IDENTIFICATION OF RAT URINARY AND BILIARY METABOLITES OF ESONARIMOD, A NOVEL ANTIRHEUMATIC DRUG, USING LIQUID CHROMATOGRAPHY/ELECTROSPRAY IONIZATION TANDEM MASS SPECTROMETRY WITH POSTCOLUMN ADDITION OF 2-(2-METHOXYETHOXY)ETHANOL, A SIGNAL-ENHANCING MODIFIER

JUN-ICHI YAMAGUCHI, MARI OHMICHI, MASATOSHI HASEGAWA, HIDEO YOSHIDA, NAOYOSHI OGAWA, AND SHOHEI HIGUCHI

Drug Metabolism Laboratory, Pharmaceutical Research Laboratories, Taisho Pharmaceutical Co., Ltd., Saitama, Japan

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ABSTRACT:

The biotransformation of esonarimod (KE-298) \([±]2\text{-acetylthioethylmethyl}-4\text{-}(4\text{-methylphenyl})4\text{-oxobutanoic acid}\), a new antirheumatic drug, was investigated in rats. Urinary and biliary excretions within 24 h after oral administration of 5 mg/kg \([14C]\)esonarimod accounted for 89 and 10% of the dose, respectively. Initial metabolite analysis by liquid chromatography/electrospray ionization tandem mass spectrometry with negative ion mode, in which a mobile phase of 20 mM ammonium acetate (pH 4.6)/methanol with gradient-elution mode was used, failed to obtain any structural information on most of the metabolites due to poor sensitivity. To overcome this problem, postcolumn addition of 2-(2-methoxyethoxy)ethanol, a powerful signal-enhancing modifier, to the mobile phase was used, allowing pronounced signal enhancement and structural elucidation of urinary and biliary metabolites. The results of metabolite analysis suggested that esonarimod is predominantly biotransformed to a pharmacologically active metabolite, thiol-containing deacetyl-esonarimod (M-I), and subsequently undergoes extensive metabolism, mainly S-methylation followed by the combination of S-oxidation and oxidative conversion of the aromatic methyl group. No disulfide metabolites, such as M-I-cysteine mixed disulfide and M-I-dimer, were found in the excreta. This finding is probably evidence supporting the notion that the reactivity of the thiol moiety of M-I with macromolecules in vivo is extremely lower than that of common thiol-containing drugs.

Immunomodulators that can improve both decreased and augmented immunological function are expected to provide fundamental treatment of rheumatoid arthritis. A series of thiol-containing immunomodulators such as D-penicillamine (Otomo et al., 1981; Nakaike et al., 1985), SA-96 (Fujimura et al., 1980), and tiopronin (Pasero et al., 1993) have exhibited various adverse effects, including thrombocytopenia, rash, and proteinuria (Craig and Buchanan, 1980; Suda et al., 1993). These toxic profiles are thought to be due to the high reactivity of thiol groups in the parent drugs or their metabolites with macromolecules in vivo (Coleman et al., 1988; Park and Kitteringham, 1990), and have resulted in the limited therapeutic potential of such agents.

Esonarimod (KE-298) \([±]2\text{-acetylthioethylmethyl}-4\text{-}(4\text{-methylphenyl})4\text{-oxobutanoic acid}\), has been shown to be an effective immunomodulator with inhibitory effects on inflammatory cytokine production (Kameo et al., 1988; Takeshita et al., 1988; Nagai et al., 1996; Takahashi et al., 1998). This compound contains a thioacetyl group and is easily deacetylated in vivo to form a pharmacologically active metabolite (thiol-containing deacetyl-esonarimod, M-I) that is a principal circulating metabolite (Yasuda et al., 1996; Yoshida et al., 1996). The toxicity of esonarimod is significantly lower than that of D-penicillamine in rats (unpublished data). It is suggested that the low toxicity may be closely related to the low reactivity of the thiol moiety of M-I with macromolecules in vivo (Yoshida et al., 1996). To support the low reactivity of M-I in vivo, the metabolic profile of esonarimod should be clarified. The objective of this study was to investigate the in vivo metabolism of esonarimod in rats by identifying its urinary and biliary metabolites using liquid chromatography/electrospray ionization-tandem mass spectrometry (LC/ESI-MS/MS\(^1\)) with postcolumn addition of 2-(2-methoxyethoxy)ethanol (2-MEE), a powerful signal-enhancing modifier (Yamaguchi et al., 1999).

**Materials and Methods**

**Chemicals.** \([14C]Esonarimod** (Fig. 1) with a specific radioactivity of 762 kBq/mg was obtained from Amersham Pharmacia Biotech UK, Ltd. (Buckinghamshire, UK). Its radiochemical purity determined by radio-HPLC was not less than 98%. Unlabeled esonarimod and the following metabolites were synthesized at the Pharmaceutical Research Laboratories of Taisho Pharmaceutical Co., Ltd. (Saitama, Japan): KE-748 (M-II) \([±]4\text{-}(4\text{-methylphenyl})2\text{-methylthiomethyl}-4\text{-oxobutanoic acid}\); KE-749 (M-III) \([±]4\text{-}(4\text{-methylphenyl})2\text{-methylsulfinylmethyl}-4\text{-oxobutanoic acid}\); KE-767 (M-IV) \([±]4\text{-acetate acid; AoD}, acetate anion; [M-H], deprotonated molecular ion; b.p., boiling point; CID, collision-induced dissociation; u, unified atomic mass unit; Glc, glucuronide.

\(^1\) Abbreviations used are: LC/ESI-MS/MS, liquid chromatography/electrospray ionization-tandem mass spectrometry; 2-MEE, 2-(2-methoxyethoxy)ethanol; HPLC, high-performance liquid chromatography; AcONH\(_4\), ammonium acetate; AcOH, acetic acid; AcO, acetate acid; AoD, acetate anion; [M-H], deprotonated molecular ion; b.p., boiling point; CID, collision-induced dissociation; u, unified atomic mass unit; Glc, glucuronide.
Three rats and one additional rat were administered [14C]esonarimod, a dose of 5 mg/kg was given orally to the animals as a suspension in 5% gum arabic and the ESI source.

Mobile phase was delivered with Pump 1 and/or Pump 2 at a flow rate of 100 μl/min, was 2% solvent B for 10 min, and subsequent linear ramps from 2 to 15% (25 min), from 15 to 20% (25 min), from 10 to 10% (25 min), from 0 to 10% (2–25 min), from 10 to 15% (25–70 min), from 15 to 20% (70–85 min) followed by a 15-min holding time (85–100 min), from 20 to 40% (100–120 min), and from 40 to 100% (120–135 min) followed by a 3-min holding time (135–138 min).

Metabolite Quantification. For determination of metabolite composition, urine and bile specimens obtained after oral administration of [14C]esonarimod were subjected to radio-HPLC analysis, which was performed on a Jasco Gulliver HPLC system (Tokyo, Japan) with a Raytest Ramona-90 radioisotope detector equipped with a Raytest glass scintillation flow cell. Prior to analysis, the urine samples were filtered with the Ultrafree tubes. Separation was conducted with a Jasco J’sphere ODS M-80 (150 × 4.6-mm i.d., 4 μm, Kyoto, Japan) with the following gradient elution (separating conditions-3). Mixtures of solvent A (20 mM AcONH₄, pH 4.6, with AcOH) and solvent B (methanol) were used as a mobile phase. The linear gradient program was as follows: 2% solvent B for 10 min, and subsequent linear ramps from 2 to 10% (10–30 min), and from 10 to 20% (30–65 min).

HPLC Profiles of Metabolites. After oral administration of 5 mg/kg [14C]esonarimod to rats, most (89%) of the radioactivity was excreted in urine over 24 h, whereas a small percentage (10%) of the radioactivity was recovered from bile, indicating that urinary excretion was the predominant route of elimination of this drug. Typical gradient reversed phase HPLC profiles of the urinary and biliary metabolites, excreted within 24 h after oral administration, with separating conditions-1 described under Materials and Methods are shown in Fig. 2. A and B, respectively. At least eight peaks were found in the urine, while only one predominant peak was detected in the bile. The radioactive peak designated peak 1 in Fig. 2A was completely separated into two peaks using separating conditions-2 (Fig. 3).

Optimization of ESI. To optimize ESI, the effects of postcolumn addition of various water-miscible organic solvents with higher boiling points (b.p.) than AcOH (b.p. 118°C) to the mobile phase on the sensitivity of KE-766, a putative metabolite of esonarimod, was examined (Fig. 4). Although higher modifier b.p. tended to increase signal-enhancing ability, the effects of 2-methoxyethanol (b.p. 124°C) and 2-ethoxyethanol (b.p. 135°C) were not significant compared with
those of conventional modifiers, such as methanol (b.p. 64.7°C), acetonitrile (b.p. 81.6°C), and 2-propanol (b.p. 82.5°C). In contrast, 2-MEE, with a b.p. of 193°C, had 226 times the sensitivity of testing without addition of modifiers. Figure 5 compares the [M+2H]+ intensity of KE-766 with those obtained with several AcONH4 concentrations (0.5, 20, 50 mM) in the mobile phase, which were obtained without or with postcolumn addition of 2-propanol or 2-MEE. The postcolumn addition of 2-MEE prevented the decrease of the ESI responses resulting from increasing electrolyte concentrations in the mobile phase.

These findings led us to carry out metabolite analysis of esonarimod in Q1-full-scan mode, and results with and without postcolumn addition of 2-MEE were compared. The addition of 2-MEE allowed pronounced enhancement of ESI responses for all metabolites without affecting chromatographic performance. Representative, typical ESI-mass chromatograms for the urinary metabolites are depicted in Figs. 6 and 7.

**Metabolite Identification.** The structures of metabolites were elucidated by their chromatographic behavior as well as mass spectral data summarized in Table 1.

**Authentic esonarimod and KE-748.** As references, the key collision-induced dissociation (CID) fragmentation patterns of authentic esonarimod and KE-748, a synthetic S-methyl metabolite of M-I (M-II), are shown in Fig. 8. Esonarimod gave an [M−H]− at m/z 279 that underwent S-deacetylation subsequent to its dehydration by CID to yield m/z 237 (F1) and 219 (F2), respectively. The full-scan mass spectrum of M-II displayed an intense [M−H]− at m/z 251, and the ion underwent elimination of CH3SH and subsequent decarboxylation to form m/z 203 (F3) and 159 (F4), respectively. These characteristic product ions were used for structural analysis of the following in vivo metabolites.

**Metabolite M-III (peaks 4a and 4b).** The full-scan mass spectrum from peak 4a gave an [M+2H]+ at m/z 267, which was 16 unified atomic mass unit (u) greater than that of M-II. The product ion mass spectrum of the [M+2H]+ included m/z 203 (F3) and 159 (F4), which were similar to those of M-II. In addition, m/z 63 corresponding to the -SOCH3 moiety was detected. The mass spectral data for peak 4b were identical to those for peak 4a. Based on these results, the metabolite giving peaks 4a and 4b in reversed phase HPLC, i.e., M-III, was identified as the S-oxide metabolite of M-II, as a diastereomeric mixture. This was confirmed by comparison of the HPLC retention time and mass spectral data of M-III with those of synthetic standard (KE-749).

**Metabolite M-IV (peak 6).** The full-scan mass spectrum gave an [M−H]− at m/z 267, similar to that of M-III, while in the product ion mass spectrum of the [M−H]− included m/z 203 (F3) and 159 (F4), which were similar to those of M-II. The product ion underwent elimination of CH3SH and subsequent decarboxylation to form m/z 203 (F3) and 159 (F4), respectively. These characteristic product ions were used for structural analysis of the following in vivo metabolites.

**Metabolite M-V (peaks 2a and 2b).** No ion peaks from peaks 2a and 2b were detected using separating conditions-1 due to peak overlapping with biological endogenous compounds. Separating conditions-2 were therefore used to achieve good separation, as shown in Fig. 7B.
The full-scan mass spectrum for peak 2a gave an \([\text{M} - \text{H}]^-\) at \(m/z\) 283, which was 16 u greater than those of M-III and M-IV. The product ion mass spectrum of \(m/z\) 283 demonstrated ions corresponding to F3 and F4 at \(m/z\) 219 and 175, respectively, which were similar to those of M-IV. The mass spectral data for peak 2b were identical to those for peak 2a. Based on these results, the metabolite giving peaks 2a and 2b, i.e., M-V, was identified as the 4-hydroxymethylphenyl metabolite of M-III, as a diastereomeric mixture. This was confirmed by comparison of the retention time and mass spectral data of M-V with those of synthetic standard (KE-768).

**Metabolite M-VII (peaks 1a and 1b).** The full-scan mass spectrum for peak 1a gave an \([\text{M} - \text{H}]^-\) at \(m/z\) 297, which was 14 u greater than the respective mobile phases with no organic solvent contained 0.5, 20, and 50 mM AcONH₄. The experiment was carried out without (A) and with postcolumn addition of 2-propanol (B) or 2-MEE (C) at a flow rate of 40 µl/min. Data represent mean ± S.D. (n = 3).

**Fig. 5.** Influence of AcONH₄ content in the mobile phase (pH 4.6) on ESI responses of KE-766.

**Fig. 6.** ESI-mass chromatograms for rat urinary metabolites of esonarimod obtained in a Q1 full-scan mode without (A) and with (B) postcolumn addition of 2-MEE at a flow rate of 40 µl/min. The HPLC analysis was performed under separating conditions-1 described under Materials and Methods. Each arrow indicates the retention time of corresponding metabolite.
that of M-V. The product ion mass spectrum of $m/z$ 297 gave an ion corresponding to F4 at $m/z$ 189, which was 14 u greater than that of M-V. The mass spectral data for peak 1b were identical to those for peak 1a. Based on these results, the metabolite giving peaks 1a and 1b, i.e., M-VII, was identified as the 4-carboxyphenyl metabolite of M-III, as a diastereomeric mixture. This was confirmed by comparison of the HPLC retention time and mass spectral data of M-VII with those of synthetic standard (KE-766).

Metabolite M-IX (peak 5). The full-scan mass spectrum gave an $[\text{M}^2\text{H}]_2$ at $m/z$ 235, which was 16 u greater than F2 ($m/z$ 219) from esonarimod. The $m/z$ 235 underwent decarboxylation by CID to yield $m/z$ 191. Based on these results, the metabolite giving peak 5, i.e., M-IX, was identified as a 4-hydroxymethylphenyl-dihydrothiophen derivative, which was confirmed by comparison of its retention time and mass spectral data with those of synthetic standard (KE-298-19).

Metabolite M-X (peak 3). The full-scan mass spectrum included a weak $[\text{M}^2\text{H}]_2$ at $m/z$ 249, 14 u greater than that of M-IX; however, an intense ion at $m/z$ 205, which was probably formed by decarboxylation during ESI process, was detected. Under CID condition, the $m/z$ 205 also underwent decarboxylation, indicating that this metabolite has two carboxyl groups. Based on these results, the metabolite giving peak 3, i.e., M-X, was identified as the 4-carboxyphenyl derivative of M-IX, which was confirmed by comparison of its retention time and mass spectral data with those of a synthetic standard (KE-764).

### TABLE 1

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Peak No.</th>
<th>Precursor Ion $m/z$</th>
<th>Collision Energy eV</th>
<th>Major Product Ion$^a$ $m/z$ (relative intensity, %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Esonarimod$^c$</td>
<td>279</td>
<td>$[\text{M}^-]^{-}$</td>
<td>10</td>
<td>237 (100.0) 219 (10.4) 203 (9.0) 159 (100.0) 63 (4.1)$^d$</td>
</tr>
<tr>
<td>M-II$^d$</td>
<td>251</td>
<td>15</td>
<td>203 (7.8) 159 (100.0) 203 (13.2) 159 (100.0) 63 (3.7)$^d$</td>
<td></td>
</tr>
<tr>
<td>M-III$^d$</td>
<td>Peak 4a</td>
<td>267</td>
<td>15</td>
<td>219 (5.1) 175 (100.0)</td>
</tr>
<tr>
<td>M-IV$^d$</td>
<td>Peak 6</td>
<td>267</td>
<td>15</td>
<td>219 (4.9) 175 (100.0)</td>
</tr>
<tr>
<td>M-V$^d$</td>
<td>Peak 2a</td>
<td>283</td>
<td>15</td>
<td>219 (6.0) 175 (100.0)</td>
</tr>
<tr>
<td>M-VII$^d$</td>
<td>Peak 1a</td>
<td>297</td>
<td>15</td>
<td>189 (100.0)</td>
</tr>
<tr>
<td></td>
<td>Peak 1b</td>
<td>297</td>
<td>15</td>
<td>189 (100.0)</td>
</tr>
<tr>
<td>M-IX$^d$</td>
<td>Peak 5</td>
<td>255</td>
<td>15</td>
<td>203 (13.7) 159 (68.4) 251 (100.0)$^d$ 193 (82.4)$^d$ 175 (37.8)$^d$</td>
</tr>
<tr>
<td>M-X$^d$</td>
<td>Peak 3</td>
<td>205</td>
<td>20</td>
<td>161 (100.0)$^d$ 171 (2.9)$^d$ 127 (5.1)$^d$</td>
</tr>
<tr>
<td>M-II-Glu$^c$</td>
<td>Peak 7</td>
<td>427</td>
<td>15</td>
<td>203 (13.7) 159 (68.4) 251 (100.0)$^d$ 193 (82.4)$^d$ 175 (37.8)$^d$</td>
</tr>
</tbody>
</table>

$^a$ See Figs. 2 and 3.
$^b$ CID fragmentation patterns: See Fig. 8.
$^c$ Authentic standard.
$^d$ Found in urine.
$^e$ Found in bile.
$^f$ Authentic standard is not available.
$^g$ $[\text{SO}^2\text{CH}_3]^-$
$^h$ $[\text{M}^- - \text{CO}_2\text{H}]^-$
$^i$ $[\text{M}^- - 2\text{CO}_2\text{H}]^-$
$^j$ $[\text{M}^- - 2\text{CO}_2\text{SH}]^-$
$^k$ $[\text{aglycon}^- \text{H}]^-$
$^l$ $[\text{glucuronic acid}^- - \text{H}^-]$
$^m$ $[\text{glucuronic acid}^- - \text{H}^- \text{H}_2\text{O}]^-$

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**Fig. 7.** ESI-mass chromatograms for rat urinary metabolites of esonarimod corresponding to peaks 2a and 2b obtained in Q1 full-scan mode without (A) and with (B) postcolumn addition of 2-MEE at a flow rate of 40 µl/min.

The HPLC analysis was performed under separating conditions-2 described under Materials and Methods.

M-X, was identified as a 4-hydroxymethylphenyl-dihydrothiophen derivative, which was confirmed by comparison of its retention time and mass spectral data with those of synthetic standard (KE-298-19).

**Metabolite M-X (peak 3).** The full-scan mass spectrum included a weak $[\text{M}^-]^{-}$ at $m/z$ 249, 14 u greater than that of M-IX; however, an intense ion at $m/z$ 205, which was probably formed by decarboxylation during ESI process, was detected. Under CID condition, the $m/z$ 205 also underwent decarboxylation, indicating that this metabolite has two carboxyl groups. Based on these results, the metabolite giving peak 3, i.e., M-X, was identified as the 4-carboxyphenyl derivative of M-IX, which was confirmed by comparison of its retention time and mass spectral data with those of a synthetic standard (KE-764).

**Fig. 8.** Proposed key CID fragmentation schemes of esonarimod (A) and KE-748 (B).
**Metabolite M-II-Gluc (peak 7).** The full-scan mass spectrum gave an \([M - H]^-\) at \(m/z\) 427, which was 176 u greater than that of M-II. The product ion mass spectrum included an abundant ion at \(m/z\) 251 corresponding to the aglycon, as well as F3 (\(m/z\) 203) and F4 (\(m/z\) 159) ions that were similar to those of M-II. In addition, \(m/z\) 193 and 175, which were diagnostic ions for glucuronic acid moiety, were found. These results indicated that the metabolite giving peak 7 was the acyl glucuronide of M-II (M-II-Gluc).

**Metabolite Quantification.** The main urinary metabolite excreted within 24 h was M-III, accounting for 30.1 ± 0.8% of the dose, followed by M-V (16.6 ± 0.6%) and M-VII (13.2 ± 0.3%). In the case of bile, M-II-Gluc was predominant and accounted for 6.4 ± 0.3% of the dose. Each value represents mean ± S.E. for three rats.

**Discussion**

Our previous study demonstrated that \(^{14}\text{C}\)esonarimod was rapidly and completely absorbed from the gastrointestinal tract after oral administration to rats, and a pharmacologically active deacetyl-esonarimod (M-I) and its S-methyl metabolite (M-II), which accounted for approximately 50 and 25%, respectively, of the plasma total radioactivity, were found with a small amount of the unchanged drug in plasma obtained at 30 min after dosing (Yoshida et al., 1996). In this present study, identification of the urinary and biliary metabolites of esonarimod was conducted to obtain further information on the in vivo metabolism of this drug in rats.

LC/ESI-MS/MS has been widely used in drug metabolism studies (Luffer-Atlas et al., 1997; Fernández-Metzler et al., 1999; Ramanathan et al., 2000). However, this technique has the serious drawback that analyte sensitivity is dependent upon the mobile-phase composition, such as organic solvent and electrolyte contents, as well as the nature of the analyte (Ikonomou et al., 1990; Jemal et al., 1998; Kamel et al., 1999). Our initial LC/ESI-MS/MS analysis in which a mobile phase containing AcONH\(_4\)/AcOH buffer, a common ESI-compatible buffer, was used failed to obtain any structural information on most of the esonarimod metabolites due to poor ESI responses. The ESI process involves the formation of highly charged droplets from the mobile phase, ion emission from the charged droplets into the gas phase, and gas-phase ion-molecular reaction (Gaskell, 1997). The poor ESI responses probably resulted from ion competition between the analyte and coexisting acetate anion (AcO\(^-\)) in the electrosprayed droplet surface during the ionization process (Witters et al., 1996). We recently demonstrated the usefulness of 2-MEE, an effective postcolumn-adding modifier, for negative ion ESI; this regent improves ion suppression by AcO\(^-\) (Yamaguchi et al., 1999). To optimize ESI for metabolite analysis of esonarimod, the effect of 2-MEE on sensitivity of synthetic KE-766, a putative metabolite of esonarimod, was examined. Unlike conventional signal-enhancing modifiers including 2-propanol, 2-MEE did not result in ion suppression, resulting in pronounced 226-fold signal enhancement. An effective modifier in improving ion suppression caused by AcO\(^-\) should possibly have a higher b.p. than that of AcOH (b.p. 118°C) as well as a surface tension-lowering property. The solvent 2-MEE (b.p. 193°C) has the desirable property, and probably makes possible the formation of smaller droplets containing lower percentages of water and AcO\(^-\) because of effective evaporation of AcOH from the droplets prior to analyte ion emission. This hypothesis may be supported by the b.p. dependence of the signal-enhancing ability of the surface tension-lowering solvents as indicated in Fig. 4.

![Fig. 9. Possible metabolic pathways of esonarimod in rats.](image-url)
methodology, metabolite analysis was carried out. The post-column addition of 2-MEE allowed pronounced enhancement of ESI responses and structural elucidation for all metabolites without affecting chromatographic performance, indicating that this technique is very useful.

The results of the metabolite analysis demonstrated that esonarimod underwent extensive metabolism and was not excreted as the unchanged form. Moreover, M-I and M-II, which were circulating metabolites (Yoshida et al., 1996), were not clearly detected in either urine or bile specimens. The main urinary metabolite was M-III, followed by M-V and M-VII, which are S-methyl M-I-related metabolites. As minor components, dihydrothienophene derivative-related metabolites M-IX and M-X were identified, which were probably formed from M-I by intramolecular condensation of the thiol group with the C-4 carbonyl group and subsequent oxidation of the aromatic methyl group. In contrast, the biliary metabolite profile was qualitatively different from that for urine. One S-methyl M-I-related metabolite, M-II-Glu, accounted for the majority of biliary radioactivity. On the basis of these findings, possible metabolic pathways of esonarimod were proposed as shown in Fig. 9. Esonarimod is rapidly biotransformed into an active metabolite M-I after drug absorption (Yoshida et al., 1996). This metabolite undergoes partial intramolecular condensation, but mainly S-methylation, followed by extensive metabolism by combination of S-oxidation and oxidative conversion of the aromatic methyl group.

In general, thiol-containing drugs, such as d-penicillamine (Nozu et al., 1977; Pilkington and Waring, 1988), captopril (Yeung and Park, 1989), and SA-96 (Morikawa et al., 1985), tend to remain in the body longer than that of common thiol-containing drugs, supporting the low toxicity of esonarimod in rats.

References


