Tetrahedron Letters 50 (2009) 6252–6255

Contents lists available at ScienceDirect

Tetrahedron Letters

journal homepage: www.elsevier.com/locate/tetlet

Ionic probe attachment ionization mass spectrometry

Fumihiro Ito *, Tomoko Nakamura, Satoko Yorita, Hiroshi Danjo, Kentaro Yamaguchi *

Faculty of Pharmaceutical Sciences at Kagawa Campus, Tokushima Bunri University, 1314-1 Shido, Sanuki, Kagawa 769-2193, Japan

ARTICLE INFO

Article history:
Received 24 July 2009
Revised 1 September 2009
Accepted 3 September 2009
Available online 9 September 2009

ABSTRACT

A new ionization method that uses metal-complex-based ionization probes containing the 2,6-bis(oxazolinyl)pyridine (pybox) ligand is presented. This method was proven to effectively ionize large complex molecules, including biomolecules. The preparation of the charged probes and their application to the ionization of biomolecules using cold-spray ionization mass spectrometry are shown.

The continuous development of the soft ionization technique is thought to have sparked the advancement of mass spectrometry (MS). Since electron ionization (EI), the first practical ionization method used in chemistry, was coined, remarkable progress has been made in this area. Recent soft ionization technique has made it possible to analyze such biomacromolecules as proteins.[1] The technique is based on electrospray ionization (ESI)[2] and matrix-assisted laser desorption ionization (MALDI).[3] During analysis under soft ionization conditions, the higher order structure of a biomacromolecule could be preserved. Therefore, the elucidation of primary structures as well as higher order structures[4] and their dynamics has become possible, and post-translational modification analysis has become a reality.

However, problems that hinder the reliable and reproducible ionization of complex large biomolecules by these methods still remain, because of the often observed ambiguous protonation during ESI-MS analysis. The protonation seems to be more difficult in the case of cold spray ionization (CSI), a variant of ESI method operating under low temperature to ionize labile organic species.[5] Therefore, an effective adjustable-charge-state ionization technique[6] is required for these molecules. We have developed a new method called 'ionic probe attachment ionization.' We have designed ionic probes that can donate plural charges contained in the metal charged site of the probe molecule to the target compound. As regards previous studies of instrumental analysis using chemical probes, the caged lanthanide NMR probe by Ubbink and co-workers[7] and the ruthenium(II) bisterpyridine complexes of an electron spectroscopic probe by Thordarson and co-workers[8] are known. The former is used as a relaxation reagent and/or a pseudo contact-shift reagent to obtain long-distance restraints for solving solution structures. In the latter, the probe is introduced to cytochrome *c* to observe photoelectron transfer between the probe donor and the cytochrome *c* acceptor. Furthermore, ruthenium(II) bisterpyridine complex was used in various related research areas.[9] In this Letter, we present new metal-complex-based ionization probes containing the 2,6-bis(oxazolinyl)pyridine (pybox) ligand, which can ionize target molecules by donating plural charges effectively and reliably. We also describe the application of the charged probes to the ionization of biomolecules by cold-spray ionization mass spectrometry (CSI-MS).[5]

The probe comprises three functional parts: a charged site, a linker, and an anchoring site (Fig. 1), similar to biotin reagents.[10] The pybox ligand is used to enclose the charged metal in the charged site, although the terpyridine-type ligand also seems to be a good candidate for this site. Compared with the terpyridine-type ligand, the pybox ligand has structural diversity that can be easily controlled by choosing the structure of the amino alcohol used as the starting material. In addition, complexation with metal cations takes place with the pybox ligand under mild conditions. A haloalkane moiety having variable alkyl chain length was used as a linker.[11] Two compounds, succinimide (NHS) and maleimide (Mal), were used as the anchoring site to bind the probe to the target molecule. The two probes used in this study, NHS-tetramethylpybox (NHS-TMpybox) and Mal-tetramethylpybox (Mal-TMpybox), were prepared as follows.[12] 4-Chloropybox **5** was prepared from chelidamic acid (**1**) using a previously reported method for pybox synthesis.[13] Nucleophilic aromatic substitution of **5** with BnOH afforded **6** in 94% yield. 4-Hydroxypybox **7** was obtained by removing the benzyl group on **6** in 68% yield. Ethyl ester **8** was furnished from **6** in 86% yield by alkylation with ethyl 4-bromobutyrate. Finally, desired NHS-TMpybox **10** was obtained by hydrolysis of **8**

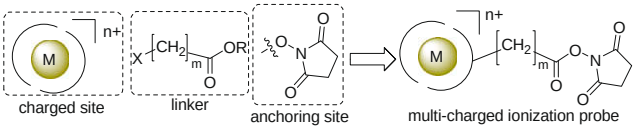
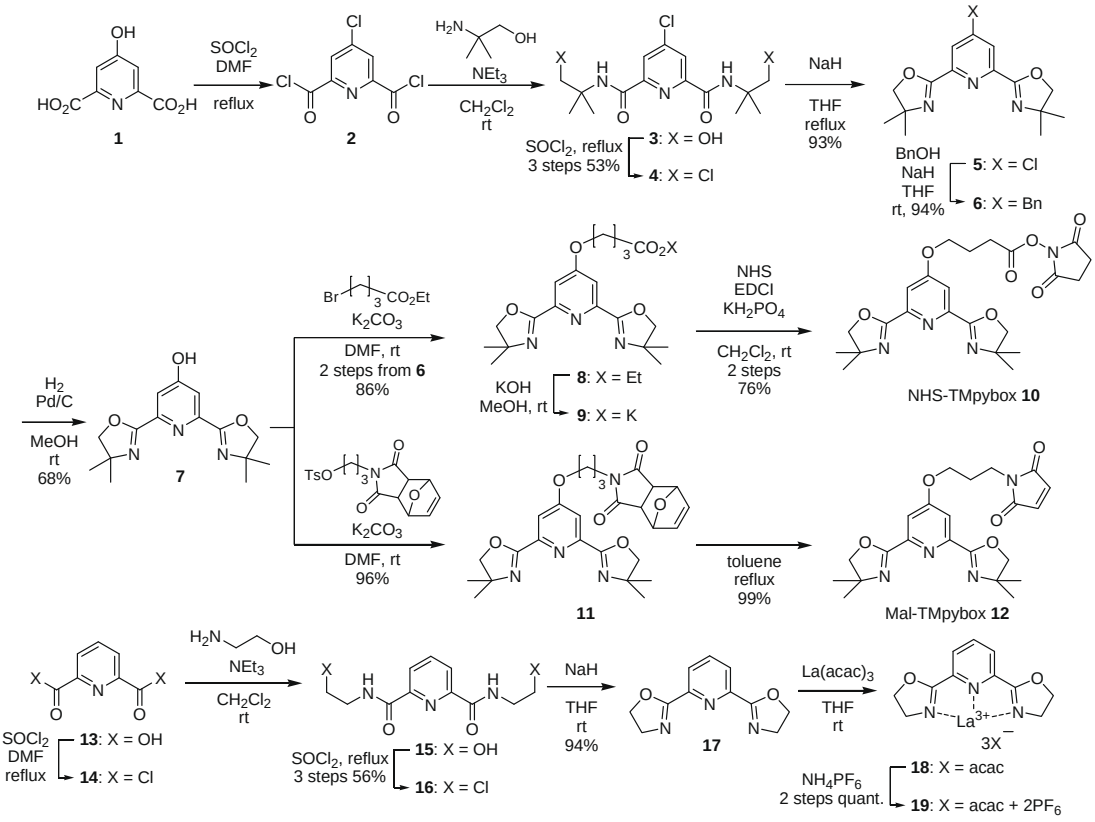


Figure 1. Constitution of the charged probe.

* Corresponding authors. Tel.: +81 87 894 5111; fax: +81 87 894 0181.
E-mail addresses: h-itoh@kph.bunri-u.ac.jp (F. Ito), yamaguchi@kph.bunri-u.ac.jp (K. Yamaguchi).

0040-4039/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.tetlet.2009.09.021



Scheme 1. Synthesis of NHS-TMpybox **10** and Mal-TMpybox **12**.

and conversion of potassium salt **9** into NHS ester **10** in two steps in 76% yield. Mal-TMpybox **12** was prepared by coupling **7** with tosylate[14] and subsequent retro Diels–Alder reaction of **11** (Scheme 1). As previously described,[13] pybox **17** was prepared from dipicolinic acid (**13**). We chose lanthanum(III) ion as the charged center because of its robustness to oxidation/reduction and low isotope abundance ratio (^{138}La:139La = 0.09:99.91). Treatment of pybox **17** with La(acac)$_3$ (acac: acetylacetonato) gave La(III) complex **18**. Finally, **19** was obtained by counter anion exchange of acac$^-$ with PF_6^- (Scheme 1).[15] Although we attempted to replace all the three acac$^-$ with PF_6^-, only two of them could be exchanged to give La(III) complex **19**. Then, **19** was mixed with **10** and **12** to give respective charged probes **20** and **21** in which the metal cation was enclosed by two pybox ligands to prevent coordination with the counter anion, which promote ionic dissociation (Fig. 2).[16] However, as the yield of charged probe **20** was low by elimination of NHS and purification of **20** and **21** was difficult, their use as probes remains a formidable task.

Then, two methods to introduce the probe to the target molecule were examined. One involved the addition of a DMSO solution of previously prepared NHS-TMpybox **10** or Mal-TMpybox **12** to the phosphate-buffered standard solution of the target molecule, followed by purification (Scheme 2, method A). Then, the acetonitrile solution of metal complex **19** was added. The other method involved the direct mixing of the acetonitrile solution of **19** with **10** or **12**-binding crude compound that was freeze-dried without further purification (Scheme 2, method B). Metal complex **19** was also added to obtain triply charged metal cation probes. Then, the synthesized probes were applied to the CSI-MS measurement[17] of several amino acids and peptides.

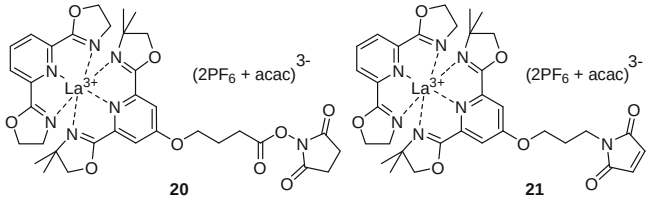


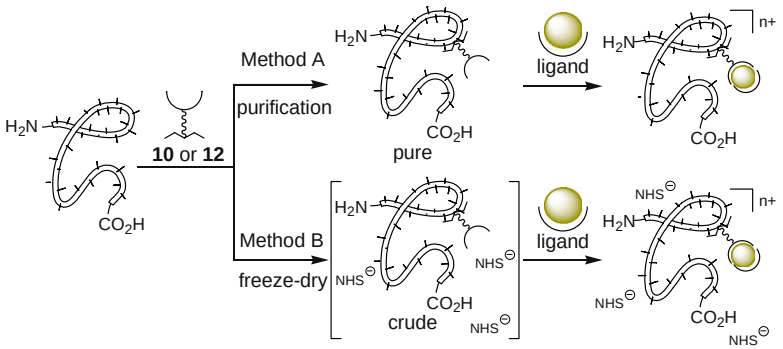
Figure 2. Two charged probes **20** and **21**.

Probe **20**-binding lysine prepared by method A exhibited a doubly charged ion peak [M+acac$^-$]$^{2+}$ in the CSI mass spectrum (Fig. 3a). The expected triply charged ion peak was not detected because the acac$^-$ moiety canceled one charge of this ion. In the CSI mass spectrum of the molecule prepared by method B, [M+acac$^-$+NHS$^-$]$^{2+}$ ion peak was detected in addition to the doubly charged ion peak that appeared in the mass spectrum of the molecule prepared by method A (Fig. 3b). Again, NHS$^-$ canceled one of the three positive charges of this ion.

In the case of charged probe **21**-binding cysteine, a doubly charged ion peak involving acac as the counter anion was detected, similar to probe **20**-binding lysine. The ion intensity in the CSI mass spectrum of the molecule prepared by method A and B were both almost identical (Fig. 4). Method B, which is simpler than method A, is thought to be more favorable for the analysis of complex large biomolecules.

The NHS-TMpybox-charged probe in method A ionized two pentapeptides, TTKTT and KTTTK. The CSI mass spectra of these peptides are shown in Figure 5. Two probes were introduced to KTTTK to give a quadruply charged ion peak, while single probe attachment to TTKTT yielded a doubly charged ion peak. The charge of the target molecule could be controlled by the number of probes attached.

This charged probe method was applied to the ionization of three bioactive peptides: glutathione (GSH), arginine vasopressin (AVP), and somatostatin (SS). Probe **21**-binding GSH prepared by method A was analyzed and the CSI mass spectrum of this com-



Scheme 2. Introduction of multiply charged ion probe.

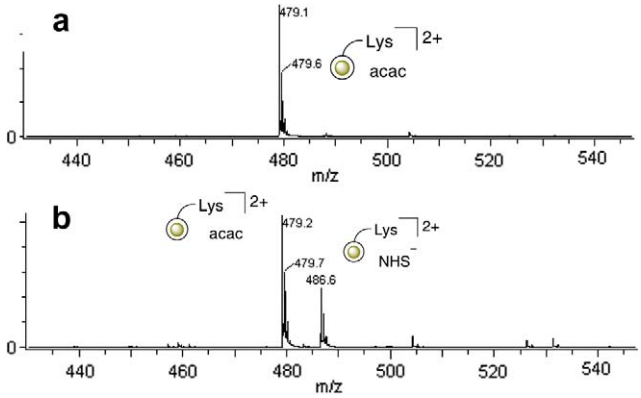


Figure 3. CSI mass spectra of probe **20**-binding lysine prepared by methods A (a) and B (b).

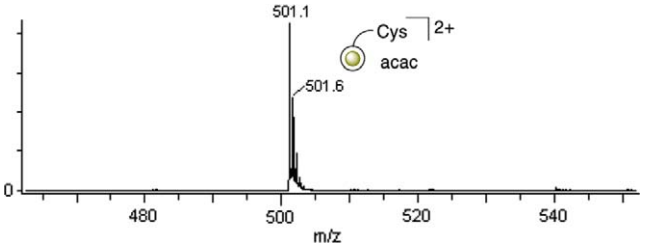


Figure 4. CSI mass spectra of probe **21**-binding cysteine prepared by methods A.

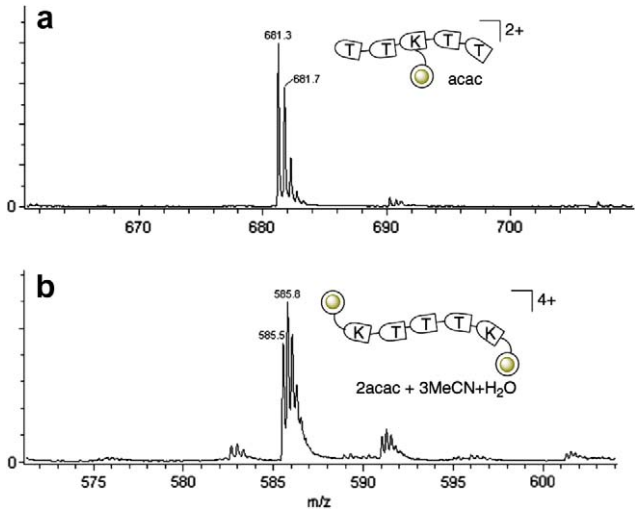


Figure 5. CSI mass spectra of probe **20**—(a) TTKTT and (b) KTTTK prepared by method A.

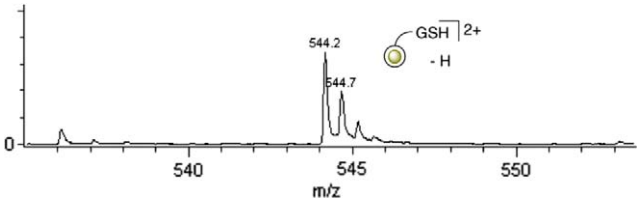


Figure 6. CSI mass spectra of probe **21**-binding GSH prepared by methods A.

pound is shown in Figure 6. A deprotonated doubly charged molecular ion peak was clearly observed, the intensity of which was three times higher than that of the ion peak observed in the mass spectrum of unmodified native GSH. Deprotonation occurred in one of the two carboxylic groups that had a tendency to release their protons.

In the case of AVP modified by charged probe **21** via method B, three major ion peaks were observed, as shown in Figure 7. Two reaction sites, arginine and glycine, of this peptide were available. A doubly charged ion peak based on NHS-TMpybox-AVP (M), in which the probe bound to arginine or glycine in AVP, was observed at *m*/*z* 947 ($[M+acac^-]^{2+}$). Interestingly, triply charged ion peaks based on 2(NHS-TMpybox)-AVP (M′) at *m*/*z* 955 ($[M'+2acac^-+NHS^-+MeCN]^{3+}$) and *m*/*z* 967 ($[M'+2acac^-+2MeCN]^{3+}$) were also observed. The observed ion intensities were almost identical to those in the mass spectrum of unmodified native AVP. However, the charge-weight ratio was clearly reduced by the multiple charge formation.

Finally, SS was analyzed by attaching charged probe **20**. Two attachment sites were available in SS because this peptide contained two lysines. Doubly charged ion peaks due to Mal-TMpybox-SS with some adducts were observed (Fig. 8). The four major

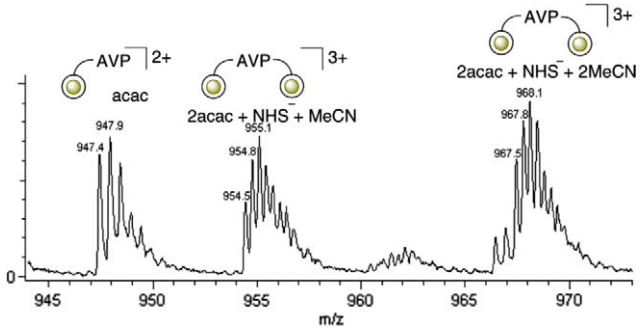


Figure 7. CSI mass spectrum of probe **21**-binding AVP prepared by method B.

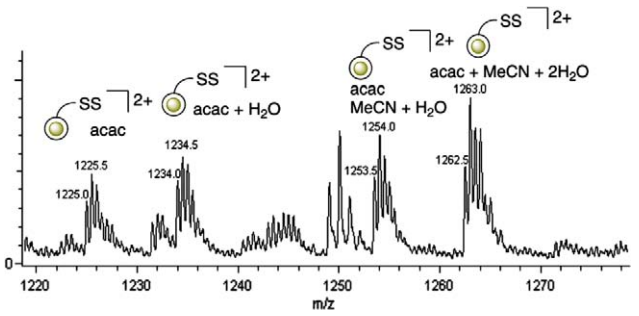


Figure 8. CSI mass spectrum of probe **20**-binding SS prepared by method B.

ion peaks were assigned as $[M+acac^-]^{2+}$ (m/z 1225), $[M+acac^-+H_2O]^{2+}$ (m/z 1234), $[M+acac^-+MeCN+H_2O]^{2+}$ (m/z 1253), and $[M+acac^-+MeCN+2H_2O]^{2+}$ (m/z 1262). These results confirm that the newly developed charged probes can ionize biomolecules.[18] In the ionization of pentapeptide by means of charged probe attachment, quadruply charged ions produced by the attached two probes were observed. This method reduced the charge-weight ratio by donating a charge using the probe.

In conclusion, an effective and reliable ionization method called ionic probe attachment ionization was presented. Two charged probes, Mal-TMpybox **10** and NHS-TMpybox **12**, were prepared and biomolecules including peptides were ionized using these probe attachments. This method was proven to be effective for ionizing various large complex molecules, including biomolecules. Using this method, multiply charged biomolecules were clearly observed by deducing the charge-weight ratio from the CSI mass spectrum. Further application of the charged probes is in progress.

Supplementary data

Supplementary data (synthetic procedures for **3** to **19**, characterization data of **3** to **17** and modification method A and B) associated with this article can be found, in the online version, at doi:10.1016/j.tetlet.2009.09.021.

References and notes

1. Siuzdak, G. *The Expanding Role of Mass Spectrometry in Biotechnology*; MCC Press: San Diego, 2003.
2. Karas, M.; Bachmann, D.; Hillenkamp, F. *Anal. Chem.* **1985**, *57*, 2935.
3. Fenn, J. B.; Mann, M.; Meng, C. K.; Wong, S. F.; Whithouse, C. M. *Science* **1989**, *246*, 64.
4. Kaltashov, I. A.; Abzalimov, R. R. *J. Am. Soc. Mass Spectrom.* **2008**, *19*, 1239.
5. Yamaguchi, K. *J. Mass Spectrom.* **2003**, *38*, 473.
6. Maki, T.; Ishida, K. *J. Org. Chem.* **2007**, *72*, 6427; Che, F.-Y.; Fricker, L. D. *J. Mass Spectrom.* **2005**, *40*, 238; Mirzaei, H.; Regnier, F. *Anal. Chem.* **2006**, *78*, 4175.
7. Vlasie, M. D.; Comuzzi, C.; van den Nieuwendijk, A. M. C. H.; Prudêncio, M.; Overhand, M.; Ubbink, M. *Chem. Eur. J.* **2007**, *13*, 1715; Keizers, P. H. J.; Desreux, J. F.; Overhand, M.; Ubbink, M. *J. Am. Chem. Soc.* **2007**, *129*, 9292.
8. Peterson, J. R.; Smith, T. A.; Thordarson, P. *Chem. Commun.* **2007**, 1899.
9. Kaneko, M.; Masui, R.; Ake, K.; Kousumi, Y.; Kuramitsu, S.; Yamaguchi, M.; Kuyama, H.; Ando, E.; Norioka, S.; Nakazawa, T.; Okamura, T.; Yamamoto, H.; Ueyama, N. *J. Proteome Res.* **2004**, *3*, 983; Ito, A.; Okamura, T.; Yamamoto, H.; Ueyama, N.; Yamaguchi, M.; Kuyama, H.; Ando, E.; Tsunasawa, S.; Ake, K.; Masui, R.; Kuramitsu, S.; Nakazawa, T.; Norioka, S. *Rapid Commun. Mass Spectrom.* **2007**, *21*, 2647.
10. Lin, P.-C.; Ueng, S.-H.; Yu, S.-C.; Jan, M.-D.; Adak, A.-K.; Yu, C.-C.; Lin, C.-C. *Org. Lett.* **2007**, *9*, 2131.
11. Three different lengths of the alkyl chain $-[-CH_2-]_n-$ were prepared. Length $n = 3$ was used in this experiment because it exhibited the highest yield (56%) compared to the other lengths ($n = 4$: 39% and $n = 5$: 25%).
12. An increase in solubility due to dimethyl substitution into the dihydrooxazole ring of both pybox probes in organic solvent was noted compared to unsubstituted or monomethyl substituted compounds.
13. Vermonden, T.; Branowska, D.; Marcelis, A. T. M.; Sudhölter, E. J. R. *Tetrahedron* **2003**, *59*, 5039.
14. Tang, F. H. Y.; Wang, M. Y. S.; Zhu, Y. L. *Adv. Funct. Mater.* **2007**, *17*, 996.
15. Although other counter ions including BF_4 were tested, PF_6 seems to be favorable for the CSI process based on solvation, because the low coordination ability of this anion may promote dissociation. Sakamoto, S.; Fujita, M.; Kim, K.; Yamaguchi, K. *Tetrahedron* **2000**, *56*, 955.
16. Unpublished data. Though a doubly charged ion was detected in the case of 1:2 (cation to pybox ligand ratio) complex, single charged ions containing solvents and counter ions were detected in the case of 1:1 complex.
17. CSI mass spectra were recorded on a JEOL JMS-T100LC mass spectrometer equipped with a cold-spray ion source. This setup makes it possible to conduct measurements at low temperatures. Measurement conditions were as follows. sprayer temperature: −20 to 20 °C; needle voltage: +3030 V; ring lens voltage: +15 V; orifice 1 voltage: +150 V; orifice 2 voltage: +15 V; concd ca. 1 mM.
18. This method was also applied to the ionization of large peptides. Multiply charged molecular ion peaks of high charge states were clearly observed in the case of nocicptine. The results will be published elsewhere.