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

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(Received for publication August 4, 1995)

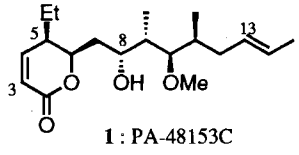
5β-Methoxy (**20**), 14-methyl (**24**), 14,14-dibromo-15-nor (**25**), 8-*O*-acyl (**26**～**45**), 8-*O*-alkyl (**46**), 8-*O*-alkoxycarbonyl (**47**, **48**), and 8-*O*-carbamoyl (**49**) derivatives of PA-48153C, a novel immunosuppressant isolated from fermentation products of *Streptomyces prunicolor* 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.[1] 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;[2] 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,[3] 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.

Scheme 1.



1: PA-48153C

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,[4] since the C-5β methoxy group could be derived from galactose. Acyclic segment **6** was prepared from **5** as shown in our previous paper.[3] 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-elimimation of the C-5β methoxy group.

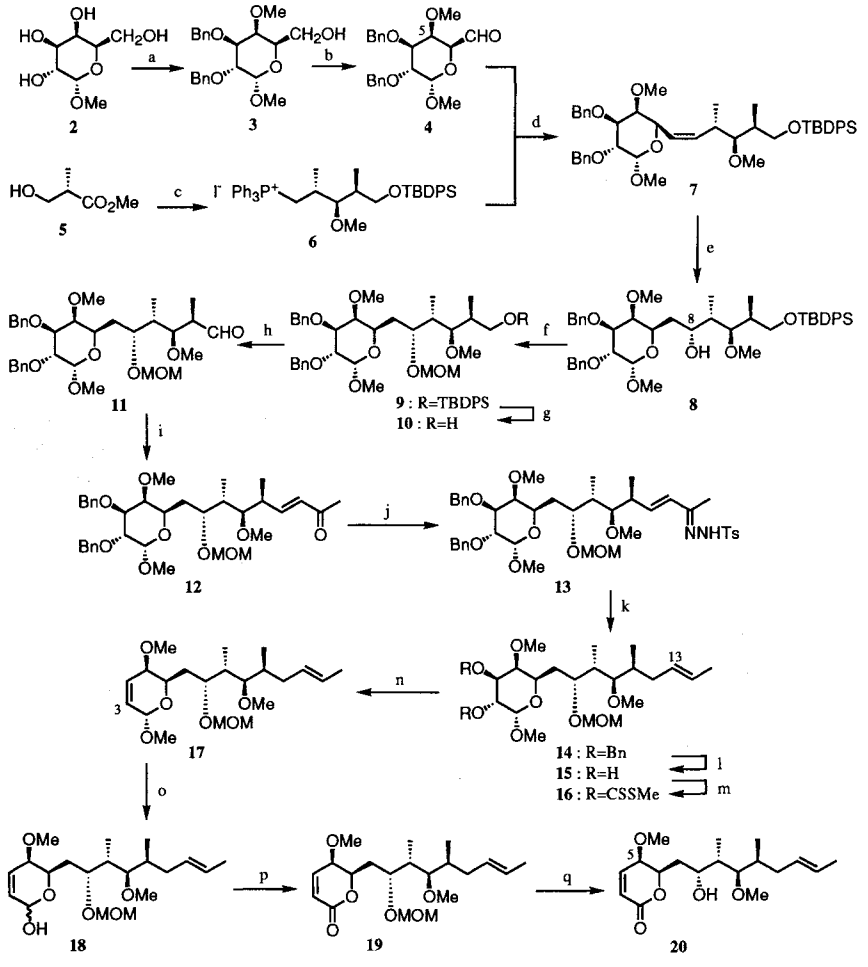
Hydroboration of **7** followed by treatment with alkaline hydrogen peroxide gave the desired C-8α alcohol **8** as the main product. Regiochemical assignment of the hydroxyl group was based on the ^{1}H NMR decoupling

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.

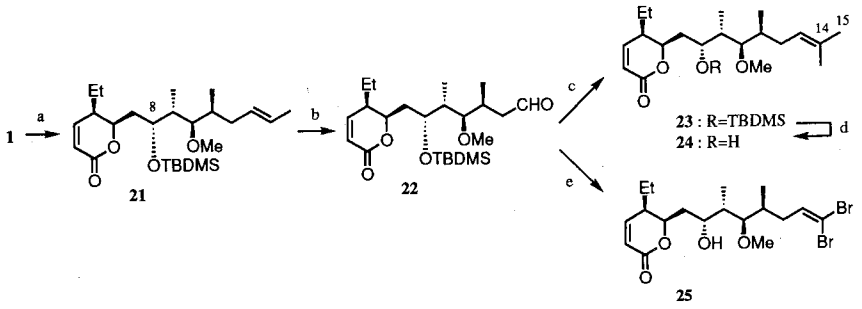
14-Methyl and 14,14-dibromo-15-nor derivatives **24** and **25** were synthesized *via* aldehyde **22** starting from natural PA-48153C as shown in Scheme 3. Since ozonolysis of **1** resulted in ring closure by intermolecular attack of C-8α hydroxyl group to aldehyde, the C-8α hydroxyl group was protected 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, $(COCl)_2$ followed by Et_3N; (c) Ref. 3; (d) *n*-BuLi (32% for b, d); (e) B_2H_6, H_2O_2; (f) MOMCl, *i*-Pr_2NEt (43% for e, f); (g) Bu_4NF (96%); (h) DMSO, $(COCl)_2$ followed by Et_3N; KO*t*-Bu, $(CH_3O)_2P(O)CH_2COCH_3$ (71% for h, i); (j) $TsNHNH_2$; (k) $NaBH_4$, HOAc (56% for j, k); (l) Na, NH_3 (92%); (m) CS_2, NaH, MeI (90%); (n) Ph_2SiH_2, AIBN (45%); (o) 75% aq. HOAc, 40°C; (p) PCC, NaOAc (33% for o, p); (q) 60% aq. HOAc, reflux (70%).

Scheme 3.



(a) TBDMSOTf, 2,6-lutidine (88%); (b) O_3, Me_2S (83%); (c) Ph_3P^+ *i*-Pr Br^-, KO*t*-Bu (61%); (d) HCl (96%); (e) CBr_4, PPh_3 (68%).

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

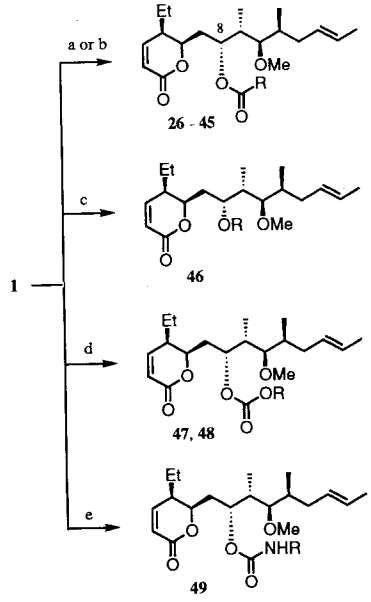
Compound	R	Yield (%)	^{1}H NMR ($CDCl_3$) δ CH_3 (*J* = Hz)
26	Et	100	0.81 (d, 7.0), 0.89 (d, 7.0), 0.97 (t, 7.4), 1.16 (t, 7.6), 1.66 (d, 5.0), 3.39 (s)
27	Pr	48	0.81 (d, 7.0), 0.88 (d, 7.0), 0.97 (t, 7.4), 0.97 (t, 7.8), 1.66 (d, 5.0), 3.39 (s)
28	$(CH_2)_4CH_3$	97	0.81 (d, 6.8), 0.88 (d, 6.8), 0.90 (t, 6.7), 0.97 (t, 7.5), 1.66 (d, 4.8), 3.39 (s)
29	CH_2Cl	74	0.82 (d, 7.0), 0.92 (d, 7.2), 0.98 (t, 7.4), 1.67 (d, 5.0), 3.40 (s)
30	$CHCl_2$	99	0.83 (d, 6.8), 0.94 (d, 7.0), 0.98 (t, 7.5), 1.65 (d, 5.2), 3.41 (s)
31	CCl_3	92	0.84 (d, 6.8), 0.97 (d, 7.0), 0.99 (t, 7.2), 1.65 (d, 4.8), 3.43 (s)
32	$(CH_2)_2CH_2Cl$	96	0.82 (d, 6.8), 0.92 (d, 7.2), 0.98 (t, 7.4), 1.67 (d, 5.0), 3.40 (s)
33	$(CH_2)_4CH_2Br$	80	0.81 (d, 6.6), 0.89 (d, 7.2), 0.97 (t, 7.6), 1.67 (d, 5.0), 3.39 (s)
34	CH_2CH=$CHEt$	93	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)
35	$(CH_2)_2CH$=CH_2	95	0.81 (d, 6.8), 0.89 (d, 6.8), 0.97 (t, 7.4), 1.67 (d, 5.0), 3.38 (s)
36	2-Cyclopentylethyl	100	0.81 (d, 6.6), 0.89 (d, 7.2), 0.97 (t, 7.4), 1.67 (d, 4.6), 3.39 (s)
37	2-Quinolyl	97	0.86 (d, 6.6), 0.96 (t, 7.4), 1.11 (d, 7.0), 1.59 (d, 4.4), 3.41 (s)
38	2-Quinoxalyl	100	0.86 (d, 6.6), 0.98 (t, 7.6), 1.12 (d, 7.0), 1.60 (d, 4.6), 3.41 (s)
39	CH_2CO_2Me	74	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)
40	$(CH_2)_2CO_2Me$	99	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)
41	CH=$CHCO_2Me$	85	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)
42	$(CH_2)_4CO_2Me$	100	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)
43	CH_2NHBoc	100	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)
44	$(CH_2)_3NHMoz$[a]	100	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)
45	$(CH_2)_5NHMoz$[a]	76	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)
46	Me	73	0.83 (6H, d, 6.8), 0.98 (t, 7.5), 1.68 (d, 4.0), 3.45 (s), 3.48 (s)
47	Bu	100	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)
48	Ph	79	0.85 (d, 7.0), 0.96 (d, 7.0), 0.98 (t, 7.0), 1.68 (d, 4.8), 3.47 (s)
49	COPh	85	0.83 (d, 6.6), 0.95 (d, 7.2), 0.97 (t, 7.3), 1.65 (d, 4.6), 3.43 (s)

[a] *p*-Methoxybenzyloxycarbonyl.

which was accompanied by deprotection of the TBDMS group.

Derivatives of the C-8α alcohol were conveniently prepared from natural PA-48153C as shown in Scheme 4 and Table 1. Since C-8α 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*-Alkoxycarbonyl 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 presense of DMAP, although bis(tributyltin) oxide was used instead of DMAP when the reaction did not proceed.

Scheme 4.



(a) RCOX, DMAP, Pyridine; (b) RCO_2H, DCC, DMAP, CH_2Cl_2; (c) RX, NaH, DMF; (d) ROCOCl, Pyridine, C_6H_6; (e) RNCO, DMAP, CH_2Cl_2.

Biological Activities

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 IC_{50} values were also listed in Table 2. In particular, derivatives of the C-8α 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 ($H\text{-}2^k$) immunized against EL4 cells ($H\text{-}2^b$) developed CTL able to lyse $H\text{-}2^b$ 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.

Table 2. Effect of PA48153C derivatives on mitogen responses and EL-4 cell growth.

Compound	IC_{50} (ng/ml)			Con A/EL-4[c]
	Con A[a]	LPS[b]	EL-4	
1	3.4	3.4	4.8	0.708
20	15.9	14.8	33.4	0.476
24	4.6	5.0	7.8	0.590
25	2.6	3.0	4.0	0.650
26	18.4	16.7	404.5	0.045
27	7.6	5.5	256.2	0.030
28	9.7	9.4	404.5	0.024
29	29.5	18.8	228.6	0.129
30	3.9	3.6	14.2	0.275
31	3.6	3.2	12.3	0.293
32	12.5	7.9	316.7	0.039
33	16.1	15.1	311.6	0.052
34	6.9	7.0	314.1	0.022
35	6.7	6.6	301.6	0.022
36	7.7	6.1	232.3	0.033
37	19.6	20.0	254.1	0.077
38	1.8	1.0	17.7	0.102
39	13.0	9.0	421.3	0.031
40	12.6	8.8	280.0	0.045
41	10.2	7.4	417.9	0.024
42	7.9	6.6	212.4	0.037
43	13.9	8.2	428.2	0.032
44	28.3	24.5	407.8	0.069
45	9.0	7.6	137.9	0.065
46	15.9	14.8	33.4	0.476
47	25	<20	160	0.156
48	6.9	5.0	145.9	0.047
49	6.1	4.3	397.9	0.015
CsA	13.1	2800	3600	0.0036

[a] Inhibitory activity against Con A-stimulated T cell proliferation.
[b] Inhibitory activity against LPS-stimulated B cell proliferation.
[c] Ratios of IC_{50} values.

Table 3. Effect of PA-48153C and **28** on generation of cytotoxic T lymphocytes.

Compound	Dose (mg/kg)	% Specific lysis	
		E/T = 50	E/T = 25
Vehicle		18.6 ± 4.1	12.4 ± 3.8
PA-48153C	5.0	7.4 ± 2.2	3.4 ± 0.8
28	2.5	10.5 ± 2.3	6.6 ± 1.0
	5.0	1.9 ± 0.6	1.6 ± 0.7
	10.0	1.1 ± 0.7	0.4 ± 0.1

E/T: effector cells/target cells.

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, but 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 CsA and FK506 selectively inhibited T cell activation. We also observed in a preliminary experiment that PA-48153C blocked the progression of cells from G_2/M back to G_1 by piling up of cells in G_2/M, while CsA and FK506 were reported to block lymphocyte activation early on at the G_0/G_1 interface in the cell division cycle.[6] Therefore, although the precise mechanism was not clear, the mode of action of PA-48153C derivatives seemed to be different from those of CsA and FK506.

Consequently, we decided to evaluate the possibility of these derivatives for new immunosuppressive drugs. The hexanoate **28** was selected from the compounds examined 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 using *m*-nitrobenzyl alcohol as a matrix. Unless otherwise stated, all reactions were carried out under a nitrogen atmosphere with anhydrous solvents that had been dried over type 4A molecular sieves. Drying of 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."

(5*R*,6*R*,2′*R*,3′*S*,4′*R*,5′*S*)-(7′*E*)-5,6-Dihydro-6-(2′-hydroxy-4′-methoxy-3′,5′-dimethyl-7′-nonenyl)-5-methoxy-2*H*-pyran-2-one (**20**)

Compound **15** was obtained from methyl-α-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 coloriess oil using a procedure similar to that described in the previous paper.[3] IR ($CHCl_3$) cm^{-1} 3420 (OH), 1750 (C=O). ^1H NMR ($CDCl_3$) δ 0.95 (3H, d, J=7.0 Hz, CH_3CH), 0.99 (3H, d, J=7.4 Hz, CH_3CH), 1.67 (3H, d, J=4.8 Hz, 9′-H_3), 3.01 (1H, dd, J=6.0 and 6.0 Hz, 4′-H), 3.41 (3H, s, OCH_3), 3.48 (3H, s, OCH_3), 3.84 (1H, dd, J=3.3 and 4.8 Hz, 5-H), 4.23 (1H, m, 2′-H), 4.68 (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 $C_{18}H_{31}O_5$ m/z 327.2170).

(5*R*,6*R*,2′*R*,3′*S*,4′*R*,5′*S*)-(7′*E*)-5-Ethyl-5,6-dihydro-6-(2′-hydroxy-4′-methoxy-3′,5′,8′-trimethyl-7′-nonenyl)-2*H*-pyran-2-one (**24**)

To a solution of **1** (600 mg, 1.85 mmol) in dichloromethane (15 ml) were added dropwise 2,6-lutidine (650 μl, 5.55 mmol) and *tert*-butyldimethylsilyl trifluoromethanesulfonate (640 μl, 2.78 mmol) at 0°C. The mixture was stirred for 1 hour at the same temperature, then poured into cold water and extracted with ethyl ether. The organic solution was washed with 5% sodium carbonate solution and brine, then dried and evaporated. The residue was chromatographed on silica gel

(eluent: EtOAc - hexane 1 : 2) to give **21** (712 mg, 88%) as a colorless oil.

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 : 4) to give **22** (577 mg, 83%) as a colorless oil.

To a suspension of isopropyltriphenylphosphonium bromide (113 mg, 0.29 mmol) in THF (1.5 ml) was added potassium *tert*-butoxide (26 mg, 0.23 mmol) at 0°C. The mixture was stirred for 40 minutes at the same temperature, then cooled to −78°C. A solution of **22** (35 mg, 0.08 mmol) in THF (0.5 ml) was added dropwise. The mixture was stirred for 40 minutes at −78°C, then allowed to warm to 20°C and stirred for 1 hour. The mixture was poured into saturated ammonium chloride 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 : 9) to give **23** (28 mg, 61%) 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. MP 93~94°C. IR ($CHCl_3$) cm^{-1} 3446 (OH), 1714 (C=O). ^1H NMR ($CDCl_3$) δ 0.96 (3H, d, J=6.6 Hz, CH_3CH), 0.97 (3H, t, J=7.0 Hz, CH_3CH_2), 1.06 (3H, d, J=7.0 Hz, CH_3CH), 1.60 (3H, s, CH_3CMe), 1.71 (3H, s, CH_3CMe), 2.99 (1H, dd, J=5.0 and 7.0 Hz, 4'-H), 3.48 (3H, s, OCH_3), 4.22 (1H, m, 2'-H), 4.74 (1H, m, 6-H), 5.11 (1H, br t, J=6.5 Hz, 7'-H), 6.03 (1H, dd, J=1.0, and 9.0 Hz, 3-H), 7.02 (1H, dd, J=6.0 and 9.0 Hz, 4-H). HR-LSI-MS m/z 339.2536 $(M+H)^+$ (Calcd for $C_{20}H_{35}O_4$ m/z 339.2534).

(5*R*,6*R*,2'*R*,3'*S*,4'*R*,5'*S*)-(7'*E*)-6-(8',8'-Dibromo-2'-hydroxy-4'-methoxy-3',5'-dimethyl-7'-octenyl)-5-ethyl-5,6-dihydro-2*H*-pyran-2-one (**25**)

To a solution of triphenylphosphine (1.47 g, 5.6 mmol) in dichloromethane (200 ml) was added carbon tetrabromide (927 mg, 2.8 mmol) at 0°C. The mixture was stirred for 15 minutes, then a solution of **22** (300 mg, 0.7 mmol) in dichloromethane (10 ml) was added dropwise at 0°C. The mixture was stirred for 1 hour at the same temperature, then diluted with EtOAc (40 ml). After bubbling of air for 30 minutes, the mixture was filtered through a Celite pad. The organic solution was 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 ($CHCl_3$) cm^{-1} 3468 (OH), 1714 (C=O). ^1H NMR ($CDCl_3$) δ 0.97 (3H, t, J=7.0 Hz, CH_3CH_2), 1.02 (3H, d, J=6.5 Hz, CH_3CH), 1.03 (3H, d, J=7.0 Hz, CH_3CH), 2.98 (1H, dd, J=5.0 and 7.0 Hz, 4'-H), 3.50 (3H, s, OCH_3), 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).

Anal Calcd for $C_{18}H_{28}O_4Br_2$: C 46.17, H 6.03.
Found: C 45.85, H 5.91.

(5*R*,6*R*,2'*R*,3'*R*,4'*R*,5'*S*)-(7'*E*)-5-Ethyl-6-(2'-hexanoyloxy-4'-methoxy-3',5'-dimethyl-7'-nonenyl)-5,6-dihydro-2*H*-pyran-2-one (**28**)

To a solution of **1** (1.51 g, 4.66 mmol) in dichloromethane (15 ml) were added pyridine (1.6 ml) and hexanoyl chloride (1.7 ml, 12.1 mmol). The mixture was stirred for 1 hour 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 (eluent: EtOAc - hexane 3 : 17) to give **28** (1.91 g, 97%) as a colorless oil. $[\alpha]_D^{22}$ −97.9° (c 1.14, $CHCl_3$). IR ($CHCl_3$) cm^{-1} 1720 (C=O). ^1H NMR ($CDCl_3$) δ 0.81 (3H, d, J=6.8 Hz, CH_3CH), 0.88 (3H, d, J=6.8 Hz, CH_3CH), 0.90 (3H, t, J=6.7 Hz, CH_3CH_2), 0.97 (3H, t, J=7.5 Hz, CH_3CH_2), 1.66 (3H, d, J=4.8 Hz, 9'-H_3), 2.31 (2H, t, J=7.5 Hz, CH_2CO), 2.88 (1H, dd, J=2.0 and 9.6 Hz, 4'-H), 3.39 (3H, s, OCH_3), 4.46 (1H, dt, J=3.4 and 6.7 Hz, 6-H), 5.21~5.56 (3H, m, 2'-H, 7'-H, and 8'-H), 6.02 (1H, dd, J=0.5, and 9.6 Hz, 3-H), 7.01 (1H, dd, J=6.2 and 9.6 Hz, 4-H). LSI-MS m/z 423 $(M+H)^+$.

Anal Calcd for $C_{25}H_{42}O_5$: C 71.05, H 10.02.
Found: C 70.74, H 10.02.

(5*R*,6*R*,2'*R*,3'*S*,4'*R*,5'*S*)-(7'*E*)-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 DMF (3.5 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%). MP 70~71°C. $[\alpha]_D^{24}$ −129.6° (c 1.00, $CHCl_3$). IR ($CHCl_3$) cm^{-1} 1715 (C=O). ^1H NMR ($CDCl_3$) δ 0.83 (6H, d, J=6.8 Hz, 2×CH_3CH), 0.98 (3H, t, J=7.5 Hz, CH_3CH_2), 1.68 (3H, d, J=4.0 Hz, 9'-H_3), 3.10 (1H, dd, J=2.0 and 9.0 Hz, 4'-H), 3.45 (3H, s, OCH_3), 3.48 (3H, s, OCH_3), 3.71 (1H, dt, J=2.0 and 6.8 Hz, 2'-H), 4.59 (1H, ddd, 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 $(M+H)^+$.

Anal Calcd for $C_{20}H_{34}O_4$: C 70.97, H 10.12.
Found: C 70.69, H 10.05.

(5*R*,6*R*,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 (eluent: EtOAc-hexane 1:3) to give **47** (1.93 g, 100%) as a colorless oil. $[\alpha]_D^{22}$ −90.9° (*c* 1.07, $CHCl_3$). IR ($CHCl_3$) cm^{-1} 1728 (C=O). ^1H NMR ($CDCl_3$) δ 0.82 (3H, d, J=6.6 Hz, CH_3CH), 0.91 (3H, d, J=7.0 Hz, CH_3CH), 0.94 (3H, t, J=7.2 Hz, CH_3CH_2), 0.97 (3H, t, J=7.2 Hz, CH_3CH_2), 1.67 (3H, d, J=4.6 Hz, 9′-H_3), 2.98 (1H, dd, J=2.0 and 9.4 Hz, 4′-H), 3.42 (3H, s, OCH_3), 4.05~4.25 (2H, m, OCH_2), 4.50 (1H, ddd, J=3.7, 5.5, and 7.7 Hz, 6-H), 5.16 (1H, dt, J=2.2 and 6.8 Hz, 2′-H), 5.30~5.56 (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* 425 $(M+H)^+$.

Anal Calcd for $C_{24}H_{40}O_6$: C 67.89, H 9.50.
Found: C 67.62, H 9.52.

(5*R*,6*R*,2′*R*,3′*R*,4′*R*,5′*S*)-(7′*E*)-6-[2′-(Benzoylcarbamoyl)oxy-4′-methoxy-3′,5′-dimethyl-7′-nonenyl]-5-ethyl-5,6-dihydro-2*H*-pyran-2-one (**49**)

To a solution of **1** (1.10 g, 3.39 mmol) in dichloromethane (15 ml) were added benzoyl isocyanate (1.3 ml, 10.3 mmol) and DMAP (825 mg, 6.75 mmol). The mixture was stirred for 7 hours at 40°C, then diluted with dichloromethane. The organic solution was washed with brine, dried, and evaporated. The residue was chromatographed on silica gel (eluent: EtOAc-hexane 1:2) to give **49** (1.36 g, 85%) as a colorless foam. $[\alpha]_D^{24}$ −96.6° (*c* 1.01, $CHCl_3$). IR ($CHCl_3$) cm^{-1} 1780 (C=O), 1716 (C=O). ^1H NMR ($CDCl_3$) δ 0.83 (3H, d, J=6.6 Hz, CH_3CH), 0.95 (3H, d, J=7.2 Hz, CH_3CH), 0.97 (3H, t, J=7.3 Hz, CH_3CH_2), 1.65 (3H, d, J=4.6 Hz, 9′-H_3), 2.99 (1H, dd, J=2.2 and 9.4 Hz, 4′-H), 3.43 (3H, s, OCH_3), 4.56 (1H, ddd, J=3.5, 5.7, and 7.4 Hz, 6-H), 5.27~5.57 (3H, m, 2′-H, 7′-H and 8′-H), 6.02 (1H, d, J=9.8 Hz, 3-H), 7.01 (1H, dd, J=6.1 and 9.8 Hz, 4-H), 7.42~7.66 (3H, m, aromatic), 7.80~7.92 (2H, m, aromatic), 8.28 (1H, br s, NH). LSI-MS *m/z* 472 $(M+H)^+$. HR-LSI-MS *m/z* 472.2697 $(M+H)^+$ (Calcd for $C_{27}H_{38}NO_6$ *m/z* 472.2697).

(5*R*,6*R*,2′*R*,3′*R*,4′*R*,5′*S*)-(7′*E*)-5-Ethyl-5,6-dihydro-6-[4′-methoxy-3′,5′-dimethyl-7′-nonenyl-2′-(substituted)oxy]-2*H*-pyran-2-one (**26**, **27**, **29**~**45**, **48**)

These compounds were synthesized by procedures similar to those described above. Yields and selected ^1H NMR spectral data are shown in Table 1.

Effect of PA-48153C Derivatives on Mitogen Responses

Splenic mononuclear cells (5×10^5) from C3H/HeN mice were suspended in PRMI 1640 medium (0.1 ml) containing 10% fetal calf serum (FCS) and 5×10^{-5} M 2-mercaptoethanol and placed in 96-well microtiter plates. To each well were added 5 μg/ml Con A (Type IV, Sigma) or 10 μg/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 μl) 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 0.02 N HCl (50 μl) 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.[7] 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 4×10^4 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 BRUNNER[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 1×10^7 cultured EL4 cells. After 7 days, the mice were rechallenged intraperitoneally (5×10^6 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 1×10^7 cells with 100 μCi of sodium chromate (New England Nuclear, specific activity=562 μCi/mg) for 1 hour at 37°C. Cells were washed three times and resuspended in RPMI 1640+10% FCS.

c) Cytotoxicity assay.

Equal volumes (0.1 ml) of radiolabelled target cells (1×10^5/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:

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

Acknowledgments

We wish to thank Dr. Y. KAWAMURA for the production of PA-48153C, and Dr. Y. IKENISHI for the determination of the MS spectra.

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