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Recent progress of the research on novel microbial metabolites*

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Abstract: Three kinds of microbial metabolites with unique structures and activities were discovered in cell-based screening systems. Lactacystin with a peptide-like structure, isolated as an inducer of neurite outgrowth of Neuro 2a cells (a mouse neuroblastoma cell line), was found to inhibit proteasome functions specifically by binding to the active site *N*-terminal threonine of the catalytic subunit. Macrospelides with a 16-membered-ring structure embodying three lactone likages were isolated as inhibitors of the adhesion of HL-60 cells to the monolayer of LPS-activated human umbilical vein endothelial cells. They blocked the cell–cell adhesion by inhibiting the endothelial cell-leukocyte adhesion molecule-1/sialyl-Lewis^x recognition system. Macrosphelides proved effective in *in vivo* models of the lung metastasis and fibrosis. Madindolines with an indoline-diketocyclopenetene connecting structure, isolated as inhibitors of the growth of IL-6 dependent cells, were found to inhibit IL-6 and IL-11 functions selectively.

LACTACYSTIN

Background

During the collaboration with Prof. Z. Itoh (Gunma University, Japan) on 'motilide', erythromycin derivatives which show gastrointestinal motilin-like activity with no anti-microbial activity [1], an idea came out that microorganisms may produce small molecules with peptide hormone-like activities. Recently, peptide neurotropic factors (NTF) including nerve growth factor (NGF) have attracted attention because of increase in patients with nervous diseases including senile dementia such as Alzheimer's disease. NTFs are known to be responsible to nerve cells for protection from anoxia, maintenance in neuronal functions, axon elongation and neuronal development. Furthermore, nonpeptide small molecules including gangliosides were reported to induce differentiation of neuroblastoma cells, and such morphological and biochemical alterations are very similar to those of NTF-treated nerve cells. In this sense, small molecules are expected to aid in the medical treatment and prevention of nervous diseases.

Screening

We screened over microbial 6000 culture broths in an assay using the murine neuroblastoma cell line Neuro 2a. An actinomycete strain OM-6519, later named *Streptomyces lactacystinaeus*, was found to produce an active metabolite designated lactacystin, that induces neurite outgrowth in Neuro 2a cells [2,3].

Isolation and structure elucidation

The supernatant of the culture broth (50 L) was passed through an Amberlite XAD-2 column and then charged onto an active-carbon column. Materials were eluted with 80% acetone. After concentration, the resulting aqueous solution was adsorbed in a Dowex 1×4 [OH^-] column. Lactacystin was eluted with 10% acetic acid, which was recovered by an adsorption-elution process using a small active-carbon

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column. The eluant (80% acetone) was concentrated *in vacuo* to yield crude lactacystin as yellowish powder (900 mg). Lactacystin was recrystalized from ethanol to obtain pure white powder (562 mg). Relative configuration of lactacystin was determined by NMR spectroscopic and X-ray cyrstallographic analyses. A degradation product obtained by the treatment of lactacystin with diazomethane was assigned as L-cysteine derivative. Consequently, the absolute configuration was determined as shown in Fig. 1 [3]. The structure consists of two α-amino acids, namely (*R*)-*N*-acetylcysteine and a novel α-substituted pyroglutamic acid, which are joined through a thioester linkage.

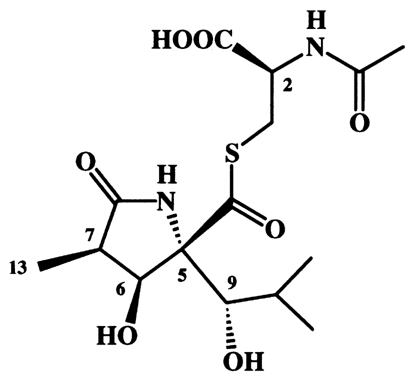


Fig. 1 Structure of lactacystin.

Biological activity and its target

The morphological changes of Neuro 2a cells in the presence of 1.3 μM of lactacystin were observed by the immunofluorescence staining against 200 kDa neurofilament, showing the formation of neurites by lactacystin [2]. The activity of acetylcholinesterase, a marker of neuritogenesis, was also induced by lactacystin. Thus, these morphological and physiological observations revealed that lactacystin induces neuritogenesis of neuroblastoma cells. Then, the mode of action of lactacystin in neuritogenesis was studied, indicating that lactacystin arrests the cell cycle at G1 or G0 and G2 phases in Neuro 2a cells [4]. However, lactacystin showed no effect on enzymes responsible for cell cycle such as protein kinases and histone deacetylase.

From 1992 to 1993, we sent the sample of lactacystin to Prof. E. J. Corey and S. L. Schreiber (Harvard University) several times. In 1995, they reported that lactacystin inhibits the proteasome function by binding to the β-type subunit X [5]. Proteasome, a large and unique protein complex, is distributed in most organisms including eukaryotic cells and archaebacteria, and catalyzes ATP- and ubiquitin-dependent proteolysis [6]. The 26S proteasome consists of catalytic 20S proteasome containing four stacked α- and β-type heptameric rings arranged synmetrically as α7β7β7α7 and regulatory 19S complex containing different ATPases, ubiquitin-isopeptidase and other functional proteins. The molecular weight of the complex is estimated to be 2000 kDa. Prof. R. Huber and his co-workers (Max Plank Institute) reported the X-ray crystalographic analyses of the yeast 20S proteasome–lactacystin complex, confirming that lactacystin is covalently bound to the *N*-terminal threonine residue of the β-type subunit PRE 2 corresponding to the human subunit X [7]. From evidence of lactacystin β-lactone showing similar activity to lactacystin and from kinetic analysis of its inhibition, lactacystin is recognized as a prodrug for proteasome inhibition. Lactacystin is converted to the active β-lactone, and the cell-permeable β-lactone rapidly enters the cells, where it can react with the *N*-terminal threonine of the proteasome subunit (Fig. 2). Prof. Corey proposed the name ‘Omuralide’ for the β-lactone in honor of discovery of lactacystin by my research group [8].

Biosynthesis and chemical synthesis

Lactacystin possesses a unique structural feature which consists of γ-lactam skeleton containing a hydroxyisobutyl and *N*-acetyl-cysteinyl thioester. The biosynthetic origin of carbon atoms of lactacystin was studied by the feeding experiment of ^{13}C-labeled precursors to the culture medium of the producing microorganism. From ^{13}C-NMR analysis, lactacystin was found to be synthesized from acetate, L-cysteine, L-leucine and isobutyrate [9,10].

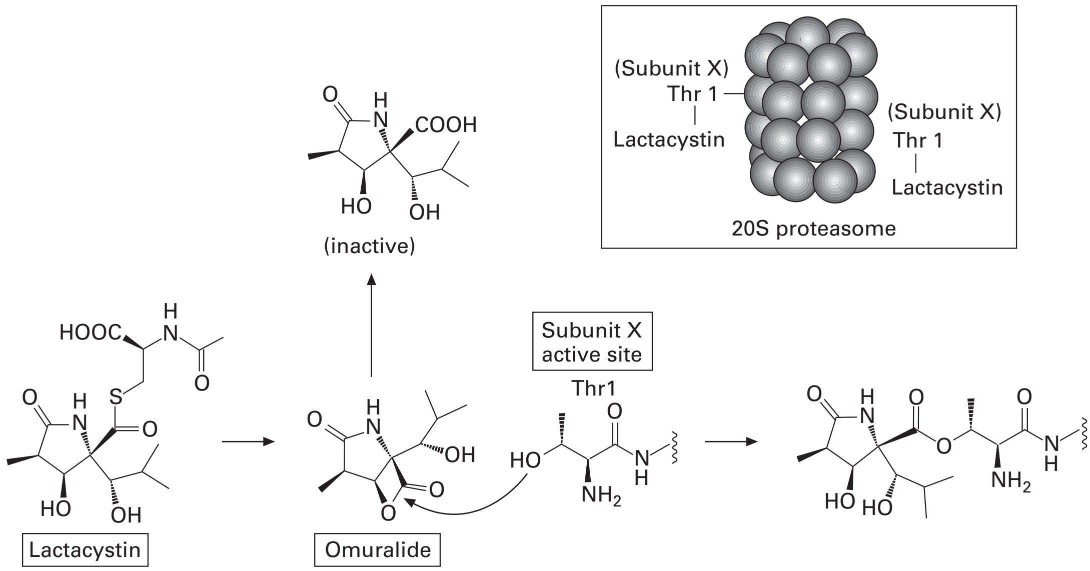


Fig. 2 Acylation of the N-terminal threonine of proteasome subunit X by lactacystin.

Total synthesis of lactacystin has been accomplished by many research groups. Corey and Reichard reported the first total synthesis [11]. The second synthesis was done by our group as shown in Fig. 3 [12,13]. Lactacystin was synthesized from (2*R*,3*S*)-3-hydroxyleucine by 10 steps with overall yield of 14%. The key reactions in the elaboration of lactam moiety include stereoselective hydroxymethylation and asymmetric allylboration to introduce the hydroxy and methyl substituents at C-6 and C-7 positions, respectively. After that, many researchers including Prof. Baldwin (Oxford University) and Prof. Chida (Keio University) synthesized lactacystin by a variety of approaches. However, in order to obtain enough amount of lactacystin, the fermentation method described above is superior to any organic syntheses reported until today. From 50 L cultured broth, 562 mg of lactacystin was obtained in a week.

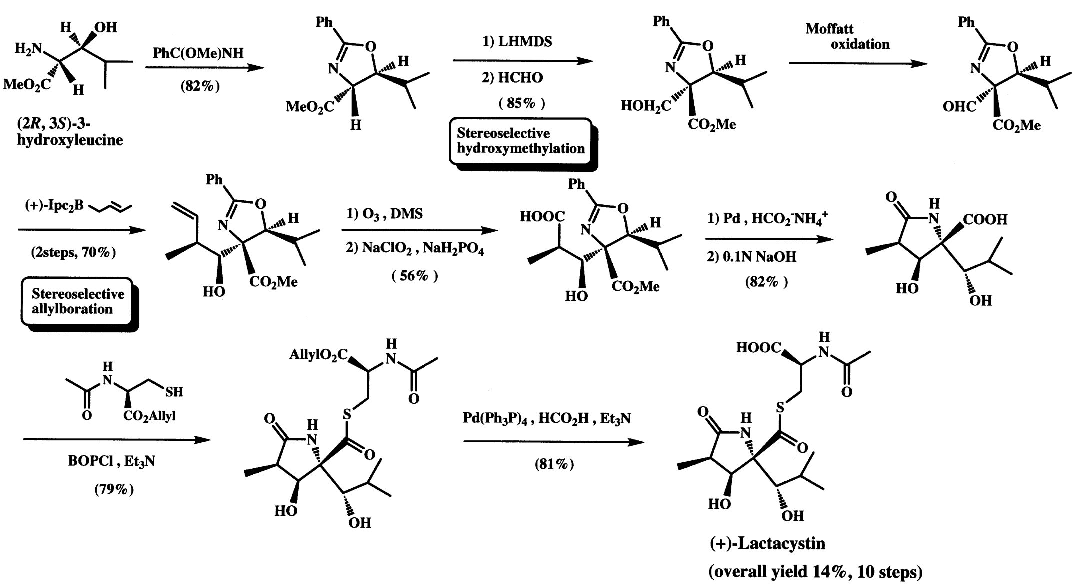


Fig. 3 Total synthesis of lactacystin.

Applications

After lactacystin was recognized as the most specific proteasome inhibitor in 1995, the sample of lactacystin has been requested by over 170 research groups. I sent it to all of these researches. As a result, it has been established that proteasome is involved in various cellular functions including regulation of the cell cycle [4], endoplasmic reticulum (ER)-associated protein degradation [14], regulation of apoptotic cell death [15], degradation of receptors [16], and degradation of regulatory proteins such as short-lived proteins and transcription factors [17].

MACROSPHELIDES

Background

Critical early events in tumor metastasis, inflammation and the allergic responses involve interaction between tumor cells and endothelial cells. A variety of cytokines and related chemical mediators control both cell–cell adhesion [18] and subsequent extravascular invasion by regulating the expression of cellular adhesion molecules. Inhibition of cell–cell adhesion thus holds promise for the treatment of diverse pathologies

Screening

We focused on binding of endothelial cell-leukocyte adhesion molecule-1 (ELAM-1, E-selectin) to the carbohydrate sialyl-Lewis^x (SLe^x). A screening system was established by using human umbilical vein endothelial cells (HUVEC) and human leukemia HL60 cells. HUVEC cultured for 24 h were treated with lipoppolysaccaride (LPS) for 5 h to express ELAM-1 on the cell surfaces. Then, fluorescence labeled-HL60 cells, which constitutively expressed SLe^x, were added to the HUVEC culture and incubated for 20 min. After removing free HL60, the extent of adhered HL60 cells was measured by fluorescence intensity. A fungal *Microsphaeropsis* sp. FO-5050 was found to produce active metabolites designated macrosphelides, which inhibited adhesion of HL60 cells to HUVEC [19].

Isolation and structure elucidation

The supernatant of the culture broth (4 L) was extracted with ethyl acetate, and the extracts (6 g) were purified by a combination with silica gel column chromatography, preparative TLC and HPLC using an ODS column to give pure macrosphelides A (580 mg), B (16 mg), C (1.8 mg) and D (8.0 mg) as white powders [19,20]. The structures including the relative and absolute stereochemistries were elucidated by a series of NMR studies, X-ray crystallographic analysis and Mosher NMR method as shown in Fig. 4 [20,21]. Macrosphelides are the first 16-membered-ring macrolides embodying three lactone linkages. The first total synthesis of macrosphelide A was achieved via highly convergent and efficient strategy as shown in Fig. 5 (11 steps, 20% overall yield) [22].

Inhibition of adhesion of HL60 cells to HUVEC

Macrosphelides inhibited the adhesion of SLex-expressing HL60 cells to ELAM-1-expressing HUVEC in a dose-dependent fashion, suggesting that they prevent the cell–cell adhesion by blocking the binding of SLe^x to ELAM-1. However, macrosphelides showed no effect on the adhesion of SLe^a-expressing WiDr cells to HUVEC. Furthermore, pretreatment of HL60 cells, not HUVEC, with macrosphelides caused inhibition of the adhesion of HL60 to HUVEC. These findings indicate that macrosphelides specifically bind to SLe^x on HL60 cells to block the cell–cell adhesion.

In vivo applications

Macrosphelides proved to be effective in several *in vivo* models. (i) In the mouse model of B16/BL6 melanoma lung metastasis, macrosphelide A (5–50 mg/kg) caused a dose-dependent decrease in lung metastatic nodules without any toxic effect including body weight loss. Furthermore, its efficacy in the combination therapy with anti-cancer drugs was demonstrated. Cisplatin (2 mg/kg/day) alone showed no reduction in lung metastatic nodules in the model, while a combination of cisplatin (2 mg/kg/day) and

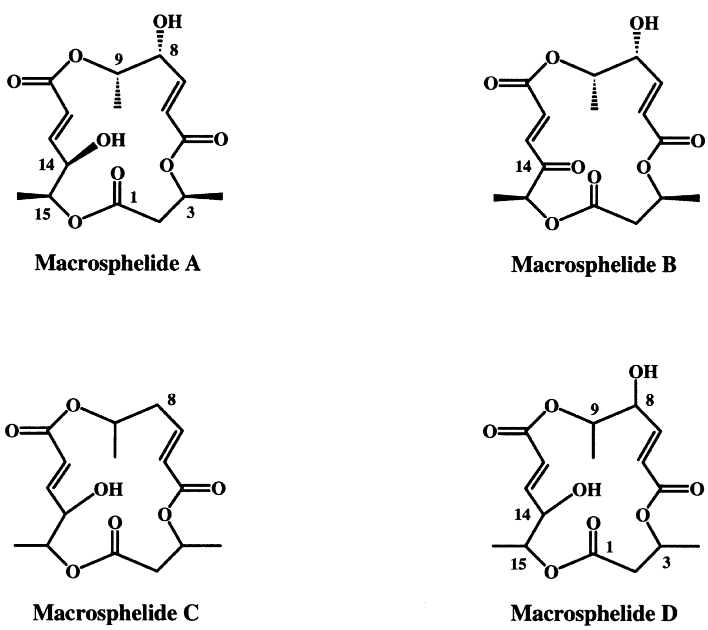


Fig. 4 Structures of macrosphelides A to D.

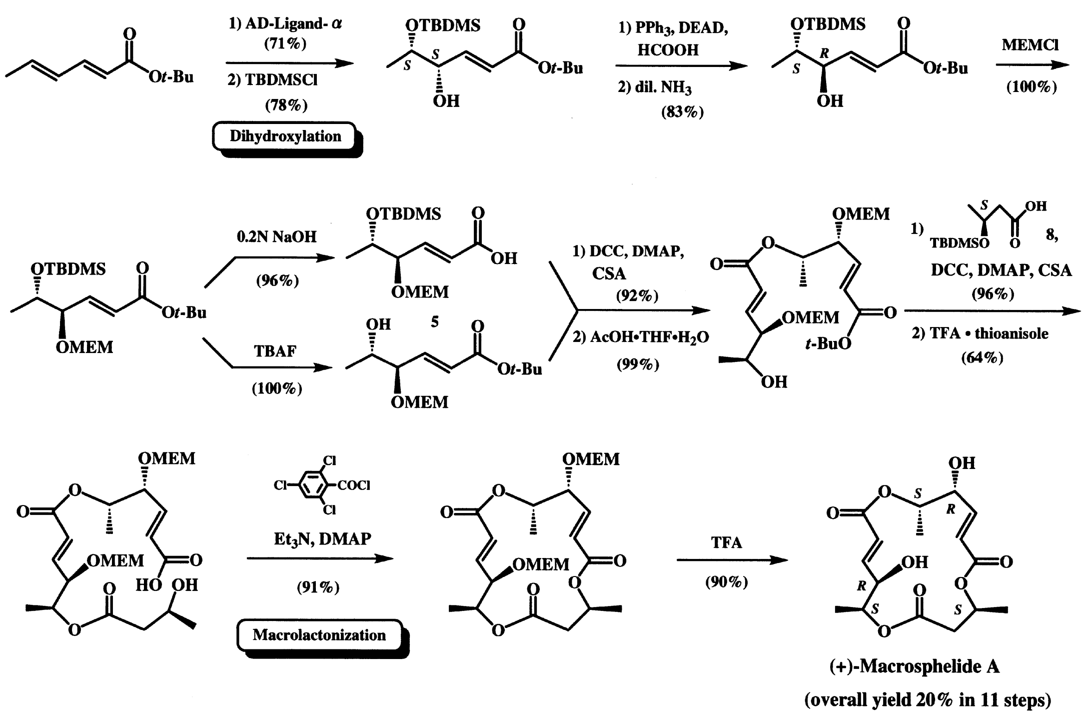


Fig. 5 Total synthesis of macrosphelide A.

macrosphelide A (50 mg/kg/day) caused a remarkable reduction in metastatic nodules without body weight loss (Fig. 6). (ii) Effect of macrosphelides was examined on an inflammation model in mice. Formation of lung fibrosis caused by accumulation of neutrophiles has been a heavy side-effect of the anti-cancer bleomycin treatment. The bleomycin-mediated lung fibrosis was significantly inhibited by the intraperitoneal administration of macrosphelide A at 50 mg/kg for 4 days.

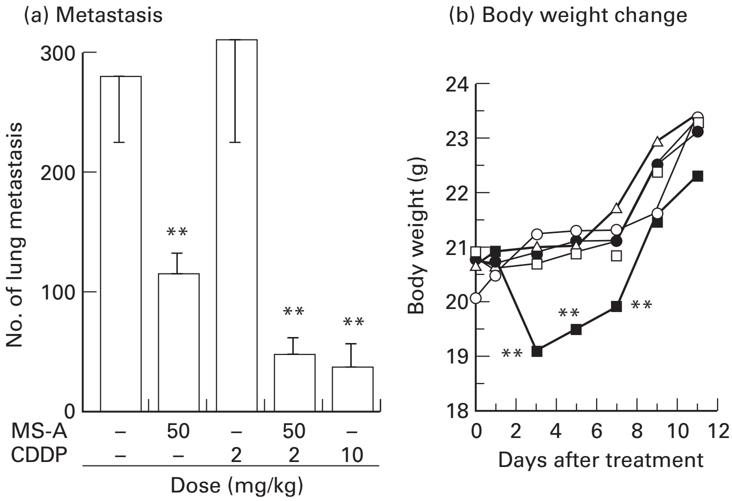


Fig. 6 Combination therapy of macrosphelide A (MS-A) and cisplatin (CDDP) on B16/BL6 melanoma-induced lung metastasis in mice. B16/BL6 melanoma (5×10^4 cells) were intravenously injected at day 0. CDDP (2(△), 10(■) mg/kg) was intraperitoneally injected at day 1. Macrosphelide A (50 mg/kg) was intraperitoneally injected at day 0, 2, 4, 6 and 8, with (●) or without (□) CDDP, 2 mg/kg ($n = 5$–8). (**; $P < 0.01$).

MADINDOLINES

Background

Cytokines play a variety of biological roles in cancer, inflammation and autoimmune diseases. IL-6 is a multifunctional cytokine involved in the regulation of immune reaction, hematopoiesis, acute-phase response and the growth of certain types of tumor cells [23,24]. However, overproduction of IL-6 causes cancer cachexia, Castleman's disease, rheumatoid arthritis, hypercalcemia and multiple myeloma [25,26]. Therefore, inhibitors or modulators of this cytokine function may be potentially effective against cancer and chronic or refractory inflammatory diseases.

Screening

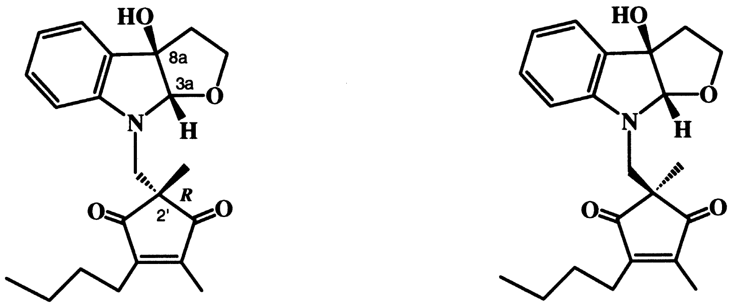
IL-6-dependent MH60 cells [27] were used in screens for inhibitors of IL-6 function. In the screening program, *Streptomyces nitrosporeus* K93-0711 was found to produce active metabolites designated madindolines, which specifically inhibit the growth of MH60 cells [28].

Isolation and structure elucidation

The supernatant of the culture broth (70 L) was extracted with ethyl acetate, and the extracts (11.5 g) were purified by silica gel column chromatography and HPLC using an ODS column to yield pure madindolines A (11.8 mg) and B (20.8 mg) as light yellow powders [28]. The structures were elucidated by NMR spectroscopic analysis [29], indicating that madindolines A and B were stereoisomers consisting of 3-hydroxyindoline and diketocyclopentene (Fig. 7). Recently, the total synthesis of madindoline A was achieved by 15 steps (Fig. 8). The key reactions include the stereoselective aldol reaction of β-hydroxyester, ring closing metathesis, reductive *N*-alkylation of indoline with cyclopentene aldehyde and asymmetric oxidative ring-closing. As a result, the absolute configuration was established as shown in Fig. 7.

Anticytokine spectrum

Madindolines A and B inhibited the growth of IL-6-dependent MH60 cells in a dose-dependent fashion with IC_{50} values of 8.0 and 30 μM, respectively. To examine the cytokine selectivity of madindoline inhibition, the effects on activities of other cytokines were also tested and the results are summarized in



Madindoline A **Madindoline B**

Fig. 7 Structures of madindolines A and B.

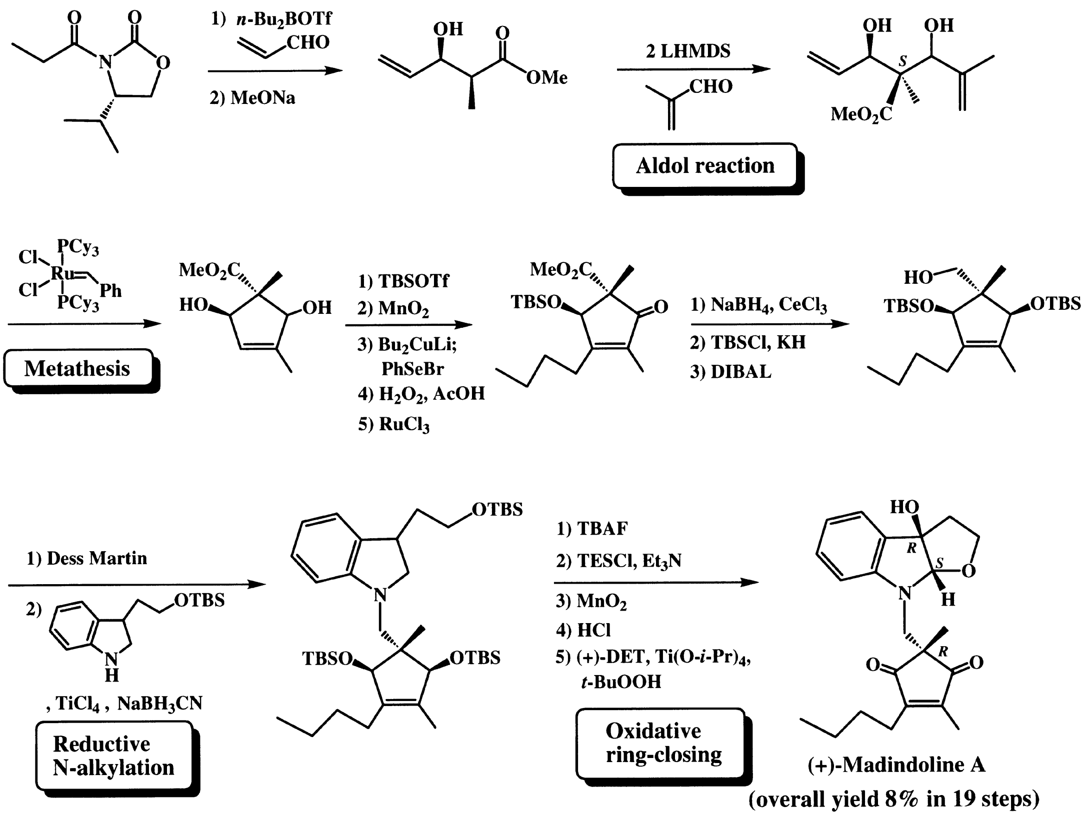


Fig. 8 Total synthesis of madindoline A.

Table 1. Madindoline was found to inhibit the IL-6-dependent cell growth, IL-6-mediated and IL-11-mediated cellular activities. However, the compound showed no effects on cell growth and cellular functions caused by other cytokines tested including IL-2, IL-3, IL-4, IL-8, TNF, LIF, NGF and G-CSF. These findings indicated that madindolines inhibit the IL-6 and IL-11 functions specifically.

Potential target

IL-6 has a unique signal transduction pathway and the receptor consists of two subunits, gp80 (α chain, an IL-6 specific subunit) and gp130 (β chain, a common subunit of IL-6 receptor family) [30]. IL-6 binds to gp80 to form a cytokine-receptor complex. The complex activates JAK2 by binding to gp130, and then

Table 1 The effect of madindoline A on cytokine activities

Cytokine	Cell line	Activity	Inhibition
IL-2	CTLL-2	Growth	−
IL-3	Baf/G-CSFR-gpl30	Growth	−
IL-4	U937	Expression (FcϵRII)	−
IL-6	MH60	Growth	+
	M1	Differentiation (Mϕ)	+
	PC12	Neuronal differentiation	+
	3T3L1	Differentiation (adipocyte)	+
	Osteoblast	Differentiation (osteoclast)	+
IL-8	PMNLs	Chemotaxis	−
IL-11	Osteoblast	Differentiation (osteoclast)	+
	3T3L1	Differentiation (adipocyte)	+
TNF	L929	Growth inhibition	−
	U937	Growth inhibition	−
NGF	PC12	Neuronal differentiation	−
G-CSF	Baf/G-CSFR-gpl30	Growth	−

further phosphorylates STAT3, which is translocated to nuclear to transcribe the target genes. Interestingly, IL-11 receptor shares the same gp130 as a receptor subunit, suggesting that the inhibition site of madindolines might be gp130. In fact, madindolines showed no effect on the binding of IL-6 to gp80 by the ELISA assay nor on the JAK2/STAT3 signal transduction by G-CSF-induced growth of Baf cells which express a chimera-receptor consisting of the extracellular domain of G-CSF receptor and the cellular domain of gp130. Other cytokines such as leukemia inhibitory factor (LIF), onkostatin M (OM), CT-1 and CNTF also utilize gp130 as a receptor subunit. However, LIF-induced macrophage differentiation was not inhibited by madindoline A (Table 1). It is noted that IL-6 and IL-11 receptors work through a homointeraction of gp130, whereas receptors for LIF, OM, CT-1 and CNTF signal through a heterointeraction between gp130 and LIF receptor [31]. From all these findings, we concluded that the target molecule of madindolines is gp130, a common receptor subunit for IL-6 and IL-11, and speculate that madindolines may inhibit the homointeraction of the activated gp130. Further study is in progress to demonstrate our speculation.

FUTURE ASPECT

Lactacystin was originally screened as an NTF-like agent. Unfortunately, it may not be useful in the treatment of the patients with nerve diseases because of its target proteasome, which is present in most cells as well as nerve cells. Very recently, lactacystin was found to show synergetic activity in combination with certain known anti-tumor agents such as topoisomerase inhibitors. Lactacystin will become more important in the basic, applied biological and biomedical sciences in near future. Macrosphelides are the first microbial inhibitors of cell–cell adhesion by blocking the ELAM-1/SLe^x recognition system. Importantly, they proved effective in animal models of the lung metastasis and fibrosis. Madindolines are the first microbial metabolites which inhibit the functions of cytokines IL-6 and -11 without any cytocidal effects on various normal and tumor cells. They will be expected as a new type of agents potentially effective against cancer cachexia and inflammatory diseases.

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