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Isolation, physico-chemical characterization and biological evaluation of compounds showing anti-microbial and immunomodulatory activities derived from *Streptomyces* sp. CS392

조선대학교 대학원

약 학 과

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Streptomyces sp. CS392 균주에서 분리한 항균, 면역 조절물질의 이화학적 특성 및 생물학적 활성 평가

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이 논문을 약학 박사학위신청 논문으로 제출함

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List of Abbreviations

DMSO	Dimethylsulfoxide
EaOAc	Ethylacetate
ELISA	Enzyme-linked immunosorbent assay
ERK1/2	Extracellular signal-related kinase1/2
FBS	Fetal bovine serum
H&E	Hematoxylin and Eosin
IκBα	Inhibitor κBα
IL	Interleukin
LDH	Lactate dehydrogenase
MAPK	Mitogen-activated protein kinase
MTT	3-(4,5-dimethylthiazol-2--2,5-diphenyltetrazolium bromide
NF-κB	Nuclear factor kappaB
PBS	Phosphate buffered saline
PMSF	Phenylmethylsulfonylfluoride
PVDF	Polyvinylidene difluoride
ROS	Reactive oxygen species
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
TNF-α□	Tumor necrosis factor-alpha

Abstract in Korean

Streptomyces sp. CS392 균주에서 분리한 항균, 면역 조절물질의 이화학적 특성 및 생물학적 활성 평가

조승식

지도교수: 유진철

조선대학교 대학원 약학과

한국 토양 방선균에서 항균, 면역조절 물질을 개발 하고자 국내 전남 등지에서 채취한 토양 및 식품유래 미생물 약 900 여종을 대상으로 항균, 면역조절 물질을 생산하는 균주를 스크리닝 하였다. 수 종의 후보 균주 중 항균효과를 가지는 CS392 균주의 에틸 아세테이트 추출물이 LPS (lipopolysaccharide)로 자극한 면역세포에서 nitric oxide 생성 억제효과를 가지며, 항산화 효과가 있음을 확인하였다.

선별한 CS392 균주는 유전학적, 배양학적, 생화학적 분석을 통하여 신규한 균주임을 확인하고 *Streptomyces* sp. CS392 라 명명하였다. 이 균주의 최적 배양조건은 탄소원으로써 glucose(1% w/v), 질소원으로써 peptone(1%

w/v), magnesium chloride(0.01% w/v), pH 7.0, 28℃ 였다. 최적배지에서 배양 후 얻은 발효액은 유기용매로 추출 후 silica gel, RP-18 silica gel column 을 수행하여 3 종의 물질을 정제하여 구조분석을 수행하였다. 구조분석 결과 주 물질은 manumycin group metabolite 인 manumycin 이었다. C1, C2 는 구조분석 중에 있다.

Manumycin 과 C1, C2 의 약리효과를 조사하였다. 세가지 물질은 그람 양성세균, 메티실린 내성 황색포도상 구균, 반코마이신 내성 장구균에 유효함을 확인하였다. 세가지 물질은 nitric oxide 소거능력과 환원력을 보여 항 산화능도 일부 가지고 있음을 확인하였다. Manumycin 및 C1, C2 는 90℃, 4 시간까지 처리시 활성이 거의 감소하지 않아 열에 안정함을 보였으며, 121℃, 15pound, 15 분 처리시 약간의 활성이 감소하였다. 또한 CS392 균주 배양액은 pH 3 에서 9 까지 안정함을 보였다.

Murine macrophage 인 Raw 264.7 cell 을 LPS (lipopolysaccharide)로 자극하여 nitric oxide 와 pro-inflammatory cytokine 생성을 유도하였을 때 세가지 물질은 모두 nitric oxide 와 pro-inflammatory cytokine 생성을 1-20 μg/ml 농도 범위에서 유의하게 억제함을 확인하였다. Stimuli 와 약물을 처리한 세포에서 얻은 단백질은 웨스턴 블럿을 수행하여 iNOS 단백질 발현이 억제됨을 확인하였다. 또한 NF-kB 의 핵 내로의 이동을 저해함을 확인하여 결과적으로 nitric oxide 나 염증성 cytokine 의 생성 억제는 NF-kB 와 연관이 있음을 확인하였다.

Manumycin 과 C1 은 SNP(sodium nitroprusside)가 방출한 nitric oxide 를 제거하였으며, iNOS 발현 억제를 통해 nitric oxide 생성을 억제하여 dual effector 로써 NO 생성 반응을 억제하여 면역세포에서 염증 반응을 효율적으로 감소시킬 것으로 생각되었다.

Manumycin group metabolite 들이 *in vivo* 에서 효과가 있는지 확인하기 위해 mouse ear edema model 과 DSS (Dextran sodium sulfate) colitis model 에서 항염증 효과를 조사하였다. 마우스 귀부종은 phorbol ester 인 TPA 를 이용하여 염증을 유도하였으며, 세가지 물질은 같은 양(50 μg/ear)을 단회 도포시 manumycin>C1>C2 의 순으로 염증 억제 효과를 보였다. 대조구 및 약물 처리군의 마우스 귀 조직의 iNOS 단백질의 발현 양상을 확인한 결과 약물 처리군은 양성, 음성 대조군과 비교시 유의한 발현억제 양상을 보였다.

DSS (Dextran sodium sulfate)로 유도된 장염모델은 IBU(intestinal bowel disease)의 대표적인 동물 모델이다. ICR mouse 에 DSS (Dextran sodium sulfate)를 자유 급여하며, 복강 내 투여로 약물을 1 일 1 회 1 주일간 투여하며 마우스를 관찰하며 DAI (Disease activity index)와 체중변화를 측정하였으며, 7 일째에 colon 을 적출하여 colon 의 조직학적 변화를 관찰하고, colon 길이 및 colon 조직에서 pro-inflammatory cytokine, TNF-α 생성량을 측정하였다. 세 물질 중 C2, manumycin 이 DSS 에 의해 증가된 염증조직을 완화시켰고, DAI 를 유의하게 감소시켰으며, DSS 에 의해

짧아지는 colon length 의 길이도 1-10 mg/kg 에서 농도에 의존하여 정상 colon 과 유사하게 회복시켰으며, colon tissue 에 증가되었던 pro-inflammatory cytokine, TNF-α 도 감소시켰다. 또한 iNOS 단백질의 발현도 유의적으로 감소하였다.

본 연구를 통해 manumycin group metabolite 가 항생제 내성 세균에 광범위한 항균 효과를 가지며, 항 산화효과로써 환원력, NO 소거능을 가지는 것을 확인하였다. 또한 *in vitro* macrophage cell 에서 manumycin 과 유도체들의 iNOS 발현억제, NF-kB translocation 억제 및 pro inflammatory cytokine 생성 억제 효과와 급성 귀 부종, 장염을 유발시킨 *in vivo* 염증 모델에서 항 염증 효과를 가져 *in vitro, in vivo* 모델에 모두 효과적임을 설명하였다. 향후 이들의 작용기전을 보다 더 자세하게 연구하면, manumycin group metabolite 의 소염작용과 그 구조와의 상관성을 이해할 수 있고, 과량의 nitric oxide 및 pro-inflammatory cytokine 의 생성과 관련한 면역질환의 이해와 치료에 도움을 줄 것이라 사료된다.

ABSTRACT

Isolation, physico-chemical characterization and biological evaluation of compounds showing anti-microbial and immunomodulatory activities derived from *Streptomyces* sp. CS392

By Seung Sik Cho
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With the goal of searching potent immonomodulatory substance with antimicrobial activity against various resistant pathogens, such as methicillin resistant *Staphylococcus aureus* (MRSA), strain CS392 was screened from several hundreds of actinomycetes strains preserved in our laboratory. Ethylacetate extract of fermentation broth of CS392 strain showed antimicrobial activity and suppressed the nitric oxide production in LPS (lipopolysaccharide) stimulated macrophage cells. Additionally ethylacetate extract showed anti-oxidative activities such as nitric oxide scavenging and reducing activity. Therefore we selected this strain for the detailed study.

Based on the morphological and biochemical characteristic, CS392 was found

related to the genus *Streptomyces*. Further, the 16S rRNA sequence analysis revealed that the strain was closely related to *Streptomyces lanatus* with 98.22% homology; therefore the strain was classified as *Streptomyces* sp. CS392.

The strain was cultivated at 28ºC and 180 rpm for 3 days and the paper disc assay technique was employed to determine the anti-MRSA effect. Antimicrobial compounds production from the strain was optimized by using several nutritional parameters. Glucose, peptone and $MgCl_2$ were found the best carbon, nitrogen and mineral source, respectively for the production of the compounds. After cultivation of CS392 strain under optimized condition for 2 days, fermentation broth was harvested, the antimicrobial compounds were extracted using ethyl acetate (1:1 v/v), and then purified using column chromatography. These compounds were finally purified and identified with reverse-phase HPLC with 60% acetonitrile (0.01% formic acid) linear gradient. Eventually, three different antimicrobial compounds (C1, C2 and C3) were achieved from the strain. Based on NMR, IR, MASS analysis, those compounds were identified as manumycin (C3) and its derivatives (C1 and C2, manumycin group metabolites).

Antimicrobial activity of these compounds against different pathogenic strains was assessed using the minimum inhibitory concentrations (MICs). It was found to be active against eight different strains, including MRSA 693E , MRSA (2-32), MRSA (4-5), MRSA (4-21), *Enterococcus faecalis* (ATCC 29212), VRE 82, VRE 89, VRE 98, *Bacillus subtilis* (ATCC 1928), *Staphylococcus aureus* (KCTC 1928) and *Mycobacterium smegmatis* (ATCC 9341). Antimicrobial activity of these compounds

were stable in desirable pharmaceutical processing conditions such as heat (up to 90 ℃/ 4 hr, 121℃ /15 pound, 15 min), freezing (-20℃) and pH treatment (pH 3 to 9). To evaluate the chemical stability of compounds, a study was designed to assess the *in vitro* endurance and effects of media and storage conditions.

These compounds were found to inhibit the production of tumor necrosis factor-a (TNF-α), interleukin-1 β (IL-1β) and interleukin-6 (IL-6). Manumycin derivatives (C1 and C2) dose dependently inhibited NO production and inducible NO synthase (iNOS) expression in Raw 264.7 macrophages activated with LPS. Besides, C1 and manumycin showed nitric oxide-scavenging effect concentration-dependently. To investigate the signaling pathway for NO inhibition by these compounds, we examined nuclear factor-κB (NF-κB) activation in Raw 264.7 cells. We found that manumycin derivatives inhibited the synthesis of pro-inflammatory cytokines and suppressed LPS-induced NF-κB activation in Raw 264.7 cells. These compounds, hence, deserved to be developed as promising anti-inflammatory drugs after further preclinical and pharmaceutical studies. Furthermore, these compounds were examined for their antioxidative activities with 2,2-diphenyl-1-pikryl hydrazyl (DPPH) radical scavenging assay, total phenolic content, Superoxide dismutase-like activity, super oxide scavenging activity and reducing power. These compounds showed nitric oxide scavenging activity but with slight reducing power. Among three, C3 showed the strongest activity.

Since these compounds inhibited the production of pro-inflammatory cytokines, NO production, and iNOS expression in Raw 264.7 macrophages upon *in vitro* experiments, it was felt necessary to investigate *in vitro* and *in vivo* relationship. Firstly, these compounds were assayed for topical anti inflammatory activity by TPA(12-O-tetradecanoylphorbol-13-acetate)-induced ear edema model in ICR mice. The anti inflammatory activities (% inhibition) in the TPA-induced ear edema test shown by C1, C2 and C3 were 22.1%, 16.7% and 29.2%, respectively. These compounds thus showed slight inhibition in edema formation without any side effects. Using each ear punch biopsies, protein samples were taken from ear whole cell lysate and further effect of these compounds on iNOS expression was investigated by western blot analysis. Three compounds were found to inhibit TPA-induced expression of iNOS.

In conclusion, these compounds have topical anti inflammatory activity mediated *via* inhibition of iNOS expression. These findings thus to gives a clear reason for the use of these compounds in the treatment of inflammation.

Secondly, we investigated protective effect of these compounds on a mice model of colitis. Colitis was induced in ICR mice by administration of dextran sulfate sodium (DSS) (3%, w/v) in drinking water. DSS colitis model was well characterized morphologically and biochemically. DSS produced decrease in colon length accompanied by mucosal edema and bloody stool. To assess the dose effect on DSS-

induced colitis, 1, 3 and 10 mg/kg/day of compounds were administered by intraperitoneal injection. Besides measuring colon length, we have estimated disease activity index (DAI) for a week, taking into account of body weight, stool consistency and gross bleeding. Pro-inflammatory mediators such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1 β), and interleukin-6 (IL-6), were determined using immunoassays. Among three compounds, manumycin and C2 notably prevented shortening of colon length and reduced DSS-induced DAI scores including decrease in weight loss, diarrhea and gross bleeding. Manumycin found to suppress iNOS expression and abnormal secretions of pro-inflammatory cytokines, such as TNF-α, interleukin-1β and IL-6.

In conclusion, three immunomodulatory compounds with strong antimicrobial activity were purified from a new *Streptomyces* strain isolated from Korean soil. One of the purified compounds was identified as manumycin and two others were as its derivatives. The results drawn from this study provide a scientific basis for the application of these compounds as antimicrobial anti-inflammatory agent. Further studies should/will be undertaken to explain the mechanism of action and roles of immune response by which these compounds exert their biological activity.

Ⅰ. Introduction

A. Production, isolation and purification of biologically active compounds from *Streptomyces* strains

Actinomycetes are important producers of antibiotics and other medicinal sources useful secondary metabolites such as antibiotics, antitumor agents and anti-inflammatory agents (1-5). *Streptomycetes* the Gram positive filamentous bacteria constituting a significant component of the microbial population in most soils. *Streptomycetes* can produce antibiotics typically. These bacteria produce about 75% of commercially and medically useful antibiotics and approximately 60% of antibiotics which have been developed for agriculture use (6-8). *Streptomyces* produce various types of antibiotics such as aminoglycosides, anthracyclins, glycopeptides, beta-lactams, macrolides, nucleosides, peptides, polyenes, polyethers and tetracyclines (Figure. 1) (9).

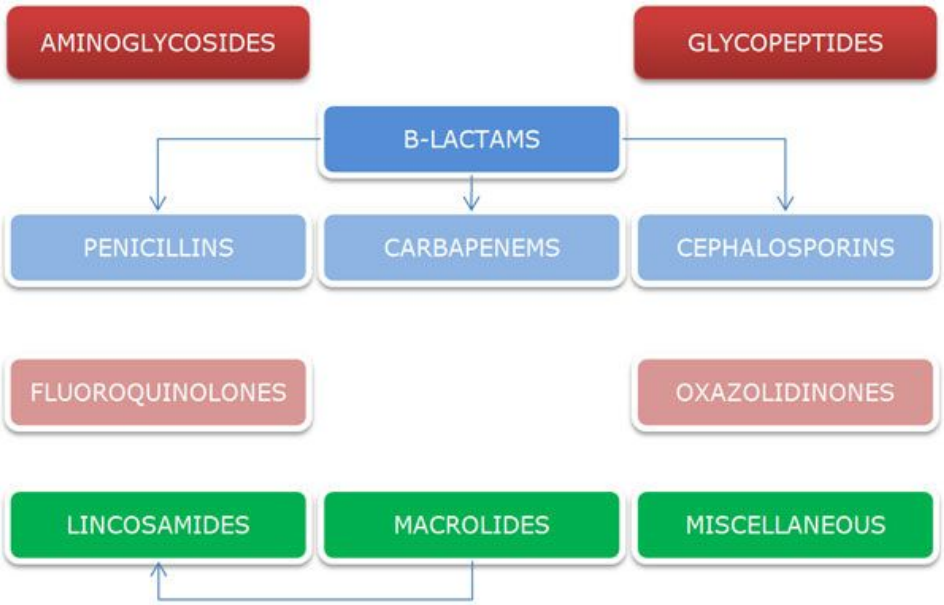

Figure 1. Classification of Antibiotic

The widespread use of antibiotics of medicine is playing a significant role in the emergence of resistant bacteria (10). They are often used in human, animals and in other industries which at least in the case of agricultural use lead to the spread of resistant strains to human populations. In human medicine the major problem of the emergence of resistant bacteria is due to misuse and overuse of antibiotics (11-12). Also unsound practices in the pharmaceutical manufacturing industry can contribute towards the likelihood of creating antibiotic resistant strains (13).

Staphylococcus aureus (colloquially known as "Staph aureus" or a *Staph infection*) is one of the major resistant pathogens. Found on the mucous membranes and the human skin of around a third of the population, it is extremely adaptable to antibiotic pressure. Methicillin and oxacillin is the antibiotic of choice. MRSA (methicillin-resistant *Staphylococcus aureus*) was first detected in Britain in 1961 and is now "quite common" in hospitals. About 50% of *S. aureus* isolates now show resistance to methicillin in areas of the USA and some European countries (14-17).

This left vancomycin as the only effective agent available at the time. However, strains with intermediate (4-8 μg/mL) levels of resistance, termed GISA (glycopeptide intermediate *Staphylococcus aureus*) or VISA (vancomycin intermediate *Staphylococcus aureus*), began appearing in the late 1990s. The first identified case was in Japan in 1996, and strains have since been found in hospitals in England, France and the US. The first documented strain with complete (>16 μg/mL) resistance to vancomycin, termed VRSA (Vancomycin-resistant *Staphylococcus aureus*) appeared in the United States in 2002 (18-24).

A new class of antibiotics, oxazolidinones, became available in the 1990s, and the first commercially available oxazolidinone, linezolid, is comparable to vancomycin in effectiveness against MRSA. Recently, Linezolid-resistance in *Staphylococcus aureus* was reported (Figure 2)(17, 25).

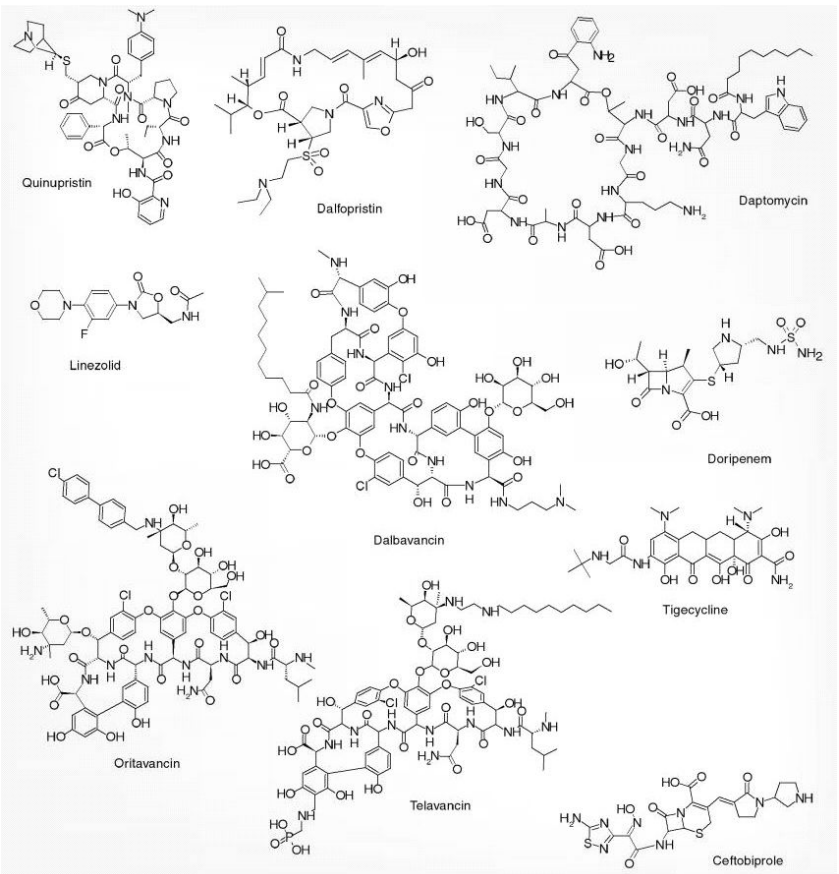

Figure 2. Novel antibiotics. chemical structure of antibiotics that have been approved or are currently under development for the treatment of serious MRSA infections.

Enterococcus faecium is another infectious strain found in hospitals. Vancomycin-resistant enterococcus (26) in 1987, and Linezolid-Resistant Enterococcus (LRE) in the late 1990s. VRE (vancomycin-resistant enterococcus) have rapidly disseminated worldwide (27-28). A surveillance study conducted in US hospitals from 1995 through to 2002 showed that 9% of nosocomial bloodstream infections were caused by enterococci and that 2% of *E. faecalis* isolates and 60% of *E. faecium* isolates were vancomycin-resistant(Figure. 3)(29).

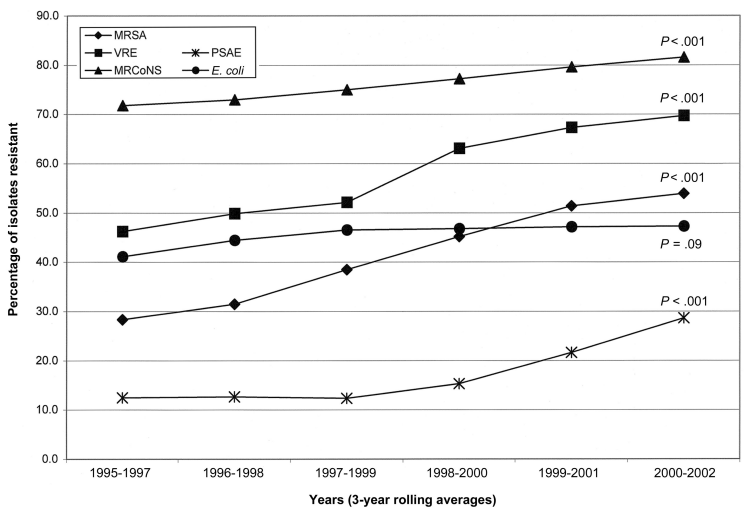

Figure 3. Comparison of antimicrobial resistance rates of antibiotic resistant bacteria. Rates of antimicrobial resistance rates over time (3-year rolling average) among gram-positive isolates (methicillin-resistant *Staphylococcus aureus* [MRSA], methicillin-resistant coagulase-negative staphylococci (MRCoNS), vancomycin-resistant *Enterococcus faecium* (26), ampicillin-resistant *Escherichia coli [E. coli],* and ceftazidime-resistant *Pseudomonas aeruginosa* [PSAE]) recovered in a series of 24,179 cases of nosocomial bloodstream infection.

So, Need for the development of new and effective antibiotics is a porioty. We focused on natural organic compounds produced by *Actinomycetes*. And our aim was to find a compound having anti-MRSA, anti-VRSA or anti-VRE activities.

In this study, we report the new *Sterptomyces* strain named *Streptomyces* sp. CS392 from Korean soil. Isolate was identified as a new s*treptomyces* from its 16s rRNA sequences analysis. We purified manumycin derivatives and analysed the structure of these compounds.

The manumycin-group is a small and discrete class of metabolites that to date combines 23 secondary metabolites all of which are of microbial origin. In 1963, the first member of the manumycin-group metabolites, manumycin A **1**, was discovered by an antibacterial screening from a *Streptomyces* strain (30.) Since Zähner and co-workers discovered manumycin, 10 years after, Schröder and Zeeck established the novel structural type of manumycin A (31).

In 1979, asukamycin was found. This was the second compound of manumycin metabolites purified from culture broth of *Streptomyces* strain. This was shortly followed by the discovery of the antibiotics U-62162 and U-56,407 (Figure 4).

The discovery of new manumycins received a boost in the late 1980s with 11 new metabolites reported up till 1995. Particulary fruitful for the growth of this class of compounds was 1996 in which 10 new manumycin-group metabolites were discovered (32).

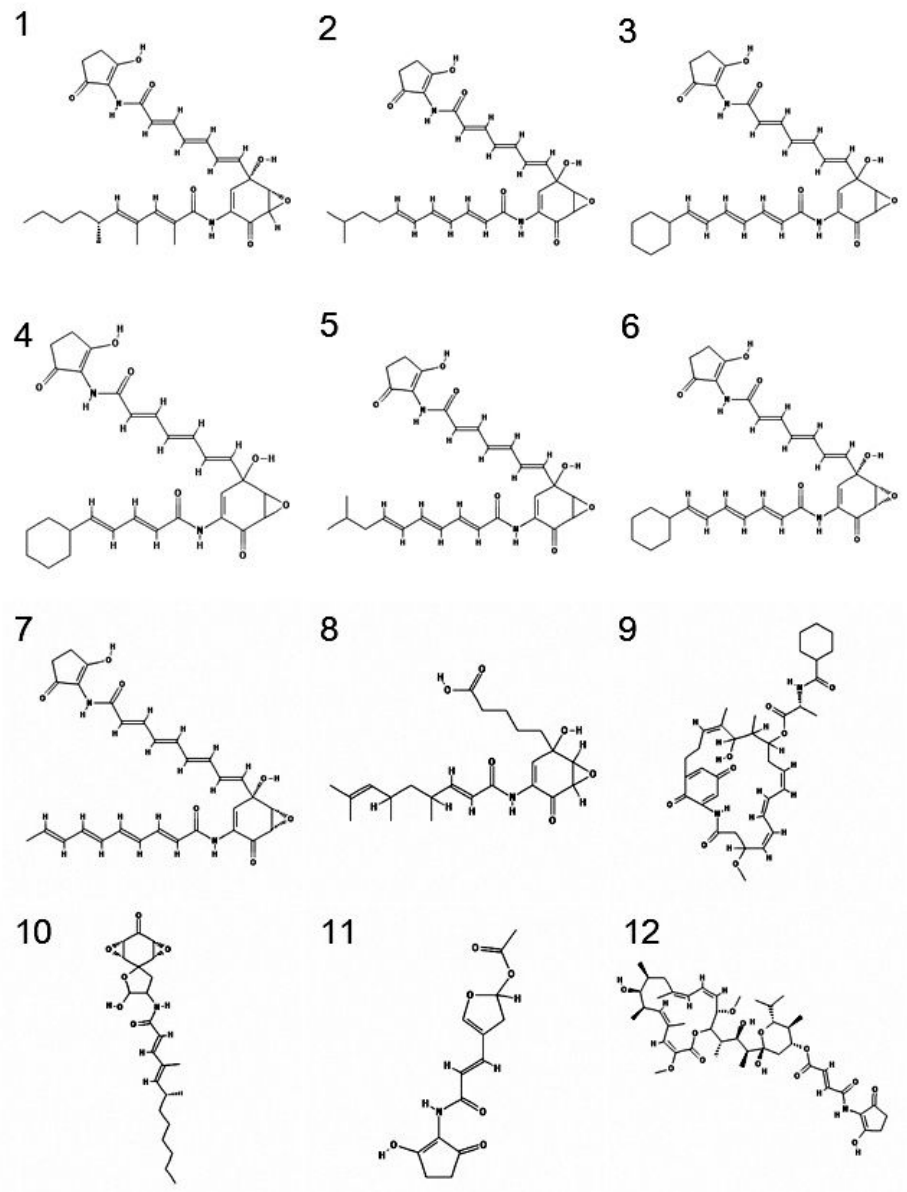

Figure 4. Structure of manumycin group metabolites

1. Manumycin A, 2. Manumycin E , 3. Manumycin F, 4. Alisamycin, 5. U-5640, 6. Asukamycin, 7.Colabomycin A 8. U-62162, 9. Ansatrienin A, 10. Aranorosin, 11. Reductinomycin, 12. Bafilomycin B1

A remarkable interest in manumycins is sparked by the discovery of novel biological activities and is indicated by the commercialization of manumycin A **1** for research purposes (32). All manumycin-group metabolites are produced by microorganisms which have been isolated from soil samples collected worldwide and are all taxonomically characterized as *Streptomycetes*.(i.e *Streptomyces nodosus* ssp. Asukaensis *Streptomyces parvulus* (strain Tu 64): manumycin producer, *Streptomyces actuosus:* alisamycin producer, *Streptomyces* AM-1042: asukamycin producer, *Streptomyces griseoflavus* : colabomycin producer. etc)

In general, manumycin-group metabolites were found by biological screening based on antibacterial activity, antitumor activity, inhibition of farnesyltransferase or inhibition of interleukin-1ß converting enzyme (Table 1) (33).

In an effort to isolate antibacterial compounds effective against resistant pathogens particularly against MRSA, VRE and VRSA from microbial sources, we have focused on *Actinomycetes*. In this study, a new such strain termed as CS392 in our collection, was selected from hundreds of strains preserved in our laboratory. We purified three manumycin group metabolites from strain CS392 and evaluated their efficacy including various characteristics.

Table 1 Producing organisms and the screening programs leading to manumycins (32)

Compound	Producing organism	Screening program	Ref
Manumycin A , B , C , D	*Streptomyces parvulus* (Tü 64)	Antibacterial screening, chemical screening	(30)
Manumycin A (=UCF1-C) , B(=UCF1-A), C (=UCF1-B)	*Streptomyces* sp. (UOF1)	Inhibitor screening with farnesyltransferase	(34)
Manumycin E , F, G	*Streptomyces* sp. (WB-8376)	Antibacterial screening	(35)
Asukamycin	*Streptomyces nodosus* subsp. *asukaensis* (AM-1042)	Antibacterial screening	(36)
Colabomycin A , D	*Streptomyces griseoflavus* (Tü 2880)	Chemical screening	(37)
U-62162	*Streptomyces verdensis* (Dietz, sp. n.; UC-8157)	Antibacterial screening	(38)
U-56,407	*Streptomyces hagronensis* (360; UC 5875)	Antibacterial screening	(39)
Alisamycin	*Streptomyces* sp. (HIL Y-88,31582)	Antibacterial screening	(40)
Nisamycin (=antibiotic 106-B) and alisamycin	*Streptomyces* sp. (K106)	Antibacterial screening	(41-43)
El-1511-3 , -5 , manumycin G ,*ent*-alisamycin , U-56,407	*Streptomyces* sp. (E-1511)	interleukin-1bßconverting enzyme Inhibitor	(44)
El-1625-2 , manumycin B , C	*Streptomyces* sp. (E-1625)	interleukin-1bßconverting enzyme Inhibitor	(44)
TMC-1A , B , C , D , manumycin D(=TMC-1E) , A (=TMC-1F) , G (=TMC-1G)	*Streptomyces* sp. (A-230)	Antitumor screening	(45)
Compound 1	*Streptomyces parvullus*	Antibacterial screening	(46)

B. Biological activities of active compounds

Antioxidant actions of bioactive components

The interest in natural antioxidants has increased considerably in recent years because many antioxidants exhibit beneficial biological effects, including antibacterial, antiviral, anti-allergic, antithrombotic and because they are linked to lower incidence of cardiovascular disease and certain types of cancer disease (47).

Free radicals, especially those of reactive oxygen species are normal products of phagocytic activity and cellular respiration. However, they may also appear under less controlled circumstances and cause reversible or irreversible damage to bio molecules.

Indeed, oxidative damage to lipids, proteins, DNA and other macromolecules has been postulated to be a major type of endogenous damage that is potentially dangerous for the cell. (Figure. 5) (48) This damage may contribute to aging and to degenerative diseases of aging such as brain dysfunction, immune system decline, cataracts, cancers and cardiovascular diseases (48-50).

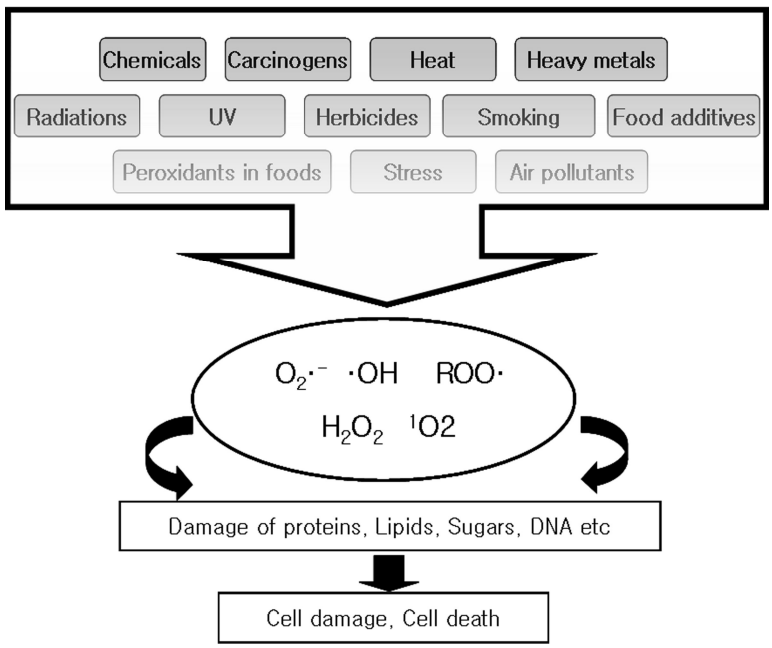

Figure 5. Oxidative damage from Free radicals on healthy cells

Increased free radical production (51-52) or reduced antioxidant defense responses (53) may increase oxidative stress. (54) These processes can induce oxidative damage (55) to membrane lipids, DNA molecules, and proteins. Researchers have screened free radical scavengers (56-57) from microorganisms that may act as therapeutic agents (58-59) for myocardial and cerebral ischemia, atherosclerosis, and inflammation. The purple supernatant (60) of *Streptomyces lincolnensis* M-20 (M-20), a lincomycin producer, showed both antioxidant and antitumor activity (61) against the human breast cancer cell line, MCF-7. Breast cancer (62-63) is the most common malignancy in women. The estrogen receptor–positive MCF-7 cell line was derived from a patient with metastatic breast chemotherapy; breast cancer cells can subsequently survive and gain

resistance to the treatment, leading to metastasis and drug resistance (64). Researchers tested the effect of purified M-20, or protocatechualdehyde (PA), on cultured MCF-7 cells (61). PA (65-66) is a phenolic compound present in many herbs.

The phenolic compounds commonly found in many plants are involved in many biological activities, including the chelation of metals, scavenging active oxygen species, and antioxidant activity. Phenolic compound are generally either in the free form or bound form. Most research has determined free soluble phenols by using aqueous methanol, ethanol, and acetone, either separately or mixed together (67).

It is well known that free radicals such as active oxygen species are involved in the pathogenesis of various diseases such as myocardial and cerebral ischemia, atherosclerosis, diabetes, rheumatoid arthritis, cancer-initiation and the aging process (68-70). These diseases have been reported to be ameliorated by free radical scavengers and thus many antioxidants of microbial origin have been searched (71-73). For free radical scavengers having the potential as protective agents against these diseases, some have investigated the metabolites of basidiomycetes and ascomycetes (74-76).

Oxidation is essential to many living organisms for the production of energy to fuel biological processes. However, oxidative metabolism continuously produces oxygen-centered free radicals and other reactive oxygen species in vivo, resulting in cell death and tissue damage. Oxidative damage caused by free radicals may be related to aging and diseases such as atherosclerosis, diabetes, cancer, and cirrhosis (77-78). Although almost all organisms possess antioxidant defense and repair systems that have evolved to protect them against oxidative damage, these systems are insufficient to prevent the damage entirely (79). These protective effects have been attributed, in part, to the various antioxidant compounds present in fruits and vegetables, for example, vitamin C and E, flavonoids, Maillard reaction products, amino acids, proteins, and beta carotene (80). Currently, synthetic antioxidants such as butylated hydroxyanisole (81), butylated

hydroxytoluene (BHT), and tertbutylhydroquinone (TBHQ) are widely used in the food industry. In general antioxidants can be classified into two groups based on their origin. The first group includes synthetic antioxidants such as tert-butylhydroxytoluene, tert-butylhydroxyquinone, and propylgallate (82). The second group includes vitamins and their derivatives such as ascorbic acid (83), α-tocopherol (84), and flavonoid (85). In addition, various products, such as carazostatin (86-87) thiazostatin (87), and benzastatin (88) that are produced by microorganisms, have been discovered. (Figure. 5) However, the use of synthetic antioxidants is being restricted because of their carcinogenicity and toxicity to the liver. The development and utilization of more effective antioxidants of natural origins are desired (89).

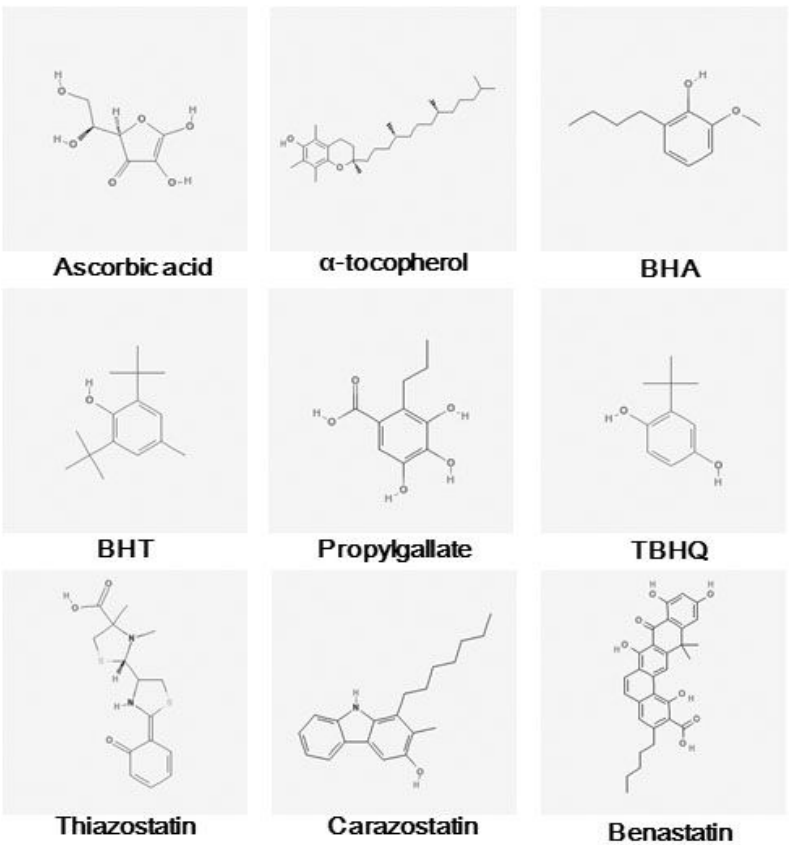

Figure 6. Molecular structure of some key antioxidants. BHA (butylated hydroxyanisole), BHT(butylated hydroxytoluene), and TBHQ (tertbutylhydroquinone)

Anti-inflammatory substances and its mechanisms of action

Macrophages and nitric oxide is closely related to inflammation and immune system. Upon inflammatory stimulation, macrophages produce and release a number of inflammatory mediators, such as nitric oxide (NO) and pro-inflammatory cytokines such as interleukin (IL)-6, interleukin (IL)-1ß and Tumor necrosis factor α(TNF-α). These pro-inflammatory cytokines mediate the development of various inflammatory diseases including rheumatoid arthritis, atherosclerosis, and hepatitis (90-94).

NO is a short-lived free radical and a very small compound that diffuses freely within cells from its site of formation to its site of action. The biosynthesis of NO in the organism is carried out from L-arginine and molecular oxygen utilizing NADPH as an electron donor and using heme, FMN, FAD and tetrahydrobiopterin (H4B) as cofactors through a reaction that consumes five electrons. The production of NO in the body is catalysed by a family of enzymes called nitric oxide synthases (95) (130–160 kDa). Three distinct isoforms of NOS have been isolated and cloned: eNOS (endothelial NOS, NOS I), iNOS (inducible NOS, NOS II) and nNOS (neuronal NOS, NOS III). inducible NOS (iNOS), is not present in resting cells but can be induced by immunostimulatory cytokines, bacterial products or infection in a number of cells, including endothelium, hepatocytes, monocytes, mast cells, macrophages and smooth muscle cells. It generates NO independently of intracellular calcium concentrations (96-98).

The regulation of NO synthesis by iNOS differs according to the strain and species of animals and depends on the inducers. Bovine and murine macrophages generate considerable amounts of iNOS in response to cytokine stimulation, but human and pig macrophages are resistant (99). Recent developments are reviewed and show that NO biosynthesis is regulated by a variety of mechanisms at the transcriptional and posttranslational levels in activated macrophages and other cells (100-

109).

Transcription factors like NF-kB (nuclear factor kappaB) mediate the expression of iNOS in immune and inflammatory responses (110). Lipopolysaccharide (LPS) is a major constituent of the outer membrane of Gram-negative bacteria and is recognized as a key molecule in the pathogenesis of Gram-negative sepsis and septic shock. LPS down-regulates the DNA binding activity of the inducible transcription factors NF-kB (111). So, NO, iNOS, pro inflammatory cytokines and NF-kB are attractive therapeutic targets for inflammation related disease.

While searching for biologically active compounds, we have found antimicrobial substances. These compounds exhibited antimicrobial, anti-inflammatory and anti-oxidative activities.

In this study, we examined the effect of three compounds on LPS-induced nitric oxide and several pro inflammatory cytokines production, expression of iNOS protein and NF-kB activity in Raw 264.7 macrophages. We investigated the topical anti-inflammatory effect of manumycin and its derivatives on mouse model of acute inflammation, using 12-*O*-tetradecanoylphorbol-13-acetate (TPA). And we investigated the anti-inflammatory effect of manumycin derivatives on experimental colitis and its underlying mechanisms.

Ⅱ. Material and Methods

A. Chemicals and reagents

All the solvent used in this experiment were extra pure grade. Hexane, ethyl acetate was purchased from J. T. Baker (Phillipsburg, NJ, USA). Silicagel for Thin layer chromatography, precoated silica gel plate (Kieselgel 60F254, Merck, NJ, USA) was used to separate manumycin and its derivatives. silcagel for silicagel column, Kieselgel 60 (70-230 mesh, Merck, NJ, USA) was used.

DMEM medium **(**Dulbecco/Vogt modified Eagle's minimal essential medium), penicillin, streptomycin and FBS(fatal bovine serum) were purchased from Invitrogen (Grand Island, NY, USA). TPA (12-*O*-tetradecanoylphorbol-13-acetate), arachidonic acid, LPS (Lipopolysaccharide), dimethyl sulfoxide (DMSO), Griess reagent and MTT (3-(4,5-dimethylthiazol-2-2,5-diphenyltetrazolium bromide) were purchased from Sigma. Mouse TNF-α ELISA kit and mouse IL-6 ELISA kit were purchased from BD Biosciences (San Diego, CA, USA). Rabbit anti-mouse iNOS polyclonal antibody was purchased from Santa cruz Biotech Inc (Santa Cruz, CA, USA). HRP-conugated donkey anti-rabbit Ig-G was purchased from Cell signaling. Alkaline phosphatase conjugated affinipure Donkey Anti mouse IgG was purchased from Jackson Immunoresearch Laboratories INC.

B. Microorganism and cells

Microorganism

Streptomyces sp. CS392 strain was used throughout this study. *Streptomyces* sp. CS392 was inoculated in 50 mL of the medium in a 250 mL round flask. The medium composition was glucose 1%, beef extract 0.4%, peptone 0.4%, yeast extract 0.1% and NaCl 0.25 % (w/v).

Fermentation was carried out at 28 °C for 5 days on a shaking machine in 400 mL of the medium in a 2 L flask. The medium composition was glucose 1%, peptone 1% and antifoam 0.1 % (w/v). Scale up fermentation was carried out in 4 L of the same medium in a 7 L fermentor inoculated with 240 mL of seed culture at 30 °C, at 180 rpm for 3 days. Antimicrobial activity was assayed by paper disc agar diffusion method using MRSA 693E as a test organism.

Cell culture

The murine macrophage cell line RAW264.7 was cultured at 37 °C under 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). The medium was routinely changed every three days. Raw 264.7 cells were passaged by trypsinization until they attained confluence.

C. Animals

Male ICR mice (5 weeks old, approximately 25 g) were purchased from the Orient Bio Inc (Seongnam, Gyeonggi,Korea). They were housed in acryl fiber cages at 22±3 °C, humidity

50±10 % and were kept on a 12 h light/dark cycle. They were fed with standard diet and water ad libitum and at least 7 days before they were used.

D. Isolation and production of biologically active compounds from S*treptomyces* sp. CS392

1. *In vitro* screening and isolation

Soil samples were collected from various locations in chonnam province. Several diverse habitats in different areas were selected for the isolation of *Streptomyces* strains. The samples were taken from up to 20 cm depth. The soil sample was incubated at 60 ℃ for 1-2 h. It was then suspended in distilled water. Test tubes containing a $10\sim10^4$ dilution of samples were inoculated on the surface of *Actinomycete* Isolation agar (OSYM medium, Oat 1%, Soy 1%, Yeast 1%, Mannitol 1%) plates. The plates were incubated at 28 ℃ until the sporulation of *Streptomyces* colonies occurred. *Streptomyces* colonies were then picked up and transferred to OSYM agar. Pure cultures were obtained from selected colonies for repeated sub-culturing. After antimicrobial activity screening, the isolated *Streptomyces* strains were maintained as suspensions of spores and mycelial fragments in 20% glycerol (v/v) at -20 ℃.

2. Screening for antimicrobial activity

In vitro, antimicrobial activity was primarily determined by the paper disk method using

paper disk (8mm, Toyo) against MRSA and the activity was determined by the diameter of clear zone.

MIC (minimal inhibitory concentration) value was determined by agar dilution method using Mueller Hinton Broth for bacteria. Observation was made after 18 h for bacteria 37 ℃ following inoculation of test organisms.

3. Taxonomic studies of *actinomycetes*

The cultural characteristics were studied in accordance with the guidelines established by the International *Streptomyces* Project (112). Strain was cultivated on an ISP 2 medium to determine the morphology. The color of the aerial mass, the substrate mycelium and the soluble pigment was determined using the Kornerup and Wanscher color scale (113). Melanin production was detected by growing the isolates on peptone-Yeast Extract ion agar (ISP 6). Utilization of different carbon and nitrogen sources were performed (114-115). Lecithinase was conducted on egg–yolk medium. Gelatinase activity, starch hydrolysis were determined by the method of Dye.(116) Also, lipases, Protease, α-amylase, chitinase and xylanase activity were monitored using standard methods (117-119).

4. Amplification and sequencing of the 16S rRNA Gene

PCR amplification of the 16S rRNA gene of the local actinomycete strain was conducted using two primers, StrepF; 5.-ACGTGTGCAGCCCAAGACA-3. and Strep R;5-

ACAAGCCCTGGAAACGG GGT-3 (120). The PCR mixture consisted of 30 pmol of each primer, 100 ng of chromosomal DNA, 200 μM dNTPs and 2.5 units of Taq polymerase, in 50 μL of polymerase buffer. Amplification was conducted for 30 cycles of 1 min at 94 °C, 1 min of annealing at 53 °C and 2 min of extension at 72 °C. The PCR reaction mixture was then analyzed via agarose gel electrophoresis and the remaining mixture was purified using QIA quick PCR purification reagents (Qiagen,USA). The 16S rRNA gene was sequenced on both strands via the dideoxy chain termination method. The 16S rRNA gene (1.5 kb) sequence of the PCR product was acquired using a Terminator Cycle Sequencing kit (ABI Prism 310 Genetic Analyzer, Applied Biosystems, USA).

5. Sequence similarities and phylogenetic analysis

The BLAST program (www.ncbi.nlm.nih.gov/blst) was employed in order to assess the degree of DNA similarity. Multiple sequence alignment and molecular phylogeny were evaluated using BioEdit software (29). The phylogenetic tree was displayed using the TREE VIEW program.

6. Optimization of fermentation process

Antimicrobial compounds production of the strain was optimized by using several cultural parameters such as carbon, nitrogen sources and minerals.

a. Carbon and nitrogen sources

To study the influence of carbon sources on cell growth and production of antimicrobial compounds, the production medium was supplemented with different carbon sources such as fructose, lactose, glucose, maltose, mannitol, sorbitol, starch and sucrose each at a level of 1% (w/v) by keeping the nitrogen source constant. Similarly, the influence of nitrogen sources on antimicrobial compounds yield was investigated by adding different nitrogen sources such as yeast extract, peptone, tryptone, beef extract, oatmeal, soy bean meal, malt extract, ammonium sulphate and ammonium chloride each at a concentration of 1% (w/v) to the production medium containing an optimum amount of the selected carbon source.

b. Minerals

Influence of minerals on the production of biomass and antimicrobial compounds was determined by supplementing various minerals such as magnesium chloride, ferric sulphate, calcium chloride, potassium chloride and sodium chloride at the rate of 0.01% (w/v) to the production medium

E. Purification and quantitative analysis of compounds

1. Purification of active compounds

Streptomyces sp. CS392 was grown on rotary shaker at 180 rpm in Emerson medium for 2 -3 days at 28 °C. Culture broth (3L) was centrifuged at 6,000rpm for 20 min. Supernatant was extracted with ethyl acetate. The extracted ethyl acetate fraction was evaporated and dried using a

rotary evaporator at 50 °C. Purification of antibiotic was carried out by silica gel column chromatography (0.8 cm x 15 cm). After washing the column with hexane, hexane-ethyl acetate (4:1), active material was eluted from the column with hexane-ethyl acetate (4:1). Active fractions were rechromatographed, using a Reverse phase-C18 silica gel column with water-acetonitrile (4: 6) to give manumycin and its derivatives.

2. Thin layer chromatography

Identification and purification of the manumycin and its derivatives was conducted by thin layer chromatography using hexane, ethyl acetate and methanol as a solvent system. Spots were detected by spraying with 10% H_2SO_4 in ethanol and baked at 80-100 °C for 10 min.

3. High-performance liquid chromatography

A reverse phase HPLC system was used for the analysis of manumycin derivatives. The liquid chromatography used throughout consisted of a Shimadzu separations module, automatic sample injector and a UV detector with peak integration at 280 nm for quantitative analysis. Data were analyzed with an integrator (Shimadzu).

A Capcelpak RP-18 column (250 x 4.6mm, 5 μm, Shisheido) was used for separation. The mobile phase was acetonitrile(solution A) and water containing 0.1% formic acid(solution B). The flow rate was 1.0 mL/min. and the injection volume was 20 μL. The gradient elution condition is summarized in Table 1. After completing the chromatographic elution, the mobile phase was programmed to its initial condition within 10 min. And a 15 min. recondition time was set before

next injection.

Table 2. HPLC gradient

Time (min)	A (%)	B (%)
0	60	40
10	60	40
20	100	0
25	60	40

4. Spectroscopic analysis

Manumycin and its derivatives were subjected to spectroscopic analysis: 300 MHz ^{1}H NMR, 75 MHz ^{13}C NMR, COSY, TOCSY, HMQC and elemental analysis was determined at the Sunmoon University, Korea.

5. Stability of compounds

a. Temperature stability

Serial two-fold dilutions of compounds (0-65 μg/mL) in Mueller–Hinton agar was prepared for MIC. Agar plates were pre-incubated at 35 °C in incubator for 0, 24, 48 and 72 h prior to being inoculated with test organisms according to reference testing method recommendations of the

National Committee for Clinical Laboratory Standards (NCCLS) (121). The thermal stability of the compounds was studied by heating the samples at various temperatures for 4 h. The residual inhibitory activity was measured as described above (using MRSA 693E as target)

b. pH stability

Five milliliters of culture filtrates were put in vials and using 0.1 N HCl or 0.1 N NaOH adjusted to the pH levels 2 to 10. Compounds were incubated for 3-4 h at room temperature and then readjusted to pH 7.0. The activity of these treated antibiotic culture filtrates was determined by the sizes of the inhibition zones using the paper disc method described earlier.

F. Biological activities of compounds

1. Antimicrobial activity

a. MIC test

The *in vitro* antimicrobial activities (MIC, μg/mL) of the compounds, which are against Gram-positive and Gram-negative bacteria, were determined by the Mueller-Hinton-agar dilution method (122-123). The results of MIC test are summarized in Table 6 and it includes those of vancomycin and oxacillin for comparison, as well.

2. Antioxidant activity

a. Determination of nitric oxide scavenging activity

The scavenging effect of compounds on nitric oxide was measured according to the method of Sreejayan et al (124). Sodium nitroprusside was freshly prepared as a 20 mM stock solution in PBS. DMSO or compounds was added to the solution. The compounds were dissolved in DMSO and then added at 5 to 20μL so as to obtain the desired concentration in 1 mL of sodium nitroprusside-PBS solution. And the samples incubated at room temperature for 2 h. Aliquots of samples were quantified for nitrite content at the stated time or time intervals. Then the nitrite concentration was determined each hour using the Griess reagent, as mentioned above.

b. Reducing power

The reaction mixture contains 200 μL of 200 mM sodium phosphate buffer (pH 6.6), 200 μL of 1% potassium ferricyanide, and 200 μL of aliquot of compounds or standard (5 mg/mL). The mixture was incubated at 50 °C for 20 minutes. 10% trichloroacetic acid 200 μL was added into the reaction mixture and centrifuged at 5000 rpm for 10 minutes. 500 μL of the upper layer was diluted with 500 μL of 0.1% ferric chloride. Absorbance was measured at 700 nm (125).

3. Anti-inflammatory activity of active compounds

a. Cell viability

Cells were seeded in a 96 well plate at a density of 0.5 x 10^4 cell/well. manumycin derivatives were added at indicated concentrations. The mitochondrial-dependent reduction of 3-(4, 5-dimethylthizaol-2yl)-2, 5-diphenyl tetrazolium bromide (MTT) to formazan was used to measure cell respiration as an indicator of cell viability (126). Briefly, after 24h incubation with or without compounds (1–10 μg/mL), a 0.5 mg/mL of MTT solution was added and the cells were incubated for 4h at 37 °C. After removing the supernatant, 100 μL of DMSO was added to the cells to dissolve the formazan. Optical density was measured using an assay reader at 590 nm. The optical density of formazan formed in control (untreated) cells was taken as 100% of viability.

b. Nitric oxide analysis and quatification of cytokine production

Raw 264.7 macrophage cells (10^6 cells/well) were cultured in 6 well plates. After incubation

for 24 h, the cells were pre-incubated with various concentrations of compounds for 30min, and then stimulated with LPS (1 μg/mL). After 24h, the supernatant was collected and Nitric oxide production was determined using the Griess reagent (mixture of equal amount of A and B. A: 1% sulphanilamide, B: 0.1% naphthylethylene diamine dihydrochloride in 5 % H_3PO_4). The supernatant (1 mL) was mixed with 1 mL of Griess reagent and incubated for 15 min. Absorbance was read at 550 nm with microplate reader (Thermo co.). Amount of nitrite of the supernatant was calculated from a sodium nitrite standard curve. Supernatant was analyzed for TNF-α, IL-1ß and IL-6 by enzyme linked immunosorbent assay (ELISA) using commercial kits (BD Biosciences) according to the manufacturer's instructions.

c. Western blot analysis

For detection of iNOS expression, Raw 264.7 cells were washed with cold PBS and lysated in a cold lysis buffer (Mixture of RIPA buffer and protease inhibitor) for 30 min. Cell was removed after centriugation (15,000 rpm 4 °C, 30min). Protein concentration of sample was determined by the Bradford method. Each sample was boiled in SDS-PAGE loading buffer. 30 μg of total protein from each samples were subjected to gel electrophoresis and electrophoretically transferred onto PVDF membranes. The membranes were blocked with 5% non-fat dried milk in Tris buffered saline-Tween (TBS-T, 10 mM Tris–HCl, 150 mM NaCl, 0.05% Tween 20, pH7.6) at room temperature for 2h. After being washed, the membranes were incubated in respective primary antibody solution (anti-iNOS or anti-ß-actin antibodies) for 1h at room temperature.

The membranes were washed with TBS-T and incubated with HRP (for iNOS antibody) or alkaline phosphatase (for anti-ß-actin antibodies) conjugated secondary antibody solution for 1h at

room temperature. For detection of iNOS expression, the blots were washed with TBS-T and incubated with ECL reagent for 2 min and viewed by chemiluminescence.

For ß-actin the blots were washed with TBS-T and incubated with AP color development buffer containing color reagent (AP Conjugatesubstrate kit, Bio-rad laboratories Inc). Band intensities were quantified by Image J program and normalized by comparison to ß-actin.

For NF-κB, Cytosolic extracts were prepared in hypotonic buffer consisting of 10 mM *N*-(2-hydroxyethyl) piperazine-*N*-2-ethanesulfonic acid (HEPES) (pH 7.6), 10 mM KCl, 2 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mM ethylenediaminetetraacetic acid (EDTA), and 0.1 mM phenylmethylsulfonyl fluoride (PMSF). Nuclear extracts were prepared in hypertonic buffer consisting of 50 mM HEPES (pH 7.9), 400 mM KCl, 0.1 mM EDTA, and 10% glycerol. After SDS-PAGE and Transferation to PVDF membrane, Membrane was blocked overnight at 4 °C with 5% non-fat dried milk in Tris buffered saline-Tween (TBS-T, 10 mM Tris–HCl, 150 mM NaCl, 0.05% Tween 20, pH7.6) and then incubated with antibodies overnight at 4 °C. The blots were washed with TBS-T, followed by 1h of incubation with the HRP-conjugated secondary antibody. Blots were washed with TBS-T and incubated with ECL reagent for 2 min and viewed by chemiluminescence.

d. Assay of 12-*O*-tetradecanoylphorbol-13-acetate induced ear edema in mice

TPA induced ear edema was performed according to Chen et al, Young et al and Rao et al (127-129) with a slight modification. Samples in acetone (25, 50 μg/ear) were painted on the left ear for 10min. And TPA (0.25 mg/mL) and dissolved in acetone were applied to the inner and outer surfaces of the ear of mice (10 μL/ear). Before the inducer application. The ear thickness was

measured with a calibrated digital thickness gauge before and and 5 h after TPA challenge, and the difference in thickness was calculated. Prednisolone at 50 μg /ear was used as positive reference

e. Assay of dextran sodium sulfate induced colitis in mice

ICR mice were used to study DSS-induced colitis. This strain is susceptible to DSS assault and has been widely used as a mouse colitis model. Acute colitis was induced in ICR mice by adding DSS (Sigma-Aldrich, St. Louis.MO) to the drinking water at a level of 3% for a period of 7 days. Mice were assigned to five different groups: non-colitic (n = 6), DSS colitic groups (n = 6). Other 4 groups consisted of mice receiving 3% DSS were administrated 5-ASA (100 mg/kg/day ip, n=6) or CS392 (1, 3, 10 mg/kg/day ip, n=6) daily for 7 days according to experimental design.

Body weight loss, stool consistency, and blood in the stool were monitored daily to assess the severity of colitis score according to the criteria proposed previously (130). Weight loss was arbitrarily scored as 1: 1%–5%, 2: 5%–10%, 3: 10%–15%, and 4: 15%–. Blood in the stool was scored as 0: negative, 1: +, 2: ++, 3: +++, 4: ++++. Diarrhea was scored as 0: normal, 1 and 2: loose stools, 3 and 4: watery diarrhea. The disease activity index was defined as the average score for these three parameters. Body weight was monitored daily.

After 7 days, mice were killed; the colons were separated and gently flushed with saline. Each colons length was measured. Mid-colon tissue was frozen in liquid nitrogen. And colons were immediately removed and fixed in 10% formaldehyde, paraffin embedded, sectioned, and stained with hematoxylin and eosin (HE).

To investigate the biochemical characteristics, the sample was then homogenized in 500 to 700 μL of a lysis buffer containing protease inhibitors. Samples were centrifuged for 30 min at 16,000 rpm, and the supernatant was frozen at -80 °C until assay. Cytokine levels were determined using ELISA

Kits (BD science). The results were expressed as pg/mg wet tissue. The iNOS Western blot from tissue was performed as previously described.

f. Protein extraction from tissue in TPA induced mouse ear edema and DSS induced coltis model

For the isolation of protein, the ears of mice derived from TPA-induced acute inflammation experiments were excised. Ear samples were homogenized for 5min in 800μL of ice-cold lysis buffer (20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4 and 1 μg/mL leupeptin) containing a protease inhibitor cocktail set (Calbiochem, Germany). The lysate was centrifuged at 15,000 rpm, for 30 min at 4 °C. The supernatant was used as total protein extracts. The protein concentration was determined by Bradford method.

Ⅲ. Results

A. Production, isolation and purification of biologically active compounds from *Streptomyces* strains

1. Identification of the *Actinomycetes*

For Molecular phylogeny, the 16S rRNA sequence of the local isolate was compared to the sequences of 32 *Streptomyces* spp. In order to determine the relation of the local isolate to these *Streptomyces* strains. The phylogenetic tree (displayed by the Tree View program) showed that the locally isolated strain is closely related to *Streptomyces lanatus* (Figure 7). Multiple sequence alignment was done between the sequences of the 16S rRNA genes of *S. lanatus, S. lucensis, S. bungoensis, S. longisporus, S. flavoviridis* and the local isolate. Computer assisted RNA searches against bacterial database similarly revealed that the 16S rRNA sequence was 98.22% identical with both *Streptomyces* sp CS392. And *Streptomyces lanatus* strain (Figure 8, Table 3).

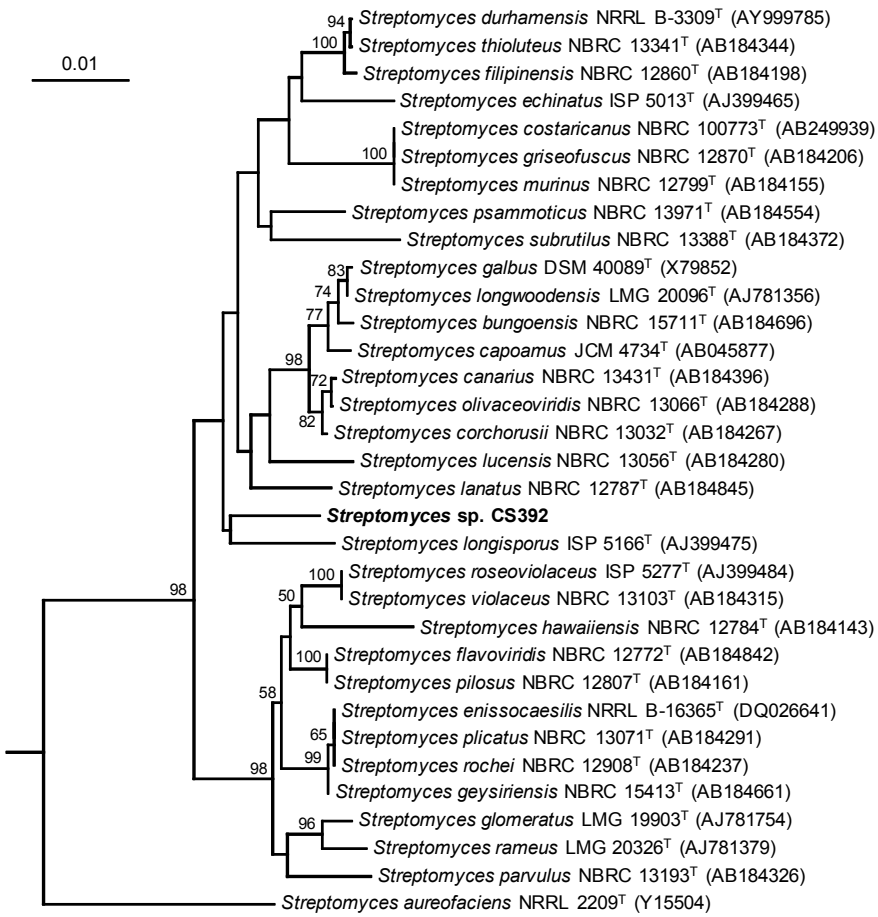

Figure 7. Phylogenetic tree of the *Streptmyces* species. Neighbor-joining tree based on nearly complete 16S rRNA gene sequences showing relationships between strain CS392 and some closely related taxa of the genus *Streptomyces.* The percentage numbers at the nodes are the levels of bootstrap support based on neighbor-joining analyses of 1000 resampled data sets. The sequence of *Nocardia asteroides* DSM 43757^T (AF430019) was used as an outgroup. Bar: 0.02 nucleotide substitution per position.

GACGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGAACGATGAACCACTTCGGTGG

GGATTAGTGGCGAACGGGTGAGTAACACGTGGGCAATCTGCCCTTCACTCTGGGACAA

GCCCTGGAAACGGGGTCTAATACCGGATATGAGCCTGGGAGGCATCTCCTGGGTTGTAA

AGCTCCGGCGGTGAAGGATGAGCCCGCGGCCTATCAGCTTGTTGGTGAGGTAATGGCTC

ACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACACTGGGACTGAG

ACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAG

CCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGC

AGGGAAGAAGCGAGAGTGACGGTACCTGCAGAAGAAGCGCCGGCTAACTACGTGCCA

GCAGCCGCGGTAATACGTAGGGCGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCT

CGTAGGCGGCTTGTCACGTCGATTGTGAAAGCCCGAGGCTTAACCTCGGGTCTGCAGTC

GATACGGGCTAGCTAGAGTGTGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAAT

GCGCAGAT

Figure 8. 16S rRNA sequence of *Streptomyces* sp. CS392 The data provided represents the 16S rRNA sequence to identify the name of the *Streptomyces* strain.

Table 3. Similarity of 16S rRNA gene sequences (CS392)

Rank	Name/Title	Strain	Accession	Pairwise Similarity	Diff/Total nt
1	*Streptomyces lanatus*	NBRC 12787(T)	AB184845	98.22	26/1462
2	*Streptomyces lucensis*	NBRC 13056(T)	AB184280	98.19	26/1433
3	*Streptomyces bungoensis*	NBRC 15711(T)	AB184696	98.15	27/1462
4	*Streptomyces longisporus*	ISP 5166(T)	AJ399475	98.12	27/1433
5	*Streptomyces flavoviridis*	NBRC 12772(T)	AB184842	98.08	28/1461
6	*Streptomyces pilosus*	NBRC 12807(T)	AB184161	98.07	28/1453
7	*Streptomyces longwoodensis*	LMG 20096(T)	AJ781356	98.05	28/1438
8	*Streptomyces galbus*	DSM 40089(T)	X79852	97.95	30/1463
9	*Streptomyces capoamus*	JCM 4734(T)	AB045877	97.88	31/1463
10	*Streptomyces psammoticus*	NBRC 13971(T)	AB184554	97.87	31/1452
11	*Streptomyces glomeratus*	LMG 19903(T)	AJ781754	97.81	32/1463
12	*Streptomyces violaceus*	NBRC 13103(T)	AB184315	97.74	33/1463
13	*Streptomyces geysiriensis*	NBRC 15413(T)	AB184661	97.74	33/1462
14	*Streptomyces rameus*	LMG 20326(T)	AJ781379	97.74	33/1462
15	*Streptomyces corchorusii*	NBRC 13032(T)	AB184267	97.74	33/1462
16	*Streptomyces olivaceoviridis*	NBRC 13066(T)	AB184288	97.74	33/1462
17	*Streptomyces filipinensis*	NBRC 12860(T)	AB184198	97.74	33/1460
18	*Streptomyces roseoviolaceus*	ISP 5277(T)	AJ399484	97.72	33/1450

2. Optimization of fermentation process

Antimicrobial compounds production of the strain was optimized by using several cultural parameters such as carbon, nitrogen sources and metal ions. The effect of carbon, nitrogen sources and metal ion was studied. The optimum conditions for the antimicrobial compounds production in *Streptomyces* sp. CS392 are pH 7.0, inoculum 3%, temperature 28 °C among parameters tested. Glucose exhibited maximum antibiotic production followed by fructose (Figure 9). Among nitrogen sources, peptone affected maximum antimicrobial activity followed by beef extract (Figure 10). A high activity of antibiotic was produced in the presence of 0.01% magnesium chloride (Figure 11). The highest yield of antibiotic production was noticed with 1% glucose as a carbon source and 1% peptone as a nitrogen source and 0.01% magnesium chloride (Figure 11). This composition was maintained in further experiments. The growth pattern of *Streptomyces* sp. CS392 was studied on optimized medium.

The antibiotic production of *Streptomyces* sp. CS392 started at 12h and reached to the maximum level after 24h of cultivation. There was a steady increase in biomass. And pH value was relatively constant at about 7.5. Effect of incubation time on growth and antibiotic production by *Streptomyces* sp. CS392 is shown in (Figure 12).

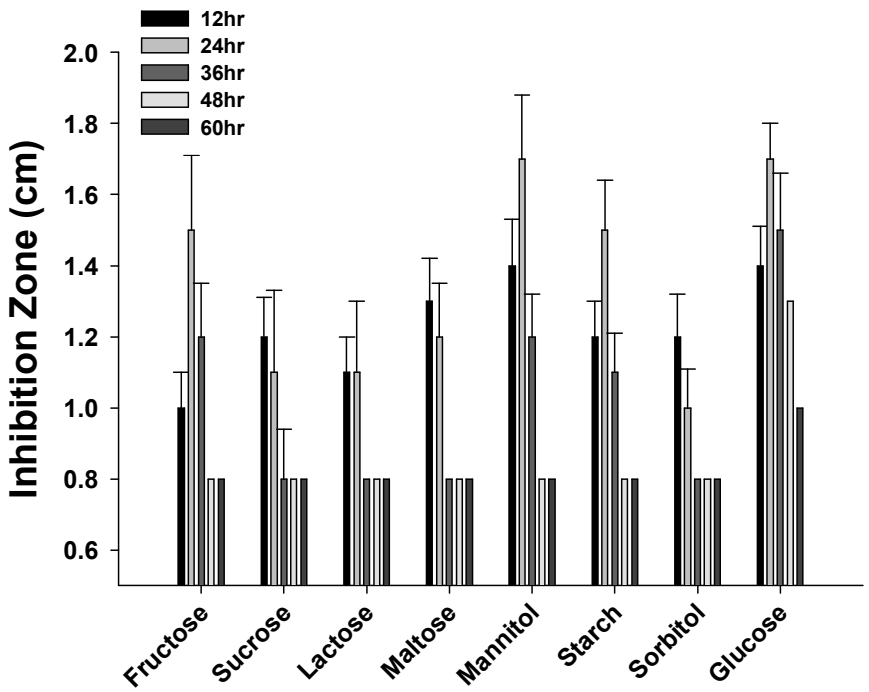

Figure 9. Effect of various carbon sources on the production of active compounds. Production medium was supplemented with various carbon sources such as fructose, lactose, glucose, maltose, mannitol, sorbitol, starch and sucrose each at a level of 1% (w/v) by keeping the nitrogen source constant

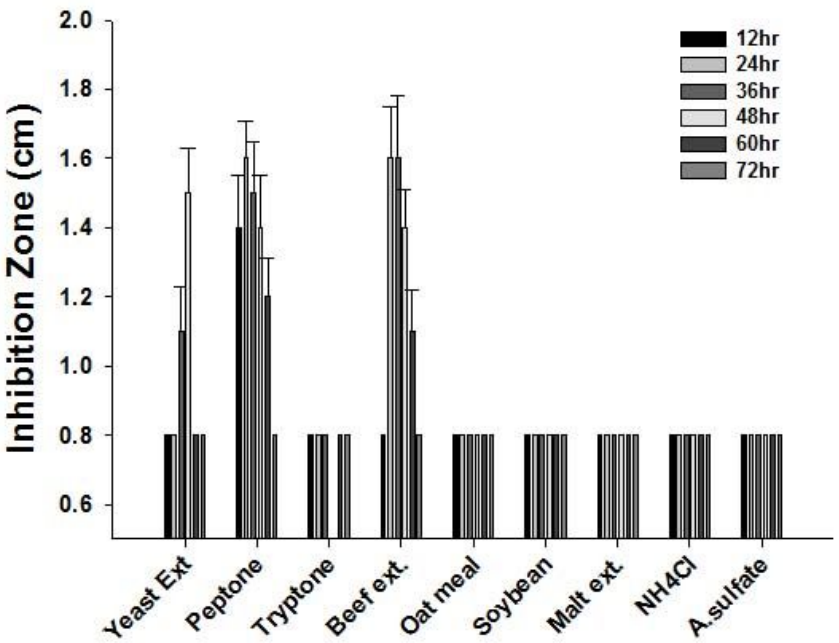

Figure 10. Effect of various nitrogen sources on the production of active compounds. Production medium was supplemented with various nitrogen sources such as yeast extract, peptone, tryptone, beef extract, oatmeal, soy bean meal, malt extract, ammonium sulphate and ammonium chloride each at a level of 1% (w/v) by keeping the carbon source

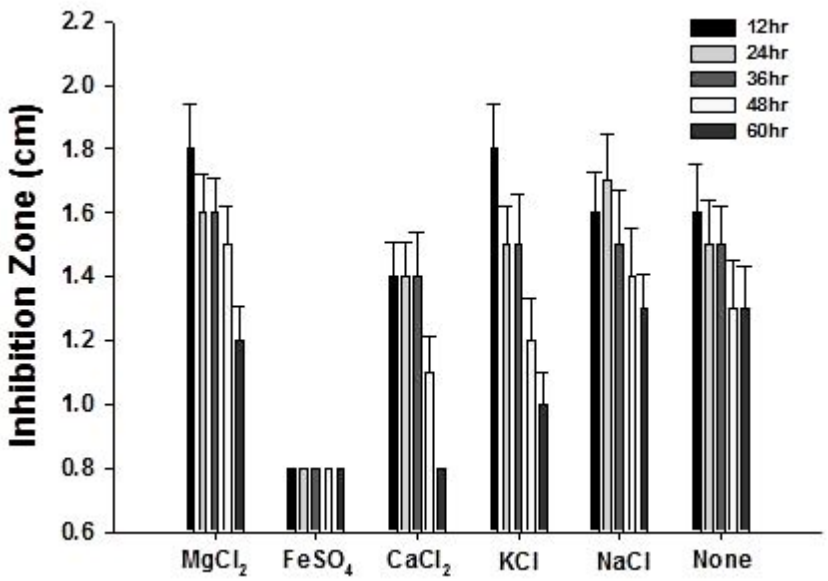

Figure 11. Effect of various metal-ion sources on the production of active compounds. Influence of minerals on the production of biomass and antimicrobial compounds was determined by supplementing various minerals such as magnesium chloride, ferric sulphate, calcium chloride, potassium chloride and sodium chloride at the rate of 0.01% (w/v) to the production medium

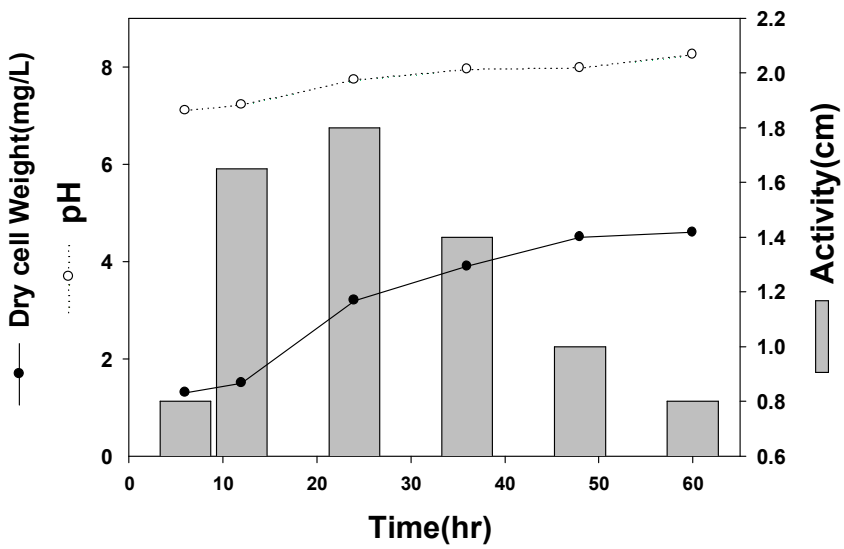

Figure 12. Fermentation profiles of *Streptomyces* sp. CS392. Strain CS392 was cultivated in 2 L of a fermentation medium at 28 °C and antimicrobial activity was assayed in a culture broth.

3. Fermentation, extraction and purification of compounds

Streptomyces sp. CS392 was grown on rotary shaker at 180 rpm in emerson medium for 2-3 days at 28 °C Culture broth (3L) was centrifuged at 6,000 rpm for 20 min. Supernatant was extracted two times with ethyl acetate (1:1, v/v). The extracted ethyl acetate fraction was evaporated and dried using a rotary evaporator at 50 °C under the reduced pressure. Purification of antibiotic was carried out by silica gel column chromatography (0.8 cm x 15 cm). After washing the column with hexane, hexane-ethyl acetate (4:1), active material was eluted from the column with hexane-ethyl acetate (4:1). Active fractions were rechromatographed, using a reverse phase-C18 silica gel column with water-acetonitrile (2: 8) to give manumycin and its analogues. Three compounds were obtained as a yellow/pale yellow powder. Sample for structure analysis, compounds were more purified with silica column chromatography (reverse phase C18) using a solvent system composed of acetonitrile and water (6:4, v/v) (Figure 13). And their structures were determined by several analysis tools. Figure 14 shows the photograph of a thin layer chromatogram with three derivatives. Asukamyicn and its derivatives had Rf values 0.7(C1), 0.5(C2) and 0.3(C3) on TLC. And figure 15 shows the HPLC chromatogram of a standard mixture.

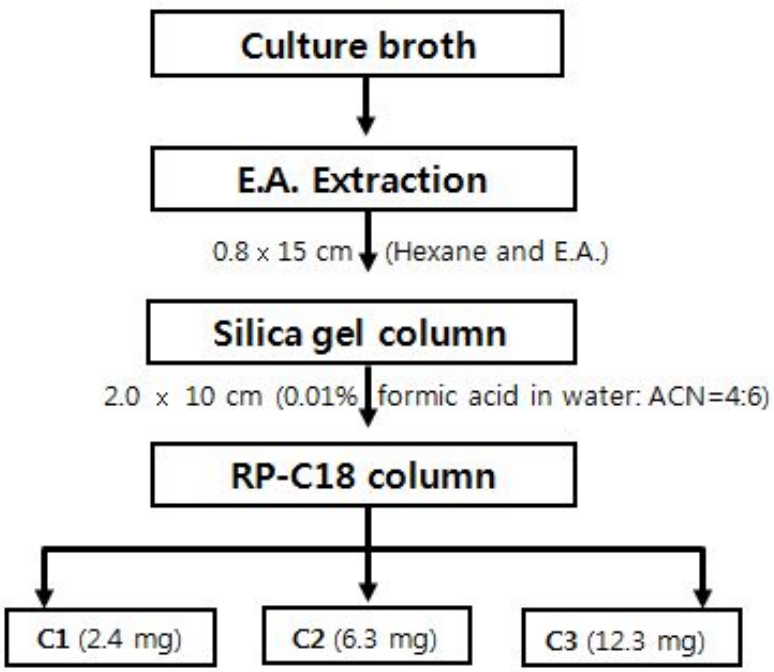

Figure 13. Purification scheme for biologically active compounds

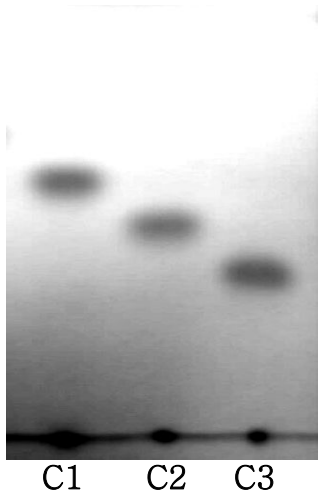

Figure 14. TLC (Thin layer chromatography) chromatogram of active compounds. The purified compounds were loaded on a TLC plate of silicagel, as described in materials and methods. The numbers noted under the TLC plate indicate the compounds code. RP-TLC (60% ACN) (Lane 1: C1, Lane 2: C2, Lane 3: C3)

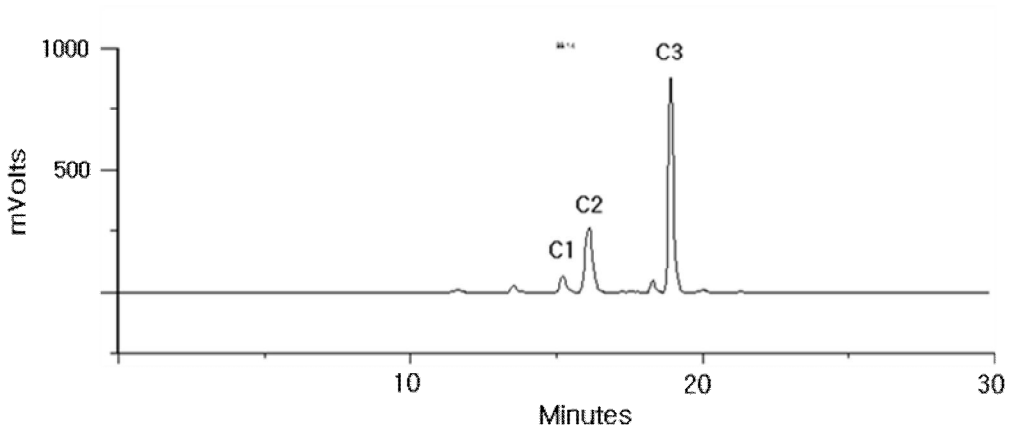

Figure 15. HPLC chromatogram of the active compounds. Separations of three compounds by a liquid chromatography in a C18 column (Capcelpak column 250× 4.6mm, 5μm) with gradient of mobile phase containing acetonitrile and Water (0.1% formic acid). The retention time of the compounds were found to be 15.2, 16.1 and 19.0 min, respectively. Three peaks were well separated and eluted within 20 min.

4. Physicochemical characteristics of *Streptomyces* sp. CS392

Streptomyces sp. CS392 grew on general media (Figure 16). The substrate mycelium of strain *Streptomyces* sp. CS392 did not fragment. The aerial mass color was red-grey on ISP 4 medium. The substrate mycelium color varied from yellow to brown depending the age of the culture. On ISP 6 medium, *Streptomyces* sp. CS392 produced melanoid pigments. And this strain produced yellow-brown diffusible pigment on ISP 4 medium (Table 4).

Streptomyces sp. CS392 grew poorly in the presence of adonitol, D-melibiose, dextran and xylitol as carbon source. As a nitrogen source, L-histidine inhibited cell growth. The strain formed rich mycelium on the media D-galactose, Sucrose, Mannitol, L-Ramnose, Raffinose, D-melezitose, L-arabinose, D-xylose, D-fructose and D-lactose. It hydrolysed starch. But hydrolysis of CMC, Egg yolk, Xylan, Tyrosine, casein, gelatin, chitin and Tween 80 was not observed (Table 5).

Figure 16. Colony morphology of *Streptomyces* sp. CS392

Table 4. Cultural characteristics of *Streptomyces* sp. CS392

Characteristics	Result
Spore mass (Inorganic salts starch agar medium , ISP 4)	Red-Grey
Spore surface (ISP 4)	Smooth
Spore chain (ISP 4)	-
Color of substrate mycelium (ISP 4)	Yellow-Brown
Diffusible pigment (ISP 4)	Yellow-Brown
Melanine production (Peptone yeast extract ion agar medium, ISP 6)	Melanine produced

Cultural characteristics were observed after cultivation on each agar plate at 28 °C for 7 days ISP 4 agar: Soluble starch 10g, $CaCO_3$ 2 g, $(NH_4)_2SO_4$ 2 g, K_2HPO_4 1 g, $MgSO_4 \cdot 7H_2O$ 1 g, NaCl 1 g, $FeSO_4 \cdot 7H_2O$ 1 mg, $MnCl_2 \cdot 7H_2O$ 1 mg, $ZnSO_4 \cdot 7H_2O$ 1 mg, Agar 20 g/ 1L Distilled water. ISP 6 agar: peptone 15g, proteose peptone 5g, K_2HPO_4 1g, Yeast extract 1g, Ferric ammonium citrate 0.5g, $Na_2S_2O_3$ agar 15g/ 1L Distilled water.

Table 5. The physiological and Biochemical characteristics of the *Streptomyces* sp. CS392

	Charateristic	Result
Carbon utilization	D-galactose	+
	Sucrose	+
	Mannitol	+
	L-Ramnose	+
	Raffinose	+
	D-melezitose	+
	Adonitol	-
	D-melibiose	-
	Dextran	-
	Xylitol	-
	L-arabinose	+
	D-xylose	+
	D-fructose	+
	D-lactose	+
	Sodium acetate	+
	Sodium citrate	+
	Sodium pyruvate	+
Nitrogen utilization	DL-α-amino-n-butyric acid	+
	L-valine	+
	L-phenylalnine	+
	L-histidine	-

	Potassium nitrate	+
Extracellular enzyme activity	Starch	+
	Tween80	-
	Casein	-
	CMC	-
	Egg Yolk	-
	Xylan	-
	Tyrosine	-
	gelatin	-
	chitin	-

5. Spectroscopic characteristics and chemical structure of compounds

The chemical structure of active compound was elucidated based on the spectroscopic data obtained from the application of MS, IR, UV, ^{1}H NMR (figure 17), ^{13}C NMR, HMQC (figure 18), COSY (figure 19) and TOCSY (figure 20) and found to be identical to the manumycin which is known to be produced by *Streptomyces parvulus, Streptomyces* sp. UOF1, *Streptomyces* sp. WB-8376(Figure 4,14, Table 1).

Structure analysis of C1 and C2 are in progress. Although the antibiotic is known, the newly isolated strain was able to produce the antibiotic as a major product.

After receiving full structural information, which is now in progress, we will study antimicrobial effects of these compounds in detail in relation to their affiliated reference antimicrobial compounds. Nevertheless, antimicrobial effects of these antibiotics, especially against various resistant pathogens, are strong so that they can be considered as potential therapeutical candidates after crucial clinical evaluation.

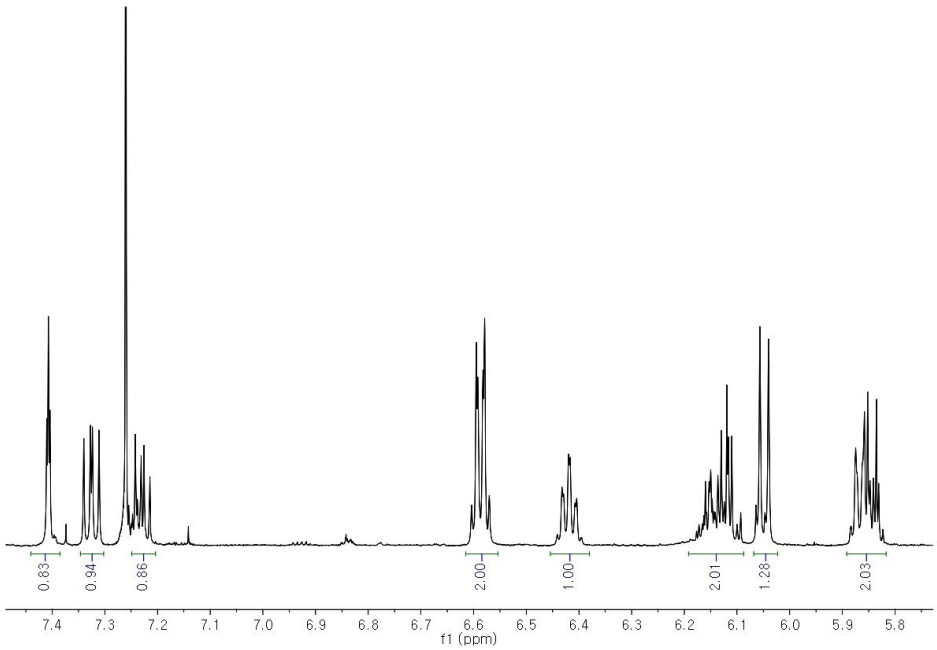

Figure 17. ^{1}H NMR spectrum of C3 (900 MHz)

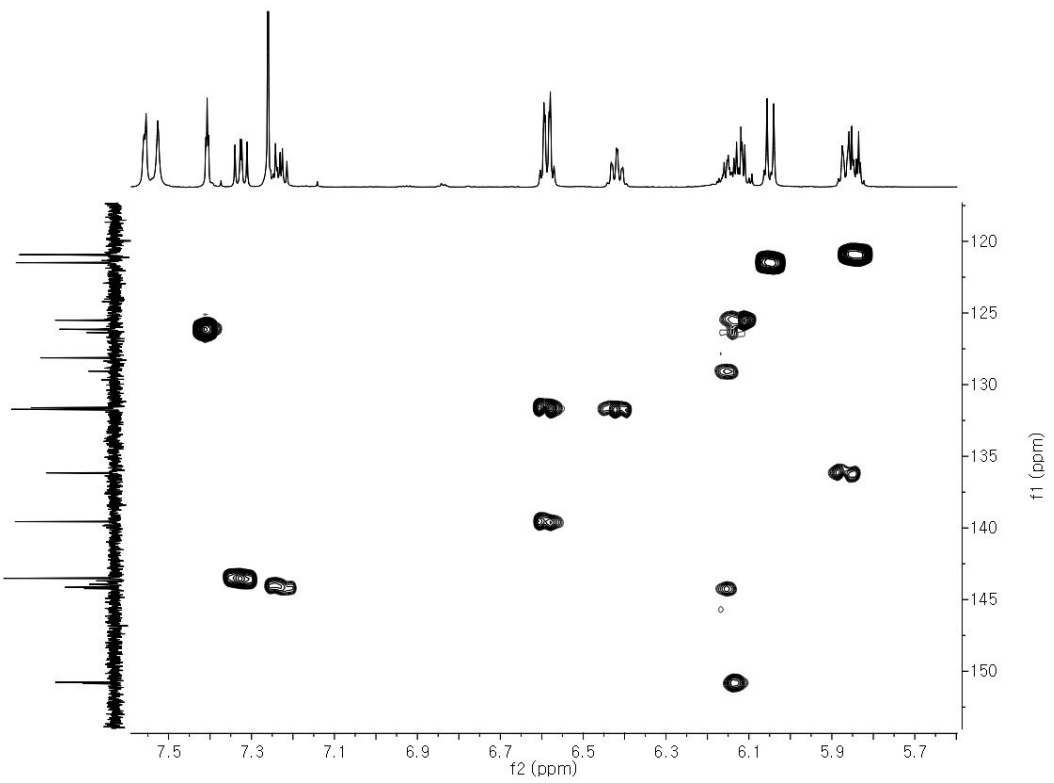

Figure 18. HMQC spectrum of C3

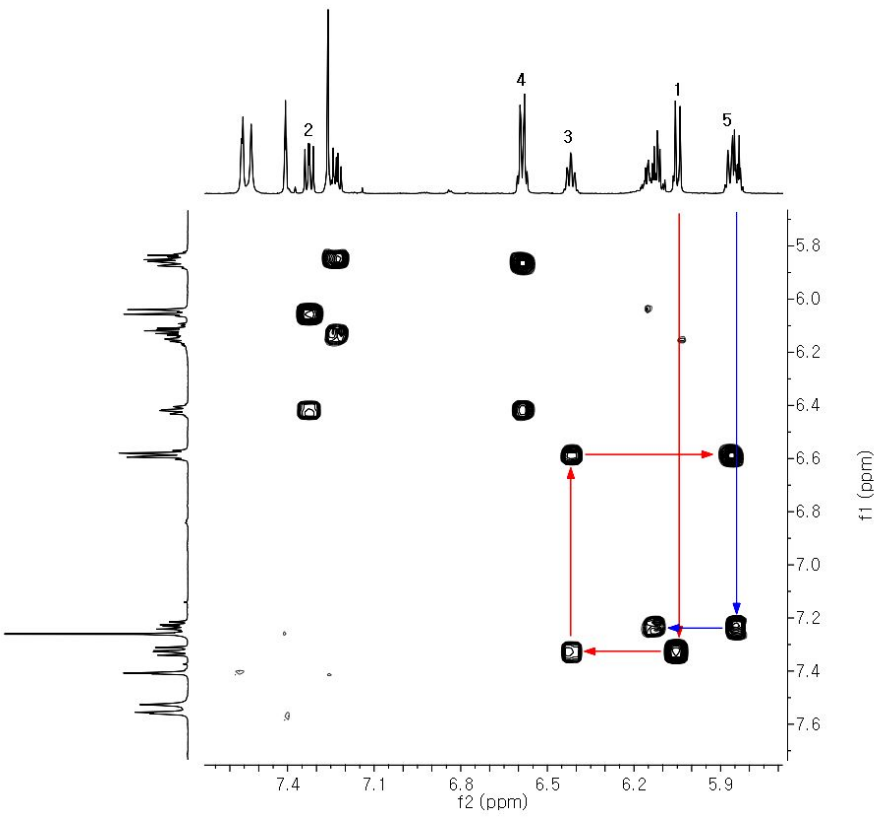

Figure 19. COSY spectrum of C3

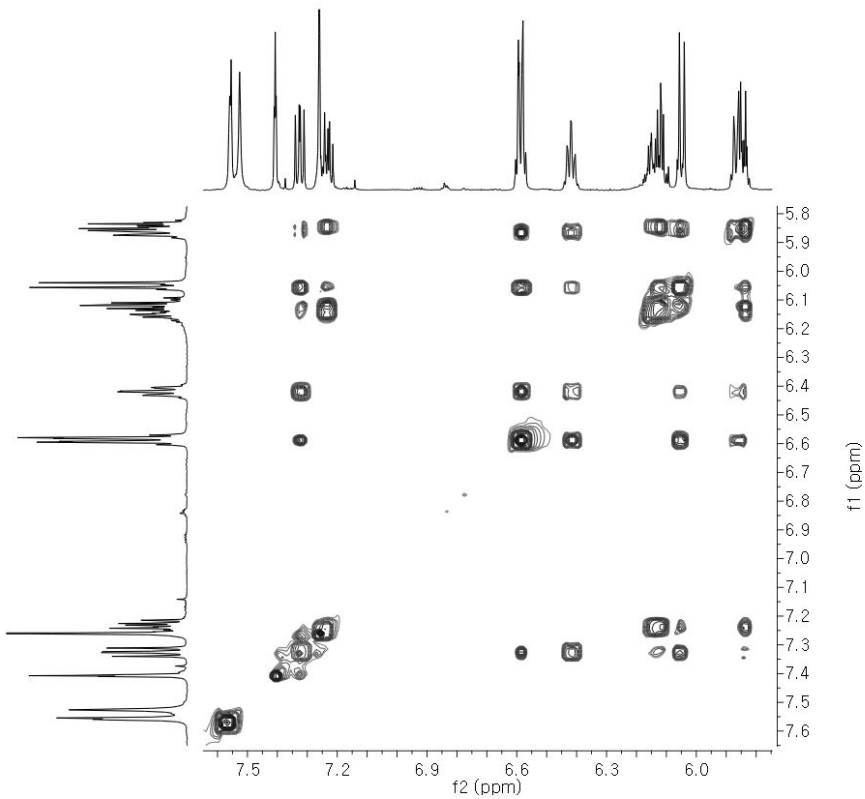

Figure 20. TOCSY spectrum of C3

B. Biological activities of active compounds

1. Antimicrobial activity of active compounds

a. Antimicrobial spectrum of active compounds

The minimum inhibitory concentrations (MICs) of the purified compounds are shown in Table 6. The compounds showed good activities against a number of Gram-positive bacteria (MIC ranged from 4 to 32 μg/mL). Especially these compound inhibited growth of VRSA, MRSA and VRE. On the other hand, Compounds exhibits no inhibitory activities against gram negative bacteria.

Table 6. MIC values of the compounds (μg/mL)

Test Organisms	Type	C1	C2	manumycin	Vancomycin	Oxacillin
***Alacligenes faecalis* ATCC 1004**	G(-)	>65	>65	>65	>65	65
***Enterococcus Faecalis* ATCC 29212**	G(+)	16.25	8.12	4.06	1.01	4.06
***Bacillus subtilis* ATCC6633**	G(+)	0.5	2.03	0.5	0.5	0.5
***Staphylococcus aureus* KCTC 1928**	G(+)	4.06	4.06	2.03	0.5	0.5
***Micrococcus luteus* ATCC 9341**	G(+)	16.25	16.12	8.1	0.5	0.5
***Mycrobacterium smegmatis* ATCC 9341**	G(+)	8.12	4.06	2.03	1.01	16.25
***Salmonella typhimrium* KCTC 1925**	G(-)	>65	>65	>65	>65	>65
***Escherrichia coli* KCTC 1923**	G(-)	>65	>65	>65	>65	>65
***Pseudomonas aeruginosa* KCTC 1637**	G(-)	>65	>65	>65	>65	>65
MRSA 693E	G(+)	4.06	4.06	2.03	0.5	16.25
MRSA 4-5	G(+)	4.06	4.06	2.03	1.01	>65
MRSA 5-3	G(+)	4.06	4.06	2.03	1.01	>65
VRE 82	G(+)	16.25	4.06	8.12	>65	65
VRE 89	G(+)	32.5	32.5	16.5	>65	>65
VRE 98	G(+)	32.5	16.25	8.12	>65	>65
VRSA	G(+)	4.06	4.06	2.03	>65	32.5

b. pH and temperature stability of active compounds

In agar dilution stability test, manumycin derivatives showed declining stability trends. manumycin were generally more stable than C1 and C2 (Table 7). MICs generally increased 2 to 8 fold (C2, manumycin) from baseline MIC over pre-incubation intervals (up to 72 h). Manumycin stability was consistently higher than C1 and C2. MIC values of these compounds were markedly increased 4 to 8 fold against VRE strains and *Micrococcus leteus* ATCC9341 within 24 h. Figure 21 and 22 shows heat and pH stability of the manumycin derivatives. The antimicrobial activity was stable at pH 3.0~9.0. The fermentation broth from *Streptomyces* sp. CS392 was stable up to 90 for 4 h. But after heating at 121 and pressure of 15 pounds for 15 min, the activity was reduced to about 1/2 (Figure 21). The compounds were also stable against freezing.

Table 7. Stability of active compounds under pre incubation time

Organism	MIC(μg /mL) Pre-incubation time			
	Compound	0h	24h	48h	72h
***Enterococcus faecalis* ATCC 29212**	C1	16	>65	>65	>65
	C2	8	32	>65	>65
	manumycin	4	8	32	>65
***Bacillus subtilis* ATCC6633**	C1	0.5	>65	>65	>65
	C2	2	4	16	65
	manumycin	0.5	2	8	32
***Staphylococcus aureus* KCTC 1928**	C1	4	65	>65	>65
	C2	8	16	65	>65
	manumycin	4	8	32	>65
***Micrococcus luteus* ATCC 9341**	C1	16	32.5	>65	>65
	C2	16	>65	>65	>65
	manumycin	8	32	>65	>65
***Mycrobacterium smegmatis* ATCC 9341**	C1	8	>65	>65	>65
	C2	4	8	65	65
	manumycin	2	4	16	32
MRSA 693E	C1	4	65	>65	>65
	C2	4	8	32	65
	manumycin	2	4	16	65
MRSA 4-5	C1	4	>65	>65	>65
	C2	4	8	32	>65
	manumycin	4	4	16	65
MRSA 5-3	C1	4	>65	>65	>65
	C2	4	8	32	>65
	manumycin	2	4	16	>65
VRE 82	C1	16	>65	>65	>65
	C2	4	16	>65	>65
	manumycin	4	8	32	>65
VRE 89	C1	32	>65	>65	>65
	C2	8	>65	>65	>65
	manumycin	8	32	>65	>65
VRE 98	C1	32	>65	>65	>65
	C2	8	65	>65	>65
	manumycin	4	32	>65	>65
VRSA	C1	4	>65	>65	>65
	C2	4	8	32	32
	manumycin	2	4	16	16

Incubation Temperature: 37℃

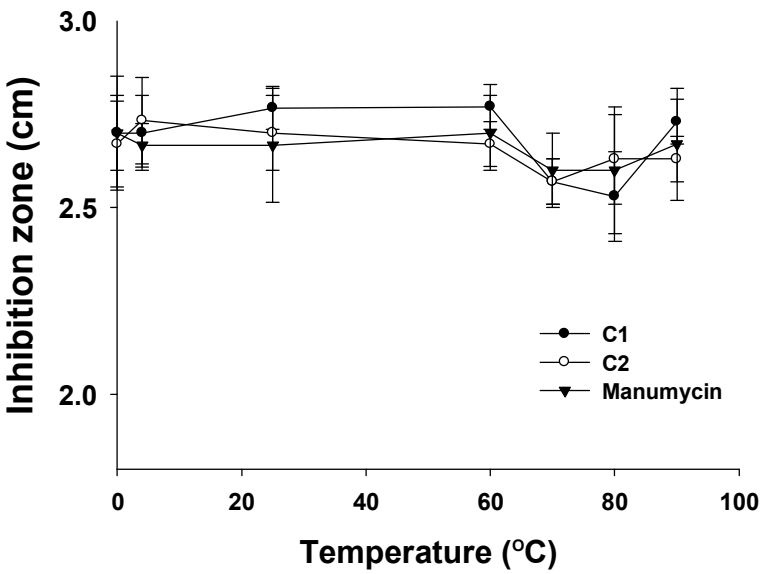

Figure 21. Stability of active compounds under various temperatures. The thermal stability of the compounds was studied by heating the samples at various temperatures for 4 h. The residual inhibitory activity was measured as described in materials and methods. Mean inhibitory diameters (cm) stored at different temperature levels.

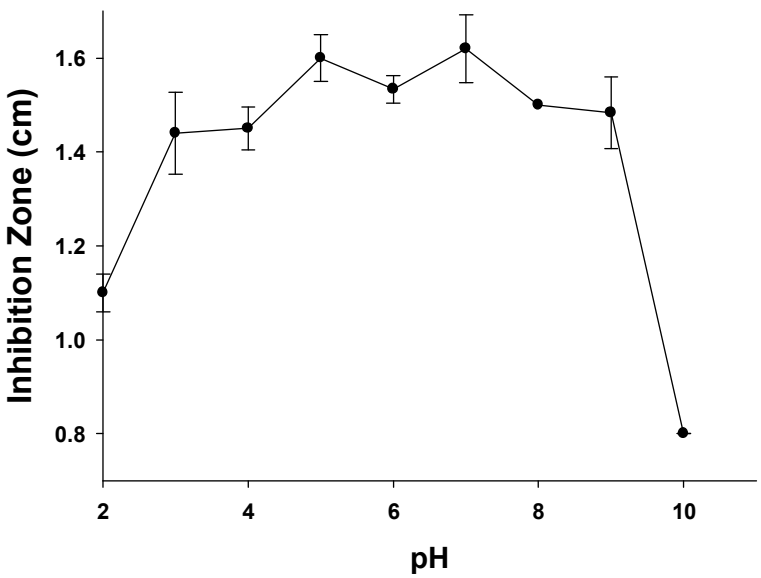

Figure 22. pH Stability of fermentation broth of *Streptomyces* sp. CS392. Fermentation broth was adjusted to pH levels 2 to 10. And the broth was incubated for 3-4 h at room temperature and then readjusted to pH 7.0. The activity of these treated antibiotic culture filtrates was determined by the sizes of the inhibition zones using the paper disc method described earlier.

2. Antioxidant activities

a. Determination of nitric oxide scavenging activity

We studied the effect of manumycin ant its derivatives on chemically generated NO using SNP. SNP is an NO donor that spontaneously generates NO in aqueous solution at physiological pH. Addition of SNP, in a course of 120 min, resulted in the production of NO in a time-dependent manner (Figure 23). However, the addition of C1 and manumycin (2 μg/mL) in methanol reduced nitrite production from SNP. For example, at 120 min, 2 μg/mL C1 and manumycin reduced the nitrite level by 28% and 19.5%, respectively. And the inhibitory rates of NO production by C2 throughout the 120 min observation period was small (10.6%). The inhibitory effect was not due to cell survival or to extracellular effects, such as direct inhibition or/and scavenging of NO (Figure 23, 26). It also did not seem to be directly due to inhibit on of iNOS activity.

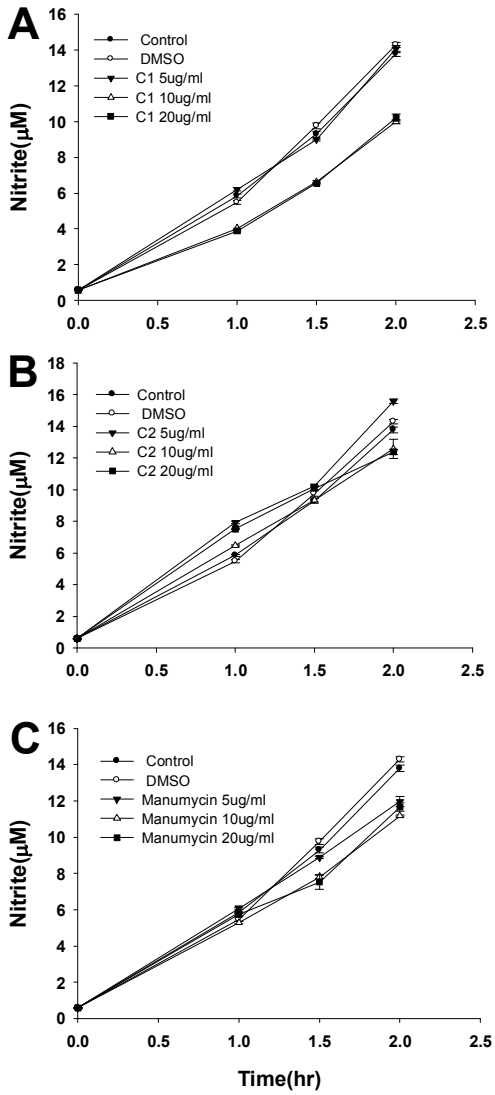

Figure 23. Effect of manumycin and its derivatives on the accumulation of nitrite upon decomposition of sodium nitroprusside (20 mM).

Data are mean±S.D. (n=3). Sodium nitroprusside was freshly prepared in PBS at 20mM. One milliliter of SNP was added into each test tube in the absence or presence of various concentrations (5–20 μg/mL) of the compounds or its vehicle, then the nitrite concentration was determined each hour using the Griess reagent, as mentioned above.

b. Reducing power

As illustrated in figure 24, Fe^{3+} was transformed to Fe^{2+} in the presence of manumycin and derivatives to measure the reductive capability. It was determined that C1 and C2 had similar differences in reducing power at concentrations from 0.01 to 1 mg/mL. And manumycin had highest activity among these compounds. At 0.25 mg/mL, the optical values of compounds were 0.328, 0.305 and 0.527. The reducing power of the treatments was in the order of: manumycin > C1≒ C2.

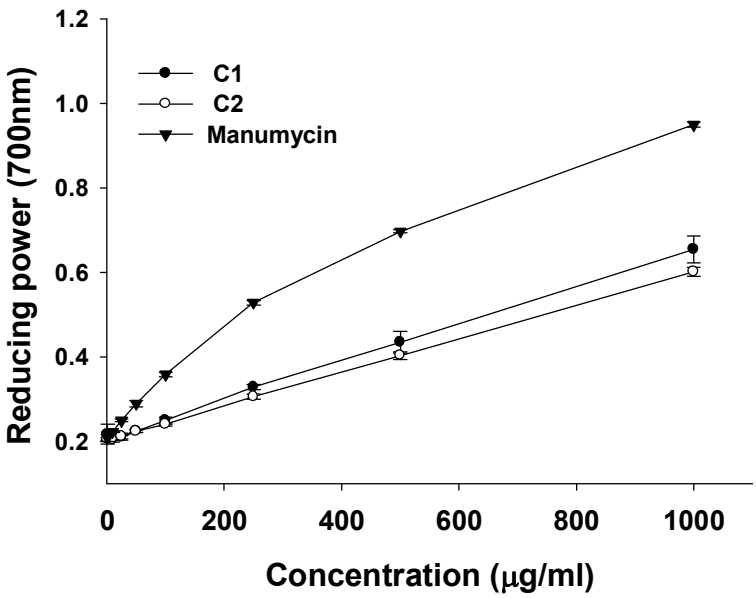

Figure 24. Reducing power of manumycin and its derivatives purified *Streptomyces* sp. CS392. The reductive abilities of active compounds. The absorbance (700nm) was plotted against concentration of sample. All values are mean ± SD of triplicates

3. Anti-inflammatory activity of active compounds

a. Cytotoxicity

Raw 264.7 cells were treated with various concentrations of manumycin and its derivatives for 24 h and the cell viability was tested by MTT assay as described above. As shown in figure 25, compounds did not exhibit cytotoxicity at the range of 1–20 μg/mL against RAW264.7 cells. Therefore compounds were used at 20 μg /mL and below for further experiments.

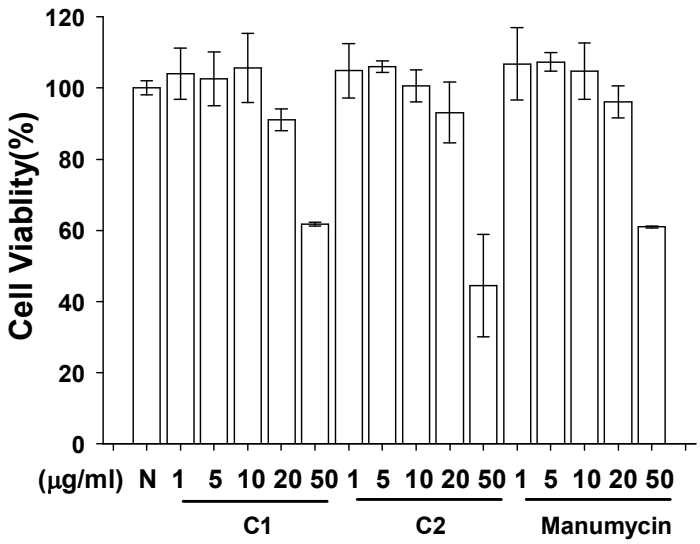

Figure 25. Effect of the active compounds on cell viability. Cell viability was measured after 24h incubation. Survival rates were tested with MTT assay in Raw 264.7 cells. Raw264.7 cells were incubated in the presence or absence of 1-20 μg/mL active compounds for 24 h. Each bar shows the mean ± S.D of three independent experiments performed in triplicate.

b. Inhibition of nitric oxide production and iNOS expression in LPS-induced Raw 264.7 macrophage cells.

Figure 26 illustrates the effect of manumycin derivatives on LPS-induced NO production and iNOS expression in Raw 264.7 macrophage cells. These compounds dose dependently inhibited NO production and iNOS expression in LPS-treated cells. When LPS treatment was not performed, manumycin derivatives alone did not affect NO production and iNOS expression in the cells. When Raw 264.7 cells were treated with 1 μg/mL of LPS, the levels of NO production and iNOS expression were markedly increased. However, treatment with compounds significantly inhibited LPS-induced production of NO and expression of iNOS protein in the range of 1-20 μg/mL. In particular, manumycin showed good inhibition of iNOS-derived NO production and expression of iNOS protein.

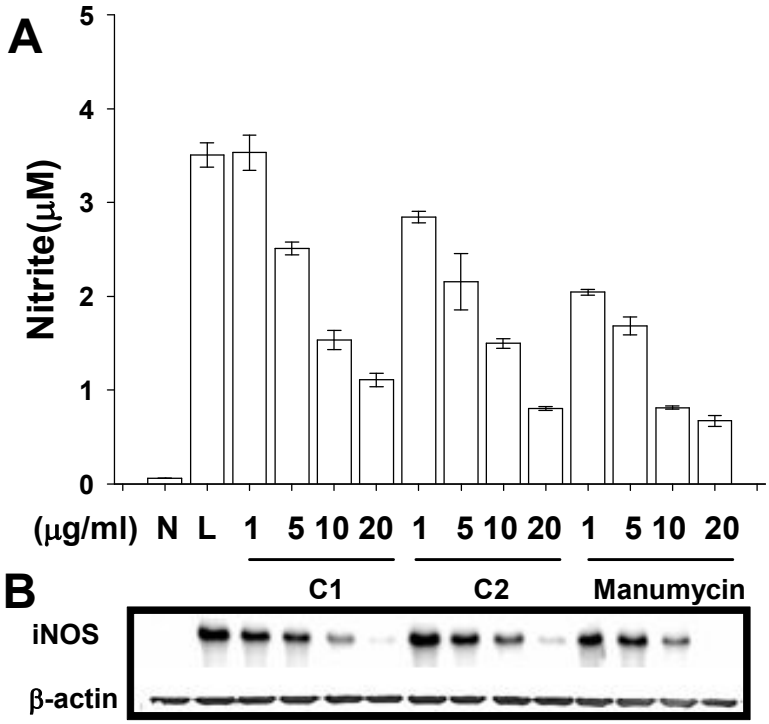

Figure 26. Effect of manumycin and its derivatives on suppression of NO production and iNOS expression in LPS-induced RAW264.7 macrophage cells.

Cells were pretreated with the various concentrations of compounds for 30 min, followed by treatment with 1 μg/mL of lipopolysaccharide(LPS). NO production in the culture media 24 h after the treatment was determined using the Griess reagent (A).Cells were pretreated with the various concentrations of compounds for 30 min, treatedwith 1 μg/mL LPS, and incubated for 24 h. Inducible nitric oxide synthase (iNOS) expression was analyzed by the western blot(B).

c. Inhibitory effects of active compounds on the productions of pro-inflammatory cytokines in LPS-induced Raw 264.7 macrophage cells

Raw 264.7 cells were treated with 1㎍/mL of LPS with or without indicated concentrations of compounds. The level of pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6 were determined by ELISA assay. As shown in Figure 27, LPS-induced cytokines release were significantly blocked by these compounds in a dose-dependent manner. C1, C2 and manumycin inhibited the release of TNF-α in LPS-activated Raw 264.7 cells. At high concentrations (20 μg/mL) C1, C2 and manumycin inhibited the TNF-α production by 40%, 48% and 40%. C1, C2 and manumycin inhibited the release of IL-1β in LPS-activated Raw 264.7 cells by 98%, 40%, and 38%. C1, C2 and manumycin inhibited the release of IL-6 in LPS-activated Raw 264.7 cells by 60%, 26%, and 32%. These results demonstrated that three compounds blocked LPS-induced pro-inflammatory mediators such as TNF-α, IL-1β, IL-6 (Figure 27) and nitric oxide (Figure 26) in macrophages, which might be responsible for its anti-inflammatory application.

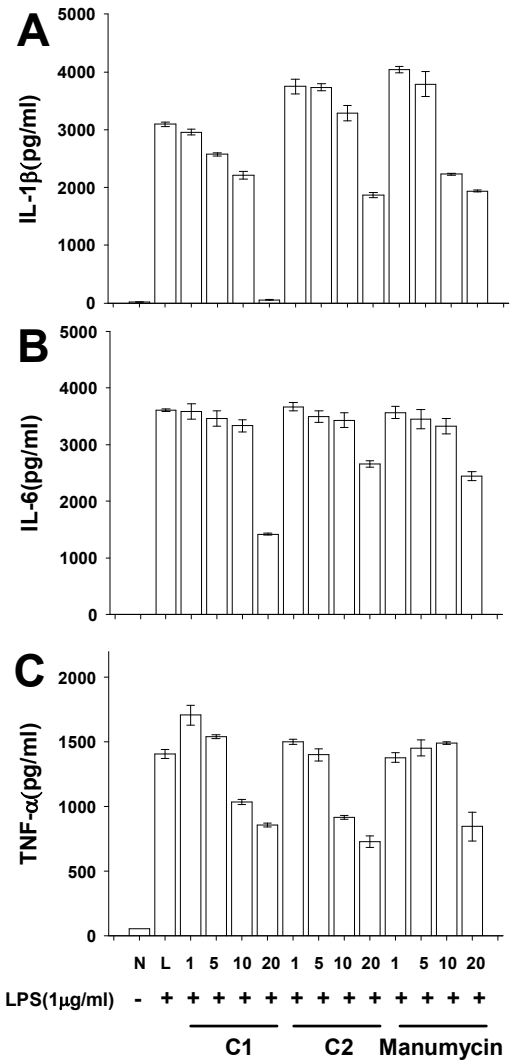

Figure 27. Effect of manumycin and its derivatives in the suppression of IL-1ß(A), IL-6(B) and TNF-α(C) productions in LPS-induced Raw 264.7 cells. Cells were pretreated with compounds for 30 min, followed by treatment with 1 μg/mL LPS for 24h. The amounts of, IL-1β, IL-6 and TNF-α released into the incubation media were measured by the ELISA assay

d. Inhibitory effects of active compounds on activation of NF-κB

The effects of manumycin and its derivatives on LPS-induced NF-kB activation, we examined western blot analysis for NF-kB p65 translocation into the nucleus. As expected, the level of p65 in the cytoplasm decreased in response to LPS treatment with an immediate increase in the nucleus up to 30 min. On the other hands, LPS-dependent change in the levels of p65 in the nucleus and cytoplasm was stopped in the macrophages treated with manumycin derivatives. These results suggest that these compounds may inhibit NF-kB binding activity by preventing the LPS-induced translocation of p65 to the nucleus (Figure 28).

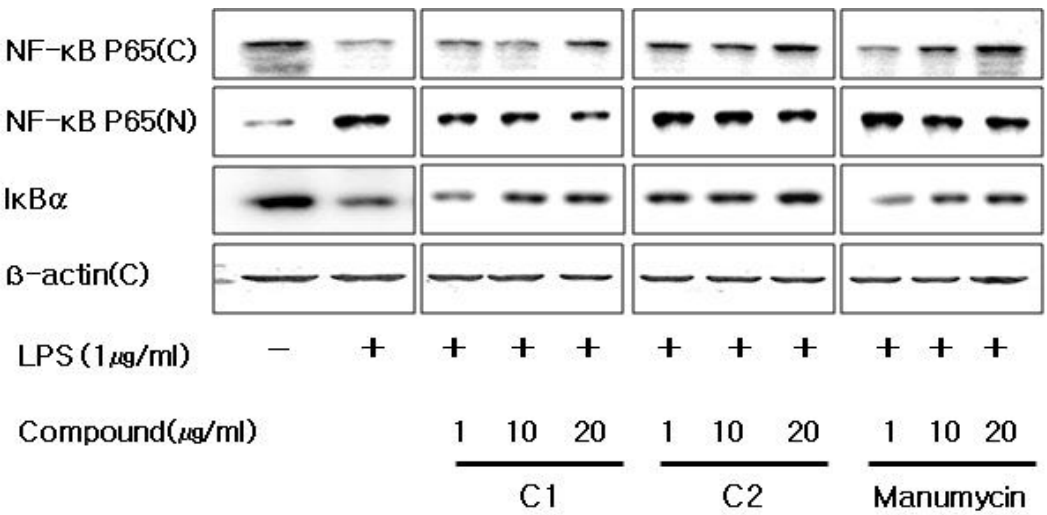

Figure 28. Effect of manumycin amd its derivatives on the activation of NF-κB in LPS-induced Raw 264.7 macrophage cells. Cells were pretreated with three compounds for 30 min, followed by treatment with 1 μg/mL LPS for 15 min. Treatment with these compounds (1~20 μg/mL) inhibited LPS-induced translocation of NF-κB p65. C (cytoplasmic extract), N (nuclear extract).

e. Effects of active compounds on TPA-induced ear edema of mice

Manumycin derivatives were submitted to the TPA induced ear edema test, at the dose of 50 μg/ear to evaluate the topical anti-inflammatory effect. These compounds were investigated in comparison to that of the control prednisolone. Prednisolone suppressed ear edema at a dose of 50 and 100 μg/ear in edema models.

As reported in Table 8, compounds and control exerted anti-inflammatory activity, inducing 22.1% (C1), 16.7% (C2), 29.2% (manumycin) and 48.5% (pred, 50 μg/ear) edema inhibition. manumycin gives the highest contribution to the anti-inflammatory activity of manumycin analogues. In this study, we further investigated the effect of manumycin derivatives on iNOS expression in each ear punch biopsies by western blot analysis.

We confirmed that iNOS expression was elevated in mouse edema with TPA for 5 h. Pre-treatment with manumycin derivatives 1h prior to the topical application of TPA resulted in decrease in the level of iNOS protein in mouse ear with TPA-induced acute inflammation (Figure 29). In short, TPA-induced up-regulation of iNOS was slightly suppressed by C1 and manumycin treatment.

Table 8. Anti-inflammatory activity of manumycin and its derivatives on TPA induced mouse ear edema (6h)

Groups	Dose (μg /ear)	Weight of left ear (mg)	Weight of Right ear (mg)	Difference (mg)	Inhibition (%)
Control	50	2.32±0.63	7.18±0.91	4.86±1.31	-
manumycin	50	6.78±0.86	5.08±0.47	1.7±0.81	29.2
C1	50	7.72±0.91	5.59±0.3	2.12±0.76	22.1
C2	50	7.26±0.63	5.98±0.65	1.28±0.78	16.7
Prednisolone	50	7.08±0.56	4.58±0.52	2.5±0.44	48.5

12-*O*-tetradecanoylphorbol-13-acetate (TPA: 1 μg/ear) alone or together with control (prednisolone : 50, 100 μg/ear) and derivatives (C1, C2, manumycin ; 50 μg/ear) in 10 ul acetone was topically applied to the right ear of ICR mice (n = 6). The weight of the ear punches were determined as described in Materials and Methods.

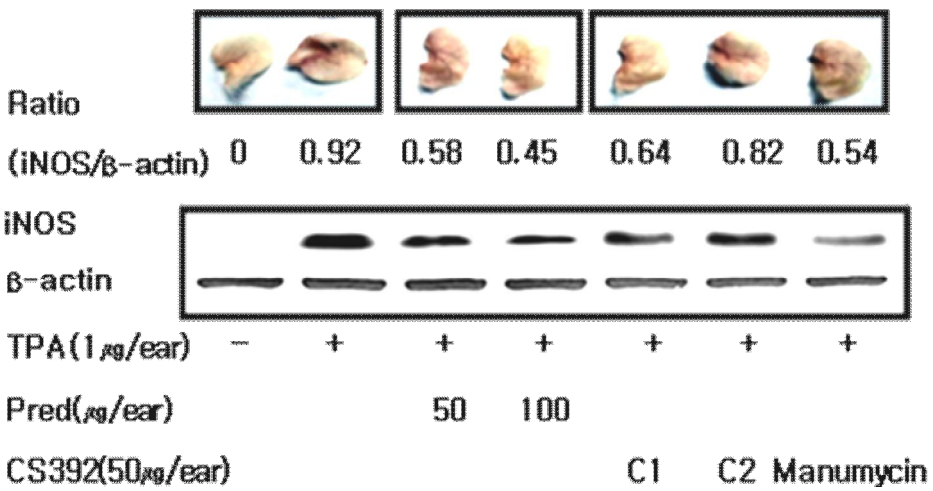

Figure 29. Effects of manumycin and its derivatives on TPA induced ear edema, and expression of iNOS in mice. Equal amounts of total proteins (30 μg /lane) were subjected to 9 % SDS-PAGE, and the expression of inducible nitric oxide synthetase (iNOS) in ICR mice ear edema tissues was detected by western blotting using specific antibodies. β-actin protein was used as an internal control.

f. Effects of active compounds in *in vivo* models of DSS-induced colitis in mice.

To confirm the anti-inflammatory effect of manumycin observed in the DSS-colitis in rats, we evaluated it in the chronic phase of the DSS model of colitis in mice, a well established model with resemblance to IBU (131).

To examine whether the beneficial effect showed by manumycin derivatives might be ascribed to a anti-inflammatory effect, we tested the effects of manumycin derivatives in the model of DSS mice colitis.

For this purpose, mice were given 3% (w/v) DSS dissolved in the drinking water for 7 days. Manumycin derivatives were given intraperitoneally to colitic mice everyday to evaluate the effects of the manumycins. At the dose of 10mg/kg/day, Manumycin derivatives showed good efficacy in this protocol.

In mice with DSS-induced acute colitis, we observed hemorrhage in the colonic lumen and a shortened colon on Day 7, marked diarrhea with bloody stools on Day 4, which is similar with a previous reports(132-133). We investigated the optimum manumycin dose and found that 1 to 10 mg/kg give dose dependent effect against DSS-induced acute colitis without observable cytotoxicity.

As a treatment, manumycin attenuated the severity of DSS-induced acute colitis, as evidenced by increased survival, decreased body weight loss (Figure. 30 A, C, E), and a reduction in disease activity index (Figure 30 B, D, F).

DSS colitis model was well characterized morphologically and biochemically. It has been suggested that the length of the colon is inversely associated with severity of DSS-induced colitis (131,134). We found that mice that received C2 and C3 (manumycin) treatment had longer colon than those treated with DSS (Figure 32 B, C). Symptoms had emerged on Day 3. The body weight

loss (Figure 30 A, C, E), and disease activity index (Figure 30 B, D, F) show that manumycin showed a good effect in reducing the severity of DSS-induced acute colitis.

Pathological examinations of colons were carried out after H&E staining and representative results are shown in Figure 31. The DSS colitis group exhibited marked erosion of the lamina propria mucosa, disappearance of glandular epithelium, inflammatory cell infiltration (Figure 31A). In the C3 (manumycin) treated group (Figure 31D) and the C2 treated group (Figure 31C), pathological inflammation was less severe than in the DSS colitis group.

Furthermore, as indicated by the proinflammatory cytokines and iNOS protein expression, manumycin administration reduced production of TNF-α (tumor necrosis factor- α), IL-6(interleukin 6), IL-1 β and expression of iNOS protein (Figure 33).

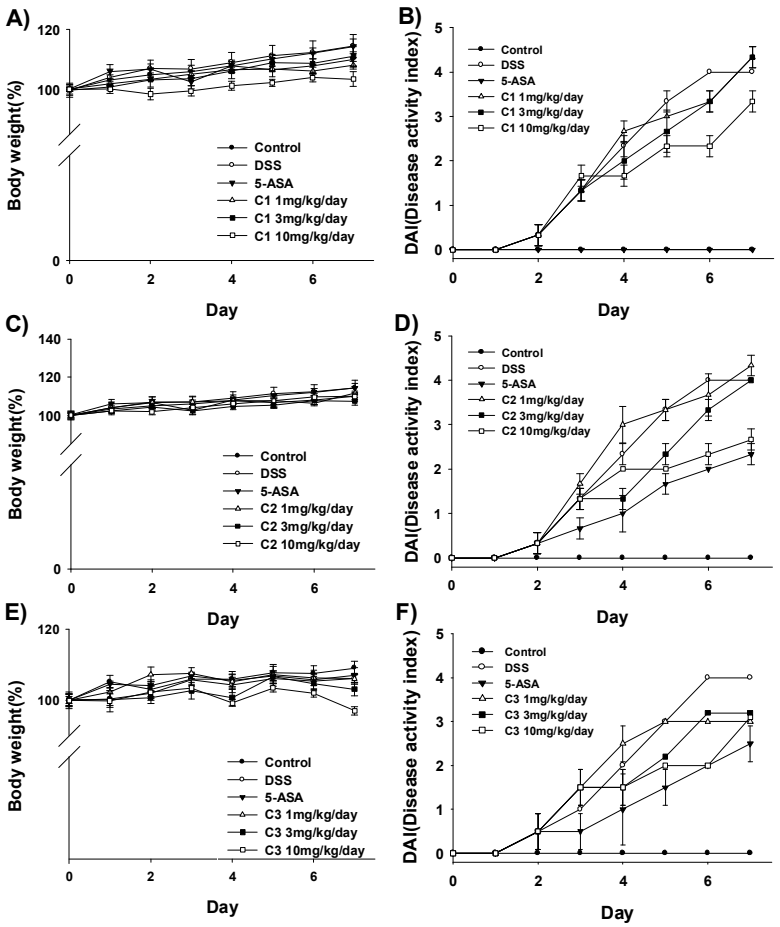

Figure 30. Preventive effects of manumycin and its derivatives treatment on the time course changes in the DAI over experimental period of 7-day in DSS model of mice colitis. The effect of manumycin(C3), C1 and C2 in DSS-induced acute colitis in ICR mice. Mice were injected intraperitoneally with vehicle or compounds from Day 0 to 7 at doses of 1, 3 and 10 mg/kg /day. 5-ASA was injected intraperitoneally at dose of 100 mg/kg/day. Body weight change and disease activity index were recorded on Day 0 to 7. (n=6 mice/group)

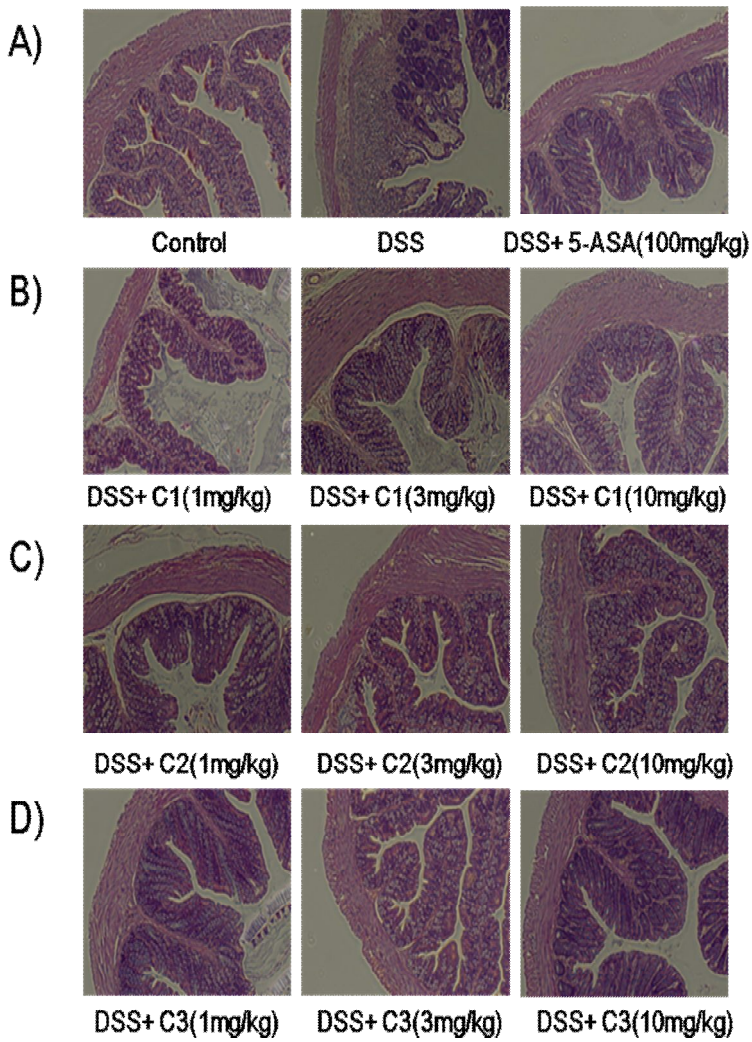

Figure 31. Effect of manumycin derivatives and 5-ASA (manumycin derivatives: 1, 3 and 10 mg/kg/day, 5-ASA: 100mg/kg/day) on the histological changes

Compounds were intraperitoneally administered starting on day 1. On day 8, mice were killed and colon biopsies were taken and fixed in 10% formalin solution. Samples were embedded in paraffin and sectioned (4μm sections). Sections were stained with hematoxylin and eosin and were viewed at either 100 magnification. (C3: manumycin)

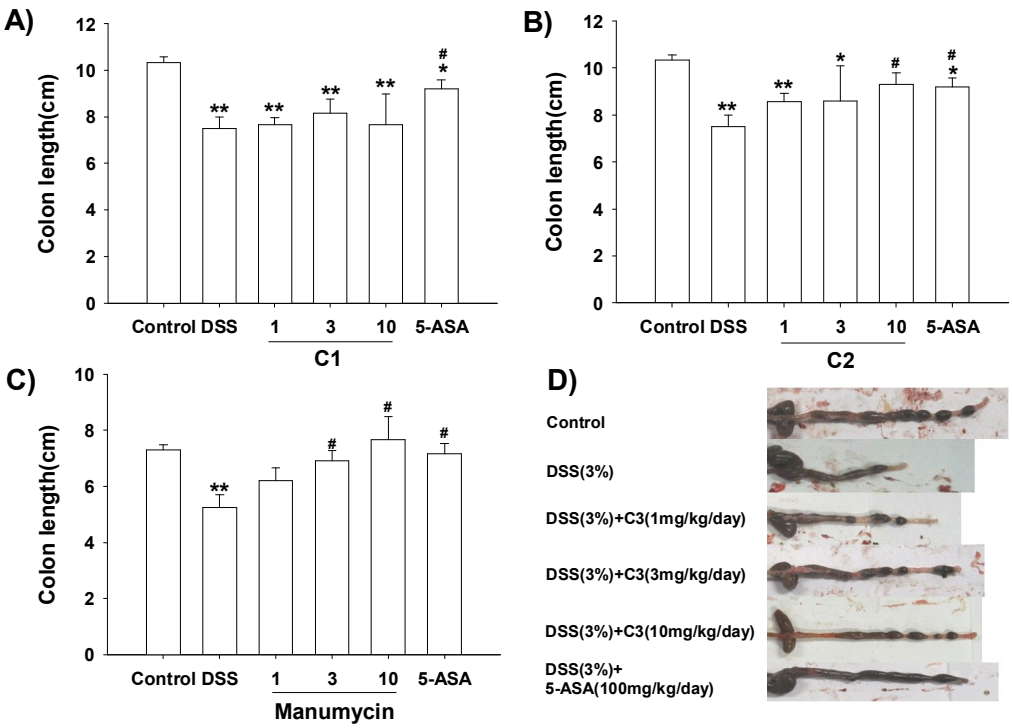

Figure 32. Effects of manumycin and its derivatives on colonic length in DSS mice colitis. Mice were administered 3% DSS in drinking water for 7 days with or without compounds (1, 3, 10mg/kg/day ip. up to 7 days). 5-ASA (100mg/kg/day ip up to 7 days) was used as a positive control. On day 7, mice were sacrificed and colons were removed. Length of colon in each group (A: C1, B: C2 and C: manumycin). Macroscopic findings of representative colon in each group (D: manumycin treatment), Data are expressed as Mean±SD. **P*<0.05 and ***P*<0.01 vs non-colitic group, #*P*<0.05 and ##*P*<0.01 vs DSS control group.

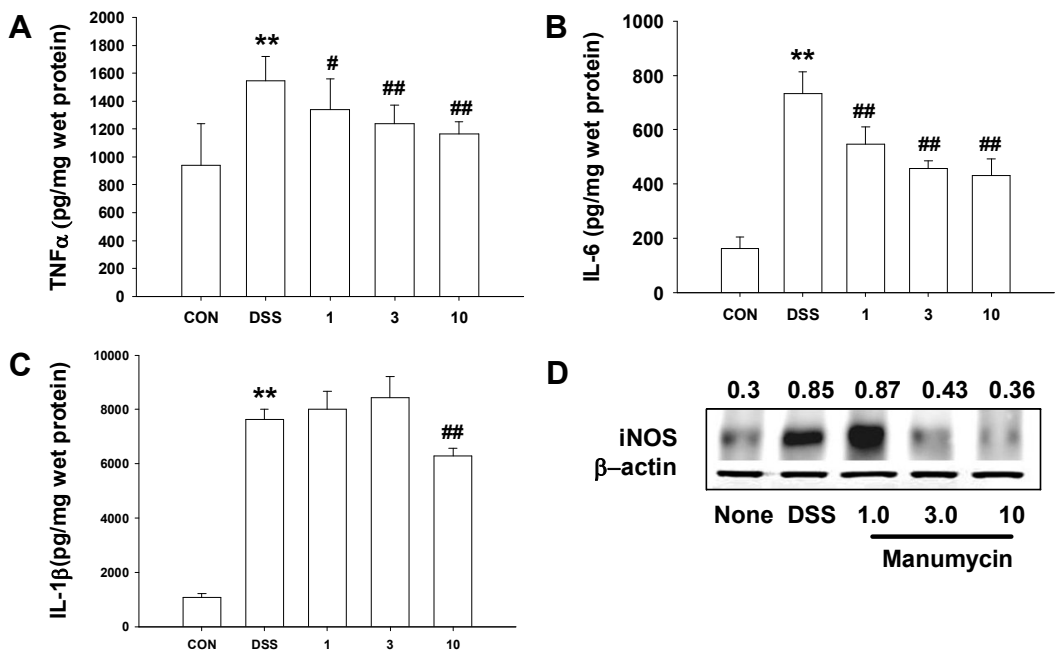

Figure 33. Effect of manumycin (1, 3, 10 mg/kg/day) in suppression of pro inflammatory cytokines (A: TNF-α, B: IL-6, C: IL-1 ß) and iNOS protein expression (D) in DSS induced colitis model. Mice were administered 5% DSS in drinking water (ad libitum) for 7 days with or without C3 (manumycin, 1, 3, 10mg/kg/day i.p .up to 7 days). The productions of (A) TNF-α, (B) IL-6, and (C) IL-1 ß were determined as described in materials and methods. (D)Western blotting was performed to detect iNOS expression.Data are expressed as Mean±SD. **P<0.01 vs non-colitic group, #P<0.05, ##P<0.01 vs DSS control group.

Ⅳ. Discussion

The actinomycete isolate, CS392 was isolated from soil sample collected from chonnam, Korea. Isolate was growing on ISP or oat meal containing medium for investigating its potency to produce antimicrobial agents. The actinomycte isolate, CS392 exhibited a wide spectrum antimicrobial activity. Identification of this strain has been carried out according to Bergey's manual (135), International Streptomyces Project (112, 136-137) and numerical taxonomic study of *Streptomyces* species program. For the purpose of identification of actinomycete isolate, the morphological characteristics and microscopic examination revealed that the spore chain was not spiral. Spore mass is red gray, spore surface was smooth, substrate mycelium is yellow brown and diffusible pigment was produced on ISP 4 media. The result of physiological, biochemical characteristics indicated that the actonomycetes isolate related to a group of *Streptomyces*. In this study, the identification of actinomycete isolate CS392 was suggested of being beloing to *Streptomyces* sp. The related sequence was aligned with available almost compete sequence of type strains of family *Streptomycetaeae*. The phylogenetic tree revealed that the local isolate is closely related *Streptomyces lanatus*. (Similarity is 98%).

The impact of carbon, nitrogen sources and metal ions on antimicrobial compounds production was studied. The optimum conditions for the antimicrobial compounds production in *Streptomyces* sp. CS392 are pH7.0, inoculum 3%, temperature 28 °C. Glucose affected maximum compounds production followed by fructose (Figure 9). Among nitrogen sources, peptone affected maximum activity followed by beef extract (Figure 10). The highest yield of antibiotic production was noticed with 1.0% glucose as a sole carbon source and 1% peptone as a sole nitrogen source and 0.01% magnesium chloride (Figure 11). Under the optimum fermentation medium, the

antimicrobial compounds production of *Streptomyces* sp. CS392 started at 12 h and reached to the maximum level after 24 h of cultivation. The gradual increase in the pH of the medium is characteristic of *Streptomyces* spp. There was a gradual increase in biomass as well as antibiotic production during log phase.

This indicates that high level of compounds production is observed during active biomass production. But generally, The manumycin-type metabolites are produced during the stationary growth phase of the organisms and are found either in the culture filtrate (*e.g.* asukamycin , antibiotic U-62162), or in the mycelium (*e.g.* manumycin A, B and C, colabomycins A and D) (31, 36-37, 138).

Effect of incubation time on growth and compounds production by *Streptomyces* sp. CS392 is shown in Figure 12. Further studies are needed to investigate scale up strategies for industrial antimicrobial compounds production.

The increase in isolates of *S. aureus* with resistance to methicillin and decreased susceptibility to vancomycin has created an urgent need for the development of new anti-staphylococcal agents.

In this study, manumycin and its derivatives demonstrated potent activity against gram positive bacteria. Bacteriocidal activity against strains of MRSA may also provide an advantage over available antibiotics used to treat antibiotic resistant strain. Especially, the in vitro bactericidal activity of manumycin and its derivatives was also superior against VRSA and VRE compared with oxacillin and vancomycin.

It was reported that manumycin group metabolite were effective against infectious microorganism (35-36, 38-39). Manumycin also showed good activity against gram positive bacteria. But until now there was no report of antimicrobial activity against antibiotic resistance bacteria.

This study evaluated the *in vitro* activity against three MRSA, three VRE and one VRSA strains. The antimicrobial activities of these compounds were remarkable. Based on this activity, one could consider manumycin analogues in the treatment of antibiotic resistant *Staphylococci* and *Enterococci* infections. However, to date, there has been no clinical experience with using manumycin analogues in the treatment of Staphylococcal and Enterococcal infections. Based on the *in vitro* activity seen in this study, one would expect manumycin derivatives to be a candidate for study against antibiotic resistant strains seen currently with increasing resistance to currently approved antimicrobial agents.

Antimicrobial compounds produced by strain CS392 in the culture supernatant were purified as illustrated in the diagrammatic sketch (Figure 13). From 3 L culture broth, 412 mg of ethyl acetate extract (dried) was achieved. Finally, 21 mg of total antimicrobial compounds were achieved. Considering ethyl acetate extract as 100%, the yield of the finally purified antimicrobial compounds (in total) becomes 5.1%. All the three compounds were in powder form and had similar but slightly different color ranged from yellow to pale yellow. Figure 14 depicts a TLC of three antimicrobial compounds, which are named as C1, C2 and manumycin, with *Rf* values of 0.7, 0.5 and 0.4, respectively.

Purification of the manumycin group metabolite follows standard procedures which involve extraction with organic solvents and column chromatography on silica gel and RP-silica gel. Alisamycin was purified from fermentation broth of *Streptomyces sp*. Y-88-31582 using by silicagel column chromatography. 6.4mg alisamycin per liter fermentation broth was obtained as a yellow crystalline powder (40). Manumycin E, F and G were purified from fermentation broth of *Streptomyces sp*. WB8376 using by counter-current distribution methods and preparative HPLC. Manumycin E (1.8mg), Manumycin F (1.3mg) and Manumycin G (1.5mg) per liter fermentation

broth was obtained. (35) Asukamycin was crystallized from antibiotic-enriched crude extracts. The extraction and crystallization procedure produced up to 140 mg of asukamycin per liter of the initial bacterial culture. But its purity was at least 85 % (183). In this study, C1 (0.8mg), C2 (2.1mg) and C3 (manumycin 4.1mg) per liter fermentation broth was obtained. Purification procedure as described in materials and methods give good yield of C1, C2 and C3 (manumycin)

In a number of cases more than one antibiotic of the manumycin-group was discovered within a producing strain. (32) In addition, a few cometabolites are reported. Apart from the manumycins A , B, C and D, *Streptomyces parvulus* (strain Tü 64) produces two red antibiotic pigments, undecylprodigiosin and metacycloprodigiosin , as well as (L)-2,5-dihydrophenylalanin.

Streptomyces verdensis (strain 360) produces U-56,407 as well as the cyclic peptide globomycin. In the colabomycin producing organism *Streptomyces griseoflavus* (strain Tü 2880) compound 2880-II was discovered by chemical screening methods as an additional biosynthetically related metabolite. (139-143) *Streptomyces* sp. CS392 produced similar type of derivatives of manumycin. Except manumycin and derivatives, there were no co metabolites in the culture broth of CS392 strain.

In HPLC analysis, C1, C2 and manumycin appeared between 15 to 20 min (Figure 15) in the solvent conditions as presented in Table 2. From the HPLC column, C1 eluted first followed by C2 and then manumycin with the peak area order manumycin>C2>C1. Among three compounds, manumycin occupies the major part with 58.6% of the total antimicrobial weight, followed by C2 (30%) and C1 (11.4%) (Figure15). Structure analysis of all the purified antimicrobial compounds are in progress, therefore, we will present their details in our future report.

Effects of compounds C1, C2 and manumycin, purified from *S*. sp. CS392 were evaluated against various pathogenic Gram-positive and Gram-negative bacteria. Antimicrobial effects, in

terms of MIC values, of all the purified compounds and two well known reference antibiotics named vanomycin and oxacilin, are illustrated in Table 6. All the antibacterial compounds displayed activities against a number of Gram-positive bacteria. Specifically, these compounds were effective against VRSA, MRSA and VRE. On the other hand, these compounds were less sensitive against Gram-negative bacteria with MIC values >65 μg/mL. Although being less sensitive, their effects against Gram-negative bacteria are comparable with vancomycin and oxacillin. Broadly speaking, manumycin showed the highest antimicrobial effect among the three purified compounds against various resistant pathogens. It is remarkable that the effect shown by manumycin against MRSA, VRE and VRSA was comparable with vancomycin and even better than that of oxacillin. In case of other Gram-positive pathogens, effects were in the order of manumycin>C2>C1, with the exception for *B. subtilis* Anti-VRE effects of manumycin (MIC, 8-16 μg/mL) determined with various strains were comparable and found even better than that of CSU-1 (MIC, 16 μg/mL), which was reported as a potent anti-MRSA/VRE antibiotic (144). In contrast, anti-MRSA effect of manumycin (MIC, 2 μg/mL) was slightly weaker than CSU-1 (MIC, 1 μg/mL). Effect of all the 3 antimicrobial compounds (MIC, 2-4 μg/mL) were much better than that of nargenicin (MIC, >80 μg/mL), which was also reported as a potent anti-MRSA compound (145). Antimicrobial effects of C1, C2 and manumycin against various VRSA strains (MIC, 2-4 μg/mL) are comparable with CSA-8 (MIC, 4 μg/mL) (146). After receiving full structural information, which is now in progress, we will study antimicrobial effects of these compounds in detail in relation to their affiliated reference antimicrobial compounds. Nevertheless, antimicrobial effects of these antibiotics, especially against various resistant pathogens, are strong so that they can be considered as potential therapeutic candidates after crucial clinical evaluation.

The reducing power assay indicates reduction activity by reacting with active oxygen and

free radicals to make them stable. In addition, the ferricyanide complex converts to the ferrous form and is accompanied with a color change from yellow to green via reducing power. The absorbance of a sample can be measured to determine the presence of the ferrous form at 700 nm. Hence, the higher the reducing power, the higher the absorbance values (147).

Figure 24 shows the effects of the manumycin derivatives at various concentrations (10-1000 μg/mL). It was determined that C1 and C2 had similar paterns in reducing power at concentrations from 10 to 1,000 μg/mL. manumycin showed good activity among three compounds. In the range of 10-1,000 μg/mL, the reducing power of the treatments was in the order of manumycin >C1 ≒ C2. Reactions of free radicals such as superoxide radical, hydroxyl radical, peroxyl radical and other reactive oxygen and nitrogen are associated with diseases such as Alzheimer, Parkinson diseases, cardiovascular disease, atherosclerosis, dementia, and cancer. Antioxidants delay or prevent oxidative damage and thus they may be useful as therapeutics (48, 50, 55, 153-155).

Figure 24 presents the NO-scavenging effects of manumycin and its derivatives. C1 and manumycin had NO-scavenging activity. C1 was a good NO-scavenger among three compounds, whereas C2 showed NO-scavenging activity slightly. These compounds which had NO-scavenging activity ranging from 10.5% to 28% indicating that they were only moderately effective NO-scavengers.

In general, antioxidant, phenolic compounds have been shown to scavenge NO and inhibit NO production (156-157). For example, flavonoids, many active compounds reduce nitrite levels of stimulated macrophages (158). At the level of gene expression, many phenolic compounds that scavenge free radicals, such as curcumin, are inhibitors of iNOS gene expression in murine macrophages (156, 159-160).

Typically, antibiotics had no NO-scavenging activity. And some antibiotic show reduction of nitric oxide levels via suppression of expression of iNOS protein. But manumycin derivatives had dual

inhibition of nitric oxide production and iNOS expression. Elimination of NO both by NO-scavengers and iNOS inactivators is helpful to understand the basics of immune response and inflammation.

Some antimicrobial compounds have additive biological activities, antitumor, anti-oxidative and anti-inflammatory activity. A few compounds possess dual / triple activities in a single component (1, 4, 161-166). Manumycin and its derivatives also showed the multi-functional activities in our study.

Some antimicrobial and antioxidant compounds or extract isolated from various source of nature. Jung et al. reported the antioxidant effect of mixture of gallic acid and linoleic acid (148). Oliveira et al. reported antimicrobial and antioxidant compounds from Hazel (Corylus avellana L.) (149). Tepe et al. evaluated the antimicrobial and antioxidant activities of the essential oils and methanol extracts of Salvia cryptantha (Montbret et Aucher ex Benth.), Cyclotrichium origani folium (Labill.) and Salvia multicaulis (Vahl) (150-151). In soil microorganisms, *Streptomyces* strain was found to produce herbimycin A and dihydroherbimycin A as antioxidants in the culture filtrate. These compounds exhibited the potent antioxidant activities in DHHP radical scavenging assay and lipid peroxidation assay (184). In our investigation, C1, C3 (manumycin) showed antioxidant activity in nitric oxide scavenging activity and reducing power assays. Antioxidant activity of each of these compounds was less than that of ascorbic acid. Among three compounds, manumycin is known as a natural/biosynthetic compound which has shown antimicrobial activity. We report the antioxidant activity of manumycin for the first time. And we suggest that m-C7N unit which is similar to phenolic structure contributed to the antioxidant activity of manumycin. But further studies are necessary to esteblish the mechanism of antioxidant activity.

We described the purification of manumycin derivatives and its antimicrobial and anti-oxidative properties. In the present study, these compounds reduced LPS-induced nitric oxide and pro-inflammatory cytokine production in Raw 264.7 cells.

Nitric oxide (NO) is an important intracellular and intercellular signalling molecule involved in the regulation of diverse physiological and pathophysiological mechanisms in cardiovascular, nervous and immunological systems. At low concentration of NO, It acts as a biological mediator, and important host defense effector in the immune system. But, abnormal nitric oxide levels, it is a free oxygen radical (NOS) and can act as a cytotoxic agent in pathological processes, particularly in inflammatory disorders (167-170). Inhibition of iNOS may be beneficial for the treatment of inflammatory disease (168, 171-172).

In inflammatory process, stimulated macrophage produce pro-inflammatory cytokines such as interleukin (IL)-6, interleukin (IL)-1ß and Tumor necrosis factor α (TNF-α). Wu CH et al. reported that NO suppression can inhibit LPS-induced TNF- α and IL-1β in RAW264.7 cells (173). Many researchers described that anti-inflammatory compounds inhibit iNOS expression and decrease secretion of pro-inflammatory cytokines (101, 103, 174-175). MJ S reported that vitisin A dose dependently inhibited LPS-induced NO production and inducible NO synthase (iNOS) expression. In contrast, the production of pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) was not altered by vitisin A (100). In this study, all compounds inhibited iNOS dependant NO production, TNF-α, IL-6 and IL-1ß productions and iNOS protein expression (Figure 26, 27).

Transcription factors like NF-κB (nuclear factor kappa B) in one of the important regulator of gene expression such as iNOS, COX-2, VCAM-1 (Vascular Cell Adhesion Molecule-1) and ICAM-1 (Intercellular Cell Adhesion Molecule-1) in immune and inflammatory responses (176). NF-κB is present in the cytosol as an inactive complex IkB-NFkB. The IkB-NFκB complex is

phosphorylated by IkB kinase (177) through activation by stimulator such as LPS, cytokines, interferon-g (IFNg), and tumor necrosis factor-α (TNF-α) which facilitate the translocation of free NF-κB from cytosol to the nucleus and the induction of iNOS gene expression (178-181).
Because of these reasons, we conformed, by western blot that C1, C2 and C3(manumycin) blocked NF-κB p65 nuclear translocation and IκBα degradation (Figure 28).

In conclusion, first, we found that these compounds had antimicrobial and anti-oxidative activities. Second, this is the first investigation of C3 (manumycin) in its anti-inflammatory activity *in vitro*. Because of its various activities, it is expected that these compounds may be beneficial for the treatment of infectious and inflammatory diseases.

So, for further *in vivo* studies, we investigated the protective effect of the compounds on a mice model of ear edema and colitis.

To investigate a relationship between *in vitro* and *in vivo,* these compounds were assayed for topical anti inflammatory activity by TPA induced ear edema model. C1, C2 and C3 (manumycin) were topically applied to the inner and outer surfaces of the left ear of mice. The anti inflammatory activity in the TPA-induced ear edema test, was shown by these compounds (C1: 22.1%, C2: 16.7%, manumycin: 29.2% inhibition), which did not show any side effect in this examination. C1, C2 and manumycin showed slight inhibition of edema formation in this method. Using each ear punch biopsies, protein samples were taken from ear whole cell lysate, we further investigated the effect of three compounds on iNOS expression by western blot analysis. Three compounds inhibited TPA-induced expression of iNOS. In conclusion, these compounds have topical anti inflammatory activity mediated via inhibition of iNOS expression.

In the present study, we demonstrated anti-inflammatory activities of C1, C2 and C3 (manumycin) in *in vitro*, using LPS stimulated Raw 264.7 macrophages. Consistent with the *in vitro* anti-inflammatory effects, C3 (manumycin) attenuated TPA-induced mouse ear edema

(29.2%), C1 and C2 showed weaker activity than C3 (manumycin). Usually, manumycin-type compounds are unstable under acidic or basic conditions and in light. Under mild oxidation conditions the C-4/C-7 bond is selectively cleaved. Increasing the concentration of chromic trioxide and longer reaction time leads to C12-epoxybenzoquinone and (-)-(2*R*)-2-methylhexanoic acid formed by additional cleavage of the C-4 double bond (32, 36,186).

The lower solubility, poorer stability due to cleavage of C-4/C-7 bond in the six membered ring so called m-C7N unit of C3(manumycin) might explain the lower *in vivo* activity. Further structural study for C1and C2 is necessary to confirm it.

Inflammatory bowel disease (IBD) is a common disorder, probably involving an immune reaction of the body to its own intestinal tract. UC (ulcerative colitis) is one of the major types of IBD. UC is associated with chronic inflammation of the intestinal tract (colon) of unknown etiology. Histologically, damages such as mucosal accumulation of leukocytes, crypt shortening, and erosion of epithelium found in the intestine. Activation of T cells and macrophages has been regarded as an important factor in its pathogenesis. Some antibiotics are effective against experimental and clinical colitis (189-190) and drugs such as 5-aminosalicyclic acid, corticosteroids, azathioprine, mercaptopurines, and cyclosporine are also used to treat IBD (187-188).

Manumycin group metabolite has been shown to possess antimicrobial, antifungal, antitumor and interleukin-1 converting enzyme inhibition properties in *in vitro* experimental models (30, 34-46). In our experiments, C1, C2 and C3 (manumycin) reduced pro inflammatory cytokines and inducible nitric oxide synthase (iNOS) expressions in murine macrophage as well as in TPA induced ear edema model. Therefore, the use of a single compound with both immunomodulatory and

antimicrobial activities could be very interesting in the pharmacological treatment of IBD. The intraperitoneal administration of these compounds has not been reported to exert intestinal anti-inflammatory effect in DSS induced colitis model.

So, we investigated the protective effect of the main compounds from *Streptomyces*. sp. CS392 on a mice model of colitis.

Colitis was induced by administration of dextran sulfate sodium (DSS) (3% w/v) in drinking water to ICR mice (male, 5 weeks) for 7 days. DSS colitis model was well characterized morphologically and biochemically. DSS produced decrease in colon length and accompanied by mucosal edema and bloody stool. To assess the effect of the dose of C1, C2 and C3 (manumycin) on DSS-induced colitis, 1, 3, or 10 mg/kg/day of compounds were administered by intraperitoneal (ip.) injection. Sample treatment was begun at the same time as DSS was administered. For 7 days, we estimated disease activity index (182), which took into account body weight, stool consistency, gross bleeding. Colon lengths were measured.

DAI (disease activity index) is the most commonly used objective assessment for determining the state of inflammation of the colon. (132-133,189) So, DAI index was first used to evaluate the efficacy of the compounds in DSS induced colitis model. In this study, DAI index is scored from 0 to 5, Zero represents no disease symptoms.

Inflammatory symptoms were reduced after treatment C2 and C3 (manumycin). General assessment indicated that the ulcerative colitis had significantly improved. The Disease Activity Index decreased from an average of 4.0 to 2.5 in C2 and C3 treatment groups.

Histologically, the colitis control group exhibited marked colonic shortening, erosion of the lamina propria mucosa and infiltration of neutrophils in the lamina propria and other related findings.

C2 and C3 (manumycin) administration was found to protect the colonic shortening

and to reduce neutrophil infiltration in the mucosa and lamina propria induced by DSS. Pro-inflammatory mediators such as tumor necrosis factor-α (TNF-α), interleukin-1ß (IL-1ß), and interleukin-6 (IL-6), were determined using ELISA. Tumour necrosis factor α (TNF-α) has been shown to play a critical role in the pathogenesis of inflammatory bowel disease (IBD). TNF-α blockers are biological agents that specifically target this key cytokine in the inflammatory process and have become a mainstay in the therapy of inflammatory bowel diseases (191-192). C3 (manumycin) inhibited the abnormal secretions of pro-inflammatory cytokines, such as TNF- α. And IL-1ß and IL-6 are important pro inflammatory cytokines of IBD. Minocycline and tetracycline were found to suppress the pro inflammatory cytokine production (194). In addition, inosine was found to attenuate the pathological symptoms of DSS-induced colitis and pro inflammatory cytokine production such as IL-1, IL-6 (195). These findings point out the fact that reduction of pro-inflammatory cytokines may represent an effective therapeutic approach for IBD.

In the present study, it was observed that the levels of pro-inflammatory cytokines such as IL-1ß and IL-6 were increased in the colonic tissues of mice with DSS-induced colitis. And the intraperitoneally administration of C3 (manumycin) reduced these cytokines production.

Furthermore, C3 (Manumycin) administration suppressed inducible nitric oxide synthase (iNOS) expression. Treatment of mice with a synthetic inhibitor specific to iNOS efficiently attenuates the severity of experimental colitis (193). Taken together, these results show that C3 (manumycin) attenuates colitis by suppressing pro-inflammatory mediators, such as NO, TNF- α, IL-1ß , and IL-6, in the colonic tissues of mice with DSS-induced colitis. C3 (manumycin) should be considered as an alternative agent for treatment of IBD.

These results suggest that manumycin has an anti-inflammatory effect that is due to the

down-regulations of iNOS expression and the productions of inflammatory mediators. And it could be one of the possible mechanisms of action of C3 (manumycin). But, to expect the possibility of development of C3 (manumycin) as a therapeutic agent, further studies are necessary to establish the constructive mechanism of actions of the compound.

In conclusion, three immunomodulatory compounds with strong antimicrobial activity were purified from a new *Streptomyces* strain isolated from Korean soil. One of the purified compounds was identified as manumycin and two others were as its derivatives. The results drawn from this study provide a scientific basis for the application of these compounds as antimicrobial anti-inflammatory agent. Further studies should/will be undertaken to explain the mechanism of action and roles of immune response by which these compounds exert their biological activity.

Ⅴ. References

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