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Analysis of reaction products of astaxanthin and its acetate with reactive oxygen species using LC/PDA ESI-MS and ESR spectrometry

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ABSTRACT

Reaction products of astaxanthin and its acetate with hydroxy radical, superoxide anion radical, and singlet oxygen were analyzed by LC/PDA ESI-MS and ESR spectrometry. Astaxanthin epoxides were found to be major reaction products of astaxanthin with superoxide anion radical and hydroxyl radical. On the other hand, astaxanthin endoperoxides were identified as major reaction products of astaxanthin with singlet oxygen. The same results were obtained in the case of astaxanthin acetate. The ESR study revealed that astaxanthin and its acetate take up superoxide anion radical by the formation of their epoxide through peroxide and oxide radicals.

Astaxanthin, which is widely distributed in marine bacteria, green algae, crustaceans, fish, and birds,[1] is an excellent antioxidant not only for quenching singlet oxygen but also for lipid peroxidation.[2–4] Recently, Hama et al. reported that astaxanthin could scavenge hydroxyl radical in a liposome system.[5] Furthermore, it was reported that astaxanthin could take up peroxynitrite into its molecule by the formation of nitroastaxanthin.[6,7] However, there are few reports on the reaction products of astaxanthin with reactive oxygen species. In order to elucidate the chemical scavenging mechanism of astaxanthin with reactive oxygen species, we investigated reaction products of astaxanthin and its acetate with hydroxyl radical (·OH), superoxide anion radical ($\cdot O_2^-$), and singlet oxygen ($^1O_2$) using both LC/PDA ESI-MS and ESR spectrometry. This Letter reports the reaction products of astaxanthin and its acetate with reactive oxygen species and these reaction mechanisms.

Hydroxyl radical, $\cdot O_2^-$, and $^1O_2$ were generated by UV-A irradiation of hematoporphyrin, riboflavin, and hydrogen peroxide (H_2O_2) solution, respectively.[8] An acetonitrile (CH_3CN) solution of astaxanthin or its acetate was added to these reactive oxygen species-generation systems. At regular intervals of UV-A irradiation, the reaction products were analyzed by LC/PDA ESI-MS[8] and ESR spin-trapping.[9]

In the case of the reaction of astaxanthin acetate with hydroxyl radical, reaction products detected by HPLC conditions shown in Figure 1 were increased with UV-A irradiation time-dependently. The amount of reaction products reached a maximum with 6 min of UV-A irradiation. Then, astaxanthin acetate and oxidation products were markedly decreased and the reaction solution was bleached. Figure 1A shows the HPLC profiles of the reaction product of astaxanthin acetate with hydroxyl radical with 6 min of UV-A irradiation. Three reaction products (Peaks 1–3) were detected on HPLC. They were assigned to astaxanthin acetate triepoxide,[10] astaxanthin acetate 5,8-epoxide (or 7,8-epoxide),[11] and astaxanthin acetate 5,6-epoxide[12] based on the MS, MS/MS, and UV–Vis spectral data. ESI-TOF MS of Peak 3[12] showed molecular formula of $C_{44}H_{56}O_7$. This formula clearly indicated that one oxygen atom was attached to astaxanthin acetate. Its UV–Vis showed an absorption maximum at 452 nm, consisted with the loss of two conjugated double bonds from 13 conjugated double bonds of astaxanthin acetate, which shows absorption maximum at 478 nm.[13] The MS/MS spectrum of sodium adduct ions $[M+Na]^+$ of this compound showed *m/z* 627 $[M+Na-92]^+$ (elimination of toluene moiety from polyene chain) and *m/z* 613 $[M+Na-106]^+$ (elimination of xylene moiety from polyene chain), which were diagnostic fragments in EI and ESI MS of carotenoids,[14,15] along with *m/z* 659 $[M+Na-AcOH]^+$ and *m/z* 599 $[M+Na-2AcOH]^+$. These spectral data indicated that an oxygen atom was attached to the 5,6-double bond and formed an epoxide ring in astaxanthin

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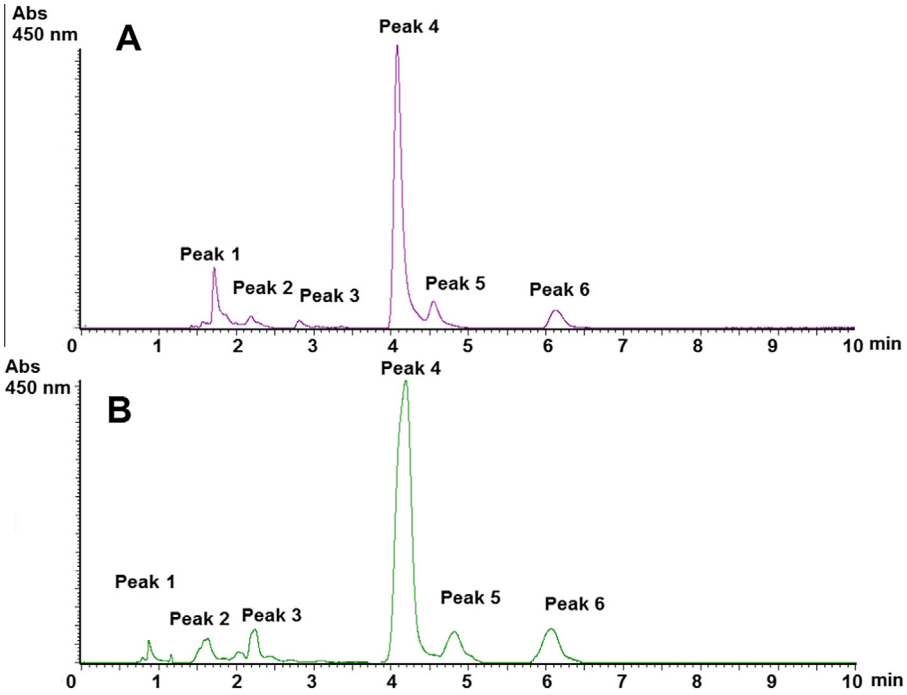

Figure 1. (A) HPLC of reaction products of astaxanthin acetate with ·OH for 6 min. Peak 1: astaxanthin acetate tri-epoxide, Peak 2: astaxanthin acetate 5,8-epoxide (or 7,8-epoxide), Peak 3: astaxanthin acetate 5,6-epoxide, Peak 4: astaxanthin acetate, Peaks 5 and 6: astaxanthin acetate *cis* isomers. (B) HPLC of reaction products of astaxanthin acetate with $^1O_2$ for 6 min. Peak 1: hematoporphyrin, Peak 2: astaxanthin acetate 5,6-endoperoxide, Peak 3: astaxanthin acetate 5,8-endoperoxide, Peak 4: astaxanthin acetate, Peaks 5 and 6: astaxanthin acetate *cis* isomers. HPLC condition ACQUITY UPLC system (Waters), column:BEH Shield RP18 (1.7 μm, 2.1 × 100 mm), mobile phase: MeOH, column temperature: 40 °C, flow rate: 0.2 ml/min, detection: 450 nm.

acetate. Peak 2[11] has the same molecular formula ($C_{44}H_{56}O_7$) as that of astaxanthin acetate-5,6-epoxide. Its UV–Vis showed an absorption maximum at 430 nm. The hypsochromic shift of the UV–Vis spectrum of 22 nm from astaxanthin acetate 5,6-epoxide indicated that this compound lacked one conjugated double bond (double bond C7 to C8) from astaxanthin acetate 5,6-epoxide.[13] Therefore, the structure of astaxanthin acetate 5,8-epoxide or 7,8-epoxide was postulated for this compound. ESI-TOF MS of Peak 1[10] showed the molecular formula of $C_{44}H_{56}O_9$. Its UV–Vis showed an absorption maximum at 400–424 nm. These data were compatible with the structure of astaxanthin acetate tri-epoxide. However, substituted positions of oxygen atoms could not be specified from these spectral data. Astaxanthin acetate 5,6-epoxide, 5,8-epoxide (or 7,8-epoxide), and tri-epoxide were also formed as major reaction products of astaxanthin acetate with $\cdot O_2^-$.

In the case of the reaction of astaxanthin acetate with $^1O_2$, astaxanthin acetate 5,6-endoperoxide[16] was obtained as major reaction product (Fig. 1B). This compound showed the molecular formula of $C_{44}H_{56}O_8$, which indicated that two oxygen atoms were attached to astaxanthin acetate. This compound showed the same UV–Vis spectrum as that of astaxanthin acetate 5,6-epoxide. These spectral data indicated that the dioxetane moiety was attached to C-5 and C-6 positions in astaxanthin acetate. MS/MS spectral data also support this structure. Furthermore, astaxanthin acetate-5,8-endoperoxide[17] was also detected. This compound showed the same MS spectral data as those of astaxanthin acetate-5,6-endoperoxide except for the hypsochromic shift of the UV–Vis spectrum of 22 nm from astaxanthin acetate 5,6-endoperoxide. These data are in agreement with the structure of astaxanthin acetate-5,8-endoperoxide for this compound. The structure of astaxanthin acetate-7,8-endoperoxide was also considered for this compound.

Similar results as those for astaxanthin acetate were obtained in the case of free-form astaxanthin with these reactive oxygen species. Namely, astaxanthin epoxides were obtained by the reaction

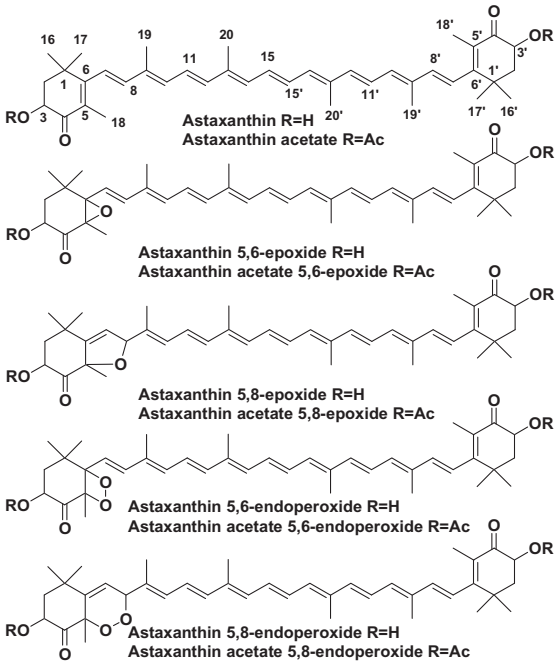

Figure 2. Structures of astaxanthin and its acetate and their major reaction products with reactive oxygen species.

of ·OH and $\cdot O_2^-$ and astaxanthin endoperoxide were produced by the reaction with $^1O_2$. It was found that oxidation products of astaxanthin acetate were more stable than those of free astaxanthin. It was assumed that acetylation might protect the oxidation of hydroxy groups of astaxanthin from reactive oxygen species and inhibit the rapid degradation of the astaxanthin molecule.

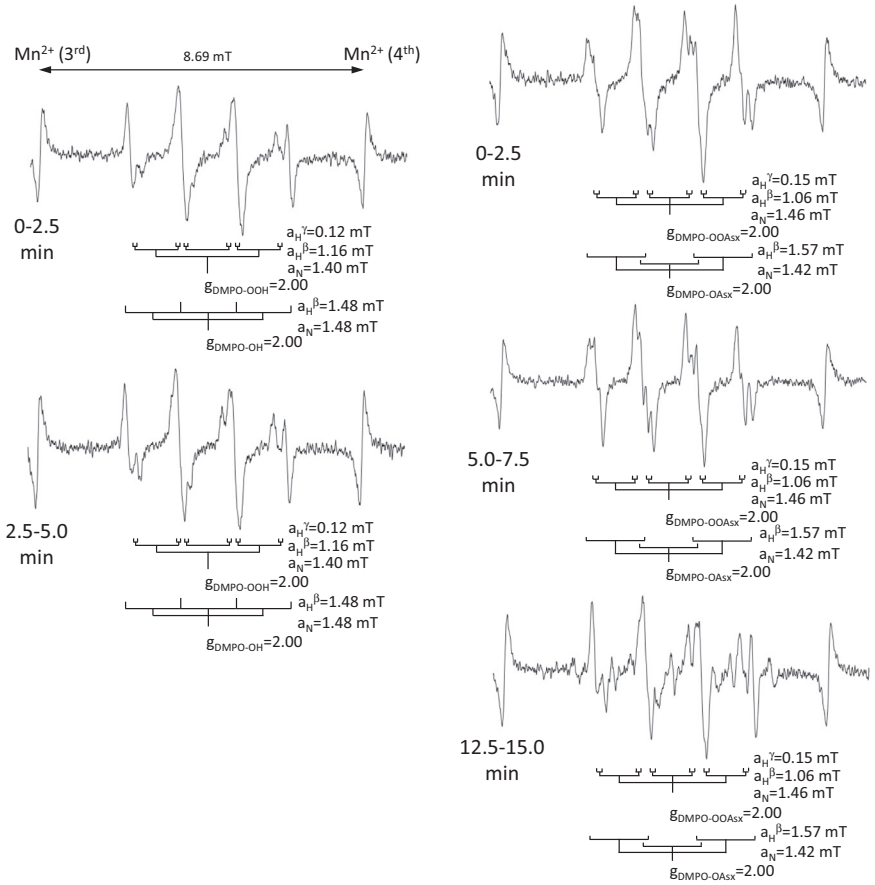

Figure 3. ESR spectra due to 8 μg/mL of astxanthin acetate in acetonitrile (100 μL), 0.025 mM of riboflavin solution (100 μL), and 250 mM of 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) (10 μL). After 2.5 min of UV-A irradiation (upper), after 7.5 min of UV-A irradiation (middle), after 15 min of UV-A irradiation (lower). ESR spectra due to both the DMPO-OOAsx (astaxanthin acetate-OO·) and DMPO-OAsx (astaxanthin acetate-O·) were observed. Initially, ESR signal of DMPO-OOAsx was dominant, and time-dependently ESR signal of DMPO-OAsx was increased. On the other hand, only ESR spectra due to both the DMPO-OOH and DMPO-OH were observed without astxanthin acetate, indicating the formation of $\cdot O_2^-$ followed by ·OH in the control RF/UV-A system. Left column: control (riboflavin+DMPO), Right column: riboflavin+astaxanthin acetate+DMPO.

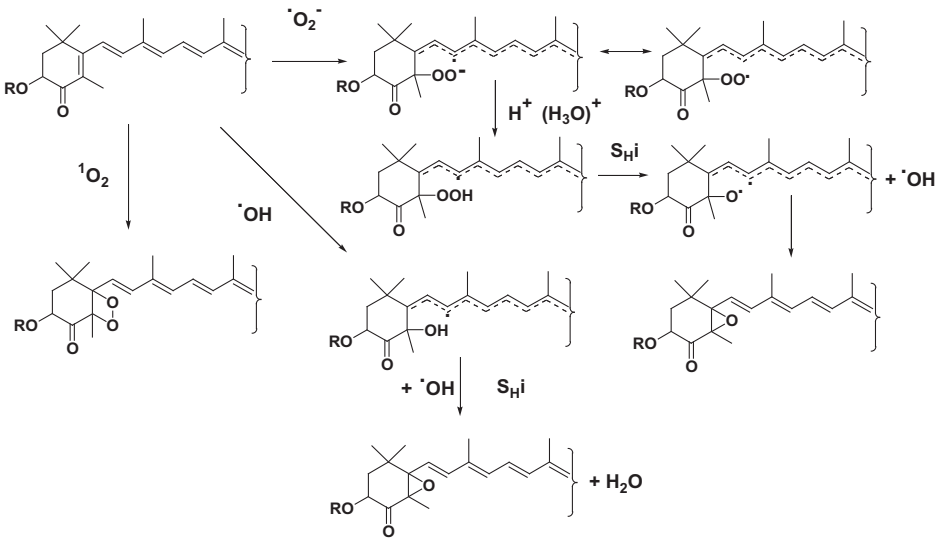

Figure 4. Reaction schemes of astaxanthin and its acetate with reactive oxygen species. R = H astaxanthin, R = Ac astaxanthin acetate. S_Hi; intra-molecular homolytic substitution reaction.

Structures of astaxanthin and its acetate and their major reaction products with reactive oxygen species are shown in Figure 2.

In order to reveal the reaction mechanism of astaxanthin and its acetate with these reactive oxygen species, these reactions were monitored with the ESR spin-trapping method.[9] Astaxanthin acetate peroxide radical (AsxOO·) and astaxanthin acetate oxide radical (AsxO·) were detected during the reaction of astaxanthin acetate with $\cdot O_2^-$ by the ESR spin-trapping method, as shown in Figure 3. Namely, a DMPO-OOAsx signal was observed in ESR for the 0–2.5 min reaction of astaxanthin acetate with $\cdot O_2^-$. The intensity of the DMPO-OOAsx signal reached it maximum with a 5 min reaction. Subsequently, the DMPO-OOAsx signal was decreased and DMPO-OAsx signal was observed for the 12.5–15.0 min reaction, as shown in Figure 3. These results indicate that astaxanthin acetate takes up $\cdot O_2^-$ together with the formation of corresponding peroxy radical. Subsequently, astaxanthin acetate alkoxy radical (AsxO·) was formed with the production of ·OH from peroxy radical. Eventually, epoxide was formed from alkoxy radical, as shown in Figure 4. The ESR spectrum showed that astaxanthin (acetate) scavenged ·OH directly. Although signals of alkoxy and/or peroxy radicals were not observed in ESR spin-trapping during the reaction of astaxanthin (acetate) with ·OH, the formation of epoxide by this reaction could be considered as shown in Figure 4. Namely, an ·OH radical was attached at C-5 of astaxanthin (acetate). Then, an epoxide ring was formed by the intra-molecular homolytic substitution reaction.

It was reported that carotenoids inhibit $^1O_2$ through a physical quench[18] and chemical reaction.[19] For example, β-carotene reacted with $^1O_2$ and formed β-carotene-endoperoxide and apo-β-carotenals.[19] In the present study, we found that astaxanthin and its acetate take up $^1O_2$ by the formation of endoperoxide. On the other hand, astaxanthin and its acetate take up $\cdot O_2^-$ by the formation of epoxide through astaxanthin peroxide radical as shown in Figure 4. Because of their unstability, there are few reports on astaxanthin endoperoxides and astaxanthin epoxides. Recently, Weesepoel et al., reported that epoxy apo-9- and epoxy-apo-13-palmitoyl astaxanthinone were the formed from palmitoyl astaxanthin with reaction of hypochlorous acid/hypochlorite ($HOCl/OCl^-$).[20] In the present study, we could detect astaxanthin endoperoxides, astaxanthin epoxides, and astaxanthin peroxy and alkoxy radicals as reaction products of reactive oxygen species by LC/PDA ESI MS and the ESR method. This is the first Letter on the detection of astaxanthin (its acetate) endoperoxides, epoxides, and peroxy and alkoxy radicals.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.tetlet.2016.03.078.

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8. After the addition of 200 μL of 8 mM H_2O_2 solution to 200 μL of 70 μg/mL of astaxanthin or astaxanthin-diacetate CH_3CN solution, ·OH was generated by UV-A irradiation using SUPERCURE-203S (SAN-Ei ELECTORIC) at room temperature. In a similar manner to that described above, $\cdot O_2^-$ and $^1O_2$ were generated by the addition of 200 μL of 0.025 mM of riboflavin and 200 μL of 0.25 mM hematoporphyrin, respectively, to 200 μL of 70 μg/mL of astaxanthin or astaxanthin-diacetate CH_3CN solution, with UV-A irradiation at room temperature. At regular intervals of UV-A irradiation, reaction products were analyzed by LC/PDA-ESI-MS. The electro-spray ionization (ESI) time-of-flight (TOF) MS spectra were acquired by scanning from *m/z* 100 to 1500 with a capillary voltage of 3.2 kV, cone voltage of 40 eV, and source temperature of 120 °C. Nitrogen was used as a nebulizing gas at a flow rate of 30 L/h. MS/MS spectra were measured with a quadrupole-TOF MS/MS instrument with argon as a collision gas at a collision energy of 30 V. UV–Visible (UV–Vis) absorption spectra were recorded from 200 to 600 nm using a photodiode-array detector (PDA). An Acquity 1.7 μm BEH UPLC C18 column (Waters Corporation) was used as a stationary phase and 90% MeOH as a mobile phase, at a flow rate of 0.2 mL/min for the HPLC system.
9. ESR spectra were recorded at room temperature on a JEOL JES-RFR30 spectrometer (Tokyo, Japan) using an aqueous quartz flat cell (Labotec, Tokyo, Japan). 5,5-Dimethoxy-1-pyrroline *N*-oxide (DMPO) was used as hydroxyl radical and superoxide anion radicals-trapping agent and 2,2,6,6-tetramethyl-4-piperidone (TMPD) was used as a singlet oxygen-trapping agent. Hydroxyl radical or $\cdot O_2^-$ were generated by addition of both the 100 μL of 8 mM H_2O_2 or 100 μL of 0.025 mM of riboflavin solution and 10 μL of 250 mM DMPO to 100 μL of 8.8 μg/mL astaxanthin or 7 μg/mL astaxanthin acetate CH_3CN solution. In a similar manner to that described above, $^1O_2$ was generated by the addition of both the 100 μL of 0.25 mM hematoporphyrin and 10 μL of 500 mM TMPD to 100 μL of 8.8 μg/mL astaxanthin or 7 μg/mL astaxanthin acetate CH_3CN solution. ESR spectrum measurements started after UVA irradiation. All spin-trapped ESR spectra were monitored between the third and fourth signals from the low magnetic field due to the external standard, Mn(II) doped MnO.
10. Astaxanthin acetate tri-epoxide, ESI TOF MS *m/z* 751.3843 ($M+Na^+$, calcd for $C_{44}H_{56}O_9Na$, 751.3822), *m/z* 729.4012 ($M+H^+$, calcd for $C_{44}H_{57}O_9$, 729.4003); Product ions of MS/MS (precursor ion $M+Na^+$) *m/z* 691 (M+Na-AcOH), 659 M +Na-toluene), 645 (M+Na-xylene), 631 (M+Na-2AcOH); UV–Vis 400–424 nm (in 90% MeOH).
11. Astaxanthin acetate 5,8-epoxide (or 7,8-epoxide), ESI TOF MS *m/z* 719.3909 ($M+Na^+$, calcd for $C_{44}H_{56}O_7Na$, 719.3924), *m/z* 697.4113 ($M+H^+$, calcd for $C_{44}H_{57}O_7$, 697.4104); Product ions of MS/MS (precursor ion $M+Na^+$) *m/z* 659 (M+Na-AcOH), 627 M+Na-toluene), 613 (M+Na-xylene), 599 (M+Na-2AcOH); UV–Vis 430 nm (in 90% MeOH).
12. Astaxanthin acetate 5,6-epoxide, ESI TOF MS *m/z* 719.3909 ($M+Na^+$, calcd for $C_{44}H_{56}O_7Na$, 719.3924), *m/z* 697.4113 ($M+H^+$, calcd for $C_{44}H_{57}O_7$, 697.4104); Product ions of MS/MS (precursor ion $M+Na^+$) *m/z* 659 (M+Na-AcOH), 627 M +Na-toluene), 613 (M+Na-xylene), 599 (M+Na-2AcOH); UV–Vis 452 nm (in 90% MeOH).
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16. Astaxanthin acetate 5,6 endperoxide, ESI TOF MS *m/z* 735.3866 ($M+Na^+$, calcd for $C_{44}H_{56}O_8Na$, 735.3873), *m/z* 713.4024 ($M+H^+$, calcd for $C_{44}H_{57}O_8$, 713.4053); Product ions of MS/MS (precursor ion $M+Na^+$) *m/z* 675 (M+Na-AcOH), 643 M+Na-toluene), 629 (M+Na-xylene), 615 (M+Na-2AcOH); UV–Vis 452 nm (in 90% MeOH).
17. Astaxanthin acetate 5,8 endperoxide, ESI TOF MS *m/z* 735.3866 ($M+Na^+$, calcd for $C_{44}H_{56}O_8Na$, 735.3873), *m/z* 713.4024 ($M+H^+$, calcd for $C_{44}H_{57}O_8$, 713.4053); Product ions of MS/MS (precursor ion $M+Na^+$) *m/z* 675 (M+Na-AcOH), 643 M+Na-toluene), 629 (M+Na-xylene), 615 (M+Na-2AcOH); UV–Vis 430 nm (in 90% MeOH).
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