Pharmacokinetic Study of Allixin, a Phytoalexin Produced by Garlic

Yukihiro Kodera,*a Makoto Ichikawa,a Jiro Yoshida,a Naoki Kashimoto,a Naoto Uda,a Isao Sumioka,a Nagatoshi Ide,a and Kazuhisa Onob
Healthcare Institute, Wakunaga Pharmaceutical Co., Ltd.,a 1624 Shimokotachi, Koda-cho, Takatagun, Hiroshima 739–1195, Japan and Department of Molecular Biotechnology, Graduate School of Advanced Science of Matter, Hiroshima University,b 1–3–1 Kagamiyama, Higashihiroshima, Hiroshima 739–8526, Japan.
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The pharmacokinetic behavior of allixin (3-hydroxy-5-methoxy-6-methyl-2-pentyl-4H-pyran-4-one) was investigated in an experimental animal, mice. Allixin was administered using an inclusion compound because the solubility of allixin in aqueous solution is very low. The allixin content in serum and in the organs of administered animals was analyzed by liquid chromatography (LC)-MS. Most of the administered allixin disappeared within 2 h, and the bioavailability of allixin was estimated to be 31% by obtained area under the blood concentration–time curve (AUC). The metabolites of allixin were studied using the metabolic enzyme fraction of liver and liver homogenate. Several new peaks corresponding to allixin metabolites were observed in the HPLC chromatographic profile. The chemical structure of the metabolites was investigated using LC-MS and NMR. Three of them were identified as allixin metabolites having a hydroxylated pentyl group.

Key words allixin; pharmacokinetic; metabolism; inclusion compound; phytoalexin

Intact constituents or transformation compounds derived from garlic (Allium sativum L.) have been investigated for more than 60 years, and numerous compounds have been isolated and investigated to reveal the relationship between the compounds and biological activities of garlic.1,2) Sulfur-containing compounds, such as allicin, diallylsulfides, dithiins, alliin and S-allylcysteine, have been well investigated by numerous researchers because garlic is rich in sulfur and its sulfur containing compounds were believed to be responsible for many biological activities of garlic.1,2) S-Allylcysteine has been investigated comprehensively, including its physical, chemical and biological properties.1—6) On the other hand, other compounds such as saponins, proteins or maillard reaction products derived from garlic, which are non-sulfur containing compounds, have also been investigated to reveal the biological properties of garlic.1,2,7—10)

Allixin (3-hydroxy-5-methoxy-6-methyl-2-pentyl-4H-pyran-4-one), a non-sulfur containing compound having a γ-pyroneskeleton structure (Fig. 1), was the first compound isolated from garlic as a phytoalexin, a product induced in plants by continuous stress.11) This compound has been shown to have unique biological properties, such as anti-oxidative effects,11) anti-microbial effects,11) anti-tumor promoting effects,12) inhibition of aflatoxin B2 DNA binding,13) and neurotrophic effects.14) Allixin showed an anti-tumor promoting effect in vivo, inhibiting skin tumor formation by TPA in DMBA initiated mice.12) Analogs of this compound have exhibited anti tumor promoting effects in in vitro experimental conditions.15) Herein, allixin and/or its analogs may be expected useful compounds for cancer prevention or chemotherapy agents for other diseases.

Analysis of the biological mechanism of action of allixin is important for the study of cancer prevention or other biological properties, and also for drug development. Nishino et al. has reported that allixin interacts with the Ca2+-calmodulin complex.12) This in vitro study suggests that allixin may play an important role in signal transduction or regulation. However, the pharmacokinetic behavior of this compound has not been evaluated, and this is an important factor for in vivo studies. Therefore, this paper presents the pharmacokinetic behavior and identified metabolites of allixin.

Result
Preparation of Allixin and Allixin-d11 Allixin and allixin-d11 were prepared as previously reported.16) The chemical structure of the obtained materials were confirmed by NMR and MS comparison with previous reporteds.11,16) Synthesized allixin-d11 was used as the internal standard for the analysis of allixin content in blood samples. The yield of allixin and allixin-d11 was 20% and 9%, respectively. Here, all
protons in the n-pentyl group of allixin were replaced with deuterium on the synthesis of allixin-d_{11} using 1-bromopen-tane-d_{11}. Allixin: Mass; [M+H]^+= 227, ^1H-NMR (CDCl₃): δ: 0.91 (3H, t, J=6.96), 1.34 (4H, m), 1.61 (4H, m), 2.34 (3H, s, –Me), 3.89 (3H, s, –OMe), 6.1—6.35 (1H, br, –OH), ^13C-NMR (CDCl₃): δ: 13.94 (C-5’), 15.06 (–Me), 22.33 (C-4’), 26.39 (C-1’), 28.31 (C-2’), 31.26 (C-3’), 60.14 (–OMe), 141.84 (C-3, C-5), 150.15 (C-2), 158.09 (C-6), 169.48 (C=O). Allixin-d_{11}: Mass; [M+H]^+= 238, ^1H-NMR (CDCl₃): δ: 6.7—6.9 (H, –OH), 3.85 (s, 3H, –OCH₃), 2.35 (s, 3H, –CH₃), ^13C-NMR (CDCl₃): δ: 17—18 (m), 19.5 (–Me), 25.2—26.2 (m), 29.6—32 (m), 31.6—32.4 (m), 34.2—35 (m), 60.0 (–OMe), 142 (C-3, C5), 151 (C-2), 158 (C-6), 169 (C=O).

Measurement of the Stability Constant of the Complex Compound  
Figure 2 showed phase solubility of allixin with maltosyl-β-cyclodextrin (M-β-Cyd). A linear relationship was observed between the concentration of allixin and M-β-Cyd from the test of solubility in water (r=0.999). This result indicated that the phase solubility diagram of allixin and M-β-Cyd was an AL type in water. The stability constant, K (M⁻¹), in water was 301, according to Eq. 1 and obtained S (=0.2291) and S₀ (=0.9886 M).^{17}

NMR Analysis of Inclusion Complex Compound  
Table 1 and Fig. 3 show the results of ^1H-NMR measurement of intact allixin and allixin in the inclusion complex compound. Each signal relating to allixin in the inclusion compound was observed at almost the original region of the intact allixin signals. However, all of the chemical shifts of the complex corresponding to allixin migrated 0.03—0.09 ppm down field from the signals of intact allixin. It was also observed that signals of M-β-Cyd were changed on the spectrum of the complex. These observations strongly indicated that the methyl, methoxyl and pentyl group of allixin interacted with the M-β-Cyd molecular structure, and that these groups might be inside the M-β-Cyd cavity (see Fig. 4).

Behavior of an Allixin Inclusion Compound in Bovine Serum Albumin (BSA) Solution and the Blood/Serum of the Experimental Animal  
Table 2 shows the results of allixin recovery in a mixture of allixin inclusion compound solution, and simulated serum and serum. Recovery with a filter unit was not more than 2% in each tested condition. Furthermore, no insoluble substance or crystalline substance observed in any ultrafiltrate; they remained as solution in each condition. The content of allixin in the mixture of whole blood and allixin solution was constant for each incubation time (data not shown).

Pharmacokinetic Study and Distribution of Allixin in Organs  
Figure 5 shows the mass chromatoprofile of selected ion monitoring and the calibration curve for allixin analysis. Allixin and internal standard allixin-d_{11} were eluted at the same time. Obtained equations showed a linear line, and the range of regression coefficient was 0.986 to 0.999.

<table>
<thead>
<tr>
<th>Group in allixin</th>
<th>Alone (ppm)</th>
<th>Complex (ppm)</th>
<th>Δδ (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pentyl 1’</td>
<td>2.751</td>
<td>2.805</td>
<td>0.054</td>
</tr>
<tr>
<td>2’</td>
<td>1.703</td>
<td>1.766</td>
<td>0.063</td>
</tr>
<tr>
<td>3’, 4’</td>
<td>1.332</td>
<td>1.387</td>
<td>0.055</td>
</tr>
<tr>
<td>5’</td>
<td>0.885</td>
<td>0.921</td>
<td>0.036</td>
</tr>
<tr>
<td>O-Methoxy</td>
<td>3.791</td>
<td>3.835</td>
<td>0.044</td>
</tr>
<tr>
<td>Methyl</td>
<td>2.407</td>
<td>2.498</td>
<td>0.091</td>
</tr>
</tbody>
</table>
Figure 6 shows allixin concentration–time profiles in mice serum after peroral and intravenous administration, respectively. Table 3 shows pharmacokinetic parameters for allixin administration in mice. Although a decrease in spontaneous motor activities was observed in the general state observation in the intravenous administration group, but not in the peroral administration group, this sign disappeared at around 30 s to 1 min after administration. In orally administered mice, allixin concentration in the serum had already reached the maximum level ($C_{\text{max}}$: 2.54 ± 1.89 µg/mL) at 5 min ($T_{\text{max}}$: 5 min) after administration, and the half-life ($t_{1/2b}$) of allixin was 66.5 ± 25.7 min. The concentration of allixin in serum quickly decreased by 10 min following intravenous adminis-

<table>
<thead>
<tr>
<th>Tested material</th>
<th>Incubation time (min)</th>
<th>Filter unit</th>
<th>Ultrafiltrate</th>
<th>Remaining solution</th>
<th>Corrected with recovered volume $^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Filter unit$^a$</td>
<td>0.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BSA solution</td>
<td>5</td>
<td>1.1</td>
<td>4.0</td>
<td>70</td>
<td>82</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>1.1</td>
<td>4.7</td>
<td>64</td>
<td>80</td>
</tr>
<tr>
<td>Filter unit$^a$</td>
<td>0.8</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum</td>
<td>5</td>
<td>0.8</td>
<td>8.8</td>
<td>69</td>
<td>92</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>1.3</td>
<td>10.5</td>
<td>68</td>
<td>94</td>
</tr>
</tbody>
</table>

$^a$ Amount of allixin trapped by filter unit in the experiment without BSA solution or serum. $^b$ sum of recovery, corrected with recovered volume of ultrafiltrate or remaining solution.

Table 3. Pharmacokinetic Parameters for Allixin Administration in Mice

<table>
<thead>
<tr>
<th>Route</th>
<th>$T_{\text{max}}$ (min)</th>
<th>$C_{\text{max}}$ (µg/mL)</th>
<th>$t_{1/2b}$ (min)</th>
<th>$AUC$ (µg min/mL)</th>
<th>$MRT$ (min)</th>
<th>Bioavailability (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>i.v.</td>
<td>43.0 ± 6.9</td>
<td>375 ± 45</td>
<td>13.9 ± 3.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p.o.</td>
<td>5</td>
<td>2.54 ± 1.89</td>
<td>66.5 ± 25.7</td>
<td>117 ± 49</td>
<td>33.1 ± 13.1</td>
<td>31</td>
</tr>
</tbody>
</table>

Allixin was administered at a dose of 50 mg/kg using an inclusion compound in which the allixin content in inclusion compound was 51.2 mg/g. Values in the table are mean ± S.E. $n=5$. 

![Figure 4. Illustration of the Hypothetical Structure of an Inclusion Compound with Allixin and Maltosyl-β-cyclodextrin](image)

![Figure 5. Mass Chromatoprofile of Selected Ion Monitoring and Calibration Curve for Allixin Analysis](image)

(a) Relationship between injected amount of standard allixin and peak area ratio. Obtained equation and regression coefficient on Fig. 5a was $Y = 0.09267 + 0.005691 \cdot X$, $r = 0.9946$, respectively. (b) Chromatoprofile of standard allixin at injection of 200 ng of allixin. (c) chromatoprofile of internal standard allixin-$d_4$ at injection of 200 ng of allixin-$d_4$. Each value in Fig. 5a represents the mean ± S.E. of 3 measurements.

![Figure 6. Allixin a Concentration–Time Profile in Mice Serum after Peroral and Intravenous Administration at a Dose of 50 mg/kg](image)

(a) Profile of peroral administration, (b) profile of intravenous administration. Each value represents the mean ± S.E. of 5 animals.
tation, and the half-life $t_{1/2B}$ was 43.0±6.9 min. Table 3 shows the distribution of allixin in mice organs. The ratios of allixin distribution in the organs against serum concentration (T/S ratio) were investigated, and the T/S ratio of the lung was higher than for other organs at one minute after intra-venous administration. The T/S ratio of organs related to metabolism and excretion, i.e. the liver and kidney, increased within 10 min, then these ratios gradually decreased over time in both administrations, except in the liver with intra-venous administration. The bioavailability of allixin in mice after peroral administration was estimated to be 31% using an inclusion compound with M-β-Cyd.

**Treatment of Allixin with Liver Homogenate and S-9 Mix**

This quantitative analysis was not accurate because it is difficult to synthesize each of the allixin metabolites. Then, 3-hydroxy-2,6-dimethyl-4H-pyran-4-one was used as the standard for quantitative analysis of allixin metabolites. The obtained sample solution was analyzed under the following conditions: column: TSK gel ODS 80TM (4.6 mm×150 mm), solvent: gradient with mixture of water and methanol (60:40) and mixture of methanol and water (90:10), detection: UV 280 nm, flow: 0.6 ml/min. Each value represents the mean±S.E. of 3 samples.

**Identification of Metabolites of Allixin**

The fractions corresponding to these new peaks were recovered by HPLC, and structure analysis was performed using MS and NMR equipment. Figure 10 shows the mass spectra of allixin and the allixin metabolite corresponding to P-5. The peak corresponding to P-2 to P-5 gave protonated molecular ions and fragment ions: $[M+H]^{+} = 243$ and $[M+H-16]^{+} = 225$, respectively, on liquid chromatography (LC)-MS analysis for each isolated fraction. $m/z = 255$ was also observed on the MS/MS function mode of MS analysis. This indicates the addition of oxygen to allixin, since the ion of 243 is 16 mass different from the ion of allixin ($[M+H]^{+} = 227$), and the ion of 225 indicates the ion of $[M+H-H_2O]^{+} = [242+1-18]^{+}$. The signal corresponding to the C-4′ bonded proton of allixin was not observed on the obtained NMR spectrum of P-2, but a new signal was observed at 3.842 ppm. Cross peak signals of protons corresponding to position C-3′ (1.724 ppm)/C-4′ (3.842 ppm) and position C-4′ (3.842 ppm)/C-5′ (1.155 ppm) were observed on $^{1}H$-H correlation spectroscopy (COSY) analysis of P-2. These results from P-2 indicate that the C-4′ position on the pentyl group of allixin would have a hydroxyl group due to an oxidative reaction.
UV spectra of P-2 to P-5 were similar to allixin, but the maximum absorption was slightly shifted to the short wavelength side (Fig. 11). These metabolites would be analogue of allixin. The UV spectrum of P-8 differed from allixin and the peaks of P-2 to P-5, and the peak corresponding to P-8 was not observed in the HPLC chromatoprofile of the liver sample upon intravenous administration (Fig. 12). Furthermore, other new peaks corresponding to P-1, P-6 and P-7 in the chromatoprofile on Figs. 8 and 12 demonstrate a different UV spectrum from P-2 to P-5 and P-8; for example, no absorption around 280 nm, or UV$_{\text{max}}$ absorption at 225 nm or 240 nm (detailed data not shown). Therefore, a detailed investigation of the structural analysis of P-1, P-6, P-7 and P-8
was not performed.

Analytical results of P-2, P-3, P-4 and P-5 on NMR and MS analysis are shown as follow, respectively.

Results of P-2: Atmospheric pressure chemical ionization (APCI)-LC-MS: m/z 243, [MS/MS]=225, $^1$H-NMR (D$_2$O) $\delta$: 1.155 (d, 3H, 6Hz), 1.483 (m, 2H), 1.724 (m, 2H), 2.374 (s, 3H), 2.747 (m, 2H), 3.764 (s, 3H), 3.842 (m, H), 1H–1H COSY cross peak: 1.155 and 3.482, 1.483 and 3.482, 1.483 and 1.724, 1.724 and 2.747

Results of P-3: APCI-LC-MS: m/z 243, [MS/MS]=225, $^1$H-NMR (D$_2$O) $\delta$: Assured results for the assignment of $^1$H-NMR signals were not obtained.

Results of P-4: APCI-LC-MS: m/z 243, [MS/MS]=225, $^1$H-NMR (D$_2$O) $\delta$: 0.914 (t, 3H, 7Hz), 1.484 (m, h), 1.553 (m, H), 1.786 (m, H), 1.925 (m, H), 2.402 (s, 3H), 2.822 (m, 2H), 3.604 (m, H), 3.783 (s, 3H) $^1$H–$^1$H COSY cross peak: and 1.484 and 1.553, 1.848 and 1.553 and 3.604, 1.786 and 1.925 and 2.822

Results of P-5: APCI-LC-MS: m/z 243, [MS/MS]=225, $^1$H-NMR (D$_2$O) $\delta$: 0.865 (t, 3H, 7Hz), 1.331 (m, 4H), 1.850 (m, 2H), 2.419 (s, 3H), 3.787 (s, 3H), 5.033 (t, 7H, 7.4 Hz), $^1$H–$^1$H COSY cross peak: 0.865 and 1.331, 1.850 and 5.033, 1.331 and 1.850

These results suggest that new peaks were observed as metabolites of allixin; P-2, P-4 and P-5 were 3-hydroxy-5-methoxy-6-methyl-2-(4-hydroxypentyl)-4H-pyran-4-one, 3-hydroxy-5-methoxy-6-methyl-2-(2-hydroxypentyl)-4H-pyran-4-one and 3-hydroxy-5-methoxy-6-methyl-2-(1-hydroxy-pentyl)-4H-pyran-4-one, respectively.

Discussion

Allixin, a non-sulfur containing compound with a $\gamma$-pyrone skeleton structure, was the first phytalexin, induced in a plant by continuous stress, isolated from garlic, and this compound has shown unique biological properties. There was no previous study relating to the pharmacokinetics of allixin, though several biological properties of allixin were investigated in both in vitro and in vivo systems as described above. Therefore, we investigated the pharmacokinetic behavior of allixin because it is well known that pharmacokinetic behaviors, parameters and metabolites of drugs are important factors for studying the action mechanisms and/or development of new drugs.

Allixin is slightly soluble in water (ca. 200 $\mu$g/ml$^{-1}$); the calculated log $P$ of allixin is 3.89±0.75. The solubilization of new drugs with poor aqueous solubility is crucial for pharmacological evaluation. The use of biologically incompatible organic solvents or surfactants in testing is undesirable for a living body. Cyclodextrins form inclusion complexes with hydrophobic compounds, and the resulting complex improves the solubility of these hydrophobic compounds in aqueous solution. Recently, several cyclodextrin derivatives have been developed to improve solubility in aqueous solution. The practical use of natural cyclodextrin as a drug carrier, however, is restricted by its low aqueous solubility. Maltosyl-$\beta$-cyclodextrin (M-$\beta$-Cyd) is a $\beta$-cyclodextrin derivative that improves solubility, and this material improved the solubility of allixin in aqueous solution (Fig. 2).

The results of $^1$H-NMR measurement of an intact substance and the inclusion complex shows an obvious interaction between allixin and M-$\beta$-Cyd. All of the chemical shifts of the complex corresponding to allixin migrated 0.03–0.09 ppm downfield from signals of intact allixin, though the migration of M-$\beta$-Cyd signals were not distinguished clearly. Observations of allixin signals in the inclusion compound indicated that methyl, methoxyl and pentyl groups of allixin interacted with the M-$\beta$-Cyd molecule. It is said that the diameter of the cyclodextrin cavity is 7–8 Å. The distance of carbon atoms between O-methoxide in position five and methyl in position six was calculated to be 3.295 Å (calculated by PM3 Hamiltonian in MOPAC97, WinMOPAC ver2.0, Fujitsu, Japan), and the distance between carbon and hydrogen is 1.1 Å. Therefore, the cavity of M-$\beta$-Cyd is large enough to include the spatial area of both of O-methoxide and methyl groups, and these groups would be inside of M-$\beta$-Cyd cavity, as shown in Fig. 4. However, the phase solubility diagram indicated the equilibrium relationship to be one of allixin and M-$\beta$-Cyd. All of these results suggest that new peaks were observed as metabolites of allixin; P-2, P-4 and P-5 were 3-hydroxy-5-methoxy-6-methyl-2-(4-hydroxypentyl)-4H-pyran-4-one, 3-hydroxy-5-methoxy-6-methyl-2-(2-hydroxypentyl)-4H-pyran-4-one and 3-hydroxy-5-methoxy-6-methyl-2-(1-hydroxy-pentyl)-4H-pyran-4-one, respectively.
several hours after preparing this inclusion compound solution at a concentration of 2 g/10 ml. Therefore, the administration was performed within 2 h after preparing the administration solution. Further investigation is necessary to reveal the detailed interaction between allixin and M-β-Cyd in aqueous solution.

It is said that the compound included by the host compound is quickly released in the body from the host compound, such as M-β-Cyd, when the inclusion compound is administered. It might be that the compound released from the host compound would crystallize inside the body with high dosage administration, thus this phenomenon is a serious problem in this pharmacokinetic study. Crystallized allixin was not trapped by a membrane filter in the experiment of the interaction with BSA and rat serum (Table 2). Furthermore, no crystalline or insoluble substance was observed in either the ultrafiltrate or remaining solution. Therefore, these results indicated that allixin released from the host compound would be circulated with the carrier protein without crystalline formation.

It appears that allixin was quickly absorbed, based on the observation of a maximum level ($C_{\text{max}}$) at 5 min ($T_{\text{max}}$) on peroral administration. The bioavailability of allixin in mice after peroral administration was estimated to be 31% using an inclusion compound with M-β-Cyd. The bioavailability of allixin itself in mice was low, but combining allixin with the administration substance improved its solubility in aqueous solution when the inclusion compound was prepared with M-β-Cyd. However, the bioavailability of allixin was about two times higher than that of allin (16.5%), which is a well-known sulfur-containing compound in intact garlic. In the preliminary administration study using an inclusion compound, allixin was not detected in rat plasma at a dose of 0.5, 5 or 10 mg/kg upon peroral administration, while allixin was detected in intravenous administration, even at dose of 0.5 mg/kg (data not shown, intact allixin in saline solution was used at a dose of 0.5 mg/kg). Most of the allixin in rat plasma disappeared within two hours upon intravenous administration, as it did in mice, and the bioavailability of allixin in rat was 1.1% based on the hypothetical estimation ($C_{\text{max}}$=6.93 μg/ml, $T_{\text{max}}$=10 min, at 10 mg/kg-dose). The time allixin disappeared from blood in mice was almost the same as S-allylcysteine, which indicates high bioavailability, but was significantly different from that in rat. Therefore, these results suggest that allixin might be experience a first-pass effect in both animals, but the details are not yet clear.

The $T/S$ ratio of the lung was higher than for other organs at one minute after intravenous administration. The extremely high content of allixin found in the lung compared to that in the serum and other organs during the early period after intravenous administration might be due to delivery through blood circulation and to the lipophilic property of allixin, because venous blood is carried first to the lung and then to other organs. Additionally, the calculated log $P$ of allixin, 3.89±0.75, indicated high lipophilicity. The content and $T/S$ ratio in the lung remained a little bit higher than that in the serum or some of the organs after both intravenous and peroral administration. Therefore, allixin might have some affinity for the lung. Its lipophilic property might be related to the high content of allixin found in the brain versus the serum upon intravenous administration. The $T/S$ ratio of those organs involved in metabolism and excretion, i.e. the liver and kidney, increased up to 10 min, then these ratios gradually decreased over time upon both peroral and intravenous administration, except for intravenous administration in the liver. It is guessed that the administered allixin was quickly distributed to liver tissue, then this allixin in the liver would return only a small amount to the blood; thus, allixin remained and accumulated in the liver, and the $k_{21}$ (transfer rate constant from blood to liver tissue) would be larger than $k_{12}$ (transfer rate constant from liver tissue to blood) in allixin distribution in liver. The $T/S$ ratio of liver in intravenous administration was extremely elevated compared to other organs, though the allixin absorbed was metabolized and/or excreted, and its concentration in serum was decreased by excretion. On the other hand, allixin was quickly absorbed in peroral administration, but not higher than with intravenous administration, and the allixin concentration in serum quickly reached the $C_{\text{max}}$ level. The allixin concentration in serum in peroral administration was much lower than intravenous administration until 15 min after administration. Therefore, the amount of allixin distributed to the liver was lower than in the case of intravenous administration. Allixin in the liver appears to be metabolized and excreted without accumulation. The $T/S$ ratio of the kidney was similar with both peroral and intravenous administration, i.e., the ratio gradually increased until 30 min and then gradually decreased. The tested organs, except the brain, retained allixin at a higher rate than serum levels, even 120 min after administration. It is considered that allixin has some affinity without specificity to tissue. The reabsorption of allixin by the kidney would be expected, but this was not investigated.

The metabolism and metabolites of allixin were investigated using rat liver homogenate and a metabolic enzyme fraction derived form rat liver microsome fraction (named S-9 Mix). Obviously, allixin was metabolized by these treatments (Fig. 8). Metabolites of allixin were isolated by HPLC, and the chemical structure of the isolated metabolites was investigated using NMR and LC-MS. Some of the allixin signals derived from the alkyl group on NMR analysis disappeared and new signals were observed at each isolated metabolite, respectively. Peaks corresponding to metabolites were also observed in chromatoprobe on HPLC analysis of liver samples following intravenous administration (Fig. 12). Based on the analytical data of metabolites, such as the shift of the UV spectrum to short wavelength, the detection of $m/z=243$ ion and $m/z=225$ ion, and the detection of cross peaks on $^1$H–$^1$H COSY NMR, allixin is likely metabolized to oxidative substances by an oxidation enzyme such as P-450 after administration. Especially, an alkyl group on the side chain would be easily oxidized. Some γ-pyrones compounds, such as maltol derivatives, were investigated for use as iron chelators for patients with hemoglobinopathic disorders. Hydroxylation of the alkyl group on the side chain of administered maltol derivatives was observed and determined to be a phase I metabolite in experimental animals and humans after oral and intravenous administration. These resulting phase I metabolites were then transformed into phase II metabolites, such as glucuroide conjugate. Additionally, the hydroxylation of an alkyl group on the side chain was observed in the pharmacokinetic study of naturally volatile compounds, such as thymol and carvacrol. Considering the
results of these reports and our obtained results, it is strongly suggested that some position in the pentyl group would undergo hydroxylation as a metabolic reaction of phase I. Identified metabolites of allixin were produced quickly, but not major metabolites, while accurate quantitative analysis of the observed metabolites was not performed. The total amount of P-2 to P-5 at 60 min incubation with S-9 Mix was less than 10% against initial allixin content in the hypothetical estimation. It is suggested that allixin might be metabolized to another kind of compound or transformed to phase II metabolites, such as glucuroide or sulfuric acid conjugates, in a living body. The biological activity and safety of hydroxylated allixin metabolites was not evaluated because the amount isolated was small and no sufficient method of chemical synthesis was established. Hydroxylation occurs as a detoxification step (phase I metabolism) in the liver, and another kind of compound or transformed to phase II metabolites, such as glucuroide or sulfuric acid conjugates. Allyl allixin metabolites was not evaluated because the mechanism of metabolism. Thus, the toxicity of allixin metabolites would be weaker than that of intact allixin. The pharmacokinetic behavior of allixin shows very little accumulation of this compound in organs.

Allixin has been reported to have various unique biological properties in both in vitro and in vivo assay systems. Namely, this compound showed strong antitumor promoting activity in the in vivo system, i.e., in skin tumor formation promoted by TPA in DMBA initiated mice. Also, the results of neurotrophic activity in the in vitro assay indicates that allixin may be beneficial in the treatment of aging problems. However, it was revealed that intact allixin is not suitable for usage in oral and intravenous administration systems targeted at internal organs. An alternative investigation, such as the modification/derivative of allixin, external application and biological evaluation of allixin metabolites, would be necessary to take advantage of this potent, beneficial compound.

Materials and Methods

Chemicals and Animals  
Allixin administered to experimental animals and allixin-<i>d<sub>4</sub></i> as an internal standard, were synthesized according to the previously reported method. M-β-Cyd, for the preparation of an inclusion compound, was purchased from Easukou-Seto (Yokohama, Japan). Previously prepared 3-hydroxy-2,6-dimethyl-4-pyrano-4-one was used as the standard for the analysis of allixin metabolites. All chemicals for analysis and synthesis, except for 1-bromopentane-<i>d<sub>4</sub></i> (99 atom% deuterium, CDN Isotope, Quebec, Canada), were purchased from Wako Pure Chemical Industries (Osaka, Japan). S-9/co-factor A set for the Ames test, named S-9 Mix, (Oriental Yeast Co., Ltd., Tokyo, Japan) was used for the investigation of allixin metabolites as a metabolism enzyme fraction. For experimental animals, 6-week old male ddY mice and 14-week old wistar male rats were used for this pharmacokinetic study.

Equipment  
The LC-MS analysis was performed using an LC-MS system composed of the Hewlett Packard HPLC piers 1100 (Hewlett Packard, U.S.A.) and MS system of Finnigan LCQ (Finnigan Corporation, U.S.A.). The NMR spectra was taken on a JNM EX-500 spectrometer (JEOL, Tokyo, Japan). The LC-10A system (Shimadzu, Kyoto, Japan) was used for the quantitative analysis of allixin and its metabolites. Absorption at UV 280 nm was used for the quantitative and/or identical analysis of allixin and its metabolites on HPLC analysis because allixin has a maximum absorption at 280 nm of the ultraviolet region.

Preparation of an Inclusion Compound of Allixin with M-β-Cyd and Analysis of Allixin Content in This Inclusion Compound  
Allixin, in the amount of 300 mg (1.33×10<sup>-3</sup> mol), was placed on an agate mortar, and 2 ml of 1 N NaOH was added. To this was added 5.8 g of M-β-Cyd (3.98×10<sup>-2</sup> mol) and 1 ml of water, then this mixture was mixed well for 30 min by agate pestle until it became a translucent paste. A few milliliters of water was added to this mixture, and it was then adjusted to pH 7.5 using diluted HCl solution. About 30 ml of water was then added to this neutralized solution. The resulting solution was kept for 1 h at room temperature, then filtered by a membrane filter (pore size: 0.45 μm). The above preparation processes were repeated two more times. These filtrates were combined then the resulting translucent solution was lyophilized. The obtained lyophilized substance was pulverized into a fine powder. About 50 mg of dry fine powder was pipetted into a 20 ml volumetric flask; 5 ml of water was added for dissolution, then methanol was added to make the 50 ml volume. Two milliliters of this solution was pipetted into a 20 ml volumetric flask and methanol was added to fill it. The resulting solution was analyzed by the following HPLC conditions to determine the allixin content in the hypothetical inclusion compound. HPLC systems: LC-10A systems (Shimadzu, Kyoto, Japan), detection: UV 280 nm absorption, solvent: mixture of methanol and 0.1% phosphate solution (72:28), solvent flow: 0.8 ml/min, column: TSK gel ODS 80T (4.6 mm×150 mm) (Tosoh, Tokyo, Japan). The yield of the inclusion compound was 15.63 g and the content of allixin in the inclusion compound was 51.9 mg/g.

NMR Analysis of the Inclusion Compound  
About 30 mg of the allixin-inclusion compound and about 25 mg of M-β-Cyd were each dissolved in about 1 ml of D<sub>2</sub>O, respectively. Each solution was filtered with a membrane filter (pore size: 0.45 μm), then the obtained filtrates were used for NMR analysis. One milliliters of D<sub>2</sub>O was added to a few milligrams of allixin, and this mixture was sonicated for 20 min to dissolve allixin. This mixture was filtered with a membrane filter (pore size: 0.45 μm), and then the obtained filtrates were measured by H-NMR.

Measurement of the Stability Constant of a Complex of Allixin and M-β-Cyd in Water  
Measurement of the stability constant of a complex of allixin and M-β-Cyd in water was performed according to the Solubility Method. Four milliliters of 0% to 30% of M-β-Cyd aqueous solution was pipetted into 10 ml glass vials with screw caps, respectively. An appropriate amount of allixin to M-β-Cyd content was added to each vial. The vials containing of resulting mixture were shaken constantly at 25 °C for 7 days (200 strokes/min). These storage mixtures were filtered with a membrane filter (pore size: 0.45 μm) after 7 days. One milliliter of filtrate was diluted to an appropriate concentration of allixin and M-β-Cyd, respectively. Then, the concentration of each resulting solutions was measured to determine the allixin content which interacted with M-β-Cyd using a UV-VISIBLE Spectrophotometer UV-1600 (Shimadzu, Kyoto, Japan) at a wavelength of 280 nm. The solubility diagram was prepared by plotting the relationship between the mole concentration of allixin and M-β-Cyd in the filtrate of each vial. The stability constant of the complex compound between allixin and maltosyl-β-cyclodextrin, <i>K</i> (<i>m</i><sup>-1</sup>), in water, was calculated by the following Eq. (1):

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K = S/S_o(1 - S)
\]

Where <i>S</i> and <i>S_o</i> represent the slope and intercept on the obtained solubility diagram, respectively.

Behavior of Allixin Inclusion Compound in BSA Solution and Blood/Serum of Experimental Animal  
Allixin was dissolved in phosphate buffered saline (PBS) solution, and the concentration of BSA was adjusted to 8%. This BSA solution was used as the simulated serum. One gram of alloxin inclusion compound was dissolved in purified water, and the mixture volume was made to 5 ml (allixin concentration: 10.4 mg/ml). BSA solution was added to 1300 μl of allixin inclusion compound solution to make a volume of 10 ml. This mixture was incubated at 37 °C for 5 and 15 min. After incubation, the mixture was immediately filtered with a filter unit, DISMIC-25 (pore size: 0.2 μm, ADVANTEC TOYO, Japan), and the obtained filtrate was placed in a centrifugal filter device, Centriflip YM-10 (cut off: 10<sup>3</sup>, Millipore Co., Bedford, U.S.A.). This device was centrifuged at 3000 g for 30 min, then the ultraplate and remaining solution were recovered. The filter unit, DISMIC-25, was rinsed with 5 ml of PBS, then allixin trapped with the filter was eluted with 20 ml of 90% methanol. Five hundred microliters of the ultraplate and remaining solution were placed in a 20 ml volumetric flask, and acetonitrile was added to volume, respectively. These mixtures were sonicated at around 15—25°C for 15 min, then a part of these mixtures was centrifuged at 15000 rpm for 10 min. The obtained supernatants and eluate from DISMIC-25 were analyzed by HPLC. The adhesion of allixin in the filter unit, DISMIC-25, was tested in the same manner as described in the above, without BSA solution.

The stability of the inclusion compound in serum was tested using rat serum. Blood was collected from the femoral vein of Wistar rats under ether anesthesia using a plastic heparinized/non-heparinized injector. Non-he-
parinated blood was kept at room temperature for 30 min, blood was cen-
trifuged at 2000g for 30 min, then the serum phase was used for the stability
experiment. The stability test in serum was performed in the same manner as
serum supplemented with BSA, as described above.

The stability of allixin in whole blood was tested using rat blood he-
parinated as described above. Four hundred microliters of allixin inclusion
compound were mixed with whole blood and 0.5 ml of PBS solution to attain a volume of 3 ml.
This mixture was incubated at 37°C. One hundred microliters of this mix-
ture was sampled at 5, 10, 15, 30 and 60 min after incubation, and the mi-
ture sample was placed in a 10 ml volumetric flask, then acetonitrile was added to attain the full volume. These mixtures were sonicated at 15—20°C
for 15 min, then part of these mixtures was centrifuged at 15000 rpm for 10
min. The obtained supernatants were analyzed by HPLC. Analytical HPLC
conditions were the same as in the preparation of inclusion compound of al-
lixin and internal standard, allixin-

Administration of Allixin to Experimental Animal Two grams of al-
lixin inclusion compound was dissolved in 10 ml of purified water at room
temperature. Osmo-regulation of the administration solution was controlled to
avoid lake on intravenous administration based on analytical results of
composition of the inclusion compound, such as the content of allixin, M-
β-Cyd and resulting NaCl by NaOH solution and HCl solution in preparation of
the inclusion compound. The prepared inclusion compound solution was
administered to fasted mice (25—30 g) by an intravenous and peroral route
within 2 h after preparation. The administered dose for the mice was 50 mg-
allixin/5 ml/kg.

Collection of Blood Sample and Organs on Administration Experi-
iment Blood samples were collected from cervix blood vessels at one, 5,
10, 15, 30, 60 and 120 min after intravenous and oral administration (blood
and organ samples were not collected at 1 min after peroral administration). Collector was kept for 30 mins at room temperature, and then cen-
trifuged at about 2000 g for 15 min. Serum was collected for the analytical
sample and was kept frozen at —80°C until analysis. Mice were sacrificed by
decapitation after collection of the blood, then the brain, kidneys, liver and
lungs were immediately removed. Each of the isolated organs were weighed and
then frozen by liquid nitrogen. Obtained organs were kept frozen at
—80°C until analysis.

Preparation of Sample Solution for LC-MS Analysis Serum sample
(50—100 µl) was placed into a 1.5 ml sample cup, and about 50 mg of NaCl, 20 µl
of 2 N HCl, 50 µl of internal standard solution and 1 ml of acetonitrile were
added. The resulting mixture was shaken vigorously using vortex mixer for
1 min, followed by sonication for 20 min at around 15 °C to 20 °C. This mix-
ture was centrifuged at 15000 rpm for 5 min, and the obtained supernatant
was used as a sample preparation for LC-MS analysis.

Each organ was placed into a glassware homogenizer with acetonitrile in
the vessel and homogenized at the same weight ratio of organ to volume of internal standard solution. This mixture was homogenized. The obtained
homogenate was transferred to 15 ml of plastic tube and was sonicated for
60 min at 20°C to 30°C for extraction. This mixture was centrifuged at
2000 g for 15 min, then the supernatant was transferred to 15 ml of plastic
tube. The solvent of this solution was removed in vacuo. One milliliter of
acetonitrile was added to the resulting residue, shaken vigorously and then
sonicated for 20 min at 20°C to 30°C. The resulting mixture was centrifuged
at 15000 rpm for 5 min, then the obtained supernatant was used as a sample
preparation for LC-MS analysis.

Quantitative Analysis of Allixin in Serum and Organs by the LC-MS
Method LC-MS analysis was performed using an LC-MS system com-
posed of the Hewlett Packard 1100 HPLC system and MS system of Finni-
gan LCQ. Standard solution and sample solution were separately injected
into LC-MS equipment, and chromatograms were recorded in terms of func-
tion of the selective ion mode. The calibration curve (regression curve) was
prepared using the injected amount of allixin and the peak area ratio of al-
lixin and internal standard, allixin-d6. The content of allixin in the sample
was calculated by the calibration curve thus obtained based on the peak area
ratio of allixin and the internal standard, allixin-d6. Analytical conditions
were as follows: Mobile phase: mixture of methanol—water-acetic acid (360: 135: 5), flow of mobile phase: 0.2 ml/min, column: Cadenza CD-C18 (2
mm×75 mm, Intakt, Kyoto, Japan), ionization conditions: APRI, detec-
tion: selected ion monitoring of positive ion ([M+H]+ = 227 for allixin, [M+
H]+ = 238 for internal standard allixin-d6). The limit for quantitative
analysis on this experiment was 200 ng/ml-serum.

Calculation of Pharmacokinetic Parameters The values of Cmax and
AUC were determined directly from the obtained allixin concentration–time
profile in serum. The values for area under the concentration–time curve
(AUC) were calculated by the trapezoidal method. Apparent half-lives were
estimated using computer programs for nonlinear regression analysis.

Treatment of Allixin with S-9 Mix and Preparation of Test Solution
for Analysis of Allixin Metabolism Fifty milligrams of allixin was placed
into a 100 ml of flask, and 50 ml of PBS solution was added. This mixture
was sonicated for about 1 h, and the resulting mixture was filtered by a mem-
brane filter (pore size: 0.45 µm). One milliliter of enzyme fraction and 9 ml of
solution of S-9-co-factor was mixed as a sample preparation for the analy-
sis of allixin metabolism. Analytical HPLC conditions were the same as in
the preparation of inclusion compound of allixin with M-β-Cyd and analysis
of allixin content in inclusion compound.

Collection of Blood Sample and Organs on Administration Experi-
iment Blood samples were collected from cervix blood vessels at one, 5,
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sonicated for 20 min at 20°C to 30°C. The resulting mixture was centrifuged
at 15000 rpm for 5 min, then the obtained supernatant was used as a sample
preparation for LC-MS analysis.
analysis of allixin metabolites, 3-hydroxy-2,6-dimethyl-4H-pyran-4-one was used as the standard. However, this was not accurate quantitative analysis because it is difficult to synthesize each of the allixin metabolites. The obtained sample solution was analyzed under the following conditions: column: TSK gel ODS 80TM (4.6 mm × 150 mm), solvent: gradient with mixture of water and methanol (60 : 40) and mixture of methanol and water (90 : 10), detection: UV 280 nm, flow: 0.6 ml/min.

LC-MS Analysis of Allixin Metabolites  The LC-MS analysis was performed using an LC-MS system composed of a Hewlett Packard 1100 HPLC system and MS system of Finnigan LCQ. Analytical conditions were as follows: mobile phase: mixture of water–methanol (600 : 400), flow of mobile phase: 0.6 ml/min, column: TSK gel ODS 80TM (4.6 mm × 150 mm, Tosoh, Japan). Ionization conditions: APCI, detection: positive ion.

NMR Analysis of Metabolites  Each metabolite fraction obtained was dissolved in vacuo under existing phosphorus pentoxide. The resulting dried residue was dissolved in deuterium oxide, then NMR analysis was performed at 1H-NMR and 1H-13C COSY NMR mode.

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References