SYNTHESIS OF [1-^{13}C] AND [1-^{15}N] LABELLED *DL*-HOMOPHENYLALANINE *VIA* A KEY NEBER REARRANGEMENT.

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Abstract

A synthetic route involving a key Neber rearrangement is described for the preparation of both [1-^{13}C] and [1-^{15}N] *DL*-homophenylalanine (2-amino-4-phenylbutanoic acid), using suitably labelled sodium cyanide as the source of the isotopic label. These compounds have been prepared for use in studies on the biosynthesis of phenylethyl glucosinolate in *Brassica napus*. 3-Phenylpropanaldoxime, the initial biosynthetic product formed from homophenylalanine, was also prepared in ^{15}N labelled form.

Keywords: glucosinolates, Neber rearrangement, *Brassica*, homophenylalanine

Introduction

Glucosinolates are a group of thioglycosides that occur in all members of the Cruciferae, including the *Brassica* vegetables such as cabbage, Brussels sprouts and oilseed rape (1). The role of these chemicals in the plant is for defence. The glucosinolates are hydrolysed by an enzyme, myrosinase, following cell damage by pests and pathogens. This produces isothiocyanates, which are effective antifungal and antimicrobial agents as well as deterring insects and other herbivores. However certain specialist pests and pathogens of crucifers have become adapted to glucosinolate-containing plants, and in fact use some of the volatile isothiocyanates as host recognition cues (2). Current strategies to enhance the natural crop protection of oilseed rape involve the use of genetic manipulation to remove the glucosinolates which produce isothiocyanates recognised by the pests (and it has been shown that such insects

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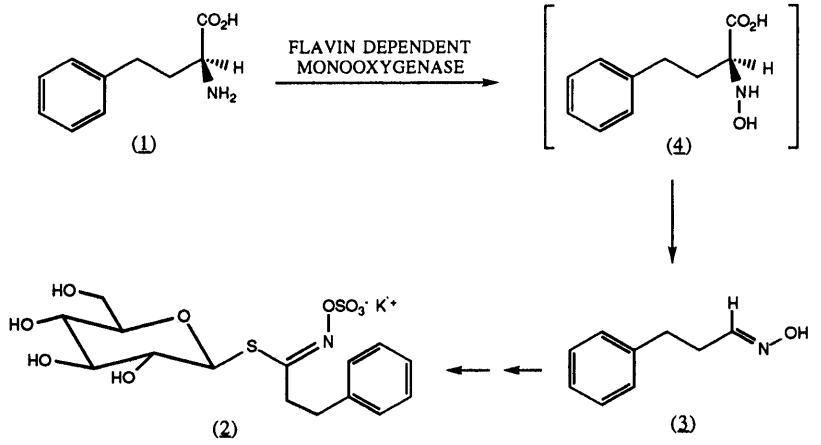
only "taste" a very few isothiocyanates, those derived from certain alkenyl glucosinolates), and perhaps introduce novel glucosinolates. Such modified plants would have little requirement for agrochemical applications, and the production costs (of great significance for industrial and energy uses of rapeseed oil, for example) would be greatly reduced. However in order for genetic modification to be rationally employed, it is essential that the biosynthetic pathways to the glucosinolates are clearly understood.

We have an ongoing collaboration with the IACR (Institute of Arable Crops Research) to investigate the early stages of the biosynthetic pathway and for these studies require access to ^{13}C and ^{15}N labelled homophenylalanine (2-amino-4-phenylbutanoic acid). This amino acid (1) is the precursor of phenethyl glucosinolate (2). The amino acid is first converted to the corresponding aldoxime (3) and in *Brassica napus* (oilseed rape) this has been shown to be catalysed by a flavin dependent enzyme (3,4) in contrast to the cytochrome P450 enzymes employed by other plant species (5). However the chemical mechanism of this transformation is not known although it has been proposed that an *N*-hydroxy amino acid (4) is an intermediate. The ^{15}N labelled homophenylalanine will be used in NMR studies to attempt to identify intermediates in this reaction.



SCHEME 1

Results and Discussion

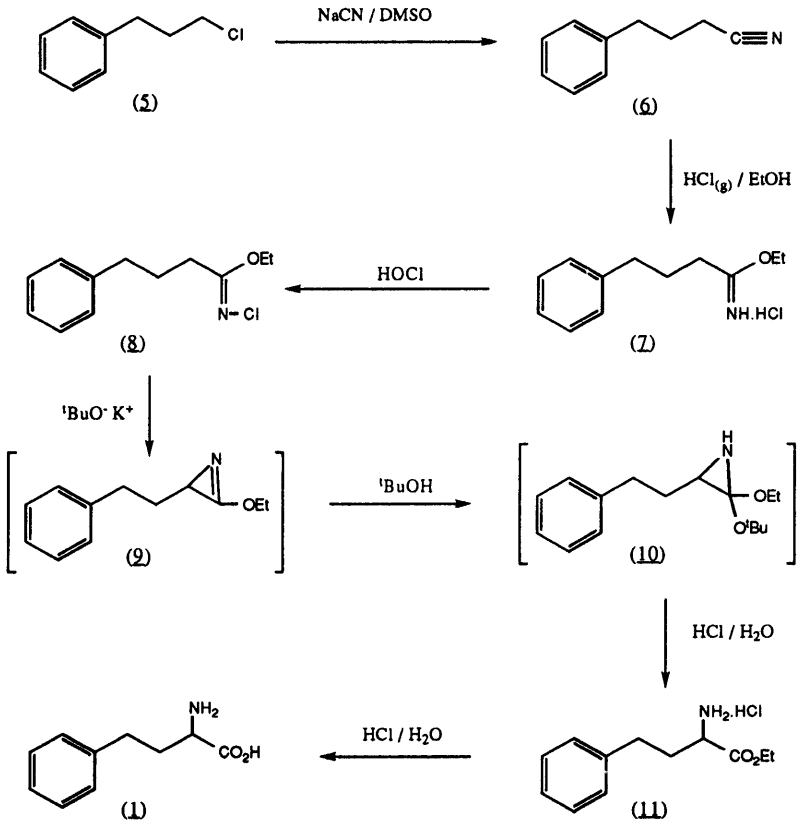
A number of approaches are available for the synthesis of amino acids labelled with ^{15}N in the amino group, including some very elegant chemo-enzymatic methods employing

dehydrogenase enzymes (6,7). For our purposes a versatile method which would allow incorporation of either ^{15}N or ^{13}C was required. Racemic homophenylalanine was employed in the initial biological work, although it was shown that only the *L*-enantiomer was active as a substrate for the oxygenase (8). It was thus intended to also prepare the labelled material in racemic form, allowing the *D*-enantiomer, which is not turned over, to serve as an internal reference for the ^{15}N NMR studies. The route chosen for the synthesis of the labelled homophenylalanine was from the alkyl cyanide utilising a key Neber rearrangement. This allows sodium cyanide to be employed as the source of the label. The same route can be used to incorporate ^{13}C and ^{15}N separately, or in combination, as all the required labelled sodium cyanide starting materials are commercially available. It could also be used to incorporate ^{14}C. The synthetic route is outlined in Scheme 2. This synthetic method has been previously employed for the preparation of amino acids (9), including vinyl glycine (10,11), but not for the production of labelled compounds.

The synthesis was first optimised with unlabelled materials. Thus 1-chloro-3-phenyl propane (5) was reacted with sodium cyanide in dimethyl sulfoxide to produce 4-phenyl butyronitrile (6) in 75 % yield, *via* nucleophilic substitution. Conversion into the corresponding ethyl imidate hydrochloride (7) was achieved in 75 % yield by reaction with gaseous HCl in ethanol. Commercial bleach was used as the source of chlorine for the *N*-chlorination to give the *N*-chloro imidate (8) in almost quantitative yield. This rather unstable compound was then used without purification for the base catalysed Neber rearrangement. Treatment with potassium ^{t}butoxide results in cyclisation to give the strained aziridene (9), which is attacked by ^{t}butanol. Acid hydrolysis followed immediately to cleave the proposed intermediate (10) and also hydrolyse the ethyl ester (11), to give the final product in 71 % yield. It was found that to obtain good yields it was essential that the potassium ^{t}butoxide was freshly prepared. The homophenylalanine was thus obtained in 40 % yield over 4 steps.

The reactions were then repeated using sodium [^{13}C] cyanide and sodium [^{15}N] cyanide to furnish the corresponding labelled derivatives. The synthesis was equally effective in both cases. [1-^{13}C] Homophenylalanine was obtained in 48 % overall yield. The identity of the compound was confirmed by electrospray mass spectrometry which showed a MH^+ ion at 181 and ^{13}C NMR where the resonance at 174.5 ppm, due to the C-1 carbon could be seen to be

enhanced. [1-^{15}N] Homophenylalanine was obtained in 50 % overall yield from sodium [^{15}N] cyanide. This compound gave the expected MH^+ ion at 181 by electrospray mass spectrometry. The ^{15}N NMR spectrum showed a single resonance at 36.45 ppm, which is consistent with an amino nitrogen from an amino acid.



The ^{15}N labelled aldoxime, 3-phenylpropanaldoxime, was also prepared as a standard for the proposed NMR studies. Reaction of [^{15}N]-hydroxylamine and 3-phenyl propionaldehyde gave the aldoxime (3) in 55 % yield. This material gave a single resonance at 354.4 ppm in the ^{15}N NMR spectrum.

It can thus be seen that the synthesis of amino acids *via* the Neber rearrangement route described presents a very useful method for the incorporation of a ^{15}N atom into the amino group, in good overall yield.

Experimental

Materials and Methods

Sodium [^{13}C] cyanide (99 %), sodium [^{15}N] cyanide (98 %+) and [^{15}N]-hydroxylamine (98 %+) were obtained from Promochem. ^{1}H, ^{13}C and ^{15}N NMR spectra were obtained using a Varian 2000 f.t spectrometer (^{1}H, 300 MHz; ^{13}C, 75.42 MHz; ^{15}N, 30.416 MHz) and a Varian Gemini f.t spectrometer (^{1}H 200 MHz; ^{13}C, 50.31 MHz). Mass spectra were recorded on a Fisons VG Platform (Electrospray) using acetonitrile as the solvent system.

4-Phenylbutyronitrile (6)

To a flask containing sodium cyanide (0.54 g, 11 mmol) in dimethyl sulfoxide (15 ml) was added 1-chloro-3-phenylpropane (1.43 ml, 10 mmol) and the mixture heated to reflux for 20 hours. After allowing the resultant mixture to cool, water (30 ml) was added and the reaction mixture extracted with diethyl ether (3 x 50 ml). The organic layers were then combined and washed with 6N HCl (2 x 50 ml) and water (50 ml). They were then dried ($CaCl_2$), filtered and concentrated at reduced pressure to yield a brown oil. Purification by column chromatography on silica gel (10 % ethyl acetate in petroleum ether) yielded the desired product (1.08 g, 75%) as a colourless oil; δ_H (200 MHz, $CDCl_3$) 7.15-7.4 (m, 5H, Ar-H), 2.78 (t, 2H, J = 7 Hz, CH_2CN), 2.35 (t, 2H, J = 7 Hz, $PhCH_2$), 2.02 (m, 2H, $PhCH_2CH_2$); δ_C (50.31 MHz, $CDCl_3$) 140.2, 129.2, 128.9, 127.0, 120.0, 34.9, 34.8, 27.3; *m/z* (EI) 145 (MH^+, 37 %).

Ethyl 4-phenylbutyl imidate hydrochloride (7)

Dry hydrogen chloride gas was bubbled through a solution of 4-phenylbutyronitrile (1.08 g, 7.5 mmol) in absolute ethanol (0.44 ml, 7.5 mmol) cooled in ice. After the solution had turned deep yellow (approx 30 min), the mixture was allowed to stand in the refrigerator overnight. Diethyl ether (6 ml) was then added and the solution stirred until the product crystallised out of solution. The resulting crystals were filtered and dried leaving the desired product (1.28 g, 75 %); δ_H (200 MHz, $CDCl_3$) 7.15-7.35 (m, 5H, Ar-H), 4.15 (q, 2H, J = 7 Hz, OCH_2CH_3), 2.68 (t, 2H, J = 7 Hz, $PhCH_2CH_2CH_2$), 2.33 (t, 2H, J = 7 Hz, $PhCH_2CH_2$), 1.85-2.05 (m, 2H, $PhCH_2CH_2$), 1.27 (t, 3H, J = 7 Hz, OCH_2CH_3); δ_C (50.31 MHz, CD_3OD) 174.0, 129.0, 128.9, 126.5, 35.7, 34.9, 33.8, 27.1, 14.8.

Ethyl *N*-chloro 4-phenylbutyl imidate (8)

To a flask containing commercial bleach (100 ml) cooled in ice was added ethyl 4-phenylbutyl imidate hydrochloride (1.28 g, 5.6 mmol) and the mixture stirred for 1 h. The resultant mixture was extracted with hexane (3 x 50 ml) and the organic layers combined, dried ($MgSO_4$), filtered and concentrated. The colourless oil obtained (1.24 g, 98 %) was used immediately.

***DL*-Homophenylalanine (11)** (2-Amino-4-phenylbutanoic acid)

Potassium metal (0.42 g, 10.7 mmol) was added to ^{t}butanol (10 ml) under a strong flow of nitrogen. The solution was then heated to reflux until all the potassium metal had dissolved. The solution was cooled to room temperature and ethyl *N*-chloro 4-phenylbutyl imidate (1.24 g, 5.5 mmol) dissolved in hexane (5 ml) was added dropwise. The reaction was then stirred at room temperature under nitrogen for 72 hours. After this time, 2N HCl (30 ml) was added and the resulting solution concentrated at reduced pressure. To the residue was added 6N HCl (50 ml) and the reaction heated to reflux for 4 hours. The reaction mixture was allowed to cool and then concentrated at reduced pressure. Water (10 ml) was added to the residue and the resultant solution readjusted to pH 7 whereupon a precipitate appeared. The solid was removed by filtration, washed with water (10 ml) and ethyl acetate (10 ml) and then dried in a vacuum desiccator leaving the desired amino acid (1.52 g, 71 %); m. p. > 300 °C (dec); (Found: C, 66.61; H, 7.20; N, 7.40; $C_{10}H_{13}NO_2$ requires C, 67.02; H, 7.31; N, 7.82 %); δ_H (200 MHz, CD_3OD) 7.15-7.35 (m, 5H, Ar-H), 3.96 (t, 1H, J = 7 Hz, CH_2CH), 2.73 (t, 2H, J = 7 Hz, $PhCH_2CH_2$), 2.15-2.3 (m, 2H, $PhCH_2CH_2$); δ_C (50.31 MHz, CD_3OD) 174.5, 141.5, 130.0, 129.7, 127.9, 49.5, 33.9, 32.4; *m/z* (electrospray) 180 (MH^+,10%), 135 (100%).

[1-^{13}C]-4-Phenylbutyronitrile.

The same procedure was used as for the above outlined synthesis but substituting sodium [13-C] cyanide (1 g, 20 mmol). The product was obtained as a colourless oil (2.1 g, 80%); Spectral data as above but with the signal at 120.0 ppm in the ^{13}C NMR enhanced and a molecular ion of 146 (MH^+) by electrospray mass spectrometry.

Ethyl [1-^{13}C]-4-phenylbutyl imidate hydrochloride.

The above procedure was repeated using [1-^{13}C]-4-phenylbutyronitrile (2.1 g, 14.5 mmol) to give the desired product (2.48 g, 80 %); Spectral data as above but with the signal at 174.0 ppm in the ^{13}C NMR enhanced.

[1-^{13}C]-*DL*-Homophenylalanine ([1-^{13}C]-2-Amino-4-phenylbutanoic acid)

The above procedure was repeated using ethyl [1-^{13}C]-4-phenylbutyl imidate hydrochloride (2.48 g, 11.6 mmol) to give the desired product (1.52 g, 75 %). Spectral data as above but with the signal at 174.5 ppm in the ^{13}C NMR enhanced and a molecular ion of 181 (MH^+) by electrospray mass spectrometry.

[1-^{15}N]-*DL*-Homophenylalanine ([2-^{15}N]-2-Amino-4-phenylbutanoic acid)

The same synthetic route was used as for the above outlined synthesis but substituting sodium [^{15}N] cyanide (0.5 g, 10 mmol) in the first reaction step to give [2-^{15}N]-homophenylalanine (0.7 g, 50 % overall yield). δ_H (200 MHz, CD_3OD) 7.15-7.35 (m, 5H, Ar-H), 3.96 (t, 1H, J = 7 Hz, CH_2CH), 2.73 (t, 2H, J = 7 Hz, $PhCH_2CH_2$), 2.15-2.3 (m, 2H, $PhCH_2CH_2$); δ_C (50.31 MHz, CD_3OD) 174.6, 141.3, 130.0, 129.6, 127.9, 49.3, 33.8, 32.4; δ_N (30.416 MHz, 1:1 $CHCl_3$: $CDCl_3$) 36.45 (referenced to saturated NH_4Cl in $CHCl_3$ at δ = 27.34 ppm); *m/z* (electrospray) 181 (MH^+, 12%), 135 (100%)

[1-^{15}N]-3-Phenylpropanaldoxime

To a solution of 3-phenyl propionaldehyde (0.63 g, 4.7 mmol), pyridine (0.5 ml) and ethanol (1 ml), was added [^{15}N] hydroxylamine hydrochloride (0.5 g, 7.1 mmol) and the mixture heated to reflux for 2 hours. The solution was allowed to cool and then concentrated at reduced pressure. Water (10 ml) was added and the resulting precipitate filtered off and washed with water. Recrystallisation gave the product as white crystals (0.39 g, 55 %) m. p. 91-93 °C (Lit. (12) 93-94.5 °C, δ_H (200 MHz, CD_3OD) 7.15-7.35 (m, 5H, Ar-H), 6.66 (2 x triplets, 1H, J = 7 Hz , CHNOH (*cis* and *trans* isomers)), 2.82 (m, 2H, $PhCH_2CH_2$), 2.67 (m, 2H, $PhCH_2CH_2$); δ_C (50.31 MHz, CD_3OD) 152.3, 129.8, 129.6, 127.4, 33.3, 27.9; δ_N (30.416 MHz, CD_3OD) 354.4 (referenced to saturated $(Me)_4NCl$ at δ = 45.68 ppm); *m/z* (electrospray) 181 (MH^+, 12%), 135 (100%)

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