Pyridine-based Strategy towards Nitrogen Isotope Exchange

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Abstract: Isotopic labeling is at the core of health and life science applications such as nuclear imaging, pharmacokinetics and bio-distribution studies and plays a central role in drug development. The rapid access to isotopically labeled organic molecules is a *sine qua non* condition to support these societally vital areas of research. Despite the relevance of pyridine as a biologically active scaffold, the nitrogen-13 labeling of this scaffold remains elusive and an almost prohibited challenge for radio-labeling (β^+ emitter, $T_{1/2}$ 9.97 min), despite its relevance in positron emission tomography. Based on a rationally driven approach, this study presents an innovative solution to access labeled pyridines by a nitrogen isotope exchange reaction based on a Zincke activation strategy. The technology conceptualizes a new opportunity in the field of nitrogen isotope labeling. ^{15}N-labeling of pyridine and other heterocycles such as pyrimidines and isoquinolines was provided on a large set of derivatives including structurally elaborated pharmaceuticals. Using [^{13}N]NH_3 as the primary nitrogen-13 source, proof-of-concept was provided to achieve examples of ^{13}N-labeling of pyridines. We believe this method will play a fundamental role for future developments of ^{13}N-based PET radiotracers.

Isotope labeling is a stringent requirement, which is of paramount importance in diverse areas constituting a multi-billion dollar global market including drugs, diagnostics, biology, toxicology and smart materials.[1] The strong industry demand elicited intensive investigations in order to develop such novel isotope labeling methods. The art of inserting an isotope into a target molecule has long fascinated radiochemists, but only over the past two decades unprecedented innovation has been witnessed, which sustained major advances in the area. In contrast with classical organic synthesis, isotope chemists must drift toward the hurdles of such specialized field and face unusual constraints, such as the high costs of building blocks, the narrow cohort of starting materials available and, in the case of radioactive isotopes, the constraints imposed by working with radioactivity, often within challenging time frames.

Among nitrogen-based heterocycles, pyridine derivatives represent the ultimate biologically active scaffold (Figure 1a). In 2014, Njardarson and co-workers have reported that pyridine is the second most common nitrogen heterocycle in U.S. FDA approved drugs[2] and agrochemicals contain this core in their structure as well.[3] Not surprisingly, radiochemists have been interested in labeling such a ubiquitous pharmacophore. In its essence, the pyridine moiety is composed of three elements: carbon, hydrogen and nitrogen (Figure 1b). Hydrogen isotope labeling is a gold standard for the insertion of deuterium and tritium (β^- emitter, $T_{1/2}$ 12.43 years) into pyridine scaffolds.[4] Hydrogen isotope exchange (HIE) using tritium has proven to be the most straightforward technology for labeling this scaffold in a single radioactive step.[5] This technology is used on a daily basis in pharmaceutical companies for drug development purposes. Noteworthy, the moderate metabolic stability at position 2 of pyridine must be taken into consideration when hydrogen labeling of this scaffold in performed.[6] In stark contrast, carbon and nitrogen isotope labeling of pyridines remain challenging, as they are located at the core of the heterocycle. Carbon labeling with ^{13}C and ^{14}C (β^- emitter, $T_{1/2}$ 5730 years) is a tedious multi-step process and based on archaic strategies, which do not meet the stringent efficiency requirements of our current society.[7,8,9,10,11,12] To the best of our knowledge, there are no reports on the core labeling of pyridines with the short-lived positron emitter ^{11}C ($T_{1/2}$ 20.4 min).

Nitrogen isotope labeling of pyridine bears great promise. Two stable isotopes of nitrogen exist, ^{14}N and ^{15}N which a natural abundance of 99.636 % and 0.364 %, respectively.[13] Radioactive isotopes of this element are known, but only nitrogen-13 (^{13}N, β^+ emitter, $T_{1/2}$ 9.97 min) allows applications for designing innovative radiotracers in positron emission tomography (PET). Unfortunately, due to its very challenging short half-life requiring its clinical application within

minutes of tracer synthesis, ^{11}C or ^{18}F counterparts are rather preferred nowadays drastically reducing the ^{13}N-radiolabeling state of the art.[14,15,16,17] Consequently, few nitrogen-13 tracers have been described and used so far. [^{13}N]ammonia is the only FDA approved radiotracer and is most wildly used to perform myocardial perfusion imaging.[18] It is also one of the primary sources of nitrogen-13 along with [^{13}N]NO_2^-, another valuable ^{13}N building block used to produce a variety of ^{13}N-labeled derivatives such as amino acids.[17,19] More complex tracers such as [^{13}N]thalidomide,[20] [^{13}N]dantrolene[21] and [^{13}N]nifedipine and derivatives[22] have also been described in the literature showing the rising interest for ^{13}N-radiotracers.

Nicotinic receptor families are involved in various neurodegenerative pathologies such as Parkinson or Alzheimers' Disease.[23] Numerous tracers containing pyridine scaffolds have been described so far,[24] but only as [^{18}F]fluoropyridine labeled moieties (^{18}F, $T_{1/2}$ 109.8 min) for positron emitting radiotracers,[25,26] the FDA-approved [^{18}F]Tauvid.[27] As substituted fluoro-pyridines are minor representatives of biologically active derivatives, ^{18}F-labeling mandates the need for additional structure activity relationship that are tedious and lengthy. Gaining access to [^{13}N]pyridine scaffolds could be therefore of great interest since they will remain unmodified and, taking advantage of its short half-life, repeated PET scans could also be envisioned on the same patient over a short time period. This is particularly important with the recent advances in ultra high sensitivity 'total body' PET Scanners. To the best of our knowledge, no reliable, reproducible and rapid labeling methods to access ^{13}N-labeled pyridines have been described, to date. During the preparation of this manuscript, independent work using ^{15}N–labeled dimethyl aspartate have been described. Its application to ^{13}N-labeling was not reported and it would require the prior ^{13}N-synthesis of the aspartate partner before labeling the pyridine moiety.[28] Moreover, it clearly appears that there is no general methodology allowing [^{13}N]NH_3 to be used as the primary isotopic source for synthesising valuable pyridine organic synthons.

This gap in the literature and lack of efficient methods for the conversion of [^{13}N]NH_3 into pyridines have dramatic consequences on the development of new promising PET radiotracers for molecular *in vivo* imaging with high sensitivity and resolution.

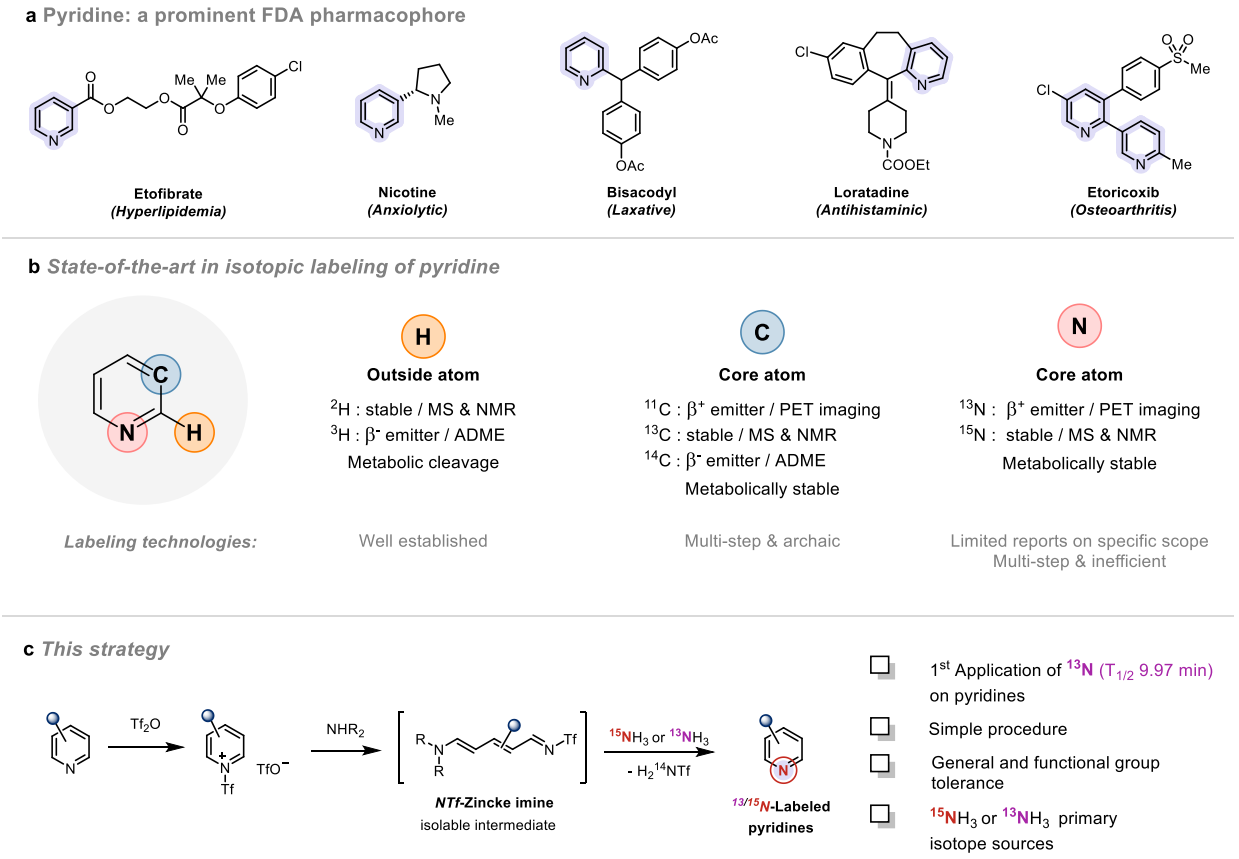


Figure 1. State-of-the-art in isotope exchange reactions. a) Pyridine: a prominent FDA pharmacophore. b) State-of-the-art for the isotopic labeling of pyridine. c) This strategy: nitrogen isotope exchange.

In order to bridge these gaps, we have investigated a pyridine-based scaffold editing technology allowing nitrogen isotope exchange (NIE) of pyridine moieties, using labeled ammonia as the primary isotopic nitrogen source (Figure 1C). The results presented herein show that by rational design and careful reactions optimization, partial to full isotope replacement can be achieved. This technology was applied to the labeling of an arsenal of substituted pyridines and pyrimidines, including structurally complex pharmaceuticals with stable ^{15}N. In addition, we provide the first proof of concept on ^{13}N-labeling of pyridines in one single radioactive step from the universal precursor [^{13}N]NH_3. This results open new avenues for multiple isotope labeling and bear unprecedented potential for the development of new ^{13}N-radiotracers.

Results and discussion

Reaction design and optimization

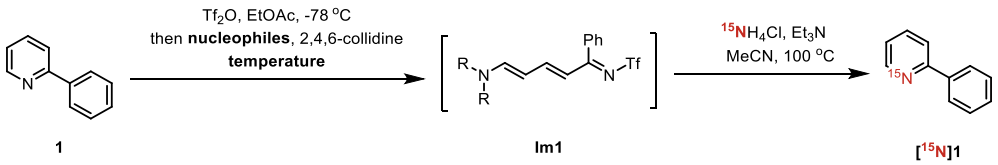
The replacement of naturally abundant ^{14}N by its isotopes (*i.e.* NIE) is an under-developed concept. Pinpointed examples exist, but are substrate specific, limited in scope, account for two-step processes and imply a structural modification between the starting material and the labeled product.[29,30,31] To the best of our knowledge, direct NIE without structural modifications is limited to nitrile metathesis in presence of molybdenum and tungsten catalysts [32] and to one single example of primary sulfonamide.[33]

Labeling of pyridine by NIE was first reported by Oppenheimer *et al.* in 1978. This procedure is based on the reaction between a Zincke pyridinium salt and labeled [^{15}N]NH_4Cl.[34] While potentially appealing, the reliability of this transformation seemed questionable, as further implementation showcased an extremely narrow synthetic application.[35,36,37,38] In addition, a series of pitfalls were identified: i) the need for a long reaction time, incompatible for applications with short-lived ^{13}N; ii) the inherent two-step nature of the procedure; iii) the well-established intolerance of the Zincke reaction towards pyridine substitution.[39,40] After preliminary investigations (see SI), we confirmed the narrow synthetic scope of the Zincke strategy and decided to move toward a more convenient approach.[41] Activation of the pyridine core in presence of trifluoromethanesulfonic anhydride (Tf_2O) has showcased outstanding potential for their late-stage functionalization under mild conditions.[42,43,44] In 1997, Toscano *et al.*[45] showed that triflypyridinium triflate (TPT) can be prepared *in situ* and underwent a ring-opening process in presence of amines to form the corresponding conjugated iminium species.[46] In 2022, Paton and McNally reported an outstanding application of this reaction manifold to perform otherwise challenging halogenation of the 3-position.[47] The same year, Sarpong and co-workers published a skeletal editing of pyrimidine with Tf_2O activation to access pyrazoles.[48] Leveraging of such strategies, we sought to achieve NIE on pyridine moieties. Aiming to provide a first entry toward ^{13}N-labeling of this pharmaceutically relevant scaffold, we recognized the mandatory use of labeled ammonia as fundamental to implement such an ambitious goal.[28]

To validate our hypothesis, 2-phenylpyridine **1** was selected as a model substrate for testing the $^{14}N/^{15}N$ exchange. As anticipated, 2-phenylpyridine **1** was converted to the corresponding *N*Tf-Zincke imine intermediate **Im1** after the nucleophilic attack of dibenzylamine (Table 1, Entry

1) on the triflic activated pyridine intermediate. [^{15}N]NH_4Cl, one of the most easily achievable ^{15}N sources, was employed to react with the *N*Tf-Zincke imine intermediate **Im1** in presence of triethylamine to generate [^{15}N]NH_3 *in situ*. After subsequent cyclization, ^{15}N-labeled 2-phenylpyridine **[^{15}N]1** was afforded in 99% yield with 68% ^{15}N-incorporation. The competition between the nucleophilic attack at the C_2 position of the Tf-pyridine intermediate and the sulfur atom of the triflic moiety could result in the desired *N*Tf-Zincke imine intermediate **Im1** and the 2-phenylpyridine **1** respectively, the latter being considered as the unlabeled component in the product. Increasing the efficiency of the conversion from starting material to the *N*Tf-Zincke imine intermediate was considered to be a key factor to further improve the isotopic enrichment (IE). A higher temperature for the nucleophilic attack of the amine on the activated pyridine was found to be beneficial to the isotopic enrichment of the product (Table 1, Entry 2-3). At 60 ^{o}C, the desired product was afforded in 87% yield with 76% of IE. Variety of nitrogen-bearing nucleophiles were therefore screened in the ring-opening step of 2-phenylpyridine since the amine substitution can affect their nucleophilicity as well as the stability of the afforded *N*Tf-Zincke imines. Representative results were illustrated in Table 1, entries 4-8 (see the supporting information for the full screening table). When tetrahydroquinoline was used, only 42% IE was obtained for the labeled product (Table 1, Entry 4). None the less, 71% IE was afforded when indoline was involved (Table 1, Entry 5). A series of tests with indolines bearing different substituents were not fruitful (see supporting information for details). *N*-methylaniline was found to be not as effective as dibenzylamine (Table 1, Entry 6) and less steric hindered nucleophiles, such as diethylamine (Table 1, Entry 7) and methylamine (Table 1, Entry 8) resulted in unidentified byproducts and a low yield of unlabeled product. Reasoning towards a full isotope replacement, we were pleased to observe that by precipitating the *N*Tf-Zincke imine intermediate **Im1** in *n*-hexane, the unlabeled 2-phenylpyridine **1** could be easily removed from the mixture. 97% ^{15}N-enriched 2-phenylpyridine **[^{15}N]1** could therefore be obtained in 64% combined yields when the precipitated *N*Tf-Zincke imine intermediate **Im1** was used in the subsequent labeling stage without further purification. This observation was a keystone for the development of our ^{13}N strategy, where isotope dilution is unsuitable, due to the limited scale of production of the radionuclide.

Table 1: Representative conditions screening of ^{15}N-labeling of pyridine.[a]



1 Im1 [^{15}N]1

Entry	Activation temperature	Nucleophiles	Isotopic Enrichment[b]	Yield[c]
1[d]	-78 to 25 °C	dibenzylamine	68%	99%
2[d]	40 °C	dibenzylamine	71%	88%
3	60 °C	dibenzylamine	76%	87%
4	60 °C	tetrahydroquinoline	42%	99%
5	60 °C	indoline	71%	99%
6	60 °C	*N*-methylaniline	62%	97%
7	60 °C	diethylamine	<5%	51%
8	60 °C	methylamine	<5%	65%
9[e]	60 °C	dibenzylamine	97%	64%[f]

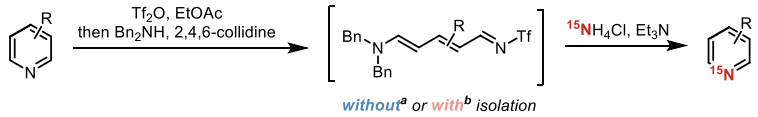
Reaction conditions: [a] 2-Phenylpyridine (0.2 mmol, 1.0 equiv.), Tf_2O (0.2 mmol, 1.0 equiv.), ethyl acetate (1.0 mL), -78 °C, 0.5 h, then nucleophiles (0.24 mmol, 1.2 equiv.), 2,4,6-collidine (0.2 mmol, 1.0 equiv.), indicated temperature, 1.0 h, then $^{15}NH_4Cl$ (0.6 mmol, 3.0 equiv.), triethylamine (1.2 mmol, 6.0 equiv.), acetonitrile (2.0 mL), 100 °C, 1 h. [b] Measured by LC-MS. [c] ^{1}H NMR yields using dibromomethane as an internal standard. [d] dichloromethane was used instead of ethyl acetate. [e] Imine intermediate was isolated by precipitation and then used in the labeling step. [f] Combined yields of isolated product was shown.

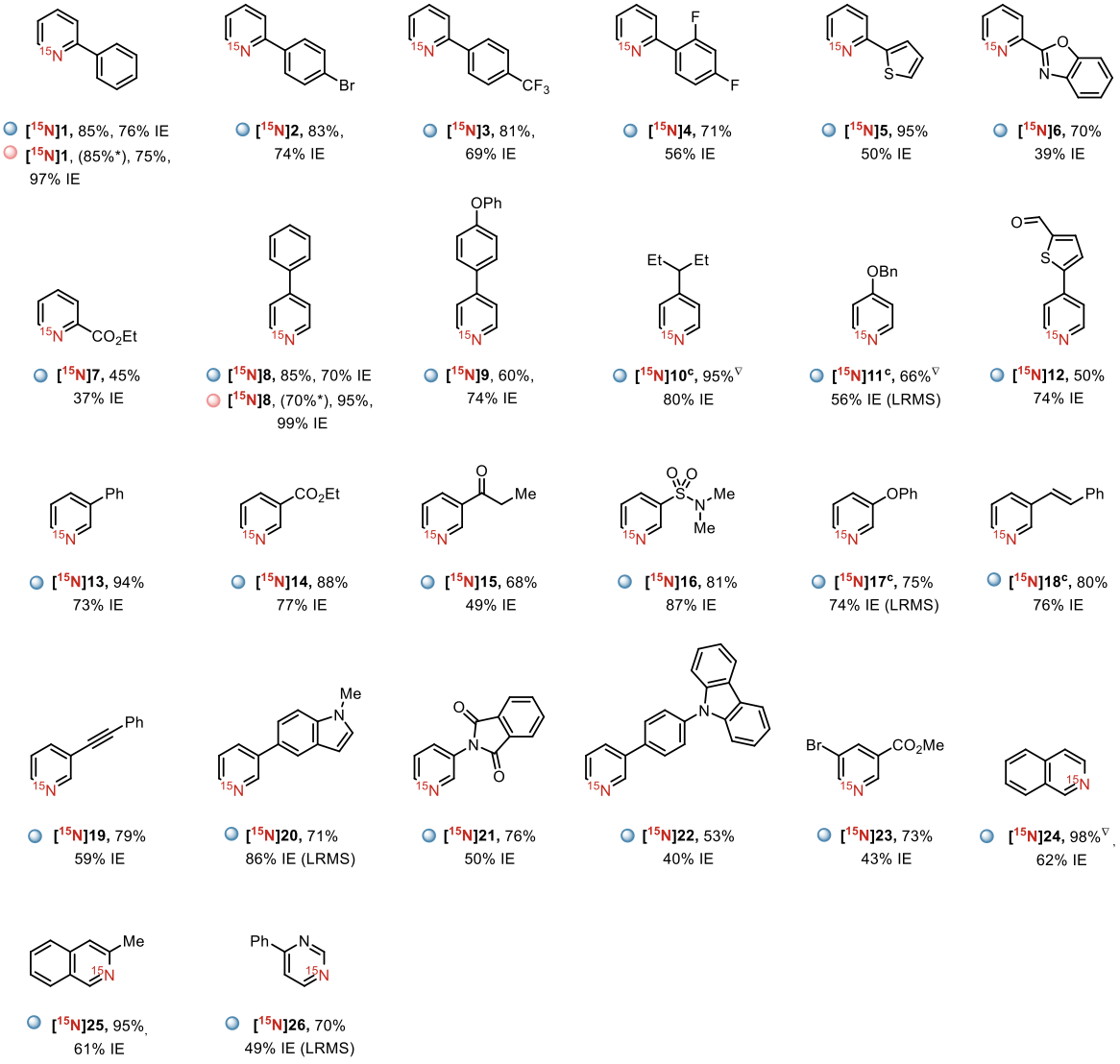
Reaction scope

With the optimized conditions in hand, we sought to explore the scope of this one-pot pyridine-based nitrogen isotope exchange strategy. Benefitting from the excellent chemoselectivity of triflic anhydride activation, this transformation displayed excellent functional group tolerance. Pyridines bearing substituents at positions 2, 3 and 4 could be efficiently converted to their corresponding ^{15}N-labeled counterparts. In contrast to the traditional Zincke strategy,[49] this procedure is fully compatible with a variety of aryl substituents at position 2 (Scheme 1, **1** to **4**) to give the corresponding ^{15}N-labeled pyridines (Scheme 1, **[^{15}N]1** to **[^{15}N]4**) with moderate to good IE. Heterocyclic moieties such as thiophene (Scheme 1, **[^{15}N]5**), benzo[*d*]oxazole (Scheme 1, **[^{15}N]6**) substituted pyridines are tolerated in this transformation. However, 2-

(pyridin-2-yl)benzo[*d*]oxazole **6** afforded the product with a lower isotopic enrichment (39%) since benzo[*d*]oxazole might react with Tf_2O. Ester moiety at position 2 of pyridine is tolerated as well (Scheme 1, **[^{15}N]7**). Pyridines with 4-substitued alkyl and aryl moieties are effective substrates (Scheme 1, **[^{15}N]8 to [^{15}N]10**). Electron donating group at position 4 (Scheme 1, **[^{15}N]11**) does not affect the reaction. Notably, the aldehyde moiety was tolerated, delivering the desired ^{15}N-lableled pyridine **[^{15}N]12** in 50% yield and 74% IE. Pyridines with a variety of substituents at position 3, including aryl (**[^{15}N]13**), ester (**[^{15}N]14**), ketone (**[^{15}N]15**), sulfonamide (**[^{15}N]16**), phenoxy group (**[^{15}N]17**), alkene (**[^{15}N]18**), alkyne (**[^{15}N]19**) as well as other heterocycles (**[^{15}N]20** to **[^{15}N]22**), proved to be efficient in this one-pot ^{15}N-labeling procedure, affording the labeled pyridines with 40% to 87% IE. To our delight, we were also able to achieve the ^{15}N-labeling on 3,5-disubstituted pyridine (**23**), isoquinolines (**24, 25**) and 4-phenylpyrimidine (**26**).

Scheme 1: ^{15}N-Labeling substrate scope.



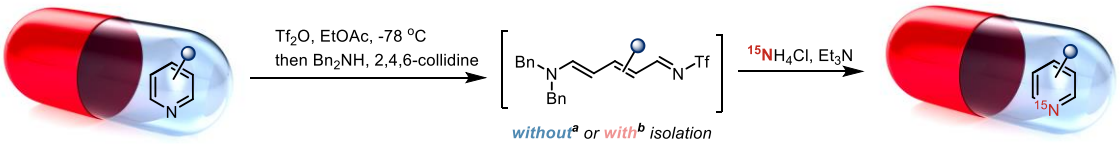


Reaction conditions: [a] Pyridines (0.2 mmol, 1.0 equiv.), Tf_2O (0.2 mmol, 1.0 equiv.), ethyl acetate (1.0 mL), -78 °C, 0.5 h, then dibenzylamine (0.24 mmol, 1.2 equiv.), 2,4,6-collidine (0.2 mmol, 1.0 equiv.), 60 °C, 1.0 h, then $^{15}NH_4Cl$ (0.6 mmol, 3.0 equiv.), triethylamine (1.2 mmol, 6.0 equiv.), acetonitrile (2.0 mL), 100 °C, 1 h. Yields of isolated products are shown. [b] Imine intermediate was isolated by precipitation and then used in the labeling step. *Yields of isolated imines. ▽^{1}H NMR yields using dibromomethane as an internal standard. [c] Dichloromethane was used instead of ethyl acetate in the first step. IE : isotopic enrichment

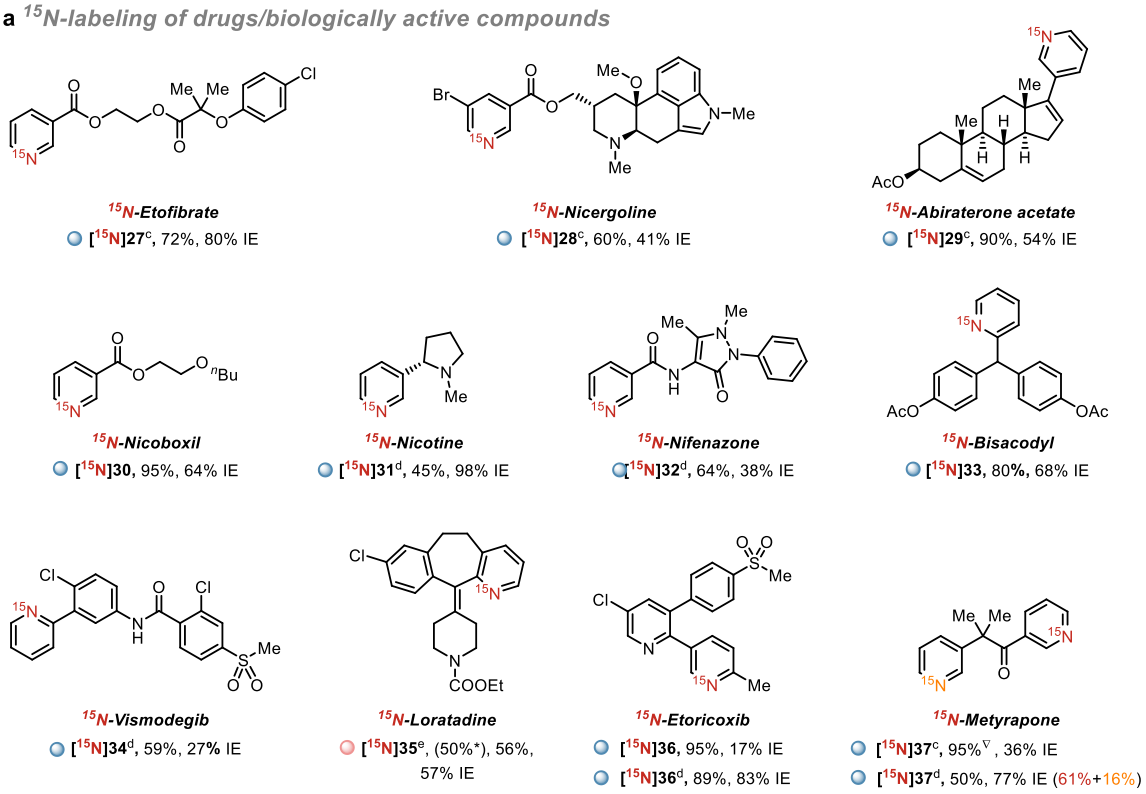
The application to late-stage ^{15}N-labeling of pyridine-containing elaborated pharmaceuticals and biologically active molecules was then realized using the developed protocol. As shown in Scheme 2a, a variety of ^{15}N-labeled drugs and biologically active compounds were labeled straightforwardly by using the corresponding starting material without the need of any functionalization. When other nucleophilic moieties presented in the same structure, an adapted procedure, with an increased number of Tf_2O equivalents and dibenzylamine, was applied to afford the product with improved isotopic enrichments (Scheme 2a, **[^{15}N]31**, **[^{15}N]32**, **[^{15}N]34,** **[^{15}N]36**, **[^{15}N]37**). Notably, ^{15}N-enriched Nicotine (Scheme 2a, **[^{15}N]31**) was obtained with 98% IE from unfunctionalized Nicotin through this adapted one-pot procedure. Secondary amides, which have the potential to react with Tf_2O, are well-tolerated in this transformation (Scheme 2a, **[^{15}N]32**, **[^{15}N]34**). While the standard process gave low isotopic enrichment of Loratadine, **[^{15}N]35** could be obtained in 57% IE by precipitating the corresponding *N*Tf-Zincke imine intermediate. Etoricoxib, a marketed selective COX-2 inhibitor, has two pyridine subunits in its structure. The nitrogen of the disubstituted pyridine was selectively labeled without affecting the trisubstituted one (Scheme 2a, **[^{15}N]36**). In another example, a 3.8:1 regioselectivity was observed for the ^{15}N-labeling of Metyrapone, favoring the electron-deficient pyridine scaffold (Scheme 2a, **[^{15}N]37**).

To assess the utility of the nitrogen isotopic exchange in the field of PET radiochemistry, we next explored its application to ^{13}N-labeling. Despite its massive potential, ^{13}N is still an underused radionuclide for PET imaging. By cyclotron mediated proton bombardment of a pure water target containing trace amounts of ethanol, [^{13}N]NH_3 was produced in aqueous solution. As a proof-of-concept, the isolated *N*Tf-Zincke imines (**Im1** and **Im8**) from 2-phenylpyridine **1** and 4-phenylpyridine **8** were treated with cyclotron-produced [^{13}N]NH_3 at 100 ^{o}C for 6 minutes (Scheme 2b). The desired [^{13}N]2-phenylpyridine **[^{13}N]1** and [^{13}N]4-phenylpyridine **[^{13}N]8** were afforded successfully with moderate radiochemical yields of 20% and 78%, respectively. These results demonstrate that our nitrogen isotopic exchange strategy can be applied to radioactive ^{13}N-labeling, and further expands the scope of new ^{13}N-labeling methods.

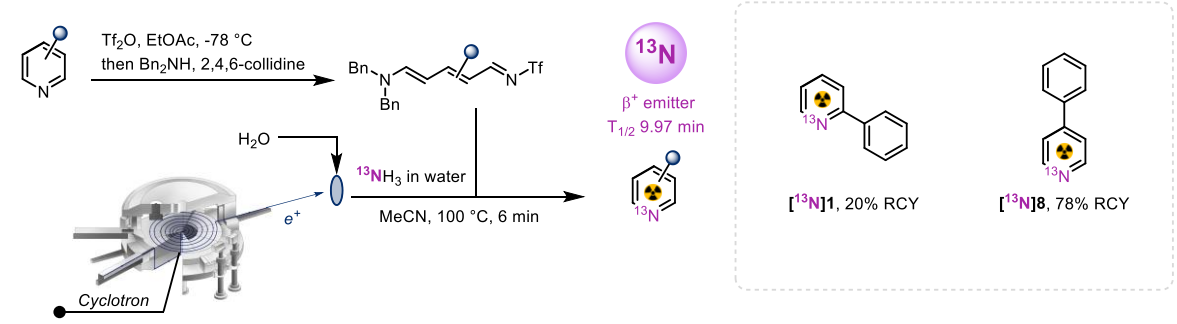
Scheme 2: ^{15}N-Labeling of pharmaceutical molecules and radioactive ^{13}N-labeling.



a *^{15}N-labeling of drugs/biologically active compounds*



b *^{13}N-labeling experiments*



Reaction conditions: [a]Pyridines (0.1 to 0.2 mmol, 1.0 equiv.), Tf_2O (1.0 equiv.), ethyl acetate (0.5 to 1.0 mL), -78 °C, 0.5 h, then dibenzylamine (0.24 mmol, 1.2 equiv.), 2,4,6-collidine (1.0 equiv.), 60 °C, 1.0 h, then $^{15}NH_4Cl$ (3.0 equiv.), triethylamine (6.0 equiv.), acetonitrile (1.0 to 2.0 mL), 100 °C, 1 h. Yields of isolated products are shown. [b] Imine intermediate was isolated by precipitation and then used in the labeling step. * Yields of isolated imines. [c] Dichloromethane was used instead of ethyl acetate in the first step. [d] Pyridines (0.2 mmol, 1.0 equiv.), Tf_2O (2.0 equiv.), dichloromethane (1.0 mL), -78 °C, 0.5 h, then dibenzylamine (4.8 equiv.), 2,4,6-collidine (2.0

equiv), 25 °C, 1.0 h, then $^{15}NH_4Cl$ (3.0 equiv.), triethylamine (6.0 equiv.), acetonitrile (2.5 mL), 100 °C, 1 h. [e] Reaction stirred for 24 hours for the labeling-cyclization step. IE: Isotopic enrichment

In conclusion, this study presents an innovative solution to access labeled pyridines by NIE based on a Zincke activation strategy. The technology conceptualizes a new opportunity in the field of isotope labeling and prospects to move forward in the challenging isotope labeling realm. ^{15}N-labeling of pyridine was possible with up-to-full isotope incorporation into a large variety of derivatives, including pyrimidines and isoquinolines. Using labeled ammonia as primary isotopic source, this method proved to be compatible with late-stage NIE of complex pharmaceutical derivatives and proof-of-concept on the application of this technology toward PET suitable ^{13}N-labeling was provided. We believe this novel method will play a fundamental role for the development of ^{13}N-based PET radiotracers.

Acknowledgments:

The authors thank T. D'Anfray, S. Lebrequier and D.-A. Buisson (DRF-JOLIOT-SCBM, CEA) for the excellent analytical support. We thank F. Taran and G. Pieters for helpful discussion.

Funding:

This work was supported by CEA and the European Union's Horizon 2020 research and innovation program under the European Research Council (ERC-2019-COG – 864576, D.A.) and the European Union's Horizon 2020 research innovation program FET-OPEN No 862179. EPSRC are gratefully acknowledged for the Programme Grant (EP/S032789/1) and Standard Research (EP/W021307/1) funding that supported this work.

Author contributions:

S. F. and D. A. conceived the idea and supervised project. M. F. and D. A. designed the experiments. M. F., M. N., and B. G. performed the experiments, synthesized and characterized the molecules, analyzed the data discussed the results. S. K. performed the nitrogen-13 labeling. S. K., A. G. analyzed and characterized the nitrogen-13 labeling experiments. P. T. performed the analysis of X-ray diffraction. M. F., S. F. and D. A. prepared the manuscript with contributions from all authors.

Competing interests:

The authors declare no competing interests.

Data and materials availability:

Experimental procedures and characterization data are provided in the supplementary material. Correspondence and requests for materials should be addressed to S. F. and D.A.

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