Simple and Effective 3D Recognition of Domoic Acid Using a Molecularly Imprinted Polymer

8 AUTHORS, INCLUDING:

Koji Nemoto
National Institute of Advanced Industrial Sci…
21 PUBLICATIONS   139 CITATIONS

SEE PROFILE

Takuya Kubo
Kyoto University
91 PUBLICATIONS   972 CITATIONS

SEE PROFILE

Ken Hosoya
Kyoto Prefectural University
200 PUBLICATIONS   5,860 CITATIONS

SEE PROFILE

Simple and Effective 3D Recognition of Domoic Acid Using a Molecularly Imprinted Polymer

Koji Nemoto,[†] Takuya Kubo,*[,†] Makoto Nomachi,[†] Tomoharu Sano,[‡]
Takatoshi Matsumoto,[§] Ken Hosoya,[†] Tetsutaro Hattori,[†] and Kunimitsu Kaya[†]

Contribution from the Graduate School of Environmental Studies, Tohoku University, Aoba 6-6-20, Aramaki, Aoba-ku, Sendai 980-8579, Japan, Laboratory of Intellectual Fundamentals for Environmental Studies, National Institute for Environmental Studies, Onogawa 16-2, Tsukuba, Ibaraki 305-8506, Japan, and Institute of Multidisciplinary Research for Advanced Materials, Tohoku University, 2-1-1, Katahira, Aoba-ku, Sendai 980-8577, Japan

Received June 6, 2007; E-mail: kubo@mail.kankyo.tohoku.ac.jp

Abstract: We have developed a simple and effective molecular imprinting technique to target compounds with flexible structure. Domoic acid (DA), an amnesic shellfish poison, was used as the target compound while many acidic compounds (mono-, di-, and tricarboxylic acids) were used as template molecules for molecularly imprinted polymers (MIPs). Evaluation of selective recognition abilities using liquid chromatography revealed that the highest selective recognition ability for DA was found when pentane-1,3,5-tricarboxylic acid (1,3,5-PeTA) was used as the template. Computer modeling studies of the DA structure suggested that the observed selective recognition depended on the structural changes in DA at the recognition site of the MIPs as well as spatial distance between the COOH groups in DA and 1,3,5-PeTA. Using the 1,3,5-PeTA-MIP, we could easily purify DA from blue mussel extracts by solid-phase extraction.

Introduction

Selective separation technologies are very important for the purification and/or quantitative analysis of toxins, drugs, biological materials, and chemicals. In general, it is difficult to achieve selective separation of target compounds from environmental or biological samples due to the complex nature of the sample matrices that interfere with the separation and detection of specific compounds in these samples. Therefore, we urgently require a separation methodology with built-in selectivity for the target compounds.

The molecular imprinting technique is one of the most attractive methods for obtaining selective recognition abilities.[1,2] In this technique, the cross-linking agent, polymerization initiator, template molecule, and functional monomer (interacting with the template molecule) are simultaneously polymerized. Then, the template molecules are removed from the prepared polymer. As a result, selective recognition based on the imprinting effect can be achieved. Usually, noncovalent bonding (such as hydrogen bonding), ionic interactions, and hydrophobic interactions are utilized in the preparation of molecularly imprinted polymers (MIPs). This technique is moderately relatively easy, and several approaches have been reported including methods involving artificial antibodies, biosensors, and stationary solid phases in liquid chromatography.[1−4] In addition to the field of analytical chemistry, this technique has also been applied to the construction of asymmetric reaction in the field of organic chemistry.[5]

However, this technique has some problems for applications involving environmental samples and/or biological samples. For example, highly toxic and/or rare compounds cannot be used as template molecules since the target molecule itself is used in the procedure for the preparation of MIPs. "Template molecule leakage" is another serious problem since the template molecule usually cannot be removed completely from the MIPs even after exhaustive repeated washing with organic solvents. This is because the imprinted sites can be formed not only on the surface but also deeply inside the cross-liked polymer network where it is difficult for the solvents to reach.[6] Therefore, contrary to expectations, the template molecules may leak from the inside of the polymer: this becomes a serious problem and interferes with the correct quantitative determination and purification.

With regard to the above-mentioned problems, several studies have been reported;[7−9] we have also reported some alternative imprinting techniques (fragment imprinting technique).[10−12]

† Graduate School of Environmental Studies, Tohoku University.
‡ National Institute for Environmental Studies.
§ Institute of Multidisciplinary Research for Advanced Materials, Tohoku University.
(1) Wulff, G.; Sarhan, A.; Zabrocki, K. *Tetrahedron Lett.* **1973**, *44*, 4329−4332.
(2) Sellergren, B.; Ekberg, B.; Mosbach, K. *J. Chromatogr.* **1985**, *347*, 1−10.
(3) Spivak, D.; Shea, K. J. *J. Org. Chem.* **1999**, *64*, 4627−4634.
(4) Sellergren, B. *Molecular Imprinted Polymers*; Elsevier Science: 2001.
(5) Tada, M.; Sasaki, T.; Iwasawa, Y. *J. Phys. Chem. B* **2004**, *108*, 2918−2930.
(6) Haginaka, J.; Sanbe, H. *Anal. Chem.* **2000**, *72* (1), 5206−5210.
(7) Rachkov, A.; Minoura, N. *J. Chromatogr. A* **2000**, *889*, 111−118.
(8) Matsui, J.; Fujiwara, K.; Takeuchi, T. *Anal. Chem.* **2000**, *72*, 1810−1813.
(9) Quaglia, M.; Chenon, K.; Hall, A. J.; Lorenzi, E. D.; Sellergren, B. *J. Am. Chem. Soc.* **2001**, *123*, 2146−2154.
(10) Kubo, T.; Hosoya, K.; Watabe, Y.; Ikegami, T.; Tanaka, N.; Sano, T.; Kaya, K. *J. Chromatogr. A* **2003**, *987*, 389−394.
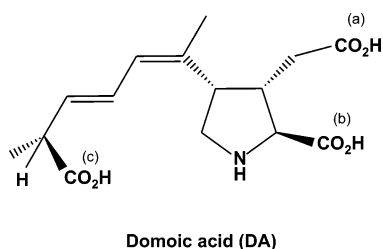
Figure 1. Structure of domoic acid (DA).

Further, we need to obtain the selective recognition ability for endocrine disrupters, halogenated aromatic compounds, and some natural toxins. However, these targeting compounds have inflexible and relatively rigid chemical structures. Therefore, in order to apply the fragment imprinting technique to a larger number of compounds, we have to establish a novel concept to obtain the selective recognition ability for compounds having flexible chemical structures.

In this study, we report 3D recognition for the flexible structural compound DA (Figure 1) by using fragment imprinted polymers. DA is a water-soluble tricarboxylic amino acid with a molecular weight of 311: it acts as an analogue of the neurotransmitter glutamate and is a potent glutamate receptor agonist. Its first identification was reported from the rhodophyta *Chondria armata* as an insecticide.[13] Finally, the cell died and symptoms such as memory loss (which is also a typical feature of ASP) were observed.[14−16] Additionally, some biological researchers have reported that DA is assembled with a kainate-type subunit of the glutamate receptor.[17] According to that study, the author has described that multipoint recognition worked effectively toward selective holding and three COOH groups were selectively recognized. In our previous study, we have reported "two-point" recognition of COOH groups on DA by MIPs.[18] In this study, we have investigated "three-point" recognition. We prepared MIPs using many simple and commercially used template molecules. The selectivity of MIPs for DA was evaluated using liquid chromatography. Additionally, in order to verify the selective recognition results, we also investigated the conformational alternation of DA by computer modeling. Finally, we basically tested the availability of MIP as a solid-phase extraction medium and achieved the selective concentration of DA from the blue mussel extract.

Experimental Section

Chemicals. Ethylene glycol dimethacrylate (EDMA) as the cross-linking agent and 4-vinylpyridine (4VP) as the functional monomer were obtained from Wako Chemicals (Osaka, Japan), and they were distilled under vacuum for removing the polymerization inhibitors.[19]

The template molecules, acetic acid (AcOH), HPLC-grade acetonitrile (AN), 2,2′-azobis(2,4-dimethyl-valeronitrile) (ADVN) as the initiator, and toluene as the porogen agent were also purchased from Wako Chemicals. DA and dihydrokainic acid were purchased from BioVectra (Prince Edward Island, Canada). Aliphatic di- and tricarboxylic acids were purchased from Sigma Aldrich (U.S.A).

Preparation of MIP Particles. To prepare a polymer-based separation medium, we applied the multistep swelling and polymerization method; polystyrene seed particles were prepared by using emulsifier-free emulsion polymerization.[20,21] The polymerization was carried out at 50 °C for 24 h using 2.0 wt % of ADVN as a radical initiator. After the polymerization procedures, the polymer particles were washed with pure water, methanol, and tetrahydrofuran to remove the template molecules and/or unreacted compounds. The polymer particles had a diameter of approximately 5.0 μm with excellent size uniformity. The compositions of the prepared polymer particles are as follows: EDMA (26.5 mmol), 4VP (4.4 mmol), template molecule (0.14 mmol), and toluene (5.0 mL). Additionally, a BLANK polymer (without templates) and a BASE polymer (without templates and the functional monomer 4VP) were also prepared under the same polymerization conditions. The abbreviations of the template molecules are defined as follows: *o*-phthalic acid (OPA), dihydrokainic acid (DKA), 2,3-naphthalene dicarboxylic acid (2,3-NDA), 1,4-naphthalene dicarboxylic acid (1,4-NDA), 2,6-naphthalene dicarboxylic acid (2,6-NDA), diphenylether-4,4′-dicarboxylic acid (EDA), 2,2′-bis(4-carboxyphenyl)hexafluoropropan (FDA), benzophenone-4,4′- dicarboxylic acid (KDA), oxalic acid (2DA), malonic acid (3DA), succinic acid (4DA), glutaric acid (5DA), adipic acid (6DA), 1,7-heptanedioic acid (7DA), 1,8-octanedioic acid (8DA), 1,9-nonanedioic acid (9DA), 1,10-decanedioic acid (10DA), propane-1,2,3-tricarboxylic acid (1,2,3-PrTA), butane-1,2,4-tricarboxylic acid (1,2,4-BTA), pentane-1,3,5-tricarboxylic acid (1,3,5-PeTA). Similarly, the polymer abbreviations are defined as **template abbreviation-MIP**.

Evaluation of Polymers Using Liquid Chromatography. The polymer particles were packed into stainless steel columns for evaluation of the selective recognition effect toward to DA. At first, AN/0.05% aqueous trifluoroacetic acid (7/3, v/v) was eluted to remove the template molecules. For the actual chromatographic evaluation, AN/0.05% aqueous acetic acid (7/3, v/v) was utilized and the void volume was estimated with a retention time of acetone.

Chromatographic data were acquired by using an LC system (Shimadzu Co., Japan) comprising LC-10 AD as a pump, SPD-M10A as a photodiode array detector, and CTO-10AC as a column oven. As the evaluation index, the retention factor k' and the separation factor α were defined as follows:

$$k' = (V(\text{retention volume of solute}) -$$
$$V(\text{void volume}))/V(\text{void volume})$$

$$\alpha = k'a/k'b \qquad (\text{where } k'a = k' \text{ of } a \text{ and } k'b = k' \text{ of } b)$$

Conformational Calculation. For an appropriate conclusion, we continuously performed structural calculations. As shown in the figures, we obtained some structural information. To obtain the initial 3D molecular coordinates of DA, CS Chem 3D version 9.0 was used. In order to obtain all the conformational isomers of several flexible dissociation states, CONFLEX 2000 was used.[22] All the geometrical optimizations were fully carried out by MMFF94 in CONFLEX 2000. The distributions of these flexible conformational isomers were obtained from the Boltzmann distribution based on the Gibbs free energies calculated by using a vibrational analysis. Each isomer was used for the structural calculation by RHF/6-31G(d), SCI-PCM in Gaussian 03W.

(11) Kubo, T.; Hosoya, K.; Sano, T.; Nomachi, M.; Tanaka, N.; Kaya, K. *Anal. Chim. Acta* **2005**, *549*, 45−50.
(12) Kubo, T.; Matsumoto, H.; Shiraishi, F.; Nomachi, M.; Nemoto, K.; Hosoya, K.; Kaya, K. *Anal. Chim. Acta* **2007**, *589*, 180−185.
(13) Takeuchi, H.; Watanabe, K.; Nomoto, K.; Ohfune, Y.; Takemoto, T. *Eur. J. Pharmacol.* **1984**, *102*, 325−332.
(14) Wright, J. L. C.; Falk, M.; McInnes, A. G.; Walter, J. A. *Can. J. Chem.* **1990**, *68*, 22−25.
(15) Hampson, D. R.; Huang, X.; Wells, J. W.; Walter, J. A.; Wright, J. L. C. *Eur. J. Pharmacol.* **1992**, *218*, 1−8.
(16) Perl, T. M.; Bedard, L.; Kosatsky, T.; Hockin, J. C.; Todd, E. C. D.; Remic, R. S. *N. Engl. J. Med.* **1990**, *322*, 1775−1780.
(17) Nanao, M. H.; Green, T.; Sterm-Bach, Y.; Heinemann, S. F.; Choe, S. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 1708−1713.
(18) Kubo, T.; Nomachi, M.; Nemoto, K.; Sano, T.; Hosoya, K.; Tanaka, N.; Kaya, K. *Anal. Chim. Acta* **2006**, *577*, 1−7.
(19) Perrin, D. D.; Armarego, W. L. F.; Perrin, D. R. *Purification of Laboratory Chemicals*; Pergamon Press: Oxford, 1980.

(20) Ugelstad, J.; Kaggerud, K. H.; Hansen, F. H.; Perge, A. *Macromol. Chem.* **1979**, *180*, 737−744.
(21) Smigol, V.; Svec, F.; Hosoya, K.; Wang, Q.; Frechet, J. M. J. *Angew. Makromol. Chem.* **1992**, *195*, 151−164.
(22) Goto, H.; Osawa, E. *J. Am. Chem. Soc.* **1989**, *111*, 8950−8951.

Table 1. Compositions of Polymers and Retention Factor k' on Their Columns[a]

abbreviation	template	k' domoic acid	k' benzoic acid
BASE[b] (nontemplates, 4VP)	−	0.10	0.25
BLANK[c] (nontemplates)	−	1.10	2.07
	Dicarboxylic Acid		
OPA-MIP	*o*-phthalic acid	9.62	5.30
DKA-MIP	dihydrokainic acid	7.95	4.55
2,3-NDA-MIP	2,3-naphthalene dicarboxylic acid	13.7	4.47
1,4-NDA-MIP	1,4-naphthalene dicarboxylic acid	7.24	4.65
2,6-NDA,MIP	2,6-napthalene dicarboxylic acid	5.94	3.96
EDA-MIP	diphenylether-4,4′- dicarboxylic acid	12.6	4.26
FDA-MIP	2,2′-bis(4-carboxyphenyl)- hexafluoropropan	11.6	4.31
KDA-MIP	benzophenone-4,4′- dicarboxylic acid	14.6	4.75
2DA-MIP	oxalic acid	12.1	6.29
3DA-MIP	malonic acid	12.4	5.22
4DA-MIP	succinic acid	14.1	5.71
5DA-MIP	glutaric acid	13.7	4.97
6DA-MIP	adipic acid	13.4	4.45
7DA-MIP	1,7-heptanedioic acid	13.3	4.40
8DA-MIP	1,8-octanedioic acid	14.6	5.17
9DA-MIP	1,9-nonanedioic acid	15.3	6.73
10DA-MIP	1,10-decanedioic acid	13.8	6.27
	Tricarboxylic Acid		
1,2,3-PrTA-MIP	propane-1,2,3-tri- carboxylic acid	11.5	8.09
1,2,4-BTA-MIP	butane-1,2,4-tri- carboxylic acid	23.5	5.95
1,3,5-PeTA-MIP	pentane-1,3,5-tri- carboxylic acid	53.5	8.15

[a] Chromatographic conditions: Mobile phase: AN/0.05%AcOH aqueous = 7/3 (v/v); Flow rate: 1.0 mL min^{-1}; Temperature: 30 °C; Detection: Photodiode array; Column size: 150 mm × 4.6 mm i.d. [b] Only crosslinker (EDMA) was used for the preparation of BASE polymer without a functional monomer (4VP) and any template molecules. [c] EDMA and 4VP were used for preparation of BLANK polymer without any template molecules.

Finally, from the above-mentioned investigation, we obtained a stable structure and/or metastable structures were obtained.

Selective Pretreatment of DA from Mussel Extracts. Blue mussel (17.7 g) was extracted with 200 mL of 50% aqueous methanol (v/v) according to the method proposed by Lopez-Rivera et al.[23] Then, the extract was treated in a centrifuge to remove the insoluble compounds, and the solvent was distilled: the residue was redissolved into 20 mL of AN/0.1% aqueous acetic acid (7/3, v/v). Then, 50 µg/mL of authentic DA was added into the extract solution. The DA-containing extract was inserted into a cartridge containing polymers imprinted with pentane-1,3,5-tricarboxylic acid, BLANK polymer, and commercially used C$_{18}$ cartridges. The adsorbed compounds were collected with AN/20 mM phosphate buffer (7/3, v/v, pH = 3), and the concentration of DA in the collected sample was determined by a conventional chromatographic analysis.

Results and Discussion

Selectivity for DA. The polymer aberrations, the corresponding template molecules, and retention factors of DA and benzoic acid on the MIPs based on chromatographic evaluation are listed
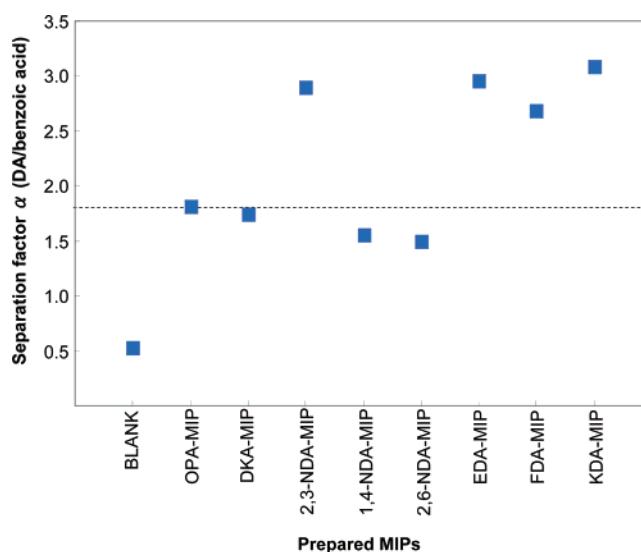
(23) Lotierzo, M.; Henry, O. Y. F.; Piletsky, S.; Tothill, I.; Cullen, D.; Kania, M.; Hock, B.; Turner, A. P. F. *Biosens. Bioelectron.* **2004**, *20*, 145−152.

Figure 2. Separation factor α (k' of DA/k' of benzoic acid) on MIPs prepared with aromatic dicarboxylic acid as templates. Chromatographic conditions were the same as those mentioned in Table 1.
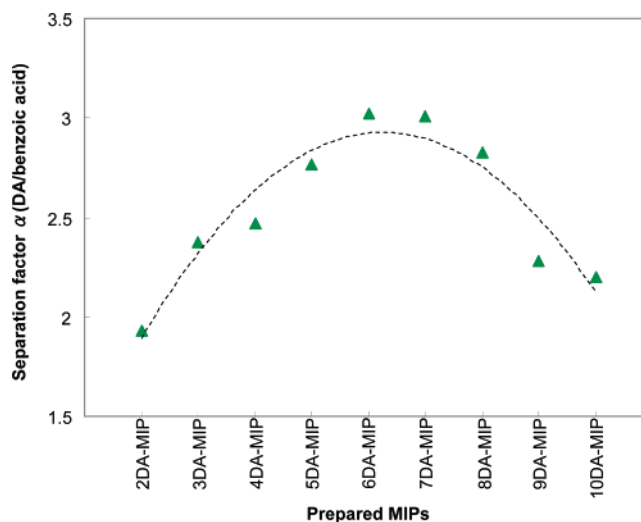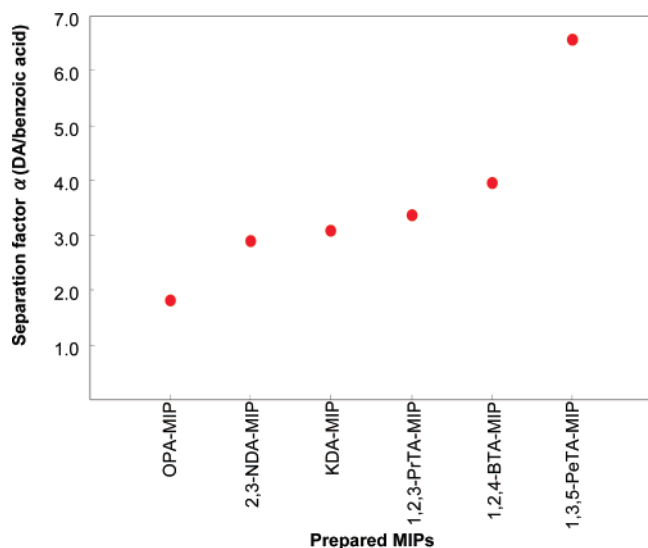

Figure 3. α on MIPs prepared with aliphatic dicarboxylic acid as templates. Chromatographic conditions were the same as those mentioned in Table 1.

Table 1. In a previous study, we described the selective separation of DA using *o*-phthalic acid imprinted polymer and suggested that the acidity and spatial location of the COOH groups in the template molecules were important for the selective recognition of DA.[18] We also considered the ortho COOH groups on the template aromatic rings in term of selectivity facilitation. However, detailed examinations of the spatial location of the template COOH groups were not discussed in our previous report. In the current study, we present results of detailed investigations on the relationship between the spatial location of COOH groups on the template molecules and DA-selectivity of resulting MIPs.

Figure 2 shows the separation factor of DA against benzoic acid on MIPs prepared using aromatic dicarboxylic acids as templates. In this figure, the broken line indicates the value of the separation factor for OPA-MIP as a reference for selectivity comparison. As shown, the selectivity for DA was considerably higher for 2,3-NDA-MIP compared to that of OPA-MIP, DKA-MIP, and 2,3-NDA-MIP. The difference in the selectivity between OPA-MIP and 2,3-NDA-MIP can easily be understood

Figure 4. α on MIPs prepared with aliphatic tricarboxylic acid as templates. Chromatographic conditions were the same as those mentioned in Table 1.
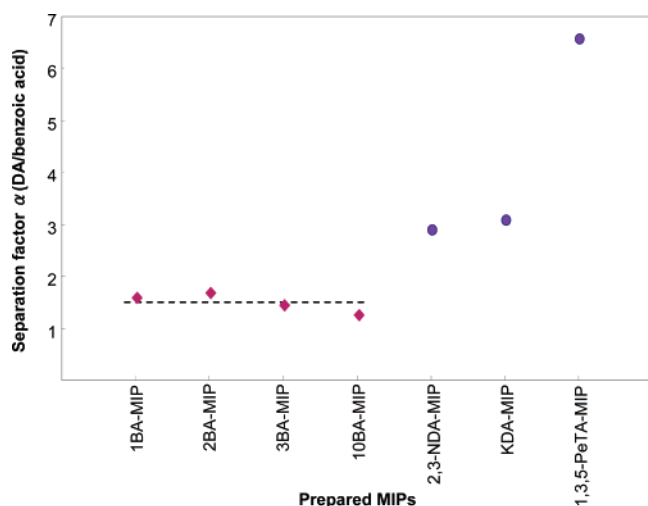

Figure 5. Comparison of selectivities for DA in each mole ratio of the template (BA). The mole ratio of the template molecule (BA) of 1BA-MIP, 2BA-MIP, 3BA-MIP, and 10BA-MIP were 1, 2, 3, and 10 times those of the other MIPs.
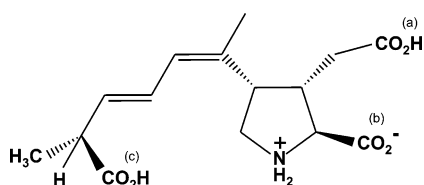

Figure 6. Dissociation structure of DA in the mobile phase condition.

Figure 7. Stable conformation of DA in the mobile phase condition.

Figure 8. Distance of COOH groups of template molecules.

Figure 9. Ratio of dissociation of each COOH group and NH group on DA depend on pH.

by the difference in the effective molecular volume at the recognition site. Interestingly, although the structure of dihydrokainic acid (DKA) is a part of DA, the selectivity for DA was higher for 2,3-NDA-MIP. This suggested that the molecular volume of the template in addition to the ortho COOH groups in the template aromatic rings was very important for the selective two-point recognition of DA ((a) and (b) in Figure 1).

On the other hand, the selectivity of DA for EDA-MIP, FDA-MIP, and KDA-MIP was almost the same as that for 2,3-NDA-MIP. However, ortho COOH groups are not contained in the template molecules on these MIPs. Therefore, we assumed that these results i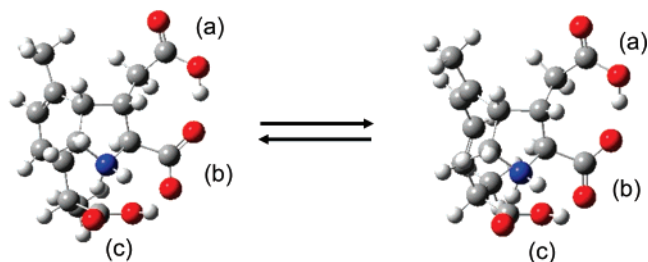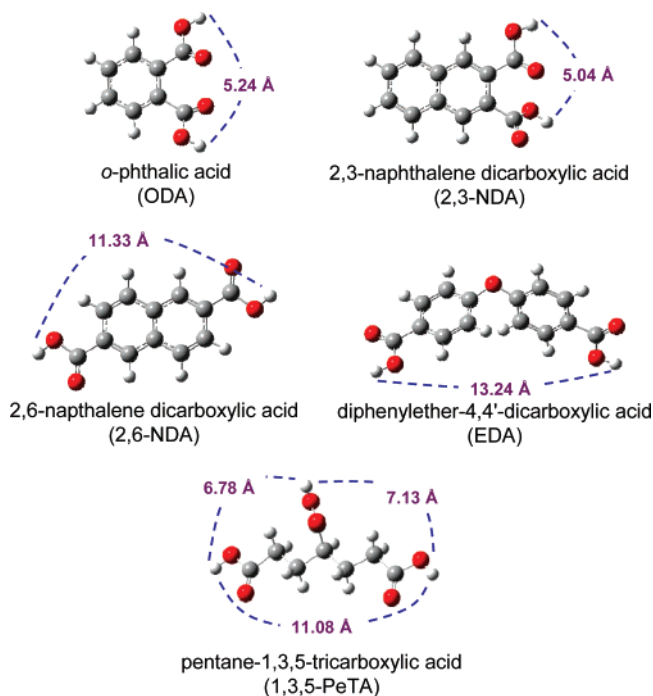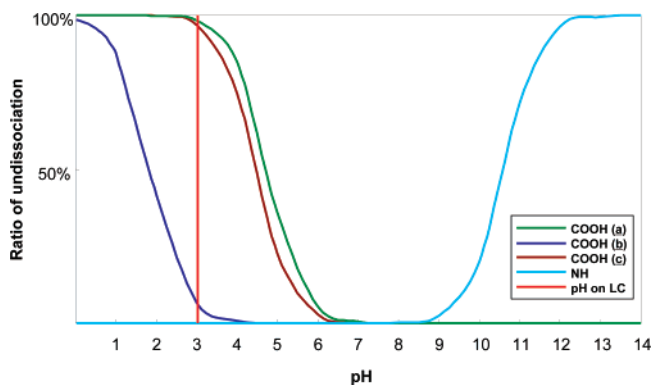ndicated possibilities for another two-point recognition of DA ((a) and (c), (b), and (c) in Figure 1). Thereafter, we examined the above possibilities using comparison of the selectivity for DA on several MIPs prepared using simple aliphatic dicarboxylic acids. The chromatographic results are shown in Figure 3. As shown in this figure, the selectivity dramatically changed by the differences in the distance of aliphatic dicarboxylic acids used as the template molecules. It is interesting to note that the plots of the separation factors had an inflection point, and we could easily control the effect of another two-point recognition. To discuss these differences in
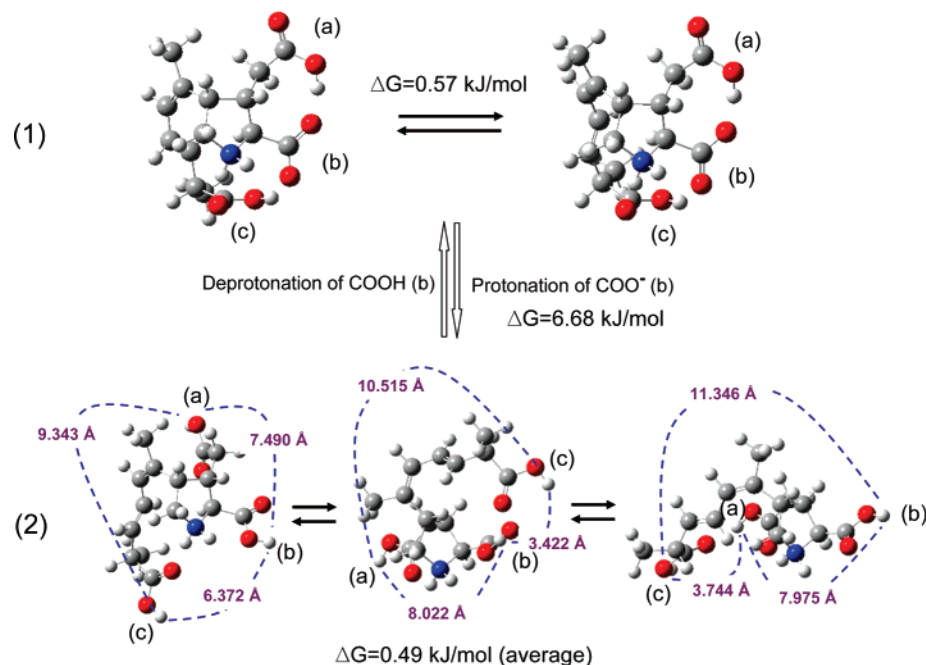
Figure 10. Alteration of ΔG by conformation changes in DA. (1) Most stable conformation of DA and (2) conformation of DA after protonation of COOH (b).

Table 2. Gibbs Free Energies of DA on Chromatographic Evaluation

polymers	K' of DA	ΔG of DA (kJ/mol)	ΔG − ΔG(on BASE) (kJ/mol)	ΔG − ΔG(on BLANK) (kJ/mol)
BASE	0.10	−5.80	−	−
BLANK	1.10	0.24	6.04	−
OPA-MIP	9.62	5.70	11.5	5.46
2,3-NDA-MIP	13.7	6.59	12.4	6.35
2,6-NDA-MIP	5.94	4.49	10.3	4.25
EDA-MIP	12.6	6.38	12.2	6.14
FDA-MIP	11.6	6.17	12.0	5.93
KDA-MIP	14.5	6.75	12.6	6.51
1,3,5-PeTA-MIP	53.5	10.0	15.8	9.79

the selectivity for DA, we had to give consideration to the conformational changes in DA so that the spatial distance of three COOH groups on DA were calculated in detail during computer modeling. The results regarding the spatial distance and the selectivity for DA are described in the next paragraph.

Following the above discussion, we showed the possibilities of the recognition of three COOH groups on DA ((a), (b), and (c) in Figure 1). Therefore, we anticipated that the three-point recognition of DA could also be achieved by the simultaneous recognition of three COOH groups of DA. Then, the simple aliphatic tricarboxylic acids were used as the template molecules, and the prepared polymers were evaluated using liquid chromatography under the same mobile phase conditions. The results are shown in Figure 4. As a result, the selective recognition for DA was also observed on MIPs prepared using tricarboxylic acids as the template. In particular, the highest selectivity was obtained for 1,3,5-PeTA-MIP. Although the acidities of these tricarboxylic acids are close, 1,3,5-PeTA-MIP has a larger volume than that of any other tricarboxylic acid. Therefore, it was probably assumed that the conformation of 1,3,5-PeTA was suitable for the three-point recognition of DA. However, the conformational information of DA had to be considered to discuss the three-point recognition as well as the above

discussion, and the conformational examinations of DA are elaborated in the next paragraph.

In the discussions pertaining to multipoint recognitions (as mentioned above), we need to consider the number of COOH groups in the template molecules. All template molecules shown in Table 1 were used at the same mole ratio in the preparation of the MIPs. Therefore, the number of COOH groups included in the templates was different among the template molecules. In fact, we noted that these differences in the selectivity for DA were due to the advancement of the recognition of simple COOH groups. Consequentially, we prepared additional MIPs with benzoic acid (BA) as the template molecule at a 1−10-fold mole ratio as against MIPs mentioned in Table 1, and the MIPs were evaluated under the same chromatographic conditions (Figure 5). The chromatographic results evidently suggested that the number of COOH groups did not affect the selectivity for DA at all. In other words, it was strongly suggested that the multipoint recognitions for DA observed in our studies were relevant to the structure and spatial distance of the COOH groups of the template molecules and/or DA.

Conformational Analysis for DA. As mentioned above, several template molecules having two or three COOH groups were evaluated for MIPs and a few MIPs had the selective recognition ability for DA. However, the correlations between the template molecules and DA were not understood; therefore, we tried to examine the conformational alteration of DA as well as the Gibbs free energies by using computer modeling.

The pK_a values of DA have been reported[24] as follows: pK_{a1} = 1.85 (b), pK_{a2} = 4.47 (c), pK_{a3} = 4.75 (a), and pK_b = 10.6 (NH). Therefore, under the chromatographic conditions (pH = 3), a greater part of the dissociation of DA was presumed as that shown in Figure 6. Thereafter, we carried out an exhaustive conformation search of DA, and the most stable conformation was estimated from the expected 20 000 conformations. The

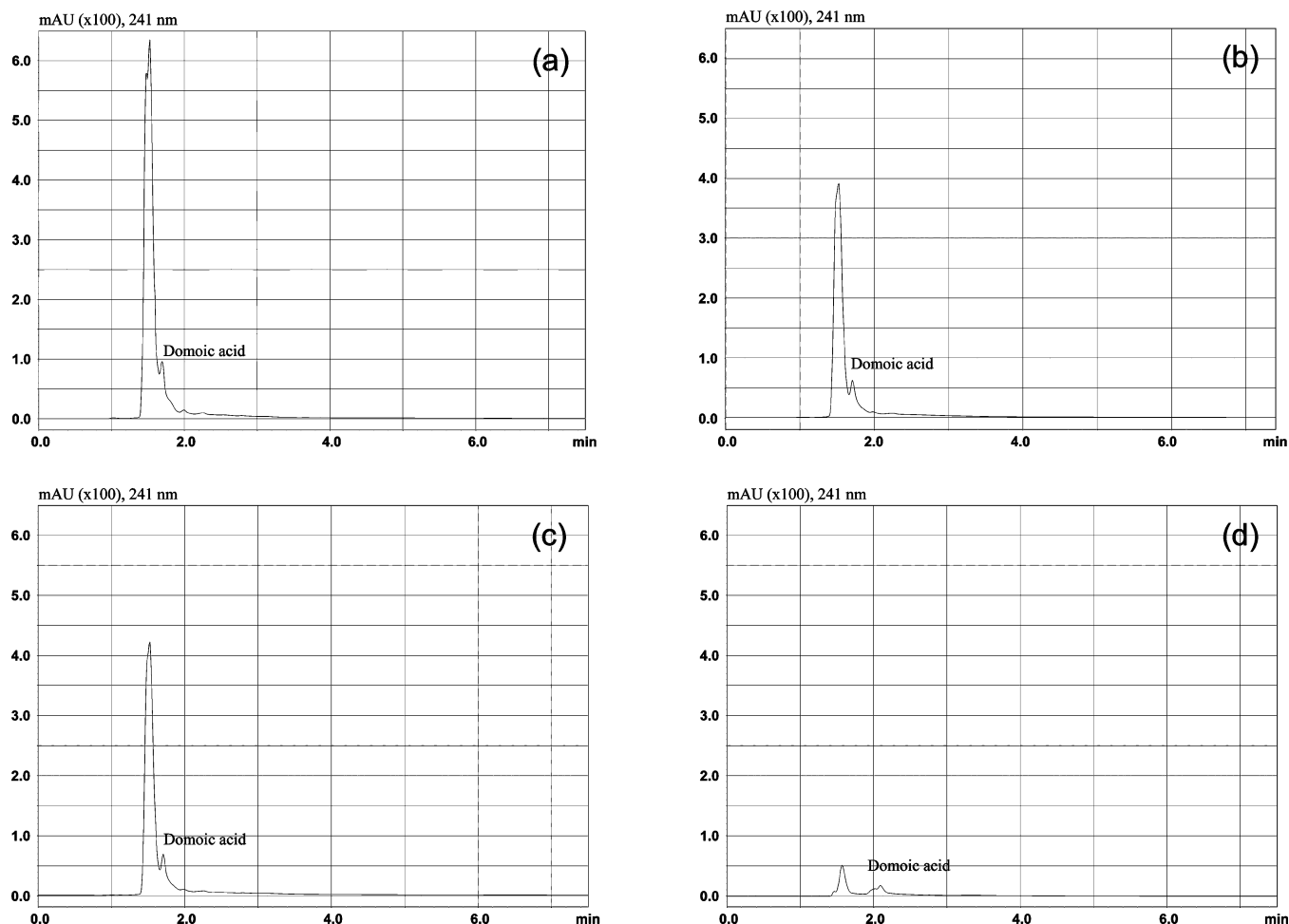(24) Walter, J. A.; Leek, D. M.; Falk, M. *Can. J. Chem.* **1992**, *70*, 1156−1161.

Figure 11. Chromatograms of blue mussel extracts containing DA. (a) non-pretreated sample, (b) passed-through fraction from C$_{18}$, (c) passed-through fraction from BLANK, and (d) collected fraction from BLANK. *Chromatographic conditions for determination of DA.* Column: Mightysil (Kanto Chemical, Japan, 150 mm × 4.6 mm i.d.); Mobile phase: AN/0.05% AcOH aqueous = 3/7; Flow rate: 1.0 mL min^{-1}; Temperature: 30 °C; Detection: Photodiode array; Injection volume: 1 μL.

result of the estimated stable conformation is shown in Figure 7. In each isomer, the structures were stabilized by the intramolecular hydrogen bonding so that the spatial distance of the COOH groups were appreciably close.

On the other hand, the distance of the COOH groups of the template molecules in vacuum are shown in Figure 8. First, the distance of the COOH groups on the stable conformation of DA were compared to that of the template molecules having ortho COOH groups. As shown in Figure 7, the distance of (a) to (c) was estimated to be from 5.105 to 5.109 Å. Results of the distance of the COOH groups on the templates and DA can easily be understand the two-point recognition for OPA-MIP and 2,3-NDA-MIP as well as that in our previous study.[18] However, in the case of the other template molecules, it was propounded that the multipoint recognitions cannot be understood by the result of the stable conformation of DA. Therefore, we thought that another equilibrium state of DA was also present in the mobile phase condition. Consequently we reexamined the conformational alteration of DA in detail. As shown in Figure 9, the COOH group (*b*) is protonated although the protonation ratio is approximately 6.6%; in contrast, the NH group is completely protonated.

Accordingly, another state without the dissociated COOH group was also calculated, and the three major isomers were estimated. The estimated isomers and the alteration of the Gibbs

free energies are shown in Figure 10. In this term, the average free energy is indicated on the state without dissociated COOH group. Additionally, the Gibbs free energies in the chromatographic analysis can easily be estimated by the equation $\Delta G = -RT \ln K$, where $k' = \beta K$ (k' is the retention factor and β is a chemical equilibrium constant). Moreover, $\Delta\Delta G$ between two stationary phases can also be estimated by the equation $\Delta\Delta G = -RT \ln \alpha$ (α is the separation factor).[25] Therefore, we calculated the alteration of free energy on each MIP: the results are listed in Table 2. As shown in Figure 10 and Table 2, the results indicated that the Gibbs free energies were more advantageous on the prepared MIPs if the conformation of DA acquired the state without dissociated COOH groups (the equilibrium is shown below (*2*) in Figure 10). Actually, the ΔG value transition of DA from (1) to (2) in Figure 10 was also lower than $\Delta G - \Delta G$ (on BASE) or $\Delta G - \Delta G$ (on BLANK). Therefore, it was provably possible to recognize DA at the state without dissociated COOH groups (2) on each MIP. In this case, the recognition of two COOH groups as (a)−(c) and (b)−(c) occurred on the MIPs prepared with aromatic dicarboxylic acid such as 2,6-NDA-MIP, EDA-MIP, FDA-MIP, and KDA-MIP. Furthermore, along with the above discussion, we can also comprehend that 1,3,5-PeTA-MIP had the most selective

---

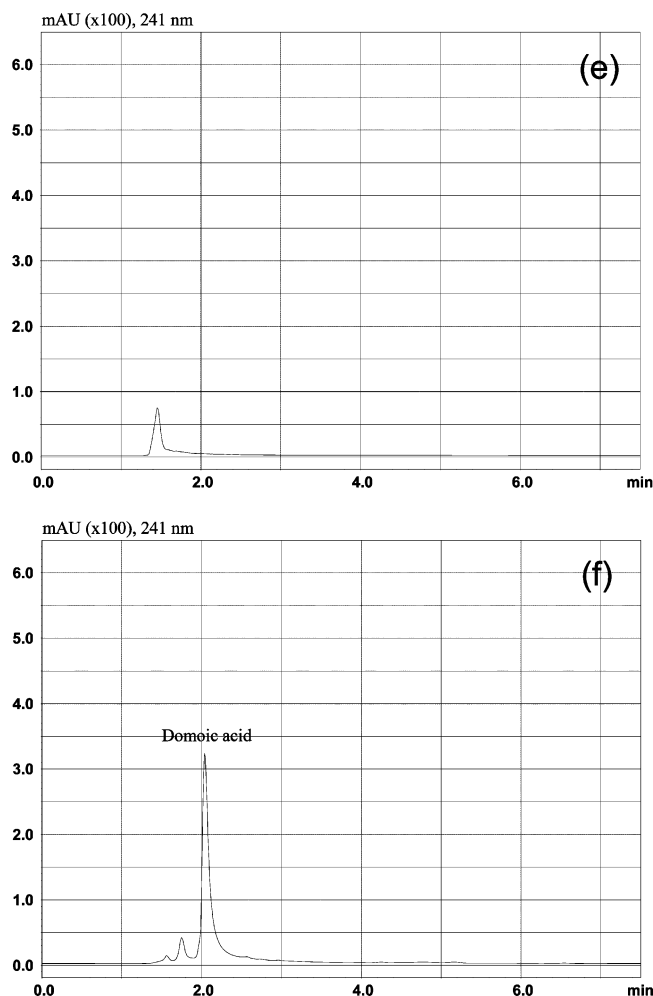(25) Neue, U. D. *HPLC Columns*; Wiley-VCH: 1997.

Figure 12. Chromatograms of blue mussel extracts containing DA with MIP. (e) Passed-through fraction from 1,3,5-PeTA-MIP and (f) collected fraction from 1,3,5-PeTA-MIP.

recognition ability for DA in the chromatographic evaluations. Afterall, the recognition sites constructed on 1,3,5-PeTA-MIP were able to recognize DA by the flexible conformational changes of DA: these conformational changes were incorporated into the recognition sites. In other words, these results suggested that three-point recognition was achieved by the conformation change of DA onto the MIPs using the optimized template molecule.

Here, we proposed a new concept of the selective 3D recognition for the flexible compounds by the molecular imprinting technique using a simple template molecule. We expect that this concept will be applied to a greater number of compounds having several functional groups and flexible conformation changes.

Application of MIP for the Pretreatment of DA. To examine the possibility as a pretreatment medium for DA, we carried out the solid-phase extraction using the prepared MIPs. Blue mussel extracts containing DA were treated by the solid-phase extraction method using a commercially available C_{18} cartridge, BLANK polymer, and 1,3,5-PeTA-MIP. The passed-through fraction and the collected fraction were evaluated by conventional liquid chromatography. The chromatograms of the nontreated sample, passed-through fraction, and collected fraction are shown in Figures 11 and 12. These chromatograms clearly show that the DA could not be purified using the C_{18} cartridge as well as the BLANK polymer, although the peak of DA was slightly identified in the latter. On the other hand, DA could clearly be purified on 1,3,5-PeTA-MIP. Moreover, as a result of the recovery examination on 1,3,5-PeTA-MIP by the analytical curve ($R_2 = 0.998$), high recovery was obtained (89%). These results strongly suggested that the prepared MIP (1,3,5-PeTA-MIP) can be utilized as a pretreatment medium for the purification of DA from natural samples.

Conclusion

In this study, we investigated a simple and effective 3D recognition methodology for compounds with a flexible structure. Simple aliphatic tricarboxylic acid was used as the template molecule of MIP for the selective recognition of DA, an amnesic shellfish poison. It was clearly revealed that the selective recognition was due to the spatial distance of the COOH groups on the template molecules and the flexible conformation of the target molecule. We believe that the concept described in this paper will contribute to the development of a usable method for the selective recognition.

Supporting Information Available: The structures of the template molecules used for the preparation of the MIPs and the results of the exhaustive conformation searching. This material is available free of charge via the Internet at http://pubs.acs.org.