Synthesis of Selectively Multi-Labelled Histidines with Stable Isotopes and Chiral Synthesis of L-Histidine from L-Aspartic Acid

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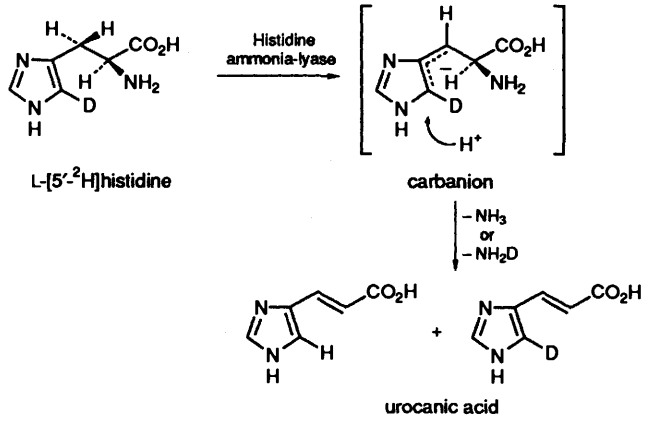
An efficient and concise synthesis of three types of multi-labelled histidines with stable isotopes to be used for investigating pharmacokinetics and enzymic reaction mechanisms *in vivo* is described. Selective deuteriation at C-3 and C-5 of DL-[5-^{15}N]diamino acid **4** was achieved by hydrogen exchange to give DL-[3,3,5,5-$^{2}H_4$,5-^{15}N]diamino acid **5**. The imidazole ring was constructed by heating of compound **5** with NaSC^{15}N in D_2O to give labelled 2'-mercapto-DL-histidine **6**, which was oxidized at C-2' to give the desired L-[3,3,5'-$^{2}H_3$,1',3'-$^{15}N_2$]histidine L-**7** after enzymic resolution. To replace deuterium at C-5' with hydrogen, the labelled histidine **7** was heated in water (pH 5.0) at 180 °C, and subsequent enzymic resolution gave L-[3,3-$^{2}H_2$,1',3'-$^{15}N_2$]histidine L-**8**. A similar sequence of reactions carried out on the diamino acid **5** with KS^{13}C^{15}N gave DL-[2'-^{13}C,3,3,5'-$^{2}H_3$,1',3'-$^{15}N_2$]histidine **7**-^{13}C. Deuteriation at C-2 and C-2' of **7**-^{13}C with DCl–D_2O (pD 5.0) at 180 °C and subsequent back-exchange of deuterium at C-2' with water (pH 7.0) at 120 °C gave DL-[2'-^{13}C,2,3,3,5'-$^{2}H_4$,1',3'-$^{15}N_2$]histidine **10**. Synthesis of optically pure L-histidine starting from L-aspartic acid is also described. The optical purity of the synthesized L-histidine was estimated to be 93.8% (e.e.).

The enzymic reaction mechanisms catalysed by mammalian histidine ammonia-lyases, both *in vitro* and *in vivo*, are poorly understood. Detailed studies of L-histidine metabolism have recently been the subject of biochemical and clinical investigations in connection with patients with typical and atypical histidinaemia caused by histidine ammonia-lyase deficiency.[1–4]

Histidine ammonia-lyase (EC 4.3.1.3) catalyses β-elimination of ammonia from L-histidine to produce (*E*)-urocanic acid.[5] We have recently demonstrated direct evidence for a stepwise mechanism *via* a carbanion intermediate in the reaction of L-histidine to urocanic acid *in vitro* catalysed by histidine ammonia-lyase from *Pseudomonas fluorescens* (Scheme 1).[6–8]



Scheme 1

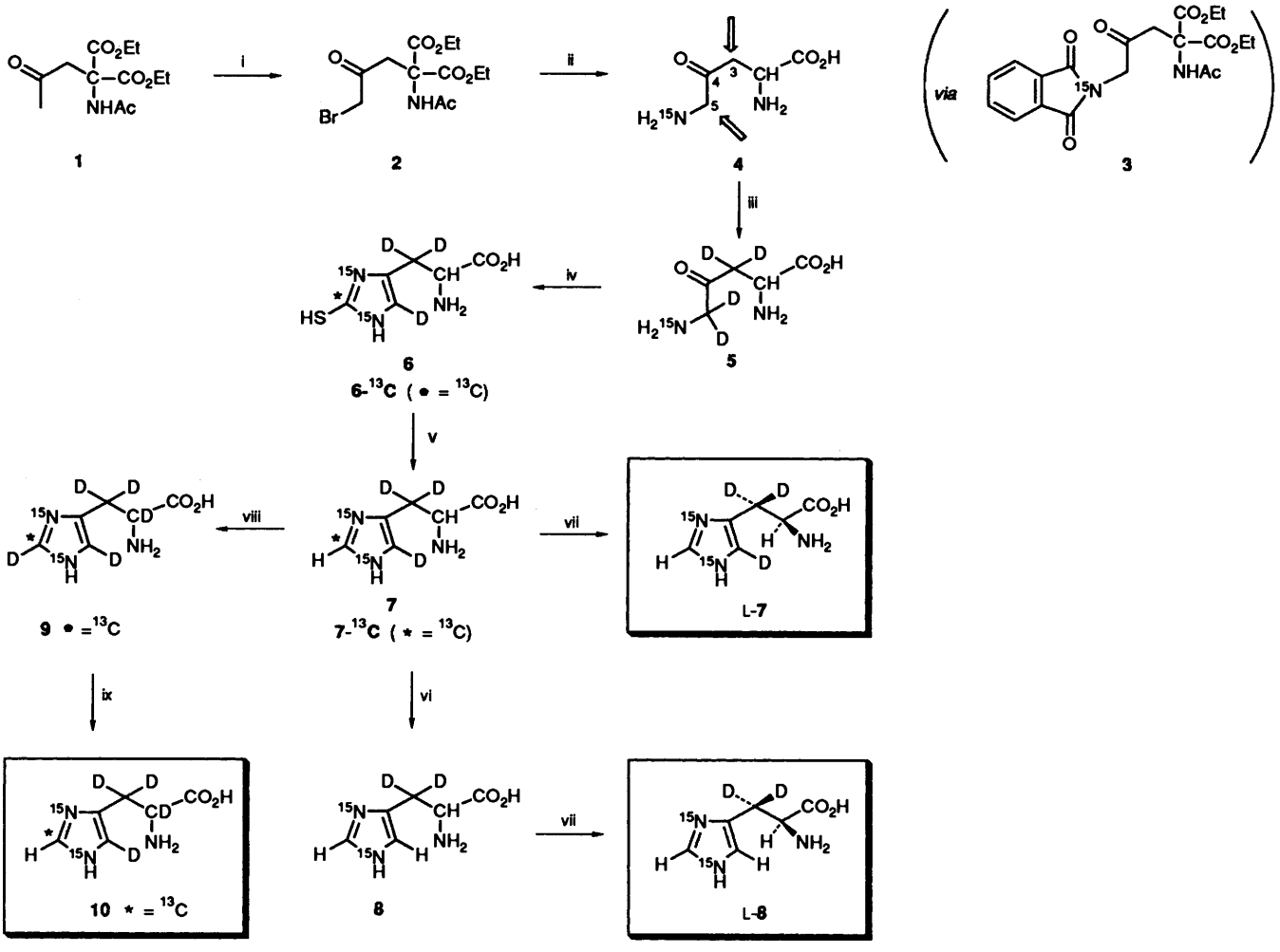
The reaction mechanism was rationalized based upon the observation that enzyme-catalysed hydrogen exchange occurred at C-5' of the imidazole ring in the reaction of labelled L-histidine (L-[5'-^{2}H]histidine) with the enzyme. This finding led to our interest in investigating the enzymic reaction mechanism of L-histidine metabolism *in vivo*. We then required, as a mechanistic probe, multi-labelled histidine containing deuterium at C-5' with three or more additional stable isotope labels.

Multi-labelled L-histidine with stable isotopes finds another field of application in the investigation of pharmacokinetics of L-histidine in patients. For the labelled L-histidine to serve as a biological internal standard, the label has to be chemically and metabolically inert to the conditions employed. Several methods have been reported for the synthesis of labelled histidine with stable isotopes.[9–11] These methods, however, do not provide suitable compounds that satisfy the requirement for biochemical and pharmacokinetic studies. In the present study, we have developed a general method that allows for the synthesis of any combination of selectively ^{13}C-, ^{15}N- and ^{2}H-labelled histidines from readily available labelled materials. Three types of multi-labelled histidines containing at least four stable isotopes with high isotopic purity were synthesized in order to allow us to investigate the pharmacokinetics of L-histidine and the reaction mechanisms catalysed by histidine ammonia-lyase *in vivo*. In addition, this paper describes the synthesis of optically pure L-histidine starting from L-aspartic acid.

Results and Discussion

Synthesis of Selectively Multi-labelled Histidines with Stable Isotopes.—Successful application of stable isotope methodology to biochemical and clinical investigations is always dependent upon the availability of compounds labelled at predesigned positions which are often obtained only at great expense or after lengthy synthetic sequences. In our previous communication,[12] we described an efficient and concise 5-step synthesis of multi-labelled DL-histidine involving the selective deuteriation at the C-3 methylene and the C-5' methine. The synthetic route also offers a convenient method of introducing stable isotopes (^{15}N and/or ^{13}C) during the construction of the imidazole ring of histidine.

In an attempt to develop an efficient route for the introduction of deuterium into C-5' and C-3 in the histidine molecule, selective deuteriation at C-3 and C-5 of DL-2,5-diamino-4-oxopentanoic acid **4** was a prerequisite (Scheme 2).



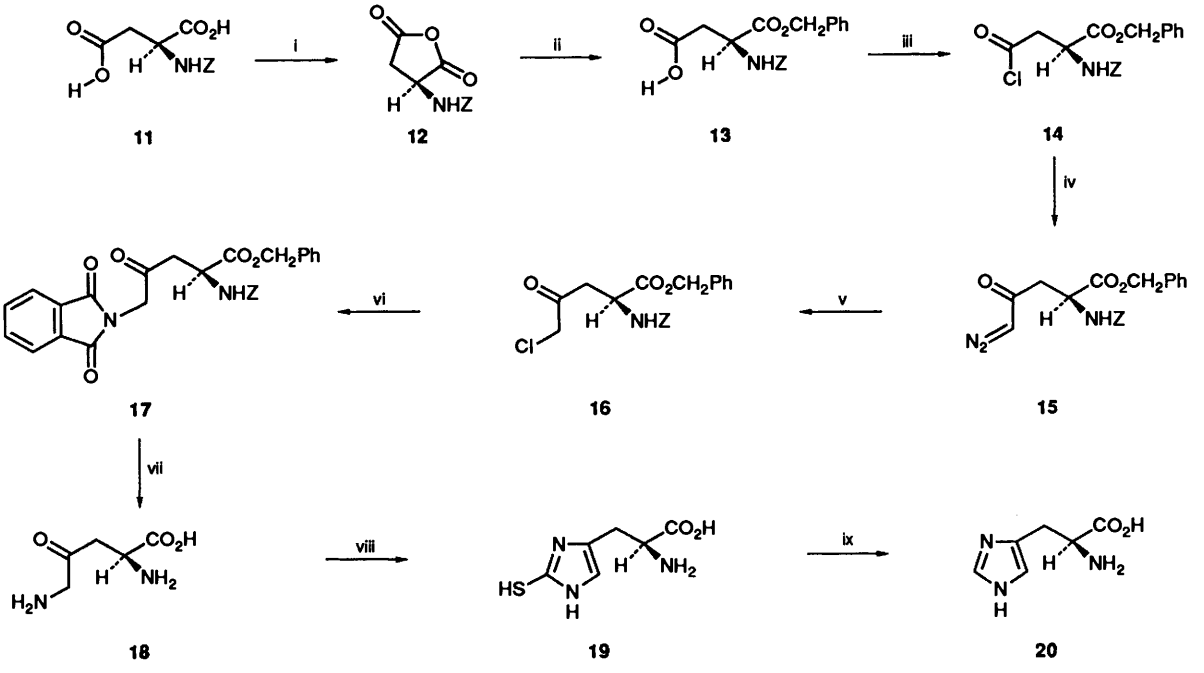
Scheme 2 *Reagents and conditions:* i, Br_2; ii, potassium [^{15}N]phthalimide (Gabriel synthesis); iii, D_2O, 80 °C; iv, NaSC^{15}N (KS^{13}C^{15}N), D_2O, 95 °C; v, $Fe_2(SO_4)_3$, H_2O, 95 °C; vi, H_2O, 180 °C; vii, resolution; viii, D_2O, 180 °C; ix, H_2O, 120 °C

The synthesis was started with the preparation of DL-[5-*amino*-^{15}N]-2,5-diamino-4-oxopentanoic acid **4** *via* a Gabriel synthesis. Treatment of the bromomethyl ketone **2**[13] with potassium [^{15}N]phthalimide (99.5 atom%) in anhydrous dimethylformamide (DMF) gave [^{15}N]phthalimido compound **3** in 62% yield. Hydrolysis of ester **3** with 37% HCl under reflux gave DL-[^{15}N]diamino acid **4** in 78% yield. Two active methylenes at C-3 and C-5 of compound **4** were easily deuteriated by hydrogen exchange. The DL-[^{15}N]diamino acid **4** was heated twice in D_2O (99.75 atom%) to 80 °C for 12 h in a sealed tube under nitrogen to give DL-[3,3,5,5-$^2H_4$,5-*amino*-^{15}N]diamino acid **5** quantitatively as needles. ^{1}H NMR spectroscopy revealed that approximately 90% deuteriation had occurred at C-3 (δ 3.28 in D_2O) and 60% at C-5 (δ 4.18) after a reaction time of 5 h. Termination of the reaction after 24 h led to complete hydrogen exchange with deuterium at both C-3 and C-5. The C-2 hydrogen (δ 4.11 in D_2O) was essentially unaffected under the reaction conditions employed.

The imidazole ring was constructed[14] by heating of the labelled DL-diamino acid **5** with dry NaSC^{15}N, prepared from $^{15}NH_4NO_3$ (99.5 atom%), in D_2O at 85–95 °C for 2.5 h. The reaction mixture was then adjusted to pD 5.0 with a saturated solution of NaOAc in D_2O to give the 2′-mercapto-DL-histidine **6** in 61% yield. The labelled 2′-mercaptohistidine **6** was oxidised with aq. $Fe_2(SO_4)_3$ at 95 °C for 1 h to give DL-[3,3,5′-$^2H_3$,1′,3′-$^{15}N_2$]histidine **7** as quadrilateral plates in 86% yield. The resolution of labelled DL-histidine **7** was carried out by the stereospecific hydrolysis of its *N*-acetyl compound with hog renal acylase I at 37 °C for 72 h to give the L-isomer L-**7**. The isotopic purity of the [$^2H_3$, $^{15}N_2$]-form was calculated to be 96.5 atom%, based upon the ion intensities in the region of the molecular ions of respective gas chromatographic derivatives, *i.e.*, $^{\alpha}$*N*-(trifluoroacetyl)-^{im}*N*-(ethoxycarbonyl)-L-histidine butyl ester,[15–17] measured by GC-MS.

Treatment of unlabelled L-histidine with D_2O (pD 5) at 120 °C for 2 h or at 80 °C for 12 h provided selective deuteriation at C-2′ of the imidazole ring. When unlabelled L-histidine was treated with DCl–D_2O (pD 5) at 180 °C for 15 h, hydrogens at C-2′, C-5′ (the imidazole ring) and C-2 (the side chain) were completely exchanged with deuteriums with concomitant racemization at C-2 to give DL-[2,2′,5′-$^2H_3$]-histidine.[8,12] The reaction conditions employed left the hydrogen at C-3 unaffected. For the replacement of deuterium at C-5′ with hydrogen, DL-[3,3,5′-$^2H_3$,1′,3′-$^{15}N_2$]histidine **7** was then heated twice in water (pH 5.0) at 180 °C for 12 h and the subsequent enzymic hydrolysis of its *N*-acetyl compound gave L-[3,3-$^2H_2$,1′,3′-$^{15}N_2$]histidine L-**8**. The isotopic purity of the [$^2H_2$, $^{15}N_2$]-form was 97.3 atom%. Optical purity of the labelled L-histidines L-**7** and L-**8** was determined to be >99.9% by high-performance liquid chromatography (HPLC) with a chiral stationary phase.

A similar sequence of reactions carried out on the [3,3,5,5-$^2H_4$,5-*amino*-^{15}N]-2,5-diamino-4-oxopentanoic acid **5** with KS^{13}C^{15}N, prepared from K^{13}C^{15}N (99 atom% for ^{13}C and 99.5 atom% for ^{15}N), gave 2′-^{13}C-labelled DL-histidine, *i.e.*, DL-[2′-^{13}C,3,3,5′-$^2H_3$,1′,3′-$^{15}N_2$]histidine **7**-^{13}**C**. Treatment of **7**-



Scheme 3 *Reagents and conditions:* i, Ac_2O, 100 °C; ii, $PhCH_2OH$, 90 °C; iii, $SOCl_2$, 40 °C; iv, CH_2N_2, −78 °C; v, HCl (gas), 0 °C; vi, potassium phthalimide, 40 °C; vii, 6 mol dm^{-3} HCl, 80 °C; viii, NaSCN, 95 °C; ix, $Fe_2(SO_4)_3$, 95 °C

^{13}C with DCl–D_2O (pD 5.0) at 180 °C for 12 h in a sealed tube resulted in the quantitative deuteriation at C-2 and C-2′ (ring) to give DL-[2′-^{13}C,2,3,3,2′,5′-$^{2}H_5$,1′,3′-$^{15}N_2$]histidine **9** in ~80% yield. Selective back-exchange of deuterium at C-2′ of the perdeuteriated histidine **9** with water (pH 7.0) at 120 °C for 2 h gave DL-[2′-^{13}C,2,3,3,5′-$^{2}H_4$,1′,3′-$^{15}N_2$]histidine **10** in 95% yield (isotopic purity as [$^{2}H_4$, $^{15}N_2$, ^{13}C]-form, 95.2 atom%).

The present route provides an efficient and concise synthesis of multi-labelled L-histidines containing at least four stable isotopes with high isotopic purity. L-[3,3,5′-$^{2}H_3$,1′,3′-$^{15}N_2$]-Histidine L-**7** containing deuterium at C-5′ in the imidazole ring will provide useful information on the mechanistic aspects of L-histidine metabolism *in vivo*. Deuterium at C-3 of L-[3,3-$^{2}H_2$,1′,3′-$^{15}N_2$]histidine L-**8** is chemically inert and the labelled histidine L-**8** can be used as a biological internal standard for investigation of the pharmacokinetic behaviour of L-histidine in patients with histidinaemia. DL-[2′-^{13}C,2,3,3,5′-$^{2}H_4$,1′,3′-$^{15}N_2$]-Histidine **10** showed a mass difference of 3 a.m.u. from L-[3,3-$^{2}H_2$,1′,3′-$^{15}N_2$]histidine L-**8** and is appropriate for use as an analytical internal standard in the assay of unlabelled L-histidine (endogenous) and labelled L-histidine L-**8** in biological fluids by stable isotope dilution mass spectrometry.

Synthesis of L-*Histidine from* L-*Aspartic Acid.*—In the present synthetic sequence for multi-labelled L-histidines, there was no hydrogen exchange at C-2 of DL-diamino acid **4** or 2′-mercapto-DL-[3,3,5′-$^{2}H_3$,1′,3′-$^{15}N_2$]histidine **6** on treatment with D_2O at 95–120 °C. In addition, treatment of unlabelled L-histidine with D_2O at 120 °C afforded L-[2′-^{2}H]histidine and left the hydrogen at C-2 unaffected. This indicates that these amino acids were not racemized. These observations suggest that the present route directly provides L-histidine from L-diamino acid without resolution. This prompted us to synthesize optically pure L-histidine starting from L-aspartic acid that possesses the structural element as well as one chiral centre necessary for the construction of L-histidine (Scheme 3).

A suspension of *N*-Z-L-aspartic acid* **11**, $[\alpha]_D^{20}$ +9.8† (*c* 7, AcOH, Tokyo Kasei), in Ac_2O was heated at 100 °C for 5 min to give the aspartic anhydride **12** in 95% yield. Heating of the anhydride **12** in benzyl alcohol at 90 °C for 3.5 h gave *N*-Z-L-aspartic acid α-benzyl ester **13** in 82% yield. The ester **13** was treated with excess of $SOCl_2$ at 40 °C for 30 min to give the β-acid chloride **14**. Introduction of a methylene function into compound **14** was carried out by adding an excess of diazomethane to compound **14** in anhydrous Et_2O at −78 °C to give diazo ketone **15**,[18] and subsequent treatment of diazo ketone **15** in $CHCl_3$–Et_2O (1:1) with dry HCl gas gave chloromethyl ketone **16**[19,20] as crystals in 51% yield; $[\alpha]_D^{25}$ +15.0 (*c* 0.01, $CHCl_3$). Introduction of the amino nitrogen functionality leading to the imidazole nitrogen at N-1′ of L-histidine was achieved *via* another Gabriel synthesis. The chloromethyl ketone **16** was treated with potassium phthalimide in anhydrous DMF to give the phthalimido compound **17**, $[\alpha]_D^{25}$ +18.3 (*c* 0.01, $CHCl_3$), in 59% yield. Hydrolysis of compound **17** with 6 mol dm^{-3} HCl at 80 °C for 20 h gave hygroscopic L-diamino acid **18** almost quantitatively, which did not crystallize as seen in the racemic compound.

Conversion of L-diamino acid **18** into L-histidine **20** was carried out according to the procedure for the preparation of racemic labelled histidine as described above. Heating of compound **18** with NaSCN gave 2′-mercapto-L-histidine **19** in 73% yield, $[\alpha]_D^{20}$ −10.0 (lit.,[14] −9.5). Subsequent oxidation of compound **19** with $Fe_2(SO_4)_3$ gave L-histidine **20** as crystals in 85% yield. The synthesized L-histidine **20** was identical with the natural compound as confirmed by ^{1}H NMR and MS spectrometry and GC and TLC mobilities. Optical purity was estimated to be 93.8% (e.e.) as determined by HPLC with a chiral stationary phase.

Experimental

M.p.s were determined on a Yanagimoto micro melting point apparatus and are uncorrected. ^{1}H NMR spectra were determined on Varian Gemini-300 300 MHz and -90 90 MHz spectrometers and a Bruker AM-400 400 MHz spectrometer for samples in $CDCl_3$ with tetramethylsilane as internal reference. Chemical shifts of samples in D_2O are reported relative to the residual HDO peak (δ 4.75). EI mass spectra

* Z = benzyloxycarbonyl.

† New IUPAC recommendation suggests that $[\alpha]_D$-values be given in units of 10^{-1} deg cm^2 g^{-1}.

were recorded on a Hitachi M-80 mass spectrometer. Capillary gas chromatographic–mass spectrometric (GC-MS) analysis was carried out on a Shimadzu QP2000 GC-MS apparatus equipped with a data-processing system. GC-MS employed a Durabond (DB-5) fused-silica capillary column (30 m × 0.32 mm i.d., film thickness 0.1 μm; J & W Scientific Inc.). Optical rotations were measured on a JASCO DIP-360 polarimeter. HPLC for the determination of optical purity was carried out on a chiral stationary-phase column (Chiralpak WH column, 25 × 0.45 cm i.d., Daicel Chemical) in a solution of 0.25 mmol dm^{-3} $CuSO_4$ as the mobile phase. Column chromatography was performed on silica gel C-200 (74–149 μm, Wakogel). DMF, acetone, EtOH, MeOH and Et_2O were redistilled before use and all other chemicals and reagents were of analytical reagent grade and were used without further purification. Light petroleum refers to the fraction boiling in the range 30–60 °C.

Sodium [^{15}N]*Thiocyanate* ($NaSC^{15}N$).—To a mixture of CS_2 (12 cm^3) and iron(III) hydroxide (8 g, 75 mmol) in absolute MeOH (20 cm^3) was added $^{15}NH_4NO_3$ (99.5 atom%; 5.0 g, 61.7 mmol). The mixture was stirred in a closed bottle at room temperature. Sodium hydroxide (5 g, 125 mmol) was added in 10 portions at 2 h intervals for 18 h. After the mixture had been stirred for an additional 24 h, it was diluted with water (200 cm^3) and centrifuged. The precipitate was extracted with water (100 cm^3 each) several times and then centrifuged. The combined supernatant was saturated with H_2S gas at room temperature to remove the excess of iron(III) ion. After filtration the solution was acidified with 3.7% HCl (pH 3) and heated to boiling for 10 min to remove the excess of H_2S. The solution was neutralized with 5% aq. NaOH (pH 8) and then evaporated to dryness under reduced pressure. The residue was extracted four times with absolute EtOH (100 cm^3 each). The combined solution was evaporated to dryness. The resulting material was dissolved in absolute EtOH (50 cm^3) and then filtered. The solution was evaporated to dryness to give $NaSC^{15}N$ (3.8 g, 75%).

Potassium [^{13}C,^{15}N]*Thiocyanate* ($KS^{13}C^{15}N$).—A solution of sulfur (2.5 g, 78.1 mmol) and $K^{13}C^{15}N$ (^{13}C; 99 atom%, ^{15}N; 99 atom%, 2.0 g, 29.8 mmol) in anhydrous acetone (40 cm^3) was refluxed for 1 h. After cooling, the excess of cyanides was filtered off and the solution was evaporated to dryness under reduced pressure. The residue was dissolved in water (20 cm^3) and the resulting sulfur was filtered off. After the solution was evaporated to dryness again, the residue was dissolved in absolute EtOH (20 cm^3) and then filtered. The solution was evaporated to dryness to give $KS^{13}C^{15}N$ (2.3 g, 78%).

Diethyl 2-Acetamido-2-acetonylmalonate **1**.—To a solution of diethyl acetamidomalonate (72 g, 365 mmol) in NaOEt (150 cm^3) at 10–15 °C was added dropwise a solution of bromoacetone (50 g, 365 mmol) in absolute EtOH (100 cm^3). The reaction mixture was stirred for 12 h at room temperature. The resulting NaBr was then filtered off. The solution was evaporated to dryness and extracted with $CHCl_3$. The extract was washed with water and then dried over anhydrous Na_2SO_4. After removal of the solvent under reduced pressure, the residue was crystallized from Et_2O to give the crude product. Recrystallization from EtOH gave pure compound **1** (62 g, 62%) as crystals: m.p. 105 °C (lit.,[13] 104–107 °C); δ_H(300 MHz; $CDCl_3$) 4.23 (4 H, q, *J* 7.1, CO_2CH_2Me × 2), 3.73 (2 H, s, $COCH_2C$), 2.13 (3 H, s, $MeCOCH_2$), 2.01 (3 H, s, NH*Ac*) and 1.24 (6 H, t, *J* 7.1, CO_2CH_2Me × 2).

Diethyl 2-Acetamido-2-(3-bromo-2-oxopropyl)malonate **2**.—To a solution of compound **1** (85 g, 311 mmol) in AcOH (250 cm^3) at 40–45 °C was added dropwise a solution of Br_2 (45 g, 281 mmol) in AcOH (100 cm^3). After cooling, the mixture was evaporated to dryness under reduced pressure. To the residue was added water and the mixture was extracted with $CHCl_3$. The extract was washed with water and dried over anhydrous Na_2SO_4. After removal of the solvent under reduced pressure, the residue was crystallized from diisopropyl ether to give the crude product. Recrystallization from EtOH gave pure compound **2** (44 g, 40%) as crystals; m.p. 97 °C (lit.,[13] 95–96 °C); δ(300 MHz; $CDCl_3$) 4.25 (4 H, q, *J* 7.1, CO_2CH_2Me × 2), 3.92 (2 H, s, $BrCH_2$), 3.87 (2 H, s, $COCH_2C$), 2.01 (3 H, s, NH*Ac*) and 1.25 (6 H, t, *J* 7.1, CO_2CH_2Me × 2).

Diethyl 2-Acetamido-2-(2-oxo-3-[^{15}N]*phthalimidopropyl)malonate* **3**. To a solution of compound **2** (36 g, 102 mmol) in anhydrous DMF (150 cm^3) was added potassium [^{15}N]phthalimide (20 g, 107 mmol; 99.2 atom%) in four portions while the mixture was stirred at 40–45 °C for 0.5 h. After the final addition of potassium [^{15}N]phthalimide the mixture was stirred at 40–45 °C for 1 h. After the mixture had cooled, $CHCl_3$ (150 cm^3) was added and the solution was washed successively with brine (100 cm^3 × 2), 3% aq. NaOH (20 cm^3), 3% aq. HCl (10 cm^3), and brine (50 cm^3 × 2), and dried over anhydrous Na_2SO_4. After removal of the solvent under reduced pressure, the residue was crystallized from Et_2O to give the almost pure product (26.6 g, 62%). Recrystallization from Et_2O gave compound **3** as crystals; m.p. 170 °C (lit.,[13] 170–171 °C); δ(300 MHz; $CDCl_3$) 7.81 (4 H, m, C_6H_4), 4.48 (2 H, s, $^{15}NCH_2$), 4.25 (4 H, q, *J* 7.1, CO_2CH_2Me × 2), 3.84 (2 H, s, $COCH_2C$), 2.07 (3 H, s, NH*Ac*) and 1.25 (6 H, t, *J* 7.1, CO_2CH_2Me × 2).

DL-[5-*Amino*-^{15}N]-2,5-*diamino*-4-*oxopentanoic Acid Dihydrochloride* **4**.—A suspension of compound **3** (37.5 g, 89.5 mmol) in 37% HCl (50 cm^3) was refluxed for 6 h. After the reaction was complete the precipitated phthalic acid was filtered off and the solution was evaporated under reduced pressure. The residue was recrystallized from aq. EtOH to give compound **4** (15.3 g, 78%) as crystals, δ(300 MHz, D_2O) 4.11 (1 H, t, *J* 5.5, 2-H), 4.18 (2 H, s, 5-H_2) and 3.28 (2 H, d, *J* 5.5, 3-H_2).

DL-[3,3,5,5-^{2}H_4,5-*Amino*-^{15}N]-2,5-*diamino*-4-*oxopentanoic Acid Dideuteriochloride* **5**.—A solution of compound **4** (15.3 g, 69.5 mmol) in D_2O (100 cm^3; 99.75 atom%) was heated at 80 °C for 12 h in a nitrogen-filled, sealed tube. After lyophilization the residue was redissolved in D_2O and heated again as described above. After lyophilization the product **5** (15.3 g, 97%) was used without further purification for the preparation of labelled 2′-mercapto-DL-histidine **6**. Recrystallization from aq. EtOH gave the pure compound **5**, δ(400 MHz; D_2O) 4.11 (1 H, s, 2-H). Other proton signals had completely disappeared.

DL-2′-*Mercapto*-[3,3,5′-^{2}H_3,1′,3′-^{15}N_2]*histidine* **6**.—To a solution of the labelled diamino acid **5** (11.0 g, 48.7 mmol) in D_2O (15 cm^3) at 95 °C was added dry $NaSC^{15}N$ (8.3 g, 102 mmol) in four portions at 30 min intervals. After the final addition of $NaSC^{15}N$ the reaction mixture was heated at 85 °C for 1 h. The solution was then treated with a saturated solution of NaOAc in D_2O (pD 5.0) and was left then at 0 °C for 12 h. The precipitate was collected, and washed with a small volume of water and EtOH to give the crude product **6** (5.7 g, 61%). Recrystallization from water gave the pure compound **6** δ(400 MHz; D_2O) 3.88 (1 H, s, 2-H). Other proton signals had completely disappeared.

DL-2′-*Mercapto*-[2′-^{13}C,3,3,5′-^{2}H_3,1′,3′-^{15}N_2]*histidine* **6**-^{13}C.—According to the procedure for the preparation of compound **6**, the labelled diamino acid **5** (2.9 g, 12.8 mmol) in

D_2O (2 cm^3) was allowed to react with dry $NaS^{13}C^{15}N$ (2.3 g, 27.7 mmol) to give **6**-^{13}C (1.7 g, 69%); δ(400 MHz; D_2O) 3.89 (1 H, s, 2-H). Other proton signals had completely disappeared.

DL-[3,3,5′-$^2H_3$,1′,3′-$^{15}N_2$]*Histidine* **7**.—A mixture of labelled 2′-mercaptohistidine **6** (5.7 g, 29.7 mmol) and aq. $Fe_2(SO_4)_3$ (~60 g, 150 mmol in 120 cm^3) was heated at 95 °C for 1 h. The reaction mixture was then poured into boiling water (200 cm^3), followed by treatment with $Ba(OH)_2$·$8H_2O$ (50 g, 158 mmol). The resulting precipitate was collected, and suspended in boiling water (150 cm^3), then filtered off, and the process was repeated. The combined filtrate (pH 8) was heated to boiling and neutralized to pH 7.2 with dil. H_2SO_4. After removal of $BaSO_4$, the solution was concentrated to a volume of ~50 cm^3 under reduced pressure and then filtered. After removal of the solvent under reduced pressure, the residue was recrystallized from aq. EtOH to give the labelled DL-histidine **7** (4.1 g, 86%) as quadrilateral plates, m.p. (decomp.) 285 °C; δ(400 MHz; D_2O) 7.75 (1 H, dd, *J* 8.3, 10.2, 2′-H) and 3.98 (1 H, s, 2-H). Other proton signals had completely disappeared.

DL-[2′-^{13}C,3,3,5′-$^2H_3$,1′,3′-$^{15}N_2$]*Histidine* **7**-^{13}C.—According to the procedure for the preparation of compound **7**, the ^{13}C-labelled 2′-mercaptohistidine **6**-^{13}C (1.7 g, 8.8 mmol) in water (30 cm^3) was allowed to react with $Fe_2(SO_4)_3$ (~20 g, 50 mmol) to give **7**-^{13}C (1.2 g, 85%), δ(400 MHz; D_2O) 7.75 (1 H, ddd, *J* 8.3, 10.2, 209.2, 2′-H) and 3.98 (1 H, s, 2-H). Other proton signals had completely disappeared.

DL-[3,3-$^2H_2$,1′,3′-$^{15}N_2$]*Histidine* **8**.—A solution of compound **7** (1.6 g, 10.0 mmol) in water (80 cm^3) was acidified with 37% HCl to pH 5.0 and heated at 180 °C for 12 h in a nitrogen-filled, sealed tube. After removal of the solvent under reduced pressure, the residue was redissolved in water (80 cm^3) and heated again as described above. The solution was neutralized to pH 7.2 with 1 mol dm^{-3} LiOH and evaporated to dryness. The residue was washed with abs. EtOH and recrystallized from aq. EtOH to give compound **8** (1.27 g, 80%) as quadrilateral plates, m.p. (decomp.) 285 °C; δ(400 MHz; D_2O) 7.76 (1 H, dd, *J* 8.3, 10.2, 2′-H), 7.06 (1 H, d, *J* 8.8, 5′-H) and 3.98 (1 H, s, 2-H). Other proton signals had completely disappeared.

DL-[2′-^{13}C,2,3,3,5′-$^2H_4$,1′,3′-$^{15}N_2$]*Histidine* **10**.—A solution of **7**-^{13}C (400 mg, 2.5 mmol) in D_2O (40 cm^3) was acidified with 37% DCl (99 atom%) to pD 5.0 and heated at 180 °C for 12 h in a nitrogen-filled, sealed tube to give DL-[2′-^{13}C,2,2′,3,3,5′-$^2H_5$,1′,3′-$^{15}N_2$]histidine **9**. After lyophilization the residue was redissolved in D_2O (40 cm^3) and heated again as described above. After removal of the solvent under reduced pressure, the product **9** was dissolved in water (50 cm^3) and the solution was heated at 80 °C for 12 h, neutralized to pH 7.2 with 1 mol dm^{-3} LiOH, and evaporated to dryness under reduced pressure. The residue was washed with abs. EtOH and recrystallized from aq. EtOH to give compound **10** (300 mg, 75%) as quadrilateral plates, m.p. (decomp.) 285 °C; δ(400 MHz; D_2O) 7.75 (1 H, ddd, *J* 8.3, 10.2, 209.2, 2′-H). Other proton signals had completely disappeared.

Resolution to L-**7** *and* L-**8**.—A solution of compound **7** (1.0 g, 6.3 mmol) in AcOH–Ac_2O (22 cm^3; 10:1) was heated at 80 °C for 10 min under a stream of nitrogen. After removal of the solvent at less than 50 °C under reduced pressure, the residue was taken up twice in water (20 cm^3) and the solution was evaporated to dryness each time to give the *N*-acetyl derivative of compound **7** (~98%). The almost pure material was dissolved in water (30 cm^3) and the solution was neutralized to pH 7.2 with 1 mol dm^{-3} LiOH. Hog renal acylase I was added in three portions (75 mg, 75 000 U each) at 24 h intervals, being incubated at 37 °C for 72 h. The enzymic hydrolysis was terminated by acidification with 1 mol dm^{-3} AcOH (pH 5.0), and EtOH (~50 cm^3) was added. After the protein had been filtered off with the aid of charcoal, the solution was neutralized to pH 7.2 with 1 mol dm^{-3} LiOH. The reaction mixture was purified by cation-exchange column chromatography (IRC-50, 50 mesh, 25 × 2.5 cm i.d.), followed by anion-exchange column chromatography (DOWEX I-X8, 200–400 mesh, 5 × 1.5 cm i.d.) to give the L-isomer L-**7** as the free base (400 mg, 80%), δ(400 MHz; D_2O) 7.75 (1 H, dd, *J* 8.3, 10.2, 2′-H) and 3.98 (1 H, s, 2-H). Other proton signals were absent. Optical purity (>99.9%) was determined by HPLC with a chiral stationary phase column (mobile phase 0.25 mmol dm^{-3} $CuSO_4$).

According to the procedure described above, resolution of the labelled DL-histidine **8** (1.1 g, 6.9 mmol) was carried out to give the L-isomer L-**8** as the free base (400 mg, 73%), δ(400 MHz; D_2O) 7.76 (1 H, dd, *J* 8.3, 10.2, 2′-H), 7.06 (1 H, d, *J* 8.8, 5′-H) and 3.98 (1 H, s, 2-H). Other proton signals were absent. Optical purity (>99.9%) was determined by HPLC with a chiral stationary phase column.

N-*Z*-L-*Aspartic Acid* α-*Benzyl Ester* **13**.—A suspension of *N*-Z-L-aspartic acid **11** {5.0 g, 18.7 mmol; $[\alpha]_D^{20}$ +9.8 (*c* 7, AcOH)} in Ac_2O (15 cm^3) was heated at 100 °C for 5 min and immediately cooled. To the solution was added Et_2O–light petroleum (100 cm^3; 1:4) and the resulting crystals were collected, and washed with anhydrous Et_2O (~5 cm^3) to give *N*-Z-L-aspartic anhydride **12** (4.4 g, 95%), m.p. 84–86 °C.

A solution of the anhydride (4.4 g, 17.7 mmol) in freshly distilled benzyl alcohol (3 cm^3) was heated at 90 °C for 3.5 h. After removal of the excess of benzyl alcohol under reduced pressure, Et_2O (100 cm^3) was added. The syrup, dissolved in Et_2O, was extracted with 5% aq. $NaHCO_3$ (twice) and the aq. solution was washed with Et_2O and acidified with 10% HCl. The resulting oil was extracted with $CHCl_3$, washed with water, and dried over anhydrous Na_2SO_4. After removal of the solvent under reduced pressure, the resulting solid was crystallized from hexane to give compound **13** (5.2 g, 82%).

Benzyl L-2-*Benzyloxycarbonylamino*-5-*chloro*-4-*oxopentanoate* **16**.—A solution of acid ester **13** (10.5 g, 29.4 mmol) in $SOCl_2$ (30 cm^3) was heated at 40 °C for 30 min. The excess of $SOCl_2$ was immediately evaporated off at room temperature under reduced pressure to give the acid chloride α-benzyl ester **14** as an oil.

The oily residue **14** was then dissolved in anhydrous Et_2O (60 cm^3) and cooled to −78 °C. A solution of diazomethane in Et_2O, prepared from *N*-methyl-*N*-nitrosotoluene-*p*-sulfonamide (20 g, 83 mmol), was added rapidly. The reaction mixture was kept at −78 °C for 12 h and then was gradually allowed to warm to room temperature. After removal of the solvent under reduced pressure at room temperature, the residue was crystallized from anhydrous Et_2O to give benzyl L-2-benzyloxycarbonylamino-5-diazo-4-oxopentanoate **15** quantitatively. The almost pure compound **15** was then dissolved in anhydrous $CHCl_3$–Et_2O (30 cm^3; 1:1). Hydrogen chloride gas was bubbled slowly into the solution at 0 °C for 5 min. After removal of the solvent under reduced pressure, the residue was crystallized from anhydrous Et_2O. The product was purified by column chromatography, and subsequent recrystallization from anhydrous Et_2O gave the pure product **16** (5.8 g, 51%) as crystals, δ(300 MHz; $CDCl_3$) 7.18–7.31 (10 H, m, C_6H_5 × 2), 5.09 (2 H, s, OCH_2Ph), 5.03 (2 H, s, OCH_2Ph), 4.61 (1 H, t, 2-H), 3.93 (2 H, s, 5-H_2) and 3.10–3.30 (2 H, m, 3-H_2); m.p. 107 °C (lit.,[20] 105–107 °C); $[\alpha]_D^{25}$ +15.0 (*c* 0.01, $CHCl_3$).

Benzyl L-2-*Benzyloxycarbonylamino*-4-*oxo*-5-*phthalimidopentanoate* **17**.—To a solution of compound **16** (2.0 g, 5.1 mmol)

in anhydrous DMF (15 cm^3) was added potassium phthalimide (1.0 g, 5.4 mmol) in four portions, while the mixture was stirred at 40–45 °C for 0.5 h. After the final addition of potassium phthalimide, the mixture was stirred at 40–45 °C for another 1 h. After the mixture had cooled, $CHCl_3$ (30 cm^3) was added and the solution was washed successively with brine (20 cm^3 × 2), 3% NaOH (5 cm^3), 3% HCl (1 cm^3), and brine (30 cm^3 × 2), and dried over anhydrous Na_2SO_4. After removal of the solvent under reduced pressure, the residue was crystallized from Et_2O to give the almost pure product (1.5 g, 59%). Recrystallization from MeOH–hexane (1:1) gave compound **17** as crystals: δ(300 MHz; $CDCl_3$) 7.64–7.80 (4 H, m, C_6H_4), 7.21–7.30 (10 H, m, C_6H_5 × 2), 5.07 (2 H, s, OCH_2Ph), 5.03 (2 H, s, OCH_2Ph), 4.59 (1 H, t, 2-H), 4.39 (2 H, s, 5-H_2) and 3.00–3.25 (2 H, m, 3-H_2); $[\alpha]_D^{25}$ +18.3 (*c* 0.01, $CHCl_3$).

L-2,5-*Diamino*-4-*oxopentanoic Acid Dihydrochloride* **18**.—A suspension of compound **17** (1.2 g, 2.4 mmol) in 6 mol dm^{-3} HCl (50 cm^3) was heated at 80 °C for 20 h. After cooling, the precipitated phthalic acid was filtered off and the solution was evaporated to dryness under reduced pressure to give compound **18** (~0.5 g, 95%). The clear residue was not crystallized because of its hygroscopicity but it was identical with the corresponding unlabelled racemic diamino acid **4**, in terms of TLC and ^1H NMR data. The crude product **18** was used without further purification for the preparation of 2′-mercapto-L-histidine **19**.

2′-*Mercapto*-L-*histidine* **19**.—According to the procedure for the preparation of compound **6**, the L-diamino acid **18** (~500 mg, 2.3 mmol) in water (5 cm^3) was allowed to react with NaSCN (200 mg, 2.5 mmol) to give compound **19** (315 mg, 73%), δ(90 MHz; 1 mol dm^{-3} DCl) 6.94 (1 H, s, 5′-H), 4.40 (1 H, t, *J* 7.1, 2-H) and 3.28 (2 H, d, *J* 7.1, 3-H_2); $[\alpha]_D^{25}$ −10.0 (*c* 0.01, 1 mol dm^{-3} HCl (lit.,[14] $[\alpha]_{5461}$ −9.5).

L-*Histidine* **20**.—According to the procedure for the preparation of compound **7**, 2′-mercapto-L-histidine **19** (100 mg, 0.53 mmol) in water (20 cm^3) was allowed to react with $Fe_2(SO_4)_3$ (~1.5 g, 3.8 mmol) to give compound **20** (~70 mg, 85%), δ(400 MHz; D_2O) 7.75 (1 H, s, 2′-H), 7.05 (1 H, s, 5′-H), 3.98 (1 H, dd, *J* 4.8, 8.0, 2-H), 3.22 (1 H, dd, *J* 4.8, 15.4, 3-H) and 3.11 (1 H, dd, *J* 8.0, 15.4, 3-H); MS {$^{\alpha}$*N*-(trifluoroacetyl)-^{im}*N*-(ethoxycarbonyl)-L-histidine butyl ester};[15–17] *m/z* 379 (M^+, 38) and 278 (M^+ − BuOCO, 100). Optical purity of the synthesized L-histidine was estimated to be 93.8% (e.e.) as determined by HPLC with a chiral stationary phase.

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