^{15}N-Labeled ionic probes for bioanalytical mass spectrometry†

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An effective La-complex-based probe ionization method is reported. Novel stable isotopically labeled probes containing the ^{15}N-labeled 2,6-bis(oxazolin-2-yl)pyridine (pybox) ligand, succinimide-tetramethylpybox (NHS-TMpybox), maleimide-tetramethylpybox (Mal-TMpybox), and 4-(tetramethylpybox)-butyl bromoacetate (BrAc-TMpybox), have been synthesized and their value in analyzing large complex molecules has been studied. The value of the ^{15}N-labeled pybox-La complex in ionizing various compounds, including bioactive peptides by cold-spray ionization mass spectrometry is emphasized.

Introduction

The combination of stable isotope labeling and mass spectrometry (MS) using electrospray ionization (ESI)[1] and matrix-assisted laser desorption ionization (MALDI)[2] has been developed.[3] This technique has made it possible to solve various problems in bioanalytical research by means of metabolic tracer experiments. Moreover, isotope labeling plays a significant role in the analysis of large complex molecules by NMR and MS. As regards previous studies that used isotope labeling in MS, the isotope-coded affinity tag (ICAT)[4] and the isobaric tag for relative and absolute quantitation (iTRAQ)[5] have been demonstrated to be useful for proteomics. MS probes that react with various functional groups to increase the relative abundance of ions formed from compounds.[6] Previously, we developed new MS probes **1** and **2**, which can donate plural charges contained in the metal charged site of the probe molecule to the target compound, in order to analyze large complex molecules, including biomolecules, in soft ionization conditions (Fig. 1).[7] The ionic probes could be an appropriate target of isotope labeling. Then, we planned to use ^{15}N-labeled pybox-La complex **4** to furnish labeled probes (**1**+**4**) and (**2**+**4**) from **1** and **2**, respectively, to compare the isotopic mass shifts between labeled and non-labeled ionic probe attached biomolecules (Scheme 1). We also prepared a new MS probe, BrAc-TMpybox **3**, which can react with the carboxyl group in biomolecules. In this paper, we describe the preparation of the ^{15}N-labeled ionic probes including BrAc-TMpybox **3** for the isotope labeling of biomolecules and the analysis of the biomolecules in soft ionization conditions by cold-spray ionization mass spectrometry (CSI-MS).[8]

Results and discussion

BrAc-TMpybox **3**, an MS probe that can react with a carboxyl group, and ^{15}N-labeled pybox-La complex **4** were prepared as follows. 4-Benzyloxypybox **6** was prepared from chelidamic acid (**5**) in five steps in 46% yield.[5] 4-Hydroxypybox **7** was obtained by removing the benzyl group on **6**, and **7** was alkylated with 4-bromobutyl acetate to yield **8**. Hydrolysis of the acetate on **8** furnished **9** (three steps, 97% yield). Finally, the desired BrAc-TMpybox **3** was obtained in 72% yield by conversion of the hydroxyl group into α-bromoacetate (Scheme 1). The nitrogen in each oxazoline ring of the pybox was labeled with ^{15}N to produce a charged complex (Scheme 1). Because the yield of the complex prepared by a conventional method that used ^{15}N-labeled glycine was low, we examined a new method to obtain ^{15}N-labeled La complex (^{15}N-**4**). The ethyl ester **12** was prepared from pyridine 2,6-dicarbonyl dichloride (**11**)[7] and glycine-^{15}N ethyl ester[9] in 77% yield. After **12** was reduced with $LiBH_4$ to furnish diol **13** in 67% yield,[10] diol **13** containing two hydroxyl groups was converted into the dichloride **14** in 70% yield. Finally, ^{15}N-**4** was obtained by construction of an oxazoline ring under basic conditions in a total yield of 32% from **10**. Synthesized probe **3**, ^{15}N-**4**, and **1** or **2** were then introduced to biomolecules and CSI-MS measurement was conducted.[11]

1- or **2**-bound biomolecules were prepared by two methods, one involving purification (A) and the other, no purification (B).[12] **3**-bound molecules were prepared by method C. **3** (1.5 Meq.), Na_2CO_3 (2 Meq.) as base, and DMF as solvent were mixed at room temperature[13] and DMF solution of the derivative was poured into an MeCN solution of ^{14}N- or ^{15}N-**4**.

At first, the probes were introduced to three amino acids (lysine, arginine, and cysteine). Similar to ^{14}N-(**1**+**4**)-bound lysine[7] (Fig. 2a), ^{15}N-(**1**+**4**)-bound lysine exhibited a doubly charged ion. The reduction of the triple charge at the metal of the probe by acetylacetonato ($acac^-$) as the counter anion led to the observation of $[M+acac^-]^{2+}$ (m/z: 480) in the CSI mass spectrum (Fig. 2b). Then, ^{15}N-(**1**+**4**)-bound arginine was detected also as a doubly charged ion of $[M+acac^-]^{2+}$ (m/z: 494) (Fig. 2d). In the case of ^{15}N-(**2**+**4**)-bound cysteine, a doubly charged ion of $[M+acac^-]^{2+}$ was observed as well (Fig. 2f). These three amino acids to which the ^{15}N-labeled probe was attached were detected as doubly charged ions as observed in the non-labeled ones with a definite isotopic shift of 1 u.

In order to confirm the specific probe attachment of the BrAc-TMpybox **3** and the ^{15}N isotope labeling, cholic acid (CA), 3,5-dihydroxybenzoic acid (3,5-DHBA), and pepstatin A (PA)

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† Electronic supplementary information (ESI) available: ^{1}H and ^{13}C NMR spectra of **3**, **8**, **9**, **12**, **13**, **14** and **15**. See DOI: 10.1039/c003784b

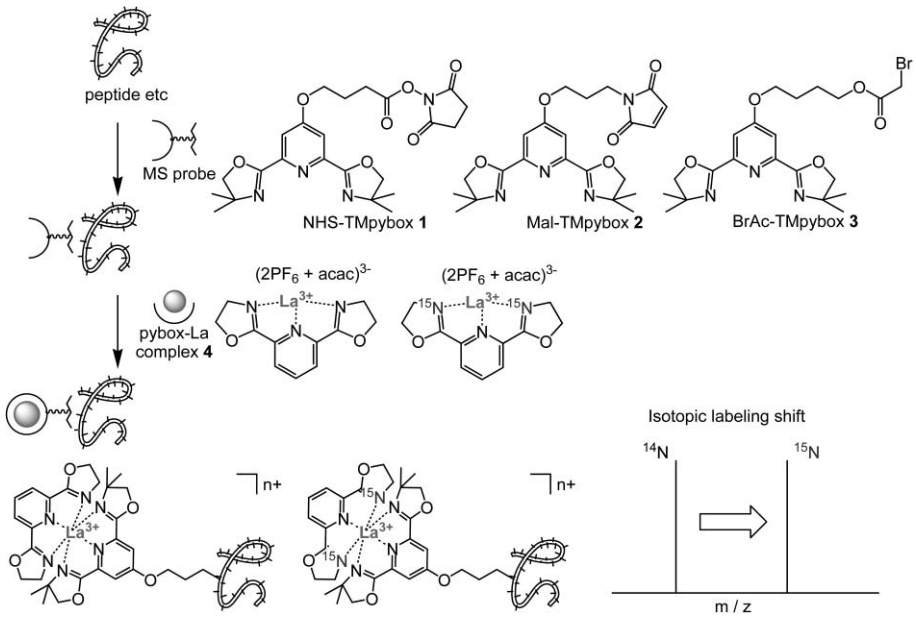
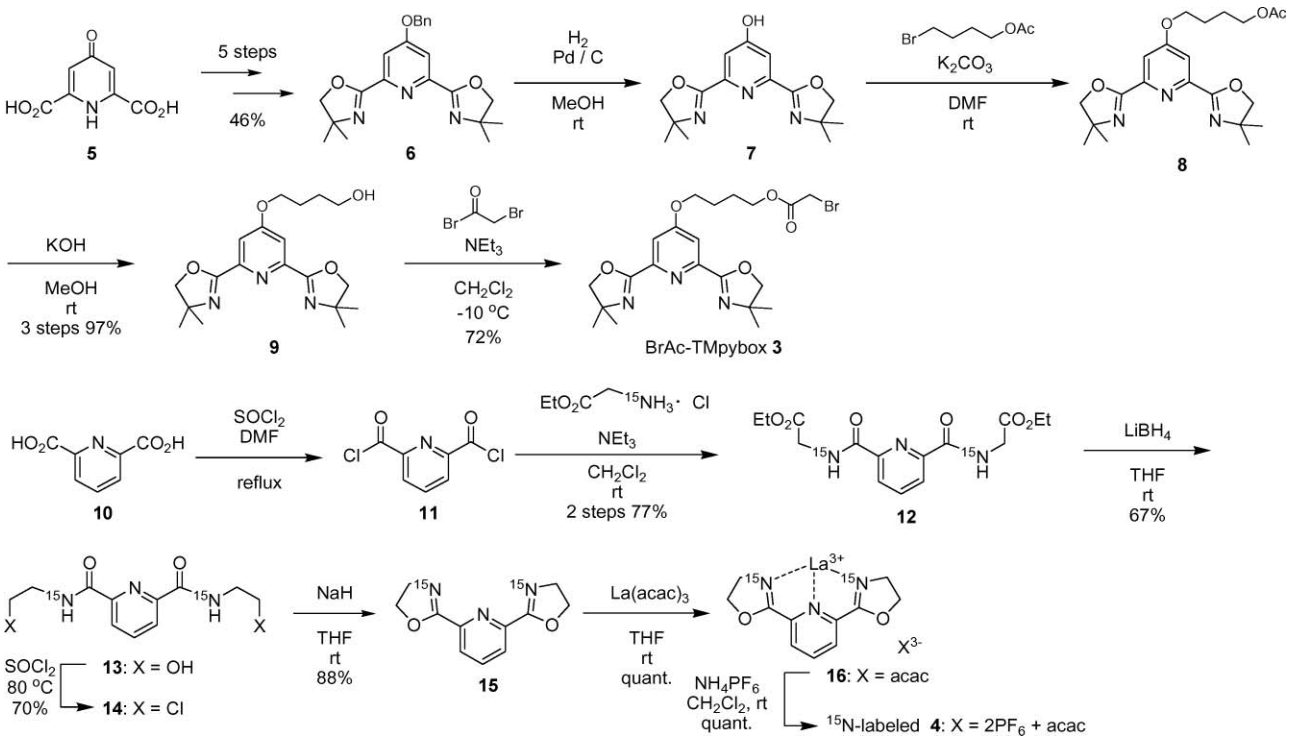


Fig. 1 Ionic probe attachment ionization mass spectrometry.



Scheme 1 Preparation of BrAc-TMpybox **3** and ^{15}N-labeled pybox-La complex **4**.

were examined using method C. These ions based on ^{14}N- and ^{15}N-**3**-bound compounds in the CSI mass spectrum corresponding to the doubly charged ions $[M–H^+]^{2+}$ (m/z: 455 and 456; 632 and 633; and 770 and 771) are seen in Fig. 3 (3a and 3b; 3c and 3d; and 3e and 3f, respectively). Clearly, a compound **3** reacted with the carboxyl group, whereas other aliphatic and aromatic hydroxyl groups in each compound, which are able to react with **3** under basic conditions, were inactive even in the presence of an excess amount of **3** by method C.

In order to induce multiply charged ions and observe the isotopic m/z shift by ^{15}N labeling, we adopted this method to various peptides [TTKTT, TTCTT, CTTTC, angiotensin-II (AT-II), bradykinin (BK), glutathione (GSH), and substance P (SP)].

The CSI mass spectra of ^{14}N- and ^{15}N-(**2**+**4**)-bound peptides prepared by method A are shown in Fig. 4. In the case of ^{14}N-(**2**+**4**)-bound GSH and TTCTT, ions of $[M–H^+]^{2+}$ (m/z: 544 and 653, respectively) were detected (Fig. 4a and c, respectively).[7,14] Deprotonation likely occurred in the maleimide of Mal-TMpybox

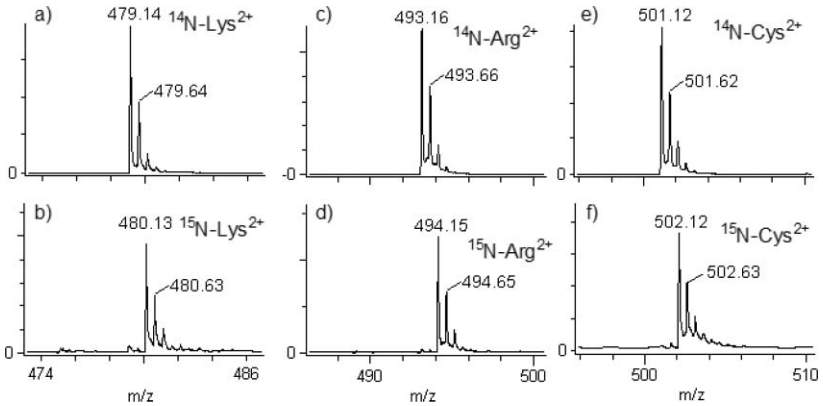


Fig. 2 CSI mass spectra of (a) ^{14}N-(**1**+**4**)-Lys, (b) ^{15}N-(**1**+**4**)-Lys, (c) ^{14}N-(**1**+**4**)-Arg, (d) ^{15}N-(**1**+**4**)-Arg, (e) ^{14}N-(**2**+**4**)-Cys, and (f) ^{15}N-(**2**+**4**)-Cys prepared by method A.

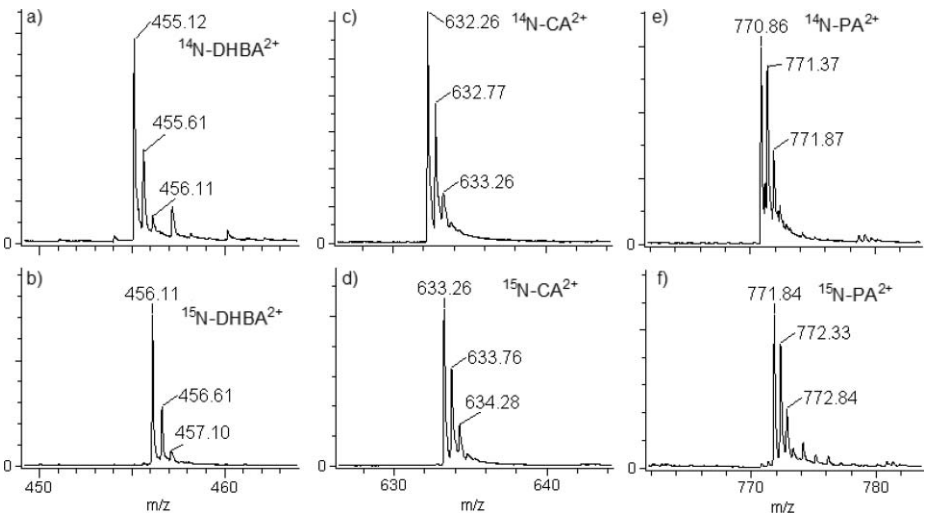


Fig. 3 CSI mass spectra of (**3**+**4**)-bound carboxylic acids (a) ^{14}N-CA, (b) ^{15}N-CA, (c) ^{14}N-3,5-DHBA, (d) ^{15}N-3,5-DHBA, (e) ^{14}N-PA, and (f) ^{15}N-PA.

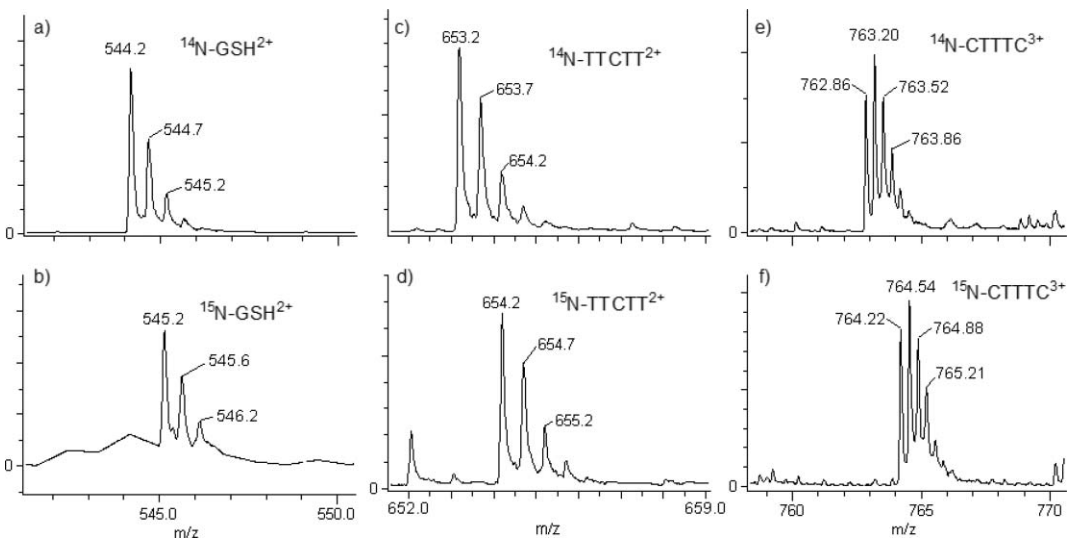


Fig. 4 CSI mass spectra of (**2**+**4**)-bound peptides prepared by method A. (a) ^{14}N-GSH, (b) ^{15}N-GSH, (c) ^{14}N-TTCTT, (d) ^{15}N-TTCTT, (e) ^{14}N-CTTTC, and (f) ^{15}N-CTTTC.

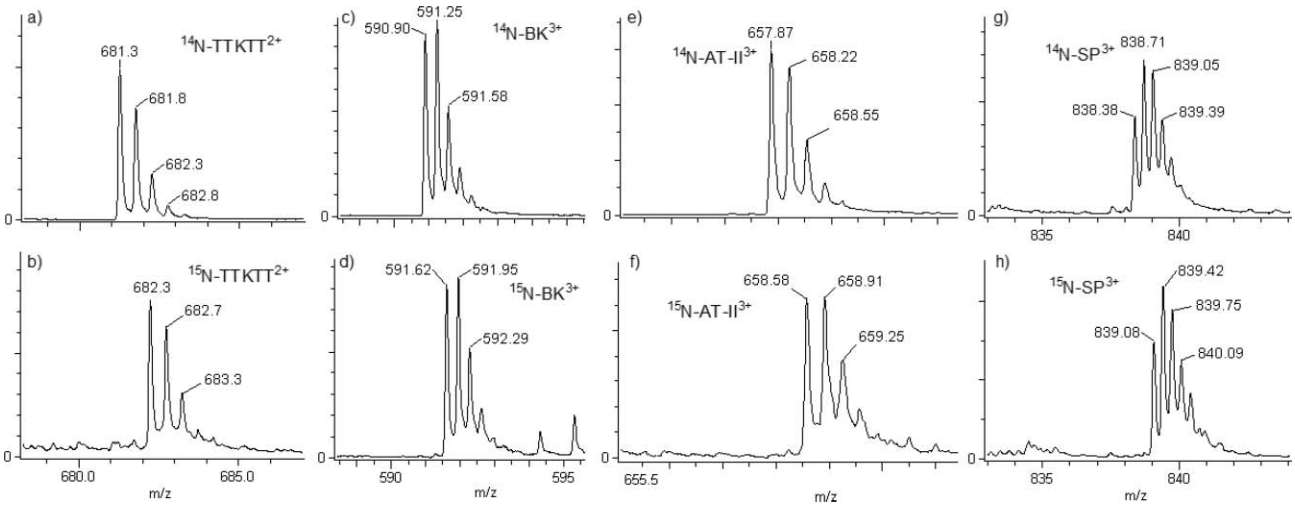


Fig. 5 CSI mass spectra of (**1**+**4**)-bound peptides: (a) ^{14}N-TTKTT, (b) ^{15}N-TTKTT, (c) ^{14}N-BK, (d) ^{15}N-BK, (e) ^{14}N-AT-II, (f) ^{15}N-AT-II, (g) ^{14}N-SP, and (h) ^{15}N-SP. (a) and (b) were prepared by method A, and (c) to (f) were prepared by method B.

2, which was caused by acidic protons in the carbonyl group or the proton in the peptide's carboxyl group. Similarly, these ions of $[M–H^+]^{2+}$ (*m*/*z*: 545 and 654) were detected in ^{15}N-(**2**+**4**)-bound GSH and TTCTT, respectively (Fig. 4b and d, respectively). The isotopic shift of 1 u was also observed in these spectra. Then, ^{14}N- and ^{15}N-(**2**+**4**)-bound CTTTC, which contain two probes in (**2**+**4**), were examined. The ions of $[M+2acac^––H^+]^{3+}$ (*m*/*z*: 763 and 764, respectively) were clearly observed (Fig. 4e and f, respectively). The isotopic shift of *ca.* 1.3 u (4/3) to higher *m*/*z*, corresponding to the increase in mass induced by four ^{15}N atoms on an ion with three charges in this molecule, was detected.

The CSI mass spectra of ^{14}N- and ^{15}N-(**1**+**4**)-bound peptides are shown in Fig. 5. Similar to the case of the amino acids, ^{14}N- and ^{15}N-(**1**+**4**)-bound TTKTT, which were prepared by method A, exhibited doubly charged ions that included $acac^–$ as the counter anion. In the case of BK, AT-II, and SP, triply charged ions of $[M]^{3+}$ (*m*/*z*: 591 and 592) (Fig. 5c and d, respectively), $[M+acetylacetone]^{3+}$ (*m*/*z*: 658 and 659) (Fig. 5e and f, respectively), and $[M+4acetylacetone+3H_2O]^{3+}$ (*m*/*z*: 838 and 839) (Fig. 5g and h, respectively) were observed, respectively, for the corresponding ^{14}N- and ^{15}N-(**2**+**4**)-bound peptides prepared by method B, in the CSI mass spectra. The isotopic shift of *ca.* 0.7 u (2/3) to higher *m*/*z*, corresponding to the increase in mass induced by two ^{15}N atoms on an ion with three charges, was detected.

Conclusions

In conclusion, we succeeded in synthesizing BrAc-TMpybox **3** and a ^{15}N-labeled pybox-La complex **4** to obtain three ionic probes, isotopically labeled metal complexes (**1**+**4**), (**2**+**4**), and (**3**+**4**). Moreover, we prepared useful ionic probes that react with major functionalities in biomolecules, such as amino acids, peptides, and bile acids. The biomolecules to which these ^{15}N-labeled ionic probes are attached were easily ionized in soft conditions and exhibited reasonable isotopic shifts. We believe that this method is useful for the analysis of proteomes and various isotopes as well as the analysis of large complex molecules by deducing the mass-to-charge ratio from the mass spectrum. The investigation of other ionic probes for large molecules, including carbon clusters and various functional probes, such as those that show fluorescence, is under way.

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Experimental

General

All melting points were measured on a Yanaco MP-500D and are uncorrected. Infrared spectra were recorded on a JASCO FT/IR 6300 spectrophotometer with an ATR (attenuated total reflectance) system. ^{1}H, ^{13}C, ^{15}N, and ^{31}P NMR spectra were recorded in $CDCl_3$ unless otherwise stated, on a JEOL JNM-ECP 400 and a Bruker 400 MHz with tetramethylsilane (TMS) as internal reference. CSI mass spectra were recorded on a JEOL JMS-T100LC mass spectrometer equipped with a cold-spray ion source. Silica gel 60 F_{254} TLC plates (Merck No. 5715) and NH plates (Fuji Silysia Chemical, Ltd., No. TO80817) were used for TLC. For column chromatography, silica gel 60 (spherical, particle size 63–210 μm, Kanto Chemical, No. 37565-84 for neutral) and NH silica gel (Fuji Silysia Chemical Ltd., particle size 100–200 mesh, No. IO61280 and 200–350 mesh, No. HU80502) were used. Combi*Flash* Companion (Teledyne Isco) was used for reversed-phase flash column chromatography. Eyela FDU-1200 was used as the vacuum freeze dryer. GSH, BK, AT-II, SP, and all anhydrous solvents were purchased from Wako Pure Chemical. DMSO was purchased from Merck. Customized peptides, TTKTT, TTCTT, and CTTTC, were purchased from Invitrogen.

Synthesis of 4-[2,6-bis(4,4-dimethyloxazolin-2-yl)pyridine-4-yloxy]butyl acetate (8)

A solution of **6** (1.01 g, 2.66 mM) in dry MeOH (10 mL) was hydrogenated over 10% Pd/C (500 mg) under hydrogen atmosphere at room temperature for 2 h. After removal of

the catalyst by filtration through a Celite pad, the filtrate was evaporated *in vacuo*. Under Ar, α-bromobutyl butyrate (0.8 mL, 5.53 mM) was added to the mixture of crude **7** and K_2CO_3 (1.12 g, 7.91 mM) in DMF (10 mL). The whole was stirred at room temperature for 24 h. The reaction was quenched with H_2O and extracted with AcOEt. The organic layer was washed with H_2O and brine, dried over Na_2SO_4, and evaporated *in vacuo*. The residue was purified by NH column chromatography (200–350 mesh, n-hexane : AcOEt = 10 : 1 to n-hexane : $CHCl_3$ = 2 : 1) to give **8** (968 mg, 97%) as a colorless oil. IR (ATR, cm^{-1}): 1737, 1642. ^1H NMR (400 MHz) δ: 1.39 (12H, s), 1.77–1.92 (4H, m, overlapped with H_2O), 2.06 (3H, s), 4.14 (4H, q, J = 6.2 Hz), 4.20 (4H, s), 7.68 (2H, s). ^{13}C NMR (100 MHz) δ: 21.1, 25.2, 25.6, 28.5, 63.9, 68.0, 68.1, 79.8, 112.2, 148.6, 161.0, 166.0, 171.2. HRCSIMS m/z 426.1985 (calcd for $C_{21}H_{29}N_3NaO_5$: 426.2005).

Synthesis of 4-[2,6-bis(4,4-dimethyloxazolin-2-yl)pyridine-4-yloxy]butan-1-ol (9)

After powdered KOH (737 mg, 13.1 mM) was added to a solution of **8** (1.06 g, 2.64 mM) in MeOH (20 mL), the whole was stirred at room temperature for 1 h. The reaction was quenched with sat. NH_4Cl aq. and extracted with CH_2Cl_2. The organic layer was washed with brine, dried over Na_2SO_4, and evaporated *in vacuo* to give colorless prisms (899 mg, 97%). This product was used without further purification. mp 128–129 °C. IR (ATR, cm^{-1}): 3276. ^1H NMR (400 MHz, CD_2Cl_2) δ: 1.39 (12H, s), 1.72, 1.92 (each 2H, quin, J = 6.7 Hz), 3.72, 4.16 (each 2H, t, J = 6.7 Hz), 4.20 (4H, s), 7.71 (2H, s). ^{13}C NMR (100 MHz) δ: 25.40, 28.5, 29.1, 62.3, 68.0, 68.6, 79.8, 112.3, 148.6, 161.1, 166.0. HRCSIMS m/z 384.1930 (calcd for $C_{19}H_{27}N_3NaO_4$: 384.1899). Anal. Calcd for $C_{19}H_{27}N_3O_4$: C, 63.14; H, 7.53; N, 11.63. Found: C, 63.02; H, 7.49; N, 11.72.

Synthesis of 4-[2,6-bis(4,4-dimethyloxazolin-2-yl)pyridine-4-yloxy]butyl bromoacetate (3)

Under Ar, after 4-bromobutyl acetate (0.4 mL, 2.76 mM) was added to a solution of 8 (300 mg, 0.83 mM) and NEt_3 (1.0 mL, 7.17 mM) in CH_2Cl_2 (10 mL) at −10 °C, the whole was stirred at −10 °C for 0.5 h. The reaction was quenched with sat. $NaHCO_3$ aq. and extracted with CH_2Cl_2. The organic layer was washed with sat. $NaHCO_3$ aq. and brine, dried over Na_2SO_4, and evaporated *in vacuo*. The residue was purified by column chromatography (AcOEt : acetone = 10 : 1) to give colorless needle-like prisms **3** (482 mg, 72%). mp 115–118 °C. IR (ATR, cm^{-1}): 1741, 1647. ^1H NMR (400 MHz) δ: 1.39 (12H, s), 1.84–1.93 (4H, m), 3.84 (2H, s), 4.16 (2H, t, J = 6.0 Hz), 4.20 (4H, s), 4.25 (2H, t, J = 6.0 Hz), 7.69 (2H, s). ^{13}C NMR (100 MHz) δ: 25.0, 25.4, 25.7, 28.4, 65.6, 67.9 (overlapped with ArOCH_2 CH_2 CH_2 CH_2O, confirmed by 2D NMR, see the ESI†), 79.8, 112.2, 148.5, 161.0, 165.8, 167.3. HRCSIMS m/z 520.0851 (calcd for $C_{21}H_{28}{}^{79}BrKN_3O_5$: 520.0849) and 522.0826 (calcd for $C_{21}H_{28}{}^{81}BrKN_3O_5$: 522.0829).

Synthesis of ethyl {6-[(ethoxycarbonylmethyl)carbamoyl]-^{15}N-pyridine-2-carbonyl}amino-^{15}N-acetate (12)

Under Ar, after DMF (20 mL) was stirred in $SOCl_2$ (75 mL) at room temperature for 10 min, dipicolic acid (**10**) (6.0 g, 36 mM) was added. The whole was stirred under reflux for 2 h. After cooling to room temperature, $SOCl_2$ was azeotroped with toluene. The residue was filtered through a Celite pad with Et_2O. The filtrate was concentrated *in vacuo* to afford crude acid chloride **11**. Under Ar, to a solution of glycine-^{15}N ethyl ester (9.3 g, 66 mM) and NEt_3 (20 mL, 144 mM) in CH_2Cl_2 (10 mL), the solution of crude **11** in CH_2Cl_2 (50 mL) was slowly added at 0 °C. The whole was stirred at room temperature for 12 h. The residue was diluted with CH_2Cl_2 and the organic layer was washed with H_2O and brine, dried over Na_2SO_4, and evaporated *in vacuo*. The residue was purified by column chromatography (n-hexane : AcOEt = 1 : 4) to obtain brownish hard oil **12** (9.4 g, 77% from **10**). IR (ATR, cm^{-1}): 3323, 1746, 1662. ^1H NMR (400 MHz) δ: 1.32 (6H, t, J = 7.1 Hz), 4.24–4.29 (4H, m), 4.27 (4H, q, J = 7.1 Hz), 7.96 (2H, t, J = 7.8 Hz), 8.24 (2H, d J = 7.8 Hz), 8.30, 8.54 (1H, t, J = 5.7 Hz). ^{13}C NMR (100 MHz) δ: 14.2, 41.4, 41.5, 61.8, 125.2, 138.9, 148.2, 163.5, 163.7, 170.3. ^{15}N NMR (40 MHz) δ: 93.1. HRCSIMS m/z 362.1079 (calcd for $C_{15}H_{19}N^{15}N_2NaO_6$: 362.1112).

Synthesis of $^{15}N^2$,$^{15}N^6$-bis(2-hydroxyethyl)-2,6-pyridine-dicarboxamide (13)

Under Ar, to a dry THF (5 mL) solution of **12** (1.1 g, 3.4 mM) was added $LiBH_4$ in THF solution (5 mL, *ca.* 15 mM) at room temperature. After the whole was stirred at room temperature for 5 h, MeOH and acetone were added at 0 °C and the whole was evaporated *in vacuo*. The residue was purified by reversed-phase flash column chromatography (H_2O : MeCN = 100 : 0 to 50 : 50) to obtain yellow powder **13** (572 mg, 67%). mp 27–31 °C. IR (ATR, cm^{-1}): 3289, 1649. ^1H NMR (400 MHz, CD_3OD) δ: 3.57 (4H, br t, J = 6.3 Hz), 3.74 (4H, br t, J = 6.3 Hz), 8.11 (1H, t, J = 7.8 Hz), 8.24 (1H, d, J = 7.8 Hz, ^{15}NH). ^{13}C NMR (100 MHz, CD_3OD) δ: 41.8, 41.9, 60.3, 124.4, 139.1, 148.8, 164.7, 164.9. ^{15}N NMR (40 MHz, CD_3OD) δ: 102.1. HRCSIMS m/z 262.1171 (calcd for $C_{11}H_{15}LiN^{15}N_2O_4$: 262.1163).

Synthesis of $^{15}N^2$,$^{15}N^6$-bis(2-chloroethyl)-2,6-pyridine-dicarboxamide (14)

Under Ar, an $SOCl_2$ (20 mL) solution of **13** (508 mg, 2.0 mM) was stirred under reflux for 1 h. After cooling to room temperature, $SOCl_2$ was azeotroped with toluene. The residue was diluted with CH_2Cl_2 and the organic layer was washed with H_2O and brine, dried over Na_2SO_4, and evaporated *in vacuo*. The residue was purified by column chromatography (n-hexane : AcOEt = 1 : 2) to obtain colorless powder **14** (408 mg, 70%). mp 155–157 °C. IR (ATR, cm^{-1}): 3303, 1646. ^1H NMR (400 MHz) δ: 3.77 (4H, br s), 3.88 (4H, br q, J = 5.7 Hz), 8.06 (1H, t, J = 7.8 Hz), 8.33, 8.38 (1H, br t, J = 5.7 Hz), 8.38 (1H, d, J = 7.8 Hz). ^{13}C NMR (100 MHz) δ: 41.2, 41.3, 44.0, 125.4, 139.3, 148.5, 163.5, 163.7. ^{15}N NMR (40 MHz) δ: 97.4. HRCSIMS m/z 316.0180 (calcd for $C_{11}H_{13}{}^{35}Cl^{37}ClN^{15}N_2NaO_2$: 316.0194).

Synthesis of 2,6-bis(oxazolin-2-yl)-$^{15}N_2$-pyridine (15)

Under Ar, amide **14** (610 mg, 2.1 mM) was added to a suspension of NaH (60%, 253 mg, 6.3 mM) in THF (10 mL). After the whole was stirred at room temperature for 0.5 h, the mixture was filtered. After this filtrate was evaporated *in vacuo*, the residue was purified by NH column chromatography (n-hexane : acetone = 1 : 1) to obtain **15** (403 g, 88%) as a brownish powder. mp 230–233 °C. IR

(ATR, cm^{-1}): 1625. ^1H NMR (400 MHz) δ: 4.13 (4H, t, $J = 9.6$ Hz), 4.54 (4H, t, $J = 9.6$ Hz), 7.88 (1H, t, $J = 7.8$ Hz), 8.17 (2H, d, $J = 7.8$ Hz). ^{13}C NMR (100 MHz) δ: 55.2, 68.4, 125.6, 137.4, 146.8, 163.5. ^{15}N NMR (40 MHz) δ: 221.1. HRCSIMS m/z 242.0684 (calcd for $C_{11}H_{11}N^{15}N_2NaO_2$: 242.0690).

Synthesis of tris(acetylacetonato)[2,6-bis(oxazolin-2-yl)-$^{15}N_2$-pyridine]lanthanum(III) (16)

Under Ar, after a mixture of **15** (118 mg, 0.54 mM) and La(acac)$_3$ (374 mg, 0.86 mM) in THF (4 mL) was stirred at room temperature for 24 h, it was filtered to give **16** (346.0 mg, quant.) as a colorless powder. This product was used without further purification. mp 216–218 °C. ^1H NMR (400 MHz, CD_2Cl_2) δ: 1.66 (18H, s), 4.00 (4H, t, $J = 9.6$ Hz), 4.55 (4H, t, $J = 9.6$ Hz), 5.13 (3H, s), 7.91 (2H, br s), 7.97 (1H, br s). ^{13}C NMR (100 MHz) δ: 28.0, 54.1, 69.7, 99.8, 124.9, 138.6, 145.5, 163.6, 188.0. ^{15}N NMR (40 MHz) δ: 223.8. HRCSIMS m/z 556.0744 (calcd for $C_{26}H_{32}{}^{139}LaN^{15}N_2O_8$–acac: 556.0748).

Synthesis of acetylacetonato(dihexafluorophosphine)[2,6-bis(oxazolin-2-yl)-$^{15}N_2$-pyridine]lanthanum(III) (4)

Under Ar, **16** (234 mg, 0.36 mM) was added to NH_4PF_6 (185 mg, 1.13 mM). After this mixture was stirred at room temperature for 24 h, it was filtered to give ^{15}N-**4** (310 mg, quant.) as a colorless powder. This product was used without further purification. mp >300 °C. ^1H NMR (400 MHz, DMSO-d_6) δ: 1.72 (6H, s), 3.98 (4H, t, $J = 9.6$ Hz), 4.46 (4H, br t, $J = 9.2$ Hz), 5.23 (1H, s), 8.08 (3H, br s). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 27.8, 54.9, 68.6, 94.7, 126.2, 139.9, 146.9, 162.7, 188.7. $^{31}P\{^1H\}$ NMR (162 MHz, DMSO-d_6) δ: −143.6 (heptet, $J = 711$ Hz). ^{15}N NMR (40 MHz, DMSO-d_6) δ: 225.0. HRCSIMS m/z 338.0520 (calcd for $C_{16}H_{18}F_{12}{}^{139}LaN^{15}N_2O_4P_2$ + pybox **15** ($C_{11}H_{12}N^{15}N_2O_2$) – $2PF_6$: 338.0547), m/z 358.5675 (calcd for $C_{16}H_{18}F_{12}{}^{139}LaN^{15}N_2O_4P_2$ + pybox **15** ($C_{11}H_{12}N^{15}N_2O_2$) – $2PF_6$ + MeCN: 358.5679).

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12 Method A: To a solution (0.5 mL) of the target compound (1 mM in phosphate pH standard solution) was added a solution (0.5 mL) of probe (**1** or **2**) (1 mM in DMSO) at room temperature. After the mixture was stirred at room temperature for 2 h, it was purified by reversed-phase flash column chromatography (H_2O–MeCN = 100 : 0 to 60 : 40). After the target compound was evaporated *in vacuo*, a solution (0.5 mL) of ligand **4** (1 mM in MeCN) was added. The mixture was stirred at room temperature for 12 h and part of the mixture was diluted with MeCN and subjected to CSI-MS. Method B: To a solution (0.5 mL) of the target compound (5 mM in H_2O) was added a solution (0.5 mL) of probe (**1** or **2**) (5 mM in DMSO) at room temperature. After the mixture was stirred at room temperature for 48 h, it was vacuum-freeze dried. A solution (1 mL) of ligand **4** (8 mM in MeCN) was added to the residue and the mixture was stirred at room temperature for 5 h. Then, part of the mixture was diluted with MeCN and subjected to CSI-MS.

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