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Enzymatic Synthesis of Isotopically Labelled Serine and Tryptophan for Application in Peptide Synthesis

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Abstract: L-[1,2-$^{13}C_2$,^{15}N]Serine was prepared from [1,2-$^{13}C_2$,^{15}N]glycine on a gram scale by the use of the enzyme serine hydroxymethyltransferase. The reaction was monitored by ^{13}C-NMR spectroscopy. This is the first simultaneously ^{13}C- and ^{15}N-labelled serine isotopomer so far reported. Part of the product was directly converted by tryptophan synthase to L-[1,2-$^{13}C_2$,^{15}N]tryptophan which could conveniently be purified and isolated as Boc-derivative in a yield of 71%. Most of the serine was isolated similarly but to remove remaining starting material in this case purification by column chromatography was required. © 1997 European Peptide Society and John Wiley & Sons, Ltd.

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Amino acids labelled with stable isotopes were first applied in metabolic investigations but have later also been used to solve various biosynthetic and mechanistic problems. A more recent application is in structural studies of proteins by NMR spectroscopy. Up to the end of June 1996 over 170 isotopomers of the 20 proteinogenic amino acids labelled with ^{13}C and/or ^{15}N were listed in *Chemical Abstracts*. A modest number are nowadays commercially available. A review on the synthesis of such amino acids has also recently been published [1].

In the Uppsala laboratory we are particularly interested in preparing amino acid derivatives labelled in the backbone with stable isotopes for application in peptide synthesis [2,3]. For simple ^{15}N-labelled amino acids we had already worked out a synthetic procedure involving amination of α-hydroxy acids of opposite configuration [4,5], whereas ^{13}C,^{15}N-labelled acids were made by asymmetric synthesis involving anionic glycine equivalents [6,7], obtained from the proper isotopomer [8]. Both procedures give useful amounts of products of very high optical purity, in most cases 99.0–99.5% ee or better.

Five ^{13}C-labelled isotopomers of serine as well as [^{15}N]serine have been reported in the literature but none containing both nuclei simultaneously. Most of them were synthesized using biosynthetic and enzymatic methods. In this work we have applied an enzymatic procedure involving serine hydroxymethyltransferase (EC 2.1.2.1.) developed in the Dublin laboratory, which was first applied several years ago to make [2-^{13}C]serine on a small scale but only briefly reported then [9,10]. Full experimental details of this procedure will now be given. Modern methods of asymmetric synthesis have only been used for isotopic labelling of serine very recently [11].

Nine tryptophan isotopomers labelled with one ^{13}C and two labelled with one ^{15}N nucleus are on

Abbreviations: The amino acid names refer to the L-configuration throughout.

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record in the literature in addition to one with both ^{13}C in the backbone and ^{15}N in the indole ring. Three papers with particular relevance to the present work in which tryptophan synthase (EC 4.2.1.20) has been used should be mentioned in this context [12–14]. In the first of these commercial *E. coli* cells are the source of the enzyme, whereas the latter two contain additional elegant syntheses of labelled indoles.

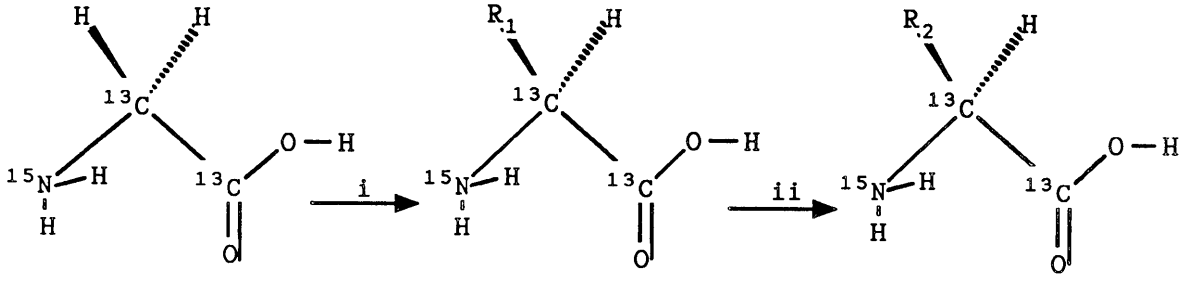
Serine hydroxymethyltransferase catalyses the formation of serine from glycine using 5,10-methylenetetrahydrofolate as a C_1 donor [15]. Serine hydroxymethyltransferase from beef liver has been used to effect the synthesis of [2-^{13}C]serine from [2-^{13}C]glycine [9,10]. In the present work serine hydroxymethyltransferase from *E. coli* was used to catalyse the synthesis of [1,2-$^{13}C_2$,^{15}N]serine from [1,2-$^{13}C_2$,^{15}N]glycine. The experimental conditions are essentially the same, except that the concentrations were increased: pyridoxal phosphate from 0.06 to 0.1 mM, formaldehyde from 30 to 50 mM, active enzyme from 1.2 to 8.0 μM (this corresponds to increasing the catalytic activity of the enzyme from 0.14 to 0.69 μmol/ml/min, using L-allothreonine as a substrate [16]); the concentration of 2-mercaptoethanol was decreased from 10 to 5 mM. The production of serine under these conditions with the *E. coli* enzyme approximated to a first order process with a half life of 2 h as was observed with the beef liver enzyme [9,10]. On adding serine hydroxymethyltransferase to the incubation mixture (see Experimental Part) there was a progressive decrease in intensity of the signals due to [1,2-$^{13}C_2$,^{15}N]glycine (δ_C (25.115 MHz, D_2O) 42.1, (dd, $J_{C,C}=53.4$, $J_{C,N}=6.3$, α-^{13}C), 173.2 (d, $J_{C,C}=53.4$, ^{13}COOH)) and a concomitant increase of the signals due to [1,2-$^{13}C_2$,^{15}N]serine (57.1 (dd, $J_{C,C}=53.0$, $J_{C,N}=6.2$, α-^{13}C), 173.1 (d, $J_{C,C}=53.0$, ^{13}COOH)). After 36 h at 37 °C, ~93% of the [1,2-$^{13}C_2$,^{15}N]glycine was converted to [1,2-$^{13}C_2$,^{15}N]serine.

Tryptophan synthase catalyses the formation of tryptophan from indole and serine [17]. We have used tryptophan synthase from *Salmonella typhimurium* to effect the formation of [1,2-$^{13}C_2$,^{15}N]tryptophan from [1,2-$^{13}C_2$,^{15}N]serine and indole (see Experimental Part). On adding tryptophan synthase to the reactants, there was a progressive decrease in intensity of the signals due to [1,2-$^{13}C_2$,^{15}N]serine and a concomitant increase of those due to [1,2-$^{13}C_2$,^{15}N]tryptophan (56.0 (dd, $J_{C,C}=53.8$, $J_{C,N}=6.1$, α-^{13}C), 175.4 (d, $J_{C,C}=53.8$, ^{13}COOH)). After 48 h at 37 °C, ~95% of the [1,2-$^{13}C_2$,^{15}N]serine had been converted to [1,2-$^{13}C_2$,^{15}N]tryptophan.

Most of the crude serine isotopomer was directly converted to the corresponding Boc-derivative [18]. In this process a slight preferential reaction of remaining glycine was noticed. The crude DCHA salt isolated contained 5% Boc-glycine. According to NMR this figure could be lowered by further recrystallization(s) but this turned out to be a dead end as amino acid analysis indicated that too much glycine still remained. Therefore, the product was finally purified by column chromatography on silica, as a result of which a completely pure product was obtained as a DCHA salt in modest 56% yield from crude serine and 31% yield from glycine.

Similarly the crude isotopically labelled tryptophan was converted to Boc-derivative. Although it contained small amounts of both glycine and serine it was more easily purified by recrystallization and DCHA-salt formation as reflected in the higher yield of product (70% from crude tryptophan). The total yield over three steps, from glycine to Boc-tryptophan, was 26%.

By making serine from glycine with serine hydroxymethyltransferase, chirality was introduced. To make serine by asymmetric synthesis would have required the use of a masked derivative of formaldehyde [11] (a formaldehyde equivalent) and therefore resulted in an OH-protected derivative. Such derivatives are often preferred in synthetic work and



Scheme 1 R_1 = $HOCH_2$- (serine) and R_2 = (3-indolyl)CH_2- (tryptophan); *Reagents:* (i) formaldehyde, serine hydroxymethyltransferase; (ii) indole, tryptophan synthase.

methods resulting in free and protected OH-functions are therefore complementary.

The conversion of glycine to serine as monitored by ^{13}C-NMR was essentially quantitative but loss of material was encountered in the further processing of the reaction mixture involving the removal of excess formaldehyde, etc. which therefore remains to be optimized in future experiments. Once this has been accomplished the whole set of ^{13}C and/or ^{15}N serine isotopomers should be available similarly on a preparative scale [8]. It might also be rational to insert an additional purification step to remove unreacted glycine [19,20], the presence of which was the major reason for the modest yield of the corresponding Boc-derivative.

Serine can be converted to β-cationic [21–23] as well as β-anionic [24] equivalents of alanine and is therefore of particular importance as a precursor for other chiral amino acids. Thus from protected iodide and tosylate [21] and serine β-lactone [22], phenylalanine and a large number of non-proteinogenic amino acids, and from the latter reagent also valine and leucine, have been made with organocuprates. Such iodides also undergo reaction with zinc. These intermediates can then be acylated and, more importantly, reacted with various aromatic compounds to provide substituted phenylalanines, etc. [24]. Consequently for the synthesis of labelled species of serine-derived amino acids it is important to have a good source for the corresponding serine isotopomer. Labelled aspartic acids have been made from serine β-lactone [25].

In their pioneering related work Lugtenburg and co-workers [13] obtained indole-labelled tryptophans in 97% yield, calculated on indole, after ion-exchange chromatography. Unkefer *et al.* [14] reported a 98% yield from labelled indole and 82% from labelled serine after a similar isolation procedure, whereas Yuan and Ajami [12] briefly stated they received two isotopomers labelled in the indole ring in 40–50% yield. Together these papers demonstrate that the enzymatic conversion of labelled serine to tryptophan by tryptophan synthase constitutes a practically very useful procedure on the preparative scale.

EXPERIMENTAL PART

(1,2-$^{13}C_2$,^{15}N)Glycine · hydrochloride

Boc-[1,2-$^{13}C_2$,^{15}N]glycine [8] (6.00 g, 33.7 mmol) was dissolved in 4 M HCl in dioxane (40 ml). After 2 h most of the dioxane was evaporated off and diethyl ether was added. The next day the product was collected by filtration, washed with ether and dried in air; yield 3.86 g (100%).

Part of the hydrochloride (3.66 g, 32 mmol) was passed through an anion exchanger resin (Amberlite IR-4B(OH)): 2.786 g was recovered, containing 9.4% Cl, corresponding to a maximum of 2.52 g (101%) of glycine. On the other hand, amino acid analysis indicated that this sample contained 86% of glycine (2.40 g, 30.7 mmol).

Preparation of (1,2-$^{13}C_2$,^{15}N)Serine from (1,2-$^{13}C_2$,^{15}N)Glycine

Samples of $\sim$0.5 g of [1,2-$^{13}C_2$,^{15}N]serine were prepared by the following procedure using serine hydroxymethyltransferase as a catalyst. The procedure was modified from that originally developed for the isolation of serine produced using the beef enzyme [10]. During the conversion of [1,2-$^{13}C_2$,^{15}N]glycine to [1,2-$^{13}C_2$,^{15}N]serine full anaerobic conditions were maintained and the reaction mixture was kept sealed under an atmosphere of nitrogen. Serine hydroxymethyltransferase (11 ml, 0.22 mM active sites) was added to a 290 ml solution containing 20 mM [1,2-$^{13}C_2$,^{15}N]glycine, 25 mM potassium phosphate buffer at pH 7.3, 50 mM formaldehyde, 0.2 mM tetrahydrofolate, 5 mM 2-mercaptoethanol and 0.1 mM pyridoxal-5-phosphate to give a 301 ml solution containing 8.0 μM serine hydroxymethyltransferase. The serine hydroxymethyltransferase was prepared and assayed as described earlier[26–28]. The conversion of [1,2-$^{13}C_2$,^{15}N]glycine to [1,2-$^{13}C_2$,^{15}N]serine was monitored by ^{13}C-NMR spectroscopy. After 36 h at 37 °C, $\sim$93% of the [1,2-$^{13}C_2$,^{15}N]glycine was converted to [1,2-$^{13}C_2$,^{15}N]serine. Then solid sodium bisulphite was added to a final concentration of 30 mM to convert the excess formaldehyde to formaldehyde bisulphite. HCl (1 M) was used to reduce the pH of this solution to 1.5. Serine hydroxymethyltransferase precipitated and it was removed by filtration using Whatman number 1 filter paper. The solution was then mixed with Dowex-50W (200 g of dry resin) which had been equilibrated with distilled water until the eluate was at $\sim$pH 7. After washing with $\sim$500 ml of distilled water the bound amino acids were eluted using $\sim$800 ml of 4 M ammonium hydroxide. Ammonia was removed by rotary evaporation and then the solution was freeze dried. By repeating this procedure three times, 2.1 g

of [1,2-$^{13}C_2$,^{15}N]glycine was converted to 2.5 g of freeze-dried material, which according to amino analysis contained 14.9 mmol serine (55%) and 1.15 mmol glycine.

Boc-(1,2-$^{13}C_2$,^{15}N)Serine DCHA Salt

Some 1.905 g of the freeze-dried solid above (11.4 mmol serine) was suspended in water (12 ml) and NaOH (1 M, 15 ml) and the dark green solution diluted with dioxane (24 ml) followed by solid Boc_2O (4.36 g, 20 mmol). After 1 h stirring, most of the dioxane was evaporated at reduced pressure, and the suspension diluted with EtOAc (125 ml) and water (25 ml). The pH of the aqueous phase was gradually lowered from 8 to 3 with ice-cold $KHSO_4$ (0.4 M, 30 ml). On extraction a solid precipitate was enriched in the EtOAc at the interface. Care was taken to avoid collecting it in the organic extract. Further extraction with EtOAc (2×100 ml + $4 \times$ 50 ml) was followed by washing with water twice before drying (Na_2SO_4). After taken to dryness, the remaining oil was dissolved in ether and mixed with dicyclohexylamine (DCHA), yielding a first fraction of salt (2.908 g). A small aliquot was left in formic acid for 4 h. After evaporation it was submitted to amino acid analysis; found, Ser 95.0% and Gly 5.0%.

The combined aqueous phase and washings were filtered by suction and the precipitate washed and dried *in vacuo* to give 53 mg of brown product. The filtrate was concentrated at reduced pressure and an aliquot analysed for free amino acids. It contained 1.44 mmol Ser and 0.012 mmol Gly, as a consequence of which it was lyophilized for recovery of additional material.

The lyophilized product was suspended in water (10 ml), whereupon pH was raised from 3 to *ca.* 10.5 with NaOH (1 M, 5 ml). The clear solution was diluted with dioxane (20 ml, formation of a precipitate) before additional Boc_2O (1.09 g, 5 mmol) was added and allowed to react for 1.5 h. The precipitate was filtered off and washed with small portions of water. Most of the dioxane was evaporated before acidification to pH 3 with $KHSO_4$ (7 ml) and extraction with EtOAc/diethyl ether (1 : 1, 4×50 ml). The combined extracts were washed twice with small volumes of water and dried (Na_2SO_4). After evaporation the residue was converted to DCHA salt as above, yielding a second fraction of 0.410 g. The combined aqueous phase and washings, containing 0.076 mmol Ser and 0.915 μmol Gly, was discarded.

The two portions of DCHA salt were combined. Half of it at a time was partitioned between EtOAc and 1 M $KHSO_4$ and the phases separated. The aqueous phase was exhaustively extracted with EtOAc and the combined extracts washed with brine and dried (Na_2SO_4). After evaporation the oil was chromatographed on a silica column (5×20 cm) in the system CH_2Cl_2–MeCOMe–HOAc (40 : 10 : 1). The Boc-Gly eluted first, followed by completely pure Boc-Ser.

Oily Boc-Ser from two identical experiments was dissolved in diethyl ether and, after filtering through a plug of cotton, was converted to DCHA salt (2.483 g, yield 56%); m.p. 139–141 °C (lit. [18] (non-labelled) 142–144 °C); $[\alpha]_D^{25}$ 12.3 and $[\alpha]_{578}^{25}$ 13.0 ($c = 1.05$, MeOH) (lit. [18] (non-labelled) $[\alpha]_D^{25.5}$ 13.3 ± 0.5 ($c = 3.044$, MeOH)); δ_H (400 MHz, $CDCl_3$) 4.05 (perturbed d, $^1J_{H,C} \approx 140$, α-^{13}CH); δ_C (100.40 MHz, $CDCl_3$) 56.54 (dd, $J_{C,C} = 52.5$, $J_{C,N} = 11.6$, α-^{13}C), 175.25 (d, $J_{C,C} = 52.5$, ^{13}CO); δ_N (40.40 MHz, $CDCl_3$) 86.9 (d, $J = 11.6$, ^{15}N).

Preparation of (1,2-$^{13}C_2$,^{15}N)Tryptophan from (1,2-$^{13}C_2$,^{15}N)Serine

[1,2-$^{13}C_2$,^{15}N]Tryptophan was prepared from [1,2-$^{13}C_2$,^{15}N]serine and indole using tryptophan synthase from *Salmonella typhimurium* as a catalyst. Some 0.58 g of the freeze-dried solid containing 0.374 g of [1,2-$^{13}C_2$,^{15}N]serine was dissolved in 10 mM potassium phosphate buffer (280 ml) at pH 7.80. After warming this solution to ~50 °C, solid indole (656 mg) was added to give a solution of 20 mM indole and 8.7 mM [1,2-$^{13}C_2$,^{15}N]serine. This solution was then equilibrated at 37 °C. Then 0.8 ml of 10 mM potassium phosphate buffer containing tryptophan synthase (0.31 mM active sites) was added to give a concentration of 0.88 μM active sites in 280.8 ml of solution. The tryptophan synthase was prepared and assayed as described previously [29]. The conversion of the serine to tryptophan was followed by ^{13}C-NMR spectroscopy. After 48 h, ~95% of the [1,2-$^{13}C_2$,^{15}N]serine had been converted to [1,2-$^{13}C_2$,^{15}N]tryptophan. The α- and β-subunits of tryptophan synthase were then removed by heat denaturation and acid precipitation, respectively [30]. The solution was then freeze dried to give 1.17 g of material, which according to amino acid analysis contained 2.33 mmol tryptophan (67%), together with 0.18 mmol glycine and 0.11 mmol serine.

Boc-(1,2-$^{13}C_2$,^{15}N)Tryptophan

The freeze-dried solid was suspended in a mixture of water (15 ml) and dioxane (30 ml), whereupon 1 M NaOH (3.0 ml) was added (no clear solution!) and the flask cooled in ice-water. Solid Boc_2O (1.09 g, 5 mmol) was allowed to react for 30 min, when pH was increased from approx. 6.0 to 6.5–7.0 with more NaOH (1 M, 0.50 ml), and then for an additional 15 min. Some slimy precipitate was sucked off and most of the dioxane was evaporated from the solution at reduced pressure before water (25 ml) and $KHSO_4$ (0.4 M, 10 ml) were added and the product taken up in EtOAc (40 ml). The urine-coloured aqueous phase (pH 2.5–3.0) was further extracted with EtOAc (3 × 40 ml) and the combined extracts washed with water (2 × 5 ml) before drying (Na_2SO_4).

An aliquot of the combined aqueous phase and washings was analysed for remaining free amino acids and was found to contain less than 5% of the original amount of Trp (together with 3 and 29% of the original Gly and Ser, respectively).

The combined organic extracts were taken to dryness and the solid residue analysed by TLC on silica (CH_2Cl_2–acetone–HOAc 40 : 10 : 1; detection with dicarboxidine): it agreed qualitatively with an artificial 90 : 5 : 5 mixture of the corresponding non-labelled Boc-amino acids. From such a mixture the two minor components can be eliminated by simple recrystallization from EtOAc–light petroleum. Therefore the crude material was next recrystallized from such a mixture (30 ml; 1 : 5), giving a solid product (582 mg) without a sharp melting point, completely free from Boc-Gly and containing only traces of Boc-Ser. This was dissolved in ether and the solution filtered through a plug of cotton to remove some brown precipitate, whereupon a small excess of DCHA was added. From the corresponding salt (841 mg, formed nearly quantitatively), m.p. 181.5–183.5 °C, after liberation with $KHSO_4$, extraction with EtOAc, washing with water, drying, evaporation and renewed crystallization from EtOAc–light petroleum, pure title compound (454 mg) was obtained; m.p. 134–136 °C (decomp.) (lit. [31] (non-labelled) varying from 135 to 144 °C); $[\alpha]_D^{25} = 19.1$ and $[\alpha]_{578}^{25} = 20.2$ ($c = 1.23$, $CHCl_3$). (An authentic sample of Boc-L-Trp prepared similarly exhibited $[\alpha]_D^{25} = 19.3$ and $[\alpha]_{578}^{25} = 20.3$ ($c = 1.23$, $CHCl_3$) (lit. [32] (non-labelled) $[\alpha]_D^{20} = 23.6$ ($c = 1.27$, $CHCl_3$)); pure by TLC in the system above; δ_H (270 MHz, DMSO) 4.14 (complex d, $^1J_{H,C} = 142$, α-^{13}CH); δ_C (67.9 MHz, DMSO) 54.4 (dd, $J_{C,C} = 58.9$, $J_{C,N} = 12.5$, α-^{13}C), 173.8 (d, $J_{C,C} = 59.2$, ^{13}CO); δ_N (27.25 MHz, DMSO) 91.1 (d, $J = 12.6$, ^{15}N); ν_{max}/(cm^1) 1687 (CO) (1721 nonlabelled).

From the mother liquor after the first crystallization from EtOAc/light petroleum additional pure crude material could be isolated by chromatography on silica and this was combined with the residue from the last mother liquor and converted to DCHA-salt (75 mg, corresponding to 47 mg of title compound); m.p. 177.5–181.5 °C; total yield 501 mg (70%).

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