Chemical Modification of PA-48153C, a Novel Immunosuppressant Isolated from Streptomyces prunicolor PA-48153

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5β-Methoxy (20), 14-methyl (24), 14,14-dibromo-15-nor (25), 8-O-acyl (26~45), 8-O-alkyl (46), 8-O-alkoxycarbonyl (47, 48), and 8-O-carbamoyl (49) derivatives of PA-48153C, a novel immunosuppressant isolated from fermentation products of Streptomyces prunicolor PA-48153, were prepared. These compounds were found to retain the inhibitory activity on the responses of both T and B cells to mitogens. Among them, the C-8 hexanoate 28 showed potent suppressive effects on mitogen responses with less cytotoxicity to EL4 cells and was selected for in vivo evaluation.

During our screening of microbial products aiming at new immunosuppressants, PA-48153C (1, Scheme 1), a novel 2-pyranone compound, was discovered in the fermentation broth of Streptomyces prunicolor PA-48153. Coincidentally, pironetin, which had the same structure as 1, was isolated from Streptomyces sp. NK10958 at the almost same time and was reported to be a plant growth regulator; its immunosuppressive activity was not described. We found that PA-48153C showed potent suppressive effects on the responses of both T and B cells to mitogens, but was fairly toxic in vivo. Consequently, we decided to pursue its chemical modification in order to obtain compounds retaining the potent immunosuppressive activity but having less toxicity.

We reported the total synthesis of (-)-PA-48153C in the previous paper, and various PA-48153C-related compounds were prepared using this synthetic route or by partial synthesis from natural PA-48153C. In most cases, even slight modifications of the 2-pyranone ring (e.g., 3,4-saturated, 3,4-epoxy, 3,4-cyclopropyl, 2-methoxy, 5-deethyl, or ring-opened derivative) or the side chain (e.g., 8β-hydroxy, 8-oxo, 13,14-epoxy, 13,14-cis-vinylene, 14-methoxycarbonyl-15-nor, or 13-oxo derivative) diminished the inhibitory activity on mitogen responses. Exceptionally, 5β-methoxy (20), 14-methyl (24), 14,14-dibromo-15-nor (25), 8-O-acyl (26~45), 8-O-alkyl (46), 8-O-alkoxycarbonyl (47, 48), and 8-O-carbamoyl (49) derivatives retained the potent inhibitory activity on the responses of both T and B cells to mitogens. Especially, derivatives of the C-8α alcohol had weakened cytotoxicity to EL4 cells, and the hexanoate 28 was selected for in vivo evaluation.

The present paper describes the preparation of PA-48153C-related compounds (20, 24~49) and their immunosuppressive activity.

Results

Chemistry

5β-Methoxy derivative 20 was prepared by convergent total synthesis from methyl-α-D-galactopyranoside (2) and (S)-(+) -methyl 3-hydroxy-2-methylpropionate (5) as shown in Scheme 2. Cyclic segment 4 was conveniently prepared from 2 by the known method, since the C-5β methoxy group could be derived from galactose. Acyclic segment 6 was prepared from 5 as shown in our previous paper. Combining both segments to get the key intermediate 7 was achieved by Wittig reaction of aldehyde 4 with a phosphorous ylide derived from phosphonium salt 6, though the yield was rather low because of β-elimination of the C-5β methoxy group.

Hydroboration of 7 followed by treatment with alkaline hydrogen peroxide gave the desired C-8α alcohol 8 as the main product. Regiochemical assignment of the hydroxyl group was based on the 1H NMR decoupling...
experiment of the corresponding ketone. The C-8α stereochemistry was confirmed later in the synthesis by evaluating the biological activity, since the C-8β hydroxy derivative of PA-48153C exhibited no inhibitory activity. Alcohol 8 was converted to (E)-olefin 15 through a sequence involving protection of the hydroxyl group of 8 as a methoxymethyl (MOM) ether 9, deprotection of the tert-butyldiphenylsilyl (TBDPS) group of 9, Swern oxidation of alcohol 10, Horner-Emmons reaction of 11, conversion of 12 to the α,β-unsaturated p-tosylhydrazone 13, reductive deoxygenation of carbonyl tosylhydrazone 13 with sodium borohydride in acetic acid, and deprotection of both benzyl groups of 14, following the previously reported synthetic route.3)

In order to introduce the C-3 double bond, diol 15 was converted to dixanthate 16 and subjected to radical dideoxygenation with diphenylsilane,5) giving the desired olefin 17. Efforts to effect the Tipson-Cohen reaction of the corresponding dimesylate were unrewarding, probably because of steric factors. We completed synthesis of 20 by selective hydrolysis of the protected δ-lactol in 17 with 75% aqueous acetic acid at 40°C, oxidation of 18 using pyridinium chlorochromate, and deprotection of the MOM group of 19 using refluxing 60% aqueous acetic acid.

14-Methyl and 14,14-dibromo-15-nor derivatives 24 and 25 were synthesized via aldehyde 22 starting from natural PA-48153C as shown in Scheme 3. Since ozonolysis of 1 resulted in ring closure by intermolecular attack of C-8α hydroxyl group to aldehyde, the C-8α hydroxyl group was protected as a tert-butyldimethylsilyl (TBDMS) ether. Ozonolysis of TBDMS ether 21, Wittig reaction of aldehyde 22, and deprotection of the TBDMS group of 23 provided 14-methyl derivative 24. 14,14-Dibromo-15-nor derivative 25 was obtained by reaction of 22 with carbon tetrabromide-triphenylphosphine,

Scheme 2.

(a) Ref. 4; (b) DMSO, (COC1)2 followed by Et3N; (c) Ref. 3; (d) n-BuLi (32% for b, d); (e) B2H6, H2O2; (f) MOMCl, i-Pr2NET (43% for e, f); (g) Bu4NF, (COC1)2, (COC1)3, (p) PCC, NaOAc (33% for o, p); (q) 60% aq. HOAc, reflux (70%).
which was accompanied by deprotection of the TBDMS group.

Derivatives of the C-8a alcohol were conveniently prepared from natural PA-48153C as shown in Scheme 4 and Table 1. Since C-8a alcohol was sterically hindered, the yield of acylation was low using bulky reagents. Therefore, in such cases, a stoichiometric amount of 4-dimethylaminopyridine (DMAP) was used to obtain 8-O-acyl derivatives 26~45. In order to prepare 8-O-alkyl derivatives by a method using sodium hydride as a base, we would have needed to convert δ-lactone in 1 to a protected δ-lactol. However, in the case of methyl derivative 46, direct alkylation proceeded in the presence of excess iodomethane at 15°C. 8-O-Alkoxy carbonyl derivatives 47 and 48 were easily prepared from 1 using chloroformates and pyridine. 8-O-Carbamoyl derivatives such as benzoylcarbamoyl derivative 49 were obtained using isocyanates in the presence of DMAP, although bis(tributyltin) oxide was used instead of DMAP when the reaction did not proceed.

Table 1. PA-48153C derivatives of C-8a hydroxyl group 26~49.

<table>
<thead>
<tr>
<th>Compound</th>
<th>R</th>
<th>Yield (%)</th>
<th>(^1^H) NMR (CDCl(_3)) 8 CH, / Hz</th>
</tr>
</thead>
<tbody>
<tr>
<td>26</td>
<td>Et</td>
<td>100</td>
<td>0.81 (d, 7.0), 0.89 (d, 7.0), 0.97 (t, 7.4), 1.16 (t, 7.6), 1.66 (d, 5.0), 3.39 (s)</td>
</tr>
<tr>
<td>27</td>
<td>Pr</td>
<td>48</td>
<td>0.81 (d, 7.0), 0.89 (d, 7.0), 0.97 (t, 7.4), 1.16 (t, 7.6), 1.66 (d, 5.0), 3.39 (s)</td>
</tr>
<tr>
<td>28</td>
<td>(CH(_2))(_2)CH(_2)</td>
<td>97</td>
<td>0.81 (d, 6.8), 0.89 (d, 6.8), 0.90 (t, 6.7), 0.97 (t, 7.5), 1.66 (d, 4.8), 3.39 (s)</td>
</tr>
<tr>
<td>29</td>
<td>CH(_2)Cl</td>
<td>74</td>
<td>0.82 (d, 7.0), 0.92 (d, 7.2), 0.98 (t, 7.4), 1.67 (d, 5.0), 3.40 (s)</td>
</tr>
<tr>
<td>30</td>
<td>CHCl(_3)</td>
<td>99</td>
<td>0.83 (d, 6.8), 0.94 (d, 7.0), 0.98 (t, 7.5), 1.65 (d, 5.2), 3.41 (s)</td>
</tr>
<tr>
<td>31</td>
<td>CCI(_4)</td>
<td>92</td>
<td>0.84 (d, 6.8), 0.97 (d, 7.0), 0.99 (t, 7.2), 1.65 (d, 4.8), 3.43 (s)</td>
</tr>
<tr>
<td>32</td>
<td>(CH(_2))(_2)CH(_2)Cl</td>
<td>96</td>
<td>0.82 (d, 6.8), 0.92 (d, 7.2), 0.98 (t, 7.4), 1.67 (d, 5.0), 3.40 (s)</td>
</tr>
<tr>
<td>33</td>
<td>(CH(_2))(_2)CH(_2)Br</td>
<td>80</td>
<td>0.81 (d, 6.6), 0.89 (d, 7.2), 0.97 (t, 7.6), 1.67 (d, 5.0), 3.39 (s)</td>
</tr>
<tr>
<td>34</td>
<td>CH(_2)CH=E(_2)</td>
<td>93</td>
<td>0.81 (d, 6.6), 0.89 (d, 7.0), 0.97 (t, 7.5), 0.99 (t, 7.4), 1.67 (d, 4.8), 3.38 (s)</td>
</tr>
<tr>
<td>35</td>
<td>(CH(_2))(_2)CH(_2)</td>
<td>95</td>
<td>0.81 (d, 6.8), 0.89 (d, 6.8), 0.97 (t, 7.4), 1.67 (d, 5.0), 3.39 (s)</td>
</tr>
<tr>
<td>36</td>
<td>2-Cyclopentylethyl</td>
<td>100</td>
<td>0.81 (d, 6.6), 0.89 (d, 7.2), 0.97 (t, 7.4), 1.67 (d, 4.6), 3.39 (s)</td>
</tr>
<tr>
<td>37</td>
<td>2-Quinolyl</td>
<td>97</td>
<td>0.86 (d, 6.6), 0.96 (t, 7.4), 1.11 (t, 7.0), 1.59 (d, 4.4), 3.41 (s)</td>
</tr>
<tr>
<td>38</td>
<td>2-Quinoxalyl</td>
<td>100</td>
<td>0.86 (d, 6.6), 0.98 (t, 7.6), 1.12 (t, 7.0), 1.60 (d, 4.6), 3.41 (s)</td>
</tr>
<tr>
<td>39</td>
<td>CH(_2)CO(_2)Me</td>
<td>74</td>
<td>0.81 (d, 6.8), 0.87 (d, 7.0), 0.97 (t, 7.5), 1.67 (d, 4.6), 3.41 (s), 3.74 (s)</td>
</tr>
<tr>
<td>40</td>
<td>(CH(_2))(_2)CO(_2)Me</td>
<td>99</td>
<td>0.81 (d, 6.8), 0.89 (d, 6.8), 0.97 (t, 7.5), 1.67 (d, 4.9), 3.39 (s), 3.68 (s)</td>
</tr>
<tr>
<td>41</td>
<td>CH=CH(_2)CO(_2)Me</td>
<td>85</td>
<td>0.82 (d, 6.8), 0.93 (d, 7.0), 0.97 (t, 7.6), 1.66 (d, 5.0), 3.36 (s), 3.82 (s)</td>
</tr>
<tr>
<td>42</td>
<td>(CH(_2))(_2)CO(_2)Me</td>
<td>100</td>
<td>0.81 (d, 6.6), 0.88 (d, 7.2), 0.97 (t, 7.4), 1.67 (d, 5.0), 3.38 (s), 3.67 (s)</td>
</tr>
<tr>
<td>43</td>
<td>CH(_2)NHBoc</td>
<td>100</td>
<td>0.81 (d, 6.6), 0.89 (d, 7.0), 0.97 (t, 7.4), 1.45 (9H, s), 1.67 (d, 4.6), 3.39 (s)</td>
</tr>
<tr>
<td>44</td>
<td>(CH(_2))(_2)NHMe(_2)</td>
<td>100</td>
<td>0.81 (d, 6.6), 0.88 (d, 7.0), 0.96 (t, 7.4), 1.66 (d, 5.0), 3.37 (s), 3.81 (s)</td>
</tr>
<tr>
<td>45</td>
<td>(CH(_2))(_2)NHMe(_2)</td>
<td>76</td>
<td>0.81 (d, 6.6), 0.88 (d, 7.2), 0.97 (t, 7.5), 1.66 (d, 4.6), 3.38 (s), 3.81 (s)</td>
</tr>
<tr>
<td>46</td>
<td>CH(_2)NMe</td>
<td>73</td>
<td>0.83 (9H, 4.6, 6.8), 0.98 (t, 7.5), 1.68 (d, 4.0), 3.45 (s), 3.48 (s)</td>
</tr>
<tr>
<td>47</td>
<td>Bu</td>
<td>100</td>
<td>0.82 (d, 6.6), 0.91 (d, 7.0), 0.94 (t, 7.2), 0.97 (t, 7.2), 1.67 (d, 4.6), 3.42 (s)</td>
</tr>
<tr>
<td>48</td>
<td>Ph</td>
<td>79</td>
<td>0.85 (d, 7.0), 0.96 (d, 7.0), 0.98 (t, 7.0), 1.68 (d, 4.8), 3.47 (s)</td>
</tr>
<tr>
<td>49</td>
<td>COPh</td>
<td>85</td>
<td>0.83 (d, 6.6), 0.95 (d, 7.2), 0.97 (t, 7.3), 1.65 (d, 4.6), 3.43 (s)</td>
</tr>
</tbody>
</table>

\(^a\) \(\rho\)-Methoxybenzoylcarbonyl.
The effects of PA-48153C derivatives and ciclosporin (CsA) on the responses of both T and B cells to mitogens were examined. As shown in Table 2, these derivatives inhibited the proliferative responses of mouse spleen cells to T cell and B cell mitogens, concanavalin A (Con A) and lipopolysaccharide (LPS), respectively. Most of the derivatives showed approximately the same potencies as that of PA-48153C.

The effects of these derivatives on the growth of EL4 cells were also examined. The inhibitory activities of most derivatives were much less potent than that of PA-48153C. The Con A/EL4 ratios of IC50 values were also listed in Table 2. In particular, derivatives of the C-8a alcohol exhibited smaller ratios than that of PA-48153C, indicating that these derivatives retained the potent immunosuppressive activity of PA-48153C but had weakened cytotoxicity.

Next, the effects of PA-48153C and the hexanoate 28, which was selected on the grounds of several preliminary experiments, on the generation of cytotoxic T lymphocytes (CTL) were examined. As shown in Table 3, C3H/HeN mice (H-2b) immunized against EL4 cells (H-2k) developed CTL able to lyse H-2b target cells. This lysis is known to be genetically restricted and to be mediated by CTL. Intraperitoneal injection of each compound produced a highly significant dose-dependent suppression of the generation of CTL. The hexanoate 28 was more effective on the suppression than PA-48153C at the same concentration of each compound (5 mg/kg) injected.
Discussion

The introduction of CsA and tacrolimus (FK506) on the market has led to remarkable improvement in human organ transplantation. Moreover, these drugs have also proved effective in the treatment of autoimmune diseases such as rheumatoid arthritis. Both CsA and FK506 block the T cell receptor-mediated signal transduction pathway by inhibiting the protein phosphatase calcineurin, the intracellular mechanism appears to be related to their significant renal toxicity. Antibody-mediated responses are also an important problem for preventing organ rejections. Therefore, much effort has been made to find new types of immunosuppressants with different mechanisms of action, and a variety of agents have been developed along this line.

In this paper, we demonstrated that the suppressive activities of PA-48153C derivatives on T cell proliferative responses were almost the same as that of CsA. The structures of these derivatives were much simpler than CsA and FK506, and derivatives of the C-8a alcohol could be conveniently prepared from natural PA-48153C which had been produced efficiently from Streptomyces prunicolor PA-48153.

In addition, PA-48153C derivatives inhibited the activity on the responses of B cells to mitogens, while the intracellular mechanism appears to be related to the generation of CTL in mice. Other in vivo examinations for in vivo evaluation, and showed inhibitory effect on the generation of CTL in mice. Other in vivo immunosuppressive activities are now under investigation.

Experimental

General Methods of Chemistry

Melting points were determined with a Yanagimoto micro melting point apparatus and are uncorrected. IR spectra were determined on a JASCO A-702 infrared spectrometer. NMR spectra were determined on a Varian Gemini-200 or Varian VXR-200 spectrometer. Liquid secondary ion mass spectra (LSI-MS) and high resolution (HR)-LSI-MS were determined on a Hitachi M-90 mass spectrometer. NMR spectra were determined on a Varian XL-200 spectrometer. IR spectra were determined on a JASCO A-702 infrared spectrometer. Melting points were determined with a Yanagimoto for an organic phase over anhydrous sodium sulfate is simply indicated by the word “dried.” Column chromatography using Merck Silica gel 60 or a Merck Lobar column is referred to as “chromatography on silica gel.”

\[ (5R,6R,2'R,3'S,4'R,5'S)-(7E)-5,6-Dihydro-6-(2'-hydroxy-4'-methoxy-3',5'-dimethyl-7'-nonenyl)-5'-methoxy-2H-pyran-2-one \]

Compound 15 was obtained from methyl-a-D-galactopyranoside (2) and (S)+(--)-methyl 3-hydroxy-2-methylpropionate (5) using a procedure similar to that described in the previous paper.3) To a solution of 15 (42 mg, 0.10 mmol) in DMF (2 ml) were added sodium hydride (24 mg, 0.10 mmol) and carbon disulfide (0.06 ml, 0.50 mmol). The mixture was stirred for 20 minutes at 20°C, then cooled to 0°C. To this mixture was added iodomethane (0.062 ml, 0.50 mmol). The mixture was stirred for 40 minutes at 20°C, then poured into 5% aqueous acetic acid (5 ml) and extracted with EtOAc. The organic solution was washed with brine, dried, and evaporated. The residue was chromatographed on silica gel (eluent: EtOAc-hexane 1:15) to give 16 (54 mg, 90%) as a yellow oil.

To a solution of 16 (9.0 mg, 0.015 mmol) in toluene (2 ml) was added diphenylsilane (0.03 ml, 0.162 mmol). The solution was heated to 100°C and treated with 2,2'-azobisisobutyronitrile (27 mg, 0.162 mmol) in toluene (1 ml). The mixture was stirred for 2 hours at the same temperature, then poured into saturated sodium bicarbonate solution and extracted with EtOAc. The organic solution was washed with brine, dried, and evaporated. The residue was chromatographed on silica gel (eluent: EtOAc-hexane 1:5) to give 17 (2.6 mg, 45%) as a colorless oil.

Compound 20 was obtained from 17 as a colorless oil using a procedure similar to that described in the previous paper.3) IR (CHCl3) cm⁻¹ 3420 (OH), 1750 (C=O). 1H NMR (CDCl3) δ 0.95 (3H, d, J = 7.0 Hz, CH3CH3), 0.99 (3H, d, J = 7.4 Hz, CH2CH3), 1.67 (3H, d, J = 4.8 Hz, 9'-H3), 3.01 (1H, dd, J = 6.0 and 6.0 Hz, 4'-H), 3.41 (3H, s, OCH3), 4.82 (3H, s, OCH3), 3.84 (1H, dd, J = 3.3 and 4.8 Hz, 5'-H), 4.23 (1H, m, 2'-H), 4.58 (1H, m, 6'-H), 5.28~5.57 (2H, m, 7'-H and 8'-H), 6.19 (1H, d, J = 9.8 Hz, 3-H), 7.01 (1H, dd, J = 5.1 and 9.8 Hz, 4-H). LSI-MS m/z 349 (M + Na)+, HR-LSI-MS m/z 327.2177 (M+H)+ (Calcd for C18H31O5 m/z 327.2177)

\[ (5R,6R,2'R,3'S,4'R,5'S)-(7E)-5,6-Dihydro-6-(2'-hydroxy-4'-methoxy-3',5'-dimethyl-7'-nonenyl)-5'-methoxy-2H-pyran-2-one \]
Ozonized oxygen was bubbled through a solution of 21 (712 mg, 1.63 mmol) in dichloromethane (30 ml) at −78°C for 5 minutes. Nitrogen was bubbled through the solution to displace ozone. To this solution was added dropwise methyl sulfide (5.0 ml) at −78°C. After standing for 12 hours at 20°C, the organic solution was evaporated. The residue was chromatographed on silica gel (eluent: EtOAc - hexane 1:2) to give 22 (712 mg, 88%) as a colorless oil.

A solution of 23 (28 mg, 0.05 mmol) in MeOH (1.2 ml) and 2 N HCl (0.2 ml) was allowed to stand at 20°C for 20 hours. The mixture was extracted with ethyl ether. The organic solution was washed with 5% sodium carbonate solution and brine, then dried and evaporated. The crystalline residue (20 mg, 96%) was recrystallized from hexane to give 24 as colorless crystals. PM 93−94°C. IR (CHCl₃) cm⁻¹ 3446 (OH), 1715 (C=O). ¹H NMR (CDCl₃) δ 0.96 (3H, d, /=6.6 Hz, C(CH₃)₂), 0.97 (3H, t, J=7.0 Hz, CH₂CH₃), 1.03 (3H, d, J=7.0 Hz, CH₂CH₃), 1.10 (3H, s, CH₃), 1.48 (3H, m, 2'-H), 1.71 (3H, s, C(CH₃)₂), 1.93 (1H, dd, J=5.0 and 7.0 Hz, 3'-H), 2.97 (1H, dd, /=5.0 and 7.0 Hz, 2'-H), 3.39 (3H, s, OCH₃), 3.48 (3H, s, OCH₃), 3.61 (1H, d, J=4.0 Hz, 9'-H), 4.28 (1H, t, J=7.5 Hz, 7-H), 4.45 (1H, d, J=6.7 Hz, 8-H), 5.00 (1H, dd, J=0.5 and 9.6 Hz, 4-H).

To a solution of 1 (700 mg, 2.16 mmol) in DMF (3.5 ml) was added iodomethane (13.4 ml, 216 mmol). The mixture was stirred for 60 minutes at 60°C, and sodium hydride (1.04 g, 43.2 mmol) was added. The mixture was stirred for 1 hour at 15°C and poured into saturated ammonium chloride solution. The product was extracted with EtOAc. The organic solution was washed with brine, dried, and evaporated. The residue was chromatographed on silica gel (eluent: EtOAc - hexane 1:4) to give 25 (225 mg, 68%) as colorless needles. MP 114−115°C. IR (CHCl₃) cm⁻¹ 3468 (OH), 1714 (C=O). ¹H NMR (CDCl₃) δ 0.97 (3H, t, J=7.0 Hz, CH₂CH₃), 1.02 (3H, d, J=6.5 Hz, CH₂CH₃), 1.03 (3H, d, J=7.0 Hz, CH₂CH₃), 2.98 (1H, dd, J=5.0 and 7.0 Hz, 4'-H), 3.50 (3H, s, OCH₃), 4.28 (1H, m, 2'-H), 4.76 (1H, m, 6-H), 6.03 (1H, dd, J=1.0, and 9.0 Hz, 3-H), 6.43 (1H, t, J=7.0 Hz, 7-H), 7.01 (1H, dd, J=6.0 and 9.0 Hz, 4-H).

Found: C 71.05, H 10.02. 

(5R,6R,2'R,3'R,4'R,5'S')-(7E)-5-Ethyl-6-(2',4'-dimethoxy-3',5'-dimethyl-7'-nonenyl)-5,6-dihydro-2'H-pyran-2-one (46)

To a solution of 1 (700 mg, 2.16 mmol) in dichloromethane (30 ml) was added iodomethane (13.4 ml, 216 mmol). The solution was cooled to −20°C, and sodium hydride (1.04 g, 43.2 mmol) was added. The mixture was stirred for 1 hour at 15°C and poured into saturated ammonium chloride solution. The product was extracted with EtOAc, washed with brine, dried, and evaporated. The residue was chromatographed on silica gel (eluent: EtOAc - hexane 1:3) to give 46 (530 mg, 73%). PM 70−71°C. [α]D²⁰ = −129.6° (c 1.00, CHCl₃). IR (CHCl₃) cm⁻¹ 1715 (C=O). ¹H NMR (CDCl₃) δ 0.83 (6H, d, J=6.8 Hz, 2 x CH₂CH₃), 0.98 (3H, t, J=7.5 Hz, CH₂CH₃), 1.68 (3H, d, J=4.0 Hz, 9'-H), 3.10 (1H, dd, J=2.0 and 9.0 Hz, 4'-H), 3.45 (3H, s, OCH₃), 3.48 (3H, s, OCH₃), 3.71 (1H, dt, J=2.0 and 6.8 Hz, 2'-H), 4.59 (1H, dd, J=3.7, 4.8, and 8.5 Hz, 6-H), 5.32−5.59 (2H, m, 7'-H and 8'-H), 6.04 (1H, d, J=9.7 Hz, 3-H), 7.02 (1H, dd, J=6.0 and 9.7 Hz, 4-H). LSI-MS m/z 339 (M+H)⁺.

Found: C 70.69, H 10.05.
NMR spectral data are shown in Table 1.

(5R,6R,2'R,3'R,4'R,5'S)-(7'E)-6-[2'-(Butoxycarbonyl)oxy-4'-methoxy-3',5'-dimethyl-7'-nonenyl]-5-ethyl-5,6-dihydro-2'H-pyran-2-one (47)

To a solution of 1 (1.53 g, 4.72 mmol) in benzene (15 ml) were added pyridine (1.5 ml) and butyl chloroformate (1.5 ml, 11.7 mmol). The mixture was stirred for 4 hours at 20°C. The mixture was diluted with ammonium hydroxide and extracted with EtOAc. The organic solution was washed with brine, dried, and evaporated. The residue was chromatographed on silica gel (eluents: EtOAc-hexane 1:3) to give 47 (1.93 g, 100%) as a colorless oil. \( [\alpha]_D^2 = -90.9^\circ \) (c 1.07, CHCl\(_3\)). IR (CHCI\(_3\)) cm\(^{-1} \) 1728 (C=O), 1716 (C=O). 1H NMR (CDCl\(_3\)) \( \delta \) 0.83 (3H, d, \( J = 6.6 \) Hz, \( C_3CH_3 \)), 0.91 (3H, d, \( J = 6.6 \) Hz, \( C_3CH_3 \)), 0.94 (3H, t, \( J = 7.2 \) Hz, \( CH_3CH_2 \)), 1.67 (3H, d, \( J = 4.6 \) Hz, \( CH_3 \)), 2.99 (1H, dd, \( J = 2.0 \) and 9.4 Hz, 4'-H), 3.42 (3H, s, OCH\(_3\)), 4.05-4.25 (2H, m, 7-H and 8'-H), 6.02 (1H, d, \( J = 9.8 \) Hz, 3-H), 7.01 (1H, dd, \( J = 6.0 \) and 9.8 Hz, 4-H). LSI-MS \( m/z \) 472.2697 (M+H)+. HR-LSI-MS found: C 67.62, H 9.52.

Effect of PA-48153C Derivatives on Mitogen Responses

Splenic monoclonal cells (5 \times 10^5) from C3H/HeN mice were suspended in PRMI 1640 medium (0.1 ml) containing 10% fetal calf serum (FCS) and 5 \times 10^{-5} M 2-mercaptoethanol and placed in 96-well microtiter plates. To each well were added 5 \mu g/ml Con A (Type IV, Sigma) or 10 \mu g/ml LPS (Difco) and PA-48153C derivative in DMSO in such a manner that the final volume was 0.2 ml. The final concentration was not more than 100 ng/ml. After 3 days incubation at 37°C in a humidified atmosphere of air containing 5% carbon dioxide, 6 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma) (25 \mu l) was added to each well. After further incubation for 4 hours under the same conditions, the formazan generated therein was dissolved by adding 20% sodium dodecylsulfonate in 0.02n HCl (50 \mu l) and allowing the mixture to stand at 37°C for 24 hours. The amount of formazan generated in proportion to the number of live cells was determined according to the optical density analyzed by an immunoreader (Sanko Junyaku) equipped with a 570-nm filter. IC_{50} (the concentration inhibiting 50% cell growth) was calculated from the correlativity of PA-48153C concentration with optical density. The results are shown in Table 2.

Effect of PA-48153C Derivatives on EL4 Cell Growth

EL4 thymoma cell line from C57BL/6 mice was put in each well of a 96-well microtiter plate in one 0.1-ml scale containing 4 \times 10^5 cells. PA-48153C derivative in DMSO (0.1 ml) was added to each well in such a manner that its final concentration was in the range of 0 to 5000 ng/ml. After 3 days incubation, IC_{50} was calculated as described above. The results are shown in Table 2.

Effect of PA-48153C and 28 on Generation of CTL

The procedure described by Brünner\(^8\) was used.

a) Tumor cells and immunization.

The EL4 cell line, derived from a C57BL/6 thymoma, has the H-2^b haplotype. It was maintained in culture in RPMI 1640+10% FCS. C3H/HeN mice were injected intraperitoneally with 1 \times 10^7 cultured EL4 cells. After 7 days, the mice were rechallenged intraperitoneally (5 \times 10^6 cells per mouse). Four groups (4 mice injected with EL4 cells per group) were treated for 2, 5, 6, 7, 8, 9, 10, 13, 14, and 15 days with PA-48153C at 5 mg/kg/day, or with 28 at doses of 2.5, 5, and 10 mg/kg/day, injected intraperitoneally in 0.2 ml of vehicle, respectively. Spleens were removed on day 16 and suspensions of cells prepared in RPMI 1640+10% FCS. These cells were used as effectors in tests for cytotoxicity.

b) Preparation of target cells.

Cultured exponentially growing tumor cells (EL4) were labelled with \( ^{51} \)Cr by incubating 1 \times 10^7 cells with 100 \mu Ci of sodium chromate (New England Nuclear, specific activity = 562 \mu Ci/mg) for 1 hour at 37°C. Cells were washed three times and resuspended in RPMI 1640+10% FCS.

Effect of PA-48153C Derivatives on Mitogen Responses

Splenic monoclonal cells (5 \times 10^5) from C3H/HeN mice were suspended in PRMI 1640 medium (0.1 ml) containing 10% fetal calf serum (FCS) and 5 \times 10^{-5} M 2-mercaptoethanol and placed in 96-well microtiter plates. To each well were added 5 \mu g/ml Con A (Type IV, Sigma) or 10 \mu g/ml LPS (Difco) and PA-48153C derivative in DMSO in such a manner that the final volume was 0.2 ml. The final concentration was not more than 100 ng/ml. After 3 days incubation at 37°C in a humidified atmosphere of air containing 5% carbon dioxide, 6 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma) (25 \mu l) was added to each well. After further incubation for 4 hours under the same conditions, the formazan generated therein was dissolved by adding 20% sodium dodecylsulfonate in 0.02n HCl (50 \mu l) and allowing the mixture to stand at 37°C for 24 hours. The amount of formazan generated in proportion to the number of live cells was determined according to the optical density analyzed by an immunoreader (Sanko Junyaku) equipped with a 570-nm filter. IC_{50} (the concentration inhibiting 50% cell growth) was calculated from the correlativity of PA-48153C concentration with optical density. The results are shown in Table 2.

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c) Cytotoxicity assay.

Equal volumes (0.1 ml) of radiolabelled target cells (1 \times 10^5/ml) and effector cells were mixed in 96 well culture plate (U-bottom, Falcon) and incubated for 4 hours at 37°C in an atmosphere of air containing 5% carbon dioxide. The ratio of effector cells to target cells (E:T ratio) was 50:1 or 25:1. Target cells, either alone (spontaneous release) or mixed with non-immune spleen cells, served as controls and were incubated for the same period. Supernatants (0.1 ml per tube) were carefully removed and counted in a gamma scintillation counter. Maximum chromium release was determined by freezing and thawing an equivalent number of labelled target cells four times, causing complete lysis of the cells.

Results are expressed in Table 3 as percentage of specific cell lysis according to the following formula:

\[
\% \text{ specific lysis} = \frac{[\text{release with effector cells} - \text{spontaneous release}]}{[\text{maximum release} - \text{spontaneous release}]} \times 100.
\]

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References


