# The Biosynthesis of Spermidine. Part 1: Biosynthesis of Spermidine from L-[3,4-$^{13}C_2$]Methionine and L-[2,3,3-$^2H_3$]Methionine

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Three mechanisms are discussed for the biosynthesis of spermidine from butane-1,4-diamine and decarboxylated adenosylmethionine catalysed by spermidine synthase: (i) enzyme-mediated $S_N2$ attack of butane-1,4-diamine at the aminopropyl group of decarboxylated adenosylmethionine; (ii) $S_N2$ attack at the aminopropyl group by a nucleophilic group of the synthase, to give an aminopropylated enzyme, which reacts with butane-1,4-diamine to give spermidine (double $S_N2$ displacement); and (iii) enzyme-induced intramolecular closure of decarboxylated adenosylmethionine to give protonated azetidine, which reacts with butane-1,4-diamine to give spermidine (alternative double $S_N2$ displacement).

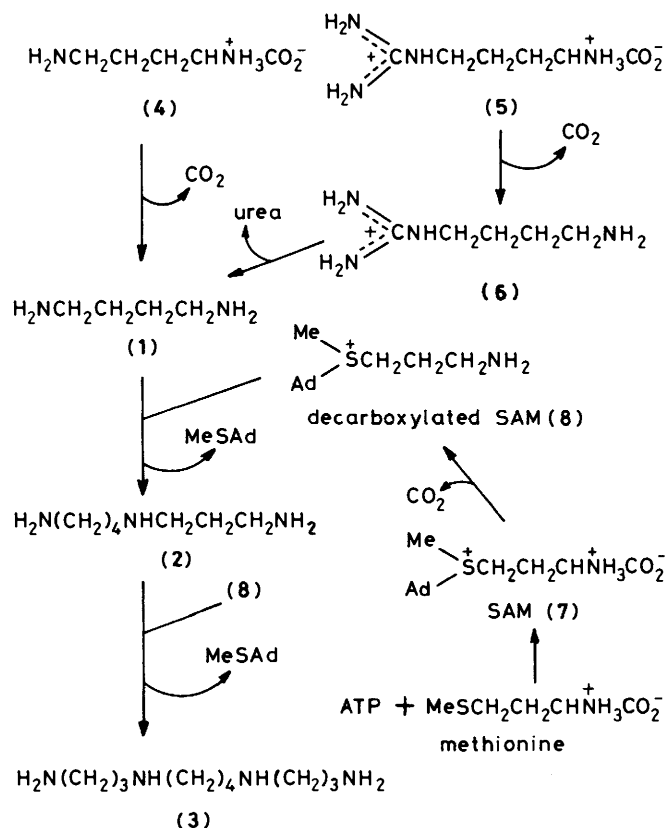
Using n.m.r. spectroscopy to determine isotope distributions in spermidines derived from *Escherichia coli* fed on [3,4-$^{13}C_2$]methionine and [2,3,3-$^2H_3$]methionine, mechanisms (i) and (ii) are shown to be tenable, but mechanism (iii) is eliminated from consideration.

The most common and important members of the class of naturally occurring aminoalkanes, known as polyamines, are putrescine (**1**) (butane-1,4-diamine), spermidine (**2**) (*N*-3-aminopropylbutane-1,4-diamine), and spermine (**3**) (*N,N'*-bis-3-aminopropylbutane-1,4-diamine). Although a spermine phosphate was first crystallised more than three centuries ago,[1] it is only relatively recently that the immense biological importance of polyamines has been recognised.[2] Thus, polyamines mediate cellular growth because they are associated with and control the functions of nucleic acids.[3] The nature of the association is a simple ionic interaction between ammonium groups of the polyamine and phosphate oxygens of the nucleic acid.[4] Levels of polyamines in the urine of persons suffering from cancer or certain other diseases are enhanced and this observation has encouraged the development of excellent methods for the clinical analyses of polyamines.[5]

The biosynthesis of putrescine in animal tissues occurs from ornithine (**4**), which is decarboxylated by ornithine decarboxylase.[2a] In bacteria, an alternative route to putrescine is from arginine (**5**) *via* agmatine (**6**).[2a] For the production of spermidine and spermine, methionine is *S*-adenosylated to *S*-adenosylmethionine (SAM), (**7**), which is then decarboxylated to (**8**). This compound is a substrate for spermidine synthase, which effects the aminopropylation of a nitrogen atom of putrescine. The enzyme has been purified to homogeneity from *Escherichia coli* and has no known co-factor.[6] The complete routes to spermidine and spermine from ornithine or arginine are summarised in Scheme 1.

The pathways of Scheme 1 were suggested by the results of experiments in which various labelled precursors were fed to *Escherichia coli* and other organisms. It was shown that [$^{14}$C]- and [$^{15}$N]-labelled ornithine gave labelled putrescine.[7] [2-$^{14}$C]Methionine gave [$^{14}$C]-labelled spermidine, whereas [1-$^{14}$C]methionine gave unlabelled spermidine, as expected.[8]

There are three distinct mechanisms possible for the aminopropyl transfer catalysed by spermidine synthase.[9-11] (i) Enzyme-mediated $S_N2$ attack of a nitrogen atom of butane-1,4-diamine at C-1 of the aminopropyl group of decarboxylated adenosylmethionine (Scheme 2). This mechanism would bring about an inversion of configuration at this carbon atom (*i.e.* at C-1' of spermidine). (ii) $S_N2$ Attack at C-1 of the aminopropyl group of decarboxylated adenosylmethionine by a nucleophilic




**Scheme 1.** Biosynthetic routes to spermidine in animal tissue [from ornithine (**4**)] and bacteria [from arginine (**5**)]. (Ad = adenosyl, ATP = adenosine triphosphate).

group of spermidine synthase, giving an aminopropylated enzyme. This reacts by an $S_N2$ mechanism with butane-1,4-diamine to give spermidine (Scheme 3). The stereochemical consequence of this mechanism would be retention of configuration at C-1 of the aminopropyl group of decarboxylated adenosylmethionine. In the terminology of enzyme kineticists this is a Ping-Pong Bi-Bi mechanism, whereas mechanism (i) is a so-called sequential Bi-Bi pathway.[12] Kinetic studies with purified spermidine synthase from *E.coli* concluded that

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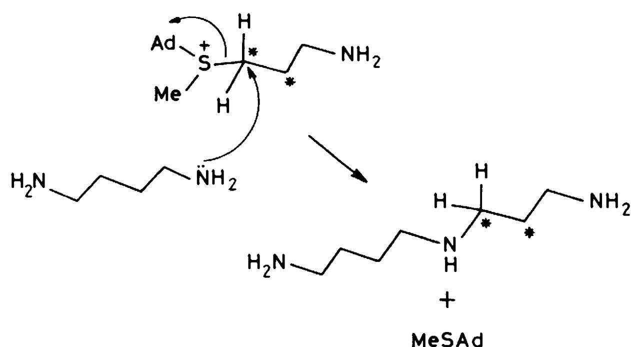
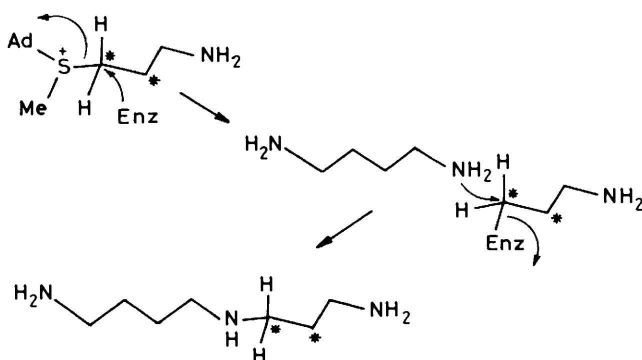
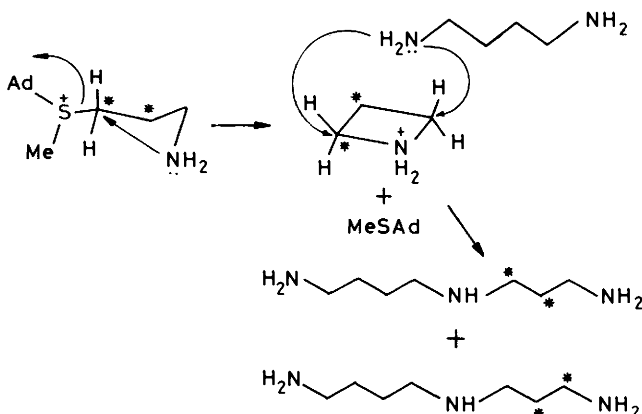



**Scheme 2.** Formation of spermidine *via* enzyme-mediated $S_N2$ attack by butane-1,4-diamine on C-1 of the aminopropyl group of decarboxylated adenosylmethionine. N.B. The asterisks denote the fate of $^{13}C$ label in this and subsequent Schemes.




**Scheme 3.** Formation of spermidine *via* $S_N2$ attack by butane-1,4-diamine on an aminopropylated enzyme produced by enzymic $S_N2$ attack on C-1 of the aminopropyl group of decarboxylated adenosylmethionine.

mechanism (ii) is correct.[9] However, this conclusion may be vitiated because product inhibition studies were not performed.[10] (iii) Enzyme-induced intramolecular closure of decarboxylated adenosylmethionine to give azetidine, which then reacts with butane-1,4-diamine by an $S_N2$ mechanism to give spermidine (Scheme 4). This mechanism would bring about retention of configuration at C-1 of the aminopropyl group of decarboxylated adenosylmethionine.




**Scheme 4.** Formation of spermidine *via* $S_N2$ attack by butane-1,4-diamine on protonated azetidine, which arises by an intramolecular $S_N2$ reaction of decarboxylated *S*-adenosylmethionine.

It is of interest to note that (iii) is the only possible intramolecular $S_N2$ reaction that decarboxylated SAM can undergo. Intramolecular *S* to *N* methyl transfer and adenosyl transfer are both forbidden, endocyclic processes.[13] Methylation of a suitable nucleophilic centre (*N*, *O* or *C*) by SAM is a very common process in the biosynthesis of natural products.[14] This *intermolecular* $S_N2$ process is expected to occur more readily at the methylsulphonium group, than at either methylenesulphonium group of SAM.[15] It was therefore attractive to consider that the intramolecular cyclisation of decarboxylated SAM to azetidine was a subtle device for overcoming the inherent greater reactivity of the methylsulphonium group to nucleophilic attack.

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. In the proposed azetidine intermediate [mechanism (iii) above], C-1′ and C-3′ are homotopic,* so if spermidine were derived from the [3,4-$^{13}C_2$]methionine *via* the azetidine (Scheme 4), this spermidine must be a mixture of two isomers, one labelled at C-2 and C-4, the other labelled at C-2′ and C-3′. We found that a single spermidine labelled at C-1′ and C-2′ was produced by *E.coli* cells from [3,4-$^{13}C_2$]methionine (see ref. 11 and below), which tended to exclude mechanism (iii).

Mechanism (i) can be distinguished from mechanisms (ii) and (iii) by using *E.coli* to convert a sample of methionine stereospecifically labelled with deuterium or tritium at C-4 into a labelled spermidine. The absolute configuration of this spermidine would have to be determined, *e.g.* by correlation with a suitable standard. An alternative approach would be to convert a sample of methionine labelled with deuterium atoms at both C-3 and C-4 and of known relative configuration, into a dideuteriated spermidine whose relative configuration would have to be determined. We adopted the second strategy. It was necessary to ensure that during the decarboxylation of SAM, hydrogen (deuterium) atoms at C-3 of the original methionine chain were not disturbed. We therefore investigated the conversion of [2,3,3-$^2H_3$]methionine into spermidine effected by cells of *E.coli*.

*Biosynthesis of Spermidine from* L-[3,4-$^{13}C_2$]*Methionine*.—L-[3,4-$^{13}C_2$]methionine[16] (containing 81% $^{13}C_2$, 18% $^{12}C^{13}C$ and 1% $^{12}C_2$) was added to a growing culture of *E.coli*. Putrescine and spermidine were isolated as described for unlabelled materials *via* their phenylaminothiocarbonyl derivatives.[17] The isolated derivatives (**9**) and (**10a**) were subjected to $^1H$ and $^{13}C$ n.m.r. spectral analyses. The $^1H$ and $^{13}C$ n.m.r. spectra of compound (**9**) were very similar to spectra of authentic unlabelled (**9**) and showed, as expected, no incorporation of $^{13}C$ into putrescine. The isolated sample of compound (**10a**) showed in its 22.6 MHz $\{^1H\}^{13}C$ 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′)] in addition to signals at natural abundance (Figure). The 22.6 MHz $^{13}C$ n.m.r. spectrum of unlabelled PATC-spermidine† (**10b**) was assigned with the aid of model compounds.[17] The signals at δ 26.86 and 48.63 in the spectrum of the unlabelled PATC-spermidine (**10b**) (Figure) were assigned[17] with the aid of data for model compounds to C-2′ and C-1′, respectively. The position of the doublets in the $^{13}C$ n.m.r. spectrum of labelled compound (**10a**)

* The C-2 and C-4 methylene groups of protonated azetidine are homotopic if the azetidine molecule can rotate once about the $C_2$ axis through *N* and C-3. It is conceivable that protonated azetidine formed at the active site of spermidine synthase would not rotate before it is subjected to nucleophilic attack by putrescine. We regarded this possibility as less likely.

† PATC = Phenylaminothiocarbonyl.

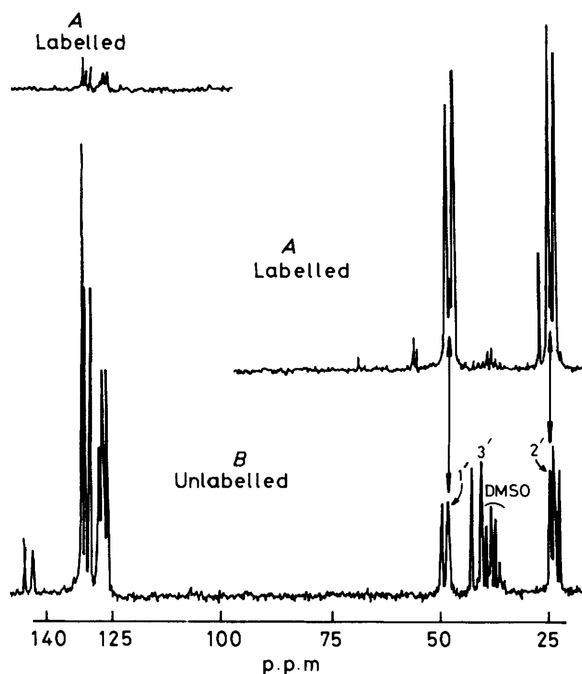
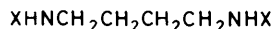
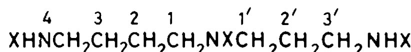



**Figure.** Lower trace, $\{^1H\}$ $^{13}C$ n.m.r. spectrum (in $[^2H_6]$dimethyl sulphoxide) of natural abundance PATC-spermidine (**10b**); inset traces, portions of the $\{^1H\}$ $^{13}C$ n.m.r. spectrum (in $[^2H_6]$dimethyl sulphoxide) of $^{13}C$-labelled PATC-spermidine (**10a**).




(9)




(10)

a; $^{13}C$-enriched at C-1′ and C-2′

b; unlabelled

c; deuteriated mainly at C-2′

X = PhNCS

confirms that C-1′ is as assigned previously[17] in the unlabelled compound (at δ 48.63). It shows also that the resonance for C-2′ is as assigned[17] in the unlabelled PATC-spermidine (**10b**) at δ 26.86.

The 220 MHz $^1H$ n.m.r. spectrum of the compound (**10a**) was similar to the spectrum of compound (**10b**) with some modification due to the $^{13}C$ atoms at C-1′ and C-2′. The resonance for 1′-H appeared at δ 3.8 (double multiplet, 0.9 × 2 H, $J_{^{13}C-^1H}$ 135 Hz and 0.1 × 2 H, m). Part of this signal was covered by the broad resonances from 4-H and 3′-H at δ 3.52. The resonance for 2′-H appeared at δ 1.95 (double multiplet, 0.9 × 2 H, $J_{^{13}C-^1H}$ 135 Hz and 0.1 × 2 H, m). Part of this signal was covered by resonances from 3-H and 2-H at δ 1.65. The residual signals for 1′-H and 2′-H at δ 3.8 and 1.95 derive from the *ca.* 10% of $^{12}C$ at both C-1′ and C-2′.

These results show that C-3 and C-4 of methionine are incorporated as an intact unit into spermidine. They support mechanisms (i) and (ii) discussed above, but exclude mechanism (iii) [see however, footnote on p. 4].

*Biosynthesis of Spermidine from* L-$[2,3,3-^2H_3]$*Methionine*.—L-$[2,3,3-^2H_3]$-methionine was prepared having a $[^2H]$ content of ≥86% (m.s. analysis).[16] By $^1H$ n.m.r. spectral analysis most (*ca.* 98%) of the C-2 positions were labelled with deuterium, and *ca.* 80% deuterium incorporation had occurred at C-3. 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 the corresponding unlabelled compounds.[17] The isolated PATC-putrescine (**9**) was subjected to 400 MHz $^1H$ n.m.r. spectral analysis and was found to be devoid of deuterium [the spectrum was very similar to that of the authentic (**9**)]. The isolated PATC-spermidine (**10c**) was also examined by 400 MHz $^1H$ n.m.r. spectroscopy and the spectrum showed many features of the 400 MHz $^1H$ n.m.r. spectrum of authentic unlabelled PATC-spermidine (**10b**). The significant differences between these spectra were consistent with the presence of deuterium atoms at C-2′ in compound (**10c**). Thus, the conversion of L-$[2,3,3-^2H_3]$methionine into spermidine had occurred with complete retention of deuterium at C-2′ of spermidine (originally C-3 in the labelled methionine). The resonance for 1′-H in PATC-spermidine (**10b**) was a triplet; the corresponding signal in the spectrum of the labelled PATC-spermidine (**10c**) appeared as a singlet due to absence of coupling between 1′-H and the adjacent 2′-H (largely deuteriated). The residual resonance at δ 2.33 (0.2 × 2 H, q, 2′-H) of the labelled PATC-spermidine (**10c**) comes from methionine containing one or two protons at C-3 [estimated total $^1H$ at C-3 *ca.* 20% (see Experimental section)]. The intensity of this signal relative to any of the unlabelled methylene groups in the spectrum of compound (**10c**) was 1:5, which correlates well with the estimate made for protons at C-3 of the labelled methionine.

The $^1H$ n.m.r. spectrum of (**10c**) showed that deuterium originally at C-2 of the labelled methionine was largely lost *en route* to spermidine. The 61.4 MHz $^2H$ n.m.r. spectrum of labelled PATC-spermidine (**10c**) confirmed that only a small portion of deuterium was present at C-3′. The integration of the resonances for deuterium at C-2′ and C-3′ in this spectrum indicated that ≥90% of deuterium originally at C-2 of the labelled methionine had been lost in the process of spermidine formation. Thus, the main product was $[2',2'-^2H_2]$spermidine rather than the expected $[2',2',3'-^2H_3]$spermidine.

In the biosynthesis of spermidine, the enzyme SAM-decarboxylase catalyses the decarboxylation of SAM (**7**) to decarboxylated-SAM (**8**).[18] According to Pankaskie and Abdel-Monem,[19] the first step in the decarboxylation of SAM is the formation of an azomethine adduct between the amino group of SAM and the ketonic carbonyl of the prosthetic group pyruvate to give enzyme-bound adenosylmethionine. This is followed by decarboxylation of the adduct to give an enzyme-bound imine. The cavity of the active site of the enzyme could have a basic functional group, which exchanges the α-H in the imine adduct, faster than it is hydrolysed to decarboxylated SAM.

For studies of the stereochemistry of spermidine synthase using stereospecifically labelled $[3,4-^2H_2]$methionines, it was very important to make sure that deuterium originally at C-3 of methionine did not exchange in the formation of spermidine, because this would have resulted in the loss of stereochemical information. Furthermore, dilution from endogenous methionine should be insignificant. In the experiments described, no exchange or dilution of deuterium originally at C-3 of methionine was observed.

**Conclusions.**—The $^{13}C$, $^1H$, and $^2H$ 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 work to demonstrate the incorporation of more than one carbon atom from the C-2 ⟶ C-4 moiety of methionine into spermidine. The intensities of the signals for C-1′ and C-2′, in the Figure, in comparison with the

natural abundance signals, suggests that the $^{13}C$ labels in [3,4-$^{13}C_2$]methionine were incorporated into spermidine without any significant dilution from endogenous methionine.

Pure spermidine can be recovered from its PATC-derivative by hydrolysis.[17] Specifically labelled spermidines, prepared as described in this paper, may be of value for studying the metabolism of spermidine,[20] its interaction with nucleic acids,[4] and the biosynthesis of spermidine-derived alkaloids.[21]

## Experimental

Solvents were either AnalaR grade or redistilled laboratory reagents. Reagents from commercial sources were either used directly, if of sufficient purity, or were purified. M.p.s are uncorrected. I.r. spectra were recorded on a Perkin-Elmer 580B spectrophotometer. $^1H$ N.m.r. spectra were recorded at either 220 MHz (Perkin-Elmer R34 spectrometer) or 400 MHz (Bruker WH-400 spectrometer); $^2H$ n.m.r. spectra were recorded at 61.4 MHz on a Bruker WH-400 spectrometer; $\{^1H\}^{13}C$ n.m.r. spectra were recorded at 22.63 MHz on a Bruker WH-90 spectrometer [internal standard, tetramethylsilane (TMS) for organic solvents, sodium 3-trimethylsilylpropanesulphonate for $D_2O$]. Mass spectra were obtained using a Kratos MS80 spectrometer. Combustion analyses were carried out by C.H.N. Analysis Ltd., Leicester.

*Isolation of* [1',2'-$^{13}C_2$]*Spermidine* (**10a**).—The standard medium[17] ($10 \times 1$ $dm^3$) 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 below for dideuteriated spermidine.[17]

*Isolation of* [2',2'-$^2H_2$]*Spermidine* (**10c**).—The standard medium[17] ($10 \times 1$ $dm^3$) in flasks of 2 $dm^3$ capacity was supplied with L-[2,3,3-$^2H_3$]methionine[16] (0.05 g $dm^{-3}$) and inoculated with *E.coli* as described.[17] 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 trichloroacetic acid. The extracted polyamines were converted into PATC-derivatives as described for the unlabelled compounds.[17]

The isolated mixture of PATC-putrescine (**9**) and PATC-spermidine (**10c**) was purified by p.l.c. [Kieselgel 60 HR reinst, $2 \times (0.5$ mm $\times 20 \times 100$ cm)]. Double elution with dichloromethane–acetonitrile (9/1, v/v) gave two bands, detected by their opacity on viewing the plates in daylight. Each band was scraped off and extracted with acetone ($2 \times 200$ $cm^3$). Evaporation of each fraction gave white residues. T.l.c. analysis of each fraction [Kieselgel 60 HR reinst, 0.25 mm thick plate, elution with dichloromethane–acetonitrile (87/13, v/v)] gave $R_F$ values of 0.34 and 0.25 corresponding to compounds (**9**) and (**10c**), respectively. The purity of each fraction was checked by h.p.l.c. which showed a single peak.[17] The yields of compounds (**9**) and (**10c**) were 50 mg and 65 mg, respectively.

The 400 MHz $^1H$ n.m.r. spectrum ([$^2H_5$]pyridine, TMS) of compound (**9**) showed resonances at $\delta$ 2.0 (4 H, m) and 4.0 (4 H, m). As expected, no deuterium was observed to have been incorporated into compound (**9**). The 400 MHz $^1H$ n.m.r. spectrum ([$^2H_5$]pyridine, TMS) of compound (**10c**) showed resonances at $\delta$ 1.74 (2 H, pent., *J* 7 Hz, 3-H), 1.94 (2 H, pent., *J* 7 Hz, 2-H), 2.33 ($0.2 \times 2$ H, q, *J* 7 Hz, 2'-H), 3.95 (4 H, m, 3'-H and 4-H), 4.07 (2 H, br, s, 1-H), 4.18 (2 H, s, 1'-H), 8.31, 8.54 and 9.67 (each 1 H, br, s, PhN*H*), and 7.0—7.7 (ArH). The 61.4 MHz $^2H$ n.m.r. (pyridine) of compound (**10c**) showed resonances at $\delta$ 2.35 (2 $^2H$, br s, 2'-$^2H$) and 4.0 ($0.1 \times$ $^2H$, br s, 3'-$^2H$). The n.m.r. spectra indicate that $\geqslant 90\%$ of the deuterium at C-3' had been lost during the formation of spermidine. For discussion of these spectra see the text.

* An undesignated methionine auxotroph of *E. coli* supplied by Dr. S. B. Primrose, Department of Biological Sciences, University of Warwick, was used for this experiment.

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