Synthesis of Spin Labels for ESR Imaging of Living Rat Head

Ryuji Niwa,* Ryusei Konaka, Midori Hiramatsu, and Hitoshi Kamada

Institute for Life Support Technology, Yamagata Technopolis Foundation, 683 Kuramonomae, Numagi, Yamagata 990, Japan. Received August 8, 1996; accepted December 10, 1996

Spin labels (7, 10, 13, 16, 22, 27) were synthesized from piperidinylloxyl (1), pyrrolidinylloxyl (2), and oxazolidinylloxyl (3). These compounds were injected into the carotid artery of anesthetized rats, and the ESR spectra of the rat brain were immediately recorded by the use of an L-band ESR spectrometer. Based on the spectra obtained, we considered whether or not these spin labels can pass the blood brain barrier and bind to brain tissue components.

Key words L-band ESR; piperidinylloxyl; pyrrolidinylloxyl; oxazolidinylloxyl; psychotropic agent

Spin labeling is a method to study the environmental status of a target into which a stable nitroxide radical (spin label) has been inserted by measuring the change of the ESR spectrum caused by the change of motion of the spin label.1–3 Typical spin labels [piperidinylloxyl (1), pyrrolidinylloxyl (2), oxazolidinylloxyl (3)]1–3 are shown in Chart 1. Spin labeling is used in various fields, e.g., studies of cell membrane state, spin label oximetry, spin-immunoassay, spin-clearance of a drug, and ESR imaging. In principle, if a spin label combines with some tissue component, the ESR spectrum should change.4 Accordingly, this method should be applicable to ESR imaging of a specific tissue.

Cytidine 5'-diphosphate choline, nordiazepam, haloperidol, and phenothiazine have been used as psychotropic agents, and are considered to pass the blood brain barrier. They have been reported5 to act at neurotransmitter receptors of acetylcholine, dopamine, norepinephrine, and serotonin.

Extensive work has been done on the synthesis of spin label reagent such as oxazolidine,6 oxazolidinylloxyl,7 pyrrolidinylloxyl,8 piperidinylloxyl (4),9 2,2,6,6-tetramethyl-4-(uridine 5'-diphospho)-1-piperidinylloxyl,10 2-chloro-10-[3-(carboxyl-2,2,5,5-tetramethyl-1-pyrrolinyl-oxyl)aminopropyl]phenothiazine.11 We utilized the procedures of Weiner9 and Berliner and Wong,10 to condense 4 with 5 and 6, affording the piperidinylloxyl (7) in 11% and 9% yields, respectively (Chart 2). When 812 was added to the diphosphate (9), 10 was obtained in 20% yield (Chart 3). Reaction of nordiazepam (11)13 with the pyrrolidinylloxyl (12) gave the pyrrolidinylloxyl (13) in 72% yield (Chart 4). Similarly, the reaction of 11 with 2-chloroethylamine monohydrochloride gave 14, which was condensed with the oxazolidinylloxyl (15) to give the oxazolidinylloxyl (16) in 69% yield (Chart 5). 19 was prepared from the reaction of 17 with ethyl isonpecotate (18) by a standard method.14 It was treated with 0.1 N NaOH to give the carboxylic acid (20) in good yield. Then 20 was allowed to react with 21, giving the piperidinylloxyl (22) in 62% yield (Chart 6).

* To whom correspondence should be addressed.

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Table 1. Analytical and Spectral Data for 7, 10, 13, 16, 19, 20, 22, 25, 26, and 27

<table>
<thead>
<tr>
<th>Compd.</th>
<th>Formula</th>
<th>C</th>
<th>H</th>
<th>N</th>
<th>IR (KBr) cm⁻¹</th>
<th>EI-MS m/z</th>
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</thead>
<tbody>
<tr>
<td>7</td>
<td>C₁₈H₁₉N₂O₅Cl₂</td>
<td>38.78</td>
<td>5.61</td>
<td>10.05</td>
<td>93.08</td>
<td>5.90</td>
</tr>
<tr>
<td>10</td>
<td>C₂₂H₁₇Cl₅O₅Cl₂</td>
<td>49.73</td>
<td>6.21</td>
<td>10.53</td>
<td>40.01</td>
<td>6.49</td>
</tr>
<tr>
<td>13</td>
<td>C₂₉H₂₃Cl₅N₄O₄</td>
<td>52.97</td>
<td>6.38</td>
<td>12.66</td>
<td>63.01</td>
<td>6.35</td>
</tr>
<tr>
<td>16</td>
<td>C₃₀H₂₃Cl₅N₄O₄</td>
<td>68.85</td>
<td>8.30</td>
<td>8.24</td>
<td>68.71</td>
<td>8.33</td>
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<tr>
<td>19</td>
<td>C₂₉H₂₃F₅N₄O₄</td>
<td>67.27</td>
<td>7.53</td>
<td>4.36</td>
<td>67.01</td>
<td>7.33</td>
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<tr>
<td>20</td>
<td>C₂₉H₂₃F₅Cl₅O₄</td>
<td>65.51</td>
<td>6.87</td>
<td>4.87</td>
<td>65.69</td>
<td>6.95</td>
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<tr>
<td>22</td>
<td>C₂₉H₂₃F₅N₄O₃</td>
<td>67.23</td>
<td>8.35</td>
<td>9.41</td>
<td>67.40</td>
<td>8.50</td>
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<td>25</td>
<td>C₁₅H₁₁Cl₄NS</td>
<td>58.07</td>
<td>4.22</td>
<td>4.52</td>
<td>58.31</td>
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<td>26</td>
<td>C₂₄H₁₇Cl₄N₄O₃</td>
<td>64.77</td>
<td>7.02</td>
<td>9.44</td>
<td>65.01</td>
<td>7.21</td>
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<tr>
<td>27</td>
<td>C₂₄H₁₇Cl₄N₄O₃</td>
<td>65.41</td>
<td>7.25</td>
<td>9.15</td>
<td>65.60</td>
<td>7.36</td>
</tr>
</tbody>
</table>

Lastly, the phenothiazine (25), prepared from 23, was allowed to react with 21 to give the piperidinylxyl (26). The reaction of 26 with methyl iodide gave 27 in 87% yield (Chart 7). Elemental analyses and spectral data for the spin labels (7, 10, 13, 16, 19, 20, 22, 25, 26, 27) are summarized in Table 1.

The X-band ESR spectra of nitroxide in the spin labels (7, 10, 13, 16, 22, 27) in dimethyl sulfoxide (DMSO) showed the characteristic three lines in a symmetrical pattern with $g = ca. 2.006$ — and the nitrogen hyperfine splitting constant ($hfsc$) $a_{N} = ca. 1.6$mT —. For example, Fig. 1 shows the ESR spectrum of 16 in DMSO ($g = 2.0061$, $a_{N} = ca. 1.68$mT).

The spin labels (7, 10, 13, 16, 22, 27) in DMSO were injected into the left carotid artery of anesthetized living rats. Immediately, the ESR spectra of the rat head were measured using L-band ESR with a loop gap resonator. The ESR spectra of 7, 10, 13, 22, and 27, but not 16, showed a three line signal, similar to that in Fig. 1, at the expected magnetic field. For example, Fig. 2 shows the ESR spectrum of 13. Thus, these compounds do not combine with any brain tissue component.

Compound 16 showed a change from the symmetrical pattern under the same ESR conditions as used for the other spin labels (Fig. 3). Usually, when a spin label combines with some high-molecular substance, the ESR spectrum shows an unsymmetrical pattern. Therefore, 16 seems to have passed the blood brain barrier and combined with some brain tissue component. This result suggests the feasibility of using ESR-CT (computed tomography) to image pathologic foci in the brain.

**Experimental**

Melting points were determined on a Yanaco model MP apparatus and are uncorrected. IR spectra were taken with a JASCO IR-810 spectrophotometer. $^1$H-NMR spectra were recorded by using tetramethylsilane as an internal standard on JEOL JNM PMX-90 spectrometers at 60 MHz. Mass spectra were recorded with a JEOL JMS-OISG-2 mass spectrometer. Wako gel (C-200) was employed for silica gel column chromatography.

**4-(Cytidine 5'-Diphosphoryl)-2,2,6,6-tetramethyl-1-piperidinylxyl (7).**

**a) Reaction of Cytidine 5'-Monophosphonomorpholinate (5) with 2,2,6,6-Tetramethyl-4-phosphoryl-1-piperidinylxyl (4)**

A solution of tri-n-octylamine (6.7 ml) in dry pyridine (10 ml) was added dropwise to a solution of 4 (6.31 g, 25.0 mmol) in dry pyridine (10 ml) at 5–10°C with stirring. This mixture was stirred at 5–10°C for 2 h, then a solution of 5 (1.96 g, 5.00 mmol) in dry pyridine (10 ml) was added dropwise at room temperature with stirring. The reaction mixture was concentrated in vacuo, and the residue was coevaporated with dry pyridine in vacuo. The reaction was allowed to proceed at room temperature in dry pyridine (15 ml) for 5 d and was terminated by the addition of water followed by lithium acetate (1.0 g). The tri-octylamine was extracted with ether. The aqueous fraction was applied to a Dowex 1 × 2 (200–400 mesh) column in the chloride form. The product was eluted as the second-to-last band using a 3-1 gradient of 0.01–0.5 M LiCl in 0.1 M NH₄Cl. The fractions were pooled and concentrated to a small volume for desalting on a Sephadex G-10 (3.0×50 cm) column. Further purification was...
achieved by repeated elution through another Dowex 1 x 2 (1 x 20 cm) column with a 1-1 gradient of 0.005–0.6 M LiCl in 0.005 n HCl (25 ml/h flow rate) prepared with stirring. The mixture was adjusted to pH 10 with LiOH, and desalted on Sephadex G-10. The solution was evaporated and reconstituted with MeOH. Precipitation with ether gave 7, colorless powder, mp 216–219 °C, 0.31 g (11%).

b) Reaction of Cytidine 5'-Monophosphate (6) with 4 Compound 6 (1.62 g, 5.02 mmol) was dissolved in dry N,N-dimethylformamide (DMF) (30 ml), then 4 (6.31 g, 25.0 mmol) and p-TsCl (0.5 g) at 5–10 °C were added with stirring. This mixture was stirred at room temperature for 1 h, then the mixture was added to Dowex 1 x 2 (200–400 mesh) column in the chloroform form. The eluted product was collected using 10 M LiCl in 0.1 M HCl, concentrated on a Sephadex G-10 (30 x 30 cm) column and precipitated with ether to give 7, 0.25 g (9%).

Cytidine 5'-Phosphophosphonooxyl-yl-N,N-dimethylaminoo- 2,2,6,6-tetramethyl-1-piperidinylxolyl (10) Following the procedure described above, 8 (0.7 g, 25.0 mmol), p-TsCl (0.5 g) was added to a solution of 9 (1.40 g, 5.00 mmol) in dry pyridine (30 ml) at 5–10 °C with stirring. Work-up as above gave 10, colorless powder, mp 234–237 °C, 0.67 g (20%).

3-[2-(7-Chloro-1,3-dihydro-2-oxo-5-pentyl-2H-1,4-benzodiazepinyl)- acetamido]-propylcarbamoyl]-2,2,5,5-tetramethyl-1-pyrrolidinilxolyl (13) A solution of 12 (1.25 g, 0.011 mol) in 0.01 M HCl was added to a suspension of 11 (1.35 g, 4.98 mmol), sodium hydride (63% dispersion in mineral oil) (132 mg, 5.50 mmol) in DMF (10 ml) with stirring and ice-cooling. The reaction was carried out for 10 min to complete. The mixture was stirred at room temperature for 4 h, then allowed to stand at room temperature overnight. It was poured into ice water, and was extracted with ethyl acetate ether. The organic layer was washed with water, dried over anhydrous sodium sulfate, and concentrated to vacuo. The residue was subjected to silica gel (100 g) column chromatography. Elution with hexane-ethyl acetate (1:1) gave 13 as colorless needles, mp 241–244 °C (from ethyl acetate). Yield, 1.99 g (72%).

2-(14-[7-Chloro-1,3-dihydro-2-oxo-5-pentyl-2H-1,4-benzodiazepinyl)- N-ethylcarbamamyl]-tetraethyl-2-ethyl-4,4-dimethyl-3-oxazolidinylxolyl (16) A solution of 2-chloroethylamine monohydrochloride (0.7 g, 6.0 mmol) in DMF (10 ml) was added dropwise to a suspension of 11 (1.35 g, 4.98 mmol) and sodium hydride (63% dispersion in mineral oil) (132 mg, 5.50 mmol) in DMF (10 ml) with stirring and ice-cooling. The mixture was stirred at room temperature overnight. A solution of triethylamine (0.61 g, 6.04 mmol) in DMF (10 ml) was added dropwise with stirring and ice-cooling. The whole was stirred for 2 h at ice-cooling. Next, a solution of 15 (2.0 g, 6.99 mmol) in ether (20 ml) was added with stirring, and then a solution of 1,3-dichloro carbodiimide (DCC) (1.65 g, 8.01 mmol) in dry ether (20 ml) was added with stirring and ice-cooling. The reaction mixture was stirred at room temperature for 4 h. The precipitate was filtered off, and the filtrate was concentrated in vacuo. The residue was subjected to silica gel (200 g) column chromatography. Elution with hexane-ethyl acetate (3:1) gave 16, colorless needles (from ethyl acetate), mp 238–240 °C. Yield, 2.34 g (4%).

ESR Measurement Male Wistar rats, 200 g, were anesthetized with an intraperitoneal injection of 50 mg/kg pentobarbital. A polyethylene tube (0.25 x 0.75 mm), filled with saline was inserted into the cervical portion of the left carotid artery. Following arterial cannulation, the head was inserted into a loop gap resonator centered between the ESR magnet. Then a spin label (10 ml of 0.4 M solution) was infused over 30 s through the intra-carotid cannula. The L-band ESR spectrometer (JEOL, Tokyo) consists of a pair of field gradient coils (Yonezawa Electric Wire Co., Ltd., Yonezawa, Yamagata) and a computer (5450, Concurrent Computer Corporation, Massachusetts, U.S.A.). ESR spectra were recorded at 700 mHz by the L-band ESR spectrometer with an electrical shield in the loop of the gap resonator. The log gap resonator was 41 mm in diameter and 10 mm in axial length.

Acknowledgements The authors are grateful to Prof. N. Katagiri, Pharmaceutical Institute of Tohoku University, for valuable comments.

References and Notes
5) "Medical Chemistry IV," ed by Yamakawa K., Kanoaka Y., Iwasa Y., Morisaki I., Kodansha, Tokyo, 1993, pp. 79–139.
13) “Progress in Medical Chemistry,” ed. by Ellis G. P., West G. B., Elsevier Science Publishers, B. V., 1983, pp. 157—163. Compound II was kindly provided by Prof. Mori (Department of Neuroscience, Institute for Molecular and Cellular Medicine, Okayama University).