



Studies of novel bioprobes isolated from rare natural sources using mutant yeasts

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Abstract

For the discovery of novel drug candidates and bioprobes, it is important to investigate rare natural sources with unique screening methods. Our group tested 1000 original MeOH extracts from natural sources, such as plants and food ingredients using six types of mutant yeasts. In our experiments, Kuji amber was used as the rare domestic natural source and the growth-restoring activity (positive screening) of mutant yeast involving Ca²⁺-signal transduction was used as a unique screening method. A prominent new anti-allergy compound, named kujigamberol (15,20-dinor-5,7,9-labdatrien-18-ol) was isolated from Kuji amber and known compounds already isolated from modern plants were identified in Baltic and Dominican ambers. Kujigamberol and the methanol extract of Kuji amber (MEKA) exerted inhibitory activity against the degranulation of rat basophilic leukemia-2H3 (RBL-2H3) cells through the inhibition of Ca²⁺-influx and inhibited the production of leukotriene C₄ (LTC₄). The effects of kujigamberol and MEKA on rhinitis model were approximately five times more potent than those of the mometasone furoate. Thus, the combination of rare natural sources and unique mutant yeasts can yield valuable bioprobes as novel drug candidates and/or basic research reagents, to benefit humankind.

Microorganisms as screening sources for drug candidates

Many human drugs have been isolated and developed from microorganisms and plants. Since Dr Alexander Fleming, who was awarded the Nobel Prize in Physiology or Medicine in 1945, discovered penicillin G from *Penicillium notatum* (now *Penicillium chrysogenum*) [1], microorganisms, such as bacteria (mainly actinomycetes) and fungi, have been invaluable in the discovery of drugs and lead compounds. In addition, Dr Selman Abraham Waksman [2], for the discovery of streptomycin for tuberculosis, and Dr Satoshi Omura [3, 4], for the discovery of avermectin/ivermectin for parasites, were also awarded the Nobel Prize

in Physiology or Medicine in 1952 and 2015, respectively. The fungal metabolite ML-236B (compactin or mevastatin), which is a lead compound for HMG-CoA reductase inhibitors and was discovered by Dr Akira Endo [5] led to the development of “statins” for the reduction of plasma cholesterol levels. The *Streptomyces* metabolite, FK506 (tacrolimus), used to suppress the immune response after transplant operations was also investigated as a bioprobe for the analysis of immune signal transduction by Dr Toshio Goto [6] and Dr Stuart L. Schreiber [7]. In addition, the antimalarial drug artemisinin was isolated from a traditional Chinese medicine, *Artemisia annua*, by Ms Youyou Tu, who received the Nobel Prize in Physiology or Medicine in 2015 with Dr Omura [8]. Thus natural products with diverse structures continue to play a highly significant role in drug discovery [9–14].

We have isolated and studied three types of novel drug candidates, liposidomycin from *Streptomyces* sp. [Fig. 1(a)] [15], SN-07 chromophore (barminomycin) from *Actinotetraspora* sp. (now *Microtetraspora* sp.) [Fig. 1(b)] [16], and propeptin from *Microbispora* sp. [Fig. 1(c)] [17] at RIKEN and in Snow Brand Milk Products Co. Ltd (now Meg-Milk Co. Ltd.). The molecular target of liposidomycin is phospho-*N*-acetylmuramoyl-pentapeptide transferase (translocase I) (EC2.7.8.13: Mra Y), which is involved in

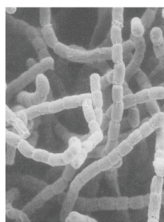
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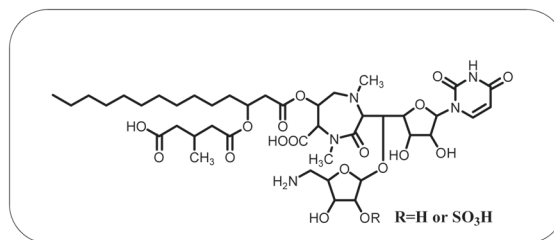
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Fig. 1 Three types of new bioprobes isolated from Actinomycetes

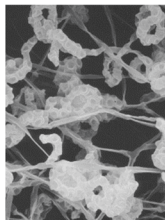
(a) *Streptomyces* sp.



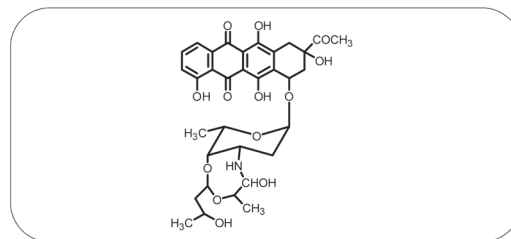
liposidomycin



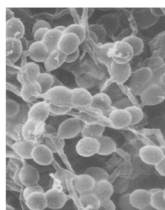
(b) *Microtetraspora* sp.



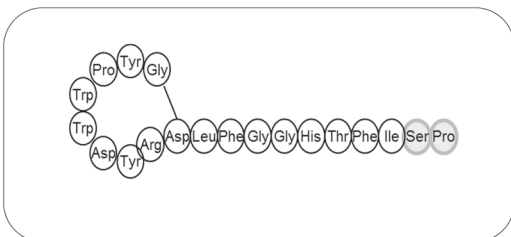
barminomycin



(c) *Microbispora* sp.



propeptin



peptidoglycan biosynthesis, and it specifically inhibits the enzyme at nanomolar concentrations. The molecular target of barminomycin is the 2-amino group of guanine in the GC sequences of DNA and it is ~1000 times more potent than daunomycin (daunorubicin), a clinically used anticancer drug. The molecular target of propeptin is prolyl oligopeptidase (the former prolyl endopeptidase: EC3.4.21.26), which is involved in Alzheimer's disease. It is a member of the group of compounds known as "lasso peptides" collectively. Propeptin also has antimicrobial activity against *Mycobacterium* sp. and the C-terminal sequence motif of Ser-Pro is known to be crucial for antimicrobial activity [Fig. 1(c)]. Although they are not yet marketed as drugs, the caprazamycin derivative CPZEN-45, which is similar to liposidomycin, has been studied as an antituberculosis agent in clinical trials in the United States of America [18].

Recently, we have identified the molecular target for three types of bioprobes (pyrrocidine A from *Neonectria* sp. [19], allantopyrone A from *Allantophomopsis* sp. [20], and neomacropholins from *Trichoderma* sp. [21]) containing an α,β -unsaturated carbonyl group. The molecular targets were phosphoinositide 3-kinase (PI3K), the Keap1-Nrf2

pathway, and the proteasome, respectively. Natural products are fascinating drug candidates from both a chemical and a biological perspective. However, many pharmaceutical companies have reduced the number of natural product projects, because they can be "treasure hunts", in which it is uncertain and laborious to isolate a pure compound from a complex mixture in suitable quantity. Instead, molecular target-based high-throughput screening with combinatorial chemistry has been the predominant approach to drug development for the past two decades. However, this approach has limitations, and phenotypic screening using yeasts, cells, and zebrafish can expand the chemical and biological spaces to the discovery of drugs and novel bioprobes, especially those from natural sources [22–25].

Unique screening method using mutant yeasts for drug discovery

Recent advances in biotechnology have afforded important information, such as the genome sequence of *Saccharomyces cerevisiae*. Owing to the technical advantages of

this organism, including simple growth conditions, rapid cell division, and the availability of genetic tools, consequently, the application of yeast as a screening tool in the field of drug discovery has expanded [26–32]. Another yeast, *Shizosaccharomyces pombe*, has also proven useful [33, 34]. Genetically mutated yeasts, such as those with gene deletions, gene overexpression, and amino acid substitutions, cannot grow in the presence of a stress such as high temperature, amino acid analogs, high salt concentration, and overproduced protein. We have collaborated with yeast molecular biologists to use the changes in growth-restoring activity in the presence of some kind of stress as an assay for drug discovery [Fig. 2A]. The changes are related to processes such as Ca^{2+} -signal transduction [35, 36], cell cycle (checkpoint) [37], ubiquitin ligase [38–40], and HSET [41].

The calcium ion (Ca^{2+}) is an important second messenger for Ca^{2+} -signal transduction. It has important roles in the regulation of diverse cellular processes, such as T-cell activation, muscle contraction, neurotransmitter release, and secretion [42] and is also involved in various molecular targets of diseases such as Ca^{2+} channel, calcineurin, mitogen-activated protein kinase (MAPK), and glycogen synthase kinase-3 β (GSK-3 β), etc [Fig. 2B] [35]. Ca^{2+} -signal transduction regulates the cell cycle in *S. cerevisiae* and the onset of mitosis is regulated by Swel that inhibits a G2 form of the Cdc28 cyclin-dependent protein kinase by phosphorylating it at Tyr-19 and delays the entry into mitosis. In the genetic background of *zds1 Δ* , SWE1 expression level is induced by high concentration of Ca^{2+} in G2/M compared to wild type [35]. Thus the inhibitors of Ca^{2+} -signal transduction were detected by their ability to stimulate the growth of mutant yeast as a growth zone around a paper disc (40 μl) or each spot site (5 μl) containing an active compound in the presence of 0.3 M CaCl_2 (Fig. 2A, B) [36]. To improve the cell permeability of drugs, a mutant yeast involving Ca^{2+} -signal transduction (YNS17 strain: *zds1 Δ erg3 Δ pdr1 Δ pdr3 Δ*) was introduced. New compounds, named eremoxylarin [43], benzophomopsin [44], and anthracobic acid [45], were isolated from the endophytic fungi, *Xyraia* sp., *Phomopsis* sp., and *Anthracobia* sp., respectively [Fig. 2C(a–c)]. The known compounds ricinoleic acid [46] and burchellin isomer [47] were isolated from the plants *Ricinus communis* and *Magnolia quinquepeta*, respectively [Fig. 2C(d, e)]. The known compounds 6-(methylsulfinyl) hexyl isothiocyanate [48] and faltarindiol [49] were isolated from the food ingredients *Oenanthe javanica* and *Wasabia japonica*, respectively [Fig. 2C(f, g)]. The unique screening method and variety of natural sources are important for the discovery of drug candidates and bioprobes.

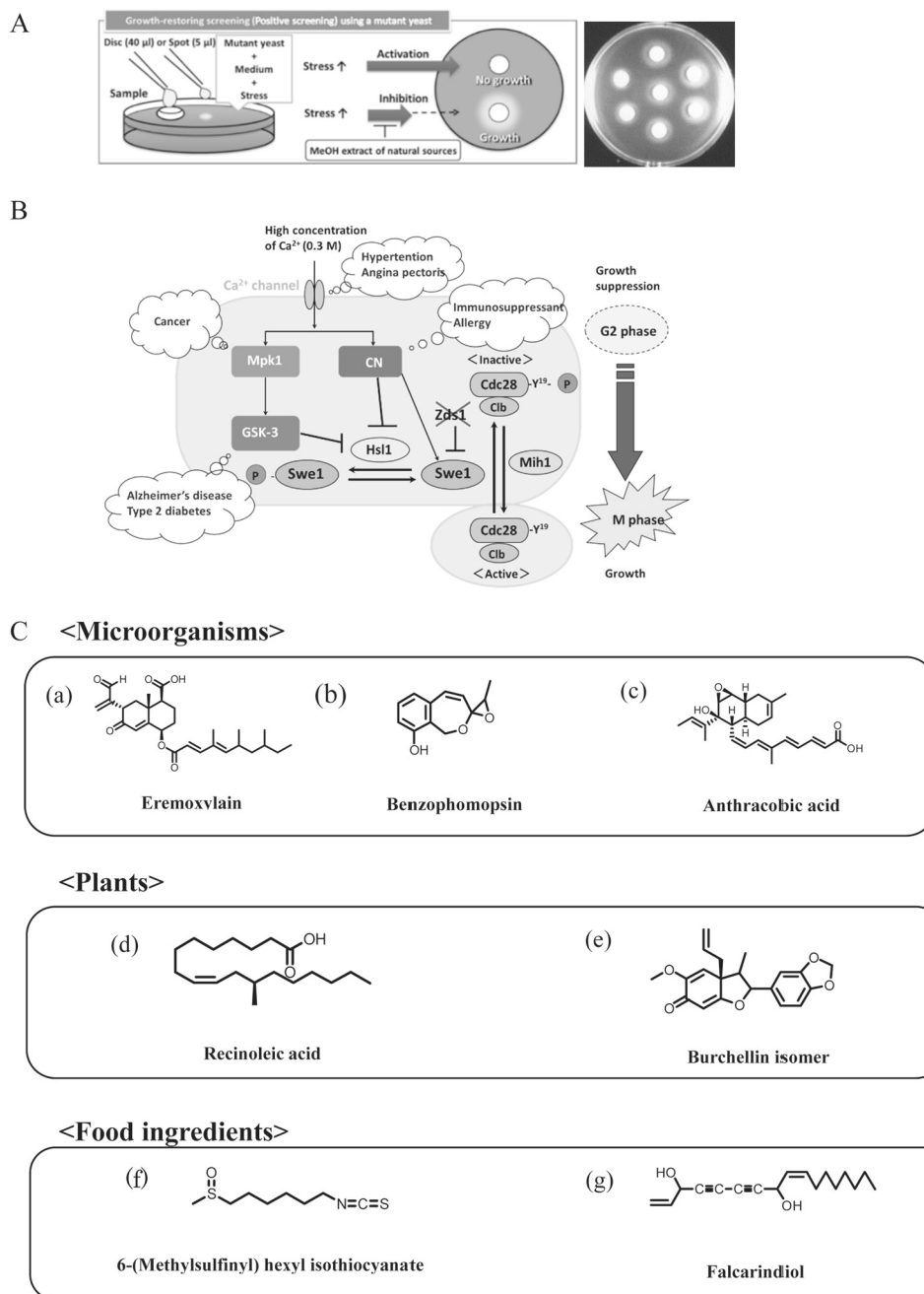
In phenotypic screening, the determination of the molecular target is most important for the development of drugs,

however, it is a complex process [27, 50]. The molecular target of eremoxylarins A and B isolated using the YNS17 strain was identified as calcineurin, owing to the physiological character, the synthetic lethal effect, and the enzyme inhibition activity [43]. 6-(Methylsulfinyl) hexyl isothiocyanate and faltarindiol were shown to have inhibitory activity against GSK-3 β [48, 49], because the phenotype of them against the YNS17 strain was similar to that of a commercial synthetic inhibitor, GSK-3 β inhibitor I [43].

Ambers and edible wild plants as rare natural sources for drug discovery

Novel natural compounds for clinical use are expected to be discovered from rare natural sources by using unique screening methods. Amber is fossilized tree resin comprising organic polymers derived from the complex maturation processes of the original plant resin and its main use is as a decorative ornament. Although it has been used for the treatment of muscle pain, headaches, and skin allergies in certain areas, no biologically active compounds from amber have been isolated prior to our studies [51, 52]. Amber has been discovered in several locations originating from different geological ages, such as Kuji [90–86 million years ago (mega-annum) (Ma) (Late Cretaceous) from Kuji-city, located on the northern Pacific coast of Japan (141°E 40°N)] (Prof. Hisao Ando, Ibaraki University, personal communication), Baltic (56–34 Ma, Poland and Russia), Dominican (45–30 Ma and 20–15 Ma, Dominican Republic), Burmese (99 Ma, Myanmar), Spanish (112–99 Ma, Spain), German (45 Ma, Germany), Dolomites (225 Ma, Italy), Lebanese (120 Ma, Lebanon), Cedar Lake (78 Ma, Canada), Mexican (26–22.5 Ma, Mexico), and New Jersey (94–90 Ma, USA) [53–55] (Fig. 3A). Kuji, Baltic, and Dominican ambers are the most common commercial ambers worldwide. Of these, Baltic amber is the most popular and has been investigated for its chemical composition by GC–MS, pyrolysis (Py)–GC–MS, and solid NMR, and it is used as a folk medicine in Russia [51, 52, 56, 57]. Other than those reported by our group, there are only a few reports of the isolation of pure compounds from ambers, such as quesnoin from Oise amber [55 Ma, Paris (France)], amberene [15,19,20-trinor-5,7,9-labdatriene (or 1,6-dimethyl-5-isopentyltetralin)] and 1-methylamberene [15,20-dinor-5,7,9-labdatriene (or 1,1,6-trimethyl-5-isopentyltetralin)] from Spanish amber, and 15-nor-cleroda-3,12-diene from Dominican ambers [Fig. 3B(a–c)] [58–60]. Furthermore, there is also no description of any biological activity and no isolation has been conducted by biological activity-guided fractionation. Hence, there is no precedent for studies focused on the biological activity of compounds

Fig. 2 Screening of the growth-restoring activity using mutant yeasts (A) and bioprobes isolated from microorganisms, plants, and food ingredients (C) by using the mutant yeast involving Ca^{2+} -signal transduction (YNS17 strain: *zds1Δ erg3Δ pdr1Δ pdr3Δ*) (B)



in amber and biological activity-guided fractionation. We have focused on the biological activity of compounds in ambers found worldwide, especially Kuji amber, and describe the use of ambers as rare natural sources for drug discovery for the first time [61].

Other rare natural sources for drug discovery are the edible wild plants from northern Japan, *Cacalia delphinifolia* [Momijigasa (Shidoke)], and *Cacalia hastate* (Bouna), both of which are food ingredients, especially in northern Japan. A sesquiterpene endoperoxide compound, 3,6-epidioxo-1,10-bisaboladiene (EDBD), was isolated from these plants by using another mutant yeast (*cdc2-1*

rad9Δ) and shown to be an inhibitor of cell cycle and a novel antitumor compound [37, 62, 63] (Fig. 3C). The *RAD9* gene has been identified as figuring in cell-cycle checkpoint control and *CDC2* encodes a catalytic subunit of DNA polymerase δ that is required for chromosomal DNA replication during mitosis and meiosis. The WCTR312A (*cdc2-1 rad9Δ* double mutant) strain rapidly lose their viability at 37°C because they lack the *RAD9* product, but they survive in the presence of the cell-cycle blockers such as thiabendazole, hydroxyurea, and mycophenolic acid. Borrelidin [64], UCS15A, copiamycin analog, and fredericamycin A [65] were all identified

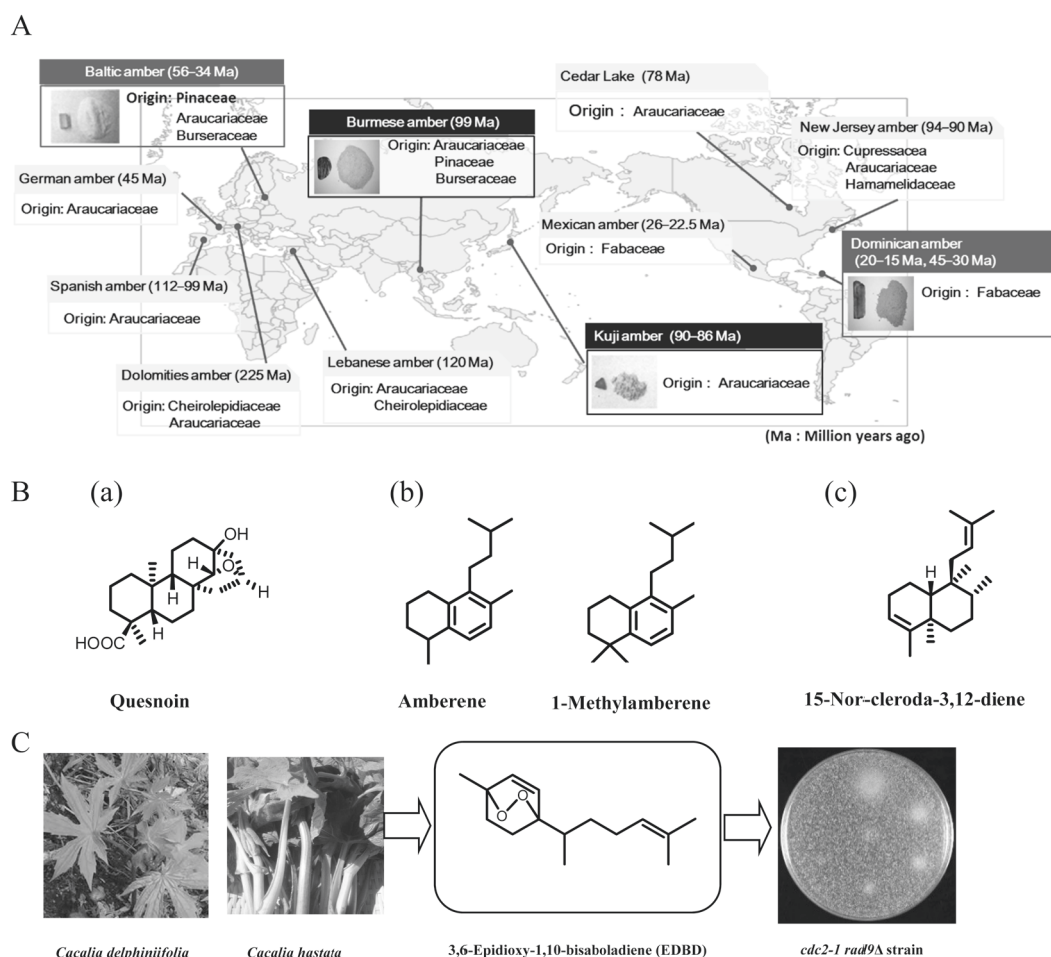


Fig. 3 Ambers of the world (a), isolated compounds from ambers without biological activity (b), and wild edible plants as rare natural resources (c)

from the fermentation broth of actinomycetes using this strain.

3,6-Epidioxy-1,10-bisaboladiene (EDBD) had cytotoxicity to the human promyelocytic leukemia cell line HL60 with an IC_{50} value of $3.4 \mu\text{M}$. Furthermore, there was no similarity between the JFCR39 (a panel of 39 human cancer cell lines) fingerprints of EDBD and dehydroartemisinin ($\gamma = 0.158$), which is a sesquiterpene endoperoxide and an analog of the antimalarial drug artemisinin. EDBD exerted antitumor effects against xenografted Lox-IMVI cells at 25 mg kg^{-1} (*i.v.*) without decrease of the body weight in vivo [66].

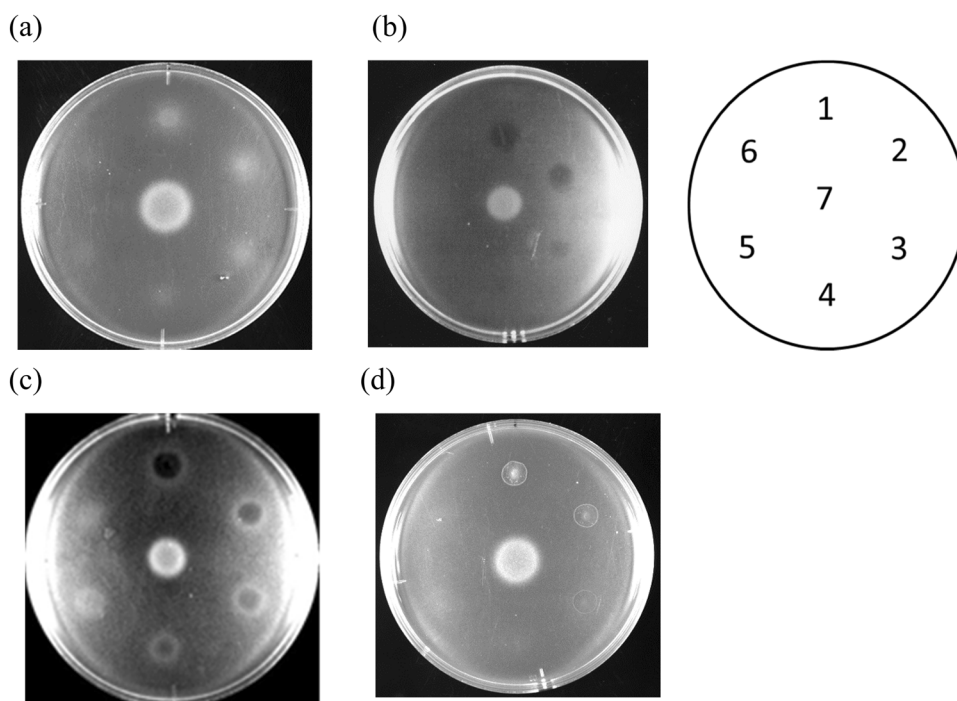
Isolation and structure elucidation of biologically active compounds from ambers

Although the extracts of all examined ambers have biological activity against the YNS17 strain, interestingly, each phenotype was distinct (Fig. 4). The MeOH extract of Kuji amber (MEKA) exerted clear growth-restoring activity in a dose-dependent manner [Fig. 4(a)]. The MeOH extract of

Baltic amber resulted in a clear inhibition zone that occurred in a dose-dependent manner [Fig. 4(b)]. The MeOH extract of Dominican amber showed growth-restoring activity and inhibitory activity at a wide range of concentrations [Fig. 4(c)]. The MeOH extract of Burmese amber showed weak growth-restoring activity [Fig. 4(d)]. These results suggested that the biologically active compounds in each MeOH extract of the Kuji, Baltic, Dominican, and Burmese ambers were different.

Kuji amber contains ~5% of the MeOH-soluble fraction and ~95% of polymers that are not soluble in MeOH. The ratio of the polymer fraction in ambers increases with the age of the sample and we first focused on the biologically active compounds of the alcohol-soluble fraction. Powdered Kuji amber (1029 g) was extracted with MeOH and the extract [33.81 g (3.3%)] was then extracted twice again in one volume of EtOAc. The organic layer [18.34 g (1.8%)] was subjected to silica gel column chromatography with hexane-EtOAc (3:1 and 2:1) as solvents and two active fractions A and B were collected. Finally, the main novel biologically active compound [52.1 mg (0.098%) and 23.6 mg (0.074%)], named kujigamberol [61, 67], was purified

Fig. 4 Growth-restoring activity of the MeOH extract of Kuji (a), Baltic (b), Dominican (c), and Burmese (d) ambers against the mutant yeast (YNS17 strain: *zds1Δ erg3Δ pdr1Δ pdr3Δ*) in the presence of 0.3 M CaCl₂. (a, b, d) 1: 5 μg/spot, 2: 2.5 μg/spot, 3: 1.25 μg/spot, 4: 0.63 μg/spot, 5: 0.31 μg/spot, 6: 0.16 μg/spot, 7: 2.5 ng/spot (FK506). (c) 1: 0.5 μg/spot, 2: 0.25 μg/spot, 3: 0.125 μg/spot, 4: 0.063 μg/spot, 5: 0.031 μg/spot, 6: 0.016 μg/spot, 7: 2.5 ng/spot (FK506)



by HPLC as a colorless oil, respectively. Two new compounds, Kujiol A (2.2 mg) and kujigamberol B (1.7 mg) were isolated as colorless oils from fraction B by HPLC, respectively [68]. These three new compounds named kujigamberol [15,20-dinor-5,7,9-labdatrien-18-ol (1)], kujiol A [13-methyl-8,11,13-podocarpatrien-19-ol (2)], and kujigamberol B [15,20-dinor-5,7,9-labdatrien-13-ol (3)] were identified by HREIMS, 1D NMR, and 2D NMR (Fig. 5). In addition, a biologically active compound with weak UV absorbance that was different in character from the aforementioned was isolated and identified as a spirolactone norditerpenoid [(4*R**, 5*S**, 8*R**, 9*R**, 10*S**)-14,15,16,19-tetranor-labdan-13,9-olide (4)] (Fig. 5) [69]. It has protein phosphatase, Mg²⁺/Mn²⁺-dependent 1A (PPM1A) [the former protein phosphatase 2C (PP2C)] activation activity in vitro and exerted growth-restoring activity against the YNS17 strain, similar to pisiferdiol [69, 70].

The use of EtOAc extraction procedures in the isolation process of biologically active compounds from Kuji amber resulted in low yields. Therefore, medium pressure liquid chromatography was explained as a more convenient procedure than EtOAc extraction. Consequently, the quantities of kujigamberol, kujiol A, and kujigamberol B were increased, and new compounds, named kujigamberol C [15-nor-8-labden-13-ol (5)] and kujigamberoic acid A [15,20-dinor-5,7,9-labdatrien-18-oic acid (6)], were isolated and identified (Fig. 5) [71, 72].

Biologically active compounds from Baltic, Dominican, and Burmese ambers were also isolated and we elucidated the structure using similar methods for those from Kuji amber. Three types of known biologically active compounds, pimaric acid (7), dehydroabietic acid (8), and agathic acid 15-monomethyl ester (9) (Fig. 5), were detected in Baltic amber and they are abundant in modern *Arucaria* and *Pinus* trees [61, 73].

One analogous new compound, 5(10)-halimen-15-oic acid [(10), 5.7 mg] and two known compounds, 3-cleroden-15-oic acid [(11), 32.1 mg] and 8-labden-15-oic acid [(12), 7.9 mg] (Fig. 5) were isolated from Dominican amber (262.4 g). 3-Cleroden-15-oic acid inhibited the degranulation of rat basophilic leukemia-2H3 (RBL-2H3) cells induced by two kinds of stimulants for Ca²⁺-influx, thapsigargin (Tg) and A23187, but not by the antigen IgE +DNP-BSA (Ag). These compounds were also isolated from modern *Himenaee* sp [74, 75].

Two types of known compounds, 16,17-bisnordehydroabietic acid [(13), 2.4 mg] and 16,17-bisnorcallitric acid [(14), 3.3 mg] (Fig. 5) were isolated from Burmese amber (912.16 g) [76]. They were also detected in Brazilian and Nigerian ambers by GC-MS analysis [77, 78].

Although their biological activity has not yet been reported, abundant labdatriene derivatives previously isolated from Spanish amber, named amberene and 1-methylamberene, were also isolated from MEKA by using HPLC [Fig. 3B (b)]. The structures elucidated by spectral analyses,

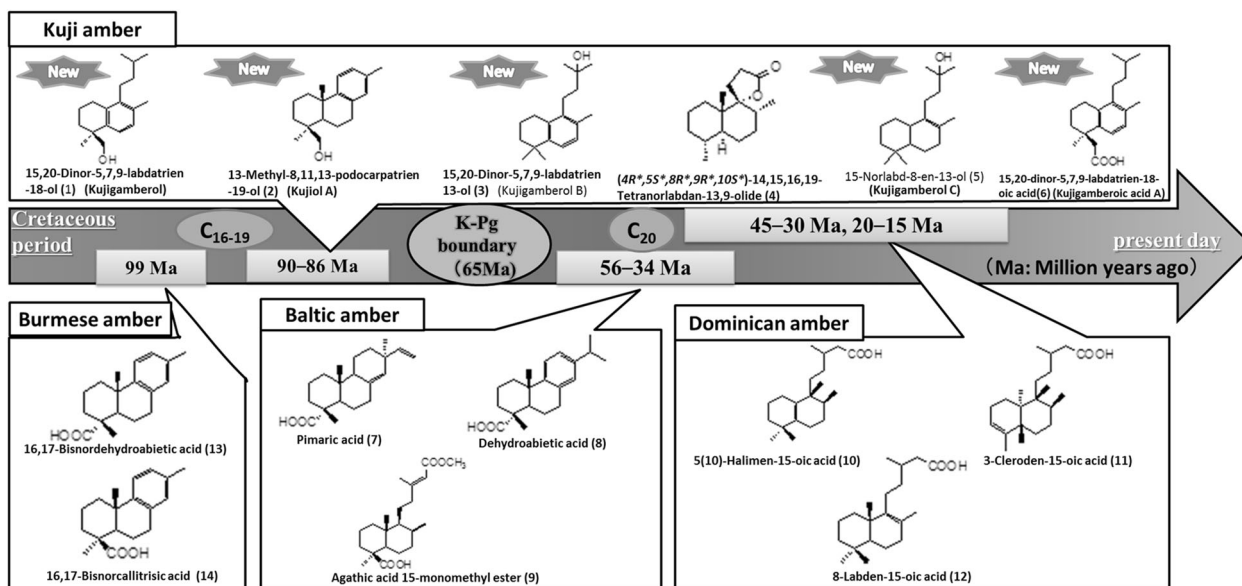


Fig. 5 Structures of biologically active compounds from Kuji, Burmese, Baltic, and Dominican ambers

including HREIMS, 1D NMR, and 2D NMR, indicated that Kuji amber was a Cretaceous amber and that the botanical origin may be Araucariaceae, Cheirolepidiaceae, or Cupressaceae [59, 79]. If the botanical origin of Kuji amber was extinct in the Cretaceous–Paleogene (K–Pg) (formerly Cretaceous–Tertiary) boundary (65 Ma), kujigamberol may also have been identified in Burmese ambers, but not in Baltic and Dominican ambers. However, kujigamberol was not detected in Spanish and Burmese ambers that were older than the K–Pg boundary [59, 76]. It is therefore an interesting question as to why new compounds have only been isolated from Kuji amber, whereas known compounds have already been isolated from modern plants and other ambers such as Baltic, Dominican, and Burmese ambers. As the environment of the earth on the K–Pg boundary changed and resulted in the destruction of most of plants and dinosaurs, the botanical origin of Kuji amber that is older than the K–Pg boundary may die out on the K–Pg boundary. In addition, the environment (diagenetic conditions) of the Kuji region in Japan may be very different from other areas in the world, because the Japanese islands were parts of the Eurasian continent in 90–86 Ma and have been split from there since approximately in 20 Ma. The Kuji area in Japan may have provided activated conditions for violent chemical reaction from 90–86 Ma to 20 Ma [80].

Anti-allergy activity of kujigamberol and MEKA

Ca^{2+} is an important second messenger in many cell types, including yeast and immune cells [42]. Ca^{2+} -signal transduction is involved in many diseases and is critical for the

degranulation, generation of eicosanoids, and production of cytokines in mast cells in response to antigens and other stimulants [81]. The incidence of type I allergies, such as allergic rhinitis, has followed an increasing trend, especially in industrialized countries and the typical symptoms of nasal itching, sneezing, rhinorrhea, and nasal congestion affect the quality of life and the productivity of patients [82]. Although the current therapeutic drugs, such as corticosteroids, for rhinitis and asthma are used worldwide, natural products are expected to lead to the development of safe and effective therapeutic agents that are alternatives to these steroids [83]. Mast cells are a key player in type I allergy and RBL-2H3 cells were used for the evaluation of anti-allergy activity through the inhibition of the β -hexosaminidase release in the medium (degranulation).

Kujigamberol, an inhibitor of Ca^{2+} -signal transduction in *S. cerevisiae*, exerted inhibitory activity against the degranulation of RBL-2H3 cells stimulated with Tg ($\text{IC}_{50} = 29.1 \mu\text{M}$) and A23187 ($\text{IC}_{50} = 24.9 \mu\text{M}$), but not by Ag ($\text{IC}_{50} > 50.0 \mu\text{M}$) without cytotoxicity ($\text{IC}_{50} > 50.0 \mu\text{M}$), respectively. It inhibited Ca^{2+} mobilization in RBL-2H3 cells stimulated with Tg and A23187 stimulations by ~100% at 50.0 μM , whereas the inhibitory activity induced by Ag stimulation was weaker. The IC_{50} values after 1000 second when stimulated by Ag, Tg, and A23187 were 10.6 μM , 6.0 μM , and $>50.0 \mu\text{M}$, which were comparable with the inhibitory activity of degranulation. MEKA also showed almost the same biological activity as kujigamberol.

Important molecules in allergy-related signal transduction are ERK1/2 and they induce a pro-inflammatory mediator LTC_4 [84, 85]. Kujigamberol suppressed the phosphorylation of ERK1/2 in a dose-dependent manner ($\text{IC}_{50} = 19.5 \mu\text{M}$). The activation of ERK1/2 is related to the

generation of LTC₄ and it inhibited the generation of LTC₄, with an IC₅₀ value of 6.1 μM. The LTC₄ production was comparable to the inhibition of the phosphorylation of ERK1/2, Ca²⁺-influx and degranulation. MEKA also showed almost the same biological activities as kujigamberol. These results suggested that kujigamberol and MEKA were expected to have anti-allergy activity against rhinitis model.

The nasal administration of ovalbumin solution to sensitized guinea pigs induced nasal blockade through an increase in sRaw (the specific airway resistance) [86]. In the ovalbumin-exposed vehicle-treated group, the percentage increase in sRaw after 10 min and 1 h was 574% ± 47% and 187% ± 26%, respectively (Table 1). The positive control, mometasone furoate (10 μg), a steroid clinical drug, resulted in sRaw of 165% ± 36% after 10 min and 47% ± 16% after 1 h. Improvements in the activity of the nasal blockade in guinea pigs were almost the same as those induced by kujigamberol (2 μg, 147% ± 31% after 10 min and 72% ± 15% after 1 h), MEKA (2 μg, 152% ± 29% after 10 min and 54% ± 15% after 1 h), and mometasone furoate (10 μg). These results indicated that the activities of kujigamberol and MEKA were approximately five times stronger than that of the clinical steroid drug, mometasone furoate [87].

Kujigamberol was approximately ten times more potent than MEKA against the mutant yeast and 5 μg MEKA was found to contain only 0.031 μg kujigamberol by HPLC analysis. The equal growth-restoring activity of 5 μg MEKA and 0.5 μg kujigamberol indicates that MEKA was ~15 times stronger than kujigamberol. The inhibitory activity of MEKA against degranulation induced by A23187 was ~50 times stronger than that of kujigamberol. LTC₄ is a typical inflammatory mediator and is closely related to asthma and nasal congestion [85]. As kujigamberol and MEKA

potently suppressed LTC₄ production, it was thought that they should exert anti-allergy effects in the rhinitis model. As expected, they had potent anti-allergy effects against rhinitis model and were approximately five times stronger than that of the steroidal clinical drug, mometasone furoate. Unexpectedly, the potency of MEKA was ~160 times stronger than that of kujigamberol against the rhinitis model. Those results suggested that MEKA includes minor compounds with potent biological activity and/or other biologically active compounds with different mechanisms of action that may act synergistically. This is another indication that the molecular target of kujigamberol was unique and is expected to be developed as a new type of anti-allergy drug.

Perspective

Ambers are a focus of geochemistry, in particular, the age, original plant(s), and the chemical constituents have been studied by using GC–MS, however, the biological activity has often been disregarded. We have focused on the biological activity and biologically active compounds of ambers from a chemical biology perspective to determine their potential as drug candidates for the first time. Ambers are a rare and fascinating natural source, as described above. To answer the two questions of the “chemistry” and “biology” considered in our study, it is important to isolate more new compounds from MEKA by using modified isolation methods or an alternative assay using enzymes such as PPM1A (PP2C). It is also necessary to identify the biologically active compounds in amber from other countries, such as Spain (El Soplao-Rabago) and Germany (Geiseltal) ambers, etc. [Fig. 3A]. The comparison of the

Table 1 The effects of the methanol extract of Kuji amber (MEKA) and kujigamberol on an ovalbumin-induced rhinitis model

Sample	Concentration (mg/mL)	<i>n</i>	10 min Increase in sRaw (%)	1 h Increase in sRaw (%)
Vehicle	0	6	574.27 ± 47.21	186.76 ± 26.42
Kujigamberol	1	6	60.09 ± 11.03**	18.76 ± 9.41**
Kujigamberol	0.1	6	147.28 ± 31.06**	71.95 ± 15.27**
Kujigamberol	0.01	6	302.86 ± 37.29**	93.39 ± 20.40*
Kujigamberol	0.001	6	473.84 ± 51.81	123.60 ± 24.98
MEKA	1	6	39.37 ± 9.89*	18.95 ± 7.31**
MEKA	0.1	6	152.25 ± 28.67*	53.56 ± 15.41**
MEKA	0.01	6	328.65 ± 82.22	115.76 ± 16.65*
MEKA	0.001	6	469.03 ± 47.59	140.36 ± 14.75
Momethazone	0.5	6	165.23 ± 35.85##	46.83 ± 15.91##

Ovalbumin solution was nasally administered to sensitized guinea pigs to induce nasal blockade, which was evaluated by the measurement of the specific airway resistance (sRaw). Airway response is the value of the percentage increase in sRaw after 10 min and after 1 h. Each value is presented as the mean ± S.E.M. of six mice.

P* < 0.05, *P* < 0.01, ## < 0.01

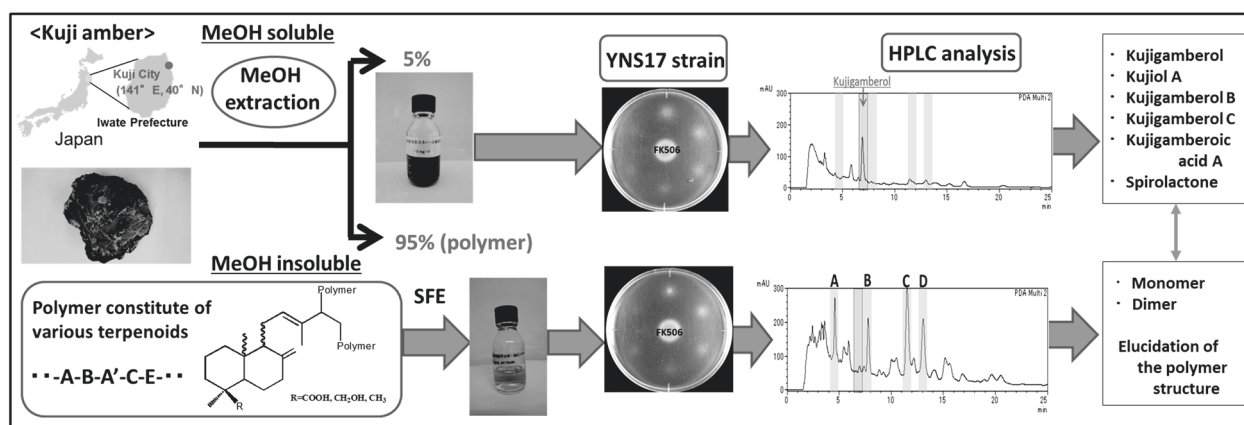


Fig. 6 Perspective study of Kuji amber and natural products discovery. Fusion of chemical biology and geochemistry

anti-allergy mechanisms of kujigamberol and MEKA may explain the potent anti-allergy activity of MEKA.

Although we have analyzed the 5% alcohol-soluble fraction of Kuji amber, 95% of the alcohol-insoluble fraction (the polymer portion) has not yet been examined (Fig. 6). The question therefore remains if novel compounds such as kujigamberol, kujiol A, kujigamberol B, C, and kujigamberoic acid A are degradation products of the polymer. We have attempted to achieve stable degradation of the polymers of Kuji amber by using supercritical fluid extraction (SFE) instead of GC-MS. Our preliminary results indicated that the greater yields of MeOH extract were obtained by SFE (MeOH) and biological activity against the YNS17 strain was observed. In addition, the HPLC analytical pattern was different from that of MEKA and novel degradation products were expected. It may be possible to show the tentative polymer structure of Kuji amber (Fig. 6). Furthermore, novel compounds such as kujigamberol C and kujigamberoic acid A were isolated from MEKA by the modified isolation procedure. Thus, then new isolation method, as well as new biological activity-guided fractionations are expected to yield other novel compounds.

Cosmetics, including MEKA, were placed on the market in 2015 to activate the local area around Iwate prefecture and to assist in the east Japan earthquake disaster reconstruction. In addition, the potent anti-allergy activity of MEKA is expected to be of use in various commodities, such as sprays, scented candles, and furniture coatings, etc. Indeed, kujigamberol and its analogs have been in development as new anti-allergic drug candidates. Several types of mutant yeasts, including Ca^{2+} -signal transduction (YNS17 strain) and rare natural sources such as ambers and/or edible wild plants have resulted in the production of various novel natural products that are beneficial to humans. In summary, Kuji amber is a splendid gift from an ancient period to modern humankind.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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