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Radiochemistry

Efficient Enzymatic Preparation of ^{13}N-Labelled Amino Acids: Towards Multipurpose Synthetic Systems

Eunice S. da Silva,[a] Vanessa Gómez-Vallejo,[b] Zuriñe Baz,[a] Jordi Llop,*[a] and Fernando López-Gallego*[c, d]

Abstract: Nitrogen-13 can be efficiently produced in biomedical cyclotrons in different chemical forms, and its stable isotopes are present in the majority of biologically active molecules. Hence, it may constitute a convenient alternative to Fluorine-18 and Carbon-11 for the preparation of positron-emitter-labelled radiotracers; however, its short half-life demands for the development of simple, fast, and efficient synthetic processes. Herein, we report the one-pot, enzymatic and non-carrier-added synthesis of the ^{13}N-labelled amino acids L-[^{13}N]alanine, [^{13}N]glycine, and L-[^{13}N]serine by using L-alanine dehydrogenase from *Bacillus subtilis*, an enzyme that catalyses the reductive amination of α-keto acids by using nicotinamide adenine dinucleotide (NADH) as the redox cofactor and ammonia as the amine source. The integration of both L-alanine dehydrogenase and formate dehydrogenase from *Candida boidinii* in the same reaction vessel to facilitate the in situ regeneration of NADH during the radiochemical synthesis of the amino acids allowed a 50-fold decrease in the concentration of the cofactor without compromising reaction yields. After optimization of the experimental conditions, radiochemical yields were sufficient to carry out in vivo imaging studies in small rodents.

Introduction

Positron Emission Tomography (PET) is a molecular imaging technique that relies on the administration of trace amounts of compounds labelled with positron emitters that enable external, non-invasive detection with unparalleled sensitivity. In recent years, PET has proved valuable in the diagnosis and prognosis of different diseases,[1] the determination of pharmacokinetic properties of new chemical entities,[2] and the investigation of the underlying molecular mechanisms of pathological processes.[3]

The application of PET requires radiolabelling of the biomarker or molecule under investigation with a positron emitter. Fluorine-18 (^{18}F) and Carbon-11 (^{11}C) have been extensively utilized because of their relatively long half-lives ($T_{1/2}$ = 109.8 min and 20.4 min, respectively) and versatile chemistries. In contrast, the use of Nitrogen-13 (^{13}N, $T_{1/2}$ = 9.97 min) has been historically more restricted, and only a few studies that describe new synthetic strategies for the incorporation of this radioisotope into bioactive molecules have been reported to date.[4] Interestingly, ^{13}N can be efficiently produced in biomedical cyclotrons in different chemical forms, and its stable isotopes (^{14}N and ^{15}N) are present in the majority of biologically active molecules. Hence, the inclusion of ^{13}N in the toolbox of PET chemists might become a valuable alternative to ^{11}C and ^{18}F, either for the preparation of new labelled compounds or the incorporation of the label in different positions. The preparation of ^{13}N-labelled amino acids for the investigation of tumour-related metabolic alterations is particularly relevant,[5] because the synthesis of natural amino acids that use other positron emitters is usually challenging.

The short half-life of ^{13}N calls for the development of simple, fast, and efficient synthetic processes. In this context, biocatalysis can offer attractive solutions because enzymes present an exquisite selectivity and high turnover numbers, which enables fast chemical conversions and yields highly pure products under extremely mild conditions. The use of enzymes for the preparation of ^{13}N-labelled radiotracers was first described in the 70s and applied to the preparation of amino acids. For example, L-[^{13}N]glutamate was synthesized by incubation of α-ketoglutarate (α-KG) with [^{13}N]NH_3 in the presence of the enzyme L-glutamate dehydrogenase and NADH as the redox cofactor.[6] Likewise, L-[^{13}N]glutamine was synthesized by glutamine synthetase by using glutamic acid as the substrate and adenosine triphosphate (ATP) as the cofactor.[6b,7] These two amino acids were synthesized with incorporation values close to 90% after 15 min of incubation.

[a] E. S. da Silva, Z. Baz, Dr. J. Llop
Radiochemistry and Nuclear Imaging Group, CIC biomaGUNE
Paseo Miramón 182, 20009 San Sebastián, Guipúzcoa, (Spain)
E-mail: jllop@cicbiomagune.es

[b] Dr. V. Gómez-Vallejo
Radiochemistry Platform CIC biomaGUNE
Paseo Miramón 182, 20009 San Sebastián, Guipúzcoa (Spain)

[c] Dr. F. López-Gallego
Heterogeneous Biocatalysis GroupCIC biomaGUNE
Paseo Miramón 182, 20009, San Sebastián, Guipúzcoa, (Spain)

[d] Dr. F. López-Gallego
IKERBASQUE, Basque Foundation for Science
Maria Diaz de Haro 3, 48013 Bilbao, Bizkaia (Spain)
E-mail: flopez.ikerbasque@cicbiomagune.es

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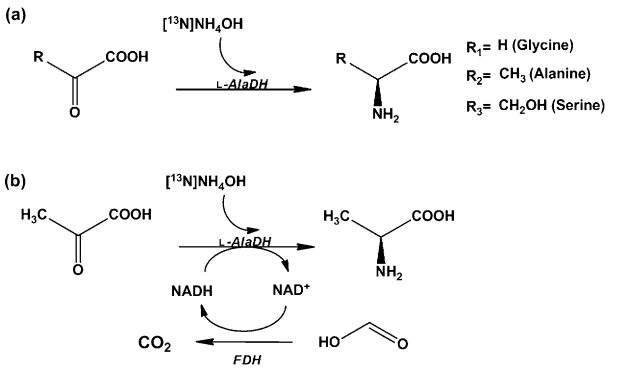
Immobilization of the enzymes on solid supports resulted in a significant qualitative improvement by simplifying the purification process. For example, immobilization of glutamate dehydrogenase enabled the preparation of L-[^{13}N]valine and L-[^{13}N]leucine.[8] Through a more sophisticated process, the production of L-[^{13}N]alanine was achieved by immobilization of the glutamine dehydrogenase and alanine transaminase on silica beads.[9] In this tandem reaction, the reductive amination of α-KG by using [^{13}N]NH_3 and NADH as the amine and hydride sources, respectively, yielded L-[^{13}N]glutamate; this was sequentially used as a co-substrate by the transaminase to transfer the radiolabelled amine to the pyruvic acid, which resulted in the formation of L-[^{13}N]alanine. Yields close to 70% were obtained in 4 min. Unfortunately, the investigation of enzyme-mediated synthesis of ^{13}N-labelled amino acids was unexpectedly discontinued, and no further works have been reported so far.

With the widespread installation of biomedical cyclotrons and small animal-dedicated cameras around the world, scientists are increasingly identifying PET as an accessible and valuable tool for the investigation of physiological or biological problems in the pre-clinical setting. This fact, together with the significant advances in the investigation of enzymatic reactions and the understanding of the precise mechanisms underlying the catalytic reactions, may open new avenues for the preparation and utilization of radiolabelled amino acids in multiple biomedical applications.

Here, in continuation of our previous works related to the application of enzyme-mediated reactions to ^{13}N-radiochemistry,[10] we report the one-pot/one-step radiosynthesis of L-[^{13}N]alanine, [^{13}N]glycine, and L-[^{13}N]serine, which is catalysed by a versatile L-alanine dehydrogenase (L-AlaDH) from *Bacillus subtilis* (Scheme 1 a). As demonstrated in this work, this enzyme catalyses the reductive amination of different α-keto acids by using NADH as the redox cofactor and cyclotron-produced, no-carrier-added [^{13}N]NH_3 as the amine source. Chromatographic yields (conversion of [^{13}N]NH_3 into the corresponding amino acid, measured from the chromatographic profile and expressed in percentage) above 90% were obtained after appropriate selection of the experimental conditions. Additionally, simultaneous utilization of the aforementioned enzyme with formate dehydrogenase (FDH) from *Candida boidinii* led to the in situ regeneration of the cofactor (Scheme 1 b), which enabled a 50-fold decrease in the concentration of the cofactor without compromising the reaction rates. This synthetic strategy resulted in ready-to-inject ^{13}N-labelled amino acids in sufficient amount to approach in vivo studies in small rodents, and paves the way towards future solid-supported, multi-purpose, flow synthetic processes.

Results and Discussion

The ultimate goal of the work reported here was the development of an efficient synthetic procedure for the preparation of ^{13}N-labelled amino acids, which will be applied in future works to the assessment of tumour-associated metabolic alterations in a mouse model of prostate cancer. The natural amino acids



Scheme 1. Schematic representation of: a) enzyme-mediated preparation of L-[^{13}N]alanine, [^{13}N]glycine, and L-[^{13}N]serine by using L-alanine dehydrogenase (L-AlaDH) from *Bacillus subtilis* as the enzyme. b) One-pot, multi-enzyme reaction for the preparation of L-[^{13}N]alanine with regeneration of NADH.

L-alanine, L-serine, and glycine are biosynthetically linked, and together, they provide the essential precursors for the synthesis of proteins, nucleic acids, and lipids that are crucial to cancer cell growth.[5b,11] Hence, these three amino acids were identified as the priority target compounds. Additionally, with the aim of investigating the versatility of our approach, the synthesis of two additional amino acids, L-phenylalanine and L-norvaline, was also investigated.

Selection, expression, and purification of an active L-AlaDH in *E. coli*

L-Alanine dehydrogenase (L-AlaDH) is an oxidoreductase (EC 1.4.1.1) that catalyses the reversible oxidative deamination of L-alanine to pyruvic acid and the reductive amination of pyruvic acid. The kinetic properties of L-AlaDHs from different microbial sources have been extensively investigated, and some of these enzymes have been cloned in heterologous hosts.[12] Although the physiological role of L-AlaDH remains controversial, it has been reported that this enzyme is involved in the utilization of alanine as an energy source during the germination process of bacillus species.[13] However, inspection of different data bases (BRENDA, KEEGG, MetaCyc) evidences that L-AlaDH is more versatile than anticipated from its physiological role, and this suggests that L-AlaDH from *Bacillus subtilis* and *Bacillus sphaericus* may enable the preparation of different ^{13}N-labelled amino acids because they present broad substrate scope for the reductive amination of α-keto acids. Among L-AlaDHs, the L-AlaDH from *Bacillus subtilis* was selected here owing to its better kinetic properties for a wider variety of amino acids. The enzyme was expressed in *E. coli* BL21 and purified by affinity chromatography based on the histidine tag inserted at its N-terminus. According to the literature,[12,14] the pure L-AlaDH is a hexamer with a subunit mass of 44 KDa, which results in a total mass of 264 KDa (see the Supporting Information, Figure S1). The pure L-AlaDH showed a specific enzyme activity of 106 ± 5 U mg^{-1} towards pyruvate.

Synthesis of amino acids under non-radioactive conditions

The amination activity towards α-keto acids with different side chains was first tested in non-radioactive conditions. With that aim, the synthesis of L-alanine, glycine, L-serine, L-phenylalanine, and L-norvaline was achieved by incubation of the appropriate substrates with ammonium chloride in the presence of NADH as the cofactor and L-AlaDH from *Bacillus subtilis* (Table 1).

Table 1. Specific enzyme activity (SEA) values for amination and deamination reactions under non-radioactive conditions.[a]

Amino acid	$SEA_{deamination}$ [μmol min^{-1} mg^{-1}]	$SEA_{amination}$ [μmol min^{-1} mg^{-1}]
alanine	5.4 ± 0.1 (L)	106 ± 5
glycine	N.D.	34.3 ± 1.6
serine	0.039 ± 0.001 (L)	22.5 ± 3.1
	0.003 ± 0.001 (D)	
norvaline	0.002 ± 0.001 (L)	18.6 ± 1.2
phenylalanine	N.D.	21.4 ± 2.1

[a] Carried out at pH 8. N.D. = not detected.

As expected, the highest activity was observed for pyruvic acid, but the enzyme also catalysed the reductive amination of other α-keto acids, such as glycolic acid, 3-hydroxypyruvic acid, 2-oxopentanoic acid, and 2-phenylpyruvic acid, to yield glycine, serine, norvaline, and phenylalanine, respectively. The specific enzyme activity towards these α-keto acids ranged from 17 to 32% of the maximum specific enzyme activity that was measured with pyruvic acid (Table 1). In general terms, bulky α-keto acids were worse substrates than small ones, as previously observed in the vast majority of the studied L-AlaDHs.[15]

As aforementioned, L-alanine dehydrogenase from *Bacillus subtilis* presented a broad substrate scope for the reductive amination towards different α-keto acids. In contrast, such substrate versatility was not observed in the oxidative deamination of L-amino acids (Table 1). The enzyme was inactive towards glycine and phenylalanine and poorly active towards serine and norvaline. These results are in good agreement with those previously reported both for the recombinant and native enzymes purified from *E. coli* and *B. subtilis*, respectively.[12,14]

The effect of the pH on the reductive amination activity of L-AlaDH towards different α-keto acids was subsequently investigated (Figure 1). Optimal specific enzyme activity values for the production of all tested amino acids were observed at pH 8, except for L-serine, which were achieved at pH 9. Notably, the specific enzyme activity of L-AlaDH towards 3-hydroxypyruvic acid at pH 9 was only 1.24 times lower than the activity towards pyruvic acid at pH 8.

The activity–pH profile of L-AlaDH for the reductive amination of pyruvic acid was wider than for other amino acid dehydrogenases.[16]

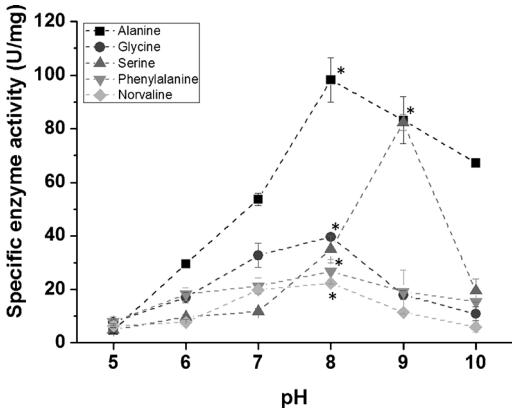


Figure 1. Activity–pH profile of L-AlaDH for the production of L-alanine, glycine, L-serine, L-phenylalanine, and L-norvaline. The reactions were carried out in the presence of NADH (0.5 mM), ammonium chloride (450 mM), and the corresponding α-keto acid (10 mM) in buffer solution (300 mM) at 25 °C. Acetate buffer was used for pH 5–6, sodium phosphate buffer for pH 7–8, and sodium bicarbonate buffer for pH 9–10. Enzyme activity measurements were carried out for 2 min, and blank experiments (without AlaDH) were performed to discard any stability issues of NADH that may lead to underestimation of the enzyme activity. Data are expressed as mean ± standard deviation ($n = 3$); (*) denotes the optimal pH value for the synthesis of the corresponding amino acid.

Synthesis of L-[^{13}N]amino acids

Translation of the reaction parameters to radioactive conditions is often not straightforward. If a carrier-free radioactive precursor is used (in our case [^{13}N]NH_3), the concentration of this species is extremely low (around 15 μM in typical experiments conducted in our set up). Hence, the kinetics of the reaction might be significantly altered, and careful refinement of the experimental conditions is usually required. In this work, we first attempted the synthesis of L-[^{13}N]alanine: Pyruvic acid (75 mM) was incubated with cyclotron-produced [^{13}N]NH_3 (aqueous solution, 20–25 MBq, 100 μL) and L-AlaDH in the presence of NADH as the cofactor (0.5 mM) in buffered solution at pH 8.0 ± 0.1. The reactions were carried out in tubes with 50 KDa cut-off membranes to enable easy separation of the enzymes from the final products by fast centrifugation. Chromatographic yield was monitored over time (Figure 2, solid line; see the Supporting Information, Figure S2 A for example of chromatographic profiles), and values were then corrected by radioactive decay to the reaction starting time (Figure 2, dotted line).

Despite the low concentration of [^{13}N]NH_3, chromatographic yields of 68 ± 5% were obtained after 5 min under the reaction conditions; this value increased to 95 ± 1% when the reaction was carried out for 20 min. Taking into account the short half-life of ^{13}N, incubation times of 10, 15, and 20 min proved to be less desirable than the 5 min period, because the gain in yield was negligible compared to the decay of Nitrogen-13 (Figure 2, dotted line). Notably, when the reaction time was set to 20 min, the amount of radiochemical impurities (mainly unreacted [^{13}N]NH_3) was < 5%, which enabled putative in vivo studies without the need for a purification step to remove unreacted [^{13}N]NH_3.

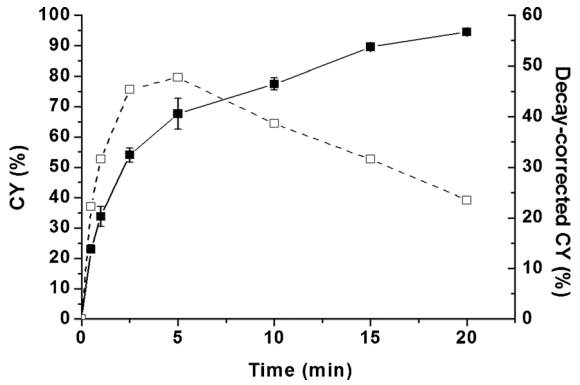


Figure 2. Chromatographic yields (CY, solid line) and decay-corrected CY (dotted line) at different reaction times for the production of L-[^{13}N]alanine. The reactions were carried out in the presence of [^{13}N]NH_4OH (20–25 MBq), L-AlaDH (15 μg mL^{-1}), NADH (0.5 mM), and pyruvic acid (75 mM) in sodium phosphate buffer solution (300 mM) at $T = 25\,^{\circ}C$.

To gain further knowledge on the effect of the amount of biocatalyst in the reaction kinetics, we performed the radiochemical synthesis of L-[^{13}N]alanine at different enzyme concentrations (see the Supporting Information, Figure S3). As expected, for a reaction time of 5 min, higher enzyme concentrations led to higher yields. Values of 68 ± 5, 78 ± 4, 85 ± 9, and 98 ± 1% were achieved when concentrations of 15, 30, 45, and 60 μg mL^{-1} of the enzyme were used, respectively.

One of the major goals of the current work was to develop a versatile synthetic process that was suitable for the efficient production of different amino acids. In addition to L-[^{13}N]alanine, the preparation of [^{13}N]glycine and L-[^{13}N]serine was a priority for future use in animal studies. Hence, and to investigate the versatility of our approach, the synthesis of these two amino acids as well as L-[^{13}N]phenylalanine and L-[^{13}N]norvaline were also attempted by using the same experimental setup that was successfully employed for L-[^{13}N]alanine. The reactions were carried out for 5 min (optimal reaction time, Figure 2) by using 60 μg mL^{-1} of L-AlaDH and a saturating concentration of the corresponding α-keto acid at the optimal pH values (Figure 1). Chromatographic yields were calculated from chromatographic profiles (Table 2; see the Supporting Information, Figure S2).

Chromatographic yields of 49 ± 3 and 42 ± 5% were obtained for [^{13}N]glycine and L-[^{13}N]serine, respectively. These values increased to > 95% when the enzyme concentration was raised to 180 and 300 μg mL^{-1} for the synthesis of [^{13}N]glycine and L-[^{13}N]serine, respectively (Table 2). The radiochemical synthesis of glycine and serine required higher enzyme concentrations because the specific enzyme activity of L-AlaDH was significantly lower towards glycolic acid and 3-hydroxypyruvic acid than towards pyruvic acid (Table 1). These results are in good agreement with our experiments under non-radioactive conditions, and they confirm that L-AlaDH from *Bacillus subtilis* is able to catalyse the reductive amination of glycolic acid and 3-hydroxypyruvic acid, although reaction rates were lower than those observed for pyruvic acid. Interestingly, the synthesis of L-[^{13}N]serine at pH 8 resulted in chromatographic yields of 7% (Table 2). The results corroborate that the synthesis of L-serine is both kinetically and thermodynamically favoured under alkaline conditions, as observed under non-radioactive conditions.

Table 2. Chromatographic yields (CY) for the synthesis of different ^{13}N-labelled amino acids.

Amino acid	pH	CY [%]
alanine	8	98 ± 1[a]
glycine	8	49 ± 3[a]/98 ± 1[b]
serine	8	7[a]
	9	42 ± 5[a]/99 ± 1[c]
norvaline	8	N.D.[a]
phenylalanine	8	N.D.[a]

[a] 60 μg mL^{-1} of protein. [b] 180 μg mL^{-1} of protein. [c] 300 μg mL^{-1} of protein. N.D. = not detected.

The preparation of L-[^{13}N]phenylalanine and L-[^{13}N]norvaline led to unexpected results. Although the enzyme was active towards the corresponding α-keto acids under non-radioactive conditions, no conversion was observed under radioactive conditions, regardless of the reaction time. The absence of formation of L-[^{13}N]phenylalanine and L-[^{13}N]norvaline and the relatively low reaction rates obtained for [^{13}N]glycine and L-[^{13}N]serine could be initially attributed to an equilibrium between amination and deamination reactions. However, the low specific enzyme activity that was observed for the deamination reactions towards the different amino acids (Table 1) discarded this hypothesis and confirmed that the reductive amination is thermodynamically favoured at pH 8, as previously reported.[15c]

To further investigate the kinetics of the enzymatic reactions, steady-state kinetic parameters for ammonia in the reductive amination of the different α-keto acids were determined under non-radioactive conditions (Table 3).

Table 3. Kinetic parameters (Michaelis–Menten model) for ammonia, for the different amino acids.

Amino acid[a]	V_{max} [U mg^{-1}]	K_M [mM]
alanine	100.46 ± 4.30	47.0 ± 11.4[a]
glycine	51.96 ± 6.05	527 ± 149[a]
serine	74.63 ± 1.26	150 ± 21[b] 2810 ± 613[a]
norvaline	114.29 ± 9.86	2231 ± 544
phenylalanine	N.D.	N.D.

[a] pH 8. [b] pH 9. N.D. = not detected.

All V_{max} values fell in the range 50–120 U mg^{-1}; however, K_M values for ammonia varied significantly from one amino acid to the next. K_M values for the synthesis of L-alanine and glycine at pH 8 were 47.0 ± 11.4 and 527 ± 149, respectively, while the K_M value for L-serine at pH 9 was 150 ± 21. However, the $K_{M(ammonia)}$ value for the synthesis of L-norvaline was 2231 ± 544 mM, and the same parameter could not be determined for the synthesis of L-phenylalanine because the substrate saturation point was never reached, regardless of the concentration

of ammonia. These values suggest that the activity of the L-AlaDH is very sensitive to the concentration of ammonia, and such sensitivity strongly depends on the nature of the α-keto acid.[17] The $K_{M(ammonia)}$ values explain that high radiochemical yields are difficult to achieve with the short reaction times that are employed for [^{13}N]glycine and L-[^{13}N]serine. In the case of L-norvaline and L-phenylalanine, a high concentration of ammonia is required for the reactions to occur at reasonable rates; consequently, no formation of the amino acid can be observed under radioactive (no-carrier-added) conditions, which is due to the low concentration of [^{13}N]NH_3 in the reaction media. A similar effect was observed for the synthesis of L-serine at pH 8, which resulted in a $K_{M(ammonia)}$ as high as the one found for L-norvaline, which supports the fact that, unlike L-[^{13}N]alanine, L-[^{13}N]serine was poorly synthesized at pH 8. The different $K_{M(ammonia)}$ values of L-AlaDH by using different α-keto acids as amine acceptors suggest that the affinity of L-AlaDH for ammonia depends on the positioning of the α-keto acid into the active site. This fact is crucial for the success of the radiochemical syntheses, for which extremely low concentrations of ammonia are used. In the light of these results, we can conclude that the enzymes or the substrates that facilitate the binding of the labelling agent to the active site (low K_M values) will lead to the best results in radiochemistry.

Overall, the above one-pot methodology is clearly superior to previously published enzymatic methods,[18] and it enables the preparation of L-[^{13}N]alanine, [^{13}N]glycine, and L-[^{13}N]serine with chromatographic yields of >95% in short reaction times.

Synthesis of L-[^{13}N]alanine with in situ NADH regeneration

With the aim of simplifying the purification process, we envisioned the possibility of using lower concentrations of the cofactor by introducing a second enzyme into the reaction media that is capable of regenerating NADH in situ. The selected enzyme was formate dehydrogenase (FDH) from *Candida boidinii*, which is known to catalyse the oxidation of formate to carbon dioxide by donating the electrons to a second substrate, such as NAD^+, and regenerating NADH (Scheme 1b).

The one-pot, bi-enzymatic reaction delivered exceptional results. The concentration of NADH in the starting solution could be decreased by 50-fold without a significant impact in chromatographic yields (Figure 3). The simultaneous addition of L-AlaDH and FDH led to the formation of L-[^{13}N]alanine with chromatographic yields of 95±2% by using NADH concentrations as low as 10 μM. These values are three times higher than those observed without FDH under identical experimental conditions. The FDH is essential for replenishing the pool of NADH by consumption of the formate as a sacrificial substrate; in this manner the reduced form remains available for the L-AlaDH to catalyse the reductive amination of pyruvate. These results substantially improve the synthetic scheme described by Baumgartner et al.;[18] with our new configuration, similar yields can be obtained but the concentration of cofactor is 100-fold lower.

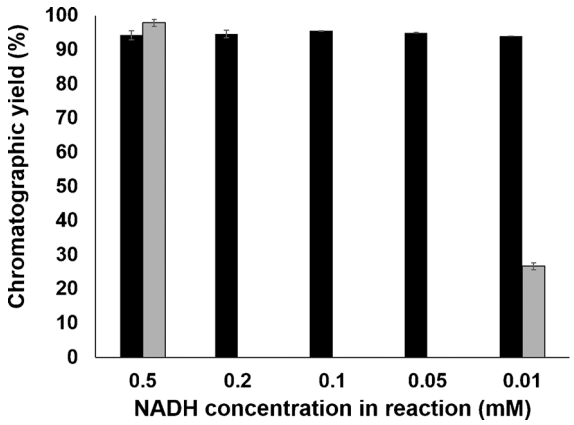


Figure 3. Chromatographic yields for the production of L-[^{13}N]alanine at different concentrations of NADH (black bars). Grey bars correspond to values obtained under identical experimental conditions in the absence of FDH. Values are expressed as mean ± standard deviation ($n=3$). The reactions were carried out in the presence of [^{13}N]NH_3 (20–25 MBq), L-AlaDH (60 μg mL^{-1}), NADH (0.01–0.5 mM), and pyruvic acid (75 mM) in sodium phosphate buffer (300 mM) at $T=25$ °C and pH 8.

In vivo studies

To prove the suitability of the labelling strategy for in vivo investigations, we carried out PET imaging with L-[^{13}N]alanine in healthy mice. Computerized Tomography (CT) images were sequentially acquired for proper localization of the radioactive signal. Starting from 220±20 MBq of cyclotron-produced [^{13}N]NH_4OH (volume = 100 μL), we obtained 85±6 MBq of pure L-[^{13}N]alanine by using the bi-enzymatic approach in an overall reaction time of 9 min (decay-corrected RCY = 73%; non-corrected RCY = 39%). Specific activity values were in the range 15–35 GBq μmol^{-1}. Acquisitions were started concomitantly with the intravenous administration of the labelled amino acid (100 μL, 16.5±3.1), and images were acquired in dynamic mode for 50 min.

As it can be seen in Figure 4, the presence of radioactivity is detected in the heart at short times after administration, which confirms the presence of the labelled amino acid in the blood. At longer time points, a progressive accumulation of radioactivity was observed in the liver, which is consistent with the anabolic function of this organ. The highest accumulation of radioactivity was found in the abdominal region, especially in the intestine; this result is consistent with biodistribution data that was previously reported for ^{11}C-labelled natural amino acids.[19] Low accumulation of radioactivity was found in the brain, which may facilitate the potential localization of tumours in this organ. Importantly, elimination by urine was negligible in the time window of the study. This is an essential finding considering the intended future application of this labelled amino acid as a potential marker for the investigation of metabolic processes in animal models of prostate cancer.

Conclusions

We have presented the fast, efficient, one-pot enzymatic synthesis of L-[^{13}N]alanine, [^{13}N]glycine, and L-[^{13}N]serine by using

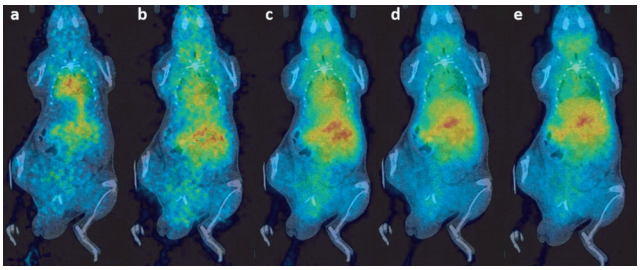


Figure 4. Representative PET-CT images obtained after intravenous administration of L-[^{13}N]alanine in mice. Coronal projections of PET images have been co-registered with representative CT slices for co-localization of the radioactive signal. Images correspond to data averaged in the following time frames: a) 0–30 s, b) 31–70 s, c) 71–230 s, d) 231–740 s, and e) 741–3020 s.

L-AlaDH and NADH as the cofactor. The synthesis of L-[^{13}N]alanine was significantly more efficient than the synthesis of [^{13}N]glycine and L-[^{13}N]serine; however, yields obtained for [^{13}N]glycine and L-[^{13}N]serine should be sufficient to approach subsequent preclinical investigations in rodents. The preparation of L-norvaline and L-phenylalanine did not work under no-carrier-added conditions owing to the high K_M values for ammonia. In the case of L-[^{13}N]alanine, the addition of FDH to the reaction media to regenerate NADH enabled a 50-fold decrease in the required concentration of NADH without compromising the radiochemical yields. The resulting labelled amino acid could be obtained in sufficient amount to carry out biodistribution studies in healthy mice by using PET-CT. This reported one-pot, multi-enzyme reaction opens new avenues for the enzymatic preparation of ^{13}N-labelled amino acids. Future efforts will pursue the co-immobilization of both enzymes on the same carrier to enable continuous flow reactions, the engineering of L-AlaDH to broaden its substrate scope, and the application of the here-reported labelled amino acids to the investigation of metabolic alterations in a mouse model of prostate cancer.

Experimental Section

Reagents

The genes of L-alanine dehydrogenase (L-AlaDH) from *Bacillus subtilis* (L-alanine: NAD oxidoreductase, $EC_{1.4.1.1}$) and formate dehydrogenase (FDH) from *Candida boidinii* (Formate: NAD^+ oxidoreductase, $EC_{1.2.1.2}$) were synthesized and cloned into pET28b by GenScript company (Piscataway, USA). β-Nicotinamide adenine dinucleotide, reduced disodium salt hydrate (β-NADH; purity > 97%), pyruvic acid ($CH_3COCOOH$, purity ≥ 98%), ammonium chloride (NH_4Cl, for molecular biology, purity ≥ 99.5%), sodium phosphate dibasic (Na_2HPO_4, for molecular biology, purity ≥ 98.5%), sodium phosphate monobasic (NaH_2PO_4, purity ≥ 99.0%), L-alanine (purity ≥ 98%), D-alanine (purity ≥ 98%), glycine (purity ≥ 99%), L-serine (purity ≥ 99.5%), D-serine (purity ≥ 98%), L-phenylalanine (purity ≥ 98%), D-phenylalanine (purity ≥ 98%), L-norvaline, and D-norvaline (purity ≥ 99%) were purchased from Sigma Chemical Co. (St. Louis, IL, USA) and used without further purification. TALON resin was purchased from Clontech (Saint-Germain-en-Laye, France). Bio-Rad Protein Assay Dye Reagent Concentrate was purchased from Bio-Rad Laboratories (Madrid, Spain). All others reagents were of analytical grade.

Production of L-AlaDH and FDH in *E. coli*

Expression

An overnight culture of *E. coli* BL21 (1 mL), transformed with the respective plasmid (pET28b-BsL-AlaDH or pET28b-CbFDH), was inoculated in a Luria–Bertani (LB) broth (50 mL) that contained kanamycin (final concentration of 30 μg mL^{-1}). For pET28b-BsL-AlaDH, the resulting culture was incubated at 37 °C with energetic shaking until the OD_{600nm} reached 0.6. 1-Thio-β-d-galactorpyranoside (IPTG) was added to the culture (final concentration of 1 mM) to induce protein synthesis. Cells were grown at 37 °C for 3 h and harvested by centrifugation at 1290 g over 30 min. For pET28b-CbFDH, the resulting culture was incubated at 21 °C with energetic shaking until the OD_{600nm} reached 0.6. 1-Thio-β-d-galactorpyranoside (IPTG) was added to the culture (final concentration of 1 mM) to induce protein synthesis. Cells were grown at 21 °C for 18 h and harvested by centrifugation at 1290 g over 30 min.

Purification

Both FDH and L-AlaDH were purified as follows: The resulting pellet was resuspended in one-tenth of its original volume of sodium phosphate buffer solution (25 mM, pH 7, binding buffer). Cells were broken by sonication (LABSONIC P, Sartorius Stedim biotech) at amplitude = 1 and cycle = 1 over 5 min. The suspension was centrifuged at 10528 g over 30 min. The supernatant that contained the enzyme was collected and passed through a TALON resin that was equilibrated with a binding buffer. Following the protein binding to the column, the column was washed three times with binding buffer prior to the protein elution with elution buffer (binding buffer supplemented with 300 mM imidazole). The eluted protein was gel-filtered by using PD-10 columns (GE healthcare) to remove the imidazole and exchange the enzyme buffer. SDS-page and Bradford assay were carried out after each production to determine the purity, concentration, and specific activity of the enzymes. Gel filtration was performed by FLPC by using a Superdex TM 200, 10/300 GL.

Protein and enzyme colorimetric assays

Determination of the protein concentration

The protein concentration of soluble L-AlaDH and FDH was carried out by Bradford's method by using bovine serum albumin (BSA) as protein standard. The soluble enzyme (5 μL) and Bradford reagent (200 μL) were mixed. The absorbance was measured at 595 nm after 5 min of reaction at $T = 25$ °C. For the standard curve, a series of BSA solutions (concentration = 0.06, 0.12, 0.25, 0.5, and 1 mg mL^{-1}) was used.

Determination of enzyme activity

The enzymatic activities were spectrophotometrically measured in 96-well plates by monitoring the absorbance at 340 nm, which varied depending on the concomitant production or consumption of NADH.

L-AlaDH: Reductive amination: L-AlaDH (10 μL), NADH (0.5 mM, 190 μL), and pyruvic acid (75 mM, or other α-keto acids) were incubated in ammonium chloride (450 mM) at pH 5–10 and $T = 25$ °C. Oxidative deamination: L-AlaDH (10 μL), NAD^+ (1 mM, 200 μL), and

alanine (10 mM L/D, or other amino acids) were incubated in sodium phosphate buffer (300 mM) at pH 8 and $T=25\,^{\circ}C$.

FDH: NAD^+ (1 mM, 200 μL) and formic acid (100 mM) in sodium phosphate buffer (25 mM, pH 7) were incubated with enzymatic solution (5 μL) at $T=25\,^{\circ}C$. One unit of activity was defined as the amount of enzyme that was needed to either reduce or oxidize 1 μmol of the corresponding nicotinamide cofactor at $T=25\,^{\circ}C$ and the corresponding pH.

Enzyme kinetic parameters

The kinetic parameters, Michaelis constant value (K_M), and maximum rate (V_{max}) were determined by activity colorimetric assay at pH 8 (pH 9 for serine) and $T=25\,^{\circ}C$ by following the method described above for L-AlaDH, with 0–1.8 M ammonium chloride as the amine source.

Production of the radiolabelling agent $[^{13}N]NH_3$

Nitrogen-13 (^{13}N) was produced in an IBA Cyclone 18/9 cyclotron by using the $^{16}O(p,\alpha)^{13}N$ nuclear reaction. The target system consisted of an aluminium insert (2 mL) covered with havar foil (thickness = 25 μm, ∅ 29 mm) and with an aluminium vacuum foil (thickness 25 μm, ∅ 23 mm). The target (containing 1.7 mL of 5 mM EtOH in H_2O) was irradiated with 18 MeV protons. The beam current was maintained at 22 μA (pressure in the range 5–10 bar into the target during bombardment) to reach the desired integrated currents (0.1–1 μAh). The resulting solution was transferred to a 10 mL vial, and the activity was measured in a dose calibrator (Capintec CRC®-25 PET, New Jersey, USA).

Synthesis of L-[^{13}N]amino acids

General procedure

Radioactive enzymatic reactions of the amino acids were carried out by adding 100 μL of a mixture that contained NADH (final concentration 0.5 mM), α-keto acid (final concentration 75 mM), and sodium phosphate buffer solution (300 mM, pH 8) to a solution that contained L-AlaDH (20 μL, concentration in the range 15–300 μg mL^{-1}) and $[^{13}N]NH_3$ (100 μL, 20–450 MBq). Reactions were conducted in tubes with 50 KDa cut-off membranes. For the preparation of L-[^{13}N]serine, sodium bicarbonate buffer (300 mM, pH 9) was used. The mixture was incubated at 25 °C under mild stirring for 2.5–20 min and filtered under centrifugation to remove the enzyme.

Enzymatic cascade reactions for regeneration of the cofactor

The reactions were conducted as above, but FDH at 5:1 (FDH/L-AlaDH) molar ratio and formic acid (100 mM) were added. 100 μL of this soluble preparation was incubated with cyclotron-produced $[^{13}N]NH_3$ solution (100 μL). All the reactions were carried out in tubes with 50 KDa cut-off membranes to easily separate the enzymes from the final products by fast centrifugation.

HPLC and radio-HPLC analysis

The determination of all radioactive/non-radioactive compounds was carried out by HPLC by using an Agilent 1100 Series system equipped with a quaternary pump and diode array detector connected in series with a radiometric detector (Gabi, Raytest, Straubenhardt, Germany). A chiral column (Phenomenex Chirex®3126 column, 50×4.6 mm; Micron Phenomenex, Madrid, Spain) was used as the stationary phase. As the mobile phase, 1 mM $CuSO_4$ solution (1 mL min^{-1}) was used for alanine, glycine, and norvaline; 2 mM $CuSO_4$ solution (0.2 mL min^{-1}) was used for serine; and 2 mM $CuSO_4$/methanol solution (70:30, 1 mL min^{-1}) was used for phenylalanine. Pure commercial enantiomers were used as standards.

For the determination of the specific activity (L-[^{13}N]alanine), the concentration of amino acid in the purified fraction was determined by HPLC-MS by using an AQUITY UPLC separation module coupled to an LCT TOF Premier XE mass spectrometer (Waters, Manchester, UK). An Acquity UPLC Glycan BEH amide column (1.7 μm×50 mm×2.1 mm) was used as the stationary phase. The elution buffers were acetonitrile (A) and 0.1 M ammonium formate (B). The column was eluted with a linear gradient: $t=0$ min, 95% B; $t=3$ min, 50% B; $t=3.5$ min, 95% B; $t=5$ min, 95% B. Total run was 5 min, injection volume was 10 μL, and the flow rate was 500 μL min^{-1}. The detection was carried out in positive ion mode, monitoring the most abundant isotope peaks from the mass spectra. Alanine was detected as a protonated molecule (m/z 89.9) with retention time = 1.81 min.

Animal experimentation

General considerations

Animals were maintained in a temperature and humidity-controlled room with a 12 h light–dark cycle. Food and water were available ad libitum. Animals were cared for and handled in accordance with the Guidelines for Accommodation and Care of Animals (European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes), and ethical protocols were approved by the internal Ethical Committee and by regional authorities.

In vivo studies

PET studies were performed by using an eXploreVista-CT small animal PET-CT system (GE Healthcare). Mice (20–22 g, C57BL/6JRJ, Janvier, France) were used ($n=2$). Mice were anesthetized with a mixture of 3–4% isoflurane in O_2 for induction, and this was reduced to 1–1.5% for maintenance. A nose cone was used to facilitate regular breathing, which was monitored by an SA Instrument Inc. (NY, USA) pressure sensor. Respiration and body temperature were monitored throughout the scan. The temperature, measured rectally, was maintained at 37±1 °C by using a water heating blanket (Homeothermic Blanket Control Unit, Bruker, Germany). For administration of ^{13}N-labelled amino acid, the tail vein was catheterized with a C10SS-MTV1301 29-gauge catheter (Instech, Laboratories Inc., Plymouth Meeting, PA, USA), and 16.5±3.1 MBq (approximately 100 μL) was injected in tandem with the start of a PET dynamic acquisition. The cannula was then rinsed with physiological saline solution (50 μL), and dynamic images (33 frames: 8×5 s, 6×15 s, 6×30 s, 6×60 s, 7×120 s) were acquired in 2 bed positions in the 400–700 keV energy window, with a total acquisition time of 50 min 20 s. After each PET scan, CT acquisitions were also performed, which provided anatomical information as well as the attenuation map for later image reconstruction. Dynamic acquisitions were reconstructed (decay, random and CT-based attenuation corrected) with OSEM-2D (2 iterations, 16 subsets). PET images were visually analysed by using PMOD image analysis software (PMOD Technologies Ltd., Zürich, Switzerland).

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Keywords: amino acids · biocatalysis · nitrogen-13 · positron emission tomography · radiochemistry

[1] *Positron Emission Tomography: Clinical Practice*, Springers – Vera, London, **2006**.
[2] a) C. M. Gomes, A. J. Abrunhosa, P. Ramos, E. K. J. Pauwels, *Adv. Drug Delivery Rev.* **2011**, *63*, 547 – 554; b) N. Gupta, P. M. Price, E. O. Aboagye, *Eur. J. Cancer* **2000**, 38, 2094 – 2107; c) E. Fernandes, Z. Barbosa, G. Clemente, F. Alves, A. J. Abrunhosa, *Curr. Radiopharm.* **2012**, *5*, 90 – 98.
[3] K. A. Frey, in *Basic Neurochemistry: Molecular, Cellular and Medical Aspects* (Ed.: B. W. A. G. J. Siegel, R. W. Albers, S. K. Fisher, M. D. Uhler), Lippincott – Raven, Philadelphia, **1999**.
[4] V. Gómez-Vallejo, V. Gaja, K. B. Gona, J. Llop, *J. Labelled Compd. Radiopharm.* **2014**, *57*, 244 – 254.
[5] a) M.-B. Tessem, M. G. Swanson, K. R. Keshari, M. J. Albers, D. Joun, Z. L. Tabatabai, J. P. Simko, K. Shinohara, S. J. Nelson, D. B. Vigneron, I. S. Gribbestad, J. Kurhanewicz, *Magn. Reson. Med.* **2008**, *60*, 510 – 516; b) M. J. Albers, R. Bok, A. P. Chen, C. H. Cunningham, M. L. Zierhut, V. Y. Zhang, S. J. Kohler, J. Tropp, R. E. Hurd, Y.-F. Yen, S. J. Nelson, D. B. Vigneron, J. Kurhanewicz, *Cancer Res.* **2008**, *68*, 8607 – 8615.
[6] a) N. Lembares, R. Dinwoodie, I. Gloria, P. Harper, K. Lathrop, *J. Nucl. Med.* **1972**, *13,* 786 – 786; b) M. G. Straatmann, M. J. Welch, *Radiat. Res.* **1973**, *56*, 48 – 56.
[7] M. B. Cohen, L. Spolter, N. S. McDonald, B. Cassen, *J. Nucl. Med.* 422.
[8] J. R. Barrio, F. J. Baumgartner, E. Henze, M. S. Stauber, J. E. Egbert, N. S. MacDonald, H. R. Schelbert, M. E. Phelps, F. T. Liu, *J. Nucl. Med.* **1983**, *24*, 937 – 944.
[9] M. B. Cohen, L. Spolter, C. C. Chang, N. S. MacDonald, J. Takahashi, D. D. Bobinet, *J. Nucl. Med.* **1974**, *15*, 1192 – 1195.
[10] E. S. da Silva, V. Gomez-Vallejo, J. Llop, F. Lopez-Gallego, *Catal. Sci. Technol.* **2015**, *5,* 2705 – 2713.
[11] I. Amelio, F. Cutruzzola, A. Antonov, M. Agostini, G. Melino, *Trends Biochem. Sci.* **2014**, *39*, 191 – 198.
[12] W. Ye, G. Huo, J. Chen, F. Liu, J. Yin, L. Yang, X. Ma, *Microbiol. Res.* **2010**, *165*, 268 – 275.
[13] a) K. J. Siranosian, K. Ireton, A. D. Grossman, *J. Bacteriol.* **1993**, *175*, 6789 – 6796; b) E. Freese, S. W. Park, M. Cashel, *Proc. Natl. Acad. Sci. USA* **1964**, *51*, 1164 – 1172.
[14] A. Yoshida, E. Freese, *Biochim. Biophys. Acta* **1965**, *96*, 248 – 262.
[15] a) P. J. Baker, Y. Sawa, H. Shibata, S. E. Sedelnikova, D. W. Rice, *Nat. Struct. Mol. Biol.* **1998**, *5*, 561 – 567; b) P. Fernandes, H. Aldeborgh, L. Carlucci, L. Walsh, J. Wasserman, E. Zhou, S. T. Lefurgy, E. C. Mundorff, *Protein Eng. Des. Sel.* **2015**, *28*, 29 – 35; c) T. Ohashima, K. Soda, *Eur. J. Biochem.* **1979**, *100*, 29 – 30.
[16] N. M. Brunhuber, J. S. Blanchard, *Crit. Rev. Biochem. Mol. Biol.* **1994**, *29*, 415 – 467.
[17] T. Ohshima, K. Soda, in *Bioprocesses and Applied Enzymology*, Springer, Heidelberg, **1990**, pp. 187 – 209.
[18] F. J. Baumgartner, J. R. Barrio, E. Henze, H. R. Schelbert, N. S. MacDonald, M. E. Phelps, D. E. Kuhl, *J. Med. Chem.* **1981**, *24*, 764 – 766.
[19] P. L. Jager, W. Vaalburg, J. Pruim, E. G. de Vries, K. J. Langen, D. A. Piers, *J. Nucl. Med.* **2001**, *42*, 432 – 445.

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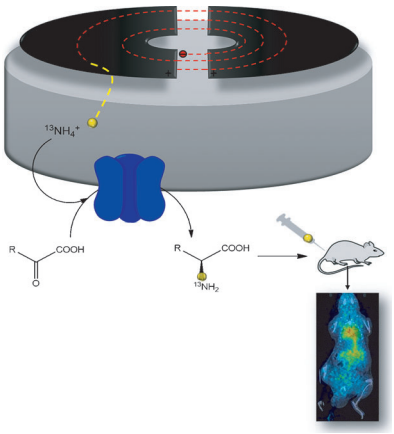
FULL PAPER

^{13}N-labelled radiotracers: L-Alanine dehydrogenase enables the fast and efficient synthesis of L-[^{13}N]alanine, [^{13}N]glycine, and L-[^{13}N]serine under mild conditions.



Radiochemistry

E. S. da Silva, V. Gómez-Vallejo, Z. Baz, J. Llop, F. López-Gallego**

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Efficient Enzymatic Preparation of ^{13}N-Labelled Amino Acids: Towards Multipurpose Synthetic Systems