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Peumusolide A, unprecedented NES non-antagonistic inhibitor for nuclear export of MEK

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ABSTRACT

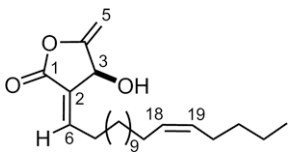
A new polyketide peumusolide A was disclosed as an unprecedented NES non-antagonistic inhibitor for nuclear export of MEK, a promising scaffold for antitumor agents with novel mechanism of action, from *Peumus boldus* Molina. The absolute stereostructure as well as optical purity was established by use of the two synthesized enantiomeric model lactones. In addition, the $\Delta\varepsilon$ values in their CD spectra were demonstrated to be the conclusive index for determination of not only configuration at C-3 but also optical purity of natural congeneric polyketides. Peumusolide A was revealed to show NES non-antagonistic action by the biotinylated probe and to inhibit proliferation of MEK-activated tumor cells selectively.

In many kinds of tumor cells, the mitogen-activated protein kinase (MAPK) cascade, one of the important signal pathways for cell proliferation, was reported to be significantly activated as compared with that in normal cells.[1] In addition, some inhibitors for MAPK/ERK kinase (MEK), one of the kinases belonging to this cascade, were shown to arrest the MAPK signal transduction to inhibit the proliferation of tumor cells.[2] MEK is transported into the nucleus after phosphorylation of MAPK in the cytoplasm, and then it forms a complex with free MAPK accomplishing transfer of phosphate groups. This complex is exported from the nucleus to the cytoplasm with the aid of the carrier protein, chromosomal region maintenance 1 (CRM1), which is the receptor for the nuclear export signal (NES) sequence in MEK.[3] Furthermore, shuttle of MEK between the nucleus and the cytoplasm was revealed to be essential for cell proliferation by the MAPK cascade. In this context, inhibitors for nuclear export of MEK were anticipated to be highly attractive seed principles toward selective antitumor agents.

Previously, the potently cytotoxic metabolite of actinomyces leptomycin B was found to be a MEK export inhibitor.[4] Moreover, leptomycin B was shown to inhibit export of MEK due to NES antagonistic action for CRM1; it inhibited binding between CRM1 and the NES in MEK.[5] On the other hand, no NES non-antagonistic inhibitors have been found in spite of the promising potential for antitumor agents with a novel mechanism of action. This circumstance prompted us to explore unprecedented NES non-antagonistic inhibitors for nuclear export of MEK from medicinal plants. Herein, we report not only the absolute stereostructure but also the biological properties of the new polyketide, peumusolide A (**1**), inhibiting nuclear export of MEK through NES non-antagonistic mode, from the Southern American medicinal plant *Peumus boldus* Molina (Fig. 1).

To search for MEK-export inhibitors, we utilized an assay to monitor distribution of MEK in HeLa cells by an indirect fluorescent antibody technique (see the Supplementary data). As a result of screening about 300 extracts of medicinal plants by this assay, the MeOH extract of *P. boldus* was found to significantly inhibit the nuclear export of MEK. Bioassay-guided separation of the extract through successive SiO_2 column chromatography, normal phase, and reverse phase HPLC disclosed the new polyketide named peumusolide A (**1**, colorless oil. $[\alpha]_D^{20}$ −15.2 in MeOH) as the MEK-export inhibitor with MIC of 5.0 μM.

The molecular formula $C_{23}H_{38}O_3$ of **1** was determined by HR FAB-MS ($[M+H]^+$ *m/z* 363.2912, calcd 363.2899) implying five degrees of unsaturation. The IR absorptions at 3418, 1770, and 1672 cm^{-1} indicated the presence of hydroxyl, carbonyl, and conjugated double bond functionalities, respectively. The ^1H NMR



peumusolide A (**1**)
(3*S*:3*R*=82:18)

Figure 1. Absolute structure of peumusolide A (**1**).

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spectrum showed an exo-olefin [δ_H 4.96 (Ha-5, dd, J = 3.1, 1.8 Hz) and 4.72 (Hb-5, dd, J = 3.1, 1.2 Hz)], a disubstituted olefin [δ_H 5.35 (H-ii, iii)], a trisubstituted olefin [δ_H 7.09 (H-6, dt, J = 2.1, 7.8 Hz)], an oxymethine [δ_H 5.26 (H-3, br s)], a terminal methyl [δ_H 0.90 (H-23, t, J = 7.3 Hz)], and an unbranched alkyl chain [δ_H 1.25–1.35 (12H, m)]. Furthermore, the ^{13}C NMR spectrum exhibited one carbonyl carbon [δ_C 166.6 (C-1)] and six sp^2 carbons [δ_C 157.7 (C-4), 150.2 (C-6), 129.9, 129.8 (C-ii, iii), 127.3 (C-2), 91.3 (C-5)] indicative of the presence of one ring in **1** by judging from the degree of unsaturation (Table 1).

The ^{1}H–^{1}H COSY presented the oxymethine (C-3) linked to the exo-olefin as well as the alkyl chain from C-6 to C-9 as partial structures. In addition, the disubstituted olefin was revealed to divide the side chain into two unbranched alkyl functions. On the other hand, the chemical shifts due to two allyl carbons [δ_C 26.9, 27.2 (C-i, iv)], which were assigned by HMQC data, determined *Z* configuration of the disubstituted olefin. The HMBC correlations from H-3 to C-2 and from H-6 to C-3 connected the two partial structures with the trisubstituted olefin, while the HMBC correlations from H-3 to C-1 and from H-6 to C-1 settled the carbonyl function adjacent to the olefinic carbon (C-2). Taking the IR absorption due to the carbonyl group and the carbon signal (δ_C 157.7) of C-4 into consideration, the left-hand structure from C-1 to C-9 including γ-lactone moiety was constructed. Furthermore, the NOE correlation between H-3 and H-7 determined 2-*E* configuration. On the basis of these physicochemical features, the gross structure of **1** except for the location of double bond in the side chain was unequivocally established as shown in Figure 2.

Next, the position of double bond in the side chain of peumusolide A (**1**) was determined by the two methods described below. In the first instance, the 18,19-bismethylsulfide prepared by treatment of **1** with dimethyldisulfide and iodine was analyzed by EI-MS. Consequently, the characteristic fragment peaks (*m/z* 339 and 117) generated by cleavage of the bond between C-18 and C-19 bearing methylthio functions were apparently detected.[6] In addition, 1,2-diol given by oxidation of **1** with osmium tetraoxide was subjected to oxidative cleavage by sodium periodate followed by reductive treatment with $NaBH_4$ to furnish 1-pentanol, which was thoroughly identified in comparison with the authentic sample by LC–MS/MS analysis. Based on these findings, the double bond in side chain of **1** was definitively clarified to be located between C-18 and C-19 (see the Supplementary data).

Finally, the configuration of C-3 in peumusolide A (**1**) was elucidated by comparison of the CD spectra with respect to both model enantiomers synthesized according to Nogami's procedure (Scheme 1).[7] Condensation of isopropyl α-(*p*-tolylsulfinyl)decanoate, prepared from isopropyl decanoate and L-menthyl (*S*)-*p*-tolylsulfinate, and propargylaldehyde afforded optically active hydroxyester **2**. Thereafter, the hydroxyester **2** was submitted to successive alkaline hydrolysis, Ag_2CO_3-mediated lactonization, and chiral HPLC separation to afford (*S*)-lactone **3**. The absolute configuration of **3** was confirmed in the following manner. DIBAL reduction of **2** followed by protection of the primary hydroxyl group as *t*-butyldiphenylsilyl (TBDPS) ether gave secondary alcohol **4**, which was applied to modified Mosher's method[8] after conversion to the corresponding MTPA esters **5a** and **5b** from the major stereoisomer of **4**. Distribution of $\Delta\delta$ value revealed stereochemistry at C-3 of **3** to be *S* configuration (see the Supplementary data). On the other hand, Mitsunobu inversion of **2** provided benzoate **6**, which was transformed into (*R*)-lactone **7** in the same protocol from **2** to **3**.

In the CD spectra of peumusolide A (**1**) and (*S*)-**3**, the negative maxima were observed at 226 nm whereas (*R*)-**7** showed the positive Cotton at the same wavelength. Thus, configuration of C-3 of **1** was undoubtedly ascertained to be *S*. However, $\Delta\varepsilon$ of **1** ($\Delta\varepsilon$ −2.1) is apparently smaller than those of **3** ($\Delta\varepsilon$ −3.6) and **7** ($\Delta\varepsilon$ +3.6). On the basis of this finding, peumusolide A (**1**) was analyzed by chiral HPLC [column: Chiracel OD 4.6 × 250 mm (DAICEL Chemical Industries), mobile phase: *n*-hexane/EtOAc = 9:1, flow rate: 1.0 mL/min, detection: UV 220 nm] to find out **1** to be a mixture of the enantiomers in a ratio of 82:18 (3*S*:3*R*). Because of the isolation of several biologically active natural congeners containing the 2-alkenyl-3-hydroxy-4-vinyl-δ-lactone portion,[9,10] the maximal $\Delta\varepsilon$ values of these models in the CD spectra should be regarded as the useful index for prediction of their optical purity.

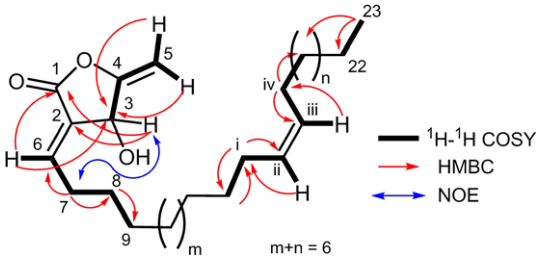
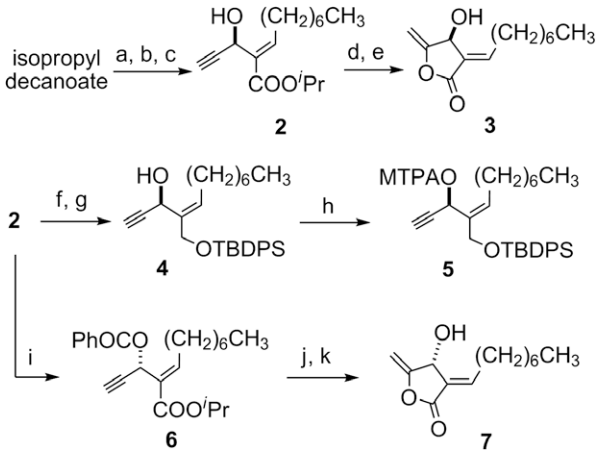


Figure 2. Gross structure of **1** by NMR data.

Table 1
^{1}H and ^{13}C NMR data for **1**

Position	^{1}H (600 MHz)	^{13}C (150 MHz)
1		166.6
2		127.3
3	5.26 (br s)	66.5
4		157.7
5a	4.96 (dd, 3.1, 1.8 Hz)	91.3
5b	4.72 (dd, 3.1, 1.2 Hz)	
6	7.09 (dt, 2.1, 7.8 Hz)	150.2
7a	2.50 (ddt-like, ca.15, 7.5, 7.5 Hz)	28.3
7b	2.43 (ddt-like, ca.15, 7.5, 7.5 Hz)	
8	1.53 (tt-like, ca. 7.5, 7.5 Hz)	27.2
9, 16	1.32 (m) 29.6,	29.8
10–15	1.26 (m)	28.3–29.8
17, 20	2.02 (m)	26.9, 27.2
18, 19	5.35 (m)	129.8[a], 129.9[a]
21	1.31 (m)	31.9
22	1.31 (m)	22.3
23	0.90 (t, 7.3 Hz)	13.9

[a] Assignments may be interchangeable.



Scheme 1. Synthesis of model lactone enantiomers. Reagents and conditions: (a) L-menthyl (*S*)-*p*-tolylsulfinate, lithium cyclohexylisopropylamide, THF, −60 °C, 44%; (b) $BrMgN^iPr_2$, propargylaldehyde, Et_2O, −40 °C; (c) $NaHCO_3$, benzene, 60 °C, 42% (two steps), 73% ee; (d) KOH, MeOH–H_2O, 60%; (e) Ag_2CO_3, benzene, 80 °C, 58%; (f) DIBAL, CH_2Cl_2, −78 °C; (g) TBDPSCl, imidazole, CH_2Cl_2, 62% (two steps); (h) (*S*)- or (*R*)-MTPA, EDCI·HCl, DMAP, CH_2Cl_2, 54% for *S*, 61% for *R*; (i) DEAD, PPh_3, benzoic acid, benzene, 60%; (j) KOH, MeOH–H_2O, 65%; (k) Ag_2CO_3, benzene, 80 °C, 63%.

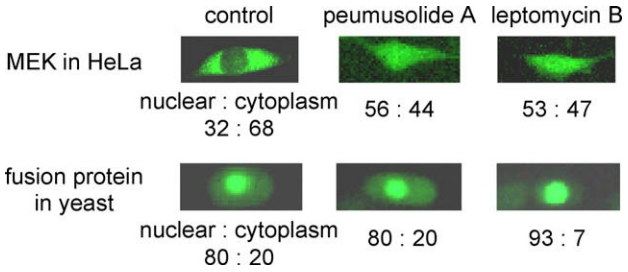


Figure 3. Difference in the mechanism of action between peumusolide A (**1**) and leptomycin B.

Next, we compared the mechanism of action between peumusolide A (**1**) and leptomycin B (LMB), an NES antagonistic inhibitor for the nuclear export of MEK. On treatment of peumusolide A (**1**) or LMB in HeLa cells at a concentration of each MIC (**1**: 5 μM, LMB: 0.02 μM), extreme change in distribution of MEK was observed by the indirect fluorescence antibody method. In brief, peumusolide (**1**) as well as LMB inhibited the nuclear export of MEK forming the complex with MAPK and CRM1 to reduce fluorescence in the cytoplasm. On the contrary, the fission yeast *Schizosaccharomyces pombe*,[4] which expresses the model fusion proteins consisting of the NES, green fluorescence protein (GFP), the NLS (nuclear localization signal), and glutathione *S*-transferase, brought about different delocalization of the fusion protein between **1** and LMB. In the fission yeast, LMB inhibited the export of the fusion protein with MIC of 0.02 μM, indicative of the fairly similar sensitivity of both assays. In contrast, treatment of peumusolide A (**1**) resulted in no alternation in distribution of the fusion protein even at a concentration of 100 μM. This difference in biological behavior intensively suggests that inhibition for the export of MEK by **1** is independent of interaction between the NES in MEK and CRM1 (Fig. 3, see the Supplementary data).

Subsequently, the mechanism of action of peumusolide A (**1**) was analyzed in further detail by use of our reported probe **8** derived from LMB. Previously, LMB was found to be bound to Cys-529 of CRM1, the receptor of NES, by a covalent bond.[5] Furthermore, the probe **8** was revealed to capture CRM1, whereas the potency almost completely disappeared in the presence of the competitive NES antagonistic inhibitors.[11] After prior addition of each MEK export inhibitor to HeLa cells, the whole was incubated in the presence of probe **8** (1.0 μM). Lysates of the harvested cells were treated with streptavidin beads, thereafter the proteins attached to the beads were detected by an immunoblotting technique (see the Supplementary data). As shown in Figure 4, pretreatment of peumusolide A (**1**) even at a concentration of 50 μM

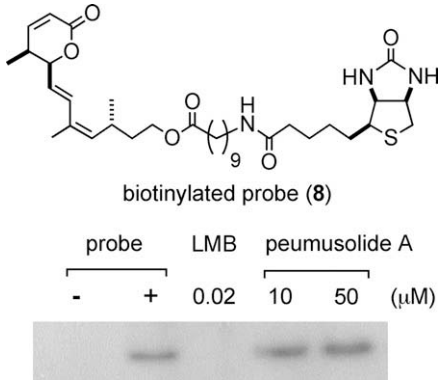


Figure 4. Comparative analysis for the mechanism of action by biotinylated probe (**8**).

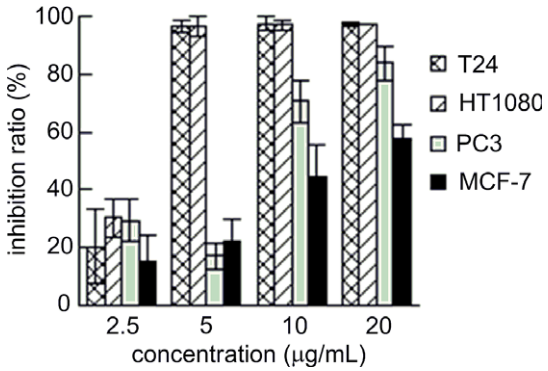


Figure 5. Cytotoxicity of **1** for MEK-activated tumor cells.

obviously exhibited the band due to CRM1 and this behavior was analogous to the treatment of only probe **8**. On the other hand, the band completely disappeared by pre-treatment of LMB. Taking these experimental outcomes together with the IC_{50} values of **1** (3.7 μM) and **8** (1.2 μM) into account, peumusolide A (**1**) was clarified to inhibit the nuclear export of MEK through NES non-antagonistic mode.

Finally, peumusolide A (**1**) was examined for cytotoxicity against MEK-activated tumor cells. Cytotoxicity of **1** was evaluated by MTT method using HT1080 and T24 as MEK-activated tumor cells and PC3 and MCF-7 as non-activated, respectively.[1] As a result, **1** displayed not only cytotoxicity for all of the four cell lines in a concentration-dependent manner but also selectivity for the MEK-activated tumor cells with IC_{50} of 3.3 μg/mL for HT1080 and that of 3.1 μg/mL for T24 cell lines. In particular, treatment of **1** at a concentration of 5 μg/mL resulted in obvious distinction; proliferation of the MEK-stimulated cells was almost inhibited, while only about 20% inhibition was observed for the non-stimulated cells (Fig. 5).

In summary, we disclosed peumusolide A (**1**) as the first NES non-antagonistic inhibitor for the nuclear export of MEK, regarded as a promising scaffold for antitumor agents with the novel mechanism of action, from the Southern American medicinal plant *P. boldus* Molina. The absolute stereostructure as well as the optical purity was unambiguously established through the synthesis of the model lactone enantiomers (**3** and **7**). In addition, we demonstrated that the $\Delta\varepsilon$ values in their CD spectra should be recognized as the conclusive index for determination of both configuration at C-3 and optical purity of natural congeneric polyketides. Not only the behavior of NES, NLS, and GFP fusion protein in the yeast but also the appearance of CRM1 in the competitive experiment between **1** and the biotinylated probe **8** clarified **1** to inhibit the export of MEK by NES non-antagonistic mode. As expected, peumusolide A (**1**) was shown to inhibit proliferation of the MEK-activated tumor cells selectively.

Acknowledgments

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Supplementary data

Supplementary data (experimental details, physicochemical property of **3** and **7**, chemical conversion for determination of

C18–19 double bond, and distribution of $\Delta\delta$ in the MTPA esters of **5**) associated with this article can be found, in the online version, at doi:10.1016/j.tetlet.2010.01.068.

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