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Enantioselective syntheses of isotopically labelled α-amino acids Preparation of specifically ^{13}C-labelled L-lysines

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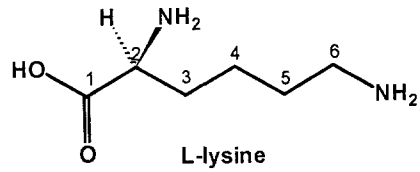
Summary. [2-^{13}C]-L-lysine, [3,4-$^{13}C_2$]-L-lysine and [5,6-$^{13}C_2$]-L-lysine are prepared from simple, commercially available, highly enriched starting materials as [2-^{13}C]-glycine, ethyl [1,2-$^{13}C_2$]-bromo acetate, and [1,2-$^{13}C_2$]-acetonitrile. The introduction of the chiral center is based on a general method starting from the bis-lactim ether of cyclo-(D-Val-Gly). The synthesis of (*2R*)-[5-^{13}C]-3,6-diethoxy-2,5-dihydro-2-isopropylpyrazine is described. The availability of our method for the preparation of specifically enriched bis-lactim ethers allows the synthesis of a great variety of site specific isotopically labelled (L- and D-) α-amino acids. Moreover, intermediate 4-[(2R,5S)-3,6-diethoxy-2,5-dihydro-2-isopropyl-5-pyrazinyl]butyronitrile is a valuable precursor in the synthesis of L-α-aminoadipic acid. The synthetic scheme in this publication makes both L-lysine and L-α-aminoadipic acid ^{13}C- or ^{15}N-labelled at any position, easily available. The isotopomers of lysine are obtained on a preparative scale in good yields, with 99% ^{13}C and high enantiomeric purity (>97% e.e.). Three isotopomers are characterized using various spectroscopic techniques, *e.g.*, ^{1}H NMR, ^{13}C NMR and Mass spectrometry.

Keywords: Amino acids – bis-lactim ether method – ^{13}C-isotope – (*2R*)-[5-^{13}C]-3,6-diethoxy-2,5-dihydro-2-isopropylpyrazine – L-α-aminoadipic acid – NMR spectroscopy – Mass spectrometry

Introduction

The experimental strategy of using isotopic substitution with stable nuclei allows the use of spectroscopic methods (NMR, FT-IR and Laser-Raman spectroscopy) in the investigation at the atomic level of otherwise unaccessible macromolecules (Lugtenburg el al., 1988). One application is the biosynthetical incorporation of specifically labelled precursors (amino acids, fatty acids, nucleotides) into fully assembled biological units (proteins, membranes, nucleic acids) and subsequently

uses the label to monitor the atomic site of interest of the macromolecule (Raap et al., 1990a). Chemical synthesis of isotopically labelled amino acids is presently the most commonly employed strategy for the production of specifically isotopically labelled proteins. For the development of specific isotope labelling as a versatile tool for protein research, it is necessary to develop synthetic routes that allow the preparation of any specifically labelled L-amino acid. Until now our group has elaborated synthetic schemes for the preparation of isotopically labelled L-tyrosine (Winkel et al., 1989), L-lysine (Raap et al., 1990b), L-tryptophan (van den Berg et al., 1988), L-glutamic acid (Cappon et al., 1991, 1992a, 1993), L-glutamine and L-proline (Cappon et al., 1992b) and L-histidine (Cappon et al., 1994). For our NMR studies on the signal-transduction mechanisms of bacteriorhodopsin (Thompson et al., 1992) and the visual pigment rhodopsin (Smith et al., 1992) we need optically pure L-lysine that is specifically mono and double labelled with ^{13}C at any carbon position. In both photoreceptors, the retinal chromophore in the active site is covalently linked to the peptide chain via the 6- (ε-)amino group of a lysine residue. For our study of the primary photoproducts of both protein pigments, detailed information about the conformation of the lysine side chain is needed. Rotational resonance (R^2) provides an NMR approach to obtain structural information from double ^{13}C-labelled samples. However, specifically ^{13}C-labelled L-lysines are not commercial available. In earlier work we published a method for the preparation of specifically ^{13}C- or ^{15}N labelled L-lysine via a synthetic scheme that allows the labelling of the C5-, C6- and N6-positions, starting from simple, highly enriched, commercial available synthons (Raap et al., 1990b). We report here the synthetic scheme (Fig. 1) which makes L-lysine ^{13}C- and ^{15}N-labelled at any position, on the gram scale available. The chiral centre is introduced in the alkylation step of the bis-lactim ether of cyclo(D-Val-Gly) (Schöllkopf, 1983). D-Valine is employed as a chiral auxiliary group, which can be removed by hydrolysis for



Starting materials for the synthesis of lysine isotopomers:

C1: [1-^{13}C]-glycine

C2: [2-^{13}C]-glycine

N2: [2-^{15}N]-glycine

C3: ethyl [1-^{13}C]-bromoacetate

C4: ethyl [2-^{13}C]-bromoacetate

C5: [2-^{13}C]-acetonitrile

C6: [1-^{13}C]-acetonitrile or potassium [^{13}C]-cyanide (Raap et al. 1990b)

N6: [^{15}N]-acetonitrile or potassium [^{15}N]-cyanide (Raap et al. 1990b)

Fig. 1. Structure of L-lysine and numbering of the positions. Commercially available isotopically labelled materials

recovery and separation from the desired product at the end of the reaction. The followed strategy has proved to be successful by the synthesis of three different lysine isotopomers: [2-^{13}C]-L-lysine (**1a**), [3,4-$^{13}C_2$]-L-lysine (**1b**) and [5,6-$^{13}C_2$]-L-lysine (**1c**). The compounds are characterized by ^{1}H- and ^{13}C-NMR spectroscopy and mass spectrometry.

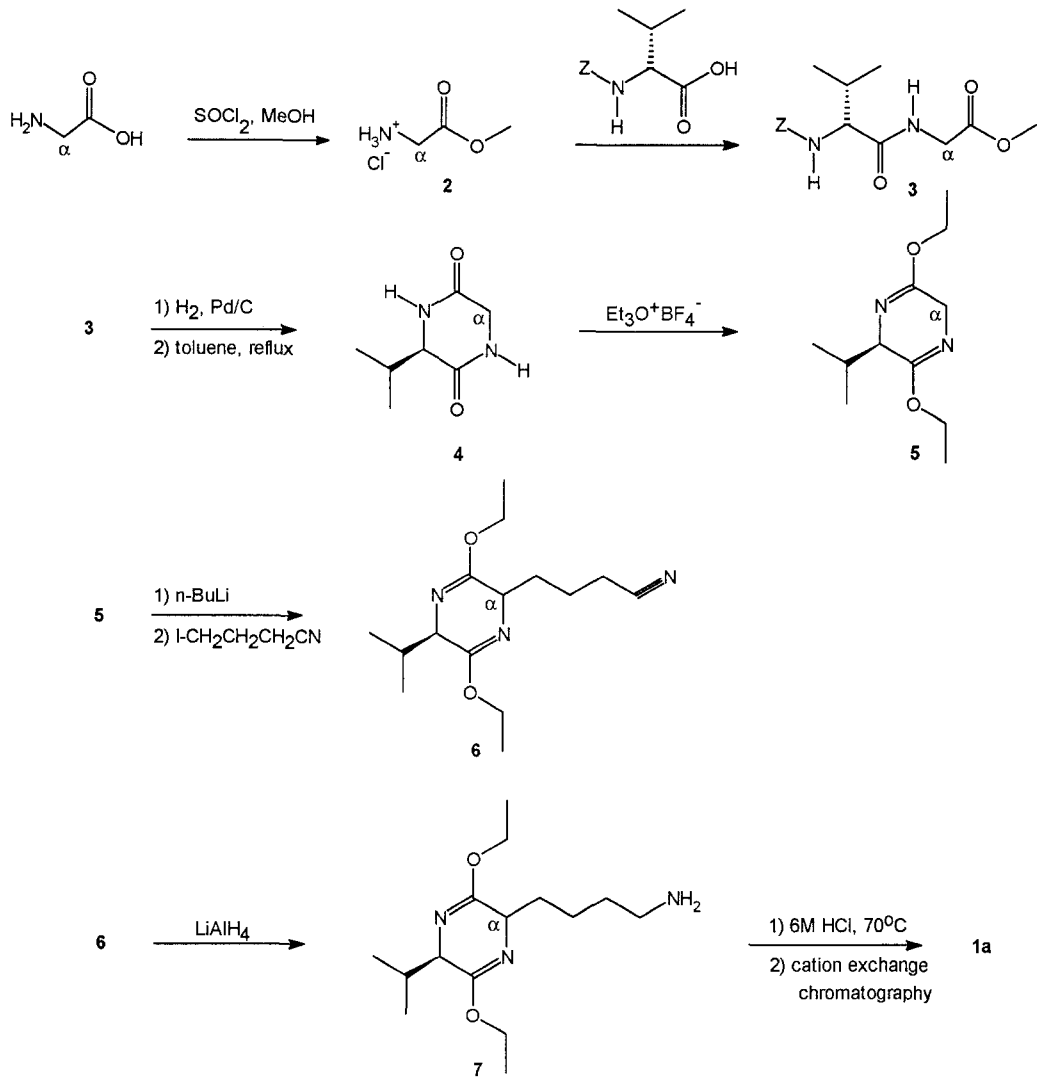
The Schöllkopf-method has also proven to be successful in the synthesis of a great variety of both L- and D-α-amino acids (Williams, 1989) and in the synthesis of specifically isotope labelled L-histidine (Cappon et al., 1994). The availability of a method for the preparation of specifically enriched bis-lactim ethers as described in this paper, offers the possibility of the labelling of other optically active α-amino acids at the 1-C, 2-C and nitrogen position and any combination of these positions.

Results

Synthesis of [2-^{13}C]-L-lysine (**1a**), *[3,4-$^{13}C_2$]-L-lysine* (**1b**) *and [5,6-$^{13}C_2$]-L-lysine* (**1c**)

For the synthesis of optically active L-lysines, labelled at the polar head group, the specifically labelled bis-lactim ethers of cyclo(D-Val-Gly) are required and labelling of the glycine residue will allow the labelling of the C1, C2 and N2 positions of L-lysine (see Scheme 1). Specifically highly ^{13}C- or ^{15}N-enriched glycines labelled at any position and combination of positions are commercial available and can function as starting material for the preparation of enriched bis-lactim ethers. For this purpose, glycine is first protected at the carboxylate function and then coupled to N-protected valine. Deprotection and ring closure affords the cyclic dipeptide, which is transformed into the bis-lactim ether by *O*-alkylation.

In the first step of the synthesis of **1a**, [2-^{13}]-glycine is esterified with thionyl chloride in methanol (98% yield; see Scheme 1). The labelled glycine methyl ester (**2**) is coupled to *N*-benzyloxycarbonyl-D-valine (Z-D-Val) using the peptide condensing reagents dicyclohexylcarbodiimide and *N*-hydroxybenzotriazole (DCC/HOBT) (92% yield). The amine function is deprotected by hydrogenation, using Pd/C as catalyst. After isolation, the product D-valine-[2-^{13}C]-glycine methyl ester is dissolved in toluene. Heating under reflux condition realizes the ring closure into the cyclic dipeptide **4** in 95% yield (based on **3**). *O*-alkylation of this diketopiperazine is effected by treatment with triethyloxonium tetrafluoroborate in dichloromethane. After stirring for four days the excess alkylating reagent is neutralized with phosphate buffer and the labelled bis-lactim ether (**5**) is isolated by extraction (85% yield). In this way **5** can be prepared from specifically enriched glycine in a 72% overall yield. The bis-lactim ether of cyclo(D-Val-Gly) was then metallated into its lithium derivative, which on reaction with 4-iodobutyronitrile and after purification by means of column chromatography, yields the adduct **6** in 80% yield. The alkyl group enters at position 5 of the dihydropyrazine ring and *trans* to the isopropyl group at the inducing centre on position 2 with high stereospecificity. In the next step, the nitrile group of compound **6** is reduced with lithium aluminium hydride to the



Scheme 1. Route for the synthesis of [2-^{13}C]-L-lysine (**1a**) starting from commercially available highly enriched glycine

amine **7**. Compound **7** is not purified but is hydrolyzed directly to D-valine and **1a** with 6 M hydrochloric acid solution at 70°C overnight. The condition during the hydrolysis of **7** are highly critical because at lower temperature or at lower hydrochloric acid concentration, the hydrolysate contains a considerable amount of α-amino-ε-caprolactam (the cyclic dehydration product of lysine). After the hydrolysis, the labelled lysine is separated from the valine efficiently by means of cation-exchange chromatography using the Biorad AG 50W-X4 resin (protonated form). In this way, **1a** is synthesized with a yield of 49% (based on compound **6**). The overall yield of **1a**, based on the labelled glycine, amounts 28%.

The enantiomeric purity of product **1a** is checked by HPLC using precolumn derivatization with *o*-phtalaldehyde and *N*-acetyl-L-cysteine, and is found to be >97% e.e. (see Fig. 2).

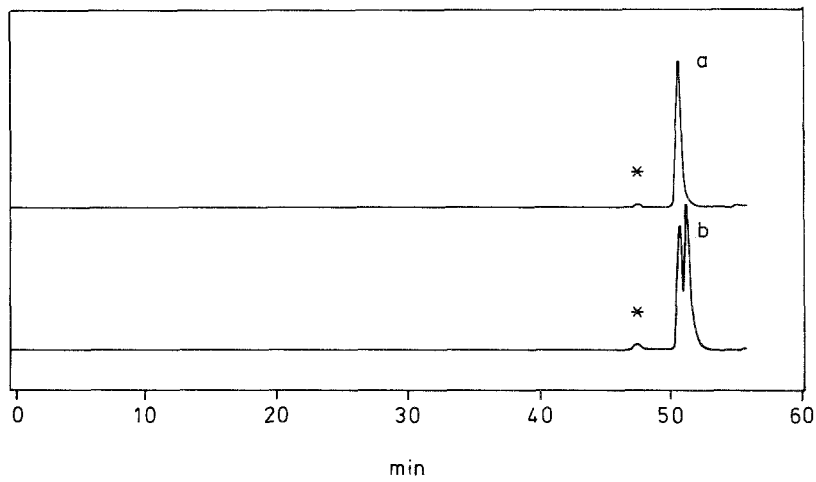
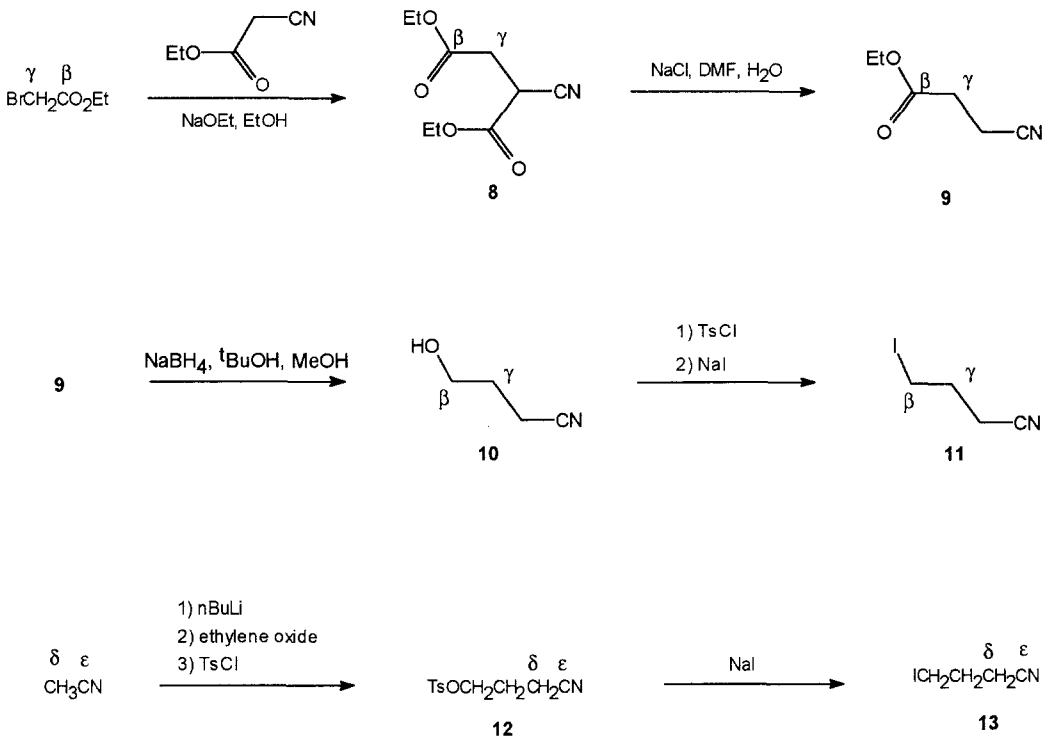


Fig. 2. Enantiomeric purity determination of lysine (**1a**) by HPLC after derivatization with OPA-NAC; **a**) synthesized L-lysine (**1a**) (e.e. >97%), **b**) 50:50 mixture of L- and D-lysine. The diastereoisomers were separated on a Spherisorb ODS-2 (5 μm) Pharmacia column (250 × 4 mm), employing a gradient of 100% buffer A (15% acetonitrile/0.05 M sodium acetate pH 4) to 25% buffer B (acetonitrile/0.05 M sodium acetate pH 7) over 30 min. (0.4 ml/min) followed by a gradient of 25%A/75%B to 100%B over 30 min. The asterisk indicates a buffer peak

Ethyl [1,2-$^{13}C_2$]-bromoacetate is the source for the introduction of the labels at the 3 (β) and/or 4 (γ) position of the lysine side chain (see Scheme 2). The synthesis is based on the alkylation of the anion of ethyl cyanoacetate with the highly enriched [1,2-$^{13}C_2$]-bromoacetate. We next subjected the isolated alkylation product **8** to reagents that might promote its decarboxylation. The method for the decarboxylation of α-cyano esters in DMSO previously reported by Krapcho et al. (1973) is successfully applied to the conversion of ethyl 2-cyanobutanedioate (**8**) in DMF to give the desired labelled ethyl cyanopropionate (**9**). The reaction is catalyzed by addition of catalytic amounts of both NaCl and H_2O. To obtain a suitable C4-reagent for the alkylation of the lithiated bislactim ether in the formation of L-lysine (see also Scheme 1), the ester group has to be transformed into a halide function. In our previous work we found that the ester function can be reduced selectively by means of $NaBH_4$ in t-butanol/methanol as a solvent (van den Berg, 1987). The anion of the reaction product **10** is quenched with tosylchloride. Treatment of the labelled tosyl-butyronitrile with sodium iodide in refluxing acetone gives labelled 4-iodobutyronitrile **11** in 61% yield based on the labelled ethyl bromoacetate.

The starting material for the synthesis of L-lysine labelled at the 5(δ)- and/or 6(ε)-position is highly enriched acetonitrile. The acetonitrile must be extended with a C2 compound to give the C4 side chain that appears in lysine. First, the lithiated acetonitrile is coupled to ethylene oxide. Secondly, tosyl *n*-butyronitrile **12** is prepared by quenching the 4-oxybutyronitrile anion with tosylchloride. Treatment of the labelled **12** with sodium iodide in refluxing acetone gives 4-iodobutyronitrile **13** in 78% overall yield based on the labelled acetonitrile.



Scheme 2. Synthetic scheme for the preparation of two isotopically enriched synthons: [3,4-$^{13}C_2$]-4-iodo-butyronitrile (**11**) and [1,2-$^{13}C_2$]-4-iodo-butyronitrile (**13**)

Spectroscopic characterization of specifically labelled lysines

Mass spectroscopy

A currently applied method for determining the percentage of labelled atoms is the analysis of amino acids by means of GC/MS. This technique requires derivatization of the amino acids to more volatile compounds. Following a standard procedure, we have converted lysine isotopomers to their trifluoroacetyl *n*-butyl esters (Gehrke et al., 1987). The mass spectrum of unlabelled *N*,*N'*-bis(trifluoroacetyl)-L-lysine *n*-butyl ester displays peaks at *m/z* 395 (0.5%, MH^+), 320 (9%, M^+-BuOH), 293 (3%), 180 (100%), 153 (4%, TFA-$NHCHCO^+$), 126 (9%, 6-$CH_2 = NH^+$-TFA), 69 (12%, CF_3^+), 57 (8%, $C_4H_9^+$). The most prominent peak in the spectrum occurs at *m/z* 180. This peak is most likely formed from the protonated molecular ion (1%, *m/z* 395) by the loss of a butoxy radical and subsequent elimination of the trifluoracetamide from the iminium ion (Fig. 3; Roessler et al., 1977). In agreement with this mechanism, base peaks of the labelled lysine derivatives **1a**, **1b** and **1c** are found at m/z 181, 182 (**1b**) and 182 (**1c**). From the base peak, an isotopic enrichment of 99% is determined for the labelled lysines that is the same enrichment as their starting materials [99%, 2-^{13}C]-glycine, ethyl [99%, 1,2-$^{13}C_2$]-bromoacetate and [99%, 1,2-$^{13}C_2$]-acetonitrile.

Another noteworthy feature of the mass spectra of the lysine derivatives is the elimination of 6-$CH_2 = NH^+$-TFA ion (*m/z* 126). Previously, it was reported

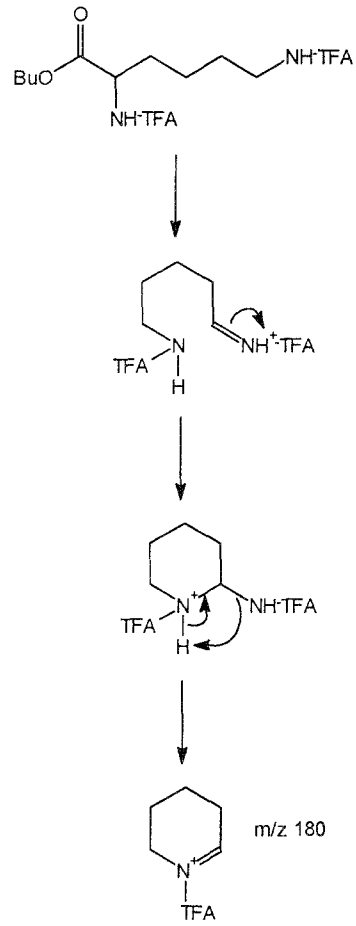


Fig. 3. Fragmentation pathway of *N,N'*-bis(trifluoroacetyl)-lysine *n*-butyl ester

that lysine ethyl ester undergoes a similar cleavage to yield $CH_2 = NH_2^+$ from the 6-(ε-)amino group (Gelpi at al., 1969). In the case of the unlabelled *N,N'*-bis(trifluoroacetyl)-L-lysine *n*-butyl ester and the corresponding derivatives of compounds **1a**, **1b** and **1c**, peaks at the respective values *m/z* 126, 126 and 127 are observed.

NMR spectroscopy

The position of the ^{13}C-labels in compounds **1a–c** has been independently ascertained by ^1H NMR spectroscopy as well as by ^1H-noise-decoupled 75.5 MHz ^{13}C NMR spectroscopy. Characterization of the specifically labelled compounds, furthermore, allows the unambiguous assignment of chemical shifts and the determination of homonuclear and heteronuclear coupling constants. As an example the ^{13}C NMR spectrum of the monolabelled [2-^{13}C]-L-lysine (**1a**) is shown in Fig. 4 together with that of the unlabelled L-lysine. The spectrum of **1a** displays a strong peak due to the labelled carbon at the chemical-shift value to be expected for the 2-carbon (55.2 ppm). The spectrum of **1b** (see Materials and Methods) shows intense peaks at 22.1 ppm (C4) and 30.6 ppm (C3). The

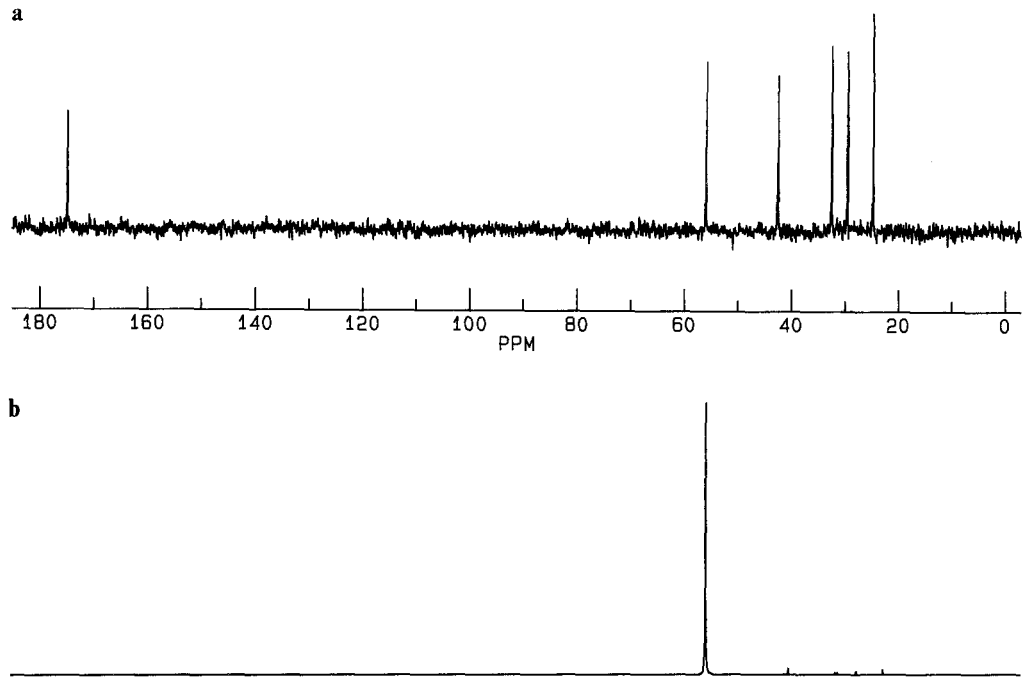


Fig. 4. ^{1}H-noise decoupled 75 MHz ^{13}C-NMR spectra in $^{2}H_2O$ (pH 7) of **a**) unlabelled L-lysine and **b**) labelled [2-^{13}C]-L-lysine **1a**

signals are split with 34.6 Hz ($^{1}J_{CC}$). The signals of **1c** at 27.0 ppm (C5) and at 39.7 ppm (C6) are split with 35.8 Hz. From the ^{1}H NMR spectra of **1a**, **1b** and **1c** (see Materials and Methods), the location of the ^{13}C-isotopes is clear via the large $^{1}J_{CH}$ couplings (**1a**: 145 Hz, **1b**: 125 Hz and **1c**: 143 Hz). No signals of natural-abundance lysine are observed in the respective spectra of **1a**, **1b** and **1c**, indicating a high degree of enrichment (99%).

Conclusion

In this paper we describe the synthesis of [2-^{13}C]-L-lysine, [3,4-$^{13}C_2$]-L-lysine and [5,6-$^{13}C_2$]-L-lysine in good yields with high isotope enrichment and optical purity. The scheme we have followed allows specific ^{13}C- and ^{15}N- enrichment of all carbon and nitrogen positions and any combination of positions. From the ^{1}H-NMR and ^{13}C-NMR spectra, it is evident that the reactions of our synthetic scheme take place without observable isotope scrambling or isotope dilution. For the enantioselective introduction of the α-amino acid moiety into L-lysine we use the bis-lactim ether method, which is a convenient method for the synthesis of a great variety of optically active α-amino acids (Williams, 1989; Raap et al., 1990b).

The availability of our method for the preparation of specifically enriched bis-lactim ethers not only allows the preparation of isotopically labelled lysine but also a large number of other α-amino acids that are labelled at the C1, C2 and N1 position (Cappon et al., 1994). Moreover, intermediate **6** is itself a useful intermediate in the enantioselective preparation of L-α-aminoadipic acid, (Raap

et al., 1990b). L-α-Aminoadipic acid is a well-known lysine metabolite and a precursor of several antibiotics, *e.g.*, penicillins and cephalosporins. The synthetic scheme in this publication makes L-α-aminoadipic acid ^{13}C- or ^{15}N-labelled at any position, easily available by hydrolysis of **6** with 4M hydrochloric acid and by anion exchange chromatography.

For labelling of the lysine side chain three different synthetic schemes have been developed for the synthesis of iodo-butyronitrile. Isotopes at the C6- (ε-) and N6-positions can be introduced by labelled potassium cyanide (Raap et al., 1990b) whereas labels at the C5- (δ-) and C6- position are incorporated by enriched acetonitrile. Site directed labelling of the C3- (β-) and C4- (γ-) position can be achieved by starting from the isotopomers of ethyl bromoacetate. In conclusion, we have developed an enantioselective total synthesis of optically pure L-lysine isotopomers from simple starting compounds that are commercially available in highly enriched form.

Materials and methods

Materials

[2-^{13}C]-glycine, ethyl [1,2-$^{13}C_2$]-bromoacetate and [1,2-$^{13}C_2$]-acetonitrile with 99% enrichment were purchased from Cambridge Isotopes Laboratories. (*2R*)-2,5-dihydro-2-isopropyl-3,6-dimethoxy-5-pyrazine, was purchased from Merck. Resin for the cation exchange column (AG 50W-X4) was obtained from Biorad. In all experiments, distilled dry solvents were used. Diethyl ether was dried over phosphorous pentoxide for one day and then distilled. The ether was stored over sodium wire. Ether refers to diethyl ether and petroleum ether refers to low-boiling petroleum ether 40–60°C. Tetrahydrofuran was refluxed with lithium aluminium hydride for several hours and freshly distilled before use.

Methods

GLC analyses were performed using a Hewlett-Packard 433, coupled to an integrator, with a capillary column (with cross-linked methyl silicone; film thickness 0.17 μ; length 25 m; internal diameter 0.31 mm) at 75 or 150°C. Ascending thin-layer chromatography were performed on Merck F_{254} silica-gel-60 sheets (0.2 mm). The following elution systems were used: I (*n*-propanol/25% ammonia 70/30 vv), II (*n*-butanol/acetic acid/pyridine/water 15/3/10/12), III (methanol/dichloromethane 5/95), IV (ether/petroleum ether 1/1) and V (ethyl acetate/petroleum ether 1/1). Spots were visualized with a UV-lamp (254 nm), iodine vapour, ninhydrin (0.2% in ethanol) for amino detection, Reindel-Hoppe reagent for secondary amine detection, 3 M hydrochloric acid followed by ninhydrin for bis-lactim ether detection. Melting points were determined with a Büchi apparatus and were uncorrected. ^1H-NMR spectra were recorded on a Bruker WM-300 in C^2HCL_3, with tetramethylsilane (TMS; $\delta = 0$ ppm) as internal standard. The isotopically labelled lysines were measured in ^2H_2O at pH = 7 (meter reading) with 3-(trimethylsilyl)pentadeuteropropionic acid (TSP) as internal standard. Assignments were made by means of 2-D-correlated spectroscopy. ^{13}C-NMR spectra were recorded on a Bruker WM-300 spectrometer at 75.5 MHz in C^2HCl_3 with TMS as internal standard. The isotopically labelled lysines were measured in ^2H_2O at pH 7. Isotopic enrichments of the amino acids were measured, after their conversion to *N*-(trifluoroacetyl) *n*-butyl esters, with a Finnigan MAT ITD 700 mass spectrometer interfacing with a Packard 438A gas chromatograph. The amino acid derivatives were separated on a 50 m CP-Sil 5 capillary column (Chrompack) that was programmed from 100 to 200°C at 3°C/min. The mass spectrometer was scanned in the "Full Scan" mode from *m/z* 50 to 450 at a rate of 1 scan (the average of 11 μscans) per second. The mass spectrometer was

coupled with an IBM AT personal computer, with ITD software version 3.0 installed, in order to control the automatic gain of the mass spectrometer and in order to acquire and integrate signals. The enantiomeric purity of the labelled lysine was determined by HPLC using a Pharmacia LKB gradient delivery system, a Rheodyne injection valve fitted with a 5-μm loop, and a Spectro-Vision FD-300 fluorescence detector. Prior to chromatography, the labelled lysines were derivatized using *o*-phtalaldehyde (OPA, Janssen) and *N*-acetyl-L-cysteine (NAC, Aldrich). The excitation and emission wavelengths for detection of OPA-NAC derivatives were 360 and 405 nm, respectively. The diastereoisomers were separated on a Spherisorb ODS-2 (5 μm) Pharmacia column (250 × 4 mm), employing a gradient of 100% buffer A (15% acetonitrile/0.05 M sodium acetate pH 4) to 25% buffer B (acetonitrile/0.05 M sodium acetate pH 7) over 30 min. (0.4 ml/min) followed by a gradient of 25%A/75%B to 100%B over 30 min. The retention time was 52 min. Optical rotations ($[\alpha]_D$) were measured using a Perkin-Elmer 141 polarimeter (Na lamp 589 nm).

[2-^{13}C]-L-lysine monohydrochloride **1a**

540 mg (1.9 mmol) of compound **7** was dissolved in 15 ml of 6.0 M hydrochloric acid at 0°C (the hydrochloric acid was deaerated by purging nitrogen gas through it). In a nitrogen atmosphere the stirred solution was heated overnight at 70°C. After evaporation of the solvent, the yellow residue was redissolved in water and treated with a small amount of charcoal to decolorize the solution. The solution was successively filtered and concentrated to 15 ml. The labeled L-lysine was separated from the D-valine by using a strongly acid cation exchanger, AG50W X4 (H^+-form). Successively, valine and lysine eluted from the column after elution with dilute ammonia (0.15 M). All fractions were analyzed by TLC (elution system I). The fractions containing lysine were combined and the solvent was evaporated. The residue was dissolved in 25 ml of water and the pH was adjusted to 7 with hydrochloric acid. The solution was then lyophilized. 170 mg of [2-^{13}C]-L-lysine monohydrochloride was obtained in an 49% yield. Enantiomeric purity (HPLC): >97% e.e.. TLC (system I): Rf(**8**) 0.13; Rb(**8**/Val) 0.32. ^{1}H-NMR (^2H_2O, 300 MHz): δ 1.44 (m, 2H, 4-CH_2), 1.68 (quintet, $^3J_{HH}$ 7.6 Hz, 2H, 5-CH_2), 1.84 (m, 2H, 3-CH_2), 2.98 (t, $^3J_{HH}$ 7.6 Hz, 2H, 6-CH_2), 3.68 (dt, $^1J_{CH}$ 145.0 Hz, $^3J_{HH}$ 6.1 Hz, 1H 2-CH). ^{13}C-NMR (^2H_2O 75.5 MHz): δ 22.1 (s, 4-C), 27.1 (d, $^3J_{CC}$ 4.6 Hz, 5-C), 30.8 (d, $^1J_{CC}$ 34.1 Hz, 3-C), 39.8 (s, 6-C), 55.2 (s, 2-^{13}C), 175.8 (d, $^1J_{CC}$ 55.7 Hz, CO).

*[3,4-$^{13}C_2$]-L-lysine (***1b***)*

In a nitrogen atmosphere, 0.6 g (15.8 mmol) of lithium aluminium hydride was suspended in 60 ml of dry diethyl ether at 0°C. 2 Gram of compound **14a** was slowly added to the stirred mixture with a syringe. The temperature of the suspension was allowed to rise to room temperature and stirring was continued for 6 h. During the reaction, the colour of the mixture changed from grey to brown. Under stirring, 10 ml of cold water was slowly added to the suspension. The organic layer was separated and successively washed with water and then dried over magnesium sulphate. Evaporation of the ether yielded 2.0 g (95%) of [3,4-$^{13}C_2$]-4-[(*2R,5S*)-2,5-dihydro-2-isopropyl-3,6-dimethoxy-5-pyrazinyl]butylamine (**15a**). The conditions for the hydrolysis of **15a** and the purification of the labelled L-lysine were both as described for **1a**. ^{1}H-NMR (^2H_2O, 300 MHz): δ 1.50 (dm, $^1J_{CH}$ 128 Hz, 2H, 4-$^{13}CH_2$), 1.75 (m, 2H, 5-CH_2), 1.96 (dm, $^1J_{CH}$ 123 Hz, 2H, 3-$^{13}CH_2$), 3.08 (t, 2H, 6-CH_2), 3.78 (m, 1H, 2-CH). ^{13}C-NMR (^2H_2O, 75.5 MHz): 22.1 (d, $^1J_{CC}$ 34.5 Hz, 4-^{13}C), 27.0 (d, $^1J_{CC}$ 34.3 Hz, 5-C), 30.6 (d, $^1J_{CC}$ 34.6 Hz, 3-^{13}C), 39.8 (s, 6-C), 55.2 (d, $^1J_{CC}$ 34.3 Hz, 2-C). All other ^{1}H-NMR and ^{13}C-NMR parameters are the same as for **1a**.

*[5,6-$^{13}C_2$]-L-lysine (***1c***)*

The reaction conditions and the working-up procedure were both as described for **1b**, but **14a** was replaced by compound **14b**. ^{1}H-NMR (^2H_2O, 300 MHz): δ 1.47 (m, 2H, 4-CH_2), 1.70

(dm, $^1J_{CH}$ 143 Hz, 2H, 5-$^{13}CH_2$), 1.88 (dm, 2H, 3-CH_2), 2.97 (dm, $^1J_{CH}$ 143 Hz, 2H, 6-$^{13}CH_2$), 3.72 (m, 1H, 2-CH). ^{13}C-NMR (^2H_2O, 75.5 MHz): δ 22.0 (d, $^1J_{CC}$ 34.6 Hz, 4-C), 27.0 (d, $^1J_{CC}$ 36.0 Hz, 5-^{13}C), 30.6 (s, 3-C), 39.7 (d, $^1J_{CC}$ 35.7 Hz, 6-$^{13}CH_2$), 55.2 (d, $^3J_{CC}$ 4.1 Hz, 2-CH_2). All other ^{1}H-NMR and ^{13}C-NMR parameters are the same as for **1a**.

Methyl[2-^{13}C]-glycinaat monohydrochloride **2**

To a stirred suspension of 2.0 g (26 mmol) of [2-^{13}C]-glycine in 25 ml of anhydrous methanol, 2.2 ml of thionylchloride was added dropwise at −15°C. After all the thionylchloride was added, the temperature of the resulting solution was refluxed during 3 h. After cooling down the volatile components were evaporated *in vacuo*. This resulted in 3.29 g of **2** (crystalline, 99% yield). M.p. 175°C. TLC (system I): Rf(**2**) 0.66, Rb (**2**/Gly) 3.3, TLC (system II): Rf(**2**) 0.40, Rb (**2**/Gly) 2.35. ^{1}H-NMR (C^2HCl_3, 400 MHz): δ 4.02 (d, $^1J_{CH}$ 146.0 Hz, 2H, 2-$^{13}CH_2$), 3.88 (s, 3H, OCH_3). ^{13}C-NMR (C^2HCl_3, 75.5 MHz): δ 40.8 (s, main peak, ^{13}C), 54.1 (s, OCH_3), 169.5 (d, $^1J_{C-C}$ 62.5 Hz, CO).

Methyl N-benzyloxycarbonyl-D-valinyl-[2-^{13}C]-glycinaat **3**

To a stirred mixture of 2.5 g (10 mmol) of N-benzyloxycarbonyl-D-valine, 1.26 g (10 mmol) **2** and 1.29 g (10 mmol) of diïsopropylethylamine in 30 ml of THF cooled at 0°C, 2.06 g (10 mmol) of dicyclohexylcarbodiïmide (DCC) in 5 ml of THF was added. After stirring for 1 h at 0°C and 1h at room temperature, the filtered solution was evaporated to dryness. The oily residue was dissolved in 75 ml of chloroform and washed successively with saturated aqueous $NaHCO_3$ solution, water, an ice cold aquous solution of $KHSO_4/K_2SO_4$ (0.2 M) and a saturated aqueous sodium chloride solution. After drying (magnesium sulphate) and evaporating the chloroform, the crystalline product was recrystallized from methanol. Yield 2.97 g of **3** (92%). M.p.: 155–157°C. TLC (system II): Rf(**3**) 0.73, Rb (**3**/Z-Val) 1.3. TLC (system III): Rf(**3**) 0.50, Rb(**3**/Z-Val) 0.99. ^{1}H-NMR (C^2HCl_3, 300 MHz): δ 0.94 (d, $^3J_{HH}$ 6.9 Hz, 3H, Val/CH_3'), 0.99 (d, $^3J_{HH}$ 6.8 Hz, 3H, Val/CH_3), 2.17 (double septet, 3.73 (s, 3H, OCH_3), 4.05 (dm, $^1J_{CH}$ 141.1 Hz, 2H, Gly/$^{13}CH_2$), 4.08 (dd, $^3J_{HH}$ 6.2 Hz, $^3J_{HH}$ 8.6 Hz, 1H, Val/CH), 5.10 (s, 2H, Ar/CH_2), $^3J_{HH}$ 13.4 Hz en $^3J_{HH}$ 6.7 Hz, 1H, Val/CH), 5.40 (d, $^3J_{HH}$ 8.7 Hz, 1H, Val/NH), 7.35 (s, 5H, Ar/H), 6.50 (s, 1H, Gly/NH). ^{13}C-NMR (C^2HCl_3, 75.5 MHz): δ 17.6 (Val/CH_3), 19.1 (Val/CH_3'), 33.9 (Val/CH), 41.1 (Gly/$^{13}CH_2$, main peak), 52.4 (OCH_3), 60.2 (Val/CH), 67.1 (Ar/CH_2), 136.1/128.5/128.1/128.0 (Ar-C's), 156.4 (Z/CO), 170.0 (d, $^1J_{C-C}$ 61.7, Gly/CO), 171.5 (Val/CO).

Cyclo-(D-valinyl-[2-^{13}C]-glycine) **4**

5.5 g of **3** (17 mmol) was hydrogenated in a suspension of 1.0 g of catalyst (Pd 10% on carbon) and methanol/acetic acid (100/4 v/v) at 40°C. The completeness of the reaction was checked by TLC (system II). The suspension was filtered over celite. The filtered solution was evaporated to yield an oily residue. This residue was dissolved in 100 ml of toluene and boiled under reflux overnight. The gelatinous precipitate formed was collected, washed with ether and dried *in vacuo* at 100°C. Yield: 2.39 g of **4** (90%). TLC (system II): Rf(**4**) 0.70. ^{1}H-NMR (^2H_2O, 400 MHz): δ 0.92 (d, $^3J_{HH}$ 6.9 Hz, 3H, Val/CH_3'), 1.02 (d, $^3J_{HH}$ 6.9 Hz, 3H, Val/CH_3), 2.26 (m, $^3J_{HH}$ 3.7, $^3J_{HH}$ 6.9 Hz, 1H, Val/2-CH), 3.92 (d, $^3J_{HH}$ 3.7 Hz, 1H, Val/2-CH), 4.15 (dd, $^1J_{CH}$ 143.3 Hz, $^2J_{HH}$ 18.5 Hz, 1H, Gly/2-$^{13}CH_2$). ^{13}C-NMR (^2H_2O, 75.5 MHz): δ 16.3 (Val/CH_3), 18.6 (Val/CH_3'), 33.6 (Val/2-CH), 44.4 (main peak, Gly/2-$^{13}CH_2$), 60.6 (Val/CH), 169.6 (d, $^1J_{CC}$ 50.4 Hz, Gly/CO), 171,0 (Val/CO).

(2R)-[5-^{13}C]-3,6-diethoxy-2,5-dihydro-2-isopropylpyrazine **5**

In a nitrogen atmosphere, 3.05 g (16 mmol) of Et_3OBF_4 in 5 ml of anhydrous dichloromethane was added to 500 mg (3.2 mmol) of **4**. After 4 days stirring at room temperature the light yellow coloured solution was diluted with 20 ml of dichloromethane. A cold buffer

pH 7 (a solution of 1.23 g KH_2PO_4 and 5.23 g K_2HPO_4 in 25 ml of water) was added to the vigorously stirred reaction mixture at 0°C. After stirring the mixture during half an hour the organic phase was separated and the aqueous phase was extracted with dichloromethane three times. The combined organic phases were dried on magnesium sulphate, filtered and the solvent was removed *in vacuo*. The residue was purified on silica column chromatography (ether/petroleumether 1/1 v/v). Yield: 510 mg of **5** (87%). $[\alpha]_D^{20} = -119°$ (c = 1, EtOH). TLC (system II): Rf(**5**) 0.70; (system IV): Rf(**5**) 0.86; (system V: Rf(**5**) 0.81. ^{1}H-NMR (C^2HCl_3, 400 MHz): δ 0.77 (d, $^3J_{HH}$ 6.9 Hz, 3H, ^{i}Pr-CH_3), 1.02 (d, $^3J_{HH}$ 6.9 Hz, 3H, ^{i}Pr-CH_3), 1.28 (t, $^3J_{HH}$ 7.2 Hz, 3H, OEt/CH_3'), 1.29 (t, $^3J_{HH}$ 7.0 Hz, 3H, OEt/CH_3), 2.23 (m, $^3J_{HH}$ 3.7, $^3J_{HH}$ 6.9 Hz, 1H, ^{i}Pr-CH), 3.95 (1H, m, 2-CH), 4.04 (dm, $^1J_{CH}$ 196.2 Hz, 2H, 5-$^{13}CH_2$), 4.14 (m, 4H, OEt/CH_2). ^{13}C-NMR (C^2HCl_3, 75.5 MHz): δ 14.3 (OEt/CH_3), 17.0 (^{i}Pr-CH_3), 19.0 (^{i}Pr-CH_3'), 32.5 (^{i}Pr-CH), 46.8 (main peak, 5-$^{13}CH_2$), 60.7 (2-CH), 61.0 (OEt/CH_2), 61.03 (OEt/CH_2'), 161.8 (d, $^1J_{CC}$ 50.1 Hz, 6-CO), 164.3 (3-CO). GC/MS: *m/z* 214 (11%, MH^+), 196 (6%), 185 (24%, M − ethene), 170 (70%, M − ^{i}Pr), 154 (9%), 142 (41%, M − ^{i}Pr − ethene), 127 (15%), 114 (84%, M − ^{i}Pr − 2 ethene), 98 (17%), 86 (100%) 70 (19%), 57 (47%).

4-[(2R,5S)-[5-^{13}C]-3,6-diethoxy-2,5-dihydro-2-isopropyl-5-pyrazinyl]butyronitrile **6**

Under a nitrogen atmosphere 500 mg (2.35 mmol) of **5** was dissolved in 2 ml of dry THF in a 10 ml two-necked flask. The solution was cooled to −70°C and 1.5 ml of *n*-butyllithium (1.6 M solution in hexane) was added dropwise by means of a syringe. After the solution was stirred for 10 minutes, 500 mg (2.35 mmol) of 4-iodobutyronitrile was added with a syringe to the solution at −70°C. After 2 h the temperature of the reaction mixture was allowed to rise to room temperature. The solvents were evaporated, yielding a brown oily residue. The oil was taken up in 25 ml of ether and washed with 25 ml of cold water. The organic layer was dried over magnesium sulphate, followed by evaporation of the ether. Yield 610 mg of a brown oil. After silica column chromatography (eluent: ether/ethyl acetate 2/1 vv) 542 mg of **6** (80%) was obtained. TLC (system V) Rf(**6**) 0.76. ^{1}H-NMR (C^2HCl_3, 400 MHz): δ 0.70 (d, $^3J_{HH}$ 6.9 Hz, 3H, ^{i}Pr/CH_3'), 1.01 (d, $^3J_{HH}$ 6.9 Hz, 3H, ^{i}Pr/CH_3), 4.13 (m, 4H, OEt/CH_2), 1.26 (m, 6H, OEt/CH_3), 1.68 (m, 2H, butyronitrile/3-CH_2), 1.90 (dm, $^1J_{CH}$ 24.0 Hz, 2H, butyronitrile/4-CH_2), 2.25 (double septet, $^3J_{HH}$ 3.4 Hz, 1H, ^{i}Pr/CH)), 2.37 (t, $^3J_{HH}$ 7.2 Hz, 2H, butyronitrile/2-CH_2), 3.91 (dt, $^3J_{HH}$ 3.5 Hz, $^5J_{HH}$ 3.5 Hz, $^4J_{CH}$ 1.5 Hz, 1H, 2-CH), 3.97 (dm, $^1J_{CH}$ 139.7 Hz, $^5J_{HH}$ 3.5 Hz, 1H, 5-^{13}CH). ^{13}C-NMR (C^2HCl_3, 75.5 MHz): δ 16.7 (^{i}Pr/CH_3), 17.1 (d, $^3J_{CC}$ 4.6 Hz, butyronitrile/2-CH_2), 19.0 (^{i}Pr/CH_3'), 21.3 (butyronitrile/3-CH_2), 32.0 (^{i}Pr/CH), 33.0 (d, $^1J_{CC}$ 36.6 Hz, butyronitrile/4-CH_2), 54.6 (main peak, 5-^{13}CH), 60.6 (2-C), 60.8 (OEt/CH_2), 60.9 (OEt/CH_2'), 119.7 butyronitrile/CN, 162.5 (d, $^1J_{CC}$ 50.4 Hz, 6-CO), 163.5 (3-CO). GC/MS: *m/z* 281 (41%, MH^+), 280 (20%, M^+), 252 (47%, M − ethene), 237 (85%, M − ^{i}Pr), 223 (22%, M − 2 − ethene − H), 212 (47%, M − $(CH_2)_3CN$), 209 (72%, M − ^{i}Pr − ethene), 181 (58%, M − $CH(CH_3)_2$ − 2 ethene), 170 (41%, M − ^{i}Pr − $(CH_2)_3CN$ + H), 166 (76%), 153 (25%), 142 (28%), 125 (47%), 109 (38%), 98 (100%), 83 (48%), 72 (28%), 55 (86%).

4-[(2R,5S)-[5-^{13}C]-3,6-diethoxy-2,5-dihydro-2-isopropyl-5-pyrazinyl]butylamine **7**

In a nitrogen atmosphere 163 mg (4.3 mmol) of lithium aluminium hydride was suspended in 15 ml of dry ether at 0°C. A solution of **6** (600 mg; 2.14 mmol) in 10 ml of ether was slowly added to the mixture at 0°C with a syringe. The suspension was stirred at room temperature during 6 h. Under stirring, 10 ml of cold water was slowly added to the suspension. After the organic phase was separated, the aqueous phase was extracted six times with ether. The combined organic phases were dried over magnesium sulphate. Evaporation of the ether yielded 540 mg (89%) of compound **7**. GC/MS: *m/z* 269 (43%), 254 (14%), 240 (18%), 226 (50%), 212 (100%), 198 (27%), 184 (18%), 170 (72%), 155 (10%), 142 (57%), 126 (11%), 114 (33%), 98 (21%), 86 (21%), 70 (21%), 55 (39%).

*Diethyl [3,4-$^{13}C_2$]-2-cyanobutanedioate (**8**)*

In a nitrogen atmosphere, 0.27 g (12 mmol) of sodium was carefully added to 40 ml of dry ethanol at 0°C. Then 0.67 g (5,9 mmol) of ethyl cyano acetate was added dropwise. After stirring during 15 minutes at 0°C, a solution of ethyl [1,2-$^{13}C_2$]bromo acetate (1.0 g (5.9 mmol)/20 ml ethanol) was slowly added and stirring was continued for 2 h. After the temperature was slowly allowed to rise to room temperature, the reaction mixture was stirred during 1 h. The reaction was stopped by adding an aqueous solution of potassium bisulphate (0.8 g) until neutral pH. The solvents were evaporated and the residue was dissolved in diethylether. De solution was successively washed with cold water and a saturated aqueous sodium chloride solution. The aqueous phase was washed with diethylether and the combined organic phases were dried over magnesium sulphate. Evaporation of the solvent yielded 1.16 g (98%) of compound **8**. ^{1}H-NMR (C^2HCl_3, 75.5 MHz): δ 1.29 (t, 3H, ethyl/CH_3), 1.34 (t, 3H, ethyl/CH_3), 3.00 (dm, $^1J_{CH}$ 134.7 Hz, 2H, 3-$^{13}CH_2$), 3.93 (dq, $^2J_{CH}$ 6.0 Hz, $^3J_{HH}$ 6.27 Hz, 1H, 2-CH), 4.21 (m, 2H, CH_2-ethyl), 4.23 (m, 2H, CH_2-ethyl). ^{13}C-NMR (C^2HCl_3, 100 MHz, main peaks): δ 33.54 (d, $^1J_{CC}$ 38.2 Hz, 3-$^{13}CH_2$), 168.88 (d, $^1J_{CC}$ 38.1 Hz, 4-^{13}CO). MS: m/z 202 (M + 1), m/z 156 ($C_7H_8O_3N^+$), m/z 83 ($C_5H_8N^+$), m/z 55 ($C_3H_4N^+$), (unlabelled 200, 154, 82, 54). GC: R_t (**8**) 14.2 min ($T_{initial}$ = 100°C; after 10 min. the temperature was slowly increased (gradiënt = 20°C/min) till 250°C).

*Ethyl [1,2-$^{13}C_2$]-3-cyanopropionate (**9**)*

After 1.16 g (5.8 mmol) of **8** was dissolved in 10 ml of *N*-dimethylformamide, successively 100 mg (1.9 mmol) of sodium chloride and 200 μl (11.6 mmol) of water was added. The reaction mixture was stirred at 150°C. The liberated carbon dioxide gas could be detected by purging the solution with nitrogen gas and by passing the carbon dioxide/nitrogen gas mixture through an aqueous solution of barium hydroxide. After 5 h an additional volume of water (100 μl) was added and stirring was continued until no barium carbonate precipitate was formed (ca. 10 h). The solvents were evaporated and the residue was taken up in diethyl ether. The ether was washed successively with cold water and a saturated solution of sodium chloride. After drying the organic phase over magnesium sulphate and removing of the volatile components, the residue (0.66 g) was purified by means of silica column chromatography (ether/petroleum ether 1/1). The fractions were collected and analyzed by TLC (spots were visualized with iodine vapour). After combining the fractions containing **9**, the solvents were evaporated yielding 0.60 g (81%).

GC: R_t (**9**) 3.6 min. (initial temperature 100°C; temperature gradiënt 20°C/min.). ^{1}H-NMR (C^2HCl_3, 200 MHz): δ 1.29 (t, $^3J_{HH}$ 7.1 Hz, 3H, OEt/CH_3), 2.65 (m, 2H, 3-CH_2), 2.69 (ddt, $^1J_{CH}$ 133.5 Hz, $^2J_{CH}$ 0.6 Hz, $^3J_{HH}$ 7.3 Hz, 2H, 2-$^{13}CH_2$), 4.20 (q, $^3J_{HH}$ 7.2 Hz, 2H, OEt/CH_2). ^{13}C-NMR (C^2HCl_3, 75.5 MHz, main peaks only) δ 29.7 (d, $^1J_{CC}$ 58.6 Hz, 2-$^{13}CH_2$), 169.9 (d, $^1J_{CC}$ 58.6 Hz, 1-^{13}CO). MS: *m/z* 130 (M + 1), 102 ($C_4H_6O_2N^+$), 84 ($C_4H_4ON^+$), 55 ($C_3H_4ON^+$), (unlabelled compound *m/z* 128, 100, 82, 54).

*[3,4-$^{13}C_2$]-4-hydroxy-butyronitrile (**10**)*

0.37 g (9.7 mmol) of sodium borohydride was added to a solution of **9** (0.5 g; 3.9 mmol in 17.5 ml of dry *tert*-butanol). The resulting reaction mixture was heated till boiling point. After adding slowly 3.5 ml of MeOH, the mixture was stirred for 1.5 h under reflux. The solvents were removed *in vacuo* and the residue was treated with 7 ml of a saturated aqueous sodium chloride solution. The aqueous phase was extracted three times with chloroform. Yield: 0.30 g (86%). ^{1}H-NMR (C^2HCl_3, 300 MHz): δ 1.90 (dm, $^1J_{CH}$ 139.2 Hz, 2H, 3-$^{13}CH_2$), 2.51 (m, 2H, 2-CH_2), 7.51 (m, $^1J_{CH}$ 142.3 Hz, $^2J_{CH}$ 2.1 Hz, $^3J_{HH}$ 5.65 Hz, 2H, 4-$^{13}CH_2$). ^{13}C-NMR (C^2HCl_3, 75.5 MHz): δ 27.9 (d, $^1J_{CC}$ 38.2 Hz, 3-$^{13}CH_2$), 59.9 (d, $^1J_{CC}$ 38.1 Hz, 4-$^{13}CH_2$). MS: *m/z* 88 (M + 1), 55 ('base peak', $[CH_2CH_2CN]^+$), (unlabelled compound *m/z* 86, 54).

*[3,4-$^{13}C_2$]-4-Iodo-butyronitrile (**11**)*

0.41 g (2.2 mmol) of tosylchloride was slowly added at 0°C to a solution of **10** (180 mg; 2.1 mmol; 2 ml of pyridine). After stirring the solution for 3 h at room temperature a mixture of water/diethyl ether (5/15 v/v) was added and the pH was adjusted until 3 with a 6 M aqueous solution of hydrochloric acid. The organic phase was separated and the aqueous phase was extracted with diethyl ether. After the combined organic phases were dried over magnesium sulphate the volatile components were evaporated *in vacuo*. Yield: 0.47 g (97%) of [3,4-$^{13}C_2$]-4-tosylbutyronitrile. 0.47 g of [3,4-$^{13}C_2$]-4-tosylbutyronitrile was dissolved in 10 ml of dry acetone. After addition of 0.60 g dry sodium iodide the stirred suspension was refluxed overnight. The reaction mixture was cooled to room temperature and the unsoluble material was filtered off. The acetone was removed *in vacuo* and the residue was taken up in diethyl ether. After the ether solution was washed successively with a solution of sodium thiosulphate and water, the organic phase was dried over magnesium sulphate and the ether was evaporated. Yield: 330 mg (87%). ^{1}H-NMR (C^2HCl_3, 400 MHz): δ 2.13 (dm, $^1J_{CH}$ 133.0 Hz, $^2J_{CH}$ 4.3 Hz, $^3J_{HH}$ 6.73 Hz, 2H, 3-$^{13}CH_2$), 2.52 (m, 2H, 2-CH_2), 3.27 (dt, $^1J_{CH}$ 151.7 Hz, $^2J_{CH}$ 2.6 Hz, $^3J_{HH}$ 6.5 Hz, 2H, 4-$^{13}CH_2$). ^{13}C-NMR (C^2HCl_3, 75.5 MHz): δ 2.9 (d, $^1J_{CC}$ 36.4 Hz, 4-$^{13}CH_2$), 18.3 (d, $^1J_{CC}$ 34.2 Hz, 2-CH_2), 28.7 (d, $^1J_{CC}$ 35.9 Hz, 3-$^{13}CH_2$), 118.2 (s, CN).

*[1,2-$^{13}C_2$]4-tosyl-butyronitrile (**12**)*

In a nitrogen atmosphere, 1.0 g (23.8 mmol) of [1,2-$^{13}C_2$]-acetonitrile was dissolved in 50 ml of tetrahydrofuran. The solution was cooled to −60°C and 15.5 ml of *n*-butyllithium (1.6 M solution in hexane) was added dropwise with a syringe. The solution was stirred for 15 min and it gradually turned into a white suspension. 1.05 g (23.8 mmol) of liquid ethylene oxide was added to the suspension at −60°C. After 1 h, the temperature of the mixture was allowed to rise to room temperature. The mixture was heated for 15 min at 30–40°C. After addition of 4.54 g (23.8 mmol) of tosyl chloride, the mixture became clear again. The tetrahydrofuran was evaporated and the residue was taken up in 30 ml of ether. The organic layer was washed with water and a saturated aqueous solution of sodium chloride. Finally, the organic layer was dried over magnesium sulphate and the ether was evaporated. The result was 5.50 g (97%) **12**. ^{1}H-NMR (C^2HCl_3, 300 MHz): δ 2.04 (m, 2H, 3-CH_2), 2.44 (ddt, $^1J_{CH}$ 136 Hz, $^2J_{CH}$ 9.8 Hz, $^3J_{HH}$ 7.1 Hz, 2H, 2-$^{13}CH_2$), 2.46 (s, 3H, CH_3), 4.14 (q, $^3J_{CH}$ 5.8 Hz, $^3J_{HH}$ 5.8 Hz, 2H, 4-CH_2), 7.60 (dd, 4H, Ar/H). ^{13}C-NMR (C^2HCl_3, 75.5 MHz, main peaks): δ 13.8 (d, $^1J_{CC}$ 57.0 Hz, 2-$^{13}CH_2$), 118.2 (d, $^1J_{CC}$ 56.7 Hz, 1-^{13}CN).

*(1,2-$^{13}C_2$)-4-Iodo-butyronitrile (**13**)*

3.7 g (15.5 mmol) of **12** was dissolved in 100 ml of dry acetone. After 4.7 g (31.0 mmol) of dry sodium iodide was added to this solution, the mixture was stirred and refluxed for 6 h. During the reaction, the colour of the mixture changed to yellow. After cooling, the white precipitate was filtered off and the acetone evaporated. The residue was dissolved in diethyl ether and successively washed with a saturated sodium bisulphite solution and water. The organic layer was dried over magnesium sulphate. Evaporation of the ether yielded 2.4 g of **13** (80%). ^{1}H-NMR (C^2HCl_3, 300 MHz): δ 2.14 (m, 2H, 3-CH_2), 2.53 (ddt, $^1J_{CH}$ 134.8 Hz, $^2J_{CH}$ 9.7 Hz, $^3J_{HH}$ 6.9 Hz, 2H, 2-$^{13}CH_2$), 3.30 (dt, $^3J_{CH}$ 5.8 Hz, $^3J_{HH}$ 6.5 Hz, 2H, 4-CH_2)

*[3,4-$^{13}C_2$]-4-[(2R,5S)-2,5-dihydro-2-isopropyl-3,6-dimethoxy-5-pyrazinyl]butyronitrile (**14a**)*

Under a nitrogen atmosphere 1 g (5.5 mmol) of (2*R*)-2,5-dihydro-2-isopropyl-3,6-dimethoxy-5-pyrazine was dissolved in 6 ml of dry THF in a 25 ml two-necked flask. The solution was cooled to −70°C and 3.75 ml of *n*-butyllithium (1.6 M solution in hexane) was added dropwise by means of a syringe. After the solution was stirred for 10 minutes, 1.0 g (5.1 mmol) of **11** was added with a syringe to the solution at −70°C. After 2 h the temperature of the

reaction mixture was allowed to rise to room temperature. The solvents were evaporated, yielding a brown oily residue. The oil was taken up in ether and washed with cold water. The organic layer was dried over magnesium sulphate, followed by evaporation of the ether. Yield 1.18 g of a brown oil. After silica column chromatography (eluent: ether/ethyl acetate 2/1 vv) 1.01 g of **14a** (80%) was obtained. TLC (system V): Rf(**14a**) 0.76. ^{1}H-NMR (C^2HCl_3, 300 MHz): δ 0.70 (d, $^3J_{HH}$ 6.8 Hz, 3H, ^{i}Pr/CH_3, 1.0 (d, $^3J_{HH}$ 6.8 Hz, 3H, ^{i}Pr/CH_3), 1.70 (m, $^1J_{CH}$ 128 Hz, 2H, butyronitrile/3-$^{13}CH_2$), 1.80 (dm, $^1J_{CH}$ 130 Hz, 1H, butyronitrile/4-$^{13}CH_A$), 2.0 (dm, $^1J_{CH}$ 130 Hz, 1H, butyronitrile/4-$^{13}CH_B$), 2.25 (m, 1H, ^{i}Pr/CH), 2.38 (m, 2H, butyronitrile/2-CH_2), 3.67 (s, 3H, OCH_3), 3.71 (s, 3H, OCH_3'), 3.96 (m, 1H, ^{i}Pr/2-CH), 4.01 (m, 1H, 5-CH). ^{13}C-NMR (C^2HCl_3, 75.5 MHz): δ 16.77 (s, ^{i}Pr/CH_3), 16.80 (d, $^1J_{CC}$ 56.3 Hz, butyronitrile/2-$^{13}CH_2$), 18.95 (s, ^{i}Pr/CH_3'), 21.22 (d, $^1J_{CC}$ 33.7 Hz, butyronitrile/3-$^{13}CH_2$), 31.88 (s, ^{i}Pr/CH), 32.92 (d, $^3J_{CC}$ 4.0 Hz, butyronitrile/4-CH_2), 52.41 (s, OCH_3), 52.48 (s, OCH_3'), 54.60 (d, J 4.5 Hz, 5-CH), 60.80 (s, 2-CH), 119.65 (d, $^1J_{CC}$ 56.3 Hz, ^{13}CN).

*[1,2$^{13}C_2$]4-[(2R,5S)-2,5-dihydro-2-isopropyl-3,6-dimethoxy-5-pyrazinyl]butyronitril (***14b***)*

The reaction of **13** with (2*R*)-2,5-dihydro-2-isopropyl-3,6-dimethoxy-5-pyrazine was carried out under similar conditions as described for **14a**. ^{1}H-NMR (C^2HCl_3, 300 MHz): δ 0.70 (d, $^3J_{HH}$ 6.8 Hz, 3H, ^{i}Pr/CH_3), 1.04 (d, $^3J_{HH}$ 6.8 Hz, 3H, ^{i}Pr/CH_3'), 1.70 (m, 2H, butyronitrile/3-CH_2), 1.80 (m, 1H, butyronitrile/4-CH_A), 2.00 (m, 1H, butyronitrile/4-CH_B), 2.25 (dm, 1H, ^{i}Pr/CH), 2.38 (ddt, $^1J_{CH}$ 134.6 Hz, $^2J_{CH}$ 9.6 Hz, $^3J_{HH}$ 7.1 Hz, 2H, butyronitrile/2-$^{13}CH_2$), 3.68 (s, 3H, OCH_3), 3.70 (s, 3H, OCH_3'), 3.96 (dd, J 3.4/3.6 Hz, 1H, 2-CH), 4.01 (m, 1H, 5-CH). ^{13}C-NMR (C^2HCl_3, 75.5 MHz): δ 16.8 (s, ^{i}Pr/CH_3), 16.8 (d, $^1J_{CC}$ 56.3 Hz, butyronitrile/2-$^{13}CH_2$), 19.0 (^{i}Pr/CH_3), 21.2 (d, $^1J_{CC}$ 33.7 Hz, butyronitrile/3-CH_2), 31.9 (s, ^{i}Pr/CH), 32.9 (d, $^3J_{CC}$ 4.0 Hz, butyronitrile/4-CH_2), 52.4 (s, OCH_3), 52.5 (s, OCH_3'), 54.6 (s, 5-CH), 60.8 (s, 2-CH), 119.7 (d, $^1J_{CC}$ 56.3 Hz, ^{13}CN). All other ^{1}H-NMR parameters are the same as for **14a**.

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