Retention Mechanism of Imidazoles in Connective Tissue. III.\(^1\)
Aldehyde Adduct Formation of a 4(5H)(or 5(4H))-Imidazolone
Product in Vitro

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2-Methylimidazole (2MI), as well as imidazole, has been thought to undergo cupro-ascorbate (Cu-VC)-catalyzed oxidative transformation in vitro to become a reactive species capable of combining with aldehydes intrinsic to connective-tissue proteins. We attempted to seize the essence of the above reaction through obtaining the structural information of an aldehyde-bonding species. As major products from 2MI in the in vitro Cu-VC system, 2-hydroxymethylimidazole (2OHMI) and 2-methyl-4(5H)(or 5(4H))-imidazolone (2MIone) were identified by mass-spectral and chromatographic comparison with the corresponding authentic standards synthesized. The in situ addition of acetaldehyde or propionaldehyde as a simple protein-aldehyde model to the system resulted in the degradable formation of an aldol condensate, 2-methyl-4(5H)-ethylenylidene(4H)-imidazolone (2MIone) or its possible analogue with a propylidene moiety, respectively. The authentic compound of 2MIone directly reacted with acetaldehyde and easily afforded the products assignable to the isomers of 2MIone through the ethylenidene moiety at physiological pH and temperature, whereas neither 2MI or 2OHMI reacted at all. These results suggest that a 4(5H)(or 5(4H))-imidazolone product, although simply a monoxygenated form, is sufficiently reactive to give aldol condensation-type covalent adducts with aldehydes, even under physiological conditions, probably having an activated methylene moiety in the ring structure. Based on the present results, we discussed the mechanism of the retention of imidazole-containing drugs in connective tissue.

Key words imidazolone; covalent binding; aldehyde adduct; connective tissue

The five-membered heterocyclic imidazole structure is found in a large number of drugs or drug candidates.\(^5\) It has been reported that, for several imidazole-containing drugs, the marked retention of their equivalents in connective tissue was observed after dosing to laboratory animals.\(^2\) However, the mechanism of the retention, which potentially involves some toxicological significance, remains unclear.

Investigations to elucidate this mechanism have been conducted in our laboratory using radiolabeled compounds with a simple structure: imidazole (1m) and its 2-methyl derivative (2MI). A previous study in rats has indicated that the in vivo retention of imidazole-containing drugs is largely attributable to irreversible binding between the imidazole moiety and elastin, a major macromolecule of the extracellular matrix, and that their interaction might be mediated through a cytochrome P450-independent biotransformation of drugs with an imidazole moiety.\(^1\)

In an in vitro study, it has also been indicated that the catalytic oxidation of the cupro-ascorbate (Cu-VC) system can be involved in the activation of both 1m and 2MI to give their reactive species capable of combining with aldehydes intrinsic to connective-tissue proteins.\(^1\) These reactions occurred even under physiological conditions.

In the present study, \(^13\)C-labeled 2MI as a model substrate was reacted in the Cu-VC system, and structural elucidation of its products was undertaken, especially focusing on identification of an aldehyde-bonding species.

MATERIALS AND METHODS

Chemicals [Ring-C2-\(^{13}\)C] 2MI (51.2μCi/μmol), synthesized by Chemsyn Science Laboratories (Kansas City, KS, U.S.A.), was purchased from Nemoto & Co., Ltd. (Tokyo). Its radiochemical purity was assessed as being more than 97% by TLC analysis. The specific activity of the incubation mixtures was adjusted through dilution with unlabeled 2MI, obtained as having more than 98% purity from Wako Pure Chemical Industries, Ltd. (Osaka). The incubation mixtures were prepared in double-distilled water to minimize metal contamination.

The following reference compounds for analytical use were prepared synthetically in our research center, and their structural analysis was performed by \(^1\)H-NMR spectrometry and thermospray liquid chromatography/tandem mass spectrometry (TSP LC/MS/MS).

2-Methyl-4(5H)(or 5(4H))-imidazolone (2MIone) was obtained by reacting ethyl acetamidate (Sigma, St. Louis, MO, U.S.A.) with glycine methyl ester (Wako), as previously reported by Jacquier et al.\(^6\) \(^1\)H-NMR (dimethyl-d<sub>4</sub> sulfoxide (DMSO-d<sub>4</sub>)) δ: 2.02 (s, 3H, CH<sub>3</sub>), 3.89 (s, 2H, CH<sub>2</sub>), 10.77 (broad s, 1H, NH). TSP-MS m/z (rel. int. %): 99 (MH<sup>+</sup>, 100). 2-Hydroxymethylimidazole (2OHMI) was synthesized from imidazole-2-carboxaldehyde (Fluka, Buchs, Switzerland) through a crossed Cannizzaro reaction. \(^1\)H-NMR (DMSO-d<sub>4</sub>) δ: 4.43 (s, 2H, C2-CH<sub>2</sub>), 6.89 (s, 2H, C4- and C5-H). TSP-MS m/z (rel. int. %): 99 (MH<sup>+</sup>, 100). Although the signals for OH and NH protons were not clearly observed in the \(^1\)H-NMR spectrum, the presence of alcohol and imidazole moieties in this compound was indicated by MS/MS analysis of the MH<sup>+</sup> ion, which exhibited peaks at m/z 81 and 69 corresponding to a dehydrated fragment and a protonated fragment of the imidazole moiety, respectively.

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2-Methyl-4(or 5)-ethylidene-4(5H)(or 5(4H))-imidazolone (2Mlone) was obtained by reacting 2Mlone with acetaldehyde (E. Merck, Darmstadt, Germany) under an alkaline condition. \(^1\)H-NMR (DMSO-\(d_6\)) \(\delta\): 1.97 (d, \(J=7.3\) Hz, 3H, CH\(\text{CH}_3\)), 2.12 (s, 3H, C2-CH\(\text{CH}_3\)), 6.19 (q, \(J=7.3\) Hz, 1H, CH\(\text{CH}_3\)), 11.04 (broad s, 1H, NH). TSP-MS \(m/z\) (rel. int. %): 125 (M\(^+\), 100). Though not evidently deduced from the above spectra, the possibility that this compound could exist as a mixture of cis and trans forms in an equilibrium is taken up later.

Deuterium-labeled compounds were purchased from Nippon Sanso Co., Ltd. (Tokyo). All other chemicals and reagents were of the highest purity available and, unless otherwise indicated, were obtained from Wako Pure Chemical Industries, Ltd.

In Vitro Reactions The reaction of \([^{14}\text{C}]2\text{MI}\) with copper ion and ascorbate was achieved in 0.1 M sodium phosphate buffer (pH 7.2). The standard reaction mixture, in a final volume of 5 mL, contained 1 mm \([^{14}\text{C}]\text{substrate}\) (2 \(\mu\)Ci/mL), 0.05 mm cupric chloride, and 5 mm L-ascorbic acid, and was incubated in a shaking water bath maintained at 37 °C under an aerobic condition up to 24 h. In addition, incubations of the substrate without copper ion and/or ascorbate were performed as controls.

The aldehyde adduct formation of \([^{14}\text{C}]2\text{MI}\)-derived products in the Cu-VC system was examined as follows. After 5 min of the aforementioned reaction, acetaldehyde or propionaldehyde was added to the mixture at a final concentration of 1 mm, then it was further incubated at 37 °C for 1 h. A control test was conducted similarly, except that buffer alone was added instead of the aldehydes.

The reactivity of either authentic 2Mlone, 2(OH)MI, or 2MI to acetaldehyde was examined by direct incubation in 0.1 M phosphate buffer (pH 7.2) at 37 °C for 1 h, each at a concentration of 1 mm.

At pre-determined intervals, aliquots (50 \(\mu\)L) were withdrawn from the incubates and immediately injected into analytical instruments.

Instrumentation HPLC was performed on a Gilson model 305 system (Middleton, WI, U.S.A.) equipped with a Raytest Ramona 93 radioisotope detector (Straubenhardt, Germany) and a Linear 206PHD UV detector (Reno, NV, U.S.A.). A C18-silica-packed column (SUMIPAX ODS A212, 5 mm, 6.0 mm i.d. \(\times 150\) mm, Sumika Chemical Analysis Service, Osaka) was maintained at 50 °C and subjected to either of the following two gradient elution systems: (A), a mobile phase of water–methanol containing 0.1% (w/v) perfluorohexanoic acid (Kanto Chemical, Tokyo) as an ion-pairing reagent, a linear ramp from 0 to 13% methanol over 20 min with a hold for 20 min, followed by a further linear ramp up to 50% over 10 min; or (B), a mobile phase of water–methanol, a 5 min hold at 0% methanol, followed by a linear ramp to 15% over 5 min with a hold for 10 min, followed by a rapid rise to 50% with a 5 min hold. Each elution system was operated at a flow rate of 1 mL min\(^{-1}\). For radioactivity quantification, the HPLC effluent was fractionated, mixed with a Flo-Scint II scintillation cocktail (Packard, Downers Grove, IL, U.S.A.), and then analyzed in a Beckman LS6000TA liquid scintillation counter (Fullerton, CA, U.S.A.). The total recovery of chromatographed \(^{14}\text{C}\) material was greater than 95% in either case for the elution systems.

TSP LC/MS/MS was performed on a Finnigan MAT TSQ70 triple-stage quadrupole mass spectrometer (San Jose, CA, U.S.A.). HPLC separations were conducted with a Shimadzu LC 9A liquid chromatograph (Kyoto) under the same conditions as those previously described, and a post-column addition of 0.6 M ammonium acetate to the mobile phase was performed for the efficient ionization of analytes by using a Yokogawa LC100 solvent delivery system (Tokyo) at a flow rate of 0.2 mL min\(^{-1}\). Detailed operating conditions were as follows: ionization mode, TSP (filament off, discharge off); ion source temperature, 250 °C; vaporizer temperature, 85 °C; repeller voltage, 20 V; collision gas, argon at a pressure of 10\(^{-3}\) Torr; collision energy, −40 to −60 eV.

\(^1\)H-NMR spectra were recorded at 200 MHz with a Varian VXR-200 instrument (Palo Alto, CA, U.S.A.). DMSO-\(d_6\) was used as a solvent, and chemical shifts are reported in ppm (\(\delta\)) downfield from the internal standard, tetramethylsilane.

RESULTS

HPLC Profile of 2MI-Derived Products in a Cu-VC System Products from \([^{14}\text{C}]2\text{MI}\) in the \textit{in vitro} Cu-VC system were analyzed by the radio-HPLC with the aid of a counterion effect (elution system A). As shown in Fig. 1, this metal/reductant-dependent catalytic reaction led to four peaks of major products, with retention times of 5, 13, 17, and 21 min (P\(_1\)→P\(_4\) in the elution order). Immediately after the reaction was initiated by the addition of ascorbic acid to the incubation mixture, P\(_1\), P\(_3\), and P\(_4\) were detectable (−2%:

![Graph](image-url)

**Fig. 1.** Representative Radio-HPLC Profiles of \([^{14}\text{C}]2\text{MI}\)-Derived Products in a Cu-VC System

HPLC separation was conducted on a C18 column eluted with gradient system A as described in Materials and Methods. Dashed and solid lines indicate the profiles of 1- and 24-h-reacted mixtures, respectively. Four peaks of major products with retention times of 5, 13, 17, and 21 min (P\(_1\)→P\(_4\) in the elution order) were observed.
of total $^{14}$C chromatographed). Also, $P_3$ was detectable after 5 min of the reaction. A gradual increase in the amount of the products was observed during the course of incubation, and their formation almost reached a plateau after 4—8 h of incubation. After 24 h, $-68\%$ of the substrate was converted, and the $P_1$—$P_4$ accounted for $-44$, $5$, $16$, and $19\%$ of the total products formed, respectively. Further, the control incubation samples lacking copper ion and/or ascorbate gave only some minor peaks other than the substrate with a baseline resolution on HPLC even after 24 h. This confirms that 2MI is chemically stable under noncatalytic conditions.

**Structural Information of 2MI-Derived Products Obtained by TSP LC/MS/MS** For $P_1$ and $P_2$, each mass spectrum displayed a protonated molecular ion at $m/z$ 99, thus indicating the addition of one oxygen atom to the parent molecule of 2MI (Figs. 2a and 3a). Collisional activation of the 99 ion of $P_3$ gave fragment ions at $m/z$ 42 (base peak) and 30 (intensity of 42.0% of the base peak) (Fig. 2b). On the other hand, for the collisional activation of the 99 ion of $P_4$, the fragments contained a dehydrated peak at $m/z$ 81 (intensity of 61.1% of the base peak at $m/z$ 99) (Fig. 3b), suggesting of the presence of an alcohol moiety. Authentic 2MI alone or 2(OMe)MI gave an identical mass fragmentation and retention time on HPLC with those of $P_1$ or $P_4$, respectively. As to $P_1$ and $P_4$, their mass-spectrometric information was not sufficient for structural characterization by reason of the presence of a large amount of interfering ions, probably derived from ascorbate or its degraded compounds.

**Trapping of a Reactive Species with Aldehydes** To detect a reactive species in the products of $[^{14}\text{C}]2\text{MI}$, either acetaldehyde or propionaldehyde as a simple protein-aldehyde model was added to the reaction mixture of the Cu-VC system, then adduct formation was traced by the radio-HPLC with elution system B. As shown in Fig. 3, the addition of acetaldehyde resulted in the formation of two peaks, $P_A$ and $P_B$, with retention times of 13.5 and 14.0 min, respectively. Their combined production accounted for $-6\%$ of the total radioactivity chromatographed. No such peaks were observed in the absence of the aldehyde. The mass spectrum of $P_A$ displayed a protonated molecular ion at $m/z$ 125, 42 mass units greater than that of 2MI (Fig. 5a), and the product ion mass spectrum of the 125 ion showed fragment ions at $m/z$ 42.
(base peak) and 56 (intensity of 11.1% of the base peak) (Fig. 5b). This was also true of \( P_B \). It was plausibly suggested that the products have carbonyl and ethylenic moieties in their molecules, and that they are isomeric with each other. The possible presence of an ethylenic moiety was also supported by an experiment with deuterium-labeled acetaldheyde (2, 2, 2-d4 form), in which the incorporation of d4 into the products, suggestive of no enamine formation with a secondary amine, was observed (mass spectra not shown). Further, when the 14C products on HPLC were carefully fractionated to be uncontaminated with each other, processed to freeze-drying or evaporation at 50 °C in vacuo, and subjected to re-chromatography under the same conditions, each gave two peaks with the same retention times as those of the origin again. The reference 2MElone also gave two peaks on HPLC, which were co-eluted with the in vitro reaction products, and they were not isolatable through chromatographic fractionation, as is the case with the products. Their mass-spectral data corresponded to those of the products. Williams et al. have pointed out that 2-phenyl-4(or 5)-arylidenem-4(5H)(or 5(4H))-imidazolones can exist as a mixture of cis and trans forms in equilibrium, and that their interconversion can occur by conventional preservation.3) This nature, thought to be subtly linked with tautomerism, could apply to the case of 2MElone. Further, when the reference 2MElone was dissolved in acidic, alkaline, or alcoholic solution, and then analyzed by HPLC/UV (210 nm) with elution system B, the relative abundance of the two peaks varied according to the kinds of solution. As a 1H-NMR observation for the reference 2MElone, the methyl proton signals of the ethylenic part and of the ring C-2 position tended to decrease in intensity with time after the addition of deuterium oxide to the solvent of DMSO-d6. Taking these data and the information of analogous compounds into account, \( P_A \) and \( P_B \) are assignable to isomers of 2MElone, and there is a likelihood of involvement of geometrical isomerism through an ethylenic moiety, though the preferential configuration under this experimental condition was difficult to clarify. Besides, when propionaldehyde was added to the reaction mixture in the Cu-VC system, the probable formation of an aldol condensate, 2-methyl-4(or 5)-propylidenem-4(5H)(or 5(4H))-imidazolone (involving two possible isomers through propylenic moiety) was indicated by LC/MS/MS (spectra not shown). Based on these results, 2MElone is considered to be a 2MI-derived reactive species sufficient to form aldol condensation-type covalent adducts with aldehydes.

**Reactivity of 2MLone to Acetaldehyde** The aldol condensation-type reaction of 4(5H)(or 5(4H))-imidazolones with aldehydes has been postulated as an intermediate key
step in the organic synthesis of numerous 2-phenyl-4-(or 5)-
arylidene-4(5H)(or 5(4H))-imidazolones.\textsuperscript{8,10} As far as we
are aware, however, no information is available concerning
this reaction under noncatalytic or physiological conditions.
Therefore, using the authentic compound of 2MIone, its re-
activity to acetaldehyde was directly examined at physiological
pH and temperature. As shown in the ion chromatograms
(using elution system B) of Fig. 6, after 1 h of incubation
with the aldehyde, the formation of two peaks of products
monitored at \( m/z \) 125 was observed with a marked decrease
of 2MIone monitored at \( m/z \) 99 (12.8\% of the initial peak
area). There was no detectable peak other than the substrate
2MIone and the products on the total ion monitoring (scan-
ing range: \( m/z \) 80—400) of this sample. Attempts to isolate
either of the products through HPLC fractionation met with
failure, and their behaviors on chromatographic and mass-
spectral inspection were identical with those of P\(_3\) and P\(_4\)
in Fig. 4, and of the reference 2MIone, indicating that they are
assignable to the isomers of 2MIone previously described.
Besides, when authentic 2MI or 2(OH)MI was incubated
with the aldehyde under the same conditions, no product was
observed at all.

**DISCUSSION**

Our experimental results demonstrated that 2MIone, a
simply monoxygenated product from 2MI found in the Cu-
VC system, can react readily with aldehydic function through
aldol-type condensation at the methylene part of the ring C-
4(or 5) position, even under noncatalytic and physiological
conditions. In the \( ^1\)H-NMR analysis of the reference 2MIone,
the addition of deuterium oxide to the solvent of DMSO-\(d_6\)
almost brought about the disappearance of the methylene
proton signal (around at 3.9 ppm), so that its portion must be
activated to cause a hydrogen-deuterium exchange with ease.
This characteristic is probably due to the electron-attracting
effect of the neighboring carbonyl group and tertiary nitro-
gen atom, as previously postulated concerning this class of
compounds in the field of organic chemistry.\textsuperscript{10} Further, when
a preliminary examination using Im as a substrate was
performed under the same conditions as those for 2MI,
LC/MS/MS data indicated that the addition of acetaldehyde
or propionaldehyde to the Cu-VC system resulted in the
probable formation of an aldol condensate, 4(or 5)-ethyli-
dene-4(5H)(or 5(4H))-imidazolone or its analogue with a
propylidene moiety, respectively (spectra not shown). This
coincidentally suggests that Im-derived nucleophilic 4(5H)
(or 5(4H))-imidazolone was generated similarly in the cat-
alytic system.

A considerable amount of \( P_i \) derived from 2MI was found
in the Cu-VC system (Fig. 1), but not enough structural in-
formation was available. Because the reaction of N-benzoyl-
histidine in the same catalytic system can yield some imida-
zole-ring-ruptured products through a series of free radical
reactions,\textsuperscript{13} a resemblant pathway may be responsible for
the \( P_i \) formation. Besides, \( P_i \) is also separable by HPLC with the
aid of a counterion effect of perfluoropentanoic acid, which is
able to be distilled away, while \( P_5, P_6, \) and \( P_8 \) remain in a
mixture. The two fractions on the HPLC were obtained from
a 4-h-reacted \( ^{14}\)C mixture of the Cu-VC system through mul-
tiple injections/peak collections, evaporated to dryness, and
then subjected to the *in vitro* examination for binding to
slices of dog aorta by a previously described method.\textsuperscript{13} As
a result of autoradiographic detection, the tissue-bound
radioactivity was observed for the mixed fraction of \( P_5-P_8 \),
whereas no radioactivity was observed for the \( P_i \) fraction. We
thus think that, although a large amount of \( P_i \) in the Cu-VC
system was formed, its fraction is not involved in the binding
of 2MI with connective tissue.

It was observed that the catalytic oxidation in the Cu-VC
system yields a reactive imidazolone product at physiological
pH and temperature. Although in the interest of obtaining a
sufficient quantity of the product for structural analysis the
concentrations of the catalytic agents were set up high in the
present study, our previous study\textsuperscript{17} has indicated that the *in
vitro* irreversible binding of Im or 2MI equivalents to con-
ective tissue is inducible by the catalysts at concentration
levels comparable with their contents of such biological fluids
as whole blood, plasma, or serum in human or laboratory
animals.\textsuperscript{12—14} It is thus conceivable that the catalytic oxida-
tion might also take place to some extent during *in vivo*
circulation. We have proposed that, as a possible interpreta-
tion of the retention of imidazole-containing drugs in connective
tissue, an imidazole nucleus could combine with aldehyde
residues found in elastin or collagen through an oxidative
form of biotransformation.\textsuperscript{1,7} The findings in the present
study coincide with and support the above proposal; hence,
the depiction of a reaction scheme shown in Fig. 7 might be
within the bounds of possibility. It is theoretically supposed
that, having no regard for steric hindrance, the reaction path-
way expands further to the reductive 1,4-Michael addition of
free forms of the nucleophilic imidazolones to an \( \alpha, \beta \)-unsat-

![Fig. 7. A Postulated Bio-Reaction Scheme for the Covalent Binding Formation of Imidazoles in Connective Tissue](image-url)
urated carbonyl system of the ready-built imidazolone-protein adducts. Such a related product could not be detected, however, in the present model experiment.

In the normal construction process of elastin or collagen fibers, the aldehydeic function plays an essential role as a basic intermediate of covalent cross-linkages that contribute importantly to elasticity or tensile strength for the respective fibers.15 Strictly speaking, the biogenic aldehyde comprises the two types of peptidyl residue, namely, allylamine (α-aminoacidic acid δ-semialdehyde) and hydroxyallysine (δ-hydroxy, α-aminoacidic acid δ-semialdehyde).16 The former is biosynthesized from peptidyl lysine and is found in both elastin and collagen,15 but its contents of these fibrous proteins have been reported to vary and to be elastin>collagen on comparison per 10⁷ amino acids.17 On the other hand, the latter aldehydic compound is biosynthesized from the hydroxylated form of peptidyl lysine, which is found not in elastin but in collagen.15 Moreover, the aldehydic function of hydroxyallysine has been suggested to be less reactive than that of allylamine through the ability to shift to a 5-keto group in a keto-enol mechanism (-CHOH–CHO ⇔ -C(OH)=CHOH ⇔ -CO–CH₂OH).15 Our previous study has shown that the in vivo retention of Im and 2MI equivalents in connective tissue is largely based on their preferential binding to elastin.7 Consequently, it is reasonable to speculate that some of the imidazoles are more likely to form a covalent adduct with the allylamine aldehyde. Additional studies are necessary to investigate this point, in order to discuss the eliciting-organ-selective toxic potential for imidazole analogues, which depends on the prevention of the normal allylamine-derived cross-linking.

In conclusion, we here present evidence for the aldehyde adduct formation of a 4(5H)-or 5(4H)-imidazolone product in vitro, which might provide a mechanistic explanation for the marked retention of several imidazole-containing drugs in connective tissue. To confirm the authenticity of this explanation, analysis of the biological samples after dosing of imidazole compounds is currently under way in the search for imidazolone-related structures as metabolites (including adducts with endogenous carbonyls).

Acknowledgments The authors are indebted to Mr. Y. Nakamura, Ms. Y. Watanabe, and Ms. H. Inuma for spectral measurement, and to Dr. Y. Ohuchi for provision of the reference compounds.

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