Biosynthesis of L-2,3-Diaminopropanoic Acid

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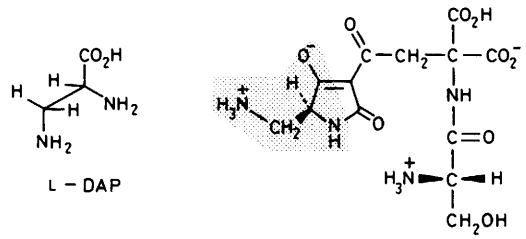
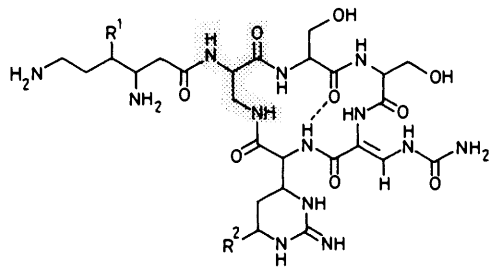
The biosynthesis of L-2,3-diaminopropanoic acid (L-DAP) was studied by means of incorporation of ^2H-labelled precursors into the simple microbial metabolite N^2-L-alanyl-N^3-fumaramoyl-L-2,3-diaminopropanoic acid (**2**). By ^2H n.m.r. spectroscopic analysis, it was established that L-serine is the biosynthetic precursor of L-DAP *via* a process in which the α-hydrogen of serine is eliminated, both β-hydrogens are retained, and ammonia is bound to C_β with retention of configuration. These findings are in accord with the mechanism of pyridoxal phosphate-dependent β-replacement reactions of serine which proceed by transient generation of an aminoacrylate intermediate and addition of a nucleophile to the double bond with retention of configuration.

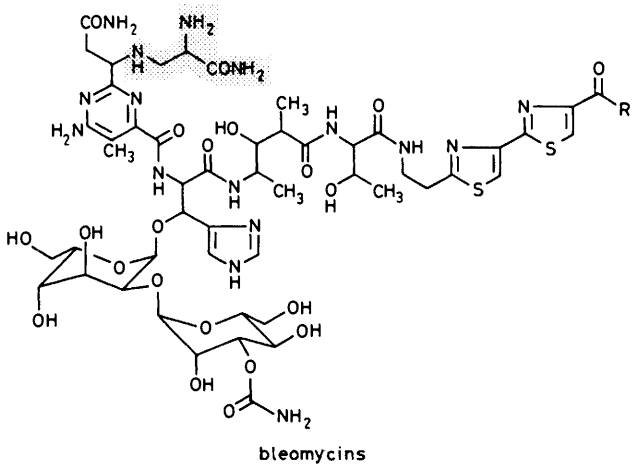
L-2,3-Diaminopropanoic acid (L-DAP) is a non-protein amino acid which occurs in its free form in many *Acacia* species.[1] Moreover, it constitutes an essential component of a number of peptide metabolites from vegetable and microbial origin with important biological activity, such as L-N^3-oxalo-2,3-diaminopropanoic acid (a neurotoxin responsible for neurolathyrism),[2] malonomicin (**1**) (active against *Trypanosoma congolense*),[3] and the tuberactinomycin[4] and bleomycin[5] families (anti-tuberculosis and antitumour antibiotics, respectively).

It has often been suggested that L-serine (or an activated derivative such as *O*-acetylserine) is the biosynthetic origin of L-DAP; nevertheless, many contradictory research results have been published. Reinbothe[6] found a good incorporation of [1-^{14}C]- and [3-^{14}C]-serine into albizziine (N^3-carbamoyl-2,3-diaminopropanoic acid); Seneviratne and Fowden,[7] however, were unable to detect any enrichment of albizziine by [U-^{14}C]serine. Likewise, studies by Roy[8] indicated that serine is not a precursor of DAP in L-N^3-oxalo-2,3-diaminopropanoic acid. Tam and Jordan[9] could not prove incorporation of serine into the DAP-containing peptide antibiotic viomycin (tuberactinomycin B); in contrast, Carter *et al.*[10] observed relatively good incorporation, particularly in the serine and DAP residues. Moreover, they found that [U-^{14}C]DAP was incorporated almost exclusively into the DAP moiety of viomycin. More recently,[11] it has been shown that *O*-acetylserine is a key intermediate in the biosynthesis of β-substituted alanines such as β-(pyrazol-1-yl)-L-alanine and β-(3-amino-1,2,4-triazol-1-yl)-L-alanine which formally are derivatives of L-DAP. Finally, we have presented unambiguous proof that both L-DAP and L-serine are excellent biosynthetic precursors of the same part of the tetramic acid nucleus of the antibiotic malonomicin (**1**).[12]

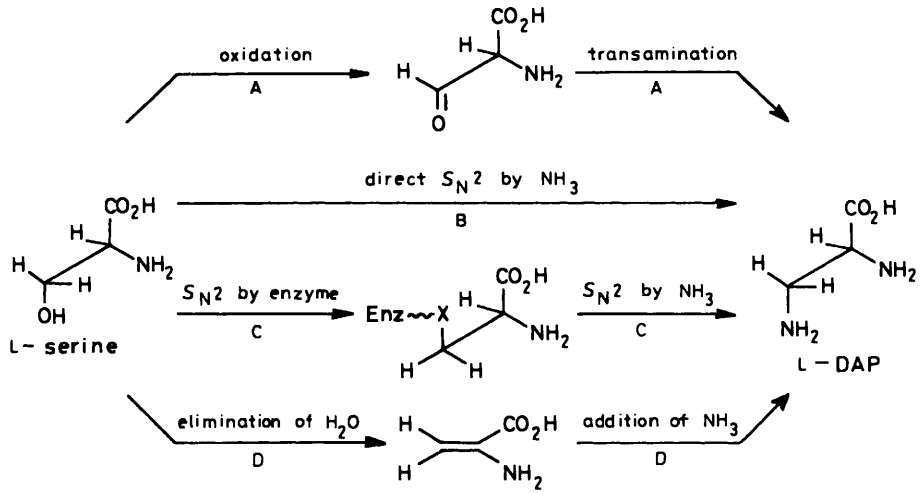
Given the fact that serine has been shown to be a precursor for DAP, several mechanistically reasonable pathways directly come to mind for this transformation (Scheme): (A) oxidation of serine to aminomalonaldehydic acid and subsequent transamination (as suggested by Reinbothe[6] without experimental evidence), (B) direct S_N2 substitution of the (activated) hydroxy group by ammonia (analogous to the recently found β-lactam ring closure in nocardicin A[13]), (C) displacement of the (activated) hydroxy group by enzyme followed by substitution by ammonia, (D) α,β-elimination of water followed by Michael addition of ammonia to the conjugated system (analogous to the biosynthesis of tryptophan[14]).







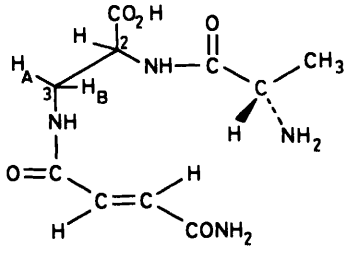
The first pathway (A) implies conservation of the α-hydrogen, but loss of one hydrogen at the β-carbon of serine; the second pathway (B) would give conservation of the α- and β-hydrogens and inversion of configuration at the β-carbon; the third pathway (C) would give conservation of the α- and β-hydrogens and retention of configuration at the β-carbon; the fourth pathway (D) would result in loss of the serine α-hydrogen and, probably,[14] retention of configuration at the β-



Scheme 1. Possible pathways for the biosynthetic conversion of L-serine into L-2,3-diaminopropanoic acid (L-DAP)

carbon. Thus, these four pathways have chemical and stereochemical characteristics which can be used to distinguish between them. Tracing the fate of the α- and β-hydrogens of serine upon incorporation into a suitable DAP-derived metabolite could therefore give a detailed picture of the mode of formation of L-DAP. In this communication we present the results of such a study.

Unfortunately, the α-hydrogen of the DAP moiety of malonomicin (**1**) is exchanged during the biosynthetic ring closure reaction leading to the heterocyclic ring of (**1**) so that malonomicin is not a suitable object with which to study in detail the mechanism of conversion of L-serine into L-DAP.[15] It was therefore necessary to look for a simple microbial metabolite containing L-DAP in an uncomplicated way because only then would the expectation be justified that L-DAP is incorporated intact and without further metabolic transformations. Such a metabolite is N^2-L-alanyl-N^3-fumaramoyl-L-2,3-diaminopropanoic acid (**2**) which we recently isolated from cultures of *Streptomyces collinus* Lindenbein.[16] In this



(**2**)

compound, only the amino groups of L-DAP are bonded to other groups in a peptide fashion while the backbone is unsubstituted. Moreover, in contrast to (**1**), the ABX-system of the $-CH_2CH-$ group of the DAP part of (**2**) is sufficiently (though not ideally) spaced (2–H, 3–H_A, and 3–H_B having chemical shifts of δ 4.16, 3.59, and 3.38, respectively, in neutral D_2O)[16] to allow a stereochemical investigation into the mechanism by means of ^{2}H-labelled precursors and ^{2}H n.m.r. spectroscopy.

A prerequisite condition for (**2**) to be a suitable object for study was that L-serine be (as expected) the biosynthetic precursor of the DAP moiety in (**2**), and, very importantly, that the α-hydrogen of L-DAP, after its formation from serine, be retained in the further metabolic processes leading to (**2**). To settle these questions, L- and D-[3-$^2H_2$]serine,[17,18] and L- and D-[2,3-$^2H_3$]DAP[19,21] were prepared and administered separately to growing cultures of *S. collinus* Lindenbein. The DAP-derived metabolite (**2**) was isolated, purified, and crystallised as described previously.[16] The sites and extents of ^{2}H-enrichment of the obtained samples of (**2**) were determined by ^{2}H n.m.r. spectroscopy at 38.39 MHz [*ca.* 80 mg of (**2**) in *ca.* 2.5 ml of deuterium-depleted water; pH 5; 25 °C] (Figure 1). Both L-serine and L-DAP were found to be incorporated into (**2**) as judged from the degree of deuterium enrichment at C-3 [enrichment 0.7 and 4.0%, respectively, calculated by integration and comparison with a known internal amount of $(CD_3)_2SO$].* The D-enantiomers, however, were not incorporated, at least not to a detectable extent (enrichment less than 0.05% above natural abundance), confirming that L-serine is indeed the biosynthetic origin of L-DAP in (**2**).

It was gratifying to find that the ^{2}H n.m.r. spectrum of (**2**) derived from fermentation in the presence of L-[2,3-$^2H_3$]DAP (Figure 1, spectrum b) clearly showed at δ 4.2 the conservation of the α-deuteron of L-DAP (*ca.* 80% relative to the β-deuterons), confirming the presumption that, in principle, the mechanism of the transformation of L-serine into L-DAP can be studied in (**2**). Moreover, it is directly clear that mechanisms such as advocated by Reinbothe,[6] implying change of oxidation level at the β-carbon of serine (Scheme 1, pathway A), cannot be correct since it is evident from the perfectly symmetrical shape of the ^{2}H n.m.r. spectrum (Figure 1, spectrum a; Figure 2, spectrum c) which is centred around δ 3.5 (whereas 3-^2H_A and 3-^2H_B have chemical shifts of δ 3.6 and δ 3.4, respectively), that both β-deuterons of serine are retained at C-3 of the DAP moiety of (**2**).

To gain insight into the mechanism of the biosynthesis of DAP from serine, knowledge of the fate of the α-hydrogen of serine is of crucial importance. Therefore, D,L-[2-^{2}]serine was prepared[22] and fed to *S. collinus* Lindenbein. In contrast to the result of feeding β-labelled serine, no trace of incorporation of ^{2}H-label into (**2**) was found. Also, in a control feeding experiment with a 1:1 mixture of D,L-[2-^{2}H]- and D,L-[3-$^2H_2$]serine, it was evident from the ^{2}H n.m.r. spectrum that the α-hydrogen of serine is completely lost in the conversion into L-

* The ^{2}H n.m.r. spectra of samples of (**2**) derived from fermentation in the presence of [3-^{2}H]serines also reveal incorporation of label into the methyl group of the alanyl moiety (*via* pyruvate) (0.4%) and, further, into the CH=CH group of the fumaramoyl part of (**2**) (probably *via* the citric acid cycle) (0.2%).

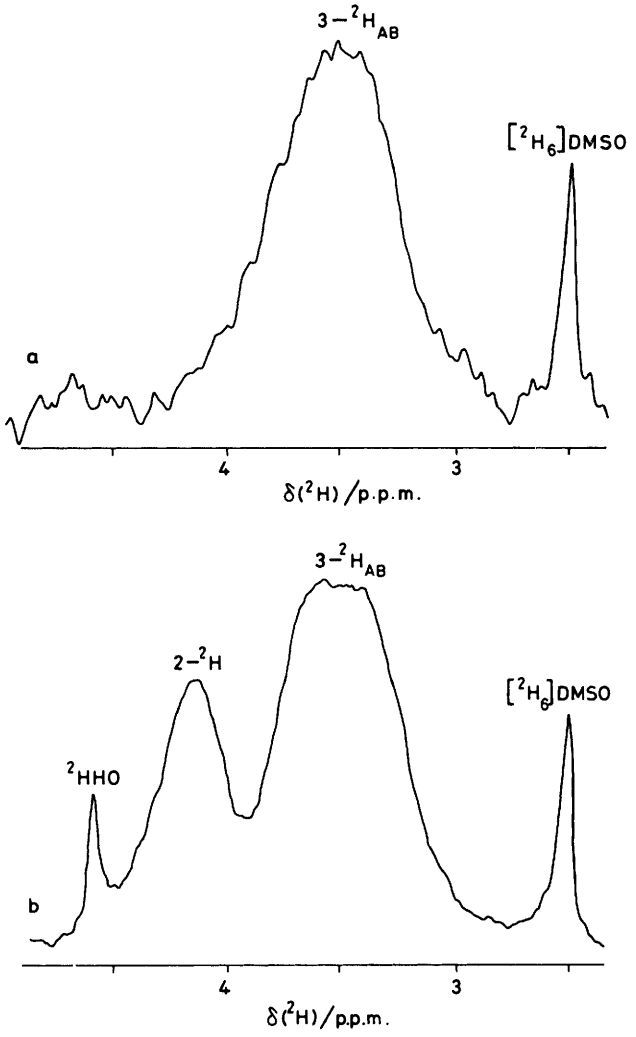


Figure 1. ^2H N.m.r. spectra of N^2-L-alanyl-N^3-fumaramoyl-L-2,3-diaminopropanoic acid (**2**) (Bruker WM-250, 38.39 MHz; spectral width 1 000 Hz, acquisition time 4.096 s; *ca.* 12 000 transients, 1.5 Hz line broadening) obtained from incorporation of (a) L-[3-$^2H_2$]serine and (b) L-[2,3-$^2H_3$]DAP

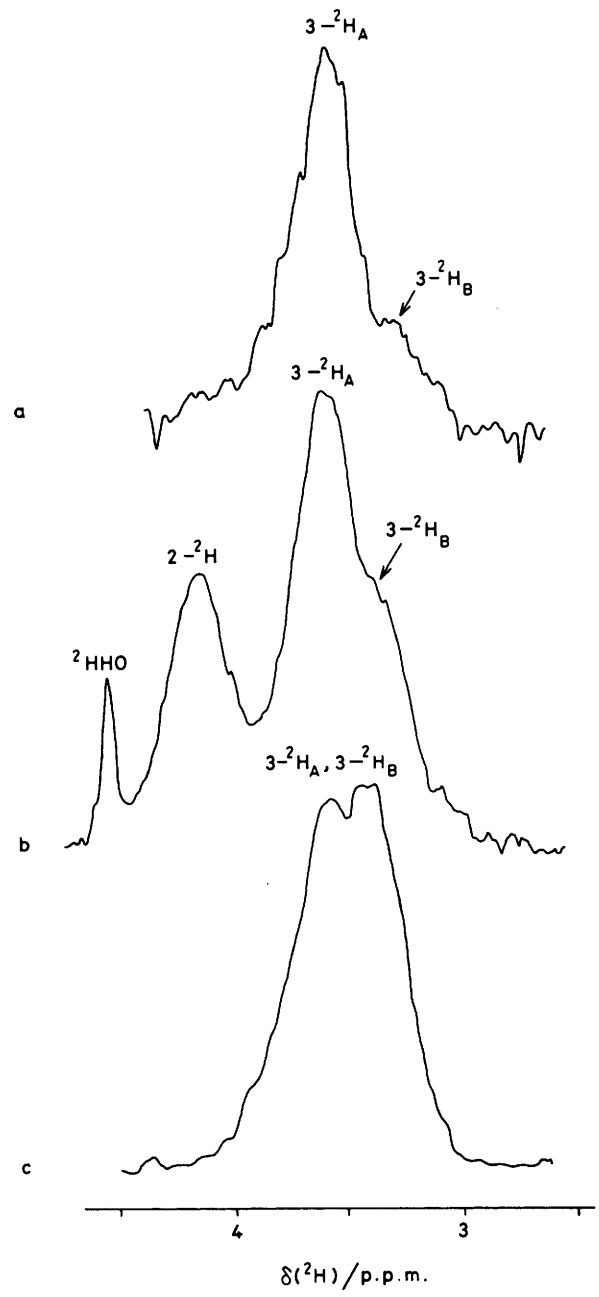


Figure 2. ^2H N.m.r. spectra of N^2-L-alanyl-N^3-fumaramoyl-L-2,3-diaminopropanoic acid (**2**) (proton broad-band decoupled; for further conditions, see Figure 1) obtained from incorporation of (a) 2*S*,3*R*-[2,3-$^2H_2$]serine, (b) 2*S*,3*R*-[2,3-$^2H_2$]DAP, and (c) L-[3-$^2H_2$]serine

DAP,† whereas both β-hydrogens are retained. The combination of these findings strongly supports the mechanistic rationale that an α,β-dehydroalanine (α-aminoacrylate) derivative is a key intermediate in the biosynthesis of L-DAP (Scheme 1, pathway D); in contrast, in all other pathways the original serine α-hydrogen is retained (Scheme 1, pathways A, B, and C).

Indeed, some other amino acids such as tryptophan[14] are also formed *via* an intermediary α-aminoacrylate derivative. The mechanism[23] involves the formation of a Schiff's base from pyridoxal phosphate (PLP) and serine, cleavage of the C_α–H bond, and subsequent elimination of OH (presumably activated) to give the PLP–aminoacrylate intermediate. Michael addition of the substituting nucleophile (indole in the case of tryptophan) at C_β, protonation at C_α, and finally hydrolysis of the product Schiff's base give a net β-replacement *via* elimination–addition. Without exception, the stereochemical course of nucleophilic β-replacement reactions of serine has been shown to be retention of configuration at C_β.[14,23] Therefore, to investigate the stereochemistry of the substitution of OH in serine by NH_2 to give DAP, and in order to affirm the intermediary role of a PLP-dependent enzyme, incorporation experiments were performed with 2*S*,3*R*-[2,3-$^2H_2$]serine and 2*S*,3*R*-[2,3-$^2H_2$]DAP. The chirally labelled serine was prepared according to the procedure of Slieker and Benkovic[24] starting with methyl (*E*)-[2,3-$^2H_2$]acrylate, followed by resolution of the *N*-acetyl derivative.[18] Mass spectral analysis of the intermediates, and 250 MHz ^1H n.m.r. spectra of the deuteriated serine at pD 12.5, indicated a diastereoisomeric purity at C_β of *ca.* 85%. The specifically deuteriated DAP was obtained by Schmidt degradation[19] of a sample of L-aspartic acid which had been prepared by the prolonged action of *Proteus vulgaris*

† Similarly, C_α of the alanyl moiety, and the CH=CH group of the fumaramoyl part of (**2**) are not labelled with ^2H.

on the ammonium salt of fumaric acid in D_2O.[25] According to the ^2H n.m.r. spectrum in D_2O at pD 12.5 (internal[$^2H_5$]-pyridine as a standard), the obtained aspartic acid contained a considerable amount of ^2H-label at C_α (38%), while the β_1- and β_2-position (3*R* and 3*S*, respectively) were enriched with ^2H-label to 72 and 8%, respectively.* ^2H N.m.r. analysis of the derived L-DAP revealed a deuterium content of 37% at C_α and 76% at C_β (the deuterons at C_β of DAP are not resolved in the ^2H n.m.r. spectrum). In view of the conservation of almost all deuterium present and of the overwhelming evidence of retention of configuration in the Schmidt degradation (and related reactions),[26] we may assume with great confidence that the distribution of ^2H-label in this sample of DAP is more or less identical with that in the corresponding L-Asp. Feeding of 2*S*,3*R*-[2,3-$^2H_2$]serine and of 2*S*,3*R*-[2,3-$^2H_2$]DAP in parallel experiments to identical cultures of *S. collinus* Lindenbein gave two samples of (**2**) which were analysed by ^2H n.m.r. spectroscopy under conditions of proton broad-band decoupling. The ^2H n.m.r. spectra obtained (Figure 2, spectra a and b) show similar characteristics with respect to the chemical shift and shape of the resonances ascribed to 3-^2H_A and 3-^2H_B. In comparison with the ^2H n.m.r. spectrum (proton broad-band decoupled) of a sample of (**2**) derived from L-[3-$^2H_2$]serine (Figure 2, spectrum c), it is clear that both L-serine and L-DAP specifically enriched with ^2H at the 3*R*-position give rise to enhanced ^2H-content at the same position of (**2**) (δ 3.6, 3-^2H_A). The distribution of label over the two diastereoisotopic positions at C-3 of (**2**) in both cases is approximately the same as in the precursors. As the carbon-bonded hydrogens of amino acids are normally not involved in peptide bond formation,† the specific incorporation of ^2H-label in the 3_A-position of (**2**) by feeding 2*S*,3*R*-[2,3-$^2H_2$]DAP is an additional indication for the stereochemistry of (**2**) as depicted, *i.e.* 3-H_A corresponds to 3-*pro*-*R*, and 3-H_B to 3-*pro*-*S*.

More importantly, however, the identical labelling of (**2**) by 2*S*,3*R*-[2,3-$^2H_2$]serine and 2*S*,3*R*-[2,3-$^2H_2$]DAP proves that the stereochemical course of the conversion of L-serine into L-DAP *in vivo* must be retention of configuration, irrespective, for that matter, as to whether or not L-DAP is subsequently incorporated into (**2**) with stereochemical integrity of the carbon-bonded hydrogens.

In summary, our results demonstrate that, at least in *S. collinus* Lindenbein, L-DAP is biosynthetically derived from L-serine by a process in which water (or acetate, if *O*-acetylserine functions as an intermediate) is eliminated and ammonia is added, with retention of configuration at C-2 and at C-3. This finding is in accord with the mechanism of PLP-dependent nucleophilic β-replacement reactions of serine.

Experimental

^1H N.m.r. spectra at 250 MHz in D_2O (99.8%; Aldrich) and ^2H n.m.r. spectra at 38.39 MHz in deuterium-depleted H_2O (%^2H = natural abundance × 0.0033; Aldrich) were recorded with a Bruker model WM-250 spectrometer; chemical shifts are reported in p.p.m. (δ) downfield from $SiMe_4$ (δ 0 p.p.m.) with [$^2H_6$]dimethyl sulphoxide ([$^2H_6$]DMSO) or [$^2H_5$]pyridine as internal reference. Mass spectral analysis of labelled compounds was performed with a Variant Mat CH 5 DF spectrometer or a Finnigan 4021 instrument.

All broth and slants were sterilised at 121 °C for 30 min prior to inoculation. *Streptomyces collinus* Lindenbein (CBS 718.79) was obtained from Centraal Bureau Schimmelcultures, Baarn, The Netherlands, and maintained at 4 °C on malt extract agar [malt extract (Oxoid L39; 15 g), bacteriological peptone (Oxoid L34; 10 g), NaCl (5 g), agar (Oxoid L11; 20 g), and tapwater (1 l)].

Incorporation of ^2H*-Labelled Precursors and Recording of* ^2H *N.m.r. Spectra.*—*S. collinus* Lindenbein (CBS 718.79), maintained on malt extract agar, was inoculated into 4 cotton-stoppered 25-ml Erlenmeyer flasks, each containing sterilised medium (6.5 ml, adjusted to pH 8 with 2M-NaOH) of the following composition: malt extract (Oxoid L39; 15 g), bacteriological peptone (Oxoid L34; 10 g), NaCl (5 g), and tapwater (1 l). After incubation for 2 d at 28 °C in a rotary shaker, the cultures were used to inoculate nine 500-ml baffled conical flasks with wadding closure, each containing fermentation broth (125 ml) of the above composition. After incubation for 18 h in a rotary shaker (New Brunswick model G-24; 300 r.p.m.; 3.8 cm eccentricity) all cultures were combined in a sterile 2-l flask containing one third of the total amount of labelled precursor to be fed [dissolved in sterile medium (25 ml), pH adjusted to 7]. After the mixture had been partitioned again equally over the nine fermentation flasks, the fermentation was continued. This procedure was repeated at 25 and at 32 h after the start of the incubation. In this way, labelled serines were administered in 3 portions of *ca.* 50 mg each, and labelled DAP in 3 portions of *ca.* 25 mg each. After 48 h total incubation time, the combined contents of the fermentation flasks were centrifuged (10 min at 11 000 r.p.m.) and the supernatant was filtered and processed as described before.[16]

The recrystallised and vacuum-dried metabolite (**2**) (on average, 80—90 mg were obtained from one fermentation run) was dissolved in a small amount of deuterium-depleted water to exchange all *O*- and *N*-bonded natural abundance deuterium (if necessary, the pH was adjusted to 5 with 1M-NaOH), the solution evaporated to dryness, and the residue redissolved in deuterium-depleted water (2.5 ml). After addition of a known amount of a 0.01M stock solution of [$^2H_6$]DMSO (99.9%; Aldrich) in deuterium-depleted water, the ^2H n.m.r. spectrum of (**2**) was measured at 38.39 MHz under the conditions specified in Figure 1 and 2. Enrichments were calculated by integration and comparison with the added internal reference.

Preparation of ^2H*-Labelled Serines.*—(*a*) D,L-[2-^2H]*Serine* was prepared exactly as described in the literature.[22]

(*b*) L- *and* D-[3-$^2H_2$]*Serine.* The procedure of King[17] was applied to the preparation of D,L-[3-$^2H_2$]serine starting with diethyl acetamidomalonate (Aldrich) and [$^2H_2$]formaldehyde (Merck, Sharp, and Dohme). Resolution of the *N*-acetyl derivative was achieved with acylase I from porcine kidneys (Serva) using published methods.[18]

(*c*) 2S,3R-[2,3-$^2H_2$]*Serine.* The method of Slieker and Benkovic[24] was followed to prepare (2*S*,3*R*)/(2*R*,3*S*)-[2,3-$^2H_2$]serine which was converted into the *N*-acetyl derivative and resolved into the antipodes.[18] Mass spectral analysis of the

* Assignment of β_1-^2H (3*R*) and β_2-^2H (3*S*) was made according to M. Kainosho and K. Ajisaka, *J. Am. Chem. Soc.*, 1975, **97**, 5630. Deuterium can be incorporated at the α-position of aspartic acid by reversible transamination (*cf.* ref. 25). The small amount of ^2H-label at the β_2-position is probably a consequence of other metabolic pathways in the micro-organism, as label at β_2 (and at α) is completely absent in a sample of L-aspartic acid prepared by the action of pure aspartase on ammonium fumarate in D_2O.

† The stereochemical integrity of the carbon-bonded hydrogens of DAP in the biosynthetic conversion into (**2**) was proven by chemical synthesis of some N^2,N^3-diacylated derivatives of 2*S*,3*R*-[2,3-$^2H_2$]DAP. They gave ^2H n.m.r. spectra very similar to the ^2H n.m.r. spectrum of (**2**) (Figure 2, spectrum b) derived from incorporation of 2*S*,3*R*-[2,3-$^2H_2$]DAP (showing in addition that the distribution of ^2H-label over the two diastereotopic positions at C-3 of (**2**) is indeed 9:1 as estimated).

synthetic precursors of labelled serine indicated that approximately 10% was non-deuteriated, 29% was monodeuteriated (probably equally partitioned over the α- and β-position), and 61% was α,β-dideuteriated (mostly due to incomplete $^2H_2$-incorporation at the Pd-catalysed deuteriation of the starting material). The total serine pool bore 76% ^2H at C-2 and 76% ^2H at C-3. Integration of the ^1H resonances (250 MHz; NaOD–D_2O, pD *ca.* 12.5) ascribed to the diastereotopic serine β-hydrogens,[13,24] gave a ratio of *hydrogen* at C-3 in the *pro-S* position (δ 3.65) to that in the *pro-R* position (δ 3.71) of 85:15 for the deuterium-labelled serine, which means an 85% diastereotopic purity of 2*S*,3*R*-[2,3-$^2H_2$]serine.

Preparation of ^{2}H-*Labelled* 2,3-*Diaminopropanoic Acids*.—(a) L- *and* D-2,3-*Diamino*[2,3-$^2H_3$]*propanoic acid*. According to the procedure of Blomquist *et al.*,[20] D,L-[2,3-$^2H_3$]aspartic acid was prepared in 52% yield from [$^2H_4$]acetic acid (99%; Merck) and diethyl phthalimidomalonate; δ (^{2}H) (38.39 MHz; H_2O–NaOH, pH *c.a.* 12.5; standard [$^2H_5$]pyridine) 2.3 [0.96 ^{2}H, 3-^{2}H (*pro-S*)], 2.7 [0.96 ^{2}H, 3-^{2}H (*pro-R*)], and 3.6 (0.94 ^{2}H, 2-^{2}H). Schmidt degradation with NaN_3–H_2SO_4–SO_3 according to Rao[19] gave D,L-[2,3-$^2H_3$]DAP in 75% yield; δ (^{2}H) (38.39 MHz; H_2O–HCl, pH ≤ 1; standard [$^2H_5$]pyridine) 3.6 (1.90 ^{2}H, 3-^{2}H) and 4.5 (0.94 ^{2}H, 2-^{2}H). The dibenzoyl derivative was prepared and resolved as described[21] to yield, after hydrolysis, L- and D-[2,3-$^2H_3$]DAP in 37 and 32% yield, respectively.

(b) 2S,3R-2,3-*Diamino*[2,3-$^2H_2$]*propanoic Acid*. The ammonium salt of fumaric acid was incubated at 37 °C in D_2O (and some toluene) with a whole-cell suspension of *Proteus vulgaris* during 48 h (instead of *ca.* 1 h as usually applied) to effect, besides specific labelling of the 3-*R* position, a high incorporation of ^{2}H at C-2 of the produced 2*S*,3*R*-[2,3-$^2H_2$]aspartic acid;[25] δ (^{2}H) [for conditions see (a) above] 2.3 (0.08 ^{2}H, 3*S*-^{2}H), 2.7 (0.72 ^{2}H, 3*R*-^{2}H), and 3.6 (0.38 ^{2}H, 2-^{2}H). Schmidt degradation[19] gave 2*S*,3*R*-[2,3-$^2H_2$]DAP which according to ^{2}H n.m.r. analysis [see (a)] revealed 37% ^{2}H at C-2 and 76% ^{2}H at C-3 (not resolved). In view of the retention of ^{2}H and of the stereochemical integrity of the Schmidt degradation, the ratio of deuterium at C-3 in the *R*- and *S*-position is assumed to be the same as in the precursor aspartic acid, *i.e.* 9:1.

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