Synthesis of ^{15}N-Labelled D-Isovaline and α-Aminoisobutyric Acid

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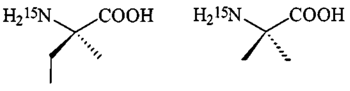
Keywords: α,α-Dialkylated amino acids / Isotopic labelling / Strecker synthesis / Solvent effects / Stereoselective hydrolysis

[^{15}N]-D-isovaline was prepared from DL-[α-^{15}N]-α-aminoisovaleramide by enzymatic resolution with *Mycobacterium neoaurum*. The ^{15}N-isotope was introduced during the Strecker synthesis of its precursor, e.g. aminoisovaleronitrile. Attempts to prepare the amino nitrile precursor of [^{15}N]-α-aminoisobutyric acid (Aib) led to a poor yield and loss of the label. Significantly, improved results were obtained when a cosolvent is present during formation of aminoisobutyronitrile.

Introduction

α,α-Dialkylated amino acids such as D-isovaline (D-Iva) and α-aminoisobutyric acid (Aib) are important constituents of peptaibol antibiotics. These linear peptides are thought to act through ion channel formation in cell membranes, thereby dissipating the *trans*-membrane potential of bacteria. ^{15}N-Isotopically enriched amino acids are important precursors for site directed labelling of peptaibol molecules[1] which can be used to investigate the structure and function of ion channels in phospholipid membranes at the molecular level by means of isotope sensitive spectroscopic techniques.[2]



[^{15}N]D-Iva [^{15}N]Aib

Figure 1. Synthesized α,α-dialkylated amino acids

Numerous methods have been developed for the preparation of α,α-dialkylated amino acids.[3] Many of these are based on the Strecker amino nitrile synthesis.[4] The aim of the present research is to exploit this strategy for quantitative incorporation of $^{15}NH_4Cl$ into Aib and the synthesis of enantiopure D-Iva.

Results and Discussion

In the first step of the route to [^{15}N]-D-Iva (see Scheme 1) the labelled amino nitrile was prepared (in quantitative yield) from 2-butanone, sodium cyanide and $^{15}NH_4Cl$ (molar ratio 2:2:1). The amino nitrile was then selectively hydrolysed to the corresponding amide with formic acid saturated with hydrogen chloride in the presence of an equimolar amount of water. On treatment of the racemic amide with whole cells of *Mycobacterium neoaurum* ATCC 25795, stereoselective hydrolysis of [^{15}N]-L-Iva-NH$_2$ occurred and gave [^{15}N]-D-Iva-NH$_2$. Since the amidase in *M. neoaurum* has only a moderate stereoselectivity for Iva-NH$_2$ (enantiomeric ratio $E=19$)[5] a conversion of more than 60% is necessary to obtain enantiopure [^{15}N]-D-Iva-NH$_2$. Separation of the labelled D-Iva-NH$_2$ from the hydrolysed L-Iva was achieved by cation exchange chromatography. Hydrolysis with 12 M HCl solution gave the isotope-substituted D-Iva (99% ^{15}N) in high enantiomeric purity ($>$ 99.5% e.e.) and in 31% isolated yield.

Our attempt to label the second naturally occurring α,α-disubstituted amino acid, Aib, by similar conditions used for the synthesis of Iva, was not successful due to a poor yield (20%) of the Strecker product, α-aminobutyronitrile, and a high level of isotope dilution (75% of the amino nitrile is unlabelled). Since the organic nitrile is the only plausible ^{14}N-source, alkaline hydrolysis of the amino nitrile must be considered as a possibile explanation for our observations (despite the fact that nitriles are relatively stable, even at pH of 9.7 in the current Strecker reaction). A decrease in pH, by addition of an extra equivalent of ammonium chloride, does not have a significant effect on the yield of the amino nitrile. However, even when the Strecker reaction is performed *in the absence* of ammonium chloride, the free amino acid Aib could be isolated in 15% yield (Aib was isolated with ion-exchange chromatography and identified by NMR) and a qualitative test showed the presence of free ammonia in the vapour phase of the closed

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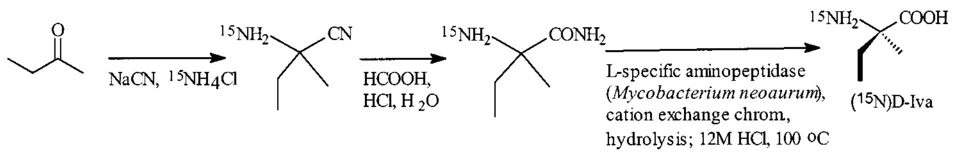
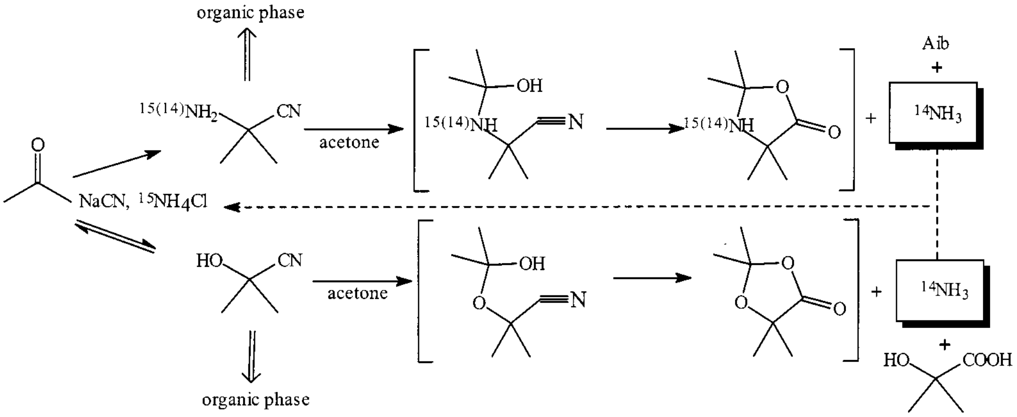


Scheme 1. Synthesis of enantiomerically pure [^{15}N]-D-Iva



Scheme 2. Mechanism of the ^{15}N-isotope dilution observed for [^{15}N]isobutyronitrile; hydrolysis of the amino nitrile is prevented by addition of dichloromethane to the aqueous reaction mixture

reaction vessel. These results are consistent with the mechanism as shown in Scheme 2.

Unlabelled ammonia is supposed to be liberated after formation of 2,2,4,4-tetramethyloxazolidine-5-one, the addition intermediate of acetone and amino nitrile. Another plausible source for unlabelled ammonia is the addition product of cyanohydrin and acetone, since it is has previously been observed that amino nitriles, as well as cyanohydrins, condense with acetone to form compounds with 5-membered ring structures (Scheme 2).[6] In addition, we have detected ammonia, when a mixture of cyanohydrin (0.1 mol) and acetone (0.1 mol) was kept in 40 mL of borate buffer (pH 10) at room temperature for two h. No ammonia was detected when the pure cyanohydrin (i.e. in the absence of acetone) was kept at pH 10.

The reason that hydrolysis of the nitrile group does not occur during the reaction with 2-butanone can be easily explained by the low water solubility of α-aminoisovaleronitrile (it separates during the course of the reaction). In order to prevent hydrolysis of α-aminoisobutyronitrile, we protected the nitrile group of this compound by adding CH_2Cl_2 to the aqueous reaction mixture and were consequently able to prepare the [^{15}N]-α-aminoisobutyronitrile intermediate with quantitative isotope incorporation. After acid hydrolysis the nitrile was converted into the final product [^{15}N]Aib in significantly improved yield (65% based on $^{15}NH_4Cl$).

The yield of amino nitrile, formed in the presence of another cosolvent, was determined for ethyl acetate (40%), diethyl ether (36%) and *n*-hexane (32%). Evidently, the partition of the amino nitrile is better in a more polar solvent such as CH_2Cl_2.

Conclusion

We have synthesized highly isotope-enriched (98% ^{15}N) and optically pure [^{15}N]-D-Iva and we have improved the Strecker conditions in order to obtain quantitative label incorporation into Aib.

Experimental Section

General Remarks: Reagents like sodium cyanide (Merck, pro analysis), propanone (Merck, pro analysis) and 2-butanone (Aldrich, A.C.S. grade) were used without further purification. Reactions were generally monitored using thin layer chromatography (TLC, on Merck F_{254} silica gel 60 coated aluminium sheets, 0.2 mm); spots were visualized by ninhydrin reagent (0.2% in ethanol). Ammonia was detected in the gas phase by means of a pH indicator. Amberlite CG-50 ion exchange resin was obtained from Bio-Rad. The HPLC apparatus consisted of a Pharmacia gradient delivery system, a Rheodyne injection valve fitted with a 5-μL loop, and a Spectro Vision FD-300 fluorescence detector. The derivatization reagent *N*-acetyl-L-cysteine was purchased from Aldrich). Fast Atom Bombardment mass spectrometry (FAB-MS) was performed with a Jeol JMS-SX/SX 102A mass spectrometer. ^{1}H-NMR and ^{13}C-NMR spectra were recorded using a Bruker WM-300 and a Bruker DMX-600 spectrometer. Chemical shifts are given relative to that of the internal standard tetramethylsilane.

General Method for Synthesis of ^{15}N-Labelled Amino Nitriles: The ketone (200 mmol) was added to a solution of [^{15}N]NH_4Cl (100 mmol) (Cambridge Isotope Laboratories) and sodium cyanide (200 mmol) in water (40 mL) and dichloromethane (40 mL). The reaction mixture was stirred in a closed vessel for 24 h at room temperature. The product was isolated by extraction with dichloromethane, followed by drying with magnesium sulfate and evaporation of the solvent.

[α-^{15}N]-α-Aminobutyronitrile: Yield 65%; – ^{1}H NMR (200 MHz, CDCl$_3$): δ 1.50 (d, 6 H, $^3J_{CH3–15N}$ = 3.2 Hz, 2×CH$_3$).

DL-[α-^{15}N]-α-Aminoisovaleronitrile: yield 100%; – ^{1}H NMR (600 MHz, CDCl$_3$): δ 1.695 (dq, 2 H, $^3J_{CH2-CH3}$ = 7.5 Hz, $^3J_{CH2–15N}$ = 1.4 Hz, CH$_2$); 1.464 (d, 3 H, $^3J_{CH3–15N}$ = 3.0 Hz, ^{α}CH$_3$); 1.097 (t, 3 H, $^3J_{CH3-CH2}$ = 7.5 Hz, ^{β}CH$_3$).

DL-[α-^{15}N]-α-Aminoisovaleramide Hydrochloride: Dry HCl gas was passed into a solution of DL-[α-^{15}N]-α-aminoisovaleronitrile (100 mmol) in 50 mL of formic acid (50 mL) for 1 h. Water (100 mmol) was then added to the reaction mixture. The mixture was allowed to stand for 1 h and then concentrated. The product, which crystallized upon addition of 2-propanol, was isolated by filtration: yield 90%. – ^{1}H NMR (300 MHz, MeOD): δ = 2.000 (dq, 1 H, $^3J_{CH-CH3}$ = 7.5 Hz, $^3J_{CH-15N}$ =3.6 Hz, CH$_{R(S)}$); 1.882 (dq, 1 H, $^3J_{CH-CH3}$ = 7.5 Hz, $^3J_{CH-15N}$ = 2.1 Hz, CH$_{S(R)}$); 1.575 (d, 3 H, $^3J_{CH3–15N}$ = 2.8 Hz, ^{α}CH$_3$); 1.000 (t, 3 H, $^3J_{CH3-CH2}$ = 7.5, ^{β}CH$_3$).

D-[α-^{15}N]Aminoisovaleramide: Whole cells of *Mycobacterium neoaurum* ATCC 25795 (700 mg) were added to a solution of DL-[α-^{15}N]-α-aminoisovaleramide (90 mmol) in water (90 mL, the pH was adjusted to 8.5 with 5 M potassium hydroxide) and the mixture incubated at 38 °C. After an incubation time of 19 h, the conversion was determined to be 66% and the reaction was stopped. The cells were separated by centrifugation and the solvent evaporated. The mixture of labelled L-isovaline and D-aminoisovaleramide was applied to a cation exchange column (diameter 5 cm, length 20 cm; containing Amberlite CG-50 (200–400 mesh, type I). L-Iva was eluted with water (yield 69%) and D-Iva-NH$_2$ was subsequently eluted with 1 M ammonia (yield 31%). The enantiomeric excess of the product was determined to >99.5% by analytical reversed phase (C18) HPLC (Spherisorb ODS-2, gradient from methanol/0.03 M NaOAc (pH 6.12) 15/85 to 100% methanol, 0.4 mL/min, derivatization with *o*-phthalaldehyde and *N*-acetyl-(*R*)-cysteine.[7]

D-[α-^{15}N]Isovaline: [α-^{15}N]-D-Iva-NH$_2$ (28 mmol) was treated with 12M hydrochloric acid (50 mL) for 2 h. The solvent was evaporated *in vacuo*, the residue redissolved in 2-propanol (20 mL) and the solvent again evaporated to give the product; yield 100%. – ^{1}H NMR (600 MHz, MeOD): δ 2.001 (dq, 1 H, $^3J_{CH-CH3}$ = 7.5 Hz, $^3J_{CH-15N}$ = 3.6 Hz, CH$_{R(S)}$); 1.897 (dq, 1 H, $^3J_{CH-CH3}$ = 7.5 Hz, $^3J_{CH-15N}$ = 2.1 Hz, CH$_{S(R)}$); 1.575 (d, 3 H, $^3J_{CH3–15N}$ = 2.8 Hz, ^{α}CH$_3$); 1.000 (t, 3 H, $^3J_{CH3-CH2}$ = 7.5, ^{β}CH$_3$). – ^{15}N NMR (60.8 MHz, MeOD): δ 24.99. – Positive FAB MS (glycerol matrix): base peak *m*/*z* 119.0813; calculated label percentage >98%.

[α-^{15}N]Aminoisobutyric Acid: A solution of [α-^{15}N]-α-aminoisobutyronitrile (65 mmol) in 12 M HCl was heated at 100 °C for 2 h. The solvent was evaporated and the residue treated with absolute ethanol in order to remove traces of water. Filtration and evaporation of the solvent afforded the product in 65% yield. – ^{1}H NMR (300 MHz, MeOD: δ 1.57 (d, 6 H, $^3J_{CH3–15N}$ = 2.7 Hz, 2×CH$_3$). – ^{15}N NMR (30.4 MHz, MeOD): δ 48.63. – Positive FAB MS (glycerol matrix): base peak *m*/*z* 105.0813; calculated label percentage >98%.

Acknowledgments

This investigation was financially supported by the Netherlands Organisation for Scientific Research (NWO); Project 047.006.009. The authors wish to thank Dr. Alexei Ogrel for his active contribution to this research. We thank Mr. A. W. M. Lefeber for measurement of the NMR spectra and Mr. K. Versluis (University of Utrecht, Utrecht, The Netherlands) for recording FAB mass spectra. Prof. Dr. J. Lugtenburg and Dr. J. Brussee are greatly acknowledged for their contribution during discussions.

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Received June 18, 1999
[O99363]