Title	Metabolites Produced by Marine and Terrestrial Bacteria Streptomycetes
Author	Dr Hnin Yu Win
Issue Date	2014

Metabolites Produced by Marine and Terrestrial Bacteria *Streptomycetes*

Hnin Yu Win*

Abstract

In the search for bioactive metabolites from microorganisms, the selection of suitable bacterial strains was carried out based on chemical and biological screening. Fermentation and extraction of the selected strains were done under standard conditions. In the present work, some interesting new compounds isolated from the selected bacterial strains were presented. The new compounds involved the mandalarpyrones, a new isatine derivative, ayeyarwadayquinoline and two new kromycin derivatives. Isolation and structure elucidation of the compounds were performed using various chromatographic methods and their spectroscopic data respectively. On the stage of pure metabolites, antibacterial, antifungal, and cytotoxic activity were measured.

Keywords: antibacterial, terrestrial, marine-derived *Streptomyces* spp.

Introduction

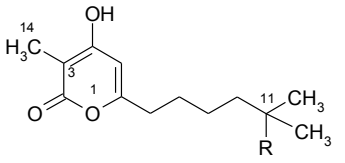
Natural products are naturally derived metabolites from microorganisms, plants or animals (Baker, et al., 2000). Over the past decade, microorganisms and especially marine bacteria have been recognized as an important source for bioactive compounds. Natural products are still an important source of new pharmaceutical compounds. Moreover, natural products provide challenges to synthetic chemists.

Streptomycetes are known to produce diverse groups of interesting bioactive secondary metabolites. In the search for pharmacologically active metabolites from microorganisms, the screening of extracts from terrestrial and marine-derived bacteria is performed continuously. In the present work, three terrestrial *Streptomyces* spp. strains and two marine-derived bacteria strains were selected according to their chemical (spot patterns on TLC) and biological screening (agar diffusion test against different microorganisms).

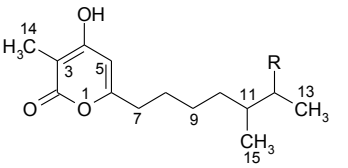
The extracts of the culture broth of *Streptomyces* sp. B8042 delivered 5 new compounds, mandalarpyrones A-D (**1-4**) and 4-chloroanthranilic acid (**6**), together with 7 known compounds. A further new α-pyrone derivative, mandalarpyrone E (**5**) was isolated from the

*Lecturer, Dr. rer. nat., Department of Chemistry, University of Mandalay

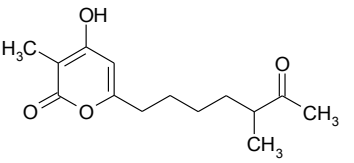
extracts of the culture broth of *Streptomyces* sp. B8480 along with 10 known compounds. New isatine derivative namely 5-methoxy 6-hydroxyisatine (**7**) was isolated from terrestrial *S*. sp. Adm 13. Another chloro containing compound, ayeyarwadayquinoline (**8**) and further known compounds were isolated from the terrestrial *S*. sp. Ank 38. Macrolide antibiotic kromycin (**11**) together with two new kromycin derivatives (**9, 10**) were isolated from the terrestrial *S*. sp. Ank 132.



1, R = OH
2, R = H

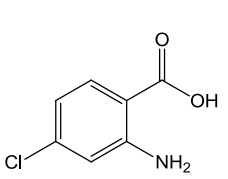


3, R = H
4, R = OH

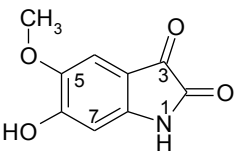


5

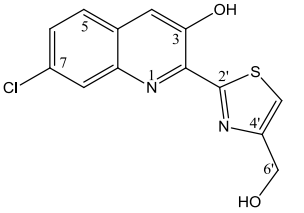
Figure 1. Structures of Mandalarpyrones A-E (**1-5**) from *Streptomyces* spp.



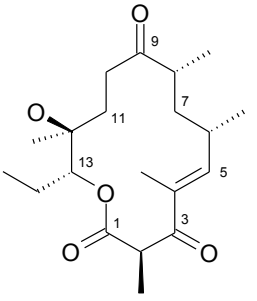
6



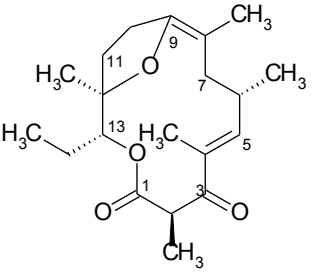
7



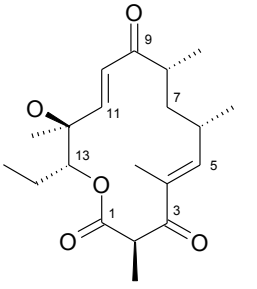
8



9



10



11

Figure 2. Structures of Compounds (**6-11**)

Materials and Methods

General Experimental Procedures

Optical rotation was measured on a Perkin-Elmer polarimeter (model 241) (Perkin-Elmer, San Jose, CA, USA). NMR spectra were measured on a Varian Inova 600 (599.740 MHz) and a Varian Unity 300 (300.145 MHz) spectrometer. ESIMS were measured on a Quattro Triple Quadrupol mass spectrometer with a Finnigan TSQ 7000 with nano-ESI API ion source. HRESIMS were measured on a Micromass LCT mass spectrometer coupled with a HP 1100 HPLC and a diode array detector. Column chromatography was carried out on MN silica gel 60, 0.05-0.2 mm; TLC was performed on Polygram SIL G/UV_{254}. All silica gel materials were purchased from Macherey-Nagel, Düren, Germany. Size exclusion chromatography was done on Sephadex LH-20 (Lipophilic Sephadex; Amersham Biosciences, Freiburg, Germany, purchased from Sigma-Aldrich Chemie, Steinheim, Germany). XAD-16 adsorber resin was obtained from Rohm and Haas (Frankfurt, Germany).

Biological screening

The crude extract was dissolved in $CHCl_3$/10% MeOH (400 μg/paper disk), in which the paper disks were dipped, dried under sterile conditions (flow box) and put on an agar plates inoculated with *Bacillus subtilis* (ATCC6051), *Staphylococcus aureus*, *Streptomyces viridochromogenes* (Tü 57), *Escherichia coli*, *Chlorella vulgaris*, *Chlorella sorokiniana*, *Scenedesmus subspicatus*, *Candida albicans* and *Mucor miehei* (Tü 284). The plates were incubated at 37 °C for bacteria (12 hours), 27 °C for fungi (24 hours), and 24-26 °C under day-light for micro-algae (96 hours). The diameter of the inhibition zones was measured by ruler.

Brine shrimp microwell cytotoxicity assay

To a 500 ml separating funnel, filled with 400 ml of artificial seawater, 0.5 g of dried eggs of *Artemia salina* was added. The suspension was aerated by bubbling air into the funnel and kept for 24 to 48 hours at room temperature. After aeration had been removed, the suspension was kept for 1 h undisturbed, whereby the remaining unhatched eggs dropped. In order to get a higher density of larvae, one side of the separating funnel was covered with aluminium foil and the other illuminated with a lamp, whereby the phototropic larvae were gathering at the illuminated side and could be collected by pipette. 30 to 40 shrimp larvae were transferred to a

deep-well microtiter plate (wells diameter 1.8 cm, depth 2 cm) filled with 0.2 ml of salt water and the dead larvae counted (number N). A solution of 20 µg of the crude extract in 5 to 10 µl DMSO was added and the plate kept at r.t. in the dark. After 24 h, the dead larvae were counted in each well under the microscope (number A). The still living larvae were killed by addition of ca. 0.5 ml methanol so that subsequently the total number of the animals could be determined (number G). The mortality rate M was calculated in %. Each test row was accompanied by a blind sample with pure DMSO (number B) and a control sample with 1 µg/test actinomycin D. The mortality rate M was calculated using the following formula:

$$M = \left[\frac{(A - B - N)}{(G - N)} \right] \cdot 100$$

with

M = percent of the dead larvae after 24 h.
A = number of the dead larvae after 24 h.
B = average number of the dead larvae in the blind samples after 24 h
N = number of the dead larvae before starting of the test.
G = total number of brine shrimps

Taxonomy

All marine-derived *Streptomyces* sp. B strains were isolated and taxonomically identified by E. Helmke from the Alfred-Wegener Institute for Polar and Marine Research in Bremerhaven, Germany. They were cultivated on M_2^+ medium (= M_2 medium + sea water). All terrestrial *Streptomyces* sp. strains were isolated and taxonomically identified by H. Anke, IBWF (Institute for Biotechnology and Drug Research, Kaiserslautern University, Germany). The strains were cultivated on M_2 medium. Taxonomically identification of strain B8042 was only mentioned in this paper.

The strain B8042 has been derived from sediment of the Laguna de Terminos at the Gulf of Mexico and was isolated on chitin agar (Weyland, 1981) containing 50% natural seawater. The sequence of the 16S rRNA gene of the strain B8042 is 98% similar to that of *Streptomyces radiopugnans* type strain R97 (accession no. DQ912930). The reference culture of *Streptomyces* sp. B8042 is kept on yeast extract-malt extract agar

in the Collection of Marine Actinomycetes at the Alfred Wegener Institute for Polar and Marine Research in Bremerhaven.

The substrate mycelium is orange/beige and the aerial mycelium is yellow with flexious (*Rectiflexibiles*) spore chains. The surface of spores is warty. Melanin pigment is neither produced on peptone yeast-extract iron agar nor on tyrosine agar (Shirling and Gottlieb, 1966)· Optimum growth temperature is at about 30 °C. The strain does neither grow at 10 °C nor at 46 °C. Growth is only obtained in media with seawater salinity from 0% up to 3%. Starch, casein, and chitin are degraded (Helmke and Weyland, 1984). Nitrate reductase is not produced.

Work Up Procedure

M_2^+ medium (250 g malt extract, 100 g yeast extract and 100 g glucose in 12.5 L tap water and 12.5 L seawater, pH 7.8) was prepared and transferred to 100 of 1 L Erlenmeyer flasks (250 mL each). The medium was autoclaved and inoculated by a well-grown agar culture of the marine-derived *Streptomyces* sp. B8042. The culture was incubated on a linear shaker (110 rpm) at 28 °C. The culture broth was harvested after five days, mixed with Celite, and filtered off under pressure. The culture filtrate was adsorbed on an XAD-16 column and the resin was washed with demineralized water and eluted with methanol to obtain a dark brown extract. The biomass was extracted exhaustively with ethyl acetate and acetone. The working procedure for all marine-derived strains was the same as for B8042, and the culture broth was harvested between five to seven days. Terrestrial strains were cultivated on M_2 medium (250 g malt extract, 100 g yeast extract and 100 g glucose in 25 L tap water, pH 7.8). Fermentation and extraction procedures were the same as for marine-derived strains.

Purification and Characterization

The combined extracts (water phase and biomass) (6.22 g) of strain B8042 were chromatographed on a silica gel column using a dichloromethane/methanol gradient, which afforded 7 fractions. Separation of fraction IV on Sephadex LH-20 afforded subfractions IVa and IVb. Subfraction IVa showed two UV absorbing spots, which turned to violet with anisaldehyde/sulfuric acid. Separation by silica gel column chromatography with a stepwise gradient of increasing polarity (CH_2Cl_2 to CH_2Cl_2/10% MeOH) resulted in mandalarpyrone A (**1**, 3.9 mg),

mandalarpyrone B (**2**, 83.3 mg) and mandalarpyrone C (**3**, 41.0 mg). Subfraction IVb was purified on RP-18 ($MeOH/H_2O$, 20:80) to give 4-chloroanthranilic acid (**6**, 11.7 mg). It showed a strong blue UV fluorescence and stained to yellow with anisaldehyde/sulfuric acid. Separation of fraction VI on a Sephadex LH-20 column followed by RP-18 ($MeOH/H_2O$, 50:50) yielded mandalarpyrone D (**4**, 56.3 mg). It showed strong UV absorption at 254 nm and turned to violet with anisaldehyde/sulfuric acid.

The brown crude extracts of strain B8480 were separated on a silica gel column using a dichloromethane/methanol gradient, which afforded three fractions. Mandalarpyrone E (**5**, 7.7 mg) was isolated from fraction II after purification on a Sephadex LH-20 column (CH_2Cl_2/MeOH, 60:40). It showed UV absorption at 254 nm and stained to violet with anisaldehyde/sulfuric acid.

The crude extract (9.07 g) of terrestrial *S*. sp. Adm 13 was subject on silica gel using an increasing dichloromethane/methanol gradient afforded three fractions. The red compound 6-hydroxy-5-methoxyisatine (**7**, 39.0 mg) was purified from fraction III by PTLC for several times.

The dark brown extract (8.31 g) of the terrestrial *S*. sp. Ank 38 was separated on silica gel to afford three fractions. Ayeyarwadayquinoline (**8,** 3.8 mg) was isolated from fraction III after purification on a silica gel with a CH_2Cl_2/MeOH.

The crude extract (5.0 g) of terrestrial *S*. sp. Ank 132 was chromatographed on a silica gel column using a gradient solvent system of CH_2Cl_2/MeOH. Purification of fraction II with Sephadex LH-20 (CH_2Cl_2:MeOH, 60:40) gave sub-fraction II-1. Subfraction II-1 was further chromatographed on RP-18 ($MeOH/H_2O$, 20:80) and afforded kromycin (**11**, 4 mg), 10,11-dihydro-kromycin (**9**, 3.0 mg), 10,11-dihydro-9,12-epoxy-8,9-anhydrokromycin (**10**, 2.7 mg).

Results and Discussion

The marine-derived *S*. sp. B8042 was cultivated on M_2^+ medium for five days. The resulting culture broth was extracted with XAD-16 resin for the filtrate, and ethyl acetate and acetone for the mycelium, respectively. As both extracts gave nearly identical chromatograms, they were combined and separated on silica gel into seven fractions. Compounds **1-3** were isolated from the middle polar fraction after purification on Sephadex LH-20

followed again by silica gel. Compound **4** was obtained from a higher polar part. The crude extracts of strain *S*. sp. B8480 delivered in a similar way the ketone **5** as the sole pyrone derivative.

Compound **1** was isolated as colorless oil. HRESIMS gave a pseudomolecular ion peak at *m/z* 239.12888 for $[M - H]^-$, corresponding to the molecular formula $C_{13}H_{20}O_4$. The ^1H NMR spectrum showed a 1H singlet in the sp^2 region; in the aliphatic region there were four methylene signals, one methyl singlet at δ 1.84 and a 6H methyl singlet at δ 1.16. In the ^{13}C NMR spectrum, 13 signals were observed: five of them were due to sp^2 hybridized carbons (four quaternary and one methine) and eight signals (three CH_3, four CH_2 and one oxygenated quaternary C) were found in the aliphatic region. The methyl at δ 1.84 may be attached to a carbonyl as acetyl or to a double bond; it showed HMBC cross signals with quaternary sp^2 carbons at δ 169.2, 168.5 and 98.8. The olefinic proton at δ 5.98 showed correlations with two of the previous signals (δ 168.5, 98.8), and with a third oxygenated carbon at δ 164.7. A search in AntiBase (Laatsch, AntiBase 2012) with these data clearly indicated a 4-hydroxy-3-methyl-α-pyrone derivative with an aliphatic side chain at C-6, which is in full agreement with further HMBC correlations (Figure 3). H-5 correlated with a methylene signal at δ 34.2; COSY and HMBC correlations extended this substituent by three further methylene groups. The singlet of the two equivalent methyl groups gave an *ipso*-correlation and coupled additionally with the terminal methylene group at δ 44.3 and with the carbon of a tertiary alcohol. It was obvious therefore, that these residual atoms were attached as isopropyl unit, resulting in structure **1**, which was named mandalarpyrone A. All further correlations confirmed this assumption.

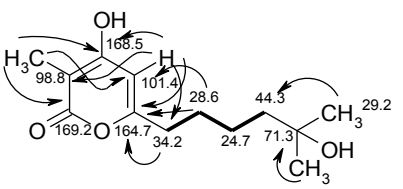


Figure 3. ^1H-^1H COSY (▬) and selected HMBC (→) correlations of Mandalarpyrone A (**1**)

Compound **2** was isolated as colorless crystals. From HRESIMS, the molecular formula was determined as $C_{13}H_{20}O_3$, containing one oxygen atom less than **1**. The H,H COSY, HSQC and HMBC correlations of **2** showed similar structural features as **1** and indicated again a substituted α-

pyrone ring. The only difference between the ^{1}H NMR spectra of **1**/**2** was one methine group more than in mandalarpyrone A (**1**) and a 6H methyl doublet instead of a 6H methyl singlet. The ^{13}C NMR spectrum revealed the expected 13 signals, but the quaternary carbon in **1** at δ 71.3 was absent. It follows that mandalarpyrone B is the respective *iso*heptyl-α-pyrone with structure **2**.

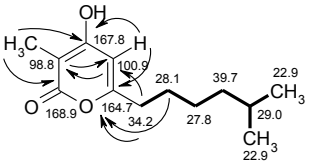


Figure 4. ^{1}H-^{1}H COSY (▬) and selected HMBC (→) correlations of Mandalarpyrone B (**2**)

Compound **3**, termed mandalarpyrone C, was not easily separated from **2**; it formed colorless crystals as well, and HRESIMS established the molecular formula as $C_{14}H_{22}O_3$. The ^{1}H NMR spectrum of **3** showed a high similarity with the previous compound, so that again an α-pyrone with a saturated side chain was expected: the only difference was that two methyls gave a doublet overlapped with a triplet instead of the 6H methyl doublet in compound **2**. The ^{13}C NMR spectrum showed five CH_2, one CH and two methyl signals. According to HMBC correlations and the molecular formula, mandalarpyrone C was obviously the *anteiso*octyl-α-pyrone **3**.

Compound **4** was isolated as colorless oil with the molecular formula $C_{14}H_{22}O_4$ (by HRESIMS). This and the NMR spectra indicated an isomer of the previous compound **1**, however, with two methyl doublets at δ 0.84 and 1.08, instead of a single 6H signal. HMBC correlations between the methyl doublets and the oxygen bearing methine carbon at δ 72.0 and further correlations (Figure 5) confirmed a hydroxylated *anteiso*octane side chain. Compound **4** was isolated as mixture of diastereomers in the ratio of 2:1. According to NMR/DFT calculations, the main isomer should have the *rel*-(*S*,*R*) configuration.

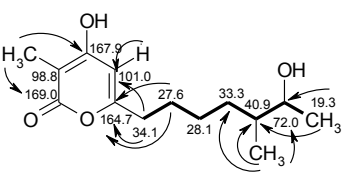


Figure 5. ^{1}H-^{1}H COSY (▬) and selected HMBC (→) correlations of Mandalarpyrone D (**4**)

4-Chloroanthranilic acid (**6**) was obtained from *S*. sp. strain B8042 as a pale yellow solid. The isotope peak in the mass spectrum indicated chlorine, and HREIMS gave the molecular formula $C_7H_6ClNO_2$. The ^1H NMR spectrum displayed three aromatic protons in 1,2,4-position by the typical coupling pattern. The ^{13}C NMR spectrum revealed only sp^2 signals, among them a carbon signal at δ 174.0 indicating the presence of an acid, ester or amide. With the help of the H,H COSY, HSQC and HMBC data (Figure 6), a 2,4-disubstituted benzoic acid derivative with nitrogen or – with respect to the ^{13}C shifts less plausible-oxygen in position 2 and chlorine at C-4 was derived. The EI mass spectrum showed a loss of water, which is an indication of an acid, not an amide, so that 4-chloroanthranilic acid was confirmed. On TLC, the compound gave a yellow spot with iron (III) chloride, but did not turn violet as expected for the phenolic salicyl amide. Compound **6** is known from synthesis, but was isolated here for the first time as natural product. The substituent pattern of **6** is found only in the few natural 6-chloroindole derivatives (Laatsch, AntiBase 2012).

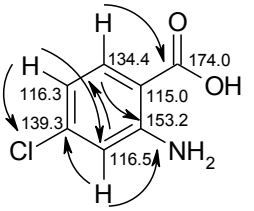


Figure 6. Selected HMBC (→) correlations of 4-chloroanthranilic acid (**6**)

The fermentation broth of a second marine-derived *S*. sp. strain B8480 delivered mandalarpyrone E (**5**) together with further known compound. Compound **5** was obtained as colorless oil. Its molecular formula was determined as $C_{14}H_{20}O_4$ by HRESIMS, which indicated five double bond equivalents, one more than in **4**. The 1D and 2D NMR data were very similar to those of mandalarpyrones B and D (**2**, **4**), so that another α-pyrone was expected. The ^1H NMR spectrum showed the singlet of a methyl group at δ 2.15, which was according to the shift attached to an sp^2 carbon as an acetyl residue, and an aliphatic methyl doublet. In comparison with the previous pyrones, the ^{13}C NMR spectrum indicated an additional ketone carbonyl at δ 213.5. In the HMBC spectrum, this carbonyl group showed two cross peaks with the methyl singlet at δ 2.15 and the methyl doublet at δ 1.09. Further spectroscopic data were related with those of the previous compounds, so that the new compound was derived as **5** and termed mandalarpyrone E; further pyrones were not found.

The NMR data of mandalarpyrones A-E (**1-5**) were comparable with those of pseudopyronine A and B (Kong, et. al., 2005), neofusapyrone (Hiramatsu, et. al., 2006), and arzanol (Appendino, G. et. al., J. Nat. Prod. 2007) which further confirmed the assignments of the α-pyrone unit.

Mandalarpyrones A-E (**1-5**) are showing a close relation with the germicidins (Aoki, et. al., 2011), hyphal elongation factors and spore germination inhibitors. Other recently published α-pyrones from *Pestalotiopsis guepinii* (Evidente, et. al., 2012) showed strong phytotoxic properties. Compounds **1-5** isolated here were tested for antibacterial and anti-tumor activity. Only mandalarpyrones B (**2**) and C (**3**) showed moderate antibacterial activity against *Staphylococcus aureus* and *Streptomyces viridochromogenes* Tü 57 by causing inhibition zones of 15 and 16 mm at 40 μg/disk. Responsible for the higher antibiotic activity of the crude extracts were mainly actinomycin or antimycins, which were known compounds isolated from this strain.

Table 1. ^{1}H (300 MHz) and ^{13}C NMR data (150 MHz) of Mandalarpyrones A-B

	Mandalarpyrone A (**1**)[a]			Mandalarpyrone B (**2**)[a]		
Position	δ_C, type		δ_H (*J* in [Hz])	δ_C, type		δ_H (*J* in [Hz])
1		O			O	
2	169.2	C		168.9	C	
3	98.8	C		98.8	C	
4	168.5	C		167.8	C	
5	101.4	CH	5.98, s	100.9	CH	5.98, s
6	164.7	C		164.7	C	
7	34.2	CH_2	2.49, t (7.3)	34.2	CH_2	2.44, t (7.5)
8	28.6	CH_2	1.64, m	28.1	CH_2	1.59, quint (7.6)
9	24.7	CH_2	1.43, m	27.8	CH_2	1.31, m
10	44.3	CH_2	1.45, m	39.7	CH_2	1.19, m
11	71.3	C		29.0	CH	1.49, m
12	29.2	CH_3	1.16, s	22.9	CH_3	0.87, d (6.6)
13	29.2	CH_3	1.16, s	22.9	CH_3	0.87, d (6.6)
14	8.3	CH_3	1.84, s	8.3	CH_3	1.83, s

[a] measured in CD_3OD.

Table 2. ^{1}H (300 MHz, CD_3OD) and ^{13}C NMR data (150 MHz, CD_3OD) of Mandalarpyrones C-D

	Mandalarpyrone C (**3**)			Mandalarpyrone D (**4**)		
position	δ_C, type		δ_H (*J* in [Hz])	δ_C, type		δ_H (*J* in [Hz])
1		O			O	
2	168.9	C		169.0	C	
3	98.8	C		98.8	C	
4	167.8	C		167.9	C	
5	100.9	CH	5.98, s	101.1	CH	5.99, s
6	164.7	C		164.7	C	
7	34.3	CH_2	2.46, t (7.5)	34.1	CH_2	2.45, t (7.4)
8	28.8	CH_2	1.61, quint (6.9)	28.1	CH_2	1.61, m
9	28.2	CH_2	1.33, m	27.6	CH_2	1.26, 1.43, m
10	37.4	CH_2	1.33, m	33.3	CH_2	1.08, 1.50, m
11	35.6	CH	1.33, m	40.9 40.7[a]	CH	1.43, m
12	30.6	CH_2	1.33, m	72.1 71.7[a]	CH	3.57, quint (6.1) 3.62 [a], quint (6.1)
13	11.8	CH_3	0.86, t (6.3)	19.3	CH_3	1.08, d (6.5)
14	8.3	CH_3	1.86, s	8.3	CH_3	1.83, s
15	19.6	CH_3	0.86, d (6.5)	15.0	CH_3	0.84, d (6.5)

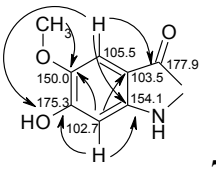
[a] minor diastereomer.

Table 3. ^{1}H (300 MHz, CD_3OD) and ^{13}C NMR data (150 MHz, CD_3OD) of Mandalarpyrones E (**5**) and 4-chloroanthranilic acid (**6**)

	Mandalarpyrone E (**5**)			4-chloroanthranilic acid (**6**)			
position	δ_C, type		δ_H (*J* in Hz)	position	δ_C, type		δ_H (*J* in Hz)
1		O		CO	174.0	CO	
2	167.0	C		1	115.0[a]	C	
3	98.6	C		2	153.2	C	
4	164.9	C		3	116.5	CH	6.70, d (2.1)
5	100.0	CH	5.95, s	4	139.3	C	
6	162.8	C		5	116.3	CH	6.49, dd, (2.0, 8.4)
7	33.1	CH_2	2.43, t (7.4)	6	134.4	CH	7.76,d (8.5)
8	26.7	CH_2	1.27, m				
9	26.4	CH_2	1.63, m				
10	32.3	CH_2	1.63, m				
11	47.0	CH	2.52, q (6.6)				
12	213.6	C					
13	28.2	CH_3	2.15, s				

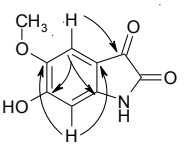
From the culture broth of terrestrial *S.* sp. Adm 13, 6-hydroxy-5-methoxyisatine (**7**) was isolated as a red compound after purification the fraction obtained from silica gel by PTLC followed by Sephadex LH-20 column. The ^{1}H NMR spectrum showed only three signals: two 1H singlets at δ 6.70 and 5.90 in the sp^2 region and one methoxy signal at δ 3.70. The ^{13}C NMR spectrum showed five downfield-shifted sp^2 signals of quaternary carbons at δ 178.0, 175.3, 168.0, 154.1 and 150.0, where the first three signals could be interpreted as carbonyls of acid, ester or amide and the other two signals could be sp^2 carbons connected with either oxygen or nitrogen atoms. In the sp^2 high field region, two methine carbon signals at δ 105.5 and 102.7 and one quaternary carbon signal at δ 103.5 appeared. In the aliphatic region, the spectrum showed one methoxy signal at δ 55.8. The molecular weight of compound (**7**) was found to be 192 Dalton by ESI MS. The HREI mass spectrum gave the molecular formula $C_9H_7NO_4$ according to seven double bond equivalents.

According to the HMBC correlations in compound **7**, the proton at δ 6.70, which is attached to carbon at δ 105.5 showed four cross peaks to δ 177.9, 175.3, 154.1 and 150.0. The proton H-7 at δ 5.46, which is attached to carbon at δ 102.7 showed two strong ^{3}J cross peaks to δ 150.0 and 103.5 and weak ^{2}J cross peaks to δ 154.1 and 175.3. The methoxy group showed correlations to a carbon at δ 150.0. From this information, the partial structure **7a** with five double bond equivalents could be drawn.



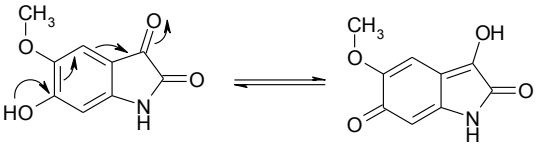
7a

No correlations appeared with the carbonyl at δ 168.1. It can be suggested as amide carbonyl, and a ring could be closed for the remaining double bond equivalent.



The carbon value of δ 175.3 for C-6 is higher than for normal oxygenated aromatic carbons. The reason is perhaps a keto-enol

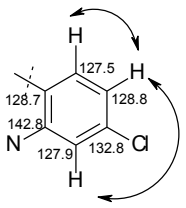
tautomerism between C-6 and C-3. Thus, C-6 carbon has also a partial carbonyl character.



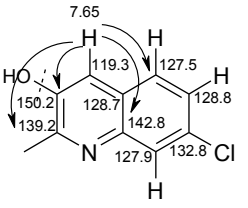
In NOE experiment for compound **7**, the methoxy group showed very strong correlation with the proton at δ 6.70. Accordingly, the isolated compound was 6-hydroxy-5-methoxyisatine (**7**). It is a known synthetic but new natural product. Some substituted isatine derivatives were synthesized and studied for their selective inhibition of carboxylesterases, which are detoxification enzymes (Cashman, et. al., 1996) and thought to be responsible for the metabolism and detoxification of xenobiotics. According to the structure activity relationship, the isatine derivatives containing hydrophobic groups attached at a variety positions have been reported as potent, specific carboxylesterases inhibitors (Hyatt, et. al., 2007). The isolated metabolite was antimicrobially and antitumor inactive.

Another chloro containing compound ayeyarwadayquinoline (**8**) was isolated from terrestrial *S*. sp. Ank 38. It was isolated as a pale yellow solid and showed green fluorescent under UV at 366 nm and visualized to orange with anisaldehyde-sulphuric acid. The ^{1}H NMR spectrum showed two *ortho*-coupled protons at δ 7.63 (d, ^{3}J = 8.8 Hz), 7.43 (dd, ^{4}J = 1.9 and ^{3}J = 8.8 Hz) and *meta*-coupled proton at δ 8.02 (d, ^{4}J = 1.4 Hz) of a 1,2,4-trisubstituted benzene ring. Furthermore, two aromatic singlets at δ 7.65 and 7.44 and one chelated OH at δ 11.68 were counted. In the aliphatic region, a methylene singlet at δ 4.89 was observed. The molar mass of 292 Dalton was established by ESI MS experiments. With respect to the isotope peak in the EI MS experiment, the compound contained a chlorine atom. The HR ESI mass spectrum gave the molecular formula $C_{13}H_9N_2O_2ClS$ with 10 double bond equivalents. The ^{13}C NMR spectrum showed 13 carbon signals, whereof those at δ 170.5 could be assigned to carbonyl of acid, amide, ester or quaternary sp^2 carbon connected with two heteroatoms; in addition, signals of 11 aromatic carbons (five methine and six quaternary) were visible. The aliphatic region showed one methylene signal. According to the HSQC, COSY and HMBC data, the carbon atoms could be assigned in the benzene ring as in fragment **8a**. Among these atoms, the two quaternary carbons at δ 142.8 and 132.8 could be linked with heteroatoms N and Cl, respectively (fragment **8a**). By analysis of the other HMBC

correlations, the proton at δ 7.65 (C-4) showed a coupling with the carbons at δ 127.5 (C-5), 142.8 (C-8a), 150.2 (C-3) and 139.2 (C-2). Based on these data, a second heteroaromatic ring could be drawn and the carbon at δ 150.2 could be assigned as oxygenated sp^2 carbon (fragment **8b**).

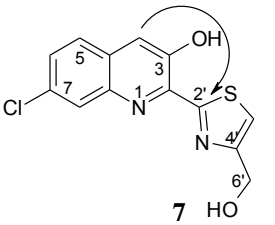


8a



8b

8c ***m/z*** **114**



7

8

In addition to these spectroscopic data, one methine singlet at δ 7.44 showed correlations to two quaternary carbons at δ 170.5 and 156.2. Furthermore, one methylene singlet at δ 4.89 showed correlations to the two carbon signals at δ 117.8 (CH) and 156.2 (C_q) (fragment **8c**). The methine proton at δ 7.65 (CH, 119.3) showed the small correlation to the quaternary carbons at δ 170.5. Therefore, the two fragments **8b** and **8c** could be connected as shown in structure **8.**

Table 4. NMR spectroscopic data for irrawadayquinoline

position	δ_C	δ_H (mult.; *J* in Hz)	position	δ_H
1	-		OH	11.68
2	139.2			
3	150.2			
4	119.3	7.65 (s)		
4a	128.7			
5	127.5	7.63 (d, 8.8 Hz)		
6	128.8	7.43 (dd, 1.9, 8.8 Hz)		
7	132.8			
8	127.9	8.02 (d, 1.4 Hz)		
8a	142.8			
1'	-			
2'	170.5			
4'	156.2			
5'	117.8	7.44 (s)		
6'	60.9	4.89 (s)		

The presence of the thiazole moiety was further confirmed by the EI mass spectrum, which gave an ion peak at *m/z* 114 of fragment **8c**. In addition, compound (**8**) showed a strong green fluorescence at 366 nm, which is typical for the thiazole moiety.

Anthranilic acid is a precursor of some quinoline and acridine alkaloids. Anthraniyl CoA, which is the CoA ester of schikimate-derived compound anthranilic acid, act as a starter unit for malonate chain extension. Aromatization of the acetate-derived portion leads to quinoline alkaloids (Paul, 2003). In a similar way, the quinoline skeleton in irrawadayquinoline can probably be derived from acetoacetyl CoA and 4-chloroanthranilic acid (**6**). 4-Chloroanthranilic acid (**6**) was isolated from the already mentioned marine-derived *Streptomyces* sp. B 8042. Successive oxidation of the methyl group at C-2 led to a carboxylic acid, which reacted with cysteine and delivered the thiazole nucleus.

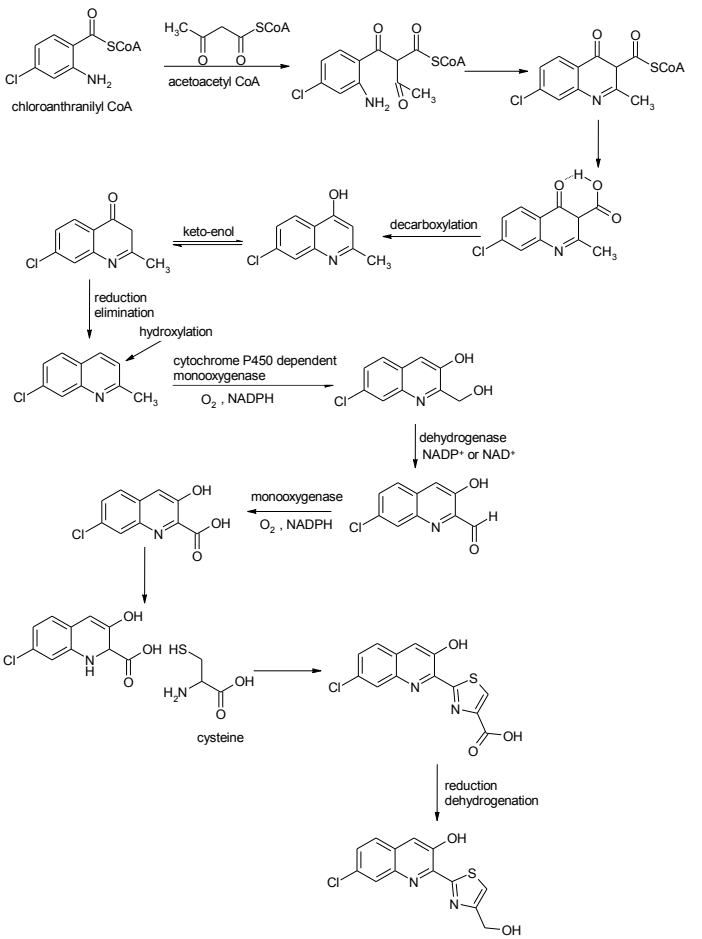


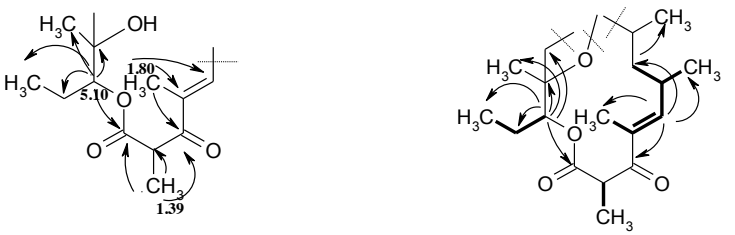
Figure 7. Hypothetical biosynthesis of irrawadayquinoline using chloroanthranilyl CoA as a starter unit

Macrolide antibiotic kromycin (**11**) was isolated from the culture broth of terrestrial *S*. sp. Ank 132. Together with kromycin, a second compound was obtained as inseparable mixture, which co-crystallized with kromycin. The ESI mass spectrum of the mixture revealed two $[M + Na]^+$ ion peaks at *m/z* 373 and 375, which fixed the molecular weight to 350 (kromycin), and 352. The HR ESI mass spectrum gave the molecular formula of $C_{20}H_{30}O_5$ (DBE = 6) and $C_{20}H_{32}O_5$ (DBE = 5), respectively. The latter compound has two more hydrogen atoms and one double bond equivalent less than kromycin. From the ^{1}H NMR spectrum, it was obvious that one α,β-unsaturated system between C-10 and C-11 in kromycin had disappeared. According to the NMR spectroscopic data, the second compound was identified as 10,11-dihydro-kromycin (**9**).

10,11-Dihydro-9,12-epoxy-8,9-anhydrokromycin (**10**) was isolated as colourless crystals. The ^{1}H NMR spectrum of this compound was very similar to that of kromycin, however, the α,β-unsaturated system had disappeared in the olefinic region; in the aliphatic region, a methyl singlet at δ 1.67 instead of a methyl doublet at δ 1.06 and two more methylene groups were visible. The molecular formula of $C_{20}H_{30}O_4$ demanded six double bond equivalents. The ^{13}C NMR and HSQC spectrum showed a ketone carbonyl at δ 196.3, an acid or ester carbonyl at δ 172.9, three sp^2 carbons (two methine and one quaternary) at δ 151.5, 148.1 and 134.1, two quaternary carbons at δ 97.3 and 86.8 and one oxygenated methine carbon at δ 76.9. In addition, there were 12 carbons (2 CH, 4 CH_2 and 6 CH_3) in the aliphatic region.

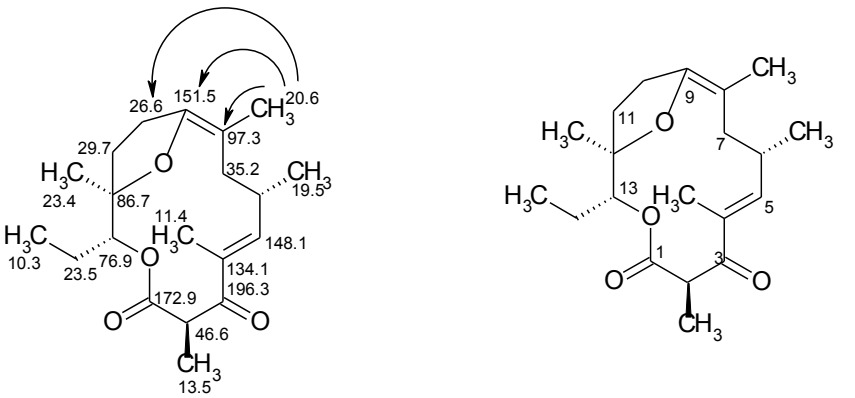
In the HMBC spectrum, a methyl singlet at δ 1.80, which is substituted to the α-position of an α,β-unsaturated carbonyl, showed correlation with the ketone carbonyl and the α- and β-carbons (fragment **10a**). The β-proton showed correlation to the methyl doublet at δ 19.5 and one methylene at δ 35.2, which correlated again with the methyl singlet at δ 20.6. The methyl doublet at δ 1.39 correlated with two carbonyls and one methine at δ 46.6.

The doublet of doublet oxygenated methine at δ 5.10 correlated with the carbonyl of ester at δ 172.9, the oxygenated quaternary carbon at δ 86.7, two methylene groups at δ 23.5 and 29.7, one methyl triplet at δ 10.3 and one methyl singlet at δ 23.4 which is attached to the oxygenated quaternary carbon (fragment **10a**). The lower part of the molecule is identical with kromycin.



10a

The methyl singlet at δ 1.67 (δ 20.6) displayed a correlation with an oxygenated quaternary sp^2 carbon at δ 151.5, a quaternary carbon at δ 97.3 and the methylene group at δ 26.6. According to these correlations, the structure was elucidated as 10,11-dihydro-9,12-epoxy-8,9-anhydrokromycin (**10**).



It seems plausible that 10,11-dihydrokromycin is unstable under acidic conditions and undergoes transformation into 10,11-dihydro-9,12-epoxy-8,9-anhydrokromycin. Except kromycin (**11**), kromycin derivatives (**9, 10**) exhibited no antimicrobial activity in our agar diffusion tests. Kromycin is a macrolide antibiotic, which was isolated and elucidated 1953 in Göttingen by Hans Brockmann. The group of James R. Pruitt synthesized this compound in 1989.

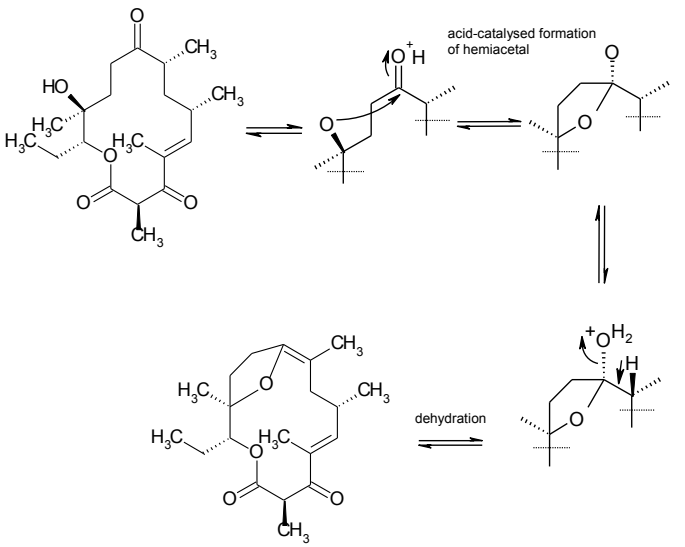


Figure 8. Transformation of 10,11-dihydrokromycin into 10,11-dihydro-9,12-epoxy-8,9-anhydrokromycin

10,11-Dihydrokromycin (**9**): colourless crystals, 3.0 mg, R_f = 0.21 (CH_2Cl_2/5% MeOH), UV absorbing band at 254 nm, violet with anisaldehyde/sulphuric acid. – **^{1}H NMR** ($CDCl_3$, 300 MHz) δ 6.48 (dd, ^4J = 1.2, ^3J = 6.3 Hz, 1H, H-5), 4.80 (dd, ^4J = 2.4, ^3J = 10.8 Hz, 1H, H-13), 4.50 (q, ^3J = 7.0 Hz, 1H, H-2), 2.91 (m, 1H, H-6), 2.50 (m, 1H, CH_2-10), 2.46 (m, 1H, H-8), 1.90 (d, ^4J = 1.3, 3H, CH_3-4), 1.83 (m, 1H, CH_2-11), 1.71 (m, 1H, CH_2-13), 1.60 (m, 1H, CH_2-11), 1.58 (m, 1 H, 7-CH2), 1.52 (m, 1H, CH_2-13), 1.52 (m, 1H, CH_2-7), 1.41 (d, ^3J = 6.9 Hz, 3H, CH_3-2), 1.40 (m, 1H, CH_2-7), 1.19 (s, 3H, CH_3-12), 1.04 (d, ^3J = 6.6 Hz, 3H, CH_3-8), 1.04 (d, ^3J = 6.3 Hz, 3H, CH_3-6), 0.93 (t, ^3J = 7.3 Hz, 3H, CH_3-13). – **^{13}C NMR** ($CDCl_3$, 125 MHz) δ 215.4 (C_q, C-9), 196.1 (C_q-3), 172.1 (C_q1), 147.4 (CH-5), 137.9 (Cq-4), 82.3 (CH-13), 73.6 (C_q-12), 45.6 (CH-2), 42.0 (CH-8), 40.7 (CH_2-7), 37.0 (CH-10), 32.6 (CH-6), 32.0 (CH-11), 23.4 (CH_3-12), 22.7 (CH_2-13), 20.9 (CH_3-6), 15.7 (CH_3-8), 14.3 (CH_3-2), 12.3 (CH_3-4), 10.9 (CH_3-13). – **(+)-ESIMS** *m/z* 727 ([2 M + Na]$^+$, 40), 375 ([M + Na]$^+$, 100). – **(+)-HRESIMS** *m/z* 375.21424 [M + Na]$^+$, (calcd. 375.21419 for $C_{20}H_{32}O_5Na$).

10,11-Dihydro-9,12-epoxy-8,9-anhydro-kromycin (**10**)**:** colourless crystals, 2.7 mg, R_f = 0.41 (CH_2Cl_2), strong UV absorbing band at 254 nm, violet with anisaldehyde/sulphuric acid. – **^{1}H NMR** ($CDCl_3$, 300 MHz) δ 6.63 (dd, ^4J = 1.2, ^3J = 9.1 Hz, 1H, H-5), 5.10 (dd, ^4J = 2.6, ^3J = 11.1 Hz,

1H, H-13), 4.00 (q, ^3J = 7.1 Hz, 1H, H-2), 2.90 (m, 1H, H-6), 2.76 (d, ^4J = 3.6, ^3J = 13.5 Hz, 1H, CH_2-7), 2.64 (m, 1H, CH_2-11), 2.56 (m, 1H, CH_2-11), 2.23 (q, ^3J = 11.9 Hz, 1H, CH_2-10), 1.80 (d, ^4J = 1.3 Hz, 3H, CH_3-4), 1.67 (s, 3H, CH_3-8), 1.67 (m, 3H, CH_2-7,10,13), 1.49 (m, 1H, CH_2-13), 1.39 (d, ^3J = 7.0 Hz, 3H, CH_3-2), 1.21 (s, 3H, CH_3-12), 0.99 (d, ^3J = 7.2 Hz, 3H, CH_3-6), δ = 0.92 (t, ^3J = 7.4 Hz, 3H, CH_3-13). – **^{13}C NMR** ($CDCl_3$, 125 MHz) δ 196.3 (C_q-3), 172.9 (C_q-1), 151.5 (C_q-9), 148.1 (CH-5), 134.1 (Cq-4), 97.3 (C_q-8), 86.7 (C_q-12), 76.9 (CH-13), 46.6 (CH-2), 35.2 (CH_2-7), 34.2 (CH-6), 29.7 (CH_2-10), 26.6 (CH_2-11), 23.5 (CH_2-13), 23.4 (CH_3-12), 20.6 (CH_3-8), 19.5 (CH_3-6), 13.5 (CH_3-2), 11.4 (CH_3-4), 10.3 (CH_3-13). – **(+)-ESIMS** *m/z* 691 ($[2\ M + Na]^+$, 40), 357 ($[M + Na]^+$, 100). – **(-)-ESIMS** *m/z* 373 ($[M - 2\ H + Na]^+$. – **(+)-HRESIMS** *m/z* 335.22166 $[M + H]^+$, (calcd. 335.22169 for $C_{20}H_{31}O_4$).

Kromycin (**11**): colourless crystals, 4.0 mg, R_f = 0.21 (CH_2Cl_2/5% MeOH), strong UV absorbing band at 254 nm, deep violet with anisaldehyde/sulphuric acid. – **^{1}H NMR** (CD_3OD, 300 MHz) δ 6.80 (d, ^3J = 16.5 Hz, 1H, H-11), 6.48 (dd, ^4J = 1.24, ^3J = 10.2 Hz, 1H, H-5), 6.07 (d, ^3J = 16.5 Hz, 1H, H-10), 4.79 (dd, ^3J = 2.2, ^3J = 10.9 Hz, 1H, H-13), 4.54 (q, ^3J = 6.9 Hz, 1H, H-2), 2.91 (m, 1H, H-6), 2.74 (m, 1H, H-8), 1.92 (m, 1H, CH_2-13), 1.85 (s, 3H, CH_3-4), 1.70 (m, 2H, CH_2-7), 1.61 (m, 1H, CH_2-13), 1.39 (d, ^3J = 6.9 Hz, 3H, CH_3-2), 1.33 (s, 3H, CH_3-12), 1.06 (d, ^3J = 6.5 Hz, 6H, CH_3-6,8), 0.90 (t, ^3J = 7.39 Hz, 3H, CH_3-13). – **^{13}C NMR** ($[D_4]$ MeOH, 125 MHz) δ 206.2 (C_q-9), 197.8 (C_q-3), 173.6 (C_q-1), 152.9 (CH-11), 148.2 (CH-5), 139.3 (Cq-4), 127.3 (CH-10), 80.3 (CH-13), 74.0 (C_q-12), 46.8 (CH-2), 44.1 (CH_2-7), 41.4 (CH-8), 34.0 (CH-6), 22.3 (CH_2-13), 21.1 ($2CH_3$-6,12), 15.2 (CH_3-8), 14.2 (CH_3-2), 12.5 (CH_3-4), 10.9 (CH_3-13). – **(+)-ESIMS** *m/z* 723 ($[2\ M + Na]^+$, 100), 373 ($[M + Na]^+$, 33). – **(+)-HRESIMS** *m/z* 373.19862 $[M + Na]^+$, (calcd. 373.19855 for $C_{20}H_{30}O_5Na$).

Conclusion

In the present study, five new α-pyrone derivatives named mandalarpyrones A-E (**1-5**), two chloro containing compounds namely 4-chloroanthranilic acid (**6**) and ayeyarwadayquinoline (**8**), 6-hydroxy-5-methoxyisatine (**7**) and the two new kromycin derivatives (**9, 10**) were isolated and characterized. Among them, mandalarpyrone B (2) and C (3) exhibited moderate antibacterial activity against *Staphylococcus aureus* and *Streptomyces viridochromogenes* Tü 57 in agar diffusion test.

Acknowledgments

The author thanks Professor Dr. Hartmut Laatsch, The Georg-August University of Goettingen, Germany for his kind supervision and the German Academic Exchange Service (DAAD) for a research fellowship.

References

Aoki, Y., Matsumoto, D., Kawaide, H., and Natsume, M., (2011)**, "**Physiological Role of Germicidins in Spore Germination and Hyphal Elongation in *Streptomyces coelicolor* A3(2)." *J. Antibiot*. vol. **64**, pp. 607-611.

Appendino, G., Ottino, M., Marquez, N., Bianchi, F., Giana, A., Ballero, M., Sterner, O., Fiebich, B. L., Munoz, E., (2007), **"**Arzanol, an anti-inflammatory and anti-HIV-1 phloroglucinol α-pyrone from *Helichrysum italicum* ssp. *Microphyllum*." *J. Nat. Prod*. vol **70**, pp. 608-612.

Baker, D., Mocek, U., and Garr, C., (2000), "In Biodiversity: New Leads for Pharmaceutical and Agrochemical." Industries; S. K. Wriegley, M. A. Hayes, R. Thomas, E. J. T. Chrystal, N. Nicholson, Eds.; The Royal Society of Chemistry: Cambridge, UK, pp. 66-72.

Cashman, J., Perroti, B., Berkman, C., and Lin, (1996), "J. Pharmacokinetics and Molecular Detoxification." *EnViron. Health Perspect*. vol. **104**, pp. 23-40.

Evidente, A., Zonno, M. C., Andolfi, A., Troise, C., Cimmino, A., and Vurro, M., (2012), "Phytotoxic α-Pyrones Produced by *Pestalotiopsis guepinii*, the Causal Agent of Hazelnut Twig Blight." *J. Antibiot*. vol. **65**, pp. 203-206.

Helmke, E., & Weyland, H., (1984), "*Rhodococcus marinonascens* sp. nov., an Actinomycete from the Sea." Int. J. Syst. Bacteriol. vol. **34**, pp. 127-138.

Hiramatsu, F., Miyajima,T., Murayama, T., Takahashi, K., Koseki, T., and Shiono,Y., (2006) "Isolation and Structure Elucidation of Neofusapyrone from a Marine-Derived *Fusarium* species, and Structural Revision of Fusapyrone and Deoxyfusapyrone." *J. Antibiot*. vol. **59**, pp. 704-709.

Hyatt, J. L., Moak, T., Hatfield, M. J., Tsurkan, L., Edwards, C. C., Wierdl, M., Danks, M. K., Wadkins, R. M., and Potter, P. M., (2007) *J. Med. Chem*. vol. **50**, pp. 1876-1885.

Kong, F, Singh, M. P, and Carter, G. T. (2005), "Pseudopyronines A and B, α-pyrones produced by a marine *Pseudomonas* sp. F92S91, and evidence for the conversion of 4-hydroxy-α-pyrone to 3-furanone" *J. Nat. Prod*. vol. **68**, pp. 920-923.

Laatsch, H. *AntiBase 2012, A Data Base for Rapid Dereplication and Structure Determination of Microbial Natural Products*; Wiley VCH: Weinheim, Germany, 2012; see http://wwwuser.gwdg.de/~hlaatsc/antibase.htm.

Paul M. Dewick, (2003), "Medicinal Natural Products, A Biosynthetic Approach", Second Edition, pp. 81.

Shirling, E. B. & Gottlieb, D. (1966), "Methods for Characterization of *Streptomyces* species. Int. J. Syst. Bacteriol." vol. **16**, pp. 313-340.

Weyland, H. (1981), "Distribution of actinomycetes on the sea floor", Zbl. Bakt. Suppl. 11**,** pp. 185-193.