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NOTE

Novel 12-membered non-antibiotic macrolides, EM900 series with anti-inflammatory and/or immunomodulatory activity; synthesis, structure–activity relationships and *in vivo* study

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Erythromycin A (EMA), first isolated in 1952,[1] is a clinically useful macrolide antibiotic, active against both Gram-positive bacteria and Gram-negative cocci and mycoplasmas. In the 1980s, EMA was found to have two other promising biological properties, gastrointestinal motor-stimulating activity and anti-inflammatory and/or immunomodulatory activity by Itoh *et al.*[2] and Kudoh *et al.*,[3] respectively. Based on these findings, our research group has been striving to create an ideal derivative, which exhibits a specific activity without showing antibacterial effect. We have already found a new motilide derivative (**EM574**, de-*N*-methyl-*N*-*iso*propyl-8,9-anhydroerythromycin A 6,9-hemiketal), which exhibited gastrointestinal motor-stimulating activity but showed no antibacterial activity.[4–7]

As a result of its anti-inflammatory and/or immunomodulatory activity, EMA is an effective treatment for diffuse panbronchiolitis,[3] chronic sinusitis and cystic fibrosis.[8] Additionally, our group reported that EMA promotes monocyte to macrophage differentiation and inhibits proliferation of T cell *in vitro*.[9,10] Likewise, 14-membered macrolides are also well known to possess anti-inflammatory and/or immunomodulatory activity, which are represented by inhibition of inflammatory cytokine production.[11–13] However, the detailed mode of action of EMA has not yet been determined. Macrolide derivatives with anti-inflammatory and/or immunomodulatory activity but lacking either antibacterial activity or gastrointestinal motor-stimulating activity would be extremely useful, as they would help avoid promotion of drug resistance, as well as help to minimize any adverse effects of EMA treatment.

In a previous paper,[14] we reported our development of the novel 12-membered (8*R*,9*S*)-8,9-dihydro-6,9-epoxy-8,9-anhydropseudoerythromycin A (**EM900**), and analogues thereof, possessing the ability to promote monocyte to macrophage differentiation but lacking antibacterial activity. This provided us with a novel, acid-stable 12-membered macrolide chemical skeleton with anti-inflammatory and/or immunomodulatory activity, although no detailed structure–activity relationships had been clarified. From our initial study, we learned that neither disconnection of the side chain bearing from the C11 position nor the cladinose moiety has an important role in the anti-inflammatory and/or immunomodulatory effects. Consequently, we became interested in synthesis of analogues of the *N*,*N*-dimethylamino group on desosamine moiety, inspired by our experience with erythromycin derivatives with a potent motiline-like activity, especially motilide **EM574**.[4] Generally, the *N*,*N*-dimethylamino group of EMA is an essential moiety for generating antibacterial activity. In contrast, in the case of motiline-like activity, modification of the *N*,*N*-dimethylamino group dramatically increases the activity.[4] Taken together, we envisioned that modification of the *N*,*N*-dimethylamino group might be possible as a means of increasing anti-inflammatory and/or immunomodulatory activity.

In this communication, we report the elucidation of structure–activity relationships of the *N*,*N*-dimethylamino group of **EM900**, as well as those of the analogues, (8*R*,9*S*)-de(3′-*N*-methyl)-3′-*N*-(*p*-chlorobenzyl)-8,9-dihydro-6,9-epoxy-8,9-anhydropseudoerythromycin A (**EM905**) and (8*R*,9*S*)-de(3′-*N*, *N*-dimethylamino)-3′-morpholino-8,9-dihydro-6,9-epoxy-8,9-anhydropseudoerythromycin A (**EM914**). The two compounds, **EM905** and **EM914**, were found to be effective for treatment in a mouse model of inflammatory bowel disease (IBD), even at considerably low doses, compared with the therapeutic sulfa drug, sulfasalazine. We propose that the ability to promote monocyte to macrophage differentiation might have potential as a reliable *in vitro* assay system to help identify and develop potent anti-inflammatory and/or immunomodulatory agents.

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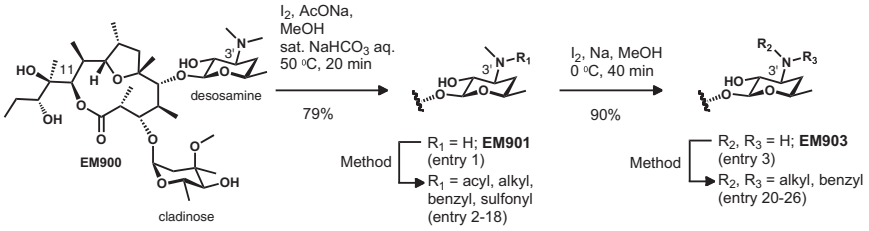
At the outset, removal of a methyl group from the nitrogen of desosamine via treatment of **EM900** with I_2, AcONa in MeOH,[15] provided the de-*N*-methyl analogue **EM901** in 79% yield (Table 1). Subsequent, de-*N*-methylation of **EM901** using I_2 and Na in MeOH[15] afforded bis-de-*N*-methyl **EM903** in 90% yield. With **EM901** and **EM903** in hand, we synthesized 24 analogues (Appendix), such as *N*-mono- and/or *N*,*N*-bis-substituted (for example, alkylated and cyclic alkylated) compounds to investigate structure–activity relationships focusing on this region. Reaction conditions (methods A–F) were as follows; (A) treatment of **EM901** with alkyl halides (RI, RBr and RCl), and *i*-Pr_2NEt in $CHCl_3$ provided *N*-mono-alkylated compounds (entry 2–15); (B) treatment of **EM901** or **EM903** with aldehydes and $NaBH(OAc)_3$ provided *N*-mono or *N*,*N*-bis-alkylated compounds (entry 18, 20, 21 and 23); (C) acetylation of **EM901** provided **EM960** (entry 16); (D) mesylation of **EM901** provided **EM961** (entry 17); (E) treatment with dibromoalkanes provided *N*,*N*-cyclic alkylated compounds (entry 22, 24–26); (F) removal of the Cbz group of **EM965** was carried out under hydrogenation reaction condition to give **EM966** (entry 26, step 2).

To investigate anti-inflammatory and/or immunomodulatory effects, we used the THP-1 assay system, which was modified according to the method of Keicho *et al.*,[10] to test for promotion of differentiation of monocytic cells to macrophages. A THP-1 cell line, derived from a patient with monocytic leukemia, was supplied by the Japanese Cancer Research Resources Bank (Tokyo, Japan). THP-1 cells (1×10^5 per well in 0.5 ml) were placed into 48-well tissue culture microplates (IWAKI, Tokyo, Japan) and cultured in the presence of phorbol myristate acetate (PMA; $2\,ng\,ml^{-1}$) or each macrolide compound (1–100 μM) alone, or both, for 4 days at 37 °C under 5% CO_2 in humidified air. The number and viability of adherent cells was measured by colorimetric

Table 1 Synthesis of *N*-substituted and *N*,*N*-bis-substituted analogues and their biological activities



Entry	*Method*	R_1	R_2	R_3	*Yield (%)*	*Compound*	ED_{50} (μM)[a]	*Cytotoxicity (μM)*
—	—	—	—	—	—	**EM900**	17.1	100
N-mono alkylated analogues								
1	—	H	—	—	79	**EM901**	15.9	100
2	A	Bn	—	—	92	**EM902**	14.5	100
3	A	*p*-ClBn	—	—	93	**EM905**	2.7	30
4	A	*p*-CF_3Bn	—	—	74	**EM919**	>100	100
5	A	*p*-BrBn	—	—	67	**EM920**	>100	100
6	A	*p*-FBn	—	—	86	**EM921**	>100	30
7	A	*o*-ClBn	—	—	98	**EM922**	1.2	100
8	A	*m*-ClBn	—	—	86	**EM923**	2.7	100
9	A	*p*-IBn	—	—	90	**EM924**	>100	30
10	A	propargyl	—	—	64	**EM929**	>100	>100
11	A	Et	—	—	86	**EM933**	>100	100
12	A	*i*-Pr	—	—	38	**EM940**	>100	100
13	A	Allyl	—	—	54	**EM957**	>100	100
14	A	*p*-MeBn	—	—	45	**EM958**	4.8	100
15	A	*p*-MeOBn	—	—	48	**EM959**	>100	100
16	C	Ac	—	—	100	**EM960**	>100	>100
17	D	Ms	—	—	64	**EM961**	44.5	>100
18	B	Pentyl	—	—	81	**EM962**	>100	>100
N,N-Bis-alkylated analogues								
19	—	—	H	H	90	**EM903**	27.6	100
20	B	—	Bn	Bn	63	**EM904**	9.5	30
21	B	—	Bn	H	31	**EM912**	>100	30
22	E	—	$-(CH_2)_2O(CH_2)_2-$		89	**EM914**	52.8	>100
23	B	—	*p*-ClBn	H	55	**EM928**	>100	30
24	E	—	$-(CH_2)_5-$		82	**EM955**	30.3	100
25	E	—	$-(CH_2)_4-$		62	**EM956**	>100	100
26	Step 1. E	—	$-(CH_2)_2N(Cbz)(CH_2)_2-$		61	**EM965**	>100	30
	Step 2. F	—	$-(CH_2)_2NH(CH_2)_2-$		52	**EM966**	>100	100

Method A: alkyl halides (for example RCl, RBr, RI), *i*-Pr_2NEt and $CHCl_3$. Method B: aldehydes, AcOH, $NaBH(OAc)_3$ and 1,2-dichloroethane. Method C: Ac_2O and DCM. Method D: MsCl and DCM. Method E: dibromoalkanes, *i*-Pr_2NEt and MeCN, 80 °C. Method F: H_2, $Pd(OH)_2$ and MeOH, room temperature, 4 h, 52%.
[a]The ED_{50} values were determined, which provided an evaluation of the promotion of monocyte to macrophage differentiation of each analogue, as compared with the result of EMA at 100 μM.

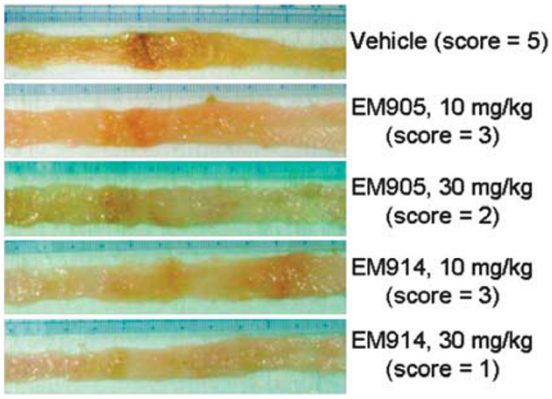
determination of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay at 550 nm. The ED_{50} values were determined, which provided an evaluation of the promotion of monocyte to macrophage differentiation of each analogue, as compared with the result of EMA at 100 μM. Cytotoxicity (μM) was determined using cell-count reagent SF (Nacalai tesque, Tokyo, Japan) according to the manufacturer's instructions.

As the bioactivity data of these analogues are summarized in Table 1, the ED_{50} of de-*N*-methyl and bis-de-*N*-methyl analogues **EM901** and **EM903** are similar compared with that of **EM900** ($ED_{50} = 17.1$ μM). Of the *N*-benzyl analogues (*N*-mono-alkylated analogues), **EM902** ($ED_{50} = 14.5$ μM) exhibited a similar effect to **EM900** ($ED_{50} = 17.1$ μM), whereas the *p*-chlorobenzyl group (for example, **EM905** ($ED_{50} = 2.7$ μM)) is five times more potent than **EM900**. Interestingly, the other *p*-substituted benzyl analogues (for example, CF_3 (**EM919**), F (**EM921**), I (**EM924**), Br (**EM920**) and OMe (**EM959**); see entry 4–6, 9 and 15) dramatically decreased the anti-inflammatory and/or immunomodulatory effects ($ED_{50} > 100$ μM) except for the *p*-methylbenzyl group (**EM958**, $ED_{50} = 4.8$ μM). In addition, the *o*- or *m*-chloro benzyl analogues, **EM922** ($ED_{50} = 1.2$ μM) and **EM923** ($ED_{50} = 2.7$ μM), also increased the anti-inflammatory and/or immunomodulatory effects as did **EM905**. These results suggested that the chloro group has a remarkably important role in the anti-inflammatory and/or immunomodulatory effect. The *N*-alkylated analogues, such as propargyl (**EM929**), ethyl (**EM933**), *i*-propyl (**EM940**) and allyl (**EM957**) (entry 10–13, $ED_{50} > 100$ μM), showed no effect, indicating that a benzyl moiety might be necessary to develop the anti-inflammatory and/or immunomodulatory effect. Likewise, conversion to the acetamide moiety (entry 16, **EM960**, $ED_{50} > 100$ μM) completely removed any anti-inflammatory and/or immunomodulatory properties. In contrast, in terms of *N*,*N*-bis-alkylated analogues, the bis-de-*N*-methyl analogue **EM903** slightly decreased the anti-inflammatory and/or immunomodulatory effects ($ED_{50} = 27.6$ μM). Conversely, the *N*,*N*-dibenzyl analogue (**EM904**) slightly increased anti-inflammatory and/or immunomodulatory effects, with cytotoxicity at 30 μM in THP-1 cells. Interestingly, *N*-mono-benzyl (**EM912**) without *N*-methyl group showed no activity ($ED_{50} > 100$ μM, cf. entry 2). Likewise, the *p*-chlorobenzyl group without the *N*-methyl group, **EM928** ($ED_{50} > 100$ μM), completely lost anti-inflammatory and/or immunomodulatory effects. These findings suggested that the *N*-methyl group has an important part in creating the anti-inflammatory and/or immunomodulatory effect. Morpholine (**EM914**) and piperidine (**EM955**) analogues expressed moderate anti-inflammatory and/or immunomodulatory effects ($ED_{50} = 52.8$ μM for **EM914** (Appendix) and 30.3 μM for **EM955**), whereas, the other three types of cyclic analogues, such as **EM956**, **EM965** and **EM966**, completely lost the activity ($ED_{50} > 100$ μM).

To demonstrate the anti-inflamamatory and/or immunomodulatory effects *in vivo* in a rat model of IBD and to confirm that our *in vitro* assay system is efficient and reliable for investigating anti-inflammatory agents, we selected two types of analogue, **EM905** and **EM914**, as model compounds. IBD is a chronic inflammatory disease, as are ulcerative colitis and Crohn's disease. Treatment of IBD requires anti-inflammatory drugs and steroids, yet few anti-inflammatory agents are suitable for IBD therapy. Thus, there is a pressing need for new drugs to treat these anti-inflammatory diseases, especially compounds that possess a new skeleton and/or a new mode of action.

Crohn's disease (Sprague–Dawley) rats (Charles River Laboratories, Japan) were fasted for 24 h with access to water *ad libitum*. On day 0, each rat was sedated by intraperitoneal injection of sodium pentobarbital (50 mg kg^{-1}). Subsequently, 3.125 ml kg^{-1} of 2,4,6-trinitrobenzene sulfonic acid (Wako Pure Chemical Industries, Tokyo, Japan), 33.3 mg ml^{-1} in 33% ethanol/saline, was infused using a polyethylene tube, inserted through the rectum into the colon to a distance of 8 cm. The 2,4,6-trinitrobenzene sulfonic acid was retained in the colon for 60 min, after which the fluid was withdrawn. On day 2, we confirmed whether a colonic lesion had been generated in each rat by fecal occult blood test, and colitis-induced rats were divided into five groups for treatment as follows: group 1: 10 mg kg^{-1} **EM905** treatment ($n = 14$); group 2: 30 mg kg^{-1} **EM905** treatment ($n = 15$); group 3: 10 mg kg^{-1} **EM914** treatment ($n = 15$); group 4: 30 mg kg^{-1} **EM914** treatment ($n = 13$); and vehicle control ($n = 15$). Test compounds were prepared by suspending in 0.5% sodium carboxymethyl cellulose (CMC-Na, Wako Pure Chemical Industries) at 2 or 6 mg ml^{-1} and orally administered twice daily by force with a microtube from day 2 to 7. On day 8, rats were killed, the colon was excised and opened longitudinally, rinsed with cold saline and colonic damage was evaluated according to a scale ranging 0–10.[16] (Scale ranging: 0, normal appearance; 1, focal hyperhemia and slight thickening (no ulcers); 2, hyperhemia and prominent thickening (no ulcers); 3, ulceration with inflammation at one site; 4, ulceration with inflammation at two or more sites; 5, tissue damage extending >1 cm length; 6–10, area of tissue damage extending >2 cm length, the score being increased by 1 for each additional cm of involvement).

The data indicated the impact on experimental colitis, 2,4,6-trinitrobenzene sulfonic acid treatment inducing severe macroscopic inflammation in the colon after rectal administration, as assessed by the colonic damage score (4.27 ± 0.38), that is, hyperemia,



Compound	Dose mg/kg/day	N	Inflammatory score in the intestinum crassum
Control (0.5%CMC-Na)	-	15	4.27 ± 0.38
EM905	10x2	14	3.29 ± 0.22
	30x2	15	2.67 ± 0.40*
EM914	10x2	15	2.93 ± 0.41
	30x2	13	2.69 ± 0.33*
sulfasalazine	300x2	12	2.70 ± 0.40*
Prednisolone	3x2	12	2.30 ± 0.40**

Figure 1 Effect of **EM905** and **EM914** with 6 days repeated oral administration on 2,4,6-trinitrobenzene sulfonic acid-induced colitis. Score means the following grade of colitis: 0, normal appearance; 1, focal hyperhemia and slight thickening; 2, hyperhemia and prominent thickening, no ulcers; 3, ulceration with inflammation at one site; 4, ulceration with inflammation at two or more site; 5, tissue damage extending >1 cm length; 6–10, area of damage extending >2 cm length, the score being increased by 1 for each additional cm of involvement. Test solutions were administered orally at the volume of 5 ml kg^{-1} for 6 days. *N* = number, $^*P<0.05$ and $^{**}P<0.01$.

thickening of the bowel and the extent of ulceration. Treatment with **EM905** and **EM914** reduced the severity of the gross lesion score in a dose-dependent manner as shown in Figure 1. Both **EM905** and **EM914** at lower dose ($10\,mg\,kg^{-1}$) had little effect (**EM905**: 3.29 ± 0.22 and **EM914**: 2.93 ± 0.41) without statistical significance, whereas the higher dosage ($30\,mg\,kg^{-1}$) had a significant effect on the intensity of the inflammatory response (**EM905**: 2.67 ± 0.40 and **EM914**: 2.69 ± 0.33). The effect was approximately equal for the two compounds. Considering their biological and chemical properties, **EM905** showed greater promotion of monocyte differentiation than **EM914** (Table 1). However, **EM905** is considerably more hydrophobic than **EM914**, so it was assumed that the bioavailability of **EM905** was much lower than **EM914** when administered orally to the rats.

In conclusion, we have developed two analogues, **EM905** and **EM914**, which exhibit similar beneficial impact on treatment of IBD in rats, dramatically decreasing dosages required when compared with therapeutic sulfa drugs such as sulfasalazine. The results suggested that the promotion of monocyte to macrophage differentiation may be a reliable and accurate assay to allow *in vitro* evaluation of potential and promising anti-inflammatory agents. The mode of action of these compounds is not yet fully elucidated and investigation of the **EM900**-binding protein is underway in our laboratory.

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APPENDIX

Experimental procedure and physico-chemical properties of EM914

To a solution of **EM903** (24.2 mg, 0.035 mmol) in CH_3CN (7.0 ml) was added with *i*-Pr_2NEt (61.0 µl, 0.350 mmol) and bis(2-bromoethyl)ether (44.0 µl, 0.350 mmol) at room temperature. The reaction was allowed to warm to 80 °C and then stirred at 80 °C for 20 h. After cooling to room temperature, *i*-Pr_2NEt (61.0 µl, 0.350 mmol) and bis(2-bromoethyl)ether (44.0 µl, 0.350 mmol) were further added to the reaction. The reaction was allowed to warm to 80 °C and then stirred at 80 °C for 6 h. The reaction was quenched with saturated aqueous $Na_2S_2O_3$ (7 ml) and extracted with $CHCl_3$ (10 ml, × 1). The organic layer was washed with saturated aqueous $Na_2S_2O_3$ (7 ml, × 1), saturated aqueous NH_4Cl (7 ml, × 1) and brine (7 ml, × 1), dried over Na_2SO_4, filtered and concentrated *in vacuo*. The crude product was purified by silica gel flash column chromatography ($CHCl_3$/MeOH/NH_4OH = 100/1/0.1–30/1/0.1) to afford **EM914** (23.6 mg, 89%) as a pale yellow solid.

EM914 $[\alpha]_D^{24}$–27.6 (*c* 1.0, $CHCl_3$), mp = 130.5–135.9 °C, IR (KBr) ν cm^{-1}; 3465, 2969, 2938, 2881, 1708, 1635, 1457, 1378, 1268, 1164, 1112, 1054 and 1018. ^1H NMR (500 MHz, $CDCl_3$) δ (p.p.m.): 5.50 (s, 1 H), 4.78 (d, *J* = 4.0 Hz, 1 H), 4.33 (d, *J* = 6.9 Hz, 1 H), 4.24 (d, *J* = 10.3 Hz, 1 H), 3.99 (dq, *J* = 9.2, 6.3 Hz, 1 H), 3.89 (d, *J* = 9.2 Hz, 1 H), 3.77–3.65 (complex m, 5 H), 3.47 (m, 1 H), 3.24 (s, 3 H), 3.24–3.21 (m, 1 H), 2.99 (t, *J* = 9.8 Hz, 1 H), 2.80 (dd, *J* = 9.8, 5.2 Hz, 1 H), 2.72–2.52 (complex m, 5 H), 2.41–2.32 (complex m, 3 H), 2.14 (m, 1 H), 2.04 (m, 1 H), 1.86 (dd, *J* = 12.3, 3.4 Hz, 1 H), 1.75 (bd, *J* = 11.5 Hz, 1 H), 1.65 (m, 1 H), 1.52 (dd, *J* = 15.2, 4.3 Hz, 1 H), 1.37 (m, 1 H), 1.34–1.33 (complex m, 4 H), 1.33 (s, 3 H), 1.29 (d, *J* = 7.0 Hz, 3 H), 1.22–1.19 (complex m, 7 H), 1.21 (s, 3 H), 1.11 (s, 3 H), 1.11–1.10 (complex m, 3 H), 1.08 (d, *J* = 7.5 Hz, 3 H), 0.96 (d, *J* = 7.2 Hz, 3 H), internal solvent peak ($CDCl_3$ 7.26 p.p.m.), ^{13}C NMR (125 Hz, $CDCl_3$) δ (p.p.m.): 177.2, 103.8, 98.1, 84.0, 83.2, 82.7, 80.6, 78.0 (2C), 75.9, 74.8, 72.4, 70.0, 69.0, 67.0 (2C), 66.0, 65.4, 49.2, 48.8 (2C), 46.6, 41.7, 36.5, 35.1, 33.8, 33.5, 30.3, 22.5, 22.2, 21.4, 21.0, 18.0, 17.5, 16.9, 16.0, 13.9, 12.0, 9.5, internal solvent peak ($CDCl_3$ 77.00 p.p.m.), HRMS FAB (PEG600 + NaI) m z^{-1}: 760.4885 $[M+H]^+$, calcd for $C_{39}H_{70}NO_{13}$: 760.4847.