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Synthesis of the proline analogue [2,3-^{3}H]azetidine-2-carboxylic acid

Uptake and incorporation in *Arabidopsis thaliana* and *Escherichia coli*

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Azetidine-2-carboxylic acid, the 4-membered ring noranalogue of proline, is regularly used in the study of proline metabolism as well as the study of protein conformation. We prepared D,L-[2,3-^{3}H]azetidine-2-carboxylic acid with an optimized 10% yield from commercially available 4-amino-[2,3-^{3}H]butyric acid. Purification was performed by fast-protein liquid chromatography. The biological activity was checked in both *Arabidopsis thaliana* and *Escherichia coli*. The obtained specific activity of 10 mCi/mmol was sufficient for most uptake and incorporation studies.

Amino acid; 4-Aminobutyric acid; *Escherichia coli*; Synthesis of radioactive compounds

1. INTRODUCTION

L-Azetidine-2-carboxylic acid (2AZ), is the 4-membered ring noranalogue of proline and occurs naturally in members of the *Liliaceae* family, where it was first isolated from *Convallaria majalis* L. [1]. 2AZ is of biochemical interest because, on the one hand, it is that similar to proline that it can be incorporated in its place into proteins but, and, on the other hand, it causes alterations in their physicochemical and biological behavior. 2AZ is toxic, even at low concentrations (<1 mM) to most plant species except some *Liliaceae* and *Agavaceae* [2], to most bacteria with the exception of e.g. the *Agrobacterium* species [3], and to mammalian cells [4]. This toxicity is due to the functional impairment of proteins containing 2AZ residues instead of proline [5,6]. It has also been reported that 2AZ inhibits proline synthesis through a false end-product feedback mechanism [7] which can also add to its toxicity. The toxicity of 2AZ has also been used in selection studies: mutants that overcome 2AZ toxicity have been isolated [4,7–12] and are a useful tool in the study of proline metabolism [13]. The importance of proline is well known in response to stress (salt, drought, cold, etc.) [14,15]. To characterize these mutants it is important to have radioactive 2AZ available.

Here, we present an adaptation of Fowden's method [1] starting from γ-aminobutyric acid, suitable for the synthesis of radioactive 2AZ with 2.2×10^{10} dpm/mM in 10% yield. Better yields have been reported starting from γ-butyrolactone (53.5%) [16] or 2-pyrrolidinone (62.5%) [16] but none of the starting materials or intermediates is available commercially as a radioactive precursor. Biosynthesis would be possible from [1-^{14}C]methionine [16,17], but its specific activity is low.

2. MATERIALS AND METHODS

2.1. *Chemicals*

These were purchased as given: 4-amino-butyric acid (Janssen, Beerse, Belgium); 4-amino-*n*-[2,3-^{3}H]butyric acid, TRK527 and L-[2,3-^{3}H]proline TRK638 (Amersham, Buckinghamshire, UK); bromine, red phosphorus, barium hydroxyde [$Ba(OH)_2 \cdot 8H_2O$], trichloroacetic acid, and pyronin G (Merck, Darmstadt, Germany); phosphopentoxide Granusic A (Baker, Deventer, The Netherlands); L-proline and L-azetidine-2-carboxylic acid (Sigma, St. Louis, USA); Picofluor 30, Solulyte, and 2,5-diphenyloxazole (Packard, Canberra, USA); TLC pre-coated cellulose sheets MN300 0.1 mm layer (Macherey-Nagel, Düren, Germany), and perhydrol (30% H_2O_2) fresh from the local drugstore.

2.2. *General methods*

An LKB Wallac 1209 Rackbeta Liquid Scintillation Counter and ReadySafe (Beckman, Fullerton, USA) were used for the assay of radioactive compounds.

Ascending thin-layer chromatography was carried out on the MN300 sheets for 5 h with butanol/pyridine/acetic acid/water, 15:12:3:10 (v:v:v:v) as solvent. Autoradiography was performed with Kodak-sensitive (X-OMAT) and amplifying screens on the thin layer dipped in 100 ml acetone, 0.5 g PPO. The R_f values of 2AZ and GABA were 0.27 and 0.39, respectively. Thin-layer electrophoresis was run at 50 V/cm for 30 min on the cellulose sheets soaked in HCOOH/CH_3COOH/H_2O, 2:8:90 (v:v:v) (pH 1.8) buffer. The migration values of 2AZ and GABA were 3.6 cm and 7.2 cm, respectively. The migration value of a colored reference (pyronin G) was 1.6 cm. Revealing of amino acids was performed by dipping dried thin-layer sheets in 100 ml acetone with 200 mg ninhydrin, 1 ml acetic acid, 1 ml pyridine, followed by 5 min at 100°C.

Abbreviations: GABA, γ-amino-butyric acid = 4-amino-butyric acid; 2AZ, azetidine-2-carboxylic acid; PPO, 2,5-diphenyloxazole; FLPC, fast-protein liquid chromatography; TCA, trichloroacetic acid; TLC, thin-layer chromatography.

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2.3. *Column chromatography*

FPLC (Pharmacia, Uppsala, Sweden) equipped with cation-exchange column mono-S 5 × 50 mm was run with HCl gradient 10^{-3} M to 10^{-1} M in 30 min, 1-ml fraction/min. Elution was monitored at 214 nm.

2.4. *Preparation of DL-[2,3-^{3}H]azetidine-2-carboxylic acid*

4-Amino-butyric acid (0.5 mCi + 36 μmol, 3.7 mg) was dissolved in 0.5 ml water, loaded on a mono-S cation exchange column, and separated with FPLC as described above. Fractions with radioactivity (between 27 and 29 min) were dried in a sealed tube (5 ml) in a Savant centrifuge speed vacuum concentrator. A trace of red phosphorus was added and the tube was put overnight over P_2O_5 in vacuum at 80°C. Bromine (4 μl) was added, the glass tube was vacuum sealed in moisture-free conditions, and put in the oven at 80°C for 16 h. After this reaction time, 0.5 ml saturated $Ba(OH)_2$ solution was added and the basic mixture was refluxed on a boiling water bath for 20 min. Subsequently, excess barium ions were precipitated by adding 0.1 N sulfuric acid up to pH 2–3 (1.8–2.2 ml). The precipitate was centrifuged in the original sealed tube, washed and discarded. The collected supernatants were loaded on a mono-S 5 × 50 mm column, washed on the column with 5 ml 10^{-3} M HCl, and submitted to FPLC gradient separation as above: 2AZ was eluted at 10–13 min (8–12% yield) and 4-aminobutyric acid at 27–29 min. Pure products were obtained by lyophilization of the respective fractions. Recycling of 4-aminobutyric acid is recommended.

2.5. *Specific activity*

0.5 mCi × 3/4 (one ^{3}H gone as HBr) for 0.036 mEq was 10.4 mCi/mmol for the racemic mixture [2,3-^{3}H]azetidine-2-carboxylic acid.

2.6. *Uptake and incorporation in Arabidopsis thaliana and* Escherichia coli

2.6.1. Preparation of plants. Seeds of *Arabidopsis thaliana* race Bensheim were surface-sterilized by dipping in 5% calcium hypochlorite for 10 min, followed by rinsing several times in water. Seeds were put on a modified Murashige and Skoog (MS) medium (K1 medium) [18] containing MS salts and vitamins, 1% sucrose, and 0.8% agar (Difco) in Petri dishes. Growth took place in a culture chamber (16-h photoperiod: 3000 lx [Philips TMV065], IP 65, 21°C, 50% relative humidity).

Ten days after sowing (C+4 stage), samples containing 10 plantlets were prepared for the uptake experiment. Groups of 10 plantlets were taken out from the solid agar medium without wounding and put into liquid culture medium for 1 h.

2.6.2. Assay for radioactivity labeled substrates in plants. The synthetic DL-[2,3-^{3}H]2AZ (racemic mixture) was prepared at a specific activity of 10 mCi/mmol. L-[2,3-^{3}H]Proline was diluted to the same specific activity. Each sample was kept for 1, 2 or 4 h in an incubation volume of 0.5 ml KI medium containing 0.5 μCi of amino acid (proline or 2AZ). After the incubation time, each sample was rinsed twice in 5 ml of water containing 5 mM amino acid. After rinsing, the samples were briefly drained on Kleenex paper before being weighted.

2.6.3. Uptake measurements in plants. Samples were dipped in 0.5 ml Solulyte for 2 h at 60°C. After cooling, 0.5 ml of 2-propanol/perhydrol, 30:70 (v/v) was added and the samples were left to decolorize overnight. Radioactivity was measured by adding 5 ml RendySafe (24 h in the dark at 4°C before counting reduced background to a minimum of 20–30 cpm).

2.6.4. Incorporation measurements. After being weighed, the samples were ground with 1 ml of trichloroacetic acid (TCA 10%) containing 5 mM proline, then filtered through GF/C Whatman paper, and washed with 20 ml TCA (5%), containing 5 mM proline. Filters were dried under an I.R. lamp and counted with 5 ml of picofluor.

2.6.5. Uptake and incorporation assay for bacteria. *E. coli* K12 was inoculated (1/50) from a fully grown preculture and grown to O.D.$_{600}$ 0.6 in 3 ml mineral medium [19] supplemented with 1.3×10^5 cpm DL-[2,3-^{3}H]2AZ or L-[2,3-^{3}H]proline 10 mCi/mmol. After growing, the bacteria were pelleted (6,000 × *g*, 15 min) and radioactivity in the supernatants was counted (1/10 + 5 ml ReadySafe). The decrease in radioactivity constitutes the uptake measurement. Pellets were resuspended in 1 ml water, sonicated on ice for 5 min at maximum intensity. Cell debris were discarded by centrifugation (15,000 × *g*, 15 min). The supernatant, acidified up to 10% TCA, was filtered through Whatman GF/C paper and washed with 20 ml TCA (5%) containing 5 mM proline. Filters were handled as above.

3. RESULTS AND DISCUSSION

Azetidine-2-carboxylic acid is formed by a base-catalyzed cyclisation reaction of 2-bromo-4-aminobutanoyl bromide. We adapted Fowden's method [1] starting from 4-aminobutyric acid for the synthesis of microamounts of radioactive material. 2-Bromo-4-aminobutanoyl bromide is formed in two steps from GABA over 4-aminobutanoyl bromide as intermediate (Fig. 1).

The optimized reaction conditions, resulting in 10% yield, were: 36 μmol of cold GABA and 0.5 mCi [^{3}H]GABA, a twofold stoichiometric excess of bromine, and a trace of red phosphorus, heated for 16 h at 80°C. The reaction was stopped and brought to basic pH with barium hydroxide. After a short reflux in a boiling-water bath, barium was precipitated as sulfate and collected supernatants were loaded onto an analytical cation-exchange column. Elution with an HCl gradient from 0 to 0.1 M resulted in 2AZ (yield 10%) and GABA separation (recovery 85%).

The progress of the synthesis could be controlled by following the radioactivity of the elution pattern of samples on the cation exchanger, and by analyzing the reaction mixture by thin-layer chromatography and electrophoresis. The thin layers were autoradiographed and also developed with ninhydrin.

The biological activity of synthesized 2AZ was checked by testing its incorporation into the proteins of *E. coli* and *A. thaliana*. It was well taken up by both systems (Tables I and II). After overnight growth, the uptake in *E. coli* of 2AZ and proline was 45% and 64%, respectively; after 4 h of incubation, the uptake in *A. thaliana* of both 2AZ and proline was 0.3%. 2AZ was incorporated into the proteins. In *E. coli* incorporation vs. uptake of the amino acids was 1% for 2AZ and 2%

Table I

Uptake and incorporation of 2AZ and proline in *E. coli*

	DL-[2,3-^{3}H]2AZ	L-[2,3-^{3}H]Proline
Uptake (cpm)	58,967 ± 2,047	83,665 ± 2,762
Uptake (%)	45	64
Incorporation (cpm)	594 ± 70	1,539 ± 65
Incorporation vs. uptake (%)	1	2

Growth until optical density at 600 nm is 0.6 O.D. in 3 ml N9 mineral medium containing 1.3×10^5 cpm amino acid (10 mCi/mmol). Three replications for each amino acid were done.

Table II

Uptake (A) and incorporation (B) of 2AZ and proline in *Arabidopsis thaliana*

Hours	D,L-[2,3-^{3}H]2AZ		L-[2,3-^{3}H]Proline	
A	(cpm)	(%)	(cpm)	(%)
1	514 ± 114	0.1	520 ± 158	0.1
2	834 ± 137	0.2	819 ± 233	0.2
4	1,441 ± 216	0.3	1,459 ± 223	0.3
B	(cpm)	(%)[1]	(cpm)	(%)[1]
1	6 ± 2	1	23 ± 7	4
2	26 ± 5	3	45 ± 11	5
4	37 ± 7	3	78 ± 5	5

Samples were incubated in K1 medium containing 5×10^5 cpm of amino acid (proline or 2AZ). Values are the means of three replications.

[1]Percentage vs. uptake.

for proline, whereas in *A. thaliana*, this ratio was 3% for 2AZ and 5% for proline. Although uptake of amino acids is an active process [20], no distinction seemed to be made in *E. coli* or *A. thaliana* between L- or D-2AZ uptake. If synthesized 2AZ was less well incorporated than proline, it is probably because proline was the L-proline and the synthesized 2AZ was a racemic mixture.

The method presented can be performed easily on a laboratory scale and produces D,L-[2,3-^{3}H]2AZ (about 10 mCi/mmol). This specific activity is convenient for most studies in bacteria or plants. The available radioactive 2AZ will be of importance for the characterization of mutants resistant to the proline analog since it makes screening for mutations in the uptake or incorporation of 2AZ very rapid and efficient.

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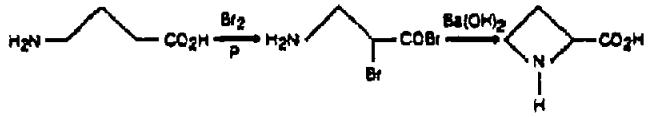


Fig. 1. Reaction scheme.

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