HOKKAIDO UNIVERSITY

Title	Zincmethylphyrins and coproporphyrins, novel growth factors released by Sphingopyxis sp., enable laboratory cultivation of previously uncultured Leucobacter sp through interspecies mutualism
Author(s)	Bhuiyan, Mohammad Nazrul Islam; Takai, Ryogo; Mitsuhashi, Shinya et al.
Citation	Journal of antibiotics, 69(2), 97-103 https://doi.org/10.1038/ja.2015.87
Issue Date	2015-08-26
Doc URL	https://hdl.handle.net/2115/61410
Type	journal article
File Information	JA69_97-103.pdf

Hokkaido University Collection of Scholarly and Academic Papers : HUSCAP

Zincmethylphyrins and coproporphyrins, novel growth factors released by *Sphingopyxis* sp., enable laboratory cultivation of previously uncultured *Leucobacter* sp. through interspecies mutualism

Mohammad Nazrul Islam Bhuiyan,[1] Ryogo Takai,[1] Shinya Mitsuhashi,[1] Kengo Shigetomi,[1] Yasuhiro Tanaka,[2] Yoichi Kamagata,[1] and Makoto Ubukata[1]*

[1]Division of Applied Bioscience, Graduate School of Agriculture, Hokkaido University, Kita-9, Nishi-9, Kita-ku, Sapporo, 060-8589, Japan.

[2]Department of Environmental Sciences, Faculty of Life and Environmental Sciences, University of Yamanashi, 4-4-37, Takeda, Kofu, Yamanashi 400-8510, Japan.

*To whom correspondence should be addressed;
E-mail: m-ub@for.agr.hokudai.ac.jp

Abstract

We have identified coproporphyrins including structurally new zincmethylphyrins I and III as growth factors A-F for the previously uncultured bacterial strain, *Leucobacter* sp. ASN212, from a supernatant of 210 liters of *Sphingopyxis* sp. GF9 culture. Growth factors A-F induced significant growth of strain ASN212 at the concentrations of picomolar to nanomolar which would otherwise be unculturable in liquid medium or on agar plate. More interestingly, we found that the growth factors functioned as self-toxic compounds for the growth-factor producing strain GF9 at the picomolar to nanomolar levels. As a variety of bacteria could potentially produce coproporphyrins, our findings suggest that these compounds function as a novel class of signal molecules across a boundary at phylum level in the complex bacterial communities.

INTRODUCTION

We have about 10,000 bacterial species in hand but numerous numbers of organisms have eluded laboratory cultivations. Why do most bacteria refuse to grow on laboratory media? This is a profound question many microbiologists and chemists have been trying to answer for a century[1]. To date, several bacteria are known to require growth-promoting factors or specific diffusible components supplied by a neighbor or partner bacterium in the same environment[2-10]. In our previous studies, we found that the supernatant of *Sphingopyxis* sp. GF9 isolated from an activated sludge significantly stimulated the growth of uncultured bacteria or bacterial organisms, which otherwise grew very poorly, including *Catellibacterium nectariphilum* AST4^T within the class *alpha-Proteobacteria*[4, 11].

Here we report novel porphyrin-type growth factors produced by strain GF9 that induce significant proliferation of a previously uncultured (here referred to as “uncultured”) actinobacterial strain, *Leucobacter* sp. ASN212 at picomolar to nanomolar levels. Even more surprisingly, the ASN212 growth factors showed self-toxicities against the growth-factor-producing bacterium, strain GF9 at picomolar to nanomolar concentrations. Co-culture experiments of strain GF9 with strain ASN212 indicated that strain ASN212 helps the survival of strain GF9 in the long-term culture. These findings imply another aspect of these growth factors to maintain a primitive mutualism existing in the bacterial consortium.

Figure 1 illustrates a growth-factor transfer network and prospective role of the ASN212 growth factors investigated in the present work. The results of this study imply that these coproporphyrins are a new class of signal molecules, which are transferred among a variety of bacteria in nature. Fig. 1

RESULTS

Interspecies signaling between cultured strain GF9 and uncultured strain ASN212 or ASTN45

To address the nature of the growth stimulation, we isolated two previously uncultivated organisms that require the diffusible growth factors from strain GF9, namely strain ASN212 related to *Leucobacter* sp. within the phylum *Actinobacteria* and strain ASTN45 related to *Bosea* sp. within the class *alpha-Proteobacteria* from activated sludge. These strains did not show significant growth in NPB broth, a nutrient-rich medium that should suffice for bacterial cell growth, but the growth was clearly enhanced by the addition of strain GF9 supernatant (Supplementary Table 1, Supplementary Figures 1, 2a and 2b). The growth of strain ASN212 was stimulated not only by the supernatant of strain GF9 but also by those of different species, namely *Sphingomonas macrogoltabidus*, *Sphingomonas sanguinis*, *Sphingomonas mali*, *Sphingobium chlorophenolica*, *Sphingopyxis terrae* and *Novosphingobium rosa*, strongly suggesting that interspecies growth factor transfer takes place across a variety of species even at phylum level. Beside the ASN212 growth factor, strain GF9 secreted two other different types of growth factors, ASTN45 growth factor and AST4^{T} growth factor (Figure 1 and Supplementary Table 1).

We first focused on the identification of ASN212 growth factor, because strain GF9 and strain ASN212 were different species at phylum level and ASN212 growth factor seemed to be relatively stable. In a two-compartment co-culture[12] of strain GF9 and strain ASN212, permeable growth factors produced by strain GF9 in an outer chamber passed through the membrane and stimulated the growth of ASN212 in an inner chamber as shown in Figure 2. Strain ASN212 did not grow in a pure culture (ASN212 single culture), whereas its cell density has increased from 96 h in the co-culture with strain GF9 (ASN212 co-culture). Strain GF9 grew well till 96 h in a pure culture, however, its cell density has continuously decreased after 96 h (GF9 single culture). In the co-culture with strain ASN212, the growth profile of strain GF9 was very unique which indicated secondary log phase after first log phase followed by first stationary phase (GF9 Fig. 2

co-culture), and the growth of strain GF9 after 96 h was approximately synchronized with that of strain ASN212 (ASN212 co-culture).

Isolation of ASN212 growth factors A, B, C, D, E and F

To identify ASN212 growth factors, we first tested the efficacy of strain GF9 supernatant to enhance the growth of strain ASN212 in NPB media. The supernatant induced significant growth of strain ASN212 with flocculation of the microbial cells, making it difficult to evaluate the efficacy in a dose-dependent manner (Supplementary Figure 1). A characteristic flocculation phenotype of strain ASN212 cells stimulated by ASN212 growth factors is shown as a magnified image of the flocks in Supplementary Figure 2c. The bioassay we established, however, allowed the easy and rapid detection of the active fractions by measuring OD_{595} at equivalent concentration (EC = dried fraction weight / culture volume of strain GF9) in a 48-well plate with a microplate reader.

Productivity of the growth factor was increased as much as around fivefold by optimizing the culture conditions of strain GF9 by using 5-L mini-jar fermentor (final conditions for 5-L mini-jar: tryptone peptone (Becton, Dickinson and Company, Sparks, MD, USA), 5.0 g L^{-1}; yeast extract, 3.0 g L^{-1}; 35 ^{o}C; 700 rpm; 72 h). The culture conditions used in the 100-L stirred-tank bioreactor were determined based on the above conditions. The culture conditions in the bioreactor and the isolation procedure of ASN212 growth factors from the strain GF9 supernatant are described in detail in Experimental Procedure, and illustrated in Figure 3 and Supplementary Figure 3. The final yields of growth factors **A**-**F** were 0.3 mg to 3.0 mg from 210 litters of the culture broth of strain GF9. The UV-visible spectra of these growth factors showed the Soret bands at 403 (**A**), 403 (**B**), 400 (**C**), 392 (**D**), 403 (**E**), 400nm (**F**) and Q-bands at 550 to 570 nm, suggesting that these compounds are porphyrins or related molecules (Figure 4).

Fig. 3

Structure identifications of growth factors A, B, C, D, E and F

Fig. 4

Although these porphyrins were very slightly soluble in any solvents at the concentration suitable for NMR measurement, growth factors **C** and **F** were soluble in dimethyl sulfoxide (DMSO)-d_6 and MeOH-d_4, respectively.

To achieve structure determination of growth factor **F**, we first attempted to assign ^{1}H and ^{13}C NMR of growth factor **C** that seemed to be closely related to growth factor **F**. ^{1}H and ^{13}C NMR assignments of growth factors **C** and **F** were made from the 2D NMR

analysis including COSY, ROESY, HSQC and HMBC (Supplementary Figures 4–6). Molecular formula of growth factor **C** was determined to be $C_{36}H_{36}O_8N_4Zn$ by ESI-MS analysis, *m/z* 715.1766 $[M–H]^-$. The numbering of the carbon atoms is shown in both Figure 4 and Supplementary Figure 4a. ^1H and ^{13}C NMR assignments of growth factor **C** are indicated in Supplementary Table 2. ^1H-^1H COSY correlations showed δ_H 4.32 (3^1-H) and 3.16 (3^2-H) ppm. ^1H-^1H ROESY correlations were observed among characteristic meso-proton [δ_H 10.08 (5-H and 10-H), 10.35 (15-H), 10.05 (20-H)], methyl proton [δ_H 3.63 (2^1-H), 3.66 (7^1-H), 3.66 (12^1-H), 3.64 (18^1-H)], and side chain proton [δ_H 4.41 (3^1-H), 3.31 (3^2-H), 4.39 (8^1-H), 3.20 (8^2-H), 4.40 (13^1-H), 3.20 (13^2-H), 4.40 (17^1-H), 3.20 (17^2-H)] as shown in Supplementary Figures 4b and 5f. ^1H-^{13}C HSQC, ^1H-^{13}C HMBC analysis was also needed to assign ^{13}C NMR of growth factor **C** whose HMBC correlations are shown in Supplementary Figures 4c and 5e. ^1H-^{15}N HMBC spectra of growth factor **C** indicated a broad singlet peak at δ_N 200 ppm correlated with meso-protons at 5-H, 10-H, 15-H and 20-H (Supplementary Figure 5g). Growth factor **C** was unambiguously identified by direct comparison with zincphyrin[13], which was synthesized from coproporphyrin III and $Zn(OAc)_2$ as described in Experimental Procedure.

LC-ESI-MS analysis of growth factor **F** in negative mode showed quasi-molecular ion, *m/z* 729 $[M–H]^-$ with *m/z* 731 $[M+2–H]^-$, and 733 $[M+4–H]^-$ suggesting that the compound was a zinc-containing porphyrin having stable isotopes,^{64}Zn, ^{66}Zn, and ^{68}Zn as in the case of zincphyrin (**C**). The molecular formula was deduced to be $C_{37}H_{38}O_8N_4Zn$ from the high-resolution ESI-MS data, *m/z* 729.1924 $[M–H]^-$ (Table 1). The difference between zincphyrin (**C**) and **F** in molecular formula was CH_2 (14 mass units), which suggested that growth factor **F** was a methyl ester of zincphyrin (**C**). The singlet peak at δ_H 3.60 of growth factor **F** was assigned to the ester methyl protons. HSQC of growth factor **F** indicated that the carbon signal at δ_C 50.8 was the ester methyl carbon (Supplementary Figure 6d). The ester carbonyl C-3^3 at δ_C 174.3 was correlated with the methyl proton at δ_H 3.60 in the HMBC spectrum. The C-3^3 carbon was correlated with C-3^1 methylene proton at δ_H 4.41, which was correlated with C-2 carbon at δ_C 136.3. The C-2 carbon was correlated with the C-20 meso-proton at δ_H 10.08 as shown in Supplementary Figures 4f and 6e. The COSY, ROESY (Supplementary

Figures 4e, 6c, and 6f) and HSQC data (Supplementary Figure 6d) supported the assignment of growth factor **F** whose ^{1}H and ^{13}C NMR assignments are shown in Supplementary Table 2. ^{1}H-^{15}N HMBC spectra of growth factor **F** indicated a broad singlet peak at δ_N 193.5 ppm correlated with meso-protons at 5-H, 10-H, 15-H and 20-H (Supplementary Figure 6g). The structure of growth factor **F** was depicted in Figure 4, and we named this new compound "zincmethylphyrin III". Growth factors **A**, **B** and **D** were identified as zinc coproporphyrin I[14], coproporphyrin I and coproporphyrin III, respectively, by comparing with their authentic samples in terms of HPLC retention time, UV-visible spectral pattern, ESI-MS and finally growth activities toward strain ASN212 (Figure 4, Table 1). Table 1

Growth factor **E** was detected as the only one isomer of growth factor **F**, which indicated the identical molecular formula of $C_{37}H_{38}O_8N_4Zn$ (Table 1). As in the case of relationship between zincphyrin (**C**) and zincmethylphyrin III (**F**), growth factor **E** was estimated to be the monomethyl ester of zinc coproporphyrin I (**A**). If growth factor **E** is a monomethyl ester of **A**, only one isomer is possible, because **A** has C_4 rotational symmetry. All data of growth factor **E** coincided with those of the monomethyl ester of authentic zinc coproporphyrin I, which was synthesized by esterification of commercially available coproporphyrin I with TMS diazomethane followed by treatment with $Zn(OAc)_2$ as described in Experimental Procedure. We named the structurally new growth factor **E** "zincmethylphyrin I" (Figure 4).

Growth-stimulating activities of growth factors A, B, C, D, E and F

The minimum effective concentrations (MECs) of zincphyrin (**C**), coproporphyrin III (**D**), zincmethylphyrin III **(F**) and the minor growth factors, zinc coproporphyrin I (**A**), coproporphyrin I (**B**) and zincmethylphyrin I (**E**), were determined by measuring the dry weight of bacterial cells from a 70-ml culture of strain ASN212, which was stimulated by each individual growth factor (Table 1). The growth-stimulating activity of zincphyrin (**C**) was most evident with a MEC value of 14 pM followed by coproporphyrin III (**D**) whose MEC was 1.5 nM. Zincmethylphyrin III (**F**) was 343-fold less potent than zincphyrin (**C**), and its MEC was 4.8 nM. As zincmethylphyrin I (**E**) and zinc coproporphyrin I (**A**) were found to be less potent growth factors whose MECs were 9.6 nM and 20 nM, respectively, and the MEC of zinc-free coproporphyrin I (**B**) was 38 nM,

a zinc ion and the relative position of the four propanoic acid groups in coproporphyrin III (**D**) seemed to be essential for the tremendous efficacy of zincphyrin (**C**) as a growth factor for an *Actinobacteria*, strain ASN212 (Table 1).

Strain GF9 produces a series of coproporphyrins and zinc coproporphyrins with variable flanking side chains that drastically affect the MEC values. Of the six growth factors identified, zincphyrin (**C**) was by far the most potent growth factor and the most abundant porphyrins secreted by strain GF9 (Experimental Procedure, Supplementary Figure 3c and d), whereas other coproporphyrins were capable of stimulating the growth of strain ASN212 at nanomolar concentrations under the standard laboratory conditions, demonstrating that all coproporphyrins represent a new class of growth factors for this *Actinobacteria* (Table 1).

Growth-stimulating activities of other porphyrins for strain ASN212

We then tested a panel of synthetic and commercial porphyrins including zinc coproporphyrin I, zincphyrin, zincmethylphyrin I, and commercially available hematin, hemin, hematoporphyrin, protoporphyrin IX, coproporphyrin I dihydrochloride, coproporphyrin III dihydrochloride, mesoporphyrin IX dihydrochloride, coproporphyrin I tetramethyl ester, coproporphyrin III tetramethyl ester, vitamin B_{12}, chlorine e6, 5,10,15,20-tetraphenyl-21H,23H-porphine, and 29H,31H phthalocyanine to explore the generality of porphyrin as growth factor for strain ASN212. Synthetic zinc coproporphyrin I, zincphyrin, and zincmethylphyrin I showed exactly the same activities as natural growth factors **A**, **C**, and **E**, respectively, while coproporphyrin I dihydrochloride and coproporphyrin III dihydrochloride stimulated growth of strain ASN212 at comparable MECs to those of natural coproporphyrin I (**B**) and coproporphyrin III (**D**), respectively. Coproporphyrin III tetramethyl ester showed significant growth stimulation at 70 nM, which was 47 times higher MEC of coproporphyrin III (**D**), and hemin and hematin showed far less growth promoting effects whose MECs were 1.2 and 2.4 μM, respectively (Table 1 and Supplementary Table 3). The rest of commercial porphyrins did not stimulate the growth of strain ASN212 even at the concentration of 1 mg ml^{-1}.

Growth factors A, B, C, D, E and F exhibit self-toxicities against strain GF9

In addition to above studies, we examined the pervasive effects of coproporphyrins on the

growth of strains ASTN45, AST4^T and GF9. Although both strains ASTN45 and AST4^T were not affected, coproporphyrins secreted by strain GF9 significantly inhibited the growth of strain GF9 itself. The results are surprising and different from what we know about previously reported growth factors. It appeared that major growth factors, zincphyrin (**C**), coproporphyrin III (**D**), zincmethylphyrin III (**F**), and minor growth factors, zincmethylphyrin I **(E**), zinc coproporphyrin I (**A**), and coproporphyrin I **(B**), completely inhibited the growth of strain GF9 at 10×MEC to 15×MEC; 0.1, 15, 48, 144, 293 and 573 nM, respectively, but the concentrations at which the growth of strain ASN212 was significantly stimulated and the order of efficacy as growth factor was exactly the same: **C** > **D** > **F** > **E** > **A** > **B** (Table 1). The half-maximal inhibitory concentration (IC_{50}) of zincphyrin (**C**) against strain GF9 was 49 pM (Figure 5). Fig. 5

DISCUSSION

Our present research has broken new ground by demonstrating that coproporphyrins produced by strain GF9 or commercially available porphyrins could have an impact on the growth of uncultured bacterial strain in laboratory conditions. To our knowledge, this is the first finding that coproporphyrins stimulate bacterial growth at the picomolar to nanomolar level. This research raises exciting possibilities for novel mechanisms on interactions of coproporphyrins with other bacteria that are very fastidious to cultivate or have yet to be cultured. Our findings indicate that not only this particular *Sphingopyxis* sp. GF9 but also other microbes may produce coproporphyrins, as the growth of *Leucobacter* sp. ASN212 is stimulated by the other microorganisms, namely, the family *Sphingomonadaceae* in the phylum *Proteobacteria* (Figure 1). The other striking feature of our study is that strain ASA212 in the phylum *Actinobacteria* required growth factor produced by an organism that is phylogenetically quite distant, indicating that interspecies growth factor transfer occurs transcending the boundaries of phylum level. As reported in previous work on secretion of coproporphyrin III from *Corynebacterium aurimucosum* and *Microbacterium oxydans*[15] and zincphyrin from *Streptomyces* sp.[13], not only Gram-negative but also various Gram-positive bacteria may secrete these coproporphyrin derivatives.

Commensalisms are well known in biology, but relations between different prokaryotic species are not well-understood and remain largely unclear. Initially, we supposed that the relation between strains GF9 and ASN212 is a kind of commensalism,

as strain GF9 grows well independently. Thus, it seemed that strain GF9 altruistically provided coproporphyrins to the partner organism.

However, considering that these coproporphyrins were toxic to strain GF9 at picomolar to nanomolar levels, the uncultured strain ASN212 might help directly strain GF9 by up-taking or cleaning up the self-toxic coproporphyrins from the vicinity of the producing strain GF9. Flocculation of strain ASN212 observed after stimulating with these ASN212 growth factors might lead to reducing their toxicities against strain GF9. The co-culture experiments supported this possibility and implied that strains GF9 and ASN212 are dependent on each other for their growth and survival in the environments (Figures 1 and 2).

In this context, microbial commensalism is not the most appropriate wording to describe the relationship, and our findings suggest that a primitive obligate mutualism exists in the bacterial communities. More importantly, we could assume that coproporphyrins function as global signal molecules that sustain the complex microbial communities as a network system. In turn, it is very likely that a number of organisms can elude cultivation without receiving this secret elixir. We therefore propose here a general strategy using these porphyrins in lieu of siderophore[5,16] to access new microorganisms. This approach could expand drug discovery efforts, because secondary metabolites from the phylum *Actinobacteria* have provided new antibiotics, therapeutic agents and chemical probes to uncover the function of gene products related to various diseases[17]. The global effect of these compounds in terms of microbial ecology, biochemistry, and genetics are currently underway.

EXPERIMENTAL PROCEDURE

General experimental procedures

Water was Milli-Q water dispensed through 0.22-micron filter with 18.2 MΩ・cm conductivity unless otherwise noted. Tryptone peptone (Becton, Dickinson and Company, Sparks, MD, USA), trimethylsilyldiazomethane (0.6 M in *n*-hexane, Tokyo Chemical Industry Co. Ltd., Tokyo, Japan) and triethylamine (Wako Pure Chemical Industries Co., Ltd (Wako), Osaka, Japan), and Centriprep YM-50 (Millipore Ireland B.V., Cork, Ireland) were purchased.

Bacterial strains and media

Sphingopyxis sp. GF9 and three uncultured strains *Leucobacter* sp. ASN212, *Bosea* sp. ASTN45, *Catellibacterium nectariphilum* AST4^T were used in this study, all of which were stored in 25% glycerol with NPB medium consisting of 10.0 g tryptone peptone, 2.0 g yeast extract (Becton, Dickinson and Company), 1.0 g $MgSO_4 \cdot 7H_2O$, 1.0 g KH_2PO_4, 5.0 g D-glucose in 1.0 liter of Milli-Q H_2O, pH 7.0 at –83 ^oC for further study.

Isolation of uncultured microbial strains ASN212 and ASTN45

Two previously uncultured microbial strains, ASN212 related to the genus *Leucobacter* within the phylum *Actinobacteria* and ASTN45 related to the genus *Bosea* within the class *alpha-Proteobacteria*, were isolated from activated sludges treating municipal wastewater and industrial effluent from a food company, respectively. Strain GF9 was cultivated in 100 ml of NPB medium and incubated for 3 days at 30 °C, and the resultant culture was centrifuged at 15,000 r.p.m for 10 min. The supernatant was added to NPB medium at final concentration of 10%. This medium (NPBGF9) was used as an isolation medium for uncultured microbes requiring growth factors. An aliquot of activated sludge sample diluted to 10^{-4} with sterilized water was inoculated on a 1.5% agar plate. After incubation at 30 °C for 4 days, colonies that emerged on the agar plates were picked and isolated. The requirement for supernatant of strain GF9 was verified by comparing growth between the NPBGF9 agar plate and NPB agar plate. As a result, strains ASN212 and ASTN45 were obtained as two microbial strains indicating significant differences between growth on NPBGF9 and NPB agar plates or in NPB liquid media with and without GF9 supernatant (Supplementary Table 1, Supplementary Figures 1 and 2).

Co-culture of *Sphingopyxis* sp. strain GF9 and *Leucobacter* sp. strain ASN212

A dialyzing co-culture of strain GF9 and ASN212 was performed as previously described[12]. Briefly, using a Centriprep YM-50 sterilized at 105^{o}C for 10 min, strain GF9 was inoculated into 5 ml of NPB medium in the outer chamber, and the test microbial strain ASN212 was inoculated into the inner chamber containing 5 ml of NPB medium. For each, initial OD_{595} was 0.01. The filter unit was incubated for 9 days at 30°C with shaking at 160 r.p.m. The pure liquid culture from a single cell as a colony of strain GF9 or strain ASN212 was performed in a Centriprep YM-50 without the inner chamber. Aliquots (200 μl) from each chamber were withdrawn at 0, 48, 96, 144, 168, 184, 191, and 206 h, and OD_{595} of each sample was measured to monitor the growth of strain GF9

or strain ASN212.

Cultivation of strain GF9 in 100-L stirred-tank bioreactor

Strain GF9 was cultivated in a 100-Liter (L) stirred-tank bioreactor (MARUBISHI MPF-100L, BE Marubishi, Tokyo, Japan) at a laboratory of Graduate School of Engineering, Hokkaido University, Japan. The bioreactor was filled with 70 liters of modified NPB media; differences were the amount of tryptone peptone (5g L^{-1}) in reverse osmosis water (RO-44P-B, Tohzai Chemical, Osaka, Japan) instead of Milli-Q water, being based on the culture conditions optimized by 5-L mini jar fermenter as described in Results. The medium was then sterilized by keeping the bioreactor in an autoclave mode for 15 min at 121 ^{o}C in 15 lbs. After autoclaving, the bioreactor was allowed to cool and 0.06% antifoam SI (Antifoam SI, Wako) suspension was added to reduce air bubble formation during the culture. The bacterial inoculum was prepared in three steps. Twenty microliters of strain GF9 stock solution were inoculated into 2.0 ml of NPB medium in culture tube, and incubated at 30 ^{o}C, 170 r.p.m for 24 h. One milliliter of the culture was inoculated into a 100 ml of NPB medium in a 300-mL baffled Erlenmeyer flask, and cultured under the above conditions. Likewise, each 5-ml aliquot from the second preculture was inoculated in two 2-L baffled Erlenmeyer flasks containing 500 ml of NPB medium, which was allowed to culture under the same conditions for 48 h. The resultant 700 ml of the GF9 culture was poured into 70 liters of NPB media and allowed to grow at 35 ^{o}C and 500 r.p.m for 78 h. The bioreactor was aerated at a constant airflow of 1 vvm (air vol. flow/unit of liquid vol. of medium/min). Samples from the bioreactor were withdrawn at intervals of 10, 22, 27, 48, 60, 70 and 78 h and optical density (OD_{595} or OD_{660}) was measured to monitor the growth of strain GF9. We made three batches of 100-L culture to obtain 210 liters of a culture broth. The extraction and isolation of ASN212 growth factors **A-F** were achieved as follows.

Extraction and isolation of ASN212 growth factors A-F

The 210 liters of GF9 culture broth were centrifuged for 20 min at 10,000*g*, and the supernatant (160 liters) was extracted with the same volume of *n*-BuOH four times. The residual mixture was then transferred into a separatory funnel to collect the aqueous layer. The aqueous layer was concentrated *in vacuo* to give 900 g of dried material and subjected to MeOH precipitation by adding 2 liters of MeOH (three times). The whole

solution was filtrated through a filter paper (ADVANTEC, ø 125 mm, Advantec Toyo Roshi, Tokyo, Japan) to afford a MeOH-soluble part. The MeOH-soluble fraction was further concentrated *in vacuo* to give 540 g of the residue, which was applied to 10 liters of a Diaion HP-20 (Mitsubishi Kasei, Tokyo, Japan) column. The column was eluted successively with water, 50% MeOH and MeOH, and the active fraction, which was determined by the bioassay described in the next section, was eluted with MeOH. The active eluate was concentrated under reduced pressure to give 25 g of a solid material, which was re-dissolved in 20 ml of MeOH and applied to a Sephadex LH-20 (2 liters, GE Healthcare Bio-Sciences, Uppsala, Sweden) column packed with MeOH-H_2O (8:2). The LH-20 column chromatography eluting with the same solvent system afforded the active fraction, which was concentrated *in vacuo* to give 125 mg of a dark-reddish solid. The solid was subjected to preparative HPLC (Mightysil RP-18 GP Aqua 20ø×250 mm, 5μm, Kanto Chemical, Tokyo, Japan, MeCN (0.1% AcOH)/H_2O (0.1% AcOH), 53:47) to give six dark-reddish solids (ca. 20 mg in total). The active samples were finally purified by using an analytical HPLC column (Mightysil RP-18 GP Aqua 4.6ø×250 mm, 5μm, Kanto Chemical, MeCN (0.1% AcOH)/H_2O (0.1% AcOH), 53:47) to yield six growth factors **A**-**F**; 0.8 mg of **A**, 0.3 mg of **B**, 3.0 mg of **C**, 1.2 mg of **D**, 0.8 mg of **E**, and 2.0 mg of **F**, respectively.

Bioassay for growth stimulating activity of chromatographic fractions

Growth activities of strain GF9 supernatant, its extractive fractions, and individual chromatographic fractions for strain ASN212 were tested in uniformly designed bioassay. Each sample dried with a centrifugal vaporizer (EYELA CVE-200D, Tokyo, Japan) was reconstituted to equivalent concentration (EC = dried fraction weight / culture volume of strain GF9) with H_2O. These experiments were performed utilizing standard 48-well micro-titer culture plate and 100 μl of each sample reconstituted to EC was added to each well followed by inoculation of 300 μl of NPB-ASN212, a mix of 20 μl of strain ASN212 stock solution and 18 ml of NPB medium. Equivalent concentration of strain GF9 supernatant and NPB medium were used as positive and negative controls, respectively. Strain GF9 supernatant and each sample were sterilized by filtration (0.2 μm Cellulose Acetate, DISMIC-13CP, Advantec Toyo Roshi, Tokyo, Japan) unless otherwise noted. The culture plates were incubated at 170 r.p.m and 28 ^{o}C for 48 h in an incubator. Bacterial cell growth of strain ASN212 was monitored by OD measurement at 595 nm.

To confirm the results, each assay was replicated at least three times.

NMR and ESI-MS measurements

NMR spectra of zincmethylphyrin III (**F**) in MeOH-d_4 were measured with a JEOL JNM-ECA600 II spectrometer using an UltraCool probe (JEOL, Tokyo, Japan) for ^{1}H, ^{13}C, ^{1}H-^{1}H COSY, HSQC, HMBC and a JNM-ECZR500 spectrometer using a Royal probe for ROESY and ^{1}H-^{15}N HMBC. NMR spectra of zincphyrin (**C**) in DMSO-d_6 were measured with a Bruker AMX-500 (Bruker, Billerica, MA, USA). Chemical shift was reported in δ ppm using tetramethylsilane as the internal standard, and coupling constants (J) were given in hertz. LC-ESI-MS and ESI-MS spectra were recorded on LTQ-Orbitrap XL (Thermo Fisher Scientific, Waltham, MA, USA) with or without a Paradigm MS2 (Michrom BioResources, Auburn, CA, USA) equipped with an InertSustain C18 column (GL Science, Tokyo, Japan).

Synthesis of zinc coproporphyrin I and its identification as growth factor A

Zinc coproporphyrin I was synthesized by a previously described method[14]. Briefly, coproporphyrin I dihydrochloride (10.0 mg, 13.7 μmol) was dissolved in 4.1 ml of DMSO and mixed with an aqueous solution of $Zn(OAc)_2 \cdot 2H_2O$ (3.1 mg, 14.1 μmol) in 0.70 ml. The incorporation of Zn^{2+} into coproporphyrin I was confirmed by changes in its UV-visible spectrum and color. The reaction mixture was extracted by a solution of equal volumes of 10 mM AcOH and *n*-BuOH. Removal of the solvent from the organic layer under reduced pressure yielded 7.2 mg of zinc coproporphyrin I. The compound was identified as growth factor **A** by HPLC, UV-visible spectral pattern, color, ESI-MS, and growth promoting activity (Table 1 and Supplementary Table 3).

Synthesis of zincphyrin and its identification as growth factor C

Coproporphyrin III dihydrochloride (2.0 mg, 2.8 μmol) was dissolved in 0.81 ml of DMSO and mixed with 0.12 ml of an aqueous $Zn(OAc)_2 \cdot 2H_2O$ solution (0.61 mg, 2.8 μmol). The incorporation of Zn^{2+} into coproporphyrin III was confirmed by changes in the UV-visible spectrum and color. The reaction mixture was extracted by using an equal volume mixture of 10 mM AcOH and *n*-BuOH. The *n*-BuOH extract was evaporated *in vacuo* to yield 1.6 mg of a dark reddish compound reported as zincphyrin[13]. Growth factor **C** was identified as zincphyrin by HPLC, UV-visible spectral pattern, color,

ESI-MS, ^{1}H and ^{13}C NMR chemical shifts, and growth promoting activity (Table 1 and Supplementary Table 3).

Synthesis of zincmethylphyrin I and its identification as growth factor E

Coproporphyrin I dihydrochloride (10.0 mg, 13.7 μmol) was dissolved in a mixed solution of 22.9 μl (1 eq.) of trimethylsilyldiazomethane (ca. 0.6 M in *n*-hexane), 1.9 μl of triethylamine (1 eq.) in 400 μl of a mixture of hexane and MeOH (1:1). The resultant solution was stirred for 24 h at room temperature, until maximum consumption of the starting material was observed on TLC and analytical HPLC. The reaction mixture was evaporated to dryness and the crude methyl ester was dissolved in 4.1 ml of DMSO and mixed with 0.7 ml of an aqueous solution of $Zn(OAc)_2 \cdot 2H_2O$ (3.1 mg, 14.1 μmol). The incorporation of Zn^{2+} into the crude methyl ester was confirmed by changes in the UV-visible spectrum and color. The reaction mixture was extracted with an equal volume mixture of 10 mM aqueous AcOH and *n*-BuOH. The compound designated as zincmethylphyrin I was recovered from the organic layer and purified by HPLC. Growth factor **E** was identified as zincmethylphyrin I by HPLC, UV-visible spectral pattern, color, ESI-MS, and growth promoting activity for ASN212 (Table 1 and Supplementary Table 3).

Porphyrin-mediated growth induction of strain ASN212

Synthetic porphyrins including zinc coproporphyrin I (**A**), zincphyrin (**C**), zincmethylphyrin I (**E**), and 13 different commercial porphyrins were tested for growth of strain ASN212 in liquid culture. Commercial porphyrins used were hematin (Alfa Aesar, Lancashire, UK), hemin (Sigma-Aldrich, St. Louis, MO, USA), hematoporphyrin (Wako), protoporphyrin IX (Sigma-Aldrich), coproporphyrin I dihydrochloride (Sigma-Aldrich), coproporphyrin III dihydrochloride (Strem Chemicals, Newburyport, MA. USA), mesoporphyrin IX dihydrochloride (Sigma-Aldrich), coproporphyrin I tetramethyl ester (Wako), coproporphyrin III tetramethyl ester (Sigma-Aldrich), vitamin B_{12} (Sigma-Aldrich), chlorine e6 (Frontier Scientific, Inc., Logan, UT, USA), 5,10,15,20-tetraphenyl-21H,23H-porphine (Sigma-Aldrich) and 29H,31H phthalocyanine (Sigma-Aldrich) (Table 1 and Supplementary Table 3).

MECs of growth factors A-F and other porphyrins.

To determine the MECs of growth factors **A**, **B**, **C**, **D**, **E**, **F**, and synthetic or commercial porphyrins, each compound stocked as 0.1% DMSO solution was mixed with 70 ml of NPB medium in a 500-ml K-1 flask (K-Techno, Toyama, Japan) adjusting it to the final concentrations in a range from 0.01 ng ml^{-1} to 5μg ml^{-1}. In the autoclaved liquid culture medium containing each respective concentration of porphyrins, 100 μl of the stock solution of strain ASN212 was inoculated. All flasks were incubated at 28 ^{o}C for 2 days at 80 r.p.m. The bacterial cells of strain ASN212 were collected on a pre-dried filter paper (ADVANTEC 4A, ø 90 mm, Advantec Toyo Roshi) by vacuum filtration, and the filtrate was removed after 5 to 6 min. The residual solid and filter paper were washed with 5 ml of H_2O under reduced pressure and dried at 60 ^{o}C in a drying oven to reach a constant weight. The dry weight of residual solid was calculated by subtracting the constant weight of filter paper before use from that of the residual solid and filter paper. To convert the dry weight of residual solid into a concentration in the media, the weight of the residual solid was divided by 70 ml to give an exact concentration of dry cell weight shown as mg ml^{-1}. The 0.1% DMSO solution was used for negative control instead of a 0.1% stock solution of growth factor. The MEC was determined by paired t test ($p < 0.05$) for comparison of the dry cell weights with and without growth factor ($n = 3$ biological replicates).

Growth inhibitory activities of ASN212 growth factors A-F against strain GF9.

The growth inhibitory activities of growth factors **A-F** against strain GF9 were measured by a uniformly designed bioassay using 48-well micro-titer plates. The concentrations of each growth factor were adjusted to equivalent concentration (EC = final weight of each growth factor isolated / culture volume of strain GF9), and sequential concentrations of 0.5×MEC to 20×MEC with H_2O. To each well containing 100 μl of each sample, 300 μl of the NPB-GF9 solution freshly prepared from NPB media (18 ml) and strain GF9 stock solution (20 μl) were added. The 0.1% DMSO aqueous solution was used as a negative control instead of each sample. Each growth factor was sterilized by filtration (0.2 μm Cellulose Acetate, DISMIC-13CP, Advantec Toyo Roshi, Tokyo, Japan) unless otherwise noted. Each plate was incubated at 28 ^{o}C for 24 h at 170 r.p.m. in an incubator. The growth of strain GF9 was monitored by OD at 595 nm. To confirm the result, each assay was replicated at least three times. The IC_{50} value of each growth factor was calculated using a GraFit 7-IC_{50} practice software (Erithacus Software Ltd., West Sussex, UK).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

The NMR measurements were conducted at the Institute of Transformative Bio-Molecules, Nagoya University, Nagoya, JEOL Resonance Inc., Tokyo, and the GC-MS & NMR Laboratory, Faculty of Agriculture, Hokkaido University, Sapporo, Japan. We sincerely thank the Institute for Fermentation, Osaka (IFO) Japan for the financial support. This work was supported in part by Grant-in-Aid for Challenging Exploratory Research (JSPS No. 25660051).

References

1. Epstein, S. S. Uncultivated Microorganisms 1-208 Microbiology Monographs Vol.10, Springer-Verlag, Berlin, Germany (2009).
2. Kaeberlein, T., Lewis, K. & Epstein, S. S. Isolating "uncultivable" microorganisms in pure culture in a simulated natural environment. *Science* **296**, 1127-1129 (2002).
3. Ogita, N., Hashidoko, Y., Limin, S.H. & Tahara, S. Linear 3-hydroxybutyrate tetramer (HB4) produced by *Sphingomonas* sp. is characterized as a growth promoting factor for some rhizomicrofloral composers. *Biosci. Biotechnol. Biochem*. **70**, 2325-2329 (2006).
4. Tanaka, Y. *et al*. *Catellibacterium nectariphilum* gen. *nov*., sp. nov., which requires a diffusible compound from a strain related to the genus *Sphingomonas* for vigorous growth. *Int. J. Syst. Evol. Microbiol*. **54**: 955-959 (2004).
5. D'Onofrio, A. *et al*. Siderophores from neighboring organisms promote the growth of uncultured bacteria. *Chem. Biol*.**17**: 254-264 (2010).
6. Diarra, M.S. *et al*. Growth of *Actinobacillus pleuropneumoniae* is promoted by exogenous hydroxamate and catechol siderophores. *Appl. Environ. Microbiol.* **62**: 853-859 (1996).
7. Ameyama, M., Matsushita, K., Shinagawa, E., Hayashi, M. & Adachi, O. Pyrroloquinoline quinone: excretion by methylotrophs and growth stimulation for

microorganisms. *Biofactors* **1**, 51–53 (1988).

8. Isawa, K. *et al*. Isolation and identification of a new bifidogenic growth stimulator produced by *Propionibacterium freudenreichii* ET-3. *Biosci. Biotechnol. Biochem.* **66**: 679–681 (2002).
9. Mori, H. *et al*. Isolation and structural identification of bifidogenic growth stimulator produced by *Propionibacterium freudenreichii*. *J. Dairy Sci*. **80**: 1959–1964 (1997).
10. Nichols, D. *et al*. Short peptide induces an "uncultivable" microorganism to grow *in vitro*. *Appl. Environ. Microbiol*. **74**: 4889–4897 (2008).
11. Tanaka, Y., Hanada, S., Tamaki, H., Nakamura, K. & Kamagata, Y. Isolation and identification of bacterial strains producing diffusible growth factor(s) for *Catellibacterium nectariphilum* strain AST4^{T}. *Microbes Environ.* **20**, 110-116 (2005).
12. Guan, L. L., Onuki H. & Kamino, K. Bacterial growth stimulation with exogenous siderophore and synthetic N-acyl homoserine lactone autoinducers under iron-limited and low-nutrient conditions. *Appl. Environ. Microbiol.* **66**, 2797-2803 (2000).
13. Toriya, M. *et al.* Zincphyrin, a novel coproporphyrin III with zinc from *Streptomyces* sp. *J. Antibiot.* **46**: 196-200 (1993).
14. Horiuchi, K. *et al*. Isolation and characterization of zinc coproporphyrin I: a major fluorescent component in meconium. *Clin. Chem*. **37**: 1173-1177 (1991).
15. Yasuma, A. *et al*. Exogenous coproporphyrin III production by *Corynebacterium aurimucosum* and *Microbacterium oxydans* in erythrasma lesions. *J. Med. Microb.* **60**: 1038-1042 (2011).
16. Ling, L.L. *et al*. A new antibiotic kills pathogens without detectable resistance. *Nature* **517**: 455-459 (2015).
17. Ubukata, M. Agricultural Sciences for Human Sustainability (eds Hashidoko, Y. *et al*.) 58-61 (Kaiseisha Press, Otsu, Japan, 2012).

Table 1 Molecular formula of ASN212 growth factors **A**-**F** and their minimum effective concentrations (MECs) on growth stimulation of *Leucobacter* sp. ASN212.

Growth factors (from GF9)	Name	MEC[a] (nM)	Molecular formula	Molecular weight (MW)	*m*/*z* $[M-H]^-$ found	*m*/*z* $[M-H]^-$ calcd.
A	zinc coproporphyrin I	2.0×10	$C_{36}H_{36}O_8N_4Zn$	716	715.1776	715.1752
B	coproporphyrin I	3.8×10	$C_{36}H_{38}O_8N_4$	654	653.2637	653.2617

C	zincphyrin	1.4×10^{-2}	$C_{36}H_{36}O_8N_4Zn$	716	715.1766	715.1752
D	coproporphyrin III	1.5	$C_{36}H_{38}O_8N_4$	654	653.2630	653.2617
E	zincmethylphyrin I	9.6	$C_{37}H_{38}O_8N_4Zn$	730	729.1912	729.1908
F	zincmethylphyrin III	4.8	$C_{37}H_{38}O_8N_4Zn$	730	729.1924	729.1908

a. The MEC that induced statistically significant growth of strain ASN212 under the conditions described in Experimental Procedure; the order of efficacy was **C** > **D** > **F** > **E** > **A** > **B**.

Legend to Figures

Figure 1 Growth factor transfer network across different species. Growth factor supplied to uncultured *Leucobacter* sp. ASN212 from cultured *Sphingopyxis* sp. GF9 is shown as ASN212 growth factor. ASN212 growth factor not only stimulates proliferation of strain ASN212 but inhibits the growth of strain GF9 itself.

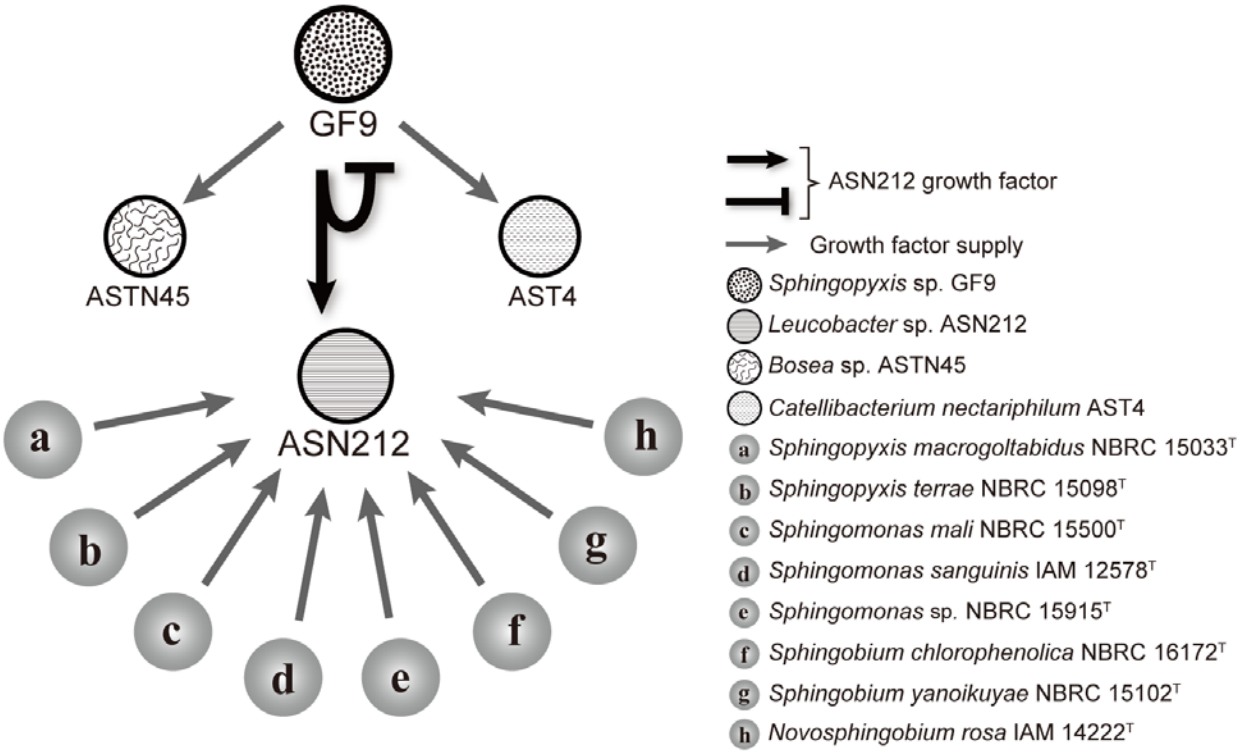
Figure 2 Co-culture of *Sphingopyxis* sp. GF9 and *Leucobacter* sp. ASN212. Using

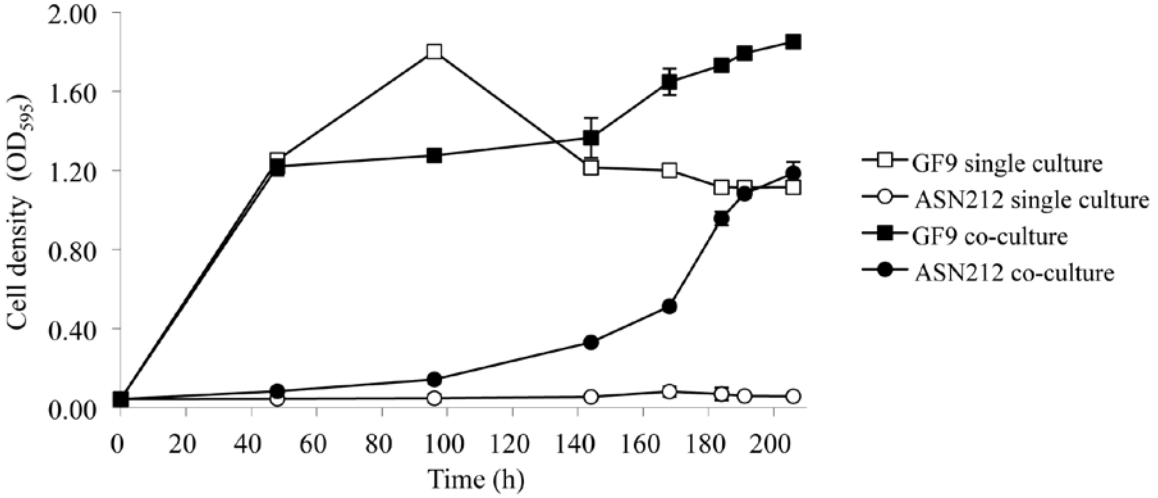
Centriprep-50, NPB medium in the inner chamber was inoculated with strain ASN212 (ASN212 co-culture), and NPB medium in the outer chamber was inoculated with strain GF9 (GF9 co-culture). Single culture of strain GF9 or strain ASN212 was performed in the outer chamber without the inner chamber (GF9 single culture or ASN212 single culture). Each bacterial cell growth was monitored by the absorbance at 595 nm of aliquot from each chamber (n = 3 biological replicates).

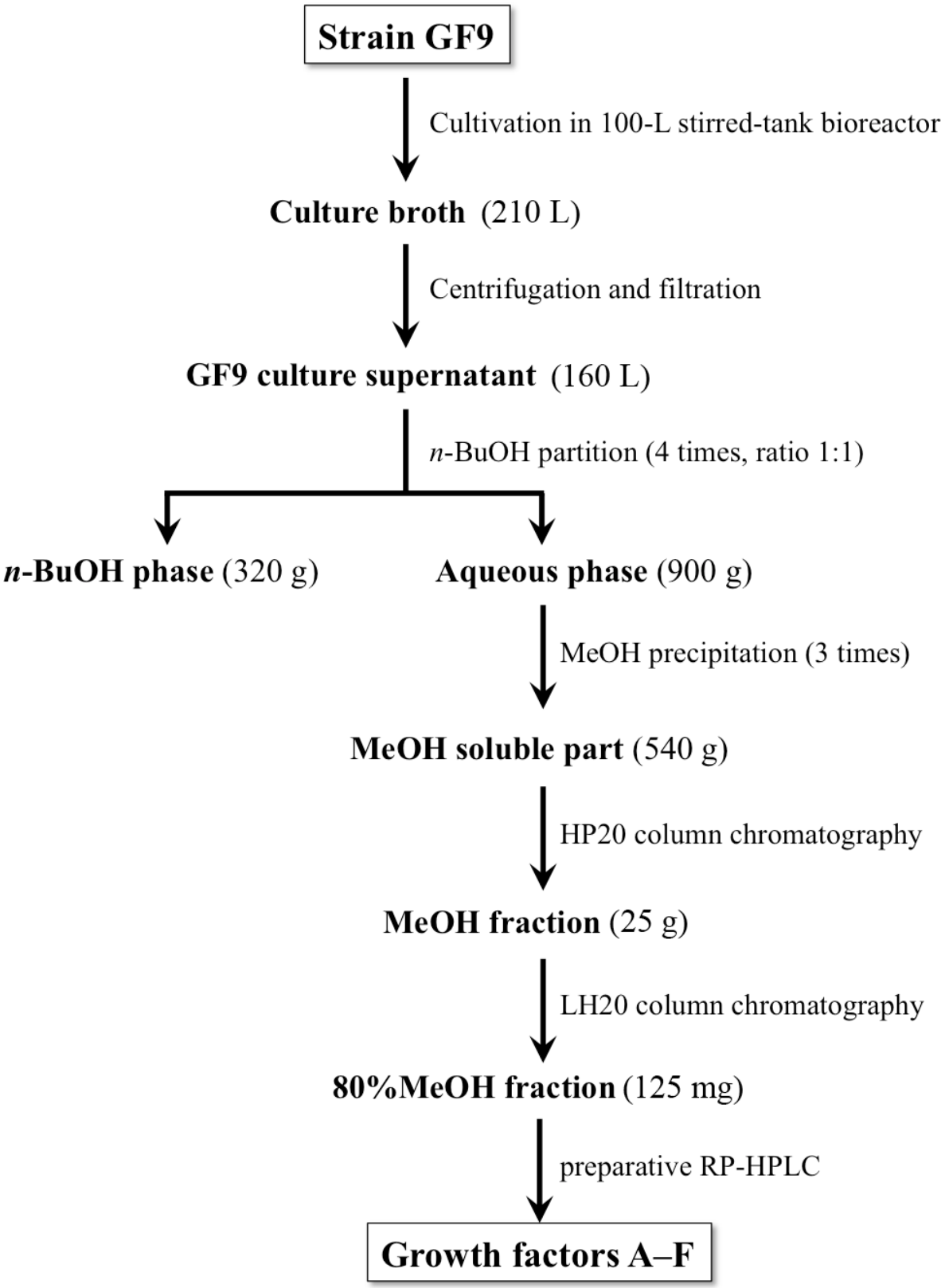
Figure 3 The entire isolation procedure of ASN212 growth factors **A**-**F**.

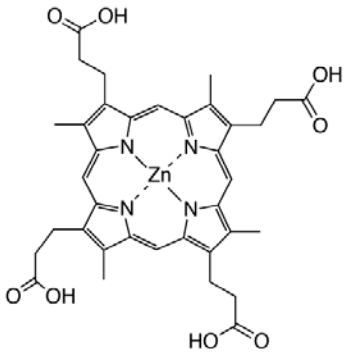
Figure 4 Structures of ASN212 growth factors **A**, **B**, **C**, **D**, **E** and **F** isolated from strain GF9 supernatant. Growth factors **A**-**F** were identified to be zinc coproporphyrin I (**A**), coproporphyrin I (**B**), zincphyrin (**C**), coproporphyrin III (**D**), zincmethylphyrin I (**E**), and zincmethylphyrin III (**F**), respectively. Growth factors **E** and **F** were structurally new compounds.

Figure 5 Growth-inhibitory activity of zincphyrin (**C**) against *Sphingopyxis* sp. GF9. The growth-inhibitory effect was determined by a uniformly designed bioassay using 48-well microplates. Each plate was incubated at 28 ^{o}C for 24 h at 170 r.p.m., and the growth of strain GF9 was determined by measuring OD_{595} (n = 3 biological replicates).

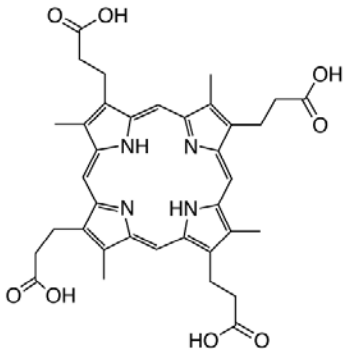

GF9
ASTN45
AST4
ASN212
a
b
c
d
e
f
g
h
ASN212 growth factor
Growth factor supply
Sphingopyxis sp. GF9
Leucobacter sp. ASN212
Bosea sp. ASTN45
Catellibacterium nectariphilum AST4
a Sphingopyxis macrogoltabidus NBRC 15033T
b Sphingopyxis terrae NBRC 15098T
c Sphingomonas mali NBRC 15500T
d Sphingomonas sanguinis IAM 12578T
e Sphingomonas sp. NBRC 15915T
f Sphingobium chlorophenolica NBRC 16172T
g Sphingobium yanoikuyae NBRC 15102T
h Novosphingobium rosa IAM 14222T


2.00
1.60
1.20
0.80
0.40
0.00
Cell density (OD595)
0
20
40
60
80
100
120
140
160
180
200
Time (h)
GF9 single culture
ASN212 single culture
GF9 co-culture
ASN212 co-culture

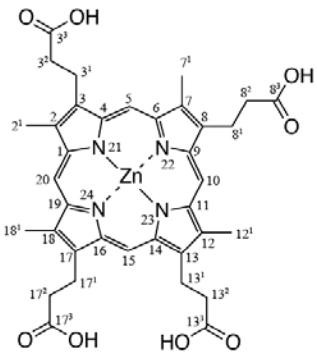

Strain GF9
Cultivation in 100-L stirred-tank bioreactor
Culture broth (210 L)
Centrifugation and filtration
GF9 culture supernatant (160 L)
n-BuOH partition (4 times, ratio 1:1)
n-BuOH phase (320 g)
Aqueous phase (900 g)
MeOH precipitation (3 times)
MeOH soluble part (540 g)
HP20 column chromatography
MeOH fraction (25 g)
LH20 column chromatography
80%MeOH fraction (125 mg)
preparative RP-HPLC
Growth factors A–F



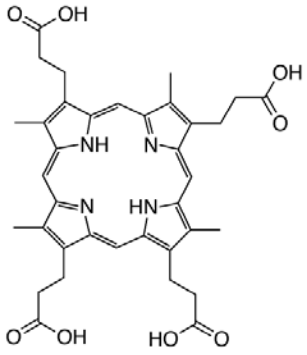
Growth factor **A**



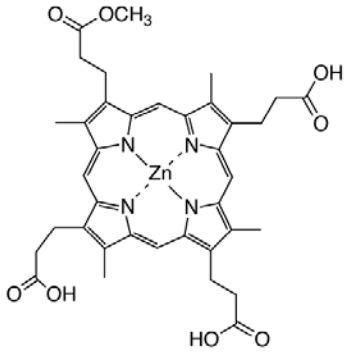
Growth factor **B**



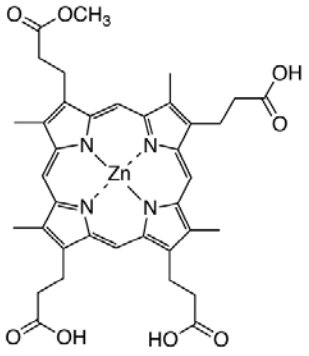
Growth factor **C**



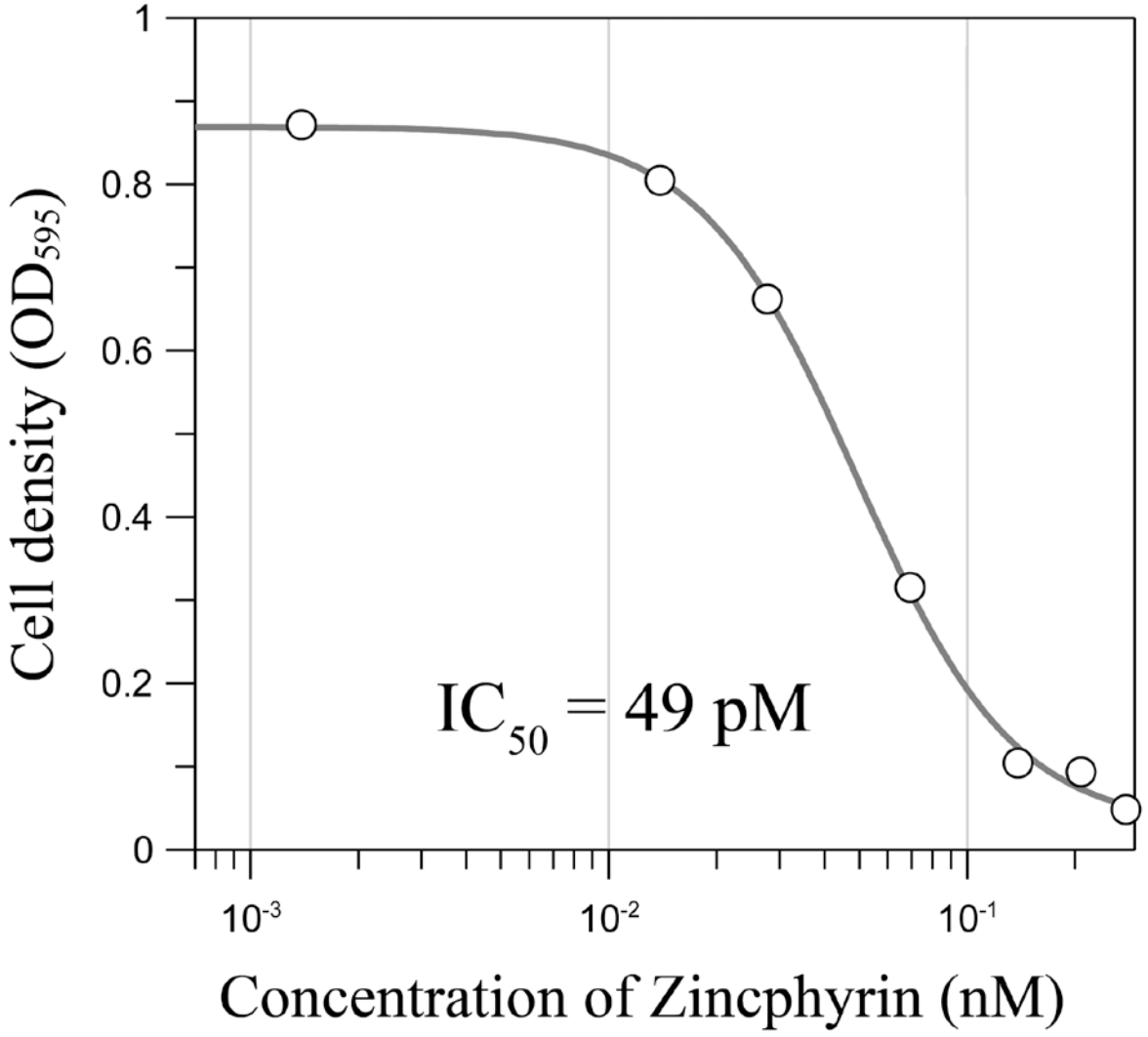
Growth factor **D**



Growth factor **E**



Growth factor **F**


1
0.8
0.6
0.4
0.2
0
Cell density (OD_{595})
IC_{50} = 49 pM
10^{-3}
10^{-2}
10^{-1}
Concentration of Zincphyrin (nM)

Supplementary information

Zincmethylphyrins and coproporphyrins, novel growth factors released by *Sphingopyxis* sp. enable laboratory cultivation of previously uncultured *Leucobacter* sp. through interspecies mutualism

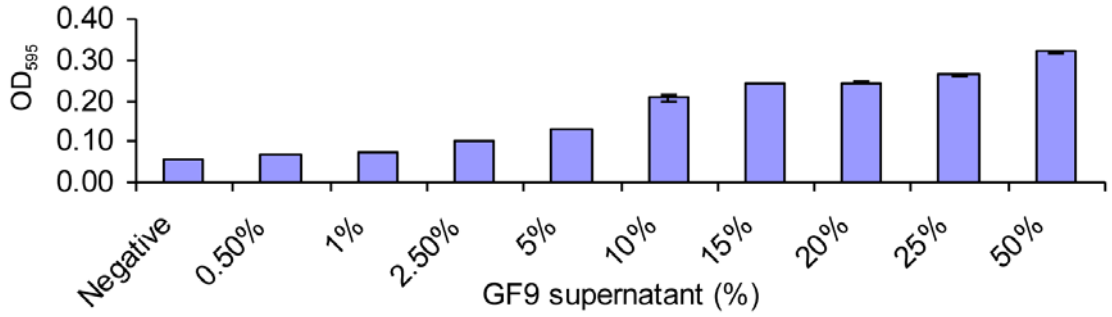
Mohammad Nazrul Islam Bhuiyan,[1] Ryogo Takai,[1] Shinya Mitsuhashi,[1] Kengo Shigetomi,[1] Yasuhiro Tanaka,[2] Yoichi Kamagata,[1] and Makoto Ubukata[1]*

[1]Division of Applied Bioscience, Graduate School of Agriculture, Hokkaido University, Kita-9, Nishi-9, Kita-ku, Sapporo, 060-8589, Japan.

[2]Department of Environmental Sciences, Faculty of Life and Environmental Sciences, University of Yamanashi, 4-4-37, Takeda, Kofu, Yamanashi 400-8510, Japan.

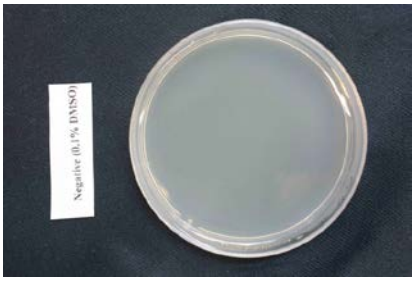
*To whom correspondence should be addressed;
E-mail: m-ub@for.agr.hokudai.ac.jp

SUPPLEMENTARY INFORMATION



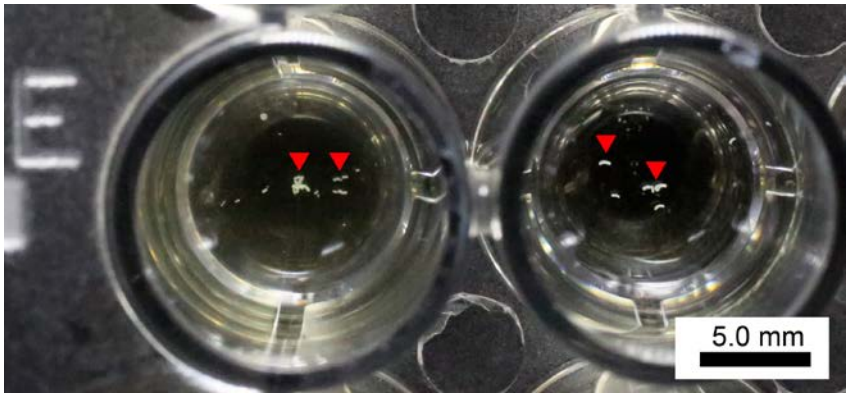
Supplementary Figure 1 Growth pattern of strain ASN212 under various concentrations of strain GF9 supernatant. The strain ASN212 was inoculated into NPB liquid media containing different concentrations of strain GF9 supernatant. Growth of strain ASN212 was evaluated with OD_{595} of each broth cultured at 28^{o}C for 24 h at 170 rpm. Although the highest growth activity was observed at 50% v/v of GF9 supernatant, accurate dose-dependency of the supernatant could not be obtained because of coagulating precipitation of the strain ASN212. Error bars, mean ± SD, n = 4. Negative: Growth of strain ASN212 without strain GF9 supernatant as a negative control.

a

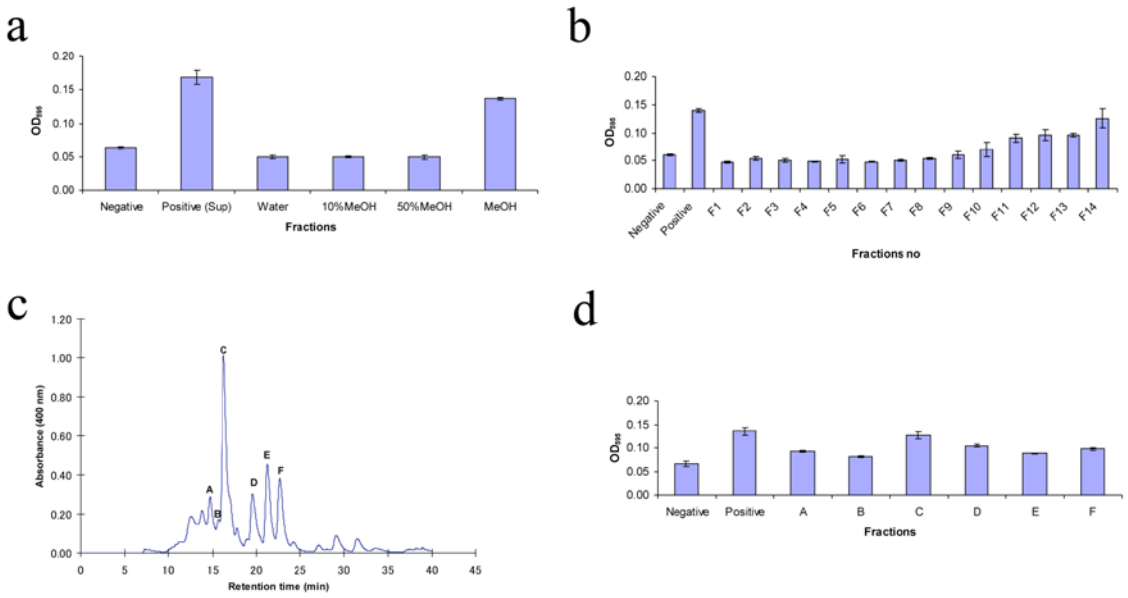


b

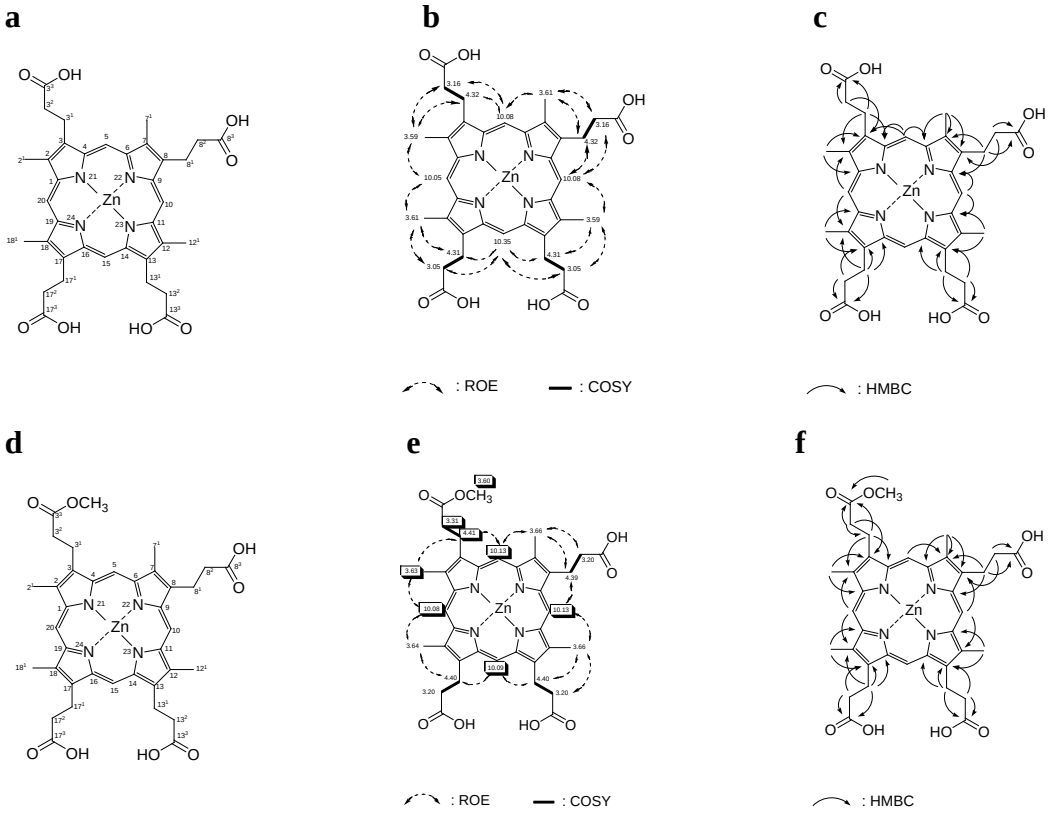
c



Supplementary Figure 2 Growth of strain ASN212 on agar plate containing strain GF9 supernatant and flocculation of strain ASN212 in liquid culture stimulated with ASN212 growth factor. (**a**) Negative control inoculated strain ASN212 on agar media without strain GF9 supernatant showed clear plate indicating no growth. (**b**) Vigorous colony formation of strain ASN212 was observed on agar media containing 10% of GF9 supernatant. (**c**) Flocculation of strain ASN212 was detected in NPB liquid media stimulated by growth factor **C** (left well) or growth factor **D** (right well). Red arrowheads show aggregates of strain ASN212 precipitated in the culture well. Scale bar = 5 mm.

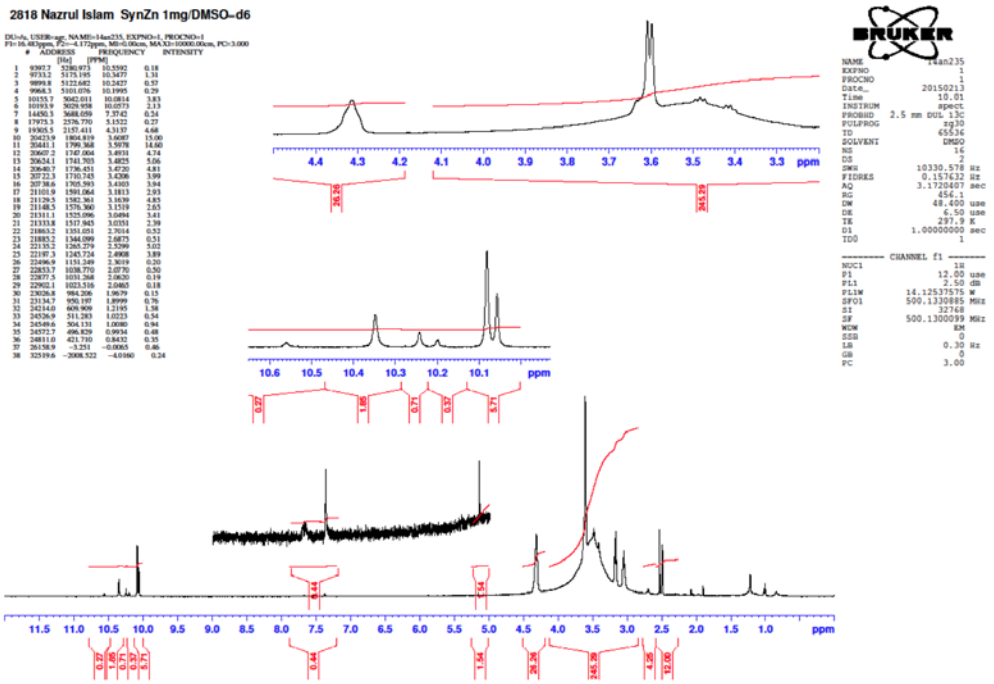


Supplementary Figure 3 The isolation procedure of ASN212 growth factors from strain GF9 supernatant. Sequential chromatographic fractionations and growth-stimulating activity of each fraction are indicated in (**a**) HP-20 chromatography showing MeOH fraction as the active eluate, (**b**) LH 20 chromatography showing active fractions of F9 to F14, (**c**) RP-HPLC (400 nm) profile of the active sample partially purified by LH20, and (**d**) the growth-stimulating activities of growth factors **A**, **B**, **C**, **D**, **E**, and **F**. Growth-stimulating activity of each fraction was evaluated by measuring optical density (OD_{595}) at equivalent concentration (EC). Error bars, mean ± SD, n = 3.

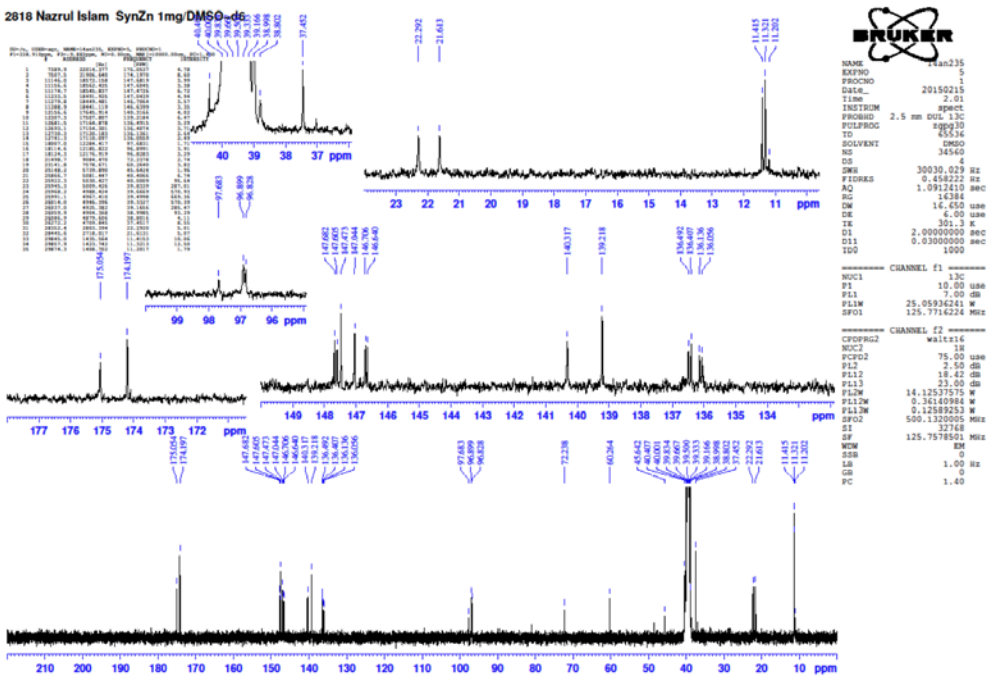


Supplementary Figure 4 2D NMR analysis of growth factors **C** and **F**. (**a**) Structure of growth factor **C** with numbering. (**b**) COSY and ROESY correlations of growth factor **C**. (**c**) HMBC correlations of growth factor **C**. (**d**) Structure of growth factor **F** with numbering. (**e**) COSY and ROESY correlations of growth factor **F**. (**f**) HMBC correlations of growth factor **F**.

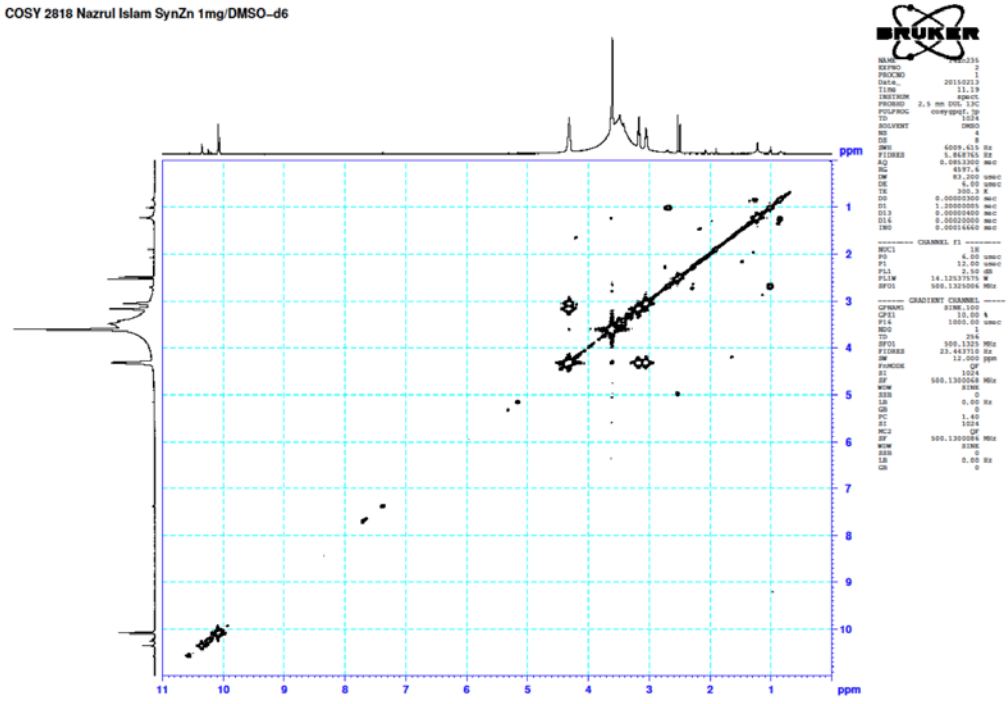
a


2818 Nazrul Islam SynZn 1mg/DMSO-d6
BRUKER

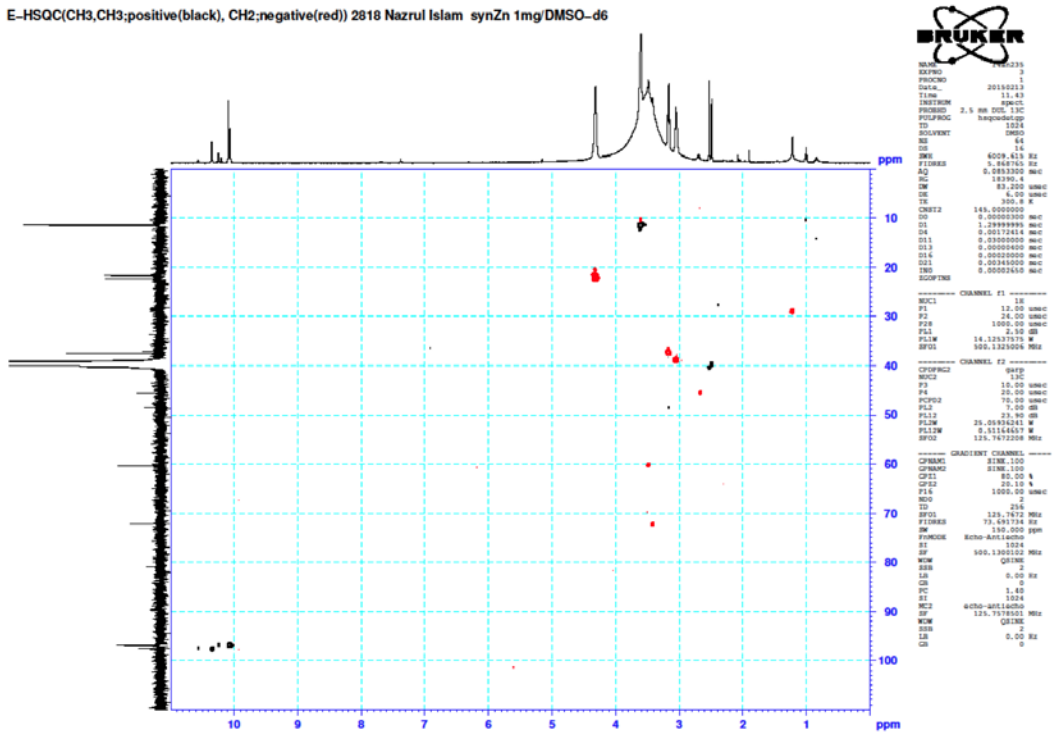
b


2818 Nazrul Islam SynZn 1mg/DMSO-d6
BRUKER

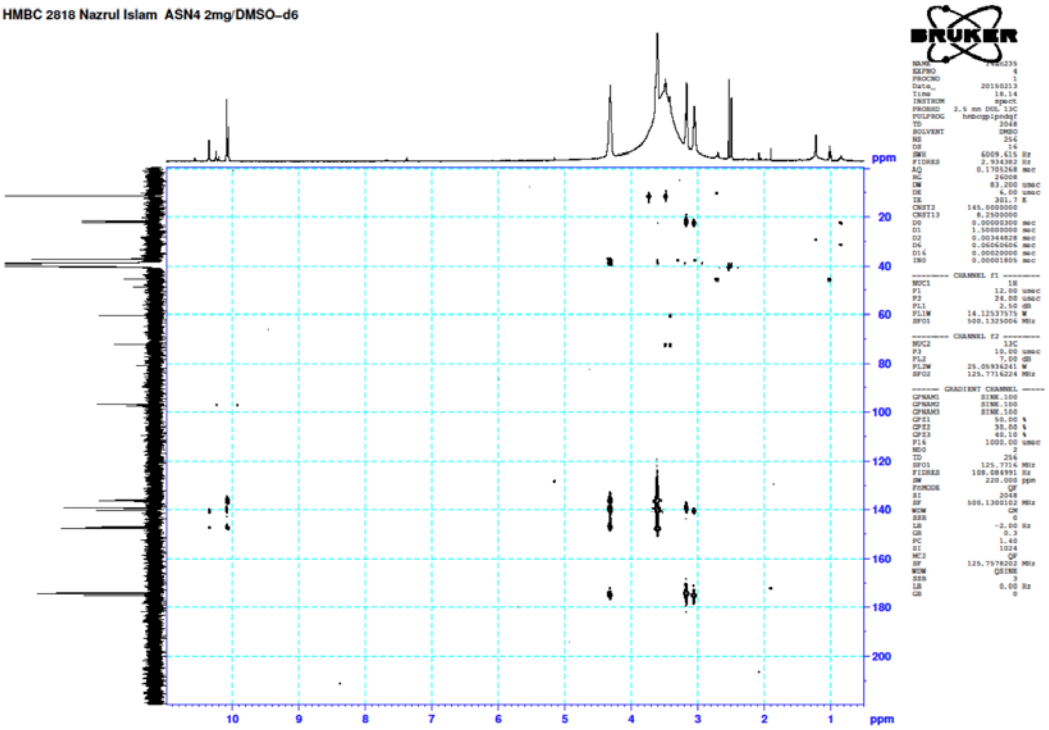
c


COSY 2818 Nazrul Islam SynZn 1mg/DMSO--d6
BRUKER
ppm
ppm

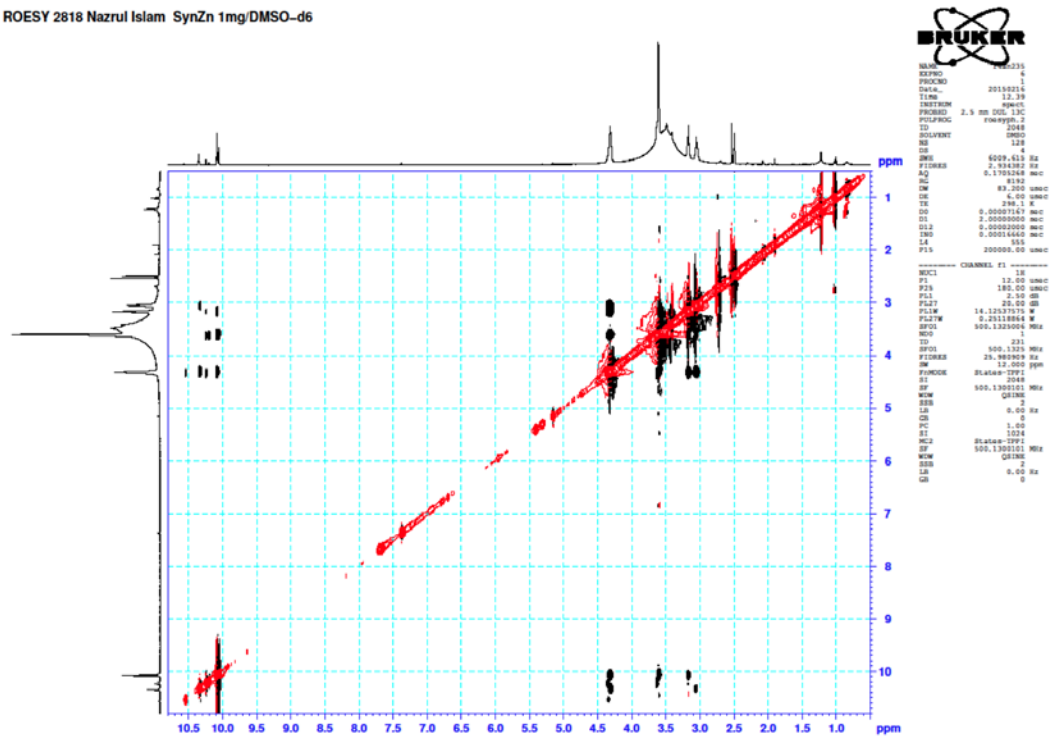
d


E-HSQC(CH3,CH3;positive(black), CH2;negative(red)) 2818 Nazrul Islam synZn 1mg/DMSO--d6
BRUKER
ppm
ppm

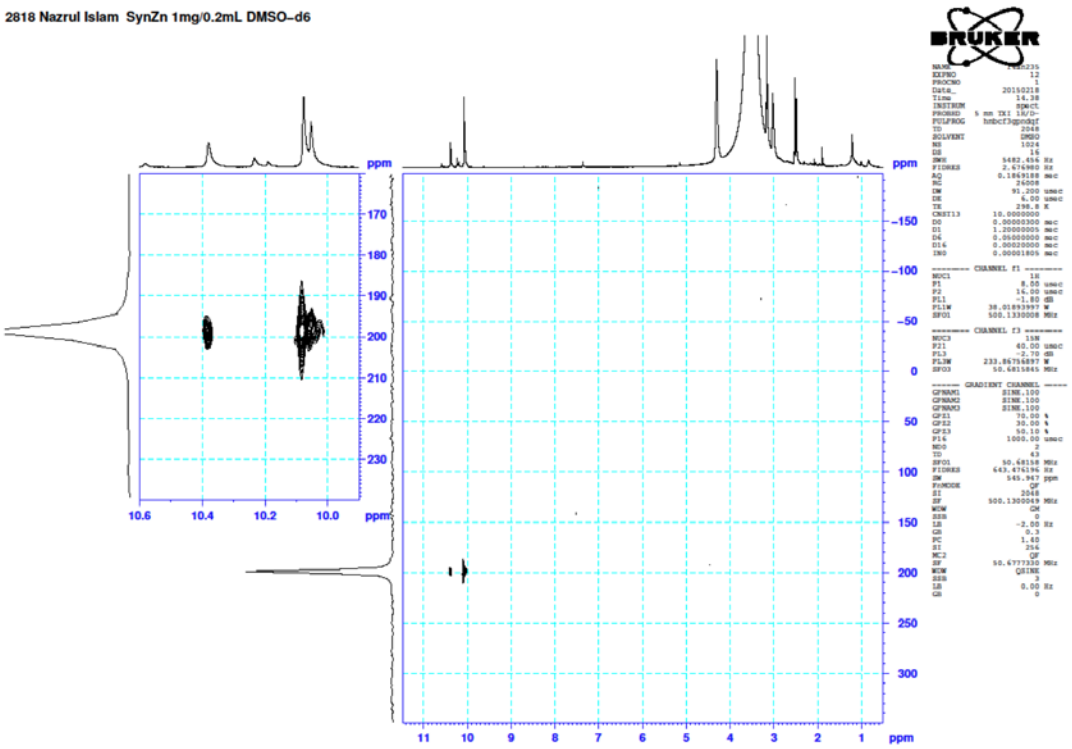
e


HMBC 2818 Nazrul Islam ASN4 2mg/DMSO-d6
BRUKER
ppm
10 9 8 7 6 5 4 3 2 1 ppm
20 40 60 80 100 120 140 160 180 200

f

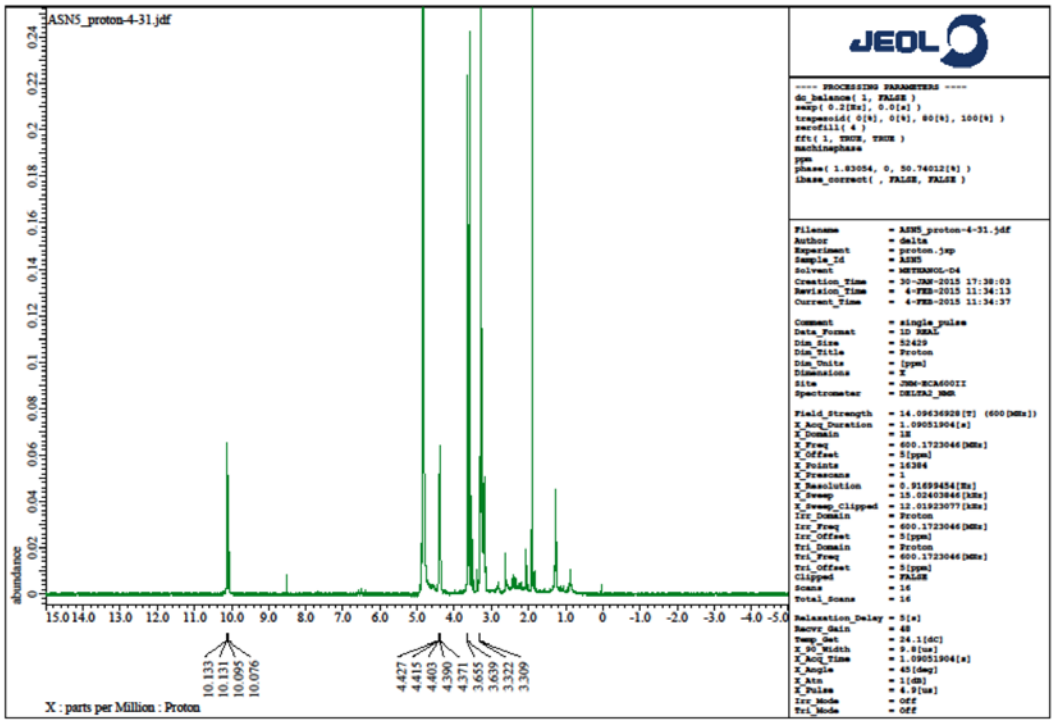

ROESY 2818 Nazrul Islam SynZn 1mg/DMSO-d6
BRUKER
ppm
10.5 10.0 9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 ppm
1 2 3 4 5 6 7 8 9 10

g

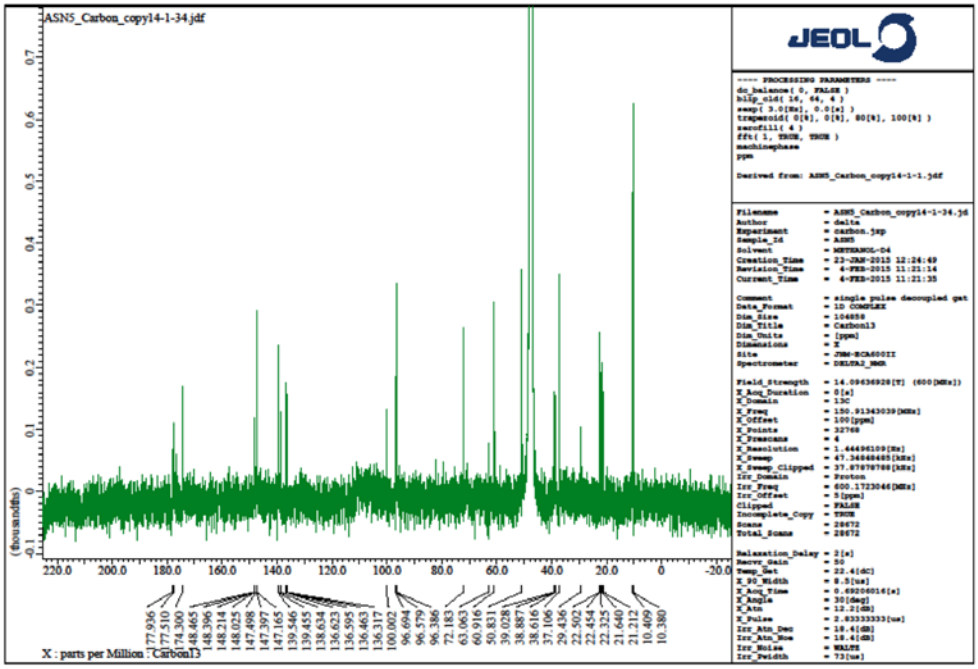


Supplementary Figure 5 NMR spectra of zincphyrin (growth factor **C**). **(a)** ^{1}H NMR spectrum (500 MHz, δ in ppm). (**b**) ^{13}C NMR spectrum (126 MHz, δ in ppm). (**c**) COSY NMR spectrum. (**d**) HSQC NMR spectrum. (**e**) HMBC NMR spectrum. (**f**) ROESY NMR spectrum. (**g**) ^{1}H-^{15}N HMBC NMR spectrum (50.7 MHz, δ in ppm).

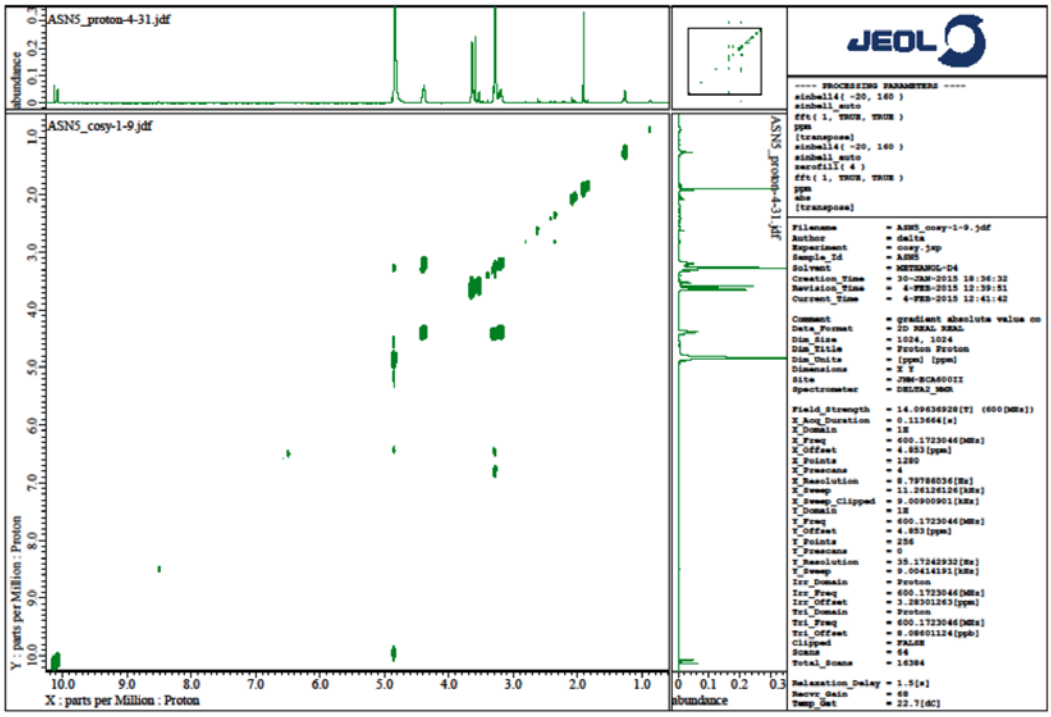
a


ASN5_proton-4-31.jdf
abundance
X : parts per Million : Proton
10.133
10.131
10.095
10.076
4.427
4.415
4.403
4.390
4.371
3.655
3.639
3.322
3.309

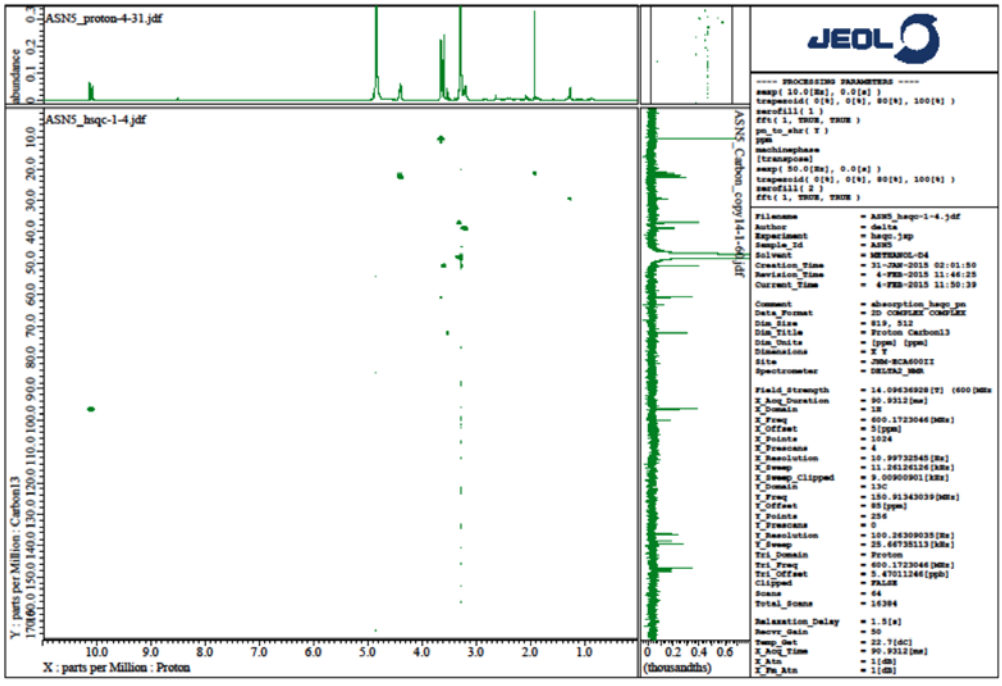
b


ASN5_Carbon_copy14-1-34.jdf
(thousandths)
X : parts per Million : Carbon13

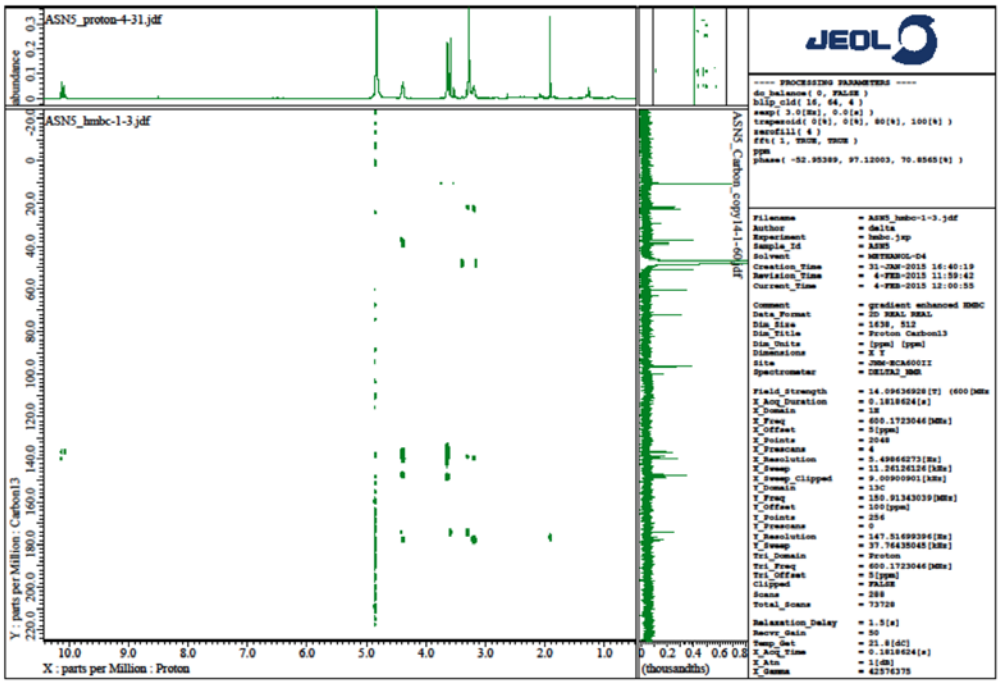
c


ASN5_proton-4-31.jdf
ASN5_cosy-1-9.jdf
Y : parts per Million : Proton
X : parts per Million : Proton
abundance
JEOL

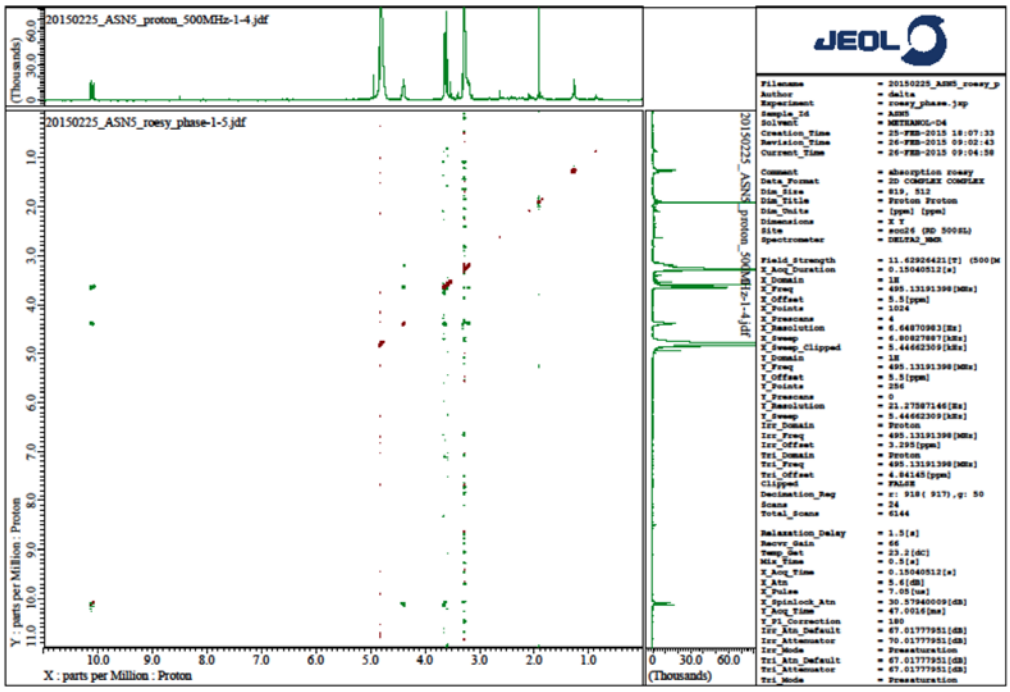
d


ASN5_proton-4-31.jdf
ASN5_hsqc-1-4.jdf
Y : parts per Million : Carbon13
X : parts per Million : Proton
abundance
(thousandths)
JEOL

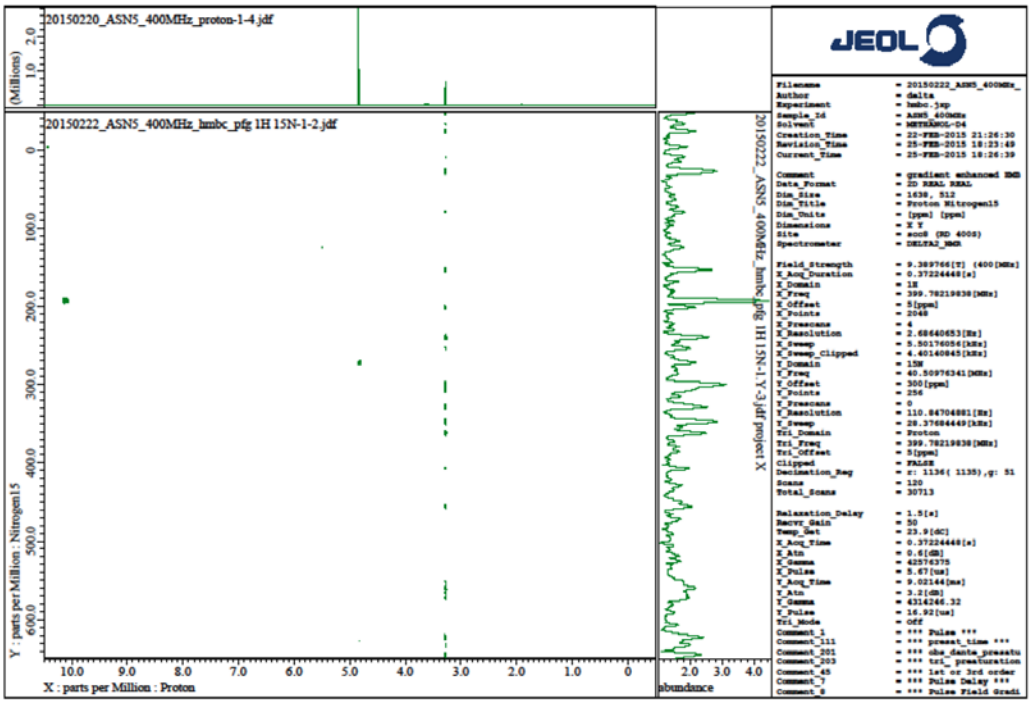
e


ASN5_proton-4-31.jdf
ASN5_hmbc-1-3.jdf
ASN5_Carbon_copy14-1-60.jdf
Y : parts per Million : Carbon13
X : parts per Million : Proton
(thousandths)
JEOL

f


20150225_ASN5_proton_500MHz-1-4.jdf
20150225_ASN5_roesy_phase-1-5.jdf
20150225_ASN5_proton_500MHz-1-4.jdf
Y : parts per Million : Proton
X : parts per Million : Proton
(Thousands)
JEOL

g



Supplementary Figure 6 NMR spectra of zincmethylphyrin III (growth factor **F**). **(a)** ^{1}H NMR spectrum (600 MHz, δ in ppm). (**b**) ^{13}C NMR spectrum (151 MHz, δ in ppm). (**c**) COSY NMR spectrum. (**d**) HSQC NMR spectrum. (**e**) HMBC NMR spectrum. (**f**) ROESY NMR spectrum. (**g**) ^{1}H-^{15}N HMBC NMR spectrum (60.8 MHz, δ in ppm).

Supplementary Table 1 Characteristics of individual growth factors for strains AST4^{T}, ASTN45 and ASN212.

Property	Growth of strain		
	ASN212	AST4^{T}	ASTN45
What stage of supernatant of GF9 culture showed the growth stimulation effect?	**lag to stationary phase**	**late log to stationary phase**	**late log to stationary phase**
Filtrate after ultrafiltration			
Nominal molecular weight limit: 50000	+++	+++	+++
Nominal molecular weight limit: 3000	+++	+++	+++
Nominal molecular weight limit: 1000	**+**	**+++**	**++++**
After charcoal column treatment	**–**	**–**	**+++**
After protease (Pronase) treatment	+++	+++	+++
After acid (pH 2.0) treatment	+++	+++	+++
After alkali (pH 10.0) treatment	+++	+++	+++
Growth stimulation by the supernatant of other strains of *Sphingomonadaceae*			
Sphingopyxis sp. strain GF9 (control)	+++	+++	+++
Sphingopyxis macrogoltabidus strain NBRC 15033^{T}	++	+	++
Sphingopyxis terrae strain NBRC 15098^{T}	++++	+++	++++
Sphingomonas mali strain NBRC 15500^{T}	++	+	++
***Sphingomonas sanguinis* strain IAM 12578^{T}**	**++**	**++**	**–**
Sphingomonas sp. strain NBRC 15915	++	++	+++
***Sphingobium chlorophenolica* strain NBRC 16172^{T}**	**++**	**–**	**+**
***Sphingobium yanoikuyae* strain NBRC C 15102^{T}**	**++**	**+++**	**+**
***Sphingobium herbicidovorans* strain NBRC 16415^{T}**	**–**	**++**	**+**
***Novosphingobium rosa* strain IAM 14222^{T}**	**++**	**+++**	**+**

++++: Over 110% of growth stimulation in comparison to strain GF9 supernatant

+++: 80–109% of growth stimulation in comparison to strain GF9 supernatant

++: 50–79% of growth stimulation in comparison to strain GF9 supernatant

+: Under 49% of growth stimulation in comparison to strain GF9 supernatant

–: no growth stimulation（≤ 2.0-fold in comparison to control without supernatant）

The result indicates that a variety of strains in Sphingomonadaceae support the growth of strains ASN212, AST4^{T} and ASTN45. Rows shown by bold fonts indicate clear differences in stimulation effect between these three strains. The strain GF9 supernatant contains at least three different growth factors, ASTN45 growth factor, ASN212 growth factor, and AST4^{T} growth factor[11].

Supplementary Table 2 ^{1}H and ^{13}C NMR data of growth factor **C** in DMSO-d_6 and growth factor **F** in MeOH-d_4.

Zincphyrin (growth factor **C**)			Zincmethylphyrin III (growth factor **F**)		
Position	δ_C	δ_H, type	Position	δ_C	δ_H, type
1	147.6		1	148.0	
2	136.1		2	136.3	
2^1	11.3	3.59, m	2^1	10.4	3.63, m
3	139.2		3	138.6	
3^1	21.6	4.32, m	3^1	21.6	4.41, m
3^2	37.5	3.16, m	3^2	37.1	3.31, m
3^3	174.2		3^3	174.3	
			3^3-OCH_3	50.8	3.60, m
4	146.6		4	147.2	
5	96.9	10.08, s	5	96.6	10.13, s
6	147.5		6	148.5	
7	136.5		7	136.6	
7^1	11.3	3.61, m	7^1	10.4	3.65, m
8	139.2		8	139.5	
8^1	21.6	4.32, m	8^1	22.3	4.39, m
8^2	37.5	3.16, m	8^2	38.6	3.20, m
8^3	174.2		8^3	177.9	
9	146.7		9	147.4	
10	96.9	10.08, s	10	96.7	10.13, s
11	147.7		11	148.4	
12	136.1		12	136.6	
12^1	11.3	3.59, m	12^1	10.4	3.66, m
13	140.3		13	139.5	
13^1	22.3	4.31, m	13^1	22.5	4.40, m
13^2	38.8	3.05, m	13^2	38.9	3.20, m
13^3	175.1		13^3	177.8	
14	147.0		14	147.4	
15	97.7	10.35, s	15	96.4	10.09, s
16	147.0		16	147.5	
17	140.3		17	139.5	4.40, m
17^1	22.3	4.31, m	17^1	22.5	3.20, m
17^2	38.8	3.05, m	17^2	39.0	
17^3	175.1		17^3	177.5	
18	136.4		18	136.5	
18^1	11.3	3.61, m	18^1	10.4	3.64, m
19	147.6		19	148.2	
20	96.8	10.05, s	20	96.6	10.08, s

Supplementary Table 3 Minimum effective concentrations (MECs) of commercial and synthetic porphyrins for growth of strain ASN212.

Porphyrins	Molecular formula	MW	MEC (nM)
Zinc coproporphyrin I (synthetic)	$C_{36}H_{36}O_8N_4Zn$	716.17	2.0×10
Zincphyrin (synthetic)	$C_{36}H_{36}O_8N_4Zn$	716.17	1.4×10^{-2}
Zincmethylphyrin 1 (synthetic)	$C_{37}H_{38}O_8N_4Zn$	730.19	9.6
Coproporphyrin I dihydrochloride	$C_{36}H_{38}O_8N_4\cdot2HCl$	727.63	4.3×10
Coproporphyrin III dihydrochloride	$C_{36}H_{38}O_8N_4\cdot2HCl$	727.63	1.8
Coproporphyrin III tetramethyl ester	$C_{40}H_{46}O_8N_4$	710.82	7.0×10
Hematin	$C_{34}H_{33}N_4O_5Fe$	633.49	2.4×10^{3}
Hemin	$C_{34}H_{32}ClFeN_4O_4$	651.94	1.2×10^{3}