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Tetrahedron 63 (2007) 9802–9809
Tetrahedron

‘Biting effect’ stabilizing gallate-type catechin/quaternary ammonium ion complexes

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Received 28 May 2007; revised 3 July 2007; accepted 3 July 2007
Available online 10 July 2007

Abstract—The relationship between the structure of catechins and their binding ability for quaternary ammonium ions was examined. From binding studies using eight catechins and benzyltrimethylammonium chloride, it was revealed that the binding ability of the gallate-type catechins is much higher than that of the non-gallate-type catechins. The quaternary ammonium ion binding site of the gallate-type catechins was determined to be the space formed by the galloyl group and the B-ring. The excellent binding ability of the gallate-type catechins is attributed to the ‘biting effect’ by the galloyl group and the B-ring.

1. Introduction

Catechins (Fig. 1) are a class of polyphenols found in the leaves and buds of the tea plant (*Camellia sinensis*). They have recently been discovered to have various physiologically modulating effects such as anti-carcinogenic,[1–7] anti-metastatic,[8–11] anti-oxidative,[12–15] anti-hypertensive,[16] anti-hypercholesterolemic,[17–19] anti-bacterial,[20] anti-dental caries,[21–23] and intestinal flora amelioration activities.[24] In each case, the various catechins exhibit a different order in their activity. Interestingly, it has been revealed that the gallate-type catechins (esterified with gallic acid at the C_3 position) generally give higher activities than the non-gallate-type catechins (without the galloyl group).[25–29] Various intermolecular interactions between the catechins and biomolecules must regulate these bioactivities. While hydrophobic bonds and hydrogen bonds are considered to be significant binding forces, CH/π or cation/π interaction is also expected to play an important role. This is because catechin molecules are composed of several aromatic rings, and a quaternary ammonium structure is present in cell membranes. The CH/π[30] or the cation/π[31–35] interaction has been recently recognized as a considerable binding force. Therefore, in order to gain an insight into the bioactivities of catechins, it is crucial to understand the interaction between the catechins and the quaternary ammonium ion. However, although several studies on artificial aromatic receptor/ammonium ion complexes have been carried out,[36–47] the interaction between the catechins and the quaternary ammonium ion has not been investigated. In the present paper, we elucidate the relationship between the structure of the catechins and their binding ability to a quaternary ammonium ion and discuss the mechanism of their molecular recognition.

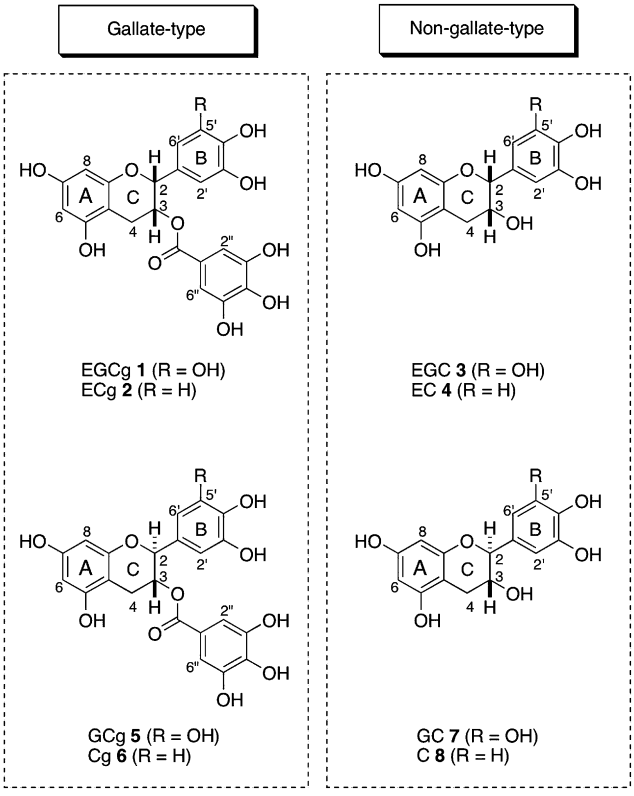

Figure 1. Structures of the catechins.

Keywords: Catechins; Quaternary ammonium ion; Binding; Galloyl group.
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doi:10.1016/j.tet.2007.07.003

2. Results and discussion

Binding studies between catechins and quaternary ammonium ions were carried out by means of standard ^{1}H NMR titration experiments in acetonitrile-d_3/chloroform-d (1:1). The following eight catechins were employed in the titration experiments: (−)-epigallocatechin-3-*O*-gallate (EGCg, **1**), (−)-epicatechin-3-*O*-gallate (ECg, **2**), (−)-epigallocatechin (EGC, **3**), (−)-epicatechin (EC, **4**), (−)-gallocatechin-3-*O*-gallate (GCg, **5**), (−)-catechin-3-*O*-gallate (Cg, **6**), (−)-gallocatechin (GC, **7**), and (+)-catechin (C, **8**). EGCg **1**, ECg **2**, EGC **3**, and EC **4** are the major catechins in green tea and have a 2,3-*cis*-stereochemistry. These 2,3-*cis*-catechins epimerize at the C_2 position in hot water to give the corresponding 2,3-*trans*-catechins (GCg **5**, Cg **6**, GC **7**, and C **8**).[48]

The concentration of each catechin was increased from 0 to 3.50 mM against a constant concentration of the quaternary ammonium ion (1.00 mM). At first, tetramethylammonium chloride (TMAC, **9**) was used as the quaternary ammonium ion source, but the titration experiments could not be carried out for all the eight catechins due to precipitation. Therefore, benzyltrimethylammonium chloride (BMAC, **10**) was adopted instead of TMAC **9**. BMAC **10** has often been used in binding studies involving artificial aromatic receptor/ammonium ion complexes.[36–39] In Figure 2, the chemical shift changes ($\Delta\delta_{obs}^{amm}$) in the methyl, benzyl, and phenyl protons ($C_{3'+5'}$-*H* and $C_{4'}$-*H*) of BMAC **10** are plotted against the total concentration of each catechin, where $\Delta\delta_{obs}^{amm}$ is the difference between the observed ^{1}H NMR chemical shift of BMAC **10** with the catechins ($\delta_{obs}^{amm+cat}$) and that without the catechins ($\delta_{obs}^{amm(free)}$): $\Delta\delta_{obs}^{amm} = \delta_{obs}^{amm+cat} - \delta_{obs}^{amm(free)}$. The other phenyl protons ($C_{2'}$-*H* and $C_{6'}$-*H*) are hidden under the residual proton signal of the solvent (chloroform-*d*). The ^{1}H NMR signals of the methyl and benzyl protons shifted upfield with increasing concentration of catechins. The chemical shift changes in the phenyl protons were much smaller than those of the methyl and the benzyl protons. These phenomena suggest that the BMAC **10** interacts with the catechin molecule at the quaternary ammonium moiety, whereas the phenyl group contributes only minimally toward the complex formation (additional evidence for this will be described later from the chemical shift changes in the catechins). Therefore, the chemical shift changes in both the methyl and benzyl protons were used for calculating the binding constants of the catechins toward BMAC **10**.

$$\Delta\delta_{obs}^{amm} = \frac{\Delta\delta_{11}^{amm}}{2K_b[C]_0}\Big[1 + K_b[A]_0 + K_b[C]_0 - \left\{\left(1 + K_b[A]_0 + K_b[C]_0\right)^2 - 4K_b^2[A]_0[C]_0\right\}^{1/2}\Big] \quad (1)$$

Eq. 1, which is the standard binding isotherm for the formation of a 1:1 complex was applied to the titration plots in Figure 2.[49] In Eq. 1, $[C]_0$ and $[A]_0$ are the total concentrations of the catechins and the ammonium ion, respectively, K_b is a binding constant, and $\Delta\delta_{11}^{amm}$ is the difference between the ^{1}H NMR chemical shift of the ammonium ion forming the 1:1 complex with the catechin molecule (δ_{11}^{amm}) and the free ammonium ion ($\delta_{obs}^{amm(free)}$), i.e., $\Delta\delta_{11}^{amm} = \delta_{11}^{amm} - \delta_{obs}^{amm(free)}$. Table 1 lists the binding constants calculated by non-linear least-squares regression procedures. These binding constants indicate that the binding ability of the gallate-type catechins (**1**, **2**, **5**, and **6**) for BMAC **10** is higher than that of the non-gallate-type catechins (**3**, **4**, **7**, and **8**).[†] In order to investigate the binding site in the catechins for BMAC **10**, the chemical shift changes in the protons of each catechin with increasing concentrations of **10** were observed. As shown in Figure 3, the protons in the gallate-type catechins (**1**, **2**, **5**, and **6**) giving the largest chemical shift changes are as follows: EGCg **1**: C_3-*H*, $C_{4\alpha}$-*H*, $C_{2'+6'}$-*H*, and $C_{2''+6''}$-*H*; ECg **2**: C_3-*H*, $C_{4\alpha}$-*H*, $C_{2'}$-*H*, $C_{6'}$-*H*, and $C_{2''+6''}$-*H*; GCg **5**: C_2-*H*, C_3-*H*, $C_{4\alpha}$-*H*, $C_{4\beta}$-*H*, $C_{2'+6'}$-*H*, and $C_{2''+6''}$-*H*; Cg **6**: C_2-*H*, C_3-*H*, $C_{4\alpha}$-*H*, $C_{4\beta}$-*H*, $C_{2'}$-*H*, $C_{6'}$-*H*, and $C_{2''+6''}$-*H*.[‡] These chemical shift changes are attributed to the interactions with the tetraalkylammonium portion of BMAC **10**, because the profiles of these changes parallel those in the gallate-type catechin/TMAC systems closely (see Fig. 4). In contrast, the ^{1}H NMR signals of the non-gallate-type catechins (**3**, **4**, **7**, and **8**) hardly shifted under the same conditions. The following cross-peaks were observed between the protons of the B-ring or the galloyl group and the protons of the ammonium moiety of **10** in the NOESY spectra of solutions with the gallate-type catechins and BMAC **10**: EGCg **1**: $C_{2'+6'}$-*H*(**1**)–N*Me*$_3$(**10**), $C_{2'+6'}$-*H*(**1**)–Me_3NCH_2(**10**), $C_{2''+6''}$-*H*(**1**)–N*Me*$_3$(**10**), and $C_{2''+6''}$-*H*(**1**)–Me_3NCH_2(**10**); ECg **2**: $C_{2'}$-*H*(**2**)–N*Me*$_3$(**10**), $C_{5'}$-*H*(**2**)–N*Me*$_3$(**10**), $C_{6'}$-*H*(**2**)–N*Me*$_3$(**10**), $C_{2''+6''}$-*H*(**2**)–N*Me*$_3$(**10**), $C_{2'}$-*H*(**2**)–Me_3NCH_2(**10**), $C_{5'}$-*H*(**2**)–Me_3NCH_2(**10**), $C_{6'}$-*H*(**2**)–Me_3NCH_2(**10**), and $C_{2''+6''}$-*H*(**2**)–Me_3NCH_2(**10**); GCg **5**: $C_{2'+6'}$-*H*(**5**)–Me_3NCH_2(**10**) and $C_{2''+6''}$-*H*(**5**)–Me_3NCH_2(**10**); Cg **6**: $C_{5''}$-*H*(**6**)–Me_3NCH_2(**10**) and $C_{2''+6''}$-*H*(**6**)–Me_3NCH_2(**10**).[§] These results suggest that the binding site of the gallate-type catechins is the space formed from the B-ring and the galloyl group (GB-space). In the GB-space, both the galloyl group and the B-ring can simultaneously interact with the tetraalkylammonium portion of **10**. Therefore, it is conjectured that this 'biting effect' stabilizes the gallate-type catechins/ammonium ion complex.

The gallate-type catechins have three aromatic ring systems (the A-ring, the B-ring, and the galloyl group) that can act as candidates for the CH-π or the cation-π interaction. Why, then, is the GB-space the main binding site? In order to further elucidate the binding mechanism, the binding abilities of these three aromatic rings to BMAC **10** were estimated by investigating the binding constants of 5-methoxyresorcinol **11**, pyrogallol **12**, pyrocatechol **13**, and methyl gallate **14** toward BMAC **10**. These compounds correspond to the A-ring, the pyrogallol-type B-ring, the pyrocatechol-type B-ring, and the galloyl group, respectively. The binding constants were determined by ^{1}H NMR titration experiments

[†] The binding constants of the gallate-type catechin/TMAC complexes were similar to those of the gallate-type catechin/BMAC complexes (EGCg/TMAC=2163±296 M^{-1}, ECg/TMAC=932±98 M^{-1}, GCg/TMAC=1289±101 M^{-1}, and Cg/TMAC=1533±319 M^{-1}).

[‡] The C_4-*H* indicating the cross-peak against the C_2-*H* in the NOESY spectra was assigned to the $C_{4\beta}$-*H* in the 2,3-*cis*-catechins and the $C_{4\alpha}$-*H* in the 2,3-*trans*-catechins.

[§] The number in the parenthesis behind each proton represents the compound number. The cross-peaks from the methyl group of **10** in the spectra of the 2,3-*trans*-catechins (**5** and **6**) are omitted, because the methyl signal overlapped with the $C_{4\beta}$-*H* signals of **5** and **6**.

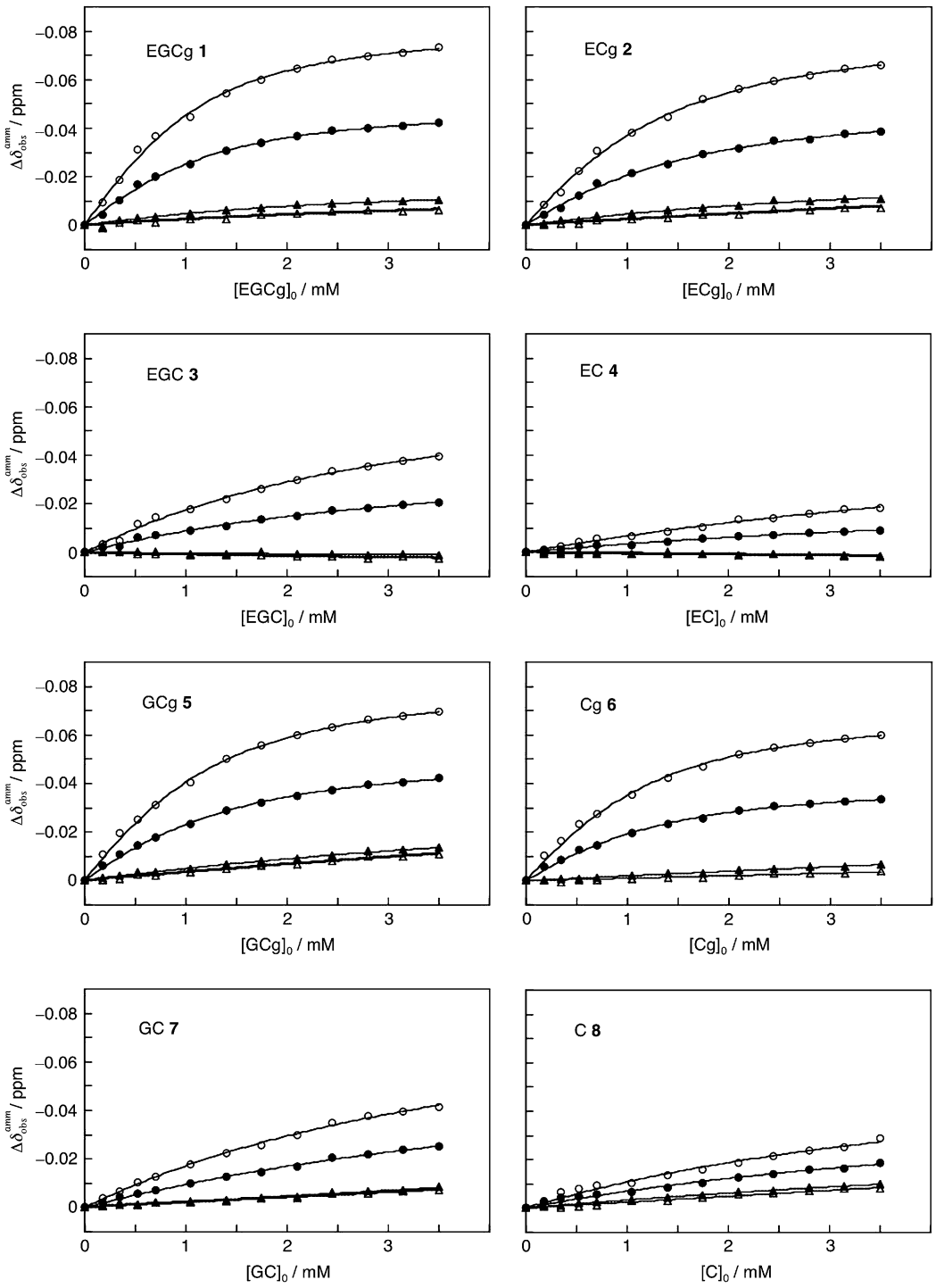


Figure 2. ^{1}H NMR titration curves between the catechins and benzyltrimethylammonium chloride **10** in $CD_3CN/CDCl_3$ (1:1) at 300 K. $\Delta\delta_{obs}^{amm}$ is the difference between the observed ^{1}H NMR chemical shifts of **10** with and without the catechins. [Catechin]$_0$ is the total concentration of the catechin. Filled circles, methyl protons; open circles, benzyl protons; filled triangles, protons at $C_{3'}$ and $C_{5'}$; open triangles, protons at $C_{4'}$.

(Fig. 5) to obtain values of 366±75 M^{-1} for **12**, 297±96 M^{-1} for **13**, and 977±222 M^{-1} for **14** as listed in Table 2. The binding constant for **11** was presumed to be extremely small from the slight chemical shift changes in the protons of **10** (the determination of K_b was impossible due to large errors). If these results are applied to the

Table 1. Binding constants (M^{-1}) of the complex between the catechins and benzyltrimethylammonium chloride (BMAC, **10**) at 300 K in $CD_3CN/CDCl_3$ (1:1)

BMAC **10**	EGCg **1**	ECg **2**	EGC **3**	EC **4**	GCg **5**	Cg **6**	GC **7**	C **8**
N–*Me*$_3$	2079±245	1115±124	379±71	196±58	1395±121	1665±190	193±31	210±80
Me_3N–*CH*$_2$	2604±330	1455±140	438±75	153±36	1810±169	1927±236	294±33	253±92
Mean	2342±288	1285±132	408±73	175±47	1603±145	1796±213	243±32	232±86

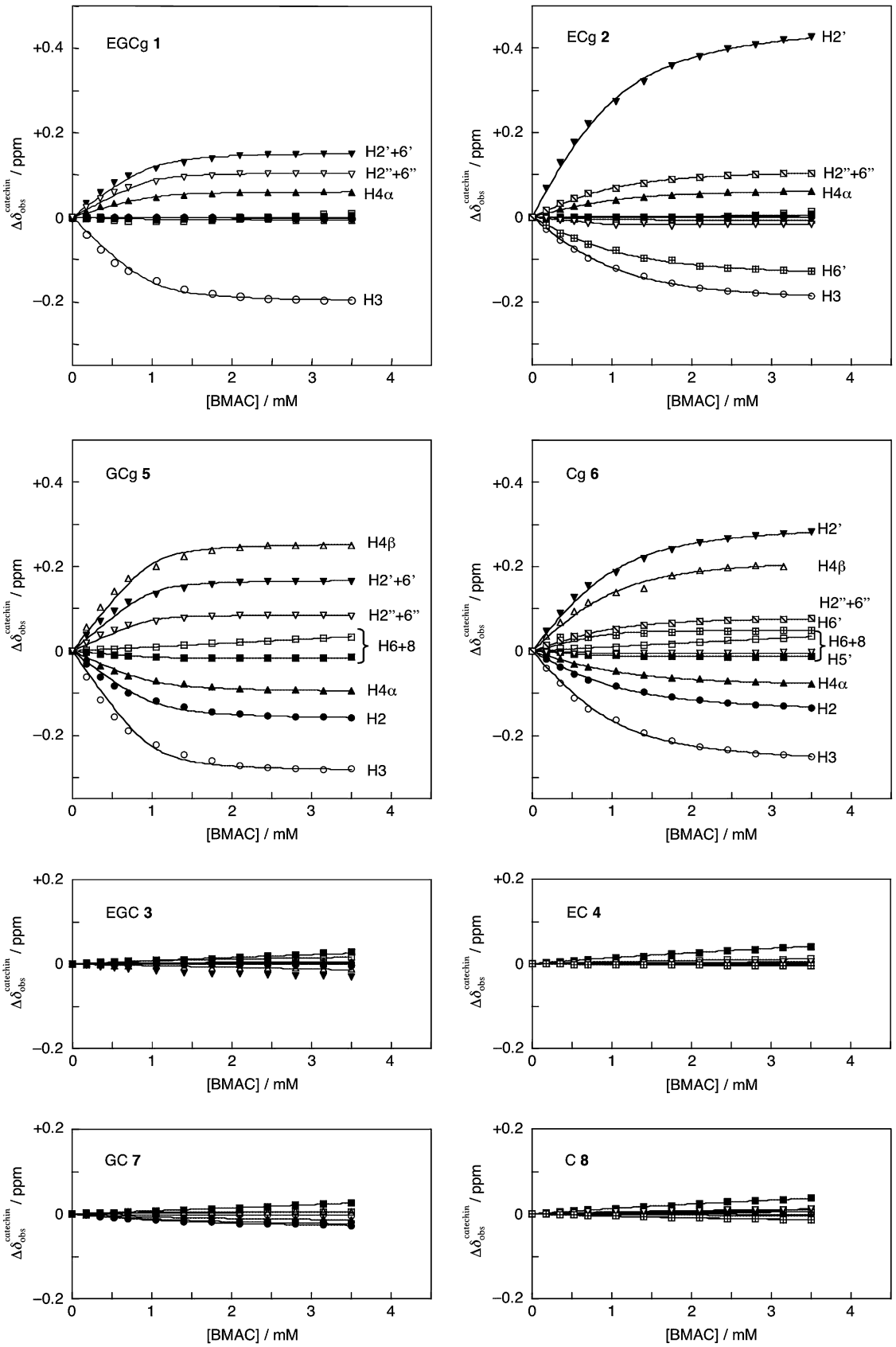


Figure 3. ^{1}H NMR chemical shift changes in the catechins (1.00 mM) by addition of benzyltrimethylammonium chloride (BMAC, **10**) in $CD_3CN/CDCl_3$ (1:1) at 300 K. $\Delta\delta_{obs}^{catechin}$ is the difference between the observed ^{1}H NMR chemical shifts of the catechins with and without **10**. $[BMAC]_0$ is the total concentration of BMAC **10**.

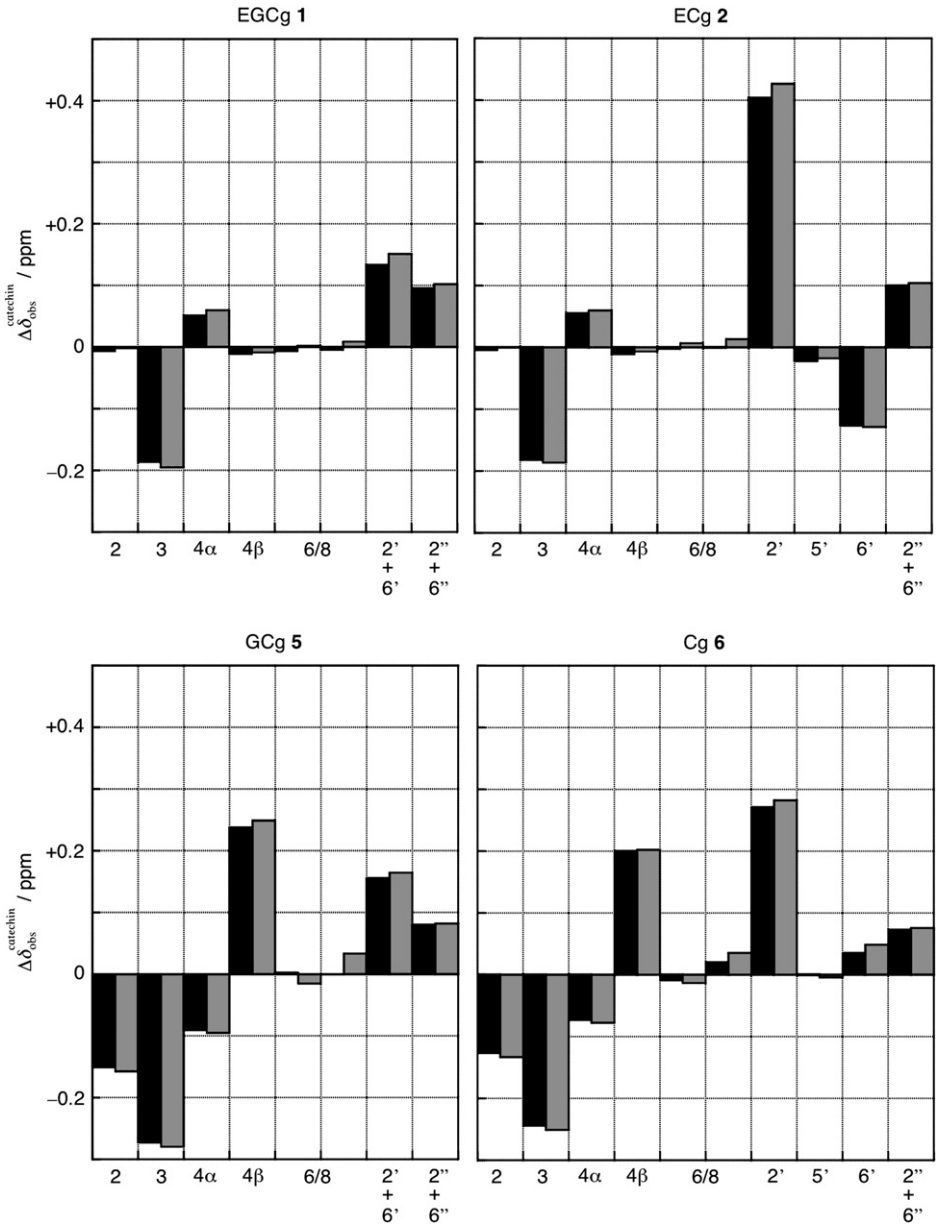


Figure 4. ^{1}H NMR chemical shift changes in the gallate-type catechins (1.00 mM) by addition of tetramethylammmonium chloride (TMAC, **9**) (3.50 mM) and benzyltrimethylammonium chloride (BMAC, **10**) (3.50 mM) in $CD_3CN/CDCl_3$ (1:1) at 300 K. $\Delta\delta_{obs}^{catechin}$ is the difference between the observed ^{1}H NMR chemical shifts of the catechins with and without the tetraalkylammonium chlorides (solid bar, TMAC **9**; shaded bar, BMAC **10**). The *x*-axis identifies the protons according to their attached carbon number.

corresponding aromatic rings of the catechin molecules, their binding abilities are estimated to be in the order of the galloyl group>the pyrogallol-type B-ring≈the pyrocatechol-type B-ring>the A-ring. Therefore, the binding site of the gallate-type catechins for BMAC **10** can be explained by the following mechanism. In the case of the gallate-type catechins, it is presumed that the galloyl group necessarily participates in the interaction with **10**, because the binding ability of the galloyl group is the highest of the three aromatic rings. The remaining question is, which of the A- and B-rings are used in the interaction. The 2,3-*cis*-gallate-type catechins (**1** and **2**) can be classified into conformers with a pseudoaxial galloyl group (Gax)/pseudoequatorial B-ring (Beq) and with a pseudoequatorial galloyl group (Geq)/pseudoeaxial B-ring (Bax). In the Gax/Beq conformer (Fig. 6a), the 'biting effect' also appears to be available from the A-ring and the galloyl group. However, the GB-space is the binding site for more stabilizing complex, because the B-ring has a higher binding ability than the A-ring. In the Geq/Bax conformer (Fig. 6b), the GB-space must be the binding site, because the A-ring is oriented in the opposite direction of the galloyl group and cannot interact with the ammonium ion. The 2,3-*trans*-gallate-type catechins (**5** and **6**) will mainly exist as Geq/Beq conformers (Fig. 6c). In this case also, the binding site is predicted to be the BG-space due to the orientation of the A-ring relative to the galloyl group. From these discussions, it is understood that the gallate-type catechins bind to BMAC **10** in the GB-space regardless of the relative stereochemistry at the C_2 and C_3 positions.

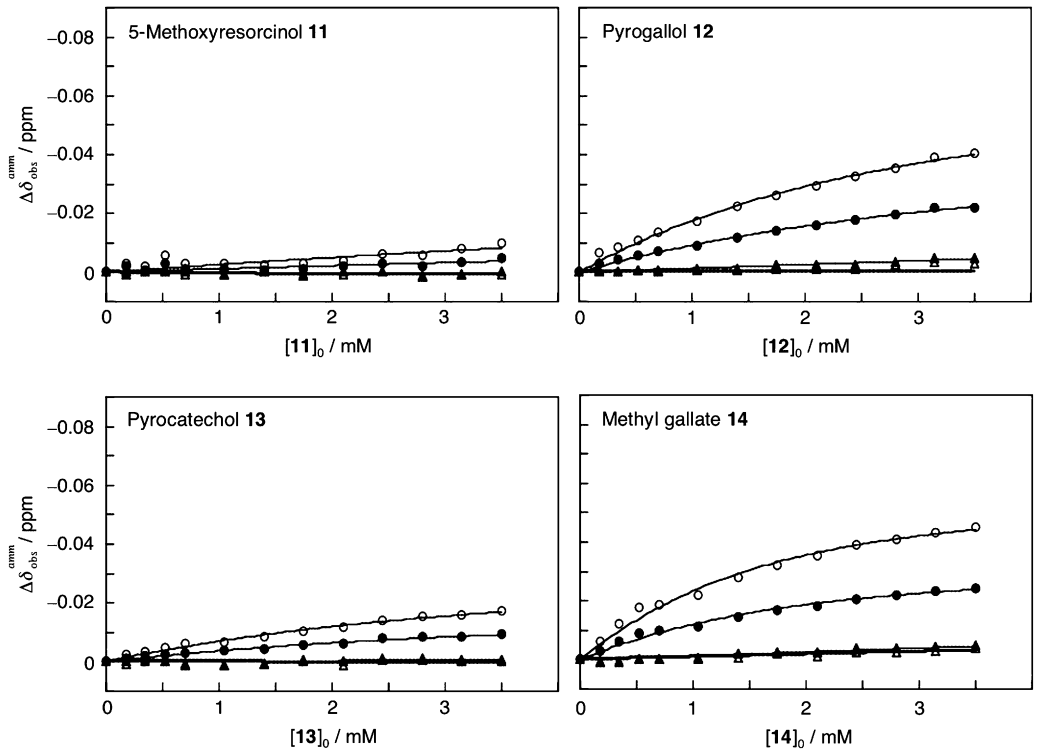
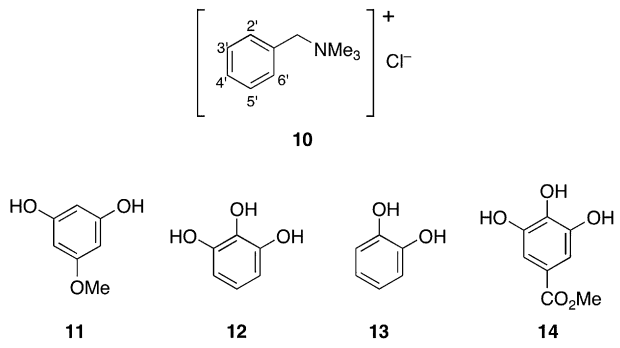


Figure 5. ^{1}H NMR titration curves between the phenolic compounds and benzyltrimethylammonium chloride **10** in $CD_3CN/CDCl_3$ (1:1) at 300 K. $\Delta\delta^{amm}_{obs}$ is the difference between the observed ^{1}H NMR chemical shifts of **10** with and without the phenolic compounds. $[X]_0$ is the total concentration of compound X. Filled circles, methyl protons; open circles, benzyl protons; filled triangles, protons at $C_{3'}$ and $C_{5'}$; open triangles, protons at $C_{4'}$.



On the other hand, the non-gallate-type catechins (**3**, **4**, **7**, and **8**) will exist as Beq conformers (Fig. 6d and e) regardless of the relative stereochemistry at the C_2 and C_3 positions. Therefore, the non-gallate-type catechins are presumed to be simply monodentate-like host molecules. This binding mode is supported by the above results, which show that the binding constants of the complexes from the non-gallate-type catechins are close to that of the complexes from the single pyrogallol **12** or pyrocatechol **13**. Consequently, the non-gallate-type catechins cannot stabilize the complex by the 'biting effect' as distinct from the gallate-type catechins. In contrast, the binding constants of the gallate-type catechins are much larger than those of the single pyrogallol **12**, pyrocatechol **13**, or methyl gallate **14**.[¶] These results support the conclusion that the complexes from the gallate-type catechins are stabilized by the 'biting effect' using the galloyl group and the B-ring.

Table 2. Binding constants (M^{-1}) of the complex between the phenolic compounds (**11**, **12**, **13**, and **14**) and benzyltrimethylammonium chloride (BMAC, **10**) at 300 K in $CD_3CN/CDCl_3$ (1:1)

BMAC **10**	**11**[a]	**12**	**13**	**14**
N–*Me*$_3$	—	305±61	273±99	864±193
Me_3N–*CH*$_2$	—	427±90	321±92	1089±252
Mean	—	366±75	297±96	977±222

[a] The binding constant of **11/10** complex cannot be determined due to large errors.

3. Conclusion

In the present study, we examined the relationship between the structures of eight catechins and their ability to form complexes with quaternary ammonium ions. It was revealed that the gallate-type catechins had much higher binding ability than the non-gallate-type catechins, and this superiority is attributed to the 'biting effect' by the galloyl group and the B-ring. In particular, EGCg **1** has the best binding ability. In fact, EGCg **1**, despite an acyclic receptor, can stabilize the complex with the BMAC **10** as well as or more than artificial aromatic receptors including cyclic compounds.[36–39]

Although non-covalent interactions are generally weak compared to covalent bonds, biomolecules achieve strong intermolecular binding forces by using several non-covalent interactions simultaneously. In a similar fashion, gallate-type

[¶] The binding constant of the ECg/BMAC complex is not large compared to those of the other gallate-type catechin/BMAC complexes. This might be due to the large deformation energy of ECg in the complex formation, because the graph in Figure 3 shows a larger chemical shift change in the C2′-*H* of ECg.

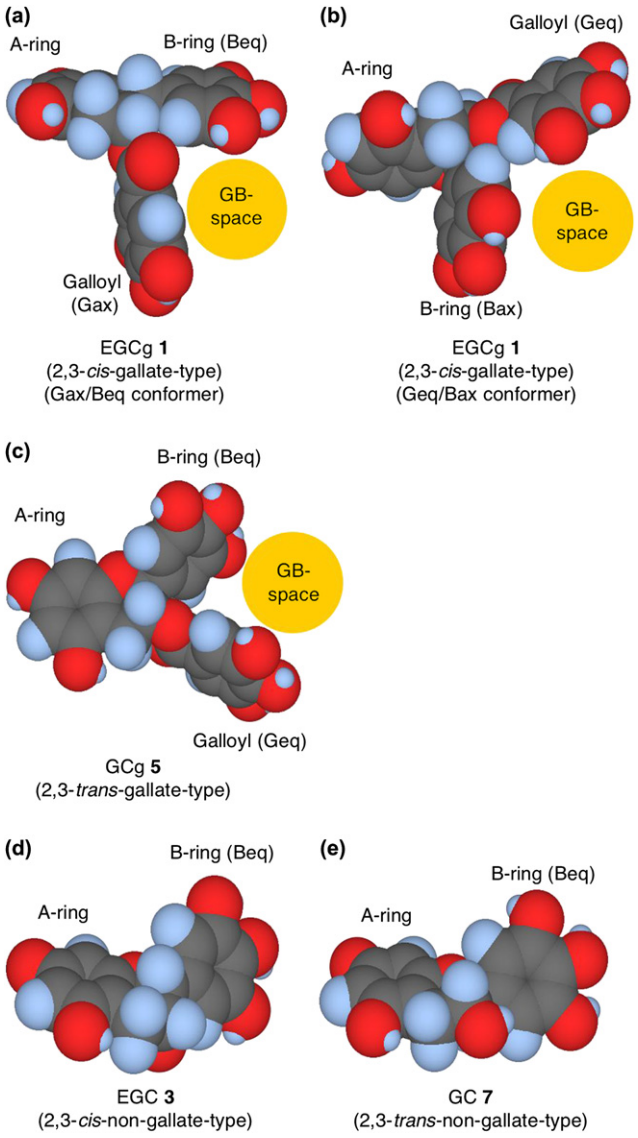


Figure 6. The conformers of the catechins and the proposed binding site for the quaternary ammonium ion (GB-space).

catechins also skillfully stabilize complexes with quaternary ammonium ions by using dual non-covalent interactions. This attractive molecular recognition of the gallate-type catechins is believed to play a significant role in biochemical processes.

4. Experimental

4.1. General

All chemicals were obtained from commercial suppliers and used without further purification. EGCg **1**, GCg **5**, and C **8** were purchased from Nagara Science. Co., Ltd. (Gifu, Japan). ECg **2**, EGC **3**, EC **4**, Cg **6**, GC **7**, and BMAC **10** were purchased from Wako Pure Chem. Ind. Ltd. (Osaka, Japan). 5-Methoxyresorcinol **11** was purchased from Sigma–Aldrich Japan Co., Ltd. (Tokyo, Japan). TMAC **9**, pyrogallol **12**, pyrocatechol **13**, and methyl gallate **14** were purchased from Tokyo Chem. Ind. Ltd. (Tokyo, Japan). NMR spectra were obtained on a JEOL JNM-LA 500 spectrometer (500 MHz). The chemical shifts were recorded with tetramethylsilane (δ=0.0000 ppm) in carbon tetrachloride as an external standard, which was inserted into an NMR tube (ϕ=5 mm) with a coaxial cell. The digital resolution of the ^{1}H NMR spectra was 0.31 Hz.

4.1.1. ^{1}H NMR titration experiments. Typical procedure for addition of the phenolic compounds (1, 2, 3, 4, 5, 6, 7, 8, 11, 12, 13, and 14) to tetraalkylammonium chlorides (9 and 10). Stock solutions of the phenolic compounds (4.28 mM) and the tetraalkylammonium chloride (5.50 mM) in acetonitrile-d_3/chloroform-d (1:1) were separately prepared. Thirteen different NMR tubes were prepared with concentrations of the phenolic compounds at 0.000, 0.175, 0.350, 0.525, 0.700, 1.05, 1.40, 1.75, 2.10, 2.45, 2.80, 3.15, and 3.50 mM against a constant concentration of the tetraalkylammonium chloride (1.00 mM) for a 0.550 mL total solution. ^{1}H NMR spectra were obtained on each sample at 300 K. $\Delta\delta_{obs}^{amm}$ (ppm) was plotted against the total concentration (mM) of polyphenols. The fitting curves for the plots were calculated by non-linear least-squares regression procedures according to Eq. 1 using a KaleidaGraph® (Synergy Software) to obtain the binding constants (K_b).[49]

4.1.2. ^{1}H NMR titration experiments. Typical procedure for addition of BMAC 10 to the catechins (1, 2, 3, 4, 5, 6, 7, and 8). Stock solutions of **10** (4.28 mM) and the catechins (5.50 mM) in acetonitrile-d_3/chloroform-d (1:1) were separately prepared. Thirteen different NMR tubes were prepared with concentrations of **10** at 0.000, 0.175, 0.350, 0.525, 0.700, 1.05, 1.40, 1.75, 2.10, 2.45, 2.80, 3.15, and 3.50 mM against a constant concentration of the catechins (1.00 mM) for a 0.550 mL total solution. ^{1}H NMR spectra were obtained on each sample at 300 K. $\Delta\delta_{obs}^{catechin}$ (ppm) was plotted against the total concentration (mM) of **10**.

4.1.3. ^{1}H NMR chemical shift changes in the gallate-type catechins (1, 2, 5, and 6) by the addition of TMAC 9. Stock solutions of **9** (7.00 mM) and the gallate-type catechins (2.00 mM) in acetonitrile-d_3/chloroform-d (1:1) were separately prepared. Two NMR tubes for each catechin were prepared with 3.50 mM of **9** and 1.00 mM of the catechins for a 0.550 mL total solution. ^{1}H NMR spectra were obtained on each sample at 300 K.

4.2. NOESY experiment

Solutions in acetonitrile-d_3/chloroform-d (1:1) (0.550 mL) including the catechins (2.20 μmol) and BMAC **10** (2.20 μmol) were prepared. NOESY spectra using pulse field gradients were acquired at 300 K under the following conditions: relaxation delay of 3.0 s, mixing time of 250 ms, 16 scans, 256×256 data points. The data were processed with a sine-bell window function.

Acknowledgements

This work was financially supported by the NIVTS priority research program.

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