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Separacenes A–C, Cytotoxic Polyene Polyketides from the Marine Actinomycete, *Streptomyces* sp.

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배 문 형

Separacenes A–C, 해양 방선균 Streptomyces 부터 분리한 세포독성 Polyene Polyketides

Separacenes A–C, Cytotoxic Polyene Polyketides from the Marine Actinomycete, *Streptomyces* sp.

지도교수 오 동 찬

이 논문을 약학석사학위논문으로 제출함

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Abstract

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During our search for new secondary metabolites with pharmaceutical potential, marine actinomycete strains, collected from Shinyang Beach in Jeju Island, were chemically analyzed by LC/MS. One of the marine actinomycetes strains, SNW10 (*Streptomyces* sp. based on 16S rDNA analysis), was revealed to produce unique tetraene / triene-bearing metabolites, which named separacenes A-C. The structures of the separacenes were determined by spectroscopic analysis of 1D and 2D NMR, MS and UV data. Separacene A possessing terminal olefinic double bond, conjugated tetraene, and two 1,2-diol groups, turned out to be a new 15-membered linear polyketide. The absolute configuration of separacene A was established by modified Mosher's method. The structures of separacene B and C, which incorporate terminal olefinic methylene, tirene, and two 1,2-diol moieties,

were also elucidated by spectroscopic analysis and chemical derivatization. Separacene A displayed cytotoxic activity against the HCT116 and A549 cancer cell line.

Key word : linear polyketides, separacene, marine actinomycete.
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Introduction

Bioactive natural products are still considered as an endless frontier in drug discovery (*1*). Even though synthetic approaches to obtain bioactive compounds via directed organic synthesis and combinatorial chemistry have been developed for recent dozen years, the successful rate for a synthetic compound to become a drug candidate is only 0.001%. (*2*) On the other hand, approximately 0.3 % of natural polyketides have been developed as drug candidates, which is a significantly high rate compared to synthetic compounds. (*2*) This statistics clearly demonstrates that natural products have considerable competitiveness in discovering and developing new drugs. Therefore natural products and chemical structures derived from or related to natural products are currently playing a significant role in pharmaceutical development. (*3*)

Secondary metabolites of actinomycetes are regarded as very prolific sources of bioactive natural products for new drug discovery. (*4*) Numerous secondary metabolites from actinomycetes were developed as clinically-used drugs, to name a few, doxorubicin, amphotericin, rapamycin, vancomycin, and daptomycin. During recent 10 years, particularly marine actinomycetes have been highlighted as a new frontier in discovering novel bioactive compounds, whereas terrestrial microorganisms repeatedly produce previously-described secondary metabolites. (*5*)

As results of pioneering studies of marine actinomycetes, new drug candidates such as salinosporamide A and thiocoraline are under clinical trial on the way to drug development, indicating the biomedical potential of marine actinomycetes. (*6*)

As search for new bioactive compounds from marine actinomycetes, we selectively isolated actinomycetes from marine-derived sediment samples collected in Korean seashores and chemically analyzed the cultures for secondary metabolite production. One of the *Streptomyces* strains, SNW10 isolated at Shinyang Beach in Jeju Island was revealed to produce polyunsaturated compounds with multiple conjugated double bonds based on the LC/MS analysis. Here we report the isolation, structure determination including absolute configurations, and biological activity of three polyunsaturated polyketides, separacenes A-C (**1**-**3**).

Results and Discussion

Separacene A (**1**) was purified as a white powder possessing the molecular formula $C_{15}H_{22}O_4$ based on FAB-HRMS (obsd $[M+Na]^+$ at *m/z* 289.1417, calcd $[M+Na]^+$ 289.1416) along with ^1H and ^{13}C NMR data (Table 1). The ^1H NMR spectrum of **1** in pyridine-d_5 displayed the characteristic polyunsaturated and polyhydroxylated signature with 10 olefinic protons between 6.80 and 5.31 ppm and 4 protons attached to oxygen-bearing carbons between 4.62 and 4.12 ppm. Further analysis of ^1H NMR spectrum of **1** identified that there is only one doublet methyl group at 1.45 ppm. The ^{13}C NMR and gHSQC spectra displayed 9 olefinic methine and one methylene carbons from 139.8 to 115.4 ppm, and 4 oxygen-bound methine carbons from 77.3 to 71.3 ppm, one methyl group at δ_C 19.6. The IR absorption at 3360 cm^{-1} indicated that some or all of the oxygenated functionalities are hydroxy groups. The observation of 10 olefinic carbons in the ^{13}C NMR spectrum and 10 olefinic protons in the ^1H NMR spectrum revealed that separacene A (**1**) bears five double bonds, which accounts for all of the unsaturation number calculated from the molecular formula. On the basis of the UV absorption maximum at 302 nm, four of the five double bonds should be conjugated.

Analysis of the gHSQC spectrum assigned all of the one-bond correlations between protons and carbons. The planar structure was then constructed mainly by the ^1H-^1H COSY and HMBC NMR spectroscopic analysis. In particular, separated two diol moieties were elucidated by the H-2 (δ_H 4.11) – H-3 (δ_H 4.43) and H-12

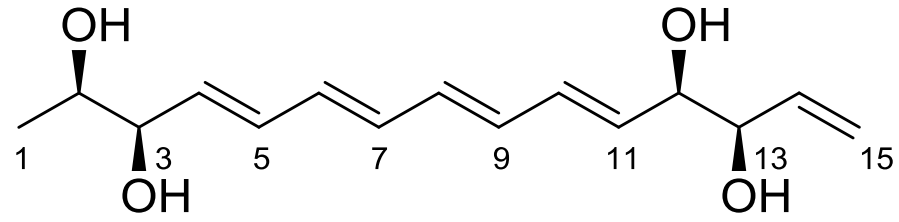
(δ_H 4.58) – H-13 (δ_H 4.54) homonuclear correlations. Besides the diols, as expected by the UV spectrum, conjugated four double bonds (C-4 to C-11) were identified by COSY correlations. Initially we acquired NMR spectroscopic data on 500 MHz but the double bond proton signals were intensively overlapped and showed second-order peaks, which hampered the unequivocal establishment of their connectivities and the double bond geometry. Thus we tried deliberate NMR analysis on higher field (900 MHz), which provides separated signals and ^{1}H-^{1}H coupling constants of the olefinic protons. COSY spectroscopic analysis confirmed the H-11 (δ_H 6.26) to H-10 (δ_H 6.79) and H-4 (δ_H 6.21) to H-5 (δ_H 6.75) correlations, which assigned C-10 – C-11 and C-4 – C-5 double bonds. Further COSY analysis revealed H-10 has a correlation with H-9 (δ_H 6.44), determining C-9 – C-10 extension. Subsequently C-9 was connected to C-8 by the COSY correlation between H-9 and H-8 (δ_H 6.38). H-8 was correlated with H-7 (δ_H 6.40), confirming the double bond between C-6 and C-7. Finally H-7 (δ_H 6.40) displayed homonuclear coupling with H-6 (δ_H 6.42), completing the C-4 to C-11 tetraene structure in separacene A (**1**). The last double bond (C-14 – C-15) with olefinic methylene was also assigned because H_2-15 (δ_H 5.58; 5.30) correlated with H-14 (δ_H 6.35) in the COSY spectrum. The first diol (C-2 and C-3) was flanked by the methyl group (C-1) and the conjugated teteraene (C-4 to C-11) on the basis of COSY and HMBC correlations. The second diol (C-12 and C-13) was connected to the tetraene on one side and the terminal double bond (C-14 – C-15) by COSY and HMBC spectroscopic analysis.

The gHMBC NMR spectrum supported the assigned structure of **1**. In details, H-

9 (δ_H 6.44) has an HMBC correlation to C-11 (δ_C 135.7) and H-11 (δ_H 6.26) has HMBC correlation to C-9 (δ_C 133.3). Additionally, the HMBC correlations from H-3 (δ_H 4.43) to C-5 (δ_C 131.6) and from H-4 (δ_H 6.75) to C-6 (δ_C 133.3) supported the C-5 – C-6 linkage. Therefore the gross structure of separacene A (**1**) was elucidated as a linear chain bearing two diols, tetraene, and a terminal olefinic methylene.

For assignment of the double bond geometries of **1**, we analyzed coupling constants in the ^{1}H NMR spectrum at 900 MHz. The large coupling constants (larger than 15.0 Hz) observed in all of the olefinic protons belonging to the tetraene moiety determined the configurations of as 4*E*, 6*E*, 8*E*, 10*E* and 14*E*.

The absolute configurations of four stereogenic centers of separacene A (**1**) were determined by the established method to assign secondary/secondary diols based on modified Mosher's method. (*7*) We derivatized separacene A with (*R*) and (*S*)-α-methoxy-α-(trifluoromethyl) phenylacetyl chloride (MTPA-Cl) to yield tetra-(*S*)- and (*R*)-MTPA esters (**4** and **5**), respectively. The ^{1}H chemical shifts around the chiral centers (C-2, C-3, C-12, and C-13) of **4** and **5** were assigned by analyzing ^{1}H and COSY NMR spectra. The $\Delta\delta_{S-R}$ values of **4** and **5** displayed the consistent sign distribution with β-*syn*-diol configurations for the two diol moieties, thus assigning the absolute configurations as 2*R*, 3*R*, 12*R*, and 13*R* (Figure 2).



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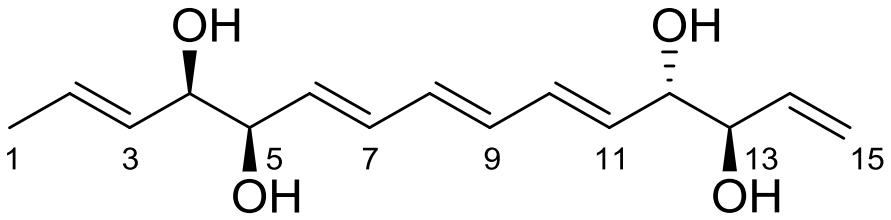

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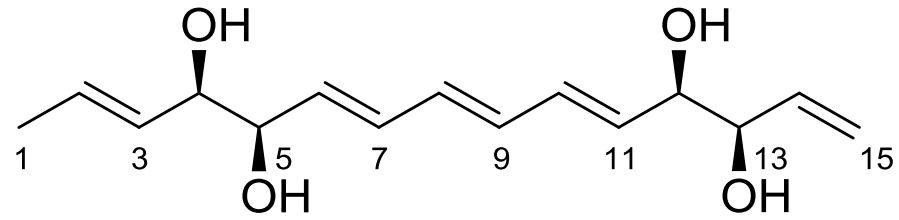

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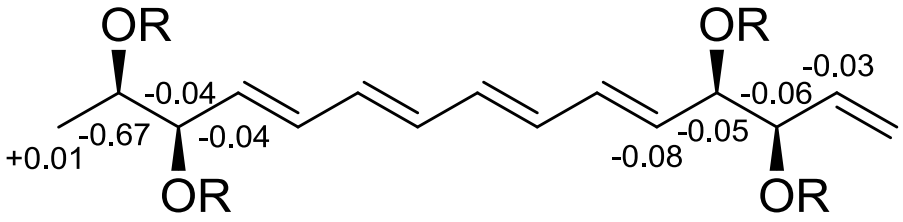
Figure 1. The structures of separacene A-C (**1**-**3**).

Table 1. NMR data of separacene A (**1**) in pyridine-d_5

C/H	δ_H [a]	mult (*J* in Hz)	δ_C [b]	
1	1.43	d (6.5)	19.6	CH_3
2	4.11	m	71.2	CH
3	4.43	dd (10.0, 6.0)	77.3	CH
4	6.21	dd (15.5, 6.0)	135.9	CH
5	6.75	dd (15.5, 10.5)	131.6	CH
6	6.42	dd (15.0, 10.5)	133.3	CH
7	6.40	m	132.9	CH
8	6.38	m	132.8	CH
9	6.44	dd (15.0, 10.5)	133.3	CH
10	6.79	dd (15.5, 10.5)	131.5	CH
11	6.26	dd (15.5, 6.0)	135.7	CH
12	4.58	dd (9.5, 6.0)	133.6	CH
13	4.54	dd (9.5, 5.5)	133.9	CH
14	6.35	ddd (17.5, 10.5, 5.5)	42.4	CH
15	5.30	dd (10.5, 1.0)	115.4	CH_2
	5.58	dd (17.5, 1.0)		

[a]900 MHz, [b]225 MHz



4: R = (*S*)-MTPA

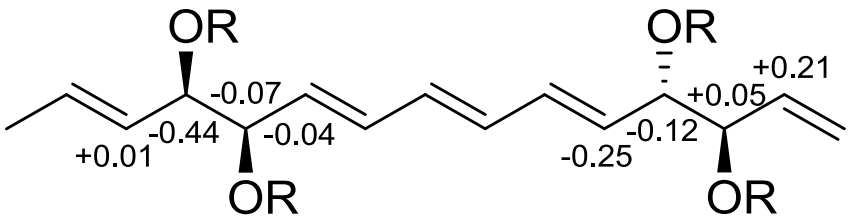
5: R = (*R*)-MTPA

Figure 2. $\Delta\delta_{S-R}$ values of **4** and **5** in pyridine-d_5.

Separacene B (**2**) was isolated as a white powder, of which molecular formula was determined as $C_{15}H_{22}O_4$ based on the analysis of HR-FAB mass (obsd $[M+Na]^+$ at *m/z* 289.1417, calcd $[M+Na]^+$ 289.1416) and ^1H and ^{13}C NMR spectroscopic data (Table 2). The IR and mass spectra of **2** were quite similar to those of **1** but separacene B (**2**) displayed the UV absorption maximum λ_{max} at 271 nm, which is shorter than that of **1** by 31 nm, indicating one less conjugated double bond and thus a triene moiety. Further investigation of ^1H, ^{13}C, COSY, HSQC, HMBC NMR spectra revealed that, as we expect, separacene B possesses three consecutive double bonds between two diols. Careful comparison between 1 and 2 revealed that separacene B (**2**) bears one double bond placed between the methyl group (C-1) and the diol (C-4 and C-5) differently from separacene A (**1**). The configurations of the double bonds were also established as 6*E*, 8*E*, and 10*E* by ^1H-^1H coupling constants.

For absolute configuration of **2**, the same procedure applied for **1** was conducted using MTPA derivatization. Based on the $\Delta\delta_{S-R}$ values of **6** and **7**, we determined the

absolute configurations of four chiral centers as 4*R*, 5*R*, 12*S*, 13*R*. (Figure 3).



6: R = (*S*)-MTPA

7: R = (*R*)-MTPA

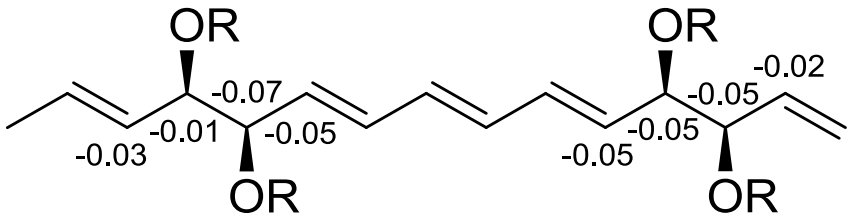
Figure 3. $\Delta\delta_{S\text{-}R}$ values of **6** and **7** in pyridine-d_5.

Table 2. NMR data of separacene B (**2**) in pyridine-d_5

C/H	δ_H [a]	mult (*J* in Hz)	δ_C [b]	
1	1.67	d (5.0)	18.7	CH_3
2	5.98	m	127.4	CH
3	5.96	dd (15.5, 5.0)	133.2	CH
4	4.48	dd (10.5, 5.0)	77.3	CH
5	4.58	dd (10.5, 6.0)	76.6	CH
6	6.23	dd (15.5, 6.0)	135.8	CH
7	6.75	dd (15.5, 10.5)	131.7	CH
8	6.42	dd (15.0, 10.5)	133.1	CH
9	6.44	dd (15.0, 10.5)	133.0	CH
10	6.79	dd (15.5, 10.5)	131.7	CH
11	6.35	dd (15.5, 6.0)	136.3	CH
12	4.67	dd (11.0, 6.0)	76.5	CH
13	4.62	dd (11.0, 5.5)	76.9	CH
14	6.48	ddd (17.0, 10.5, 5.5)	140.8	CH
15	5.30	dd (10.5, 1.0)	115.6	CH_2
	5.58	dd (17.0, 1.0)		

[a]600 MHz, [b]125 MHz

Separacene C (**3**) was obtained as white powder. The molecular formula was deduced as $C_{15}H_{22}O_4$ on the basis of the analysis of HR-FAB mass (obsd $[M+Na]^+$ at *m/z* 289.1426, calcd 289.1416) and ^1H and ^{13}C NMR spectroscopic data (Table 3). Careful analysis of ^1H, ^{13}C, COSY, HSQC, HMBC NMR spectra revealed that separacene C (**3**) has the identical planar structure with **2**. This result strongly suggested that separacene C (**3**) is a stereochemical isomer of **2**. Therefore we conducted MTPA derivatization for 3 to determine absolute configurations of the stereogenic centers of **3**. After we obtained (*S*) and (*R*)-tetra-MTPA esters (**8** and **9**), we analyzed $\Delta\delta_{S-R}$ values of **8** and **9**, which determined the absolute configurations of the four chiral oxygen-bearing carbons as 4*R*, 5*R*, 12*R*, and 13*R* (Figure 4).



8: R = (*S*)-MTPA

9: R = (*R*)-MTPA

Figure 4. $\Delta\delta_{S-R}$ values of **8** and **9** in pyridine-d_5.

Table 3. NMR data of separacene C (**3**) in pyridine-d_5

C/H	δ_H [a]	mult (*J* in Hz)	δ_C [b]	
1	1.66	d (5.0)	18.7	CH_3
2	5.98	m	133.6	CH
3	5.95	dd (15.5, 5.5)	127.8	CH
4	4.48	dd (10.5, 5.5)	77.0	CH
5	4.58	dd (10.5, 6.0)	76.5	CH
6	6.23	dd (15.0, 6.0)	132.0	CH
7	6.76	dd (15.0, 11.5)	136.0	CH
8	6.42	dd (15.0, 11.5)	133.2	CH
9	6.40	dd (15.0, 11.0)	133.0	CH
10	6.78	dd (15.5, 11.0)	131.7	CH
11	6.26	dd (15.5, 6.0)	136.3	CH
12	4.60	dd (10.0, 6.0)	76.6	CH
13	4.56	dd (10.0, 5.5)	77.1	CH
14	6.36	ddd (17.5, 10.0, 5.5)	140.3	CH
15	5.30	dd (10.5, 1.5)	115.9	CH_2
	5.58	dd (17.5, 1.5)		

[a]600 MHz, [b]125 MHz

For the biological activity of the separacences, antimicrobial activity was firstly evaluated against phylogenetically various pathogenic bacterial strains such as *Staphylococcus aureus* ATCC 6538p, *Bacillus subtilis* ATCC 6633, *Micrococcus luteus* IFO 12708, *Salmonella typhimurium* ATCC 14028, *Proteus vulgaris* ATCC 3851, *Escherichia coli* ATCC 35270 using ampicillin as a positive control compound. Separacene A (**1**) displayed weak antibacterial activity against *B. subtilis* ATCC 6633 and *P.vulgaris* ATCC 3851 with the MIC values of 50 μg/mL of 100 μg/mL, respectively. However, separacene B and C (**2** and **3**) did not show significant inhibitory activity against the tested bacteria. In addition, separacene A displayed moderate *C. albicans*-isocitrate lyase (ICL) inhibitory activity with the IC_{50} value of 45.7 μg/mL (171.7 μM) whereas separacene B and C did not show significant inhibitory in the ICL test.

Interestingly, separacene A showed considerable anticancer activity against the colon cell line HCT116 with the IC_{50} value of 14.01 μg/mL (52.7 μM) and weak cytotoxicity against the lung cancer cell line A549 with IC_{50} value of 37.6 μg/mL (141.4 μM). Unfortunately, Separacene B and C did not display remarkable inhibitory activity against the both cancer cell line.

Experimental Section

General Experimental Procedures. Optical rotations were measured by a Jasco P-1020 polarimeter with a 1 cm cell. IR spectra were acquired using a Thermo N1COLET iS10 spectrometer. UV spectra were obtained with a Perkin Elmer Lambda 35 UV/VIS spectrometer. Electrospray ionization source (ESI) low-resolution LC/MS data were recorded on an Agilent Technologies 6130 Quadrupole mass spectrometer coupled with an Agilent Technologies 1200 series HPLC using reversed-phase C_{18} column (Phenomenex Luna, 100 mm × 4.60 mm). High-resolution fast-atom bombardment (HR-FAB) mass spectra were collected with a Jeol JMS-600W High Resolution Mass Spectrometer in NCIRF (National Center for Inter-University Research Facilities). ^{1}H, ^{13}C, and 2D NMR spectra were obtained on a Bruker Avance 600 MHz in NCIRF (National Center for Inter-University Research Facilities) and 900 MHz NMR spectrometer in KBSI (Korea Basic Science Institute in Ochang).

Isolation of the Bacterial strain, SNW10. A sediment sample was collected in a 40 mL of sterilized plastic tube from Shinyang Beach in Jeju Island. The sample (1 g) was diluted in 24 mL of sterilized artificial seawater (for 1/6 dilution) and vortexed. The mixture was spread on the Actinomycete Isolation Agar, Chitin-based Agar, A4 (sea water 1 L, agar 18 g, cyclohexamide 100 mg/L), A5 (sea water 750 mL, distilled water 250 mL, agar, cyclohexamide 100 mg/L). SNW10 was isolated on A5 medium.

Identification of the strain SNW10. By using G-spin™ Genomic DNA extraction Kit, we could obtain the chromosomal DNA from *Streptomyces* sp. strain SNW10. The isolated DNA was amplified by polymerase chain reaction (PCR) using universal primers 27F and 1492R in the forward direction and 1492 in the reverse direction of the *E.coli* 16S rDNA sequence. For PCR reaction, the reaction elements including 5 μL of the template DNA, 5 μL of 10× Taq buffer, 1 μL of 10 mM mixed dATP, dTTP, dGTP, and dCTP, 1 μL of sense-27F primer (10 pmol/μL), 1 μL of antisense-1492R primer (10 pmol/μL), and 5 U of *Taq* DNA polymerase were prepared in a final volume of 50 μL. The thermal condition of PCR reaction was set as follows: pre heating for denaturation at 94 °C for 5 min, 30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 1 min 30 s, and additional extension at 72 °C for 10 min. The sequencing analysis data obtained from COSMO co, Ltd showed that SNW10 is most closely related to partial sequence of *Streptomyces sundarbansensis* (99% identity), identifying the strain as *Streptomyces* sp.

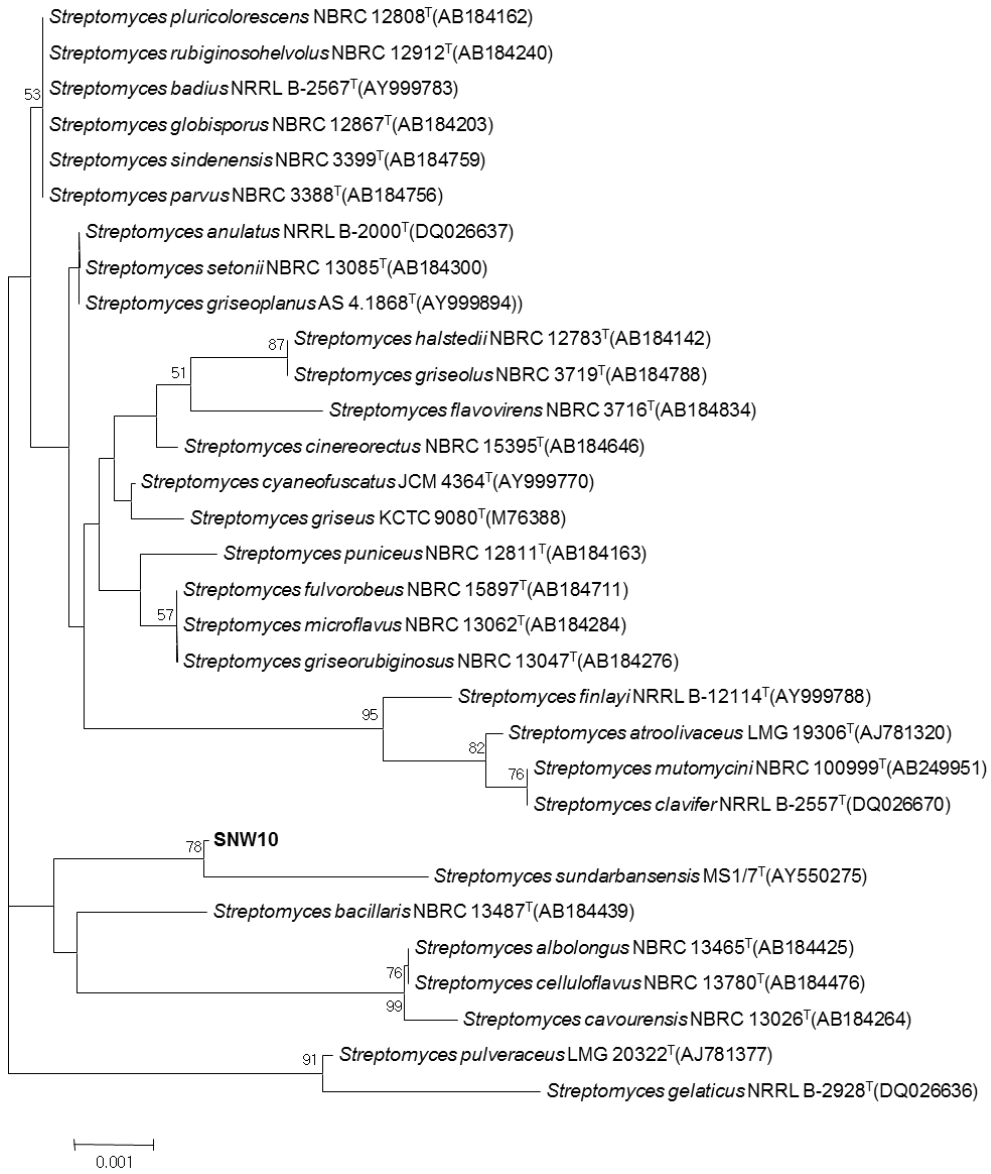


Figure 5. Phylogenetic tree based on 16S rRNA gene sequences of SNW10 (1404bp).

Cultivation and Extraction. The strain SNW10 was cultivated in 50 mL of YEME medium (4g yeast extract, 10g malt extract, and 4g glucose in 1 L of artificial seawater) in a 125 mL Erlenmeyer flask. After 3 day-cultivation on a rotary shaker at 200rpm at 30°C, 10 mL of the culture were inoculated to 1 L of YPM medium in 2.8 L Firnbach flasks (12 ea × 1 L, totally 12 L). The large culture was incubated under the same condition as used for the seed culture. After 2 days, the whole culture (12 L) was extracted with 18 L ethyl acetate twice. The ethyl acetate layer was separated and the residual water was removed by adding anhydrous sodium sulfate. The ethyl acetate extract was concentrated *in vacuo* to finally yield 1.5 g of dried material. This procedure was repeated 10 times (120 L culture in total) to obtain enough amounts of the separacenes for structure elucidation and bioassay.

Isolation of separacenes A-C (1, 2 and 3). The crude extract absorbed on celite was loaded on a 2g Sep-Pak® C18 cartridge and fractionated with 20 mL of each 20%, 40%, 60%, 80%, 100% MeOH in water, and 1:1 MeOH/dichloromethane. Among six fractions, separacenes A-C (**1**-**3**) were found in the 20% and 40% MeOH/water fractions. For purification of separacenes A-C (**1**-**3**), multiple steps of chromatography were required. At first, the fractions bearing **1**-**3** were injected to reversed-phase HPLC using Kromasil® column (5 μm C_{18} 250×10 mm) under the isocratic condition of 2:8 acetonitrile/water (UV 280nm detection, flow rate: 2 mL/min). Three peaks at retention times 20 min, 23 min, and 25 min were collected

and the peaks were further purified through a cyano HPLC column (YMC, 5 μm CN, 250×10 mm) with a gradient solvent system (30% MeOH/water to 50% MeOH/water over 40 min, flow rate: 2 mL/min). Fairly pure separacenes A-C (**1**-**3**) eluted at 13 min, 12 min, and 13.5 min, respectively. On the final purification step, normal-phase HPLC was employed (YMC, 5 μm silica 250×4.6 mm, UV 280 nm detection, flow rate: 1 mL/min). Finally separacene A (**1**) (11 mg), separacene B (**2**) (5 mg), and separacene C (**3**) (8 mg) were isolated as pure compounds at the retention times at 9 min, 11 min, and 8.5 min, respectively..

Separacene A (1): White powder, $[\alpha]_D$ -15 (c 0.075, MeOH); UV (MeOH) λ_{max} (log ε) 302 (4.3) nm; IR (neat) ν_{max} 3370, 3017, 2968, 2923, 1659, 1647, 1595, 1397 cm^{-1}, ^1H and ^{13}C NMR data, see Table 1, HRFABMS *m/z* 289.1417 $[M+Na]^+$ (calcd for $C_{15}H_{22}O_4Na]^+$ 289.1416).

Separacene B (2): White powder, $[\alpha]_D$ -4, (c 0.05, MeOH); UV (MeOH) λ_{max} (log ε) 270 (4.0) nm; IR (neat) ν_{max} 3370, 2923, 1659, 1647, 1595, 1397, 1130 cm^{-1}, For ^1H and ^{13}C NMR data, see Table 2, HRFABMS *m/z* 289.1417 $[M+Na]^+$ (calcd for $C_{15}H_{22}O_4Na$ 289.1416).

Separacene C (3): White powder, $[\alpha]_D$ -2, (c 0.05, MeOH); UV (MeOH) λ_{max} (log ε) 270 (4.0) nm; IR (neat) ν_{max} 3360, 2923, 2852, 1658, 1633 cm^{-1}, For ^1H and ^{13}C NMR data, see Table 3, HRFABMS *m/z* 289.1426 $[M+Na]^+$ (calcd for $C_{15}H_{22}O_4Na$ 289.1416).

MTPA esterification of separacenes A-C (1, 2 and 3). Separacenes A-C (**1-3**) were prepared in six 40 mL vials (two 1 mg samples for each compound) and were dried completely under high vacuum for 8 h. After adding catalytic amount of crystalline DMAP (dimethylaminopyridine) into each reaction vial, freshly distilled anhydrous pyridine (1 mL) was added under argon gas. The reaction mixtures were stirred at room temperature for 15 min. After 15min, (*R*) and (*S*)-α-methoxy trifluromethyl-phenylacetic acid (MTPA) chloride (30 μL) were separately added. The reactions were quenched by adding 50 μL of MeOH in 1 h. The reaction products then were purified by reversed-phase C_{18} column (Kromasil® 5 μm C_{18}(2) 250 × 10.0 mm) with gradient condition from 40% to 100% aqueous acetonitrile. Tetra-*S*-MTPA ester (**4**) and *R*-MTPA ester (**5**) of separacene A (**1**) eluted at 42.5 and 41.2 min respectively. *S*-MTPA ester (**6**) and *R*-MTPA ester (**7**) of separacene B (**2**) were obtained at 39 and 38.5 min and *S*-MTPA ester (**8**) and *R*-MTPA ester (**9**) of separacene C (**3**) was isolated at 43 min and 42 min. The $\Delta\delta_{S-R}$ values around stereogenic centers of the derivatives were assigned by ^{1}H NMR, ^{1}H-^{1}H COSY, HSQC, and HMBC NMR experiments.

***S*-MTPA ester (4) of separacene A (1)**: ^{1}H NMR (600 MHz, pyridine-d_5) δ 6.64 (dd, *J*= 15.5, 10.5 1H), 6.61 (dd, *J*= 15.5, 10.5 1H), 6.39-6.32 (m, 4H), 6.13 (dd, *J*= 8.0, 4.0 1H), 6.05 (m, 1H). 5.97 (m, 1H), 5.93 (ddd, *J*= 17.5, 10.5, 7.0 1H), 5.85 (dd, *J*= 15.5, 8.0 1H), 5.79 (dd, *J*= 15.5, 8.0 1H), 5.58 (m, 1H), 5.52 (dd, *J*= 17.5, 1.0

1H), 5.35 (dd, *J*= 10.5, 1.0 1H), 1.38 (dd, *J*= 6.5, 1.0 3H). The molecular formula of *S*-MTPA ester was confirmed as $C_{55}H_{50}O_{12}F_{12}$ ([M+Na]+ *m/z* 1153).

***R*-MTPA ester (5) of separacene A (1)**: ^{1}H NMR (600 MHz, pyridine-d_5) δ 6.55 (m, 1H), 6.53 (m, 1H), 6.39-6.37 (m, 4H), 6.18 (dd, *J*= 6.5, 6.5 1H), 6.11 (dd, *J*= 6.0, 6.0 1H). 6.01 (dd, *J*= 6.0, 6.0 1H), 5.96 (ddd, *J*= 17.0, 10.5, 6.5 1H), 5.87 (dd, *J*= 15.0, 7.0 1H), 5.84 (dd, *J*= 15.0, 7.5 1H), 5.65 (m, 1H), 5.46 (dd, *J*= 17.5, 1.0 1H), 5.34 (dd, *J*= 11.0, 1.0 1H), 1.37 (d, *J*= 6.5 3H). The molecular formula of *R*-MTPA ester was confirmed as $C_{55}H_{50}O_{12}F_{12}$ ([M+Na]+ *m/z* 1153).

***S*-MTPA ester (6) of separacene B (2)**: ^{1}H NMR (600 MHz, pyridine-d_5) δ 6.59 (dd, *J*= 15.5, 10.0 1H), 6.45 (dd, *J*= 15.5, 10.0 1H), 6.23-6.20 (m, 2H), 6.17-6.15 (m, *2*H), 6.11 (m, 1H). 6.06-6.04 (m, 2H), 5.99 (m, 1H), 5.86 (dd, *J*= 15.5, 8.0 1H), 5.75 (dd, *J*= 15.5, 8.0 1H), 5.62 (d, *J*= 17.0 1H), 5.58 (m, 1H), 5.52 (dd, *J*= 17.0, 1.0 1H), 5.42 (d, *J*= 10.5 1H), 1.57 (d, *J*= 6.0 3H). The molecular formula of *S*-MTPA ester was confirmed as $C_{55}H_{50}O_{12}F_{12}$ ([M+Na]+ *m/z* 1153).

***R*-MTPA ester (7) of separacene B (2)**: ^{1}H NMR (600 MHz, pyridine-d_5) δ 6.61 (dd, *J*= 15.5, 10.5 1H), 6.48 (dd, *J*= 15.5, 10.5 1H), 6.28 (dd, *J*= 15.5, 10.5 1H), 6.21 (dd, *J*= 15.5, 10.5 1H), 6.14 (dd, *J*= 8.0, 3.5 1H). 6.10 (m, 1H), 6.05 (dd, *J*= 12.0, 5.5 1H), 6.04 (m, 1H), 5.97 (dd, *J*= 15.0, 7.5 1H), 5.93 (dd, *J*= 15.5, 10.5 1H), 5.91 (dd, *J*= 15.5, 10.5 1H), 5.82 (dd, *J*= 15.0, 7.5 1H), 5.76 (ddd, *J*= 17.5, 10.5, 7.0 1H), 5.32 (dd, *J*= 17.5, 1.0 1H), 5.23 (dd, *J*= 10.5, 1.0 1H), 1.51 (d, *J*= 6.5 1H). The

molecular formula of *R*-MTPA ester was confirmed as $C_{55}H_{50}O_{12}F_{12}$ ([M+Na]+ *m/z* 1153).

***S*-MTPA ester (8) of separacene C (3)**: ^{1}H NMR (600 MHz, pyridine-d_5) δ 6.62 (dd, *J*= 15.5, 10.0 1H), 6.59 (dd, *J*= 15.5, 10.0, 1H), 6.27-6.25 (m, 2H), 6.13 (dd, *J*= 8.0, 4.0 1H), 6.07-6.05 (m, 2H). 6.02-5.98 (m, 2H), 5.95 (ddd, *J*= 17.0, 10.5, 7.0 1H), 5.86 (dd, *J*= 15.5, 7.5 1H), 5.84 (dd, *J*= 15.5, 7.5 1H), 5.52 (d, *J*= 17.0 1H), 5.35 (d, *J*= 10.5 1H), 1.57 (d, *J*= 6.5 3H). The molecular formula of *S*-MTPA ester was confirmed as $C_{55}H_{50}O_{12}F_{12}$ ([M+Na]+ *m/z* 1153).

***R*-MTPA ester (9) of separacene C (3)**: ^{1}H NMR (600 MHz, pyridine-d_5) δ 6.54 (m, 1H), 6.50 (m, 1H), 6.29-6.27 (m, 2H), 6.18 (dd, *J*= 6.5, 5.0 1H), 6.12 (dd, *J*= 11.0, 6.0 1H). 6.10 (dd, *J*= 11.0, 6.0 1H), 6.05 (dd, *J*= 7.0, 7.0 1H), 5.99 (m, 1H), 5.96 (dd, *J*= 17.0, 10.5 1H), 5.90 (ddd, *J*= 15.0, 10.5, 7.0 1H), 5.59 (dd, *J*= 7.5, 2.0 1H), 5.57 (dd, *J*= 7.5, 2.0 1H), 5.47 (d, *J*= 17.0 1H), 5.36 (d, *J*= 10.5 1H), 1.59 (d, *J*= 6.5 1H). The molecular formula of *R*-MTPA ester was confirmed as $C_{55}H_{50}O_{12}F_{12}$ ([M+Na]+ *m/z* 1153).

Antibacterial activity assay. Gram-positive bacteria (*S. aureus* ATCC 6538p, *B. subtilis* ATCC 6633, *M. luteus* IFO 12708) and Gram-negative bacteria (*S. typhimurium* ATCC 14028, *P. vulgaris* ATCC 3851, *E. coli* ATCC 35270) were used for antimicrobial activity tests. Bacteria were grown overnight in Luria Bertani (LB) broth at 37℃, harvested by centrifugation, and then washed twice with sterile distilled water. Stock solutions of separacene A-C (**1-3**) were prepared in DMSO.

Each stock solution was diluted with m Plate Count Broth (Difco) to prepare serial twofold dilution in the range of 50 to 0.8 μg/mL. Ten microliters of the broth containing about 10^5 colony-forming units (cfu)/mL of test bacteria was added to each well of a 96-well microliter plate. Culture plates were incubated for 12h at 37℃. The minimum inhibitory concentration (MIC) values were determined as the lowest concentration of test compound that inhibited bacterial growth. Ampicillin was used as a reference compound. Ampicillin inhibited *B. subtilis* ATCC 6633 with the MIC values of 0.39 μg/mL and *P. vulgaris* ATCC 3851 with the MIC values of 1.39 μg/mL.

ICL activity assay. The method is for spectrophotometrically measuring the formation of glyoxylate phenylhydrazone at 324 nm in the presence of phenylhydrazine and isocitrate. The enzyme reaction mixutre (1 mL) including 1.27 mM threo-*DS* (+) isocitrate, 3.75 mM $MgCl_2$, 4.1 mM phenylhydrazine, 20 μM sodium phosphate buffer (pH 7.0), 2.5 mg/ml of purified ICL. The reaction was performed at 37℃ for 30 min. Protein concentration was measured by using the method of Bradford with the Bio-Rad protein assay Kit (Bio-Rad, USA) and bovine serum albumin as standard. 3-nitropropionic acid was used as a positive control, inhibiting ICL with the IC_{50} value of 1.35 μg/mL.

Evaluation of anti-proliferative activity by SRB. The effect of separacene A-C (**1-3**) on the cell proliferation was evaluated by sulforhodamine B (SRB) cellular

protein-staining method with some modifications. Briefly, A549 (lung cancer), HCT116 (colon cancer), K562 (leukemia) cells (1×10^4 cells in 190μl of complete DMEM) were seeded in 96-well plate with various concentrations of separacene A-C (**1-3**) and incubated at 37℃ in a humidified atmosphere with 5% CO_2. After 72h of separacene A-C (**1-3**) treatment, cells were fixed with 10% TCA solution for 1g and stained cellular proteins with 0.4% SRB in 1% acetic acid solution. Stained cells were dissolved in 10mM Tris buffer (pH 10.0). The effect of separacene A-C (**1-3**) on cell viability was calculated as a percentage, relative to solvent-treated control and the IC_{50} values were calculated using nonlinear regression analysis (percent survival *versus* concentration).

Conclusion. Chemical analysis of marine actinomycetes strains isolated from Shinyang Beach in Jeju Island in Korea resulted in the isolation of three new linear polyene polyketide-derived natural products, separacenes A-C (1-3). The structures of the separacenes were determined by spectroscopic analysis of NMR, MS, and UV spectra and chiral derivatization based on modified Mosher's method. The separacences are structurally unique by bearing characteristic a terminal olefinic double bond, tetraene or triene, and two separated diol groups. Separacene A inhibited the HCT116 cancer cell line at the concentration of 52.67 μM and the A549 cancer cell line at the concentration of 141.6 μM. The discovery of the separacenes from a marine-derived actinomycetes strain SNW10, *Streptomyces* sp.,

provides additional evidence that marine actinomycetes are promising resources for new bioactive natural products with therapeutic significance.

GCGTGGGGGGCCATCTTTACCATGCAGTCGAACGATGAAATCACTTCGGT
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GGUAACACCCGAAGCCGGUGGCCCAACCCCUUGUGGGAGGGAGCGUC
GAAUAGGGGAUUUGCCCG

Figure 6. 16S rDNA gene sequences data of *Streptomyces* sp. SNW10.

Figure 7. ^{1}H NMR spectrum of separacene A (**1**) at 900MHz in pyridine-d_5.

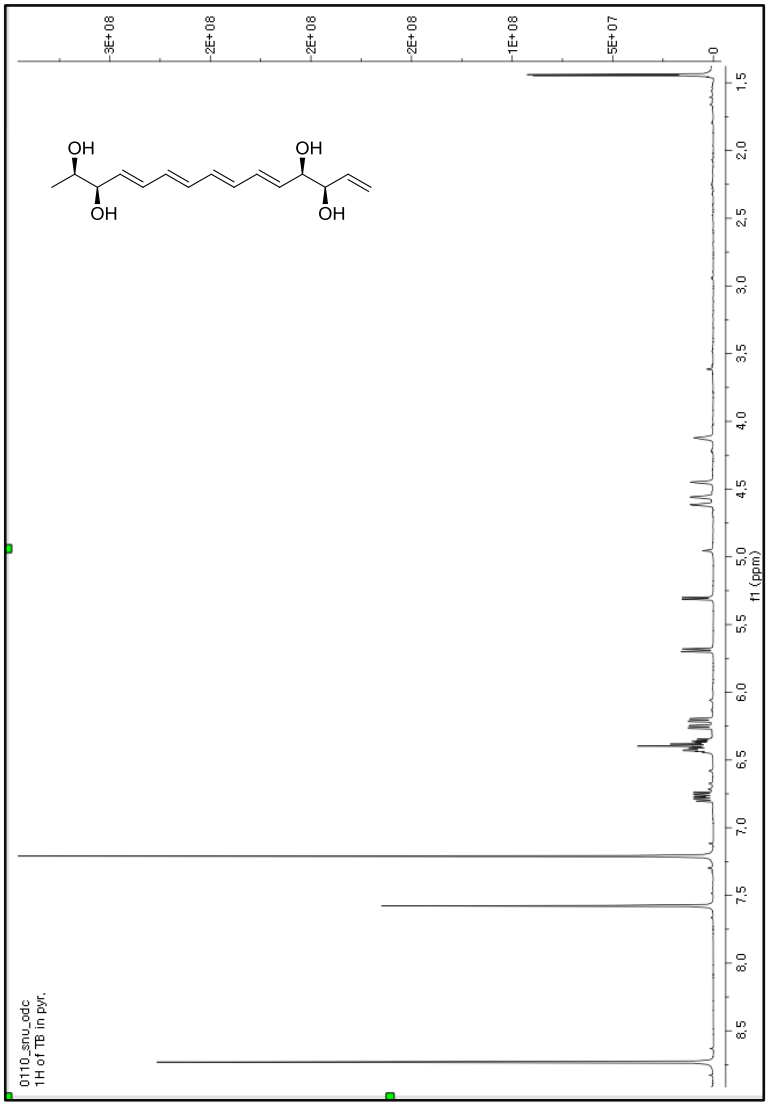


Figure 8. ^{13}C NMR spectrum of separacene A (**1**) at 225MHz in pyridine-d_5.

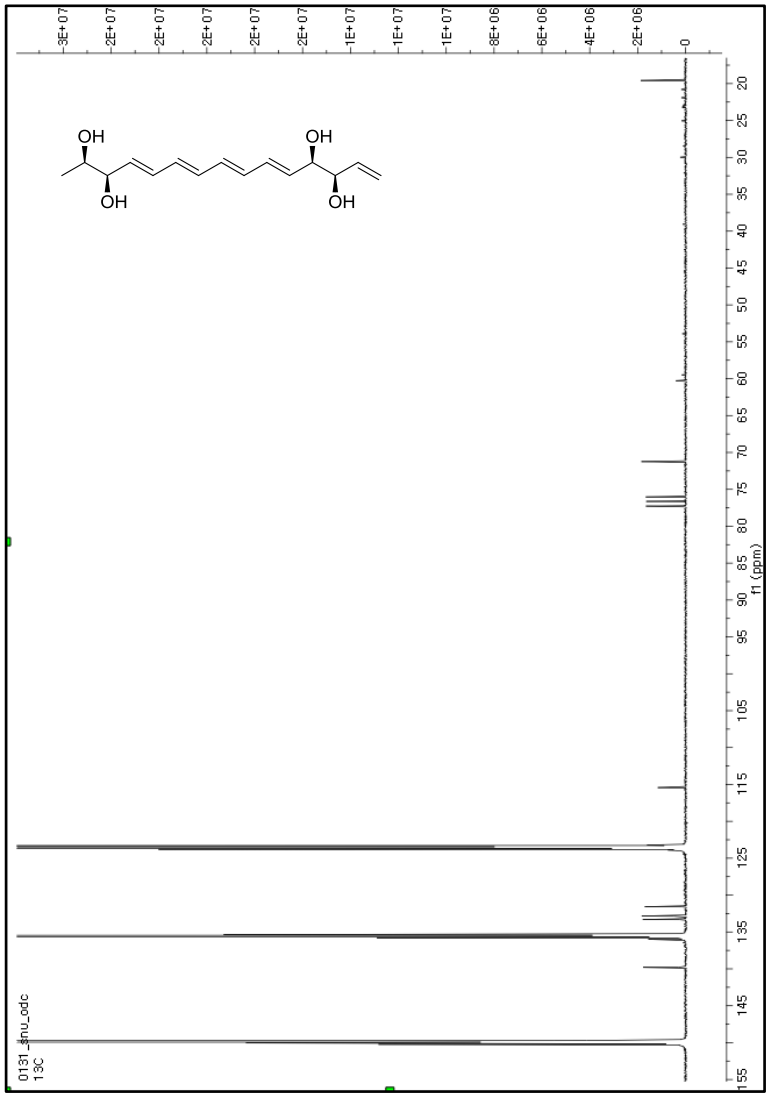


Figure 9. ^{1}H-^{1}H COSY spectrum of separacene A (**1**) at 900MHz in pyridine-d_5.

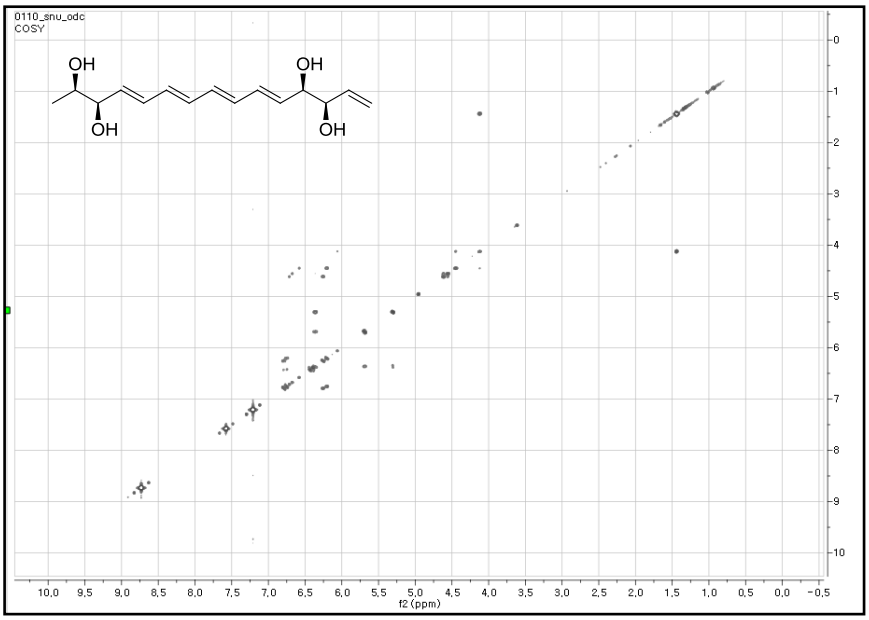


Figure 10. HSQC spectrum of separacene A (**1**) at 900MHz in pyridine-d_5.

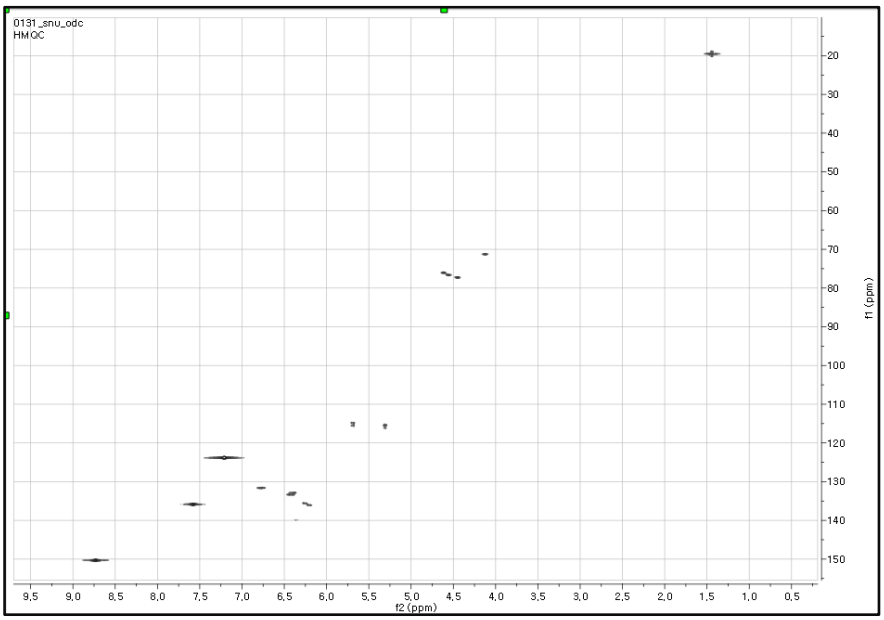


Figure 11. HMBC spectrum of separacene A (**1**) at 900MHz in pyridine-d_5.

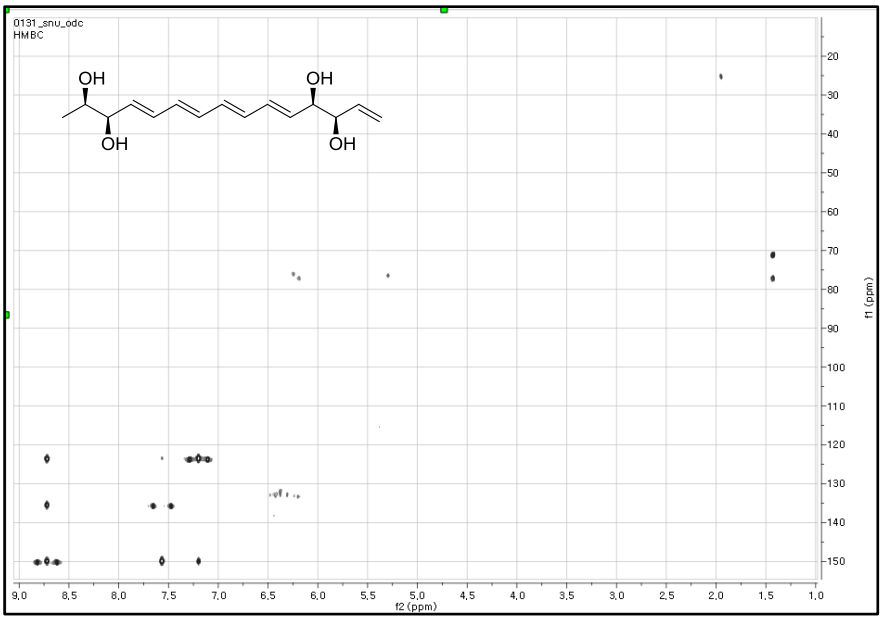


Figure 12. NOESY spectrum of separacene A (**1**) at 900MHz in pyridine-d_5.

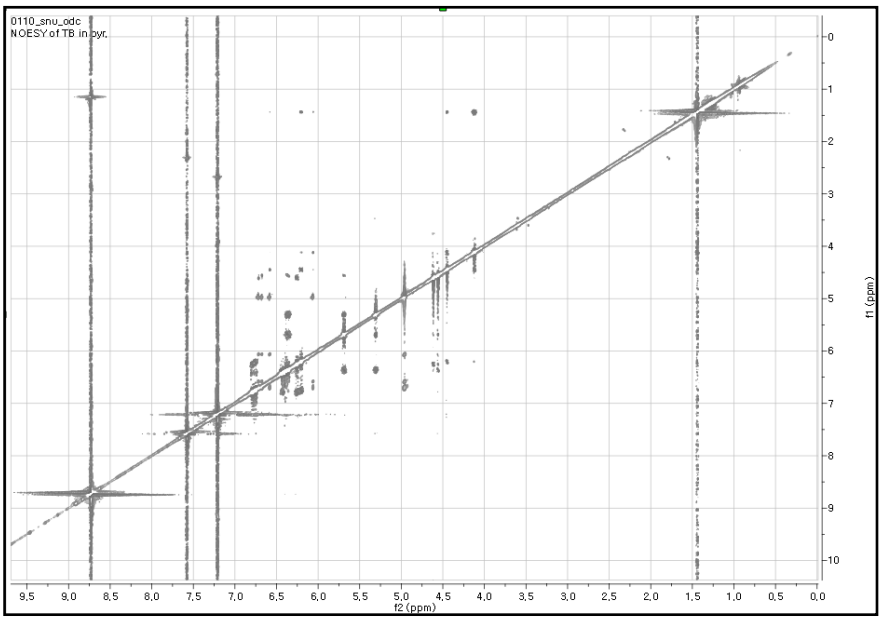


Figure 13. ^{1}H NMR spectrum of separacene B (**2**) at 600MHz in pyridine-d_5.

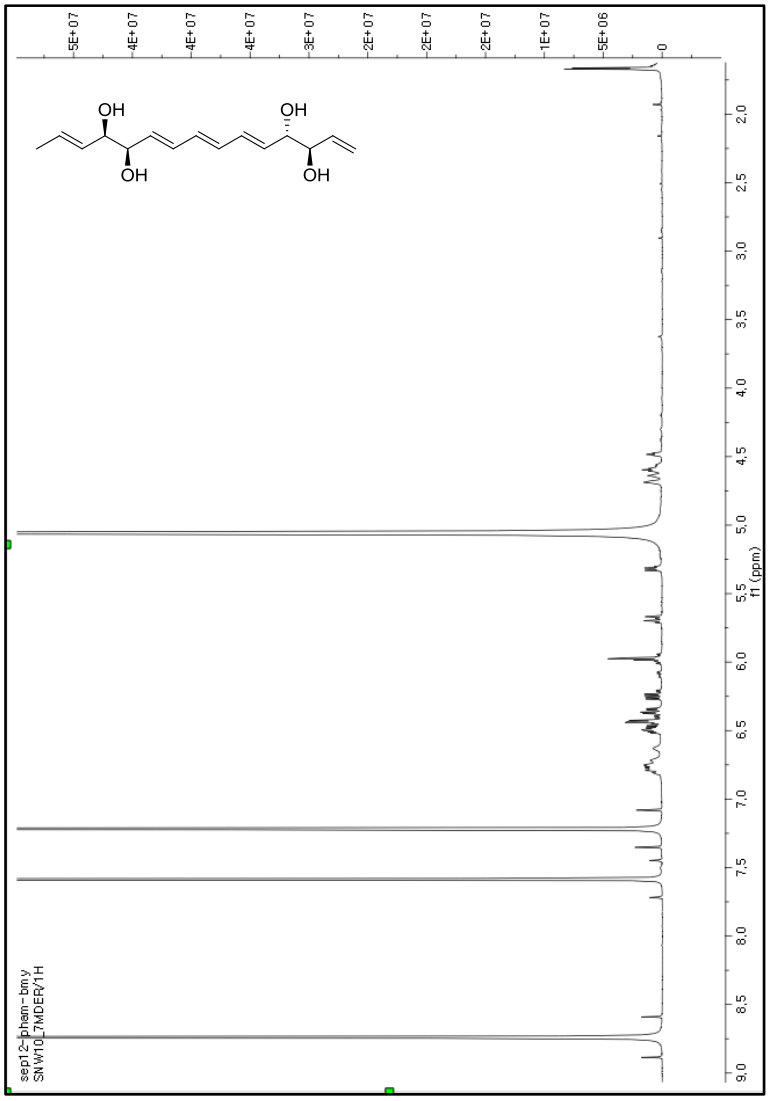


Figure 14. ^{13}C NMR spectrum of separacene B (**2**) at 125MHz in pyridine-d_5.

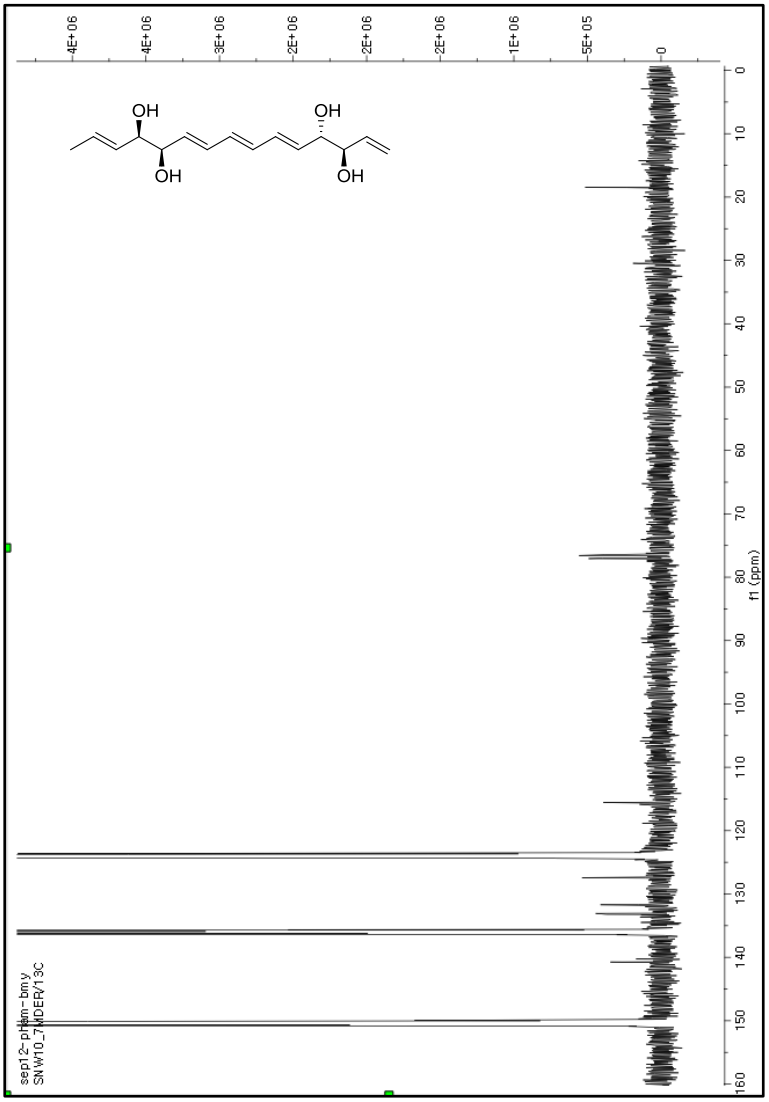


Figure 15. ^{1}H NMR spectrum of separacene C (**3**) at 600MHz in pyridine-d_5.

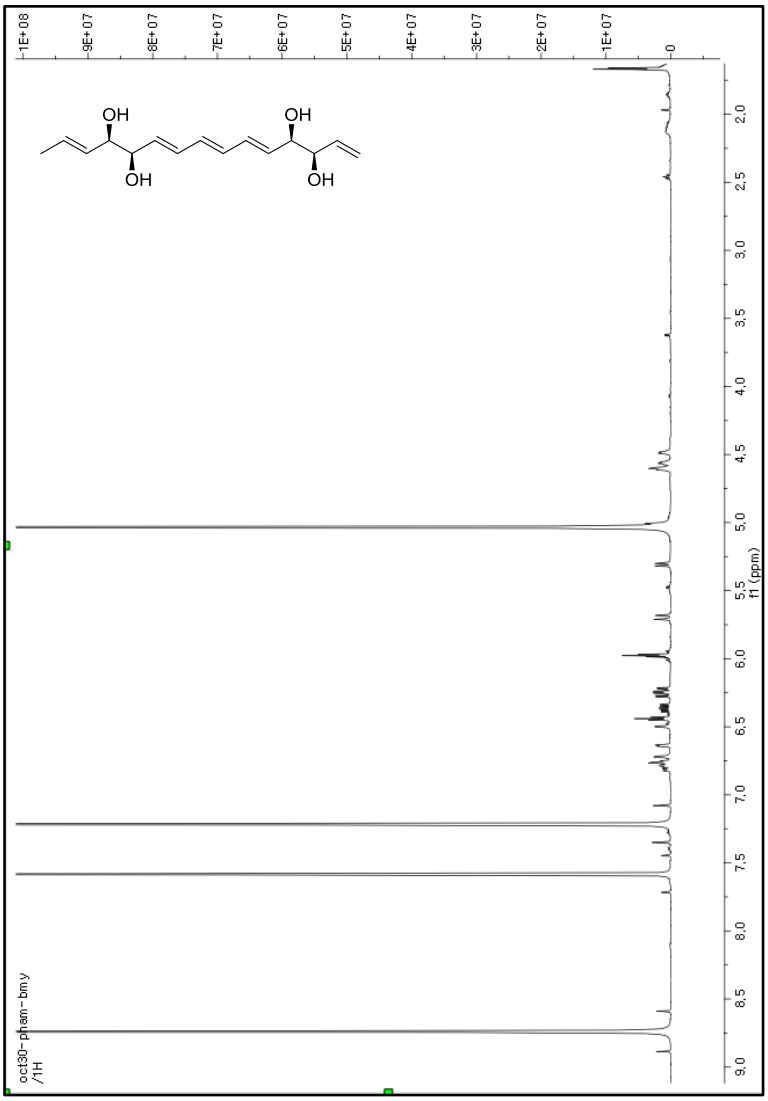


Figure 16. ^{13}C NMR spectrum of separacene C (**3**) at 600MHz in pyridine-d_5.

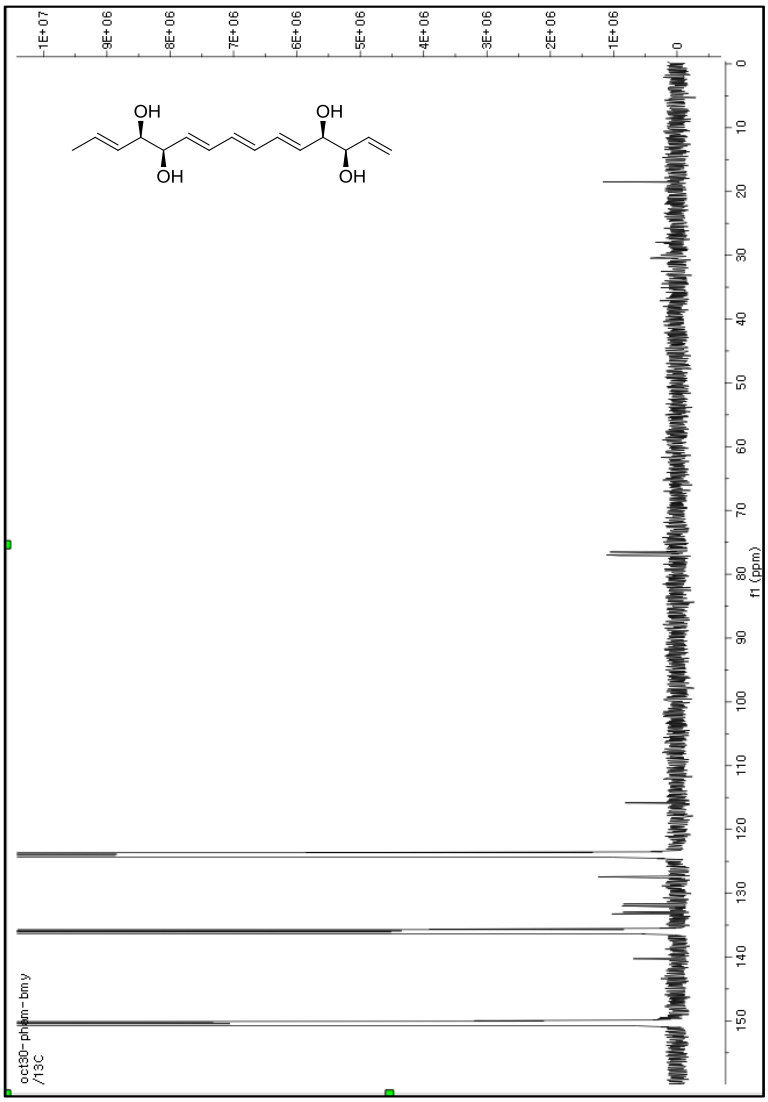


Figure 17. ^{1}H NMR spectrum of *S*-MTPA ester (**4**) for separacene A (**1**) at 600MHz in pyridine-d_5.

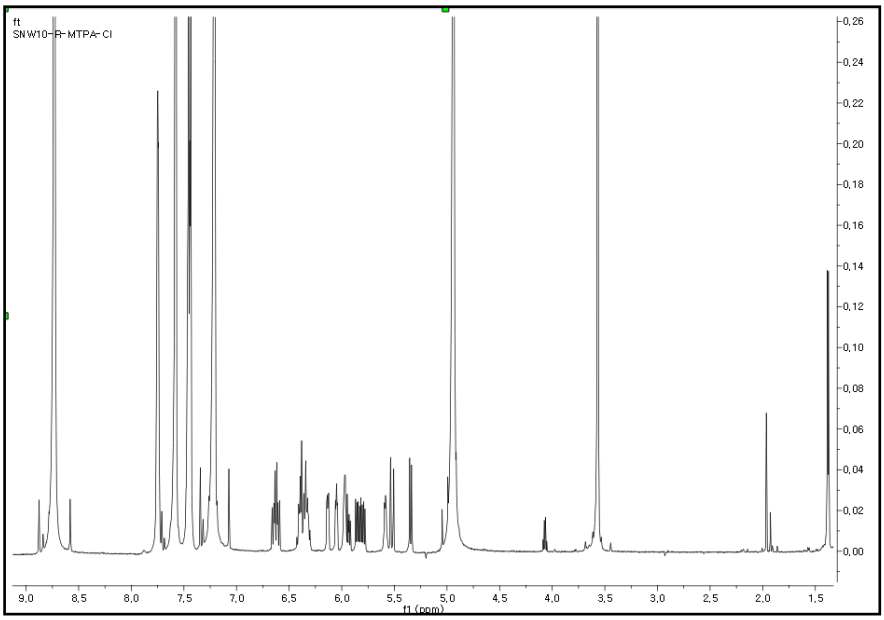


Figure 18. ^{1}H NMR spectrum of *R* MTPA (**5**) ester for separacene A (**1**) as at 600MHz in pyridine-d_5.

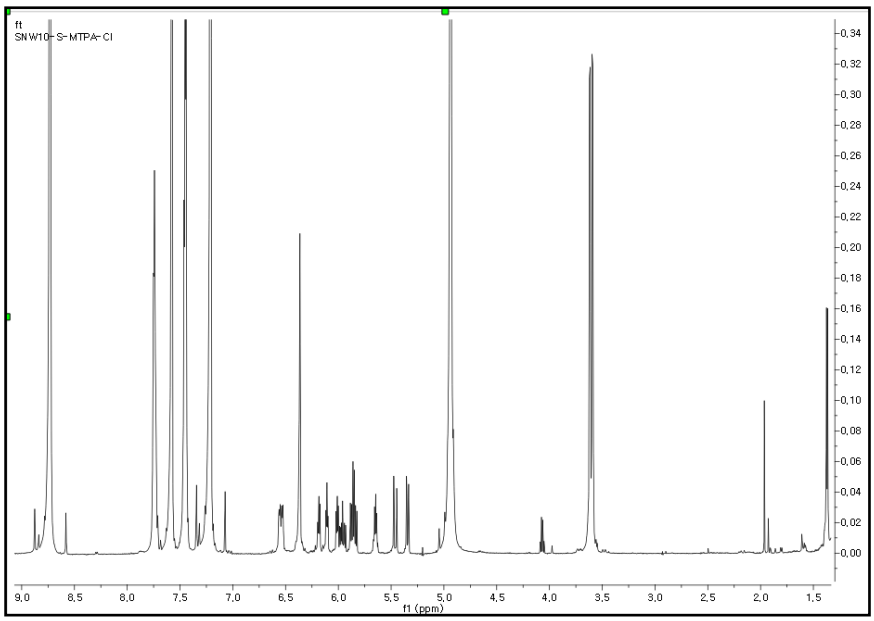


Figure 19. [1]H NMR spectrum of *S* MTPA ester (**6**) for separacene B (**2**) as at 600MHz in pyridine-d_5.

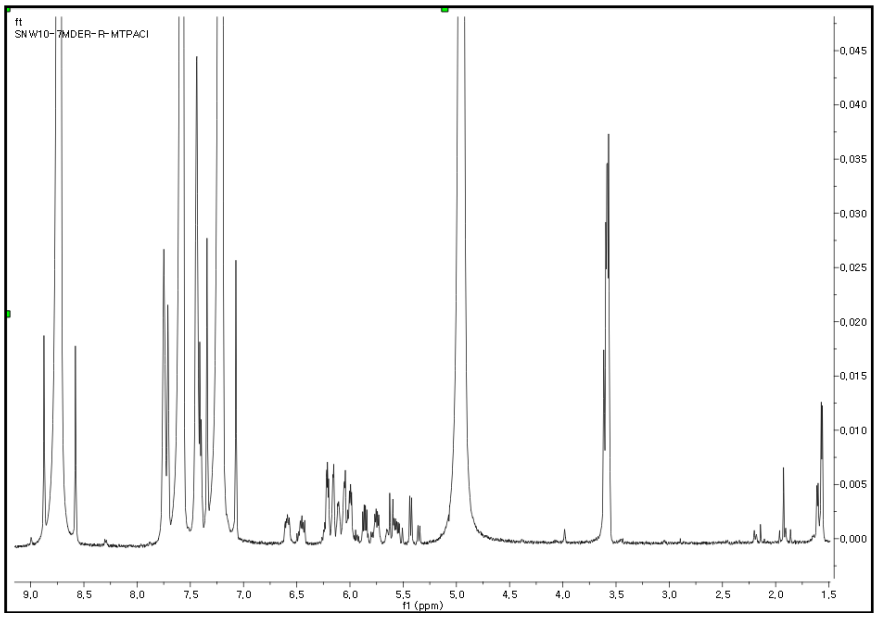


Figure 20. [1]H NMR spectrum of *R* MTPA ester (**7**) for separacene B (**2**) at 600MHz in pyridine-d_5.

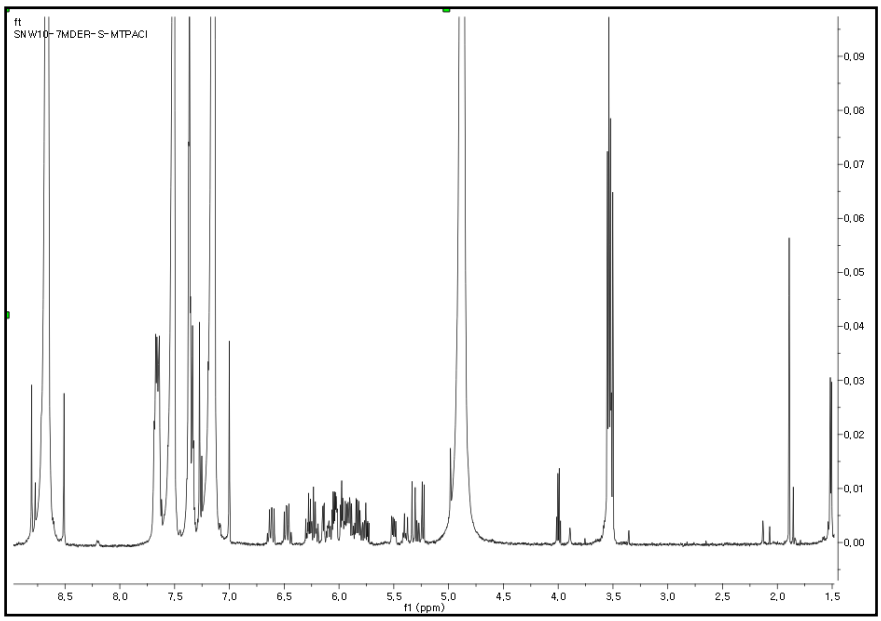


Figure 21. ^{1}H NMR spectrum of *S* MTPA ester for separacene C (**3**) at 600MHz in pyridine-d_5.

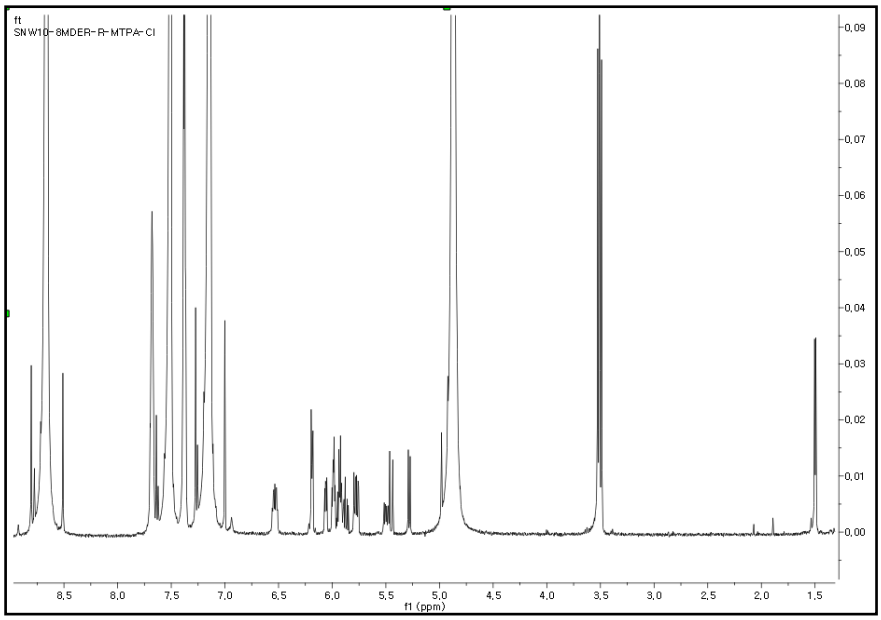
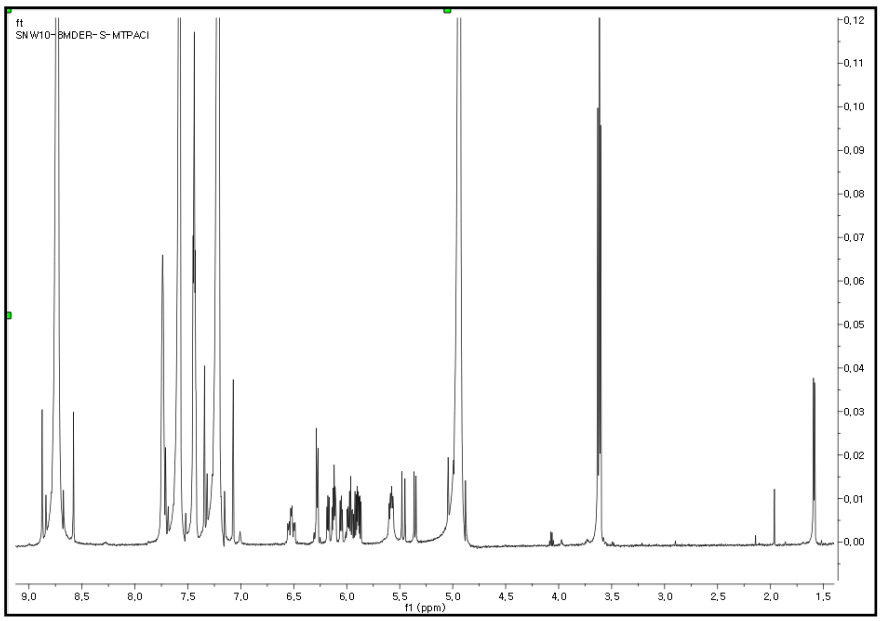


Figure 22. ^{1}H NMR spectrum of *R* MTPA ester for separacene C (**3**) at 600MHz in pyridine-d_5.



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국문초록

Separacene A-C, 해양퇴적물 유래미생물로부터 분리한 linear polyketides

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천연물과학전공

제주도의 신양 해수욕장 모래사장에서 채집된 퇴적물로부터 분리한 방선균에서 새로운 15 membered linear polyketides, separacene A-C 를 얻었다. Separacene A 는 구조의 끝에 olefinic double bonds 를 가지고 있는 특징을 보이며 2 개의 diol 사이에 4 개의 연속된 double bonds 를 포함하는 것으로 규명되었다. separacene B 와 C 는 2 개의 diol 을 지니고 있으나 separacene A 와는 다르게 3 개의 연속된 double bonds 를 포함하며 서로 streoisomer 형태임이 밝혀졌다. 이 평면구조의 결정을 위해 1D, 2D NMR, Mass, IR, UV spectra 가 사용되었고 세 물질의 입체 구조는 modified mosher's reaction 을 통하여 규명하였다. Separacene A 는 암세포의 일종인 HCT116 cell 과 A549 cell 에 대해 약한 저해 활성을 보였으나 separacene B 와 C 는 특별한 활성을 보이지 않았다.