Synthesis and Antioxidative Activity of 6-HydroxyPyridoxine

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Summary An attempt to synthesize 6-hydroxypyridoxine (OPN), hydroxylation on C-6 of pyridoxine (PN) by hydroxyl radical (OH·), was conducted. Application of two well-known OH·-generating reactions, i.e. the Fenton reaction and the Fe²⁺-EDTA/ascorbate reaction, were unsuccessful, as large amounts of by-products were formed. Although generation of OH· by autoxidation of ascorbic acid in the absence of metal ions was slow, by-products were formed in small quantities, and OPN was easily obtained in colorless crystals. Its structure was confirmed by spectral analyses. OPN was comparable to polyphenols such as (+)-catechin, rutin and gallic acid in the antioxidative activity against linoleic acid peroxidation, and was an effective DPPH radical scavenger, though the DPPH radical-scavenging activity of OPN was somewhat lower than that of the polyphenols. PN was relatively inactive under the conditions used here, indicating that the introduction of a hydroxyl group on C-6 of PN greatly enhanced both activities.

Key Words 6-hydroxypyridoxine, pyridoxine, vitamin B₆, antioxidative activity, radical-scavenging activity
and then mixed with 0.1 mL of 30% ammonium thio-
cyanate and 0.1 mL of 20 mM ferrous chloride in 3.5% 
hydrochloric acid. After the mixture was vigorously 
shaken, and allowed to stand at room temperature for 
3 min, an absorbance of 500 nm was measured. The 
antioxidative activity was calculated as \((A-B)/A\times 100\), 
where \(A\) was the absorbance in the absence of the anti-
oxidant, and \(B\) was that in its presence.

A radical-scavenging assay was performed by the 
method of Nagashima et al. (8) with a slight modifica-
tion. A reaction mixture consisted of 2 mL of 0.1M ace-
tion buffer (pH 5.5), 2 mL of ethanol and 1 mL of 
0.5 mM DPPH in ethanol, the antioxidant being dis-
solved in the buffer or ethanol. After the reaction mix-
ture was allowed to stand at room temperature for 
30 min in the dark, an absorbance of 517 nm was mea-
sured. The radical-scavenging activity was calculated as 
\((A-B)/C\times 100\), where \(A\) was the absorbance at 0 min 
of the reaction in the absence of the antioxidant, \(B\) was 
that at 30 min in its presence, and \(C\) was that at 30 min in 
its absence.

**Synthesis of OPN**

Hydroxyl radical (OH-) is known to engage in the 
ring-hydroxylation of a variety of aromatic compounds. 
The Fenton reaction, which is a one-electron reduction 
of hydrogen peroxide \((\text{H}_2\text{O}_2)\) by metal ions, generates 
OH- as shown in the Eq. (1).

\[
\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \text{OH}^- + \text{OH}^- \quad (1)
\]

This reaction was applied on a small scale to hydrox-
ylation of PN. Reaction mixtures (10 mL) consisted of 
40 mM PN; 20, 40, 100 and 200 mM \(\text{H}_2\text{O}_2\); and 20, 40, 
100 and 200 mM ferrous sulfate (a molar ratio of \(\text{H}_2\text{O}_2\) 
to \(\text{Fe}^{2+}\) of 1:1). After the reaction at 30°C in the dark, 
reaction products were analyzed at different intervals 
by HPLC. The OPN formation was completed in 1 h 
with a maximum amount of OPN at 100 mM \(\text{H}_2\text{O}_2\) and 
100 mM \(\text{FeSO}_4\), but unidentified by-products 
were formed in much larger quantities than OPN: this 
reaction was unsuccessful.

The Fe\(^{2+}\)-EDTA/ascorbate reaction is also a well-
known OH- generating one. Slivka and Cohen (9) 
showed that dopamine was hydroxylated by this reac-
tion to give three distinct products, namely 2-hydroxy-
dopamine, 5-hydroxydopamine and 6-hydroxydopa-
mine. In a preliminary experiment (10 mL of a reaction 
mixture) of hydroxylation of PN by the Slivka and 
Cohen method, by-products were formed in small quan-
tities. Accordingly, as a large scale experiment, a reac-
tion mixture (1.5 L), pH 7, containing 4 mM PN, 1 mM 
AH\(_2\), 0.24 mM EDTA and 0.2 mM ferrous ammonium 
sulfate in a 3 L-beaker was incubated and stirred at 
30°C in the dark. AH\(_2\) was completely oxidized in 3 d, 
and the brown reaction mixture was concentrated under reduced pressure to 20 mL at about 40°C. During 
centrifugation, it turned dark-brown and OPN was con-
siderably decomposed. Fe\(^{2+}\)-EDTA seemed to accelerate 
the OPN degradation. Furthermore, the presence of col-
ored substances made it difficult to obtain pure OPN: a 
yield of a crude preparation, 35 mg. The presence of 
Fe\(^{2+}\)-EDTA was considered to give this unfavorable 
result.

It is also known that OH- is generated via autoxida-
tion of AH\(_2\) in the absence of metal ions as shown in 
the Eqs. (2)-(4) (10),

\[
\begin{align*}
\text{AH}_2 + \text{O}_2 & \rightarrow \text{A} + \text{H}_2\text{O}_2 \quad (2) \\
\text{AH}_2 + \text{O}_2 & \rightarrow \text{AH}^- + \text{O}_2^- + \text{H}^+ \quad (3) \\
\text{H}_2\text{O}_2 + \text{O}_2^- & \rightarrow \text{OH}^- + \text{OH}^- + \text{O}_2 
\end{align*}
\]

where AH\(_2\) means a reduced form of ascorbic acid, AH- 
an ascorbate free radical, and A means dehydroascorbic 
acid. OPN synthesis was carried out by using this reac-
tion as follows. A mixture (1.5 L) containing 4 mM PN 
HCl and 2 mM sodium ascorbate was incubated with 
stirring at 45°C in the dark. Here, the yield of OPN was 
highest at a sodium ascorbate concentration of 2 mM. 
On day 9 of the incubation, AH\(_2\) was completely ox-
dized, and about 10% of PN was converted into OPN. 
The pale-yellow reaction mixture was concentrated to 
20 mL under reduced pressure at about 40°C. The con-
centrate was applied to a column \((1.6\times16\text{ cm})\) of 
Dowex 50 W\(^\times\)8 (H\(^+\)). PN was adsorbed on the resin, 
and OPN was eluted with distilled water, followed by 
fractionating the eluate. The fractions containing OPN 
were combined, and lyophilized. The yellowish lyophil-
sate was twice recrystallized from aqueous ethanol to 
give 90 mg of OPN in colorless needles. The present 
method gave 2.5-fold the yield of the previous method 
(1). Spectral data of the OPN specimen obtained were 
as follows. UV<sub>λ</sub>max (0.1 M HCl) nm: 323, λ<sub>λ</sub>max (0.1 M 
NaOH): 360, IR<sub>λ</sub>max, (KBr): 3100, 2850, 1640, 1538, 
1460, 1380, 1340, 1258, 1010, 922, 730, 635, EIMS 
m/z: 185 \((M^+)\), 167 (\(M^-\)-H\(_2\)O), 149 (167-H\(_2\)O), 139 
(167-CO), 138 (139-H), 122 (167-CHO\(_2\)), 121 
(149-CO), 110 (138-CO), 108 (149-CH\(_3\)CN), 80 
(121-CH\(_3\)CN). These data were identical with those 
described in the previous paper (1).

**Antioxidative and radical-scavenging activities of 
6-hydroxyppyridoxine**

Antioxidative and radical-scavenging activities of 
OPN were evaluated in comparison with those of PN, 
PL, PM, BHT, dl-\(α\)-tocopherol, (+)-catechin, rutin, gal-
lic acid, gentisic acid and catechol. The antioxidative 
activity of these compounds against LA peroxidation 
was measured in the LA system and the LA-AAPH sys-
tem at two different concentrations (Table 1). In the LA 
system, the order of the antioxidative activity was as 
follows: rutin, BHT>>dl-\(α\)-tocopherol>(+)-catechin, 
gentisic acid, gallic acid>OPN>catechol. In the LA-
AAPH system, the decrease in the antioxidative activity 
was in the following order: BHT>dl-\(α\)-tocopherol, 
rutin>OPN>gallic acid, catechol, (+)-catechacin>gen-
tisic acid. In both systems, BHT, rutin and dl-\(α\)-toc-
opherol were the most effective, and OPN was 
comparable to polyphenols in the antioxidative activity, 
though the order of the antioxidative activity of OPN 
and polyphenols was different between the two systems. 
PN, PL and PM only slightly suppressed the LA peroxi-
dation in both systems.

The radical-scavenging activity of OPN against DPPH
Table 1. Antioxidative activity of 6-hydroxypyridoxine, vitamin B₆, and some antioxidants. The antioxidative activity of 6-hydroxypyridoxine, vitamin B₆, and some antioxidants against linoleic acid peroxidation was determined at concentrations of 0.3 and 0.9 mM in the linoleic acid and linoleic acid-AAPH systems as described in the text.

<table>
<thead>
<tr>
<th></th>
<th>Linoleic acid system</th>
<th>Linoleic acid-AAPH system</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>0.3 mM</td>
<td>0.9 mM</td>
</tr>
<tr>
<td>6-Hydroxypyridoxine (OPN)</td>
<td>63</td>
<td>75</td>
</tr>
<tr>
<td>Pyridoxine</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Pyridoxal</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Pyridoxamine</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>BHT</td>
<td>95</td>
<td>95</td>
</tr>
<tr>
<td>α-Tocopherol</td>
<td>69</td>
<td>88</td>
</tr>
<tr>
<td>(+)-Catechin</td>
<td>69</td>
<td>80</td>
</tr>
<tr>
<td>Rutin</td>
<td>96</td>
<td>97</td>
</tr>
<tr>
<td>Gallic acid</td>
<td>65</td>
<td>78</td>
</tr>
<tr>
<td>Gentisic acid</td>
<td>64</td>
<td>80</td>
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<tr>
<td>Catechol</td>
<td>39</td>
<td>67</td>
</tr>
</tbody>
</table>

1 Values are the means of three determinations.

Table 2. Radical-scavenging activity of 6-hydroxypyridoxine, vitamin B₆, and some antioxidants. The radical-scavenging activity of 6-hydroxypyridoxine, vitamin B₆, and some polyphenols against DPPH was determined at concentrations of 25 and 300 μM as described in the text.

<table>
<thead>
<tr>
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<th>DPPH scavenging activity (%)²</th>
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<tbody>
<tr>
<td></td>
<td>25 μM</td>
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<tr>
<td>6-Hydroxypyridoxine (OPN)</td>
<td>42</td>
</tr>
<tr>
<td>Pyridoxine</td>
<td>0</td>
</tr>
<tr>
<td>Pyridoxal</td>
<td>0</td>
</tr>
<tr>
<td>Pyridoxamine</td>
<td>0</td>
</tr>
<tr>
<td>(+)-Catechin</td>
<td>64</td>
</tr>
<tr>
<td>Rutin</td>
<td>50</td>
</tr>
<tr>
<td>Hesperidin</td>
<td>0</td>
</tr>
<tr>
<td>Gallic acid</td>
<td>63</td>
</tr>
<tr>
<td>Gentisic acid</td>
<td>48</td>
</tr>
<tr>
<td>Catechol</td>
<td>50</td>
</tr>
</tbody>
</table>

² Values are the means of three determinations.

was compared with that of PN, PL, PM and polyphenols at concentrations of 25 and 300 μM (Table 2). OPN was an effective DPPH radical scavenger, though the activity of OPN was smaller than that of polyphenols except hesperidin. PN, PL and PM did not donate one electron to DPPH radical even at a high concentration, 300 μM.

In conclusion, introduction of a hydroxyl group on C-6 of PN greatly enhanced the antioxidative and radical-scavenging activities, and the activities of OPN were comparable to those of polyphenols.

REFERENCES