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Mechanism for odd-electron anion generation of dihydroxybenzoic acid isomers in matrix-assisted laser desorption/ionization mass spectrometry with density functional theory calculations

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RATIONALE: Proton and radical are transferred between matrices and matrix and analyte in matrix-assisted laser desorption/ionization (MALDI) and these transfers drive ionization of analytes. The odd-electron anion $[M–2H]^{\bullet-}$ was generated in dihydroxybenzoic acids (DHBs) and the ion abundance of the 2,5-DHB was the highest among six DHB isomers. We were interested in the mechanism of the ion generation of the odd-electron anion.

METHODS: The observed $[M–2H]^{\bullet-}$ and $[M–3H]^{-}$ ions, which were generated with the hydrogen radical removed from the phenolic hydroxyl groups (OH) in DHB isomers, were analyzed using negative-ion MALDI-MS. The enthalpy for ion generation and their stable structures were calculated using the density functional theory (DFT) calculation program Gaussian 09 with the B3LYP functional and the 6–31+G(d) basis set.

RESULTS: The number of observed $[M–2H]^{\bullet-}$ and $[M–3H]^{-}$ ions of the DHB isomers was dependent on the positions of the phenolic OH groups in the DHB isomers because the carboxy group interacts with the *ortho* OH group due to neighboring group participation, as confirmed from the stable structures of the $[M–2H]^{\bullet-}$ anions calculated with the Gaussian 09 program. The DHB isomers were placed into three categories according to the number of the ions.

CONCLUSIONS: Odd-electron anions ($[M–2H]^{\bullet-}$) and $[M–2H^{\bullet}–H]^{-}$ ($[M–3H]^{-}$) ions were generated from DHB isomers due to removal of the hydrogen radical from the phenolic groups. The enthalpy for ion generation revealed that ion formation proceeds via a two-step pathway through the $[M–M]^{-}$ ion as an intermediate. © 2016 The Authors. *Rapid Communications in Mass Spectrometry* Published by John Wiley & Sons Ltd.

Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) has become an established method for the structural identification of biological and biochemical molecules and industrial materials.[1,2] The ion formation mechanisms involve many complex processes in which analytes are protonated/deprotonated, which makes it difficult to fully understand these mechanisms. The models for ion formation in MALDI have been summarized in reviews.[3,4] Ion formation in MALDI generally involves irradiation of the matrix with a laser and excitation. The matrix is first desorbed and ionized, followed by the transfer of ions between the ionized matrix and neutral analytes and between desorbed neutral matrix and analyte, and finally ionization of

the analytes.[4] 2,5-Dihydroxybenzoic acid (2,5-DHB) (Fig. 1) is known as one of the most efficient matrices for large biomolecules.[1,2,5] The ion formation including proton transfer from 2,5-DHB has been well studied; however, the overall mechanism for ion formation from DHB has not yet been clearly explained.[6–13] 2,5-DHB has the largest absorption cross section among the six DHB positional isomers in the laser irradiation with a Nd:YAG laser at a wavelength of 355 nm.[14,15] Jessome *et al*. studied the ion efficiency of the different DHB isomers to reveal the ion formation mechanism for the most efficient matrix of 2,5-DHB.[13] The ion efficiency of 2,5-DHB was reported to be highest among the six DHB isomers; however, the reasons given for the superior ion efficiency of 2,5-DHB were ambiguous. No clear relationships were observed between MALDI biomolecule signals and the gas-phase basicity, proton affinity or ionization potentials of DHB isomers until now.[13]

Recently, we reported that the ion signal of odd-electron anions ($[M–2H]^{\bullet-}$) from 2,5-DHB was found to be largest among the DHB positional isomers.[16] It was detected from 2,5-DHB, besides the conventional even-electron anion $[M–H]^{-}$, as a rare case among all matrices.[6] Until now, the mechanism of the ion formation of these odd-electron anions have not been studied. We have studied the hydrogen radical

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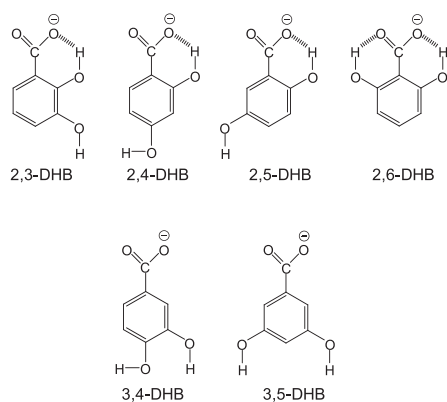
Figure 1. Structures of the [M–H]− anion form of dihydroxybenzoic acid (DHB) isomers.

removing ions (the odd-electron anion [M–2H]•− and [M–3H]−) of polyphenol compounds such as DHB and flavonoids, and the hydrogen radical can be removed from the phenolic hydroxyl groups.[16,17]

There was a phenolic OH group from which the hydrogen radical could not removed. We proposed that the hydrogen radical could not be removed from the phenolic OH groups in intramolecular interactions. In the previously reported paper, compounds such as salicylic acid and 2,5-DHB, which contain a phenolic hydroxyl group *ortho* (2-position) and a carbonyl function, often exhibit special behavior under UV irradiation, whereby UV irradiation induces an intramolecular proton shift along the intramolecular H-bond in these molecules.[9] Because of that, it was assumed that the proton at the *ortho*-phenolic OH group could be transferred to matrix and analyte from 2,5-DHB in the MALDI process in the previous papers.[3,9] In this study, we showed the hydrogen radical could not be removed from the *ortho* (2-position) phenolic OH in all six DHB positional isomers with density functional theory (DFT) calculations. Here, we attempt to reveal the mechanism of the ion formation of the odd-electron anion [M–2H]•− of 2,5-DHB and other isomers in MALDI with DFT calculations to provide clues to understanding the ionization mechanism of DHB matrix in MALDI.

EXPERIMENTAL

Materials

2,3-Dihydroxybenzoic acid (2,3-DHB), 2,4-dihydroxybenzoic acid (2,4-DHB), 2,5-dihydroxybenzoic acid (2,5-DHB), 2,6-dihydroxybenzoic acid (2,6-DHB), 3,4-dihydroxybenzoic acid (3,4-DHB), and 3,5-dihydroxybenzoic acid (3,5-DHB) (as shown in Fig. 1) were acquired from Tokyo Chemical Industry Co., Ltd (Tokyo, Japan). HPLC grade acetonitrile was obtained from Nacalai Tesque, Inc. (Kyoto, Japan). Milli Q water was used for all experiments.

Mass spectrometry

All MALDI-TOFMS spectra were acquired with an Ultraflex III MALDI TOF/TOF instrument equipped a pulsed Nd:YAG laser at a wavelength of 355 nm (fluence >250 μJ/pulse;

Bruker Daltonics, Co., GmbH, Germany) under the same experimental conditions. An aliquot of analytes was dissolved in an aqueous solution of 80% acetonitrile at a final concentration of 1 mg/mL, and 1 μL of the supernatant was deposited on the target and allowed to dry. The measurement conditions, such as laser irradiation power, were the same for each of the isomers studied. The MALDI-MS spectrum of each isomer was measured five times. The peak intensities for the [M–2H]•− and [M–3H]− ions were normalized with respect to the [M–H]− ion as 100%. The reproducibility of the data was sufficiently high to successfully determine the signal ratios.
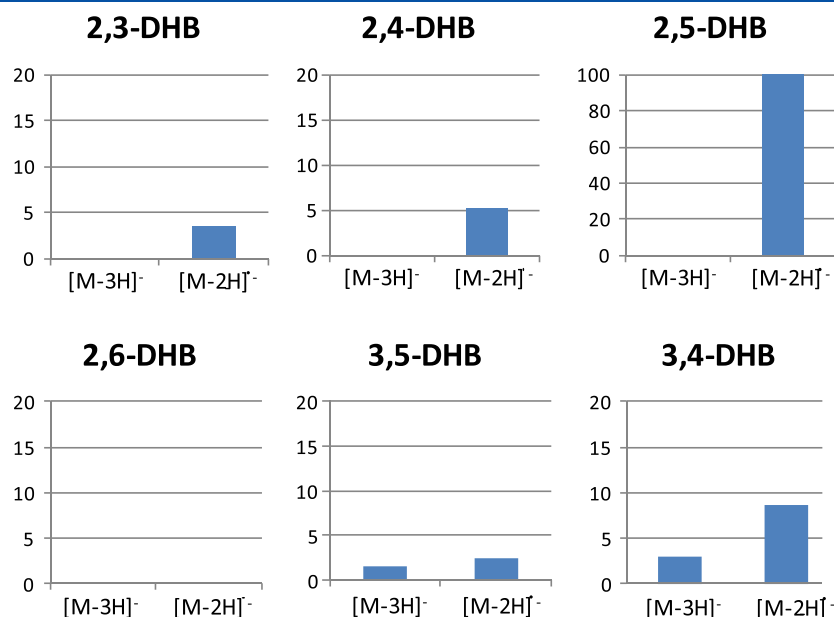
Quantum chemical calculations

Density functional theory (DFT) calculations were performed using the Gaussian 09 program.[18] The geometries were optimized using the B3LYP functional and the 6–31+G(d) basis set. Spin-unrestricted calculations were used for all open-shell systems.

RESULTS AND DISCUSSION

Figure 2 shows that the relative ion abundance for the [M–2H]•− odd-electron anion from 2,5-DHB was extremely high among the six DHB isomers, and almost as high as that for the [M–H]− even-electron anion. In contrast, the [M–2H]•− anion was not detected from 2,6-DHB, but only the [M–H]− anion. The relative ion abundances for the [M–2H]•− odd-electron anion from 2,3-DHB, 2,4-DHB, 3,4-DHB, and 3,5-DHB were much lower than those for the [M–H]− even-electron anion (<10%). 3,4-DHB and 3,5-DHB had two species of the [M–2H]•− and [M–3H]− ions. From the view point of organic chemistry, we assumed that the phenolic OH at the 2-position interacts with the carboxy group in DHBs according to neighboring group participation as salicylic acid (Supporting Information-1). The numbers of the [M–2H]•− and [M–3H]− ions observed in six DHB isomers can be explained from their structures with or without the *ortho* phenolic OH.[16] The *ortho* phenolic OH groups of 2,3-DHB, 2,4-DHB and 2,5-DHB interact with the carboxy group, and one hydrogen radical is removed from the other phenolic OH group (Fig. 3). Both of the phenolic OH groups can interact with the carboxy group in 2,6-DHB; therefore, no odd-electron anion was detected. Both of the phenolic OH groups of 3,4-DHB and 3,5-DHB cannot interact with the carboxy group, then the two hydrogen radical is removed from the phenolic groups (Fig. 3). The chemical species [M–2H•–H]− ion is very labile because of the two oxygen radicals, which changed to the [M–3H]− ion as the even-electron anion. Therefore, the [M–2H]•− and [M–3H]− ions were detected from 3,4-DHB and 3,5-DHB (Fig. 3).

To confirm our assumption, the most stable structures of the [M–2H]•− odd-electron anion were calculated using Gaussian 09 with B3LYP 6–31 + G8(d), and the results are shown in Fig. 4. In the calculated structures, one proton is trapped between the carboxy group and the *ortho* phenoxylic hydroxyl group in 2,3-DHB, 2,4-DHB, 2,5-DHB, and 2,6-DHB, which indicates that there are intramolecular hydrogen bonds between them. It is then difficult for the proton to be removed from these stable structures. In contrast, the proton of the

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2,3-DHB
2,4-DHB
2,5-DHB

2,6-DHB
3,5-DHB
3,4-DHB

Figure 2. Relative ion abundances (%) for the [M–2H]$^{\bullet-}$ and [M–3H]$^-$ ions to that of [M–H]$^-$. The relative ion abundances are normalized with respect to that for [M–H]$^-$ as 100%. The maximum value for 2,5-DHB is 100%, while that for the others is 20%.
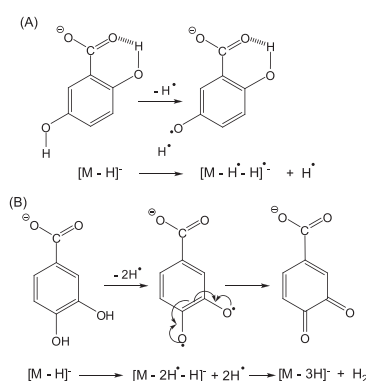

Figure 3. Scheme of [M–2H]$^{\bullet-}$ odd-electron anion generation of 2,5-DHB (A) and [M–3H]$^-$ even-electron anion generation of 3,4-DHB (B).

carboxy group in 3,4-DHB and 3,5-DHB can easily be removed in the stable structures of the [M–2H]$^{\bullet-}$ ions, as evident in Fig. 4. Both the [M–2H]$^{\bullet-}$ and [M–3H]$^-$ ions were detected in the MALDI-MS spectra for 3,4-DHB and 3,5-DHB. Thus, the calculated structures for the DHB isomers support the assumption. Therefore, the numbers of the [M–2H]$^{\bullet-}$ and

[M–3H]$^-$ ions we elucidated from the MALDI-MS data for the DHB isomers based on the intermolecular interactions between the carboxy and phenolic OH groups.

The DHB isomers were categorized into three groups according to the ion abundance of the [M–2H]$^{\bullet-}$ odd-electron anion as shown in Table 1. 2,5-DHB had an extremely high ion abundance of [M–2H]$^{\bullet-}$ among the six isomers, and no [M–2H]$^{\bullet-}$ anions were detected from 2,6-DHB, while the four other isomers, 2,3-DHB, 2,4-DHB, 3,4-DHB, and 3,5-DHB, had low abundances (<10%) of the [M–2H]$^{\bullet-}$ and [M–3H]$^-$ anions. It was difficult to explain the differences in ion abundance among these four DHBs because their [M–2H]$^{\bullet-}$ and [M–3H]$^-$ anions were present in low abundance and small differences.

To explain these tendencies for the abundance of the [M–2H]$^{\bullet-}$ odd-electron anion from the DHB isomers, especially for 2,5-DHB, the ion generation enthalpy was calculated using quantum calculations of Gaussian 09 with B3LYP 6–31 + G8(d). For instance, Fig. 5 shows the enthalpy diagram for 2,5-DHB (see enthalpy diagrams for the other DHB isomers in the Supporting Information-2). All ions from all of the DHB isomers were generated by endothermic reactions. The ion formation also included the absorbance
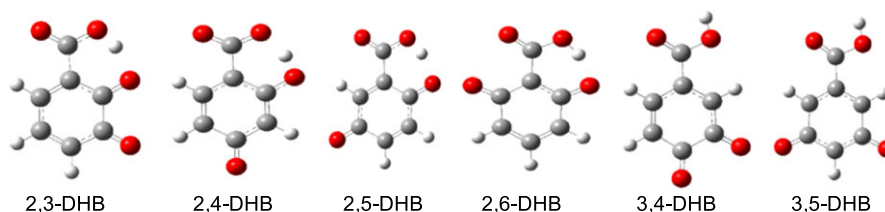
Figure 4. Calculated structures for the singly charged negative ion [M–2H]$^{\bullet-}$ of the DHB isomers.

Table 1. Enthalpy of ion generation from the DHB isomers

	2,5-DHB	2,3-DHB	3,4-DHB	2,4-DHB	3,5-DHB	2,6-DHB
Ion abundance of [M–2H]$^{\bullet-a}$	100%	less than 10%				n.d.
ΔH [M–2H]$^{\bullet-}$	366.2	370.3	374.8	393.7	448.9	447.1
ΔH [M–H]$^-$	99.7	52.2	52.9	76.1	128.8	44.5
ΔH [M-2H]$^{\bullet-}$ – ΔH [M–H]$^-$	266.5	318.8	321.8	317.6	320	402.6

(ΔH [kJ/mol])

^{a}The relative ion abundance of [M–2H]$^{\bullet-}$ normalized to the ion [M–H]$^-$.
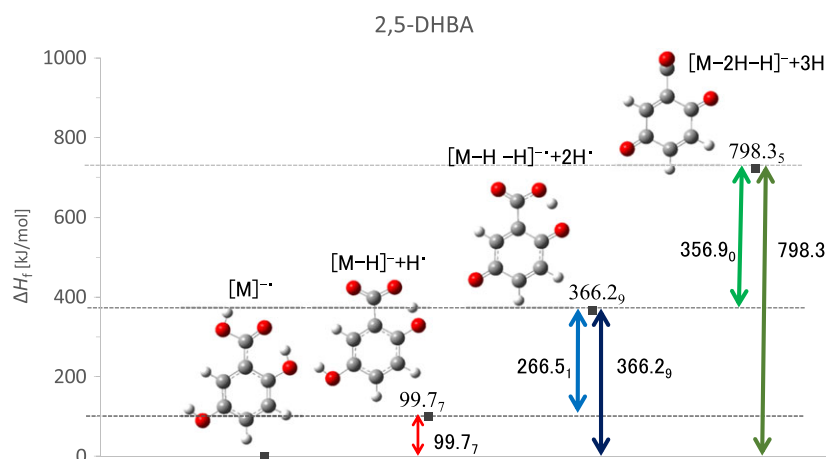
Figure 5. Enthalpy diagram for ion generation from 2,5-DHB.
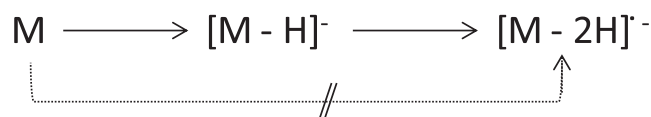
Scheme 1. Generation of the [M–2H]$^{\bullet-}$ odd-electron anion.

cross section and heat capacity factors. The heat capacity of DHB isomers was assumed to be almost the same because of their similar structures.[19] The absorbance cross section of 2,5-DHB was the highest at 355 nm as described.[14,15]

The enthalpies for the generation of the [M–2H]$^{\bullet-}$ odd-electron anion are summarized in Table 1. The differences of enthalpy between the generation of [M–2H]$^{\bullet-}$ and [M–H]$^-$ ions were reasonable and corresponded well with the ion abundance tendency for the [M–2H]$^{\bullet-}$ anions from six DHB isomers (Table 1). The enthalpy difference ([M–2H]$^{\bullet-}$ – [M–H]$^-$) for 2,5-DHB was the smallest (ΔH 266.5 kJ/mol) by far, and that for 2,6-DHB was the largest (ΔH 402.6 kJ/mol) among them. There were two gaps in the enthalpy difference (([M–2H]$^{\bullet-}$ – [M–H]$^-$) at 2,5-DHB and 2,6-DHB, which justified their separate categorization according to the experimental data.

The differences in enthalpy between the [M–2H]$^{\bullet-}$ and [M–H]$^-$ ion generation indicate that the [M–2H]$^{\bullet-}$ anion is generated mainly through the [M–H]$^-$ anion as an intermediate. This suggested that the generation of [M–2H]$^{\bullet-}$ anions is a two-step reaction, as shown in Scheme 1.

CONCLUSIONS

[M–2H]$^{\bullet-}$ ([M–H$^\bullet$–H]$^{\bullet-}$) odd-electron anions were generated from DHB isomers due to the removal of the hydrogen radical from the phenolic groups. Although there are two phenolic groups in DHBs, the [M–3H]$^-$ ions were not observed in the spectra of 2,3-, 2,4-, and 2,5-DHB because of neighboring group participation. The high abundance of the [M–H$^\bullet$–H]$^{\bullet-}$ anion for 2,5-DHB can result from two factors. One is low heat of reaction. The other is high temperature from the high absorbance. The enthalpy for ion generation revealed that ion formation proceeds via a two-step pathway through the [M–M]$^-$ ion as an intermediate. The [M–3H]$^-$ even-electron anions were generated from 3,4- and 3,5-DHB because there was no neighboring group participation. These were generated via the chemical species of [M–2H$^\bullet$–H]$^{\bullet-}$ (Fig. 3).

REFERENCES

[1] F. Hillenkamp, M. Karas, R. C. Beavis, B. T. Chait. Matrix-assisted laser desorption/ionization mass spectrometry of biopolymers. *Anal. Chem.* **1991**, *63*, 1193A.

[2] B. Stahl, M. Steup, M. Karas, F. Hillenkamp. Analysis of neutral oligosaccharides by matrix-assisted laser desorption/ionization mass spectrometry. *Anal. Chem.* **1991**, *63*, 1463.

[3] R. Zenobi, R. Knochenmuss. Ion formation in MALDI mass spectrometry. *Mass Spectrom. Rev.* **1998**, *17*, 337.

wileyonlinelibrary.com/journal/rcm

[4] I.-C. Lu, C. Lee, Y.-T. Lee, C.-K. Ni. Ionization mechanism of matrix-assisted laser desorption/ionization. *Annu. Rev. Anal. Chem.* **2015**, *8*, 21.

[5] K. Strupat, M. Karas, F. Hillenkamp. 2,5-Dihydroxybenzoic acid: a new matrix for laser desorption–ionization mass spectrometry. *Int. J. Mass Spectrom. Ion Processes* **1991**, *111*, 89.

[6] H. Ehring, M. Karas, F. Hillenkamp. Role of photoionization and photochemistry in ionization processes of organic molecules and relevance for matrix-assisted laser desorption ionization mass spectrometry. *Org. Mass Spectrom.* **1992**, *27*, 472.

[7] C. T. J. Scott, C. Kosmidis, W. J. Jia, K. W. D. Ledingham, R. P. Singhal. Formation of atomic hydrogen in matrix-assisted laser desorption ionization. *Rapid Commun. Mass Spectrom.* **1994**, *8*, 829.

[8] K. Dreisewerd, M. Schurenberg, M. Karas, F. Hillenkamp. Influence of the laser intensity and spot size on the desorption of molecules and ions in matrix-assisted laser desorption/ionization with a uniform beam profile. *Int. J. Mass Spectrom. Ion Processes* **1995**, *141*, 127.

[9] J. Krause, M. Stoeckli, U. P. Schlunegger. Studies on the selection of new matrices for ultraviolet matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *Rapid Commun. Mass Spectrom.* **1996**, *10*, 1927.

[10] E. Lehmann, R. knochenmuss, R. Zenobi. Ionization mechanisms in matrix-assisted laser desorption/ionization mass spectrometry: contribution of pre-formed ions. *Rapid Commun. Mass Spectrom.* **1997**, *11*, 1483.

[11] V. Karbach, R. Knochenmuss. Do single matrix molecules generate primary ions in ultraviolet matrix-assisted laser desorption/ionization. *Rapid Commun. Mass Spectrom.* **1998**, *12*, 968.

[12] S. Bourcier, S. Bouchonnet, Y. Hoppilliard. Ionization of 2,5-dihydroxybenzoic acid (DHB) matrix-assisted laser desorption/ionization experiments and theoretical study. *Int. J. Mass Spectrom.* **2001**, *210/211*, 59.

[13] L. Jessome, N.-Y. Hsu, Y.-S. Wang, C.-H. Chen. Matrix-assisted laser desorption/ionization mechanism study with dihydroxybenzoic acid isomers as matrices. *Rapid Commun. Mass Spectrom.* **2008**, *22*, 130.

[14] V. Horneffer, K. Dreisewerd, H.-C. Ludemann, F. Hillenkamp, M. Lage, K. Strupat. Is the incorporation of analytes into matrix crystals a prerequisite for matrix-assisted laser desorption/ionization mass spectrometry? A study of five positional isomers of dihydroxybenzoic acid. *Int. J. Mass Spectrom.* **1999**, *185/186/187*, 859.

[15] C. W. Liang, C. H. Lee, Y.-J. Lin, Y. T. Lee, C. K. Ni. MALDI mechanism of dihydroxybenzoic acid isomers: desorption of neutral matrix and analyte. *J. Phys. Chem. B* **2013**, *117*, 5058.

[16] T. Yamagaki, T. Watanabe. Hydrogen radical removal causes complex overlapping isotope patterns of aromatic carboxylic acids in negative-ion matrix-assisted laser desorption/ionization mass spectrometry. *Mass Spectrom.* **2012**, *1*, A0005.

[17] T. Yamagaki, T. Watanabe, M. Tanaka, K. Sugahara. Laser-induced hydrogen radical removal in UV MALDI-MS allows for the differentiation of flavonoid monoglycoside isomers. *J. Am. Soc. Mass Spectrom.* **2014**, *25*, 88.

[18] M. J. Frisch, G. W. Trucks, H. B. Schlegel, G. E. Scuseria, M. A. Robb, J. R. Cheeseman, J. A. Montgomery Jr, T. Vreven, K. N. Kudin, J. C. Burant, J. M. Millam, S. S. Iyengar, J. Tomasi, V. Barone, B. Mennucci, M. Cossi, G. Scalmani, N. Rega, G. A. Petersson, H. Nakatsuji, M. Hada, M. Ehara, K. Toyota, R. Fukuda, J. Hasegawa, M. Ishida, T. Nakajima, Y. Honda, O. Kitao, H. Nakai, M. Klene, X. Li, J. E. Knox, H. P. Hratchian Hratchian, J. B. Cross, C. Adamo, J. Jaramillo, R. Gomperts, R. E. Stratmann, O. Yazyev, A. J. Austin, R. Cammi, C. Pomelli, J. W. Ochterski, P. Y. Ayala, K. Morokuma, G. A. Voth, P. Salvador, J. J. Dannenberg, V. G. Zakrzewski, S. Dapprich, A. D. Daniels, M. C. Strain, O. Farkas, D. K. Malick, A. D. Rabuck, K. Raghavachari, J. B. Foresman, J. V. Ortiz, Q. Cui, A. G. Baboul, S. Clifford, J. Cioslowski, B. B. Stefanov, G. Liu, A. Liashenko, P. Piskorz, I. Komaromi, R. L. Martin, D. J. Fox, T. Keith, M. A. Al-Laham, C. Y. Peng, A. Nanayakkara, M. Challacombe, P. M. W. Gill, B. Johnson, W. Chen, M. W. Wong, C. Gonzalez, J. A. Pople. *Gaussian 09; Revision D.01*, Gaussian, Inc., Wallingford, CT, **2010**.

[19] K. Y. Chu, S. Lee, M.-T. Tsai, I.-C. Lu, Y. A. Dyakov, Y. H. Lai, Y.-T. Lee, C.-K. Ni. Thermal proton transfer reactions in ultraviolet matrix-assisted laser desorpion/ionization. *J. Am. Soc. Mass Spectrom.* **2014**, *25*, 310.

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