Antinociceptive Activities of \(\alpha\)-Truxillic Acid and \(\beta\)-Truxinic Acid Derivatives

Yu-Ming Chi,*a Motoyuki Nakamura,a Xi-Ying Zhao,a Toyokichi Yoshizawa,a Wen-Mei Yan,b Fumio Hashimoto,c Junei Kinjo,c Toshihiro Nohara,*c and Shinobu Sakurada,db

aSeiwa Pharmaceutical Ltd.; 187–11 Usuba, Hanakawa-machi, Kitabarakri, Ibaraki 319–1355, Japan; bBeijing University of Traditional Chinese Medicine and Pharmacy; Beijing 100029, China; cFaculty of Pharmaceutical Sciences, Kumamoto University; 5–1 Oe-Honmachi, Kumamoto 862–0973, Japan; and dDepartment of Pharmacology, Tohoku University; 4–4–1 Komatsushima, Aoba-ku, Sendai 981–8558, Japan.

Received August 12, 2005; accepted October 28, 2005

Our recent study demonstrated that the dimeric structure of \(\alpha\)-truxillic acid derivatives played an important role in the expression of their anti-inflammatory activities. In the present report, to investigate the correlation between the structure and anti-inflammatory activity, \(\alpha\)-truxillic acid (1) and its derivatives (2–6), \(\beta\)-truxinic acid (7) and its derivatives (8–10) were prepared, and their activities were evaluated in the formalin test. All compounds showed only weak or no activities against the neurogenic pain response, but demonstrated significant activities against the inflammatory pain response induced by formalin. The highest anti-inflammatory activities were observed for \(\alpha\)-truxillic acid (1) and its derivative 4,4'-dihydroxy-\(\alpha\)-truxillic acid (2). In addition, \(\alpha\)-truxillic acid (1) and its derivative, \(\alpha\)-truxillic acid bis(p-nitrophenyl)ester (5), showed higher anti-inflammatory activities than \(\beta\)-truxinic acid (7) and the corresponding derivative (10). Furthermore, free carboxylic acids (1, 2) showed higher activities than their dimethyl esters (3, 4) and bis(p-nitrophenyl)ester (5). These results confirmed that the \(\alpha\)-formation of dimeric structure and the free carboxylic acid were also important for the expression of anti-inflammatory activities. Otherwise, 4,4'-dichloro-\(\beta\)-truxinic acid (8) had higher activity than its parent compound 7; furthermore, 1,3-dibenzoyle-2,4-di(4-chlorophenyl)cyclobutane (6) also showed strong anti-inflammatory activity. These results suggested that substituents in the phenyl groups were also important for the expression of anti-inflammatory activity. In order to gain information about their activity intensity, the anti-inflammatory activities of 2 and 4,4'-dichlorolated derivatives (6, 8) were compared with that of indomethacin (a nonsteroidal anti-inflammatory drug) in the formalin test. As a result, compounds 2, 6 and 8 showed stronger anti-inflammatory activities than indomethacin. These results suggested that \(\alpha\)-truxillic acid and \(\beta\)-truxinic acid derivatives might be developed into a new type of anti-inflammatory drug.

Key words \(\alpha\)-truxillic acid; \(\beta\)-truxinic acid; anti-inflammatory activity; indomethacin; formalin test

In the previous report, we disclosed that \(\alpha\)-truxillic acid (1) and its derivative 4,4'-dihydroxy-\(\alpha\)-truxillic acid (2) showed significant anti-inflammatory activities in the formalin test. Their monomer components (\(E\))-cinnamic acid and (\(E\))-\(p\)-coumaric acid exhibited only weak or no activity.\(^1\) This result suggested that the dimeric structure played an important role in the expression of anti-inflammatory activity. In the course of our investigation of antinociceptive substances from natural sources, a novel monoterpene alkaloid, incarvillateine, was characterized from a traditional Chinese crude drug designed as ‘Tougucao’ (Incarvillea sinensis).\(^2\) Incarvillateine possessed the same dimeric structure as that of \(\alpha\)-truxillic acid, and demonstrated more potent antinociceptive activity than morphine in the formalin test.\(^3\) The mechanism of antinociception was regarded to be different from that of morphine, and the activity was mainly evoked by activation of \(\mu\), \(\kappa\)-opioid receptors and an adenosine receptor. Structure–activity relationship study of incarvillateine revealed that the monoterpene alkaloid moiety, incarvilline, and the dimeric structure played important roles in the expression of activities against the neurogenic and inflammatory pain responses, respectively.\(^4\) In order to investigate the structure–activity relationship between dimeric structure and anti-inflammatory activity, \(\alpha\)-truxillic acid and \(\beta\)-truxinic acid, as well as their derivatives, were prepared (Fig. 1), and their activities were evaluated in the formalin test.

MATERIALS AND METHODS

Animals Male ddy mice were used. The mice (25±5 g)

Fig. 1. Structures of Compounds 1—10

<table>
<thead>
<tr>
<th>R&lt;sub&gt;1&lt;/sub&gt;</th>
<th>R&lt;sub&gt;2&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\alpha)-truxillic acid derivatives</td>
<td>(1)</td>
</tr>
<tr>
<td>2</td>
<td>(\text{CH})</td>
</tr>
<tr>
<td>3</td>
<td>(\text{OMe})</td>
</tr>
<tr>
<td>4</td>
<td>(\text{OMe})</td>
</tr>
<tr>
<td>5</td>
<td>(\text{ON-}O\text{-}O\text{-}\text{Cl})</td>
</tr>
<tr>
<td>6</td>
<td>(\text{Cl} \text{-} N\text{-}O\text{-}N\text{-}\text{Cl})</td>
</tr>
<tr>
<td>(\beta)-truxinic acid derivatives</td>
<td>7</td>
</tr>
<tr>
<td>8</td>
<td>(\text{Cl})</td>
</tr>
<tr>
<td>9</td>
<td>(\text{Cl})</td>
</tr>
<tr>
<td>10</td>
<td>(\text{ON-}O\text{-}O\text{-}\text{Cl})</td>
</tr>
</tbody>
</table>
were allowed food and water *ad libitum* and they were housed in an air-conditioned room maintained at 22±2°C with a humidity of 55±5%. The experimental animals were allocated randomly into groups.

**Chemicals** *(E)-p-Coumaric acid, 4-chlorocinnamic acid, 3-(3-pyridyl)acrylic acid, p-nitrophenol, cinnamyl chloride, *N*,*N*-dimethylformamide, trimethylsilyldiazomethane were purchased from Tokyo Kasei (Tokyo, Japan). Thionyl chloride and lithium aluminium hydride (LiAlH₄) were bought from Kanto Kagaku (Tokyo, Japan). *(E)-*Cinnamic acid and Tween 80 (polyoxyethylene sorbitan monoleate) were purchased from Nacalai Tesque (Kyoto, Japan). Ringer solution was obtained from Fuso Pharmaceutical (Osaka, Japan).

**General Methods** Melting points were uncorrected. IR spectra were recorded on a Hitachi 270-30 spectrometer. 1H- and 13C-NMR spectra were obtained with a JEOL spectrometer, and chemical shifts were given on a scale with tetramethylsilane as an internal standard. The EI-MS and FAB-MS were measured with a JOEL DX-303 HF spectrometer. TLC was performed on precoated Kieselgel 60 F254 plates (Merck). Column chromatography was carried out on Kieselgel 60 (70—230 mesh and 230—400 mesh). All materials and reagents purchased were used without further purification.

**Synthesis of α-Truxillic Acid** *(1)*  
Compound 1 was prepared according to a previous report.  

**Synthesis of 4,4'-Dihydroxy-α-truxillic Acid (2)**  
Compound 2 was prepared according to a previous report.  

**Synthesis of α-Truxillic Acid Dimethylster (3)**  
To a solution of α-truxillic acid (1.0 g, 3.38 mmol) in methyl ethyl ketone (40 ml), trimethylsilyldiazomethane (4 ml) in ether (10 ml) was added dropwise under stirring. After 1 h reaction, the reaction solution was concentrated under reduced pressure, and the residue was purified by column chromatography on silica gel with CHCl₃—MeOH (50 : 1) and recrystallized with CHCl₃ to give α-truxillic acid dimethylster as a needle crystal (308 mg, 28.1%), mp 172—174°C (lit. 174°C). IR (KBr) cm⁻¹: 3299 [M+H]+. 1H-NMR (CDCl₃) δ: 3.79 (6H, s, COO methyl), 3.39 (2H, m, α, α'-H), 7.30 (10H, aromatic-H).  

**Synthesis of 4,4'-Dihydroxy-α-truxillic Acid Dimethylster (4)**  
To a solution of 4,4'-dihydroxy-α-truxillic acid (1.0 g, 3.05 mmol) in methyl ethyl ketone (40 ml) and MeOH (20 ml), trimethylsilyldiazomethane (4 ml) in ether (10 ml) was added dropwise under stirring. After 1 h reaction, the reaction solution was concentrated under reduced pressure, and the residue was purified by column chromatography on silica gel with hexane—AcOEt (3 : 1→1 : 2), then recrystallized with CHCl₃ to give 4,4'-dihydroxy-α-truxillic acid dimethylster as a needle crystal (892 mg, 82.2%), mp 178—181°C (lit. 174—177°C). IR (KBr) cm⁻¹: 1690 (C=O), EL-MS m/z: 357 [M+1]+. 1H-NMR (pyridine-d₅) δ: 3.37 (6H, s, COOME), 4.20 (2H, m, β, β'-H), 4.71 (2H, m, α, α'-H), 7.19 (4H, d, J=8.6 Hz, 3, 3', 5, 5'-H), 7.44 (4H, d, J=8.6 Hz, 2, 2', 6, 6'-H).  

**Synthesis of α-Truxillic Acid Bis(p-nitrophenyl)ester (5)**  
To a solution of α-truxillic acid (435 mg, 1.47 mmol) and thiouyl chloride (2.47 g) and *N*,*N*-dimethylformamide (one drop) was refluxed for 3 h. After drying, the excess thiouyl chloride was removed δ and α-truxillic acid chloride was obtained. To a solution of p-nitrophenol (230 mg) in THF (3 ml) and pyridine (0.5 g), α-truxillic acid chloride (500 mg) in THF (1 ml) was added dropwise at 0—5°C under stirring. After 2 h of stirring, the reaction mixture was poured into water (500 ml) and filtered off. The precipitate was recrystallized from methyl ethyl ketone gave α-truxillic acid bis(p-nitrophenyl)ester (580 mg, 73.4%) as a needle crystal, mp 230—231°C. IR (KBr) cm⁻¹: 1763, 1734 (C=O), 1522, 1350 (NO₂). Positive FAB-MS m/z: 539 [M+1]+ (100). 1H-NMR (CDCl₃) δ: 4.34 (2H, m, β, β'-H), 4.75 (2H, m, α, α'-H), 7.1—7.5 (10H, aromatic-H), 6.45, 8.08 (each 4H, dd, J=1.98, 6.92 Hz, p-nitrophenyl-H).  

**Synthesis of 1,3-Dibenzoyl-2,4-di(4-chlorophenyl)cyclobutane (6)**  
Chlorobenzalacetophenone (1.0 g, 4.13 mmol) was treated in a manner similar to the conversion of *(E)-*cinnamic acid to α-truxillic acid (1). After 2 d irradiation, the product was filtered off and recrystallized twice from CHCl₃ to give 1,3-dibenzoyl-2,4-di(4-chlorophenyl)cyclobutane as a needle crystal (200 mg, 10.1%), mp 208—209°C (lit. 209—210°C). IR (KBr) cm⁻¹: 3279, 1671 (C=O). Positive FAB-MS m/z: 486 [M+1]+. 1H-NMR (DMSO-d₆) δ: 4.80 (2H, m, β, β'-H), 4.87 (2H, m, α, α'-H), 7.25 (10H, aromatic-H), 7.34 (4H, d, J=7.8 Hz, 4-chlorobenzyl-3, 3', 5, 5'-H), 7.71 (4H, d, J=7.8 Hz, 4-chlorobenzyl-2, 2', 6, 6'-H).  

**Synthesis of β-Truxillic Acid (7)**  
A mixture of bis-(p-nitrophenyl) β-truxinic acid (690 mg, 1.28 mmol) and KOH (360 mg) in MeOH (7 ml) was refluxed for 3 h. After being adjusted to pH 3 with HCl, the reaction mixture was poured into water and filtered off. The precipitate was recrystallized from acetic acid to give β-truxinic acid as a needle crystal (205 mg, 53.9%), mp 208—209°C (lit. 209—210°C). IR (KBr) cm⁻¹: 3035 (OH), 1690 (C=O), EL-MS m/z: 296 [M]+. 1H-NMR (DMSO-d₆) δ: 4.01 (2H, m, β, β'-H), 4.24 (2H, m, α, α'-H), 7.15 (10H, aromatic-H).  

**Synthesis of 4,4'-Dichloro-β-truxinic Acid (8)**  
Chlorobenzalacetophenone (500 mg, 2.75 mmol) was treated in a manner similar to the conversion of *(E)-*cinnamic acid to α-truxillic acid (1). After 2 d irradiation, the product was filtered off and recrystallized twice from CHCl₃ to give 4,4'-dichloro-β-truxinic acid as a plate crystal (314 mg, 62.8%), mp 159—160°C (lit. 160°C). IR (KBr) cm⁻¹: 3102 (OH), 1719 (C=O). Positive FAB-MS m/z: 365 [M+1]+. 1H-NMR (DMSO-d₆) δ: 3.59 (2H, m, β, β'-H), 4.05 (2H, m, α, α'-H), 6.64, 7.06 (each 4H, d, J=8.25 Hz, aromatic-H).  

**Synthesis of 1,2-Bis(3-pyridyl)cyclobutane-3,4-dicarbonyl Acid (9)**  
(3-Pyridyl)acrylic acid (500 mg, 3.36 mmol) was treated in a manner similar to the conversion of *(E)-*cinnamic acid to α-truxillic acid (1). After 4 d irradiation, the product was filtered off and gave 1,2-bis(3-pyridyl)cyclobutane-3,4-dicarbonyl acid as a white powder (98.0%), mp 200°C. IR (KBr) cm⁻¹: 3398 (OH), 1578 (C=O). Negative FAB-MS m/z: 297 [M-1]-. 1H-NMR (pyridine-d₅) δ: 4.45 (2H, m, β, β'-H), 4.82 (2H, m, α, α'-H), 7.15 (10H, aromatic-H).
Synthesis of β-Truxinic Acid Bis(p-nitrophenyl)ester (10) To a solution of p-nitrophenol (6.0 g, 40.96 mmol) in THF (60 ml) and pyridine (1.9 g), cinnamyl chloride (6.8 g) in THF (2 ml) was added at 0—5 °C, and the reaction was stirred at the same temperature for 2 h. The reaction mixture was then poured into water (800 ml) and filtered off to give a precipitate (9.7 g, 88.0%). The dried precipitate (9.0 g, 33.45 mmol) was treated in a manner similar to the conversion of (E)-cinnamic acid to α-truxillic acid (1). After 10 h irradiation, the product was filtered off and recrystallized from methyl ethyl ketone to give β-truxinic acid bis(p-nitrophenyl)ester as a needle crystal (6.4 g, 62.4%), mp 195—196 °C (lit. 192—193 °C). IR (KBr) cm⁻¹: 1763, 1734 (C=O), 1522, 1350 (NO₂). Positive FAB-MS m/z: 539 [M+1]⁺ (100). ¹H-NMR (CDCl₃) δ: 4.28 (2H, m, b,b'-H), 4.62 (2H, m, a,a’-H), 7.01—7.22 (10H, m, aromatic-H), 7.26, 8.27 (each 4H, dd, J=1.98, 6.92 Hz, p-nitrophenyl-H).¹⁰

Formalin Test and Treatments This method represented a modification of that described by Dubuisson and Dennis.¹³ Male ddY mice (25±5 g) were used. The tested drugs (40 mg/kg) were prepared as suspensions with 0.5% Tween 80/saline and intraperitoneally administered 10 min prior to the injection of an inducer (1.0% formalin/saline, 20 μl). The mice were observed for 30 min, and the time the mice spent licking the injected right hindpaw was recorded. The nociceptive scores normally peaked at 0 to 10 min after formalin injection (early phase) and 10 to 30 min (late phase) after the injection, representing the neurogenic and inflammatory pain responses, respectively.¹⁴ The time spent licking the injected paw was recorded and the data are expressed as total licking time in the early phase and late phase.

Comparison with Indomethacin The tested drugs (0.625, 2.5, 10, 40 mg/kg for 2; 2.5, 10, 40 mg/kg for 6 and 8; 10, 20, 40 mg/kg for indomethacin) were prepared as suspensions with 0.5% Tween 80/saline, and were intraperitoneally administered 10 min prior to the injection of an inducer (2.0% formalin/saline, 20 μl).

Statistical Analysis All values are expressed as mean±S.E. (n=10). The ED₅₀ was determined according to the Method of Lichfield and Wilcoxon.¹⁵ For statistical analysis, one-way analysis of variance combined with Dunnett’s multiple range test for multiple comparisons were used. Differences were considered significant at p<0.01.

RESULTS AND DISCUSSION

The formalin-induced licking response has been used as a model for evaluating new analgesics.¹⁴,¹₆ The duration of the nociceptive response induced by formalin can be divided into two phases. The early phase is from 0 to 10 min after formalin injection, and the late phase is from 10 to 30 min after the injection. The pain of the early phase is evoked by direct stimulation of the nerve fibers, and that of the late phase is due to the inflammatory reaction. Centrally acting drugs such
as morphine inhibited both early and late phases equally. On the other hand, peripheral acting drugs such as indomethacin and aspirin inhibited only the late phase.

In the present study, α-truxillic acid (1) and its derivatives (2—6), as well as β-truxillic acid (7) and its derivatives (8—10) were prepared, and their activities were evaluated in the formalin test. All compounds were tested intraperitoneally for their activities at the dose of 40 mg/kg body weight by formalin test, and the results are summarized in Fig. 2. All compounds showed only weak or no activity against the neurogenic pain response (early phase), but exhibited significant activity against the inflammatory pain response (late phase) induced by formalin. The highest anti-inflammatory activities were observed for α-truxillic acid (1) and its derivative 4,4'-dihydroxy-α-truxillic acid (2). In addition, 4,4'-dichloro-derivatives 1,3-dibenzoyl-2,4-di(4-chlorophenyl)cyclobutane (6) and 4,4'-dichloro-β-truxillic acid (8) also showed strong anti-inflammatory activities. α-Truxillic acid (1) and its derivative α-truxillic acid bis(p-nitrophenyl)ester (5) had higher anti-inflammatory activities than β-truxillic acid (7) and the corresponding derivative (10). Free carboxylic acids (1, 2) showed higher activities than their dimethyl esters (3, 4). The anti-inflammatory activity of α-truxillic acid bis(p-nitrophenyl)ester (5) was weaker than that of α-truxillic acid (1), but the activity of β-truxillic acid bis(p-nitrophenyl)ester (10) was equal to that of β-truxillic acid (7). 4,4'-Dichloro-β-truxillic acid (8) had higher activity than its parent compound β-truxillic acid (7).

We then compared the anti-inflammatory activities of 4,4'-dihydroxy-α-truxillic acid (2), which showed the highest anti-inflammatory activity, and two 4,4'-dichlorolated derivatives (6, 8) which also showed strong activities, with that of indomethacin (a nonsteroidal anti-inflammatory drug) in the formalin test, with a higher concentration formalin (2.0%, 20 μl) as an inducer. All compounds exerted only weak or no activity against the neurogenic pain response (early phase), but produced marked and dose-related inhibition of the formalin-induced inflammatory pain response (late phase). The anti-inflammatory activities of 2, 6 and 8 were stronger than that of indomethacin. The ED50 values for 2, 6 and 8 were 4.93, 11.2 and 24.9 μmol/kg respectively, while the value for indomethacin was 117.0 μmol/kg (Table 1, Fig. 3). The activities of 2, 6 and 8 were about 5- to 24-fold more potent than that of indomethacin. These results suggested that 2, 6 and 8 possess very strong anti-inflammatory activity, and α-truxillic acid and β-truxillic acid derivatives might be developed into a new type of anti-inflammatory drug.

In conclusion, these results clearly indicated that α-truxillic acid and β-truxillic acid derivatives are another group of leading compounds important for developing a new type of anti-inflammatory drug. The result also supported that the dimeric structure played an important role in the expression of anti-inflammatory activity. Further investigation is required to develop more potent anti-inflammatory derivatives and to elucidate the precise mechanism underlying these activities.

REFERENCES

8) Koshino H., Terada S., Yoshitara T., Shimanuki T., Sato T., Tajimi A.,


