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Novel 12-membered non-antibiotic macrolides from erythromycin A; EM900 series as novel leads for anti-inflammatory and/or immunomodulatory agents

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Diffuse panbronchiolitis

ABSTRACT

Herein, we report the design and synthesis of the novel 12-membered non-antibiotic macrolide (8*R*,9*S*)-8,9-dihydro-6,9-epoxy-8,9-anhydropseudoerythromycin A (**EM900**), which was found to be a potent anti-inflammatory and/or immunomodulatory agent, capable of promoting monocyte to macrophage differentiation. This molecule shows improved acid stability, does not exhibit any anti-bacterial activity and has relatively low cytotoxicity against THP-1 cells. In addition, one of its analogues, (8*R*,9*S*)-4″,13-*O*-diacetyl-8,9-dihydro-6,9-epoxy-8,9-anhydropseudoerythromycin A (**EM911**), was found to be twice as effective as **EM900**.

Erythromycin A (EMA), a 14-membered macrolide, first isolated in 1952,[1] has been widely used as a safe antibiotic for 50 years. In 1984, one of the actions of EMA, gastrointestinal motor-stimulating (GMS) activity, was identified by Itoh et al.[2] In 1985, our group found an EMA derivative (**EM574**) which exhibited gastrointestinal motor-stimulating activity but which showed no antibacterial activity.[3–7] Later, Kudo et al. found that low-dose, long-term therapy with EMA is an effective treatment for diffuse panbronchiolitis (DPB),[8] suggesting that EMA expresses some anti-inflammatory and/or immunomodulatory activity. EMA is also used to treat both Chronic Sinusitis (CS) and Cystic Fibrosis (CF). However, the detailed mode of action remains unclear. Macrolide derivatives with anti-inflammatory activity but lacking either anti-bacterial activity or GMS activity would be extremely useful, as they would help avoid promotion of drug resistance, as well as helping to minimize any adverse effects of EMA treatment. We have been endeavouring to identify such compounds.

Previously, we found that the 12-membered EMA analogues, 8,9-anhydropseudoerythromycin A 6,9-hemiketal (**EM701**)[9,10] and **EM703** (Fig. 1), exhibited no anti-bacterial activity and promoted monocyte to macrophage differentiation better than EMA, while possessing a slightly motiline-like activity.[11] However, **EM701** and **EM703** readily form the corresponding 9,12-spiroketal skeleton, as well as some decomposed products, under acidic conditions, such as those found in the stomach.[12,13]

Accordingly we envisioned that a reduction of the double bond at the 6,9-enolether position could lead to a more robust structure which could prevent undesirable chemical transformation under acidic conditions,[5,14] presumably providing similar promotion of monocyte to macrophage differentiation as shown by **EM701** and **EM703**.[15] Herein, we describe the design and synthesis of the novel 12-membered (8*R*,9*S*)-8,9-dihydro-6,9-epoxy-8,9-anhydropseudoerythromycin A (**EM900**), and analogues thereof, all of which promote monocyte to macrophage differentiation but are without either anti-bacterial activity or cytotoxicity. They represent potentially new anti-inflammatory and/or immunomodulatory agents with a novel chemical structure.

Our initial efforts concentrated on preventing instability of the aglycone, 8,9-anhydropseudoerythromycin A 6,9-hemiketal, under acidic conditions. To address this issue, a reduction of 6,9-enolether, using Klein's protocol,[16] was carried out. This involved treatment of 8,9-anhydropseudoerythromycin A 6,9-hemiketal **EM701** with PtO_2 (3.0 equiv), CHF_2CO_2H, and AcOH under H_2 gas at 5 atm (50–55 psi), which afforded **EM900** in 77% yield as a single diastereomer (Scheme 1).[17]

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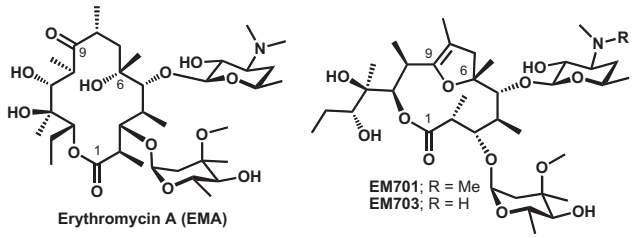
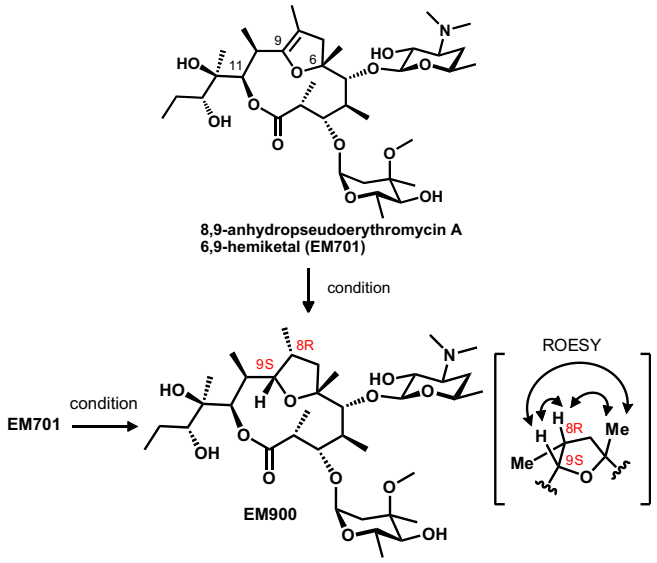


Figure 1. Structures of Erythromycin A, **EM701**, and **EM703**



Scheme 1. Synthesis of (8*R*,9*S*)-8,9-dihydro-6,9-epoxy-8,9-anhydropseudoerythromycin A **EM900**. Reagents and conditions; H_2, PtO_2, CF_2HCOOH, AcOH, 5 atm, rt, 5 h, 77%.

We determined the three-dimensional (3D) solution structure of **EM900** using a combination method of conformation analysis with high-temperature molecular dynamics (MD) and NMR spectroscopy.[15] The structural constraints derived from NMR experiments are included in the Supplementary data. The stereochemistry of C8 and C9 positions was determined as 8*R* and 9*S*, since strong ROESY correlations between 8-H and 9-H, 8-H and 6-Me, 9-H and 6-Me was observed but not seen between 8-Me and 9-H. Figure 2 shows comparison of the 3D structures of **EM900** (black) and **EM703** (green), which we determined previously.[15] This result revealed that 3D conformation of both analogues, **EM900** and **EM703**, are preserved, indicating that **EM900** could express promotional activity of monocyte to macrophage differentiation, with a robust structure under acidic condition. In fact, **EM900** shows an ED_{50} of 17.1 μM, as expected, without anti-bacterial activity (vide infra). Based on these observations, we proposed two reaction pathways; one being that hydrogenation took place via *syn* addition at the β face of the dihydropyrane ring core, the other being that the oxonium intermediate was reduced at the β face of the dihydropyrane ring core after acids facilitated protonation from the β face of the dihydropyrane ring core generated by the double bond. Moreover, we explored reducing the amount of PtO_2 used for a large-scale synthesis because the 3.0 equiv of PtO_2 was not acceptable in terms of costs. We found that PtO_2 can be reduced from 300 mol % to 150 mol % without losing the reactivity, thereby allowing us to undertake suitable scale synthesis of **EM900** (Scheme 1).

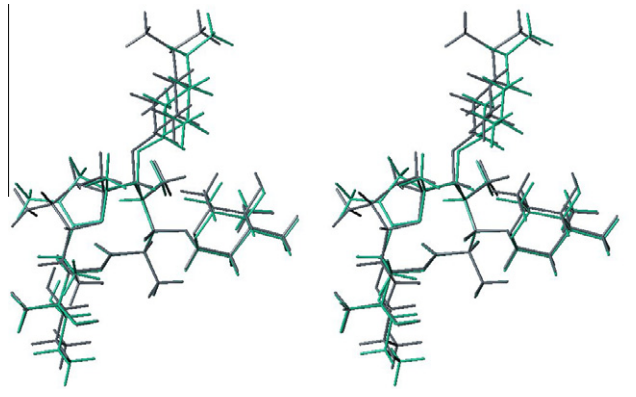


Figure 2. Stereopairs of the superposition of the 3D structures of **EM900** (black) and **EM703** (green)

To investigate anti-inflammatory and immunomodulatory effects, we used the THP-1 assay system to test for promotion of differentiation of monocytic cells to macrophages. The promotive activity was determined by modifying the method of Keicho et al.[18] 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 poured into 48-well tissue culture microplates (IWAKI, Japan) and cultured in the presence of phorbol myristate acetate (PMA; 2 ng/mL), 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 determination of MTT ((3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl)tetrazolium bromide) assay at 550 nm. The ED_{50} values were determined, which provided an evaluation of the promotion of monocyte to macrophage differentiation of analogues, as compared to the result of EMA at 100 μM. We also evaluated the anti-bacterial activity of against several representative organisms, such as *Staphylococcus aureus*, *Micrococcus luteus*, *Enterococcus faecalis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Serratia marcescens*, *Enterobacter aerogen*, *Acinetobacter calcoaceticus* using standard serial-dilution techniques.[19] Cytotoxicity (μM) of THP-1 cell was determined by using cell count reagent SF (Nacalai tesque) according to manufacturer's instructions.

Table 1
Biological activity (ED_{50}, cytotoxicity, and MIC) of EMA and **EM900**

		EMA	**EM900**
ED_{50} (μM)[a]		–	17.1
Cytotoxicity (μM)		–	100
MIC μg/mL)	*S. aureus* FDA209P[b]	⩽0.5	>128
	S. aureus Smith[b]	⩽0.5	>128
	S. aureus 8325 (pEP2104)[b]	64	>128
	S. epidermidis IFO12648[b]	⩽0.5	>128
	M. luteus ATCC9341 [b]	⩽0.5	128
	E. faecalis ATCC21212[b]	1	>128
	E. coli NIHJ JC-2[b]	64	>128
	K. pneumoniae NCTN9632[b]	32	>128
	S. marcescens IFO12648[b]	128	>128
	E. aerogen NCTC10006[b]	128	>128
	A. calcoaceticus IFO2552[b]	4	>128

[a] The ED_{50} values were determined, which provided an evaluation of the promotion of monocyte to macrophage differentiation of analogues, as compared to the result of **EMA** at 100 μM.

[b] *S. aureus; Staphylococcus aureus, M. luteus; Micrococcus luteus, E. faecalis; Enterococcus faecalis, E. coli; Escherichia coli, K. pneumoniae; Klebsiella pneumoniae, S. marcescens; Serratia marcescens, E. aerogen; Enterobacter aerogen, A. calcoaceticus; Acinetobacter calcoaceticus.*

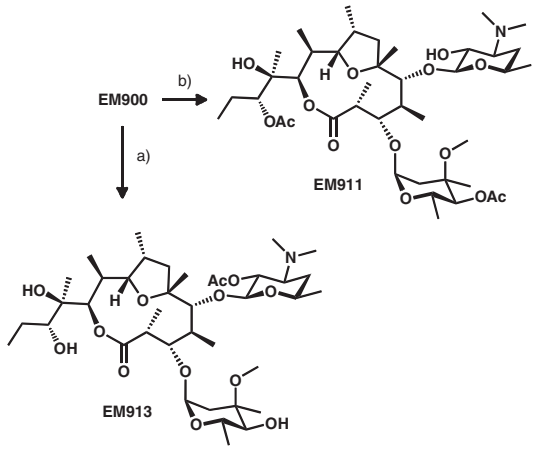
EM900 showed an ED_{50} of 17.1 μM. In addition, **EM900** did not have antibacterial activity against several representative organisms, such as *S. aureus*, *M. luteus*, *E. faecalis*, *E. coli*, *K. pneumoniae*, *S. marcescens*, *E. aerogen*, *A. calcoaceticus* (Table 1). With respect to toxicity, **EM900** showed cytotoxicity at 100 μM in THP-1 cells. Although absence of toxicity would be advantageous, we envisioned that the toxicity could be reduced using chemical modification. Consequently, we decided to use **EM900** to help elucidate structure-activity relationships (SAR). In addition, we aimed to improve potency as well as remove or minimize, as much as possible, both toxicity and anti-bacterial properties without increasing molecular weight.

At the outset, we sought to modify the three types of moiety; hydroxyl groups, the side chain at C11 position and the neutral sugar cladinose, to develop a structure-activity map. As outlined in Scheme 2, treatment of **EM900** with Ac_2O and acetone provided the 2'-O-acetylated product (**EM913**) in 89% yield. Meanwhile, treatment of **EM900** with Ac_2O, DMAP, and pyridine provided the 2′,4″,13-O-triacetylated product, followed by methanolysis to afford 4″,13-O-diacetylated product (**EM911**) in 94% yield.

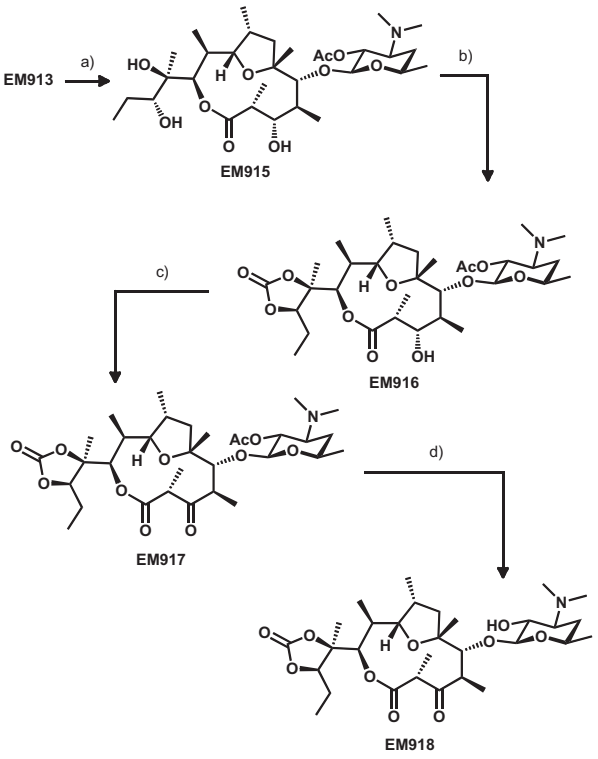
The toward 'ketolide[20] structure, **EM913,** was treated with acidic conditions to remove the cladinose moiety to generate the alcohol, **EM915**, in 46% yield, which upon cyclic-carbonation using triphosgene, **EM916** and oxidation, led to the corresponding ketolide, **EM917**. Finally, methanolysis of the acetyl group furnished the desired compound, **EM918** in 92% yield (Scheme 3). To modify the side chain of **EM900**, an epoxidation reaction was carried out with Martin's sulfurane $\{[C_6H_5C(CF_3)_2O]_2S(C_6H_5)_2\}$[21] to the produce the epoxide, **EM909**, in 70% yield. To disconnect the diol moiety, oxidative cleavage using $Pb(OAc)_4$ afforded the corresponding ketone **EM906**, followed by oxime formation to provide **EM907** in 93% yield (Scheme 4).

Based on biological evaluation (Table 2), the 2'-O-acetylated compound **EM913** (ED_{50} = 16.1 μM) exhibited the same potency as **EM900** (ED_{50} = 17.1 μM). Interestingly, the 4″,13-O-diacetylated product, **EM911**, (ED_{50} = 7.7 μM) was twofold more potent than **EM900** (ED_{50} = 17.1 μM). However, the de-cladinosyl compound, **EM915**, exhibited dramatically decreased activity (ED_{50} = > 100 μM). The cyclic carbonated **EM916** (ED_{50} = 55.7 μM) actually regained potency. Likewise, the ketolide-type molecule **EM917** (ED_{50} = 27.9 μM) slightly recovered anti-inflammatory or immunomodulatory effects. No remarkable impact was observed when the acetyl group was removed in this case **EM918**, (ED_{50} = 26.3 μM). We concluded that neither the decladinosyl-type molecule nor the ketolide-type molecule play a role in modulating the immune response. Although the other side chain derivatives, **EM906** (ED_{50} = 12.3 μM), **EM907** (ED_{50} = 12.3 μM), and **EM909** (ED_{50} = 10.2 μM) displayed slightly improved enhancement effect, the functional groups, ketone, oxime and epoxide also do not appear to play any key role.

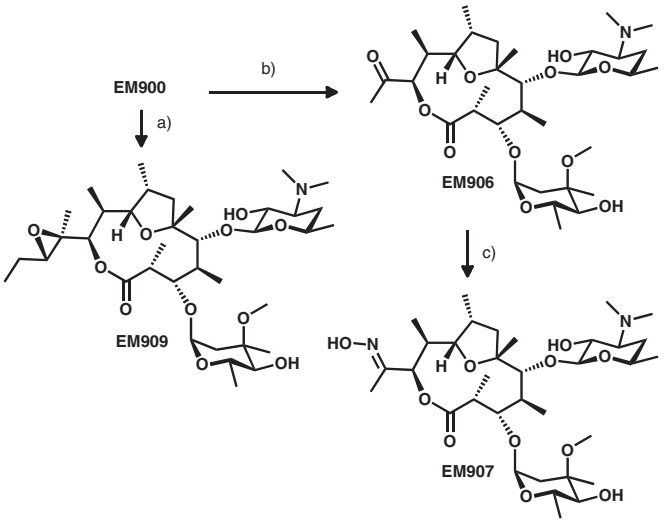
In conclusion, we have developed a novel 12-membered (8*R*,9*S*)-8,9-dihydro-6,9-epoxy-8,9-anhydropseudoerythromycin (**EM900**), which possesses potent anti-inflammatory and



Scheme 2. Acylated analogues of **EM900**. Reagents and conditions: (a) Ac_2O (6.0 equiv), acetone, rt, 30 min, 89%; (b) (1) Ac_2O, DMAP, pyridine, rt, 3 h; (2) MeOH, 50 °C, 12 h, 94% over 2 steps.



Scheme 3. Removing cladinose and modification of this region. Reagents and conditions: (a) 1.0 M HCl aq., rt, 5 h, 46%; (b) triphosgene (2.0 equiv), pyridine (12.0 equiv), DCM, −78 °C to rt, 30 min, 73%; (c) Dess–Martin periodinate (10.0 equiv), DCM, rt, 2 h, 80%; (d) MeOH, 50 °C, 30 h, 92%.



Scheme 4. Modification of the side chain. Reagents and conditions: (a) Martin's sulfurane (2.5 equiv), DCM, rt, 90 min, 70%; (b) $Pb(OAc)_4$ (2.1 equiv), DCM, 0 °C, 4 h, 75%; (c) $NH_2OH{\cdot}HCl$ (3.0 equiv), pyridine (60 equiv), EtOH, 0 °C, 4 h, 93%.

Table 2
Biological activity (ED_{50} and cytotoxicity) of **EM900** analogues

Compds	ED_{50} μM)[a]	Cytotoxicity (μM)
EM900	17.1	100
EM906	12.3	100
EM907	12.3	>100
EM909	10.2	100
EM911	7.7	100
EM913	16.1	100
EM915	>100	>100
EM916	55.7	>100
EM917	27.9	>100
EM918	26.3	>100

[a] The ED_{50} values were determined, which provided an evaluation of the promotion of monocyte to macrophage differentiation of analogues, as compared to the result of EMA at 100 μM.

immunomodulatory activity via promotion of monocyte to macrophage differentiation but which has no apparent anti-bacterial properties. The molecule has improved acid stability and lower cytotoxicity in THP-1 cells than EMA. Moreover, SAR study revealed that one of its analogues, **EM911** (bearing an acetylated moiety), was twice as effective as **EM900**. Further investigation of these compounds is currently underway in our laboratory.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2011.04.004.

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17. *(8R),(9S)-8,9-Dihydro-6,9-epoxy-8,9-anhydropseudoeryth-romycin A (EM900)*: To a pressure vessel equipped with a magnetic stirring bar was charged AcOH (7.0 mL), PtO_2 (476.0 mg, 2.10 mmol), and CF_2HCOOH (299 μL, 4.75 mmol). The resulting reaction mixture was purged with H_2 under 5 atm and stirred at room temperature for 1 h to activate the catalyst. After that, to the mixture was added a solution of **EM701** (1.00 g, 1.40 mmol) in AcOH (7.0 mL). Then the mixture was again purged with H_2 under 5 atm and stirred at room temperature for 4 h. After being quenched with $AcONH_4$ (7.0 g), the mixture was stirred at room temperature for 30 min and filtered, concentrated in vacuo. The resulting mixture was extracted with $CHCl_3$ (50 mL × 3), then the combined organic layers were washed with satd aq $NaHCO_3$ (50 mL × 2), and brine (50 mL × 1), dried over Na_2SO_4, and concentrated. The crude product was purified by flash column chromatography ($CHCl_3$/MeOH/30%NH_4OH = 50/1/0.02 to 30/1/0.02) to afford **EM900** (768.0 mg, 77%) as a colorless solid. R_f = 0.53 ($CHCl_3$/MeOH /30% NH_4OH = 15:1:0.2), mp = 129.7–139.8 °C, $[\alpha]_D^{24}$ −34.1 (*c* 1.0, $CHCl_3$); IR (KBr) ν cm^{-1}: 3459, 2973, 2937, 2881, 2788, 1704, 1637, 1459, 1380, 1272, 1168, 1112, 1051, 1020; ^1H NMR (500 MHz, $CDCl_3$) δ (ppm) 5.46 (s, 1H, 11-H), 4.75 (d, *J* = 4.6 Hz, 1H, 1″-H), 4.28 (d, *J* = 6.9 Hz, 1H, 1′-H), 4.23 (d, *J* = 10.3 Hz, 1H, 3-H), 3.97 (dq, *J* = 6.3, 9.2 Hz, 1H, 5″'-H), 3.86 (d, *J* = 8.9 Hz, 1H, 9-H), 3.75 (br s, 1H), 3.64 (d, *J* = 10.5 Hz, 1H, 5-H), 3.42 (m, 1H, 5′-H), 3.21 (s, 3H, 3″-*O*-CH_3), 3.13 (dd, 1H, *J* = 6.9, 9.7 Hz, 2′-H), 2.96 (d, *J* = 9.2 Hz, 1H, 4″-H), 2.78 (d, *J* = 9.7 Hz, 1H, 13-H), 2.68 (dq, *J* = 6.3 Hz, 10.3 Hz, 1H, 2-H), 2.46 (m, 1H, 3′-H), 2.35-2.33 (m, 1H, 8-H), 2.31 (d, *J* = 14.9 Hz, 1H, 2″a-H), 2.22 (s, 6H, 3′-N(CH_3)$_2$), 2.11 (m, 1H, 10-H), 2.02 (dq, *J* = 6.9, 9.7 Hz, 1H, 4-H), 1.84 (dd, *J* = 8.6, 12.6 Hz, 1H, 7a-H), 1.64 (m, 1H, 13-*CH*$_2$$CH_3$), 1.60 (m, 1H, 4′a-H), 1.49 (dd, *J* = 4.6, 15.5 Hz, 1H, 2″b-H), 1.39–1.35 (m, 1H, H-13-*CH*$_2$$CH_3$), 1.32 (s, 3H, 6-$CH_3$), 1.32 (m, 3H, 7b-H, the peak is overlapped with the proton of 6-CH_3), 1.31 (d, *J* = 6.3 Hz, 3H, 10-CH_3), 1.26 (d, *J* = 6.3 Hz, 3H, 5″-CH_3), 1.22 (m, 1H, 4′b-H), 1.18 (d, *J* = 5.7 Hz, 3H, 2-CH_3), 1.18 (s, 3H, 3″-CH_3), 1.16 (d, *J* = 5.7 Hz, 3H, 5′-CH_3), 1.08 (app. s, 6H, 4-CH_3, 12-CH_3, 2 peaks are overlapped), 1.06 (d, *J* = 6.9 Hz, 3H, 8-CH_3), 0.93 (t, *J* = 7.5 Hz, 3H, 13-CH_2*CH*$_3$); ^{13}C NMR (125 Hz, $CDCl_3$) δ (ppm) 177.1 (C-1), 103.9 (C-1′), 98.0 (C-1″), 83.9 (C-9), 83.2 (C-6), 82.4 (C-5), 80.6 (C-3), 78.0 (C-4″), 77.2 (C-12), 75.8 (C-13), 74.7 (C-11), 72.3 (C-3″), 70.8 (C-2′), 68.9 (C-5′), 65.3 (C-3′), 65.1 (C-5″), 49.1 (3″-*O*-CH_3), 46.6 (C-2), 41.7 (C-7), 40.1 (2C, 3′-N(CH_3)$_2$), 36.5 (C-4), 35.1 (C-2″), 33.7 (C-10), 33.5 (C-8), 28.5 (C-4'), 22.4 (13-*CH*$_2$$CH_3$), 22.1 (6-$CH_3$), 21.4 (3″-$CH_3$), 21.0 (5′-$CH_3$), 17.9 (5″-$CH_3$), 17.4 (8-$CH_3$), 16.9 (12-$CH_3$), 16.0 (10-$CH_3$), 13.9 (2-$CH_3$), 11.9 (13-$CH_2$*CH*$_3$), 9.5 (4-$CH_3$); HRMS (FAB); *m/z*: 718.4767 [M+H]$^+$, calcd for $C_{37}H_{68}NO_{12}$: 718.4742 [M+H].
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