallisation from dichloromethane gave pure (**11**) (1.7 g, 90% yield), m.p. 114—115 °C, δ_H 1.84 (2 H, pent., $CH_2CH_2CH_2CONH$), 2.25 (2 H, t, CH_2CH_2CONH), 2.6 (1 H, br s, $CONHCHDCHDCN$), 3.26 (2 H, q, $PhCH_2OCONHCH_2CH_2$), 3.45 (1 H, br s, $CONHCHDCHDCN$), 5.1 (2 H, s, $PhCH_2OCONH$), 5.11 (1 H, br s, $CH_2CONHCH_2$), 6.71(1 H, br s, $PhCH_2OCONH$), and 7.36 (5 H, s, ArH). The ^1H n.m.r. spectrum of an unlabelled sample showed resonances similar to those of the labelled compound (**11**), except for the signals of the methylene groups in the aminopropyl unit δ_H 2.6 (2 H, t, $CONHCH_2CH_2CN$) and 3.46 (2 H, q, $CONHCH_2CH_2CN$) [Found (unlabelled sample): C, 62.45; H, 6.6; N, 14.4. $C_{15}H_{19}O_3$ requires C, 62.25; H, 6.6; N, 14.5%]. The c.i. m.s. analysis (NH_4^+) for the unlabelled compound showed a peak at *m/z* 290 $(M + 1)^+$. The labelled compound (**11**) showed peaks at *m/z* 291 (10%) and 292 (90) corresponding to the ions M^+ and $(M + 1)^+$, respectively.

Hydrogenation and Reduction of Compound (**11**)† *to give* (1′R,2′R)/(1′S,2′S)-[1′,2′-$^2H_2$]*Spermidine* (**12**).—The nitrile (**11**) (0.3 g, 1 mmol) 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 1M-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 reaction was evaporated under reduced pressure. To the residue was added pre-cooled, dry ethanol (30 cm^3) and 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 (**12**) was filtered off and recrystallised from ethanol to give the pure trihydrochloride as white crystals (0.16 g, 62% yield), m.p. 254—257 °C (lit.,[25] m.p. 256—258 °C), δ_H (^2H_2O, TSS) 1.8 (4 H, m, 2 × 2-H and 2 × 3-H], 2.1 (1 H, m, 2′-H) and 3.12 (7 H, m, 1′-H, 2 × 3′-H, 2 × 1-H, and 2 × 4-H). Free spermidine was obtained by running the compound through a column of a basic

* The release of the oxirane 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.

† The detailed procedure for the hydrogenolysis and reduction of compound (**11**) was kindly supplied by Dr. D. J. Robins (Chemistry Department, University of Glasgow).

ion exchange resin (Amberlite IR-400, $^-$OH form). The first 100 cm^3 of the eluant (water) were collected and evaporated to dryness to leave behind the hydrochloride-free (1′*R*,2′*R*)/(1′*S*,2′*S*)-[1′,2′-$^2H_2$]spermidine (**12**). δ (220 MHz; $CDCl_3$, TMS) 1.5 (10 H, m, 2′-H, 2 × 2-H, 2 × 3-H, and 5 × NH), and 2.7 (7 H, m, 2 × 1-H, 2 × 4-H, 1′-H, and 2 × 3′-H). 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%.

Biosynthesis of Stereospecifically Labelled [1′,2′-$^2H_2$]*Spermidines from* (3R,4S)- *and* (3R,4R)-[3,4-$^2H_2$]*Methionines*.—Two standard media (each 10 × 1 dm^3 in 10 flasks of 2 dm^3 capacity), were prepared.[6] Each medium was supplied with one of the following: (3*R*,4*R*)- or (3*R*,4*S*)-[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). Dideuteriated spermidine was extracted from each batch of cells with trichloroacetic acid. The extracted spermidines were then converted into PATC-derivatives.[6]

The PATC-derivatives of dideuteriated spermidine from each experiment were purified by p.l.c. [Kieselgel 60 HR reinst 2 × (0.5 × 20 × 100 cm)] (*cf.* ref. 6).

The PATC-derivatives of the two samples of the dideuteriated 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 dideuteriated spermidine was isolated.[6] The hydrochloride-free dideuteriated spermidines were obtained by running each sample through an ion exchange column [Amberlite IR-400, $^-$OH form (1 × 10 cm)], using water as eluant. Evaporation of the first fraction (100 cm^3) under reduced pressure gave 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 400 MHz ^{1}H n.m.r. spectra of iminohexahydropyrimidines, prepared in $CDCl_3$ from these spermidines according to the literature procedures [5] are discussed in the text.

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