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Novel Streptopyrroles from *Streptomyces rimosus* with Bacterial Protein Histidine Kinase Inhibitory and Antimicrobial Activities

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A series of halogenated pyrrolo [2,1-b] [1,3] benzoxazines (**1**~**9**) was isolated from fermentations of an actinomycete strain X10/78/978 (NCIMB40808), identified as *Streptomyces rimosus*, during a microbial extract screening programme to identify inhibitors of bacterial histidine kinase. The structures of these compounds were elucidated by spectroscopic methods including the HMQC, HMBC and INADEQUATE NMR experiments. The structure of **1** was confirmed by X-ray crystallographic studies. Compounds **5** and **6** were produced in fermentations in the presence of NaBr and NaI respectively. The most abundant member of the series, streptopyrrole, **1**, inhibited the nitrogen regulator II (NRII) histidine kinase from *Escherichia coli* with an IC_{50} of 20 μM and exhibited antimicrobial activity against a range of bacteria and fungi.

Protein histidine kinases form the sensory element of two component signal transduction systems. These systems are widespread in prokaryotes, show a clear homology to one another and play an important role in regulation of gene expression to enable bacteria to adapt to stressful or changing conditions.[1,2] They have also been detected in lower eukaryotes[3~5] and plants.[6] Inhibitors of these systems could severely limit the ability of the bacteria to colonise and cause disease in the host organism and so they provide a good target for antibacterial therapy. The first inhibitor of a two-component signal transduction system with potent antimicrobial activity was recently reported.[7]

We have isolated a series of halogenated pyrrolo [2,1-b] [1,3] benzoxazines (Fig. 1) produced in fermentations of *Streptomyces rimosus*. Members of this series inhibit the NRIIc protein histidine kinase from *Escherichia coli* and also show potent antibacterial and antifungal activity. The present paper describes the taxonomy of the producing organism, its fermentation and the isolation, structure elucidation and biological activities of this series of compounds. After this work was completed, compound **1**, streptopyrrole, was reported as a metabolite from *Streptomyces armeniacus* with antimicrobial properties.[8]

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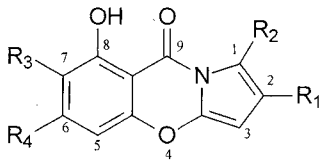
Fig. 1. Structures of compounds **1** to **9**.



Compound	R_1	R_2	R_3	R_4
1	Cl	H	Pr	OH
2	Cl	H	Et	OH
3	Cl	Cl	Pr	OH
4	Cl	H	Pr	OMe
5	Br	H	Pr	OH
6	Cl	Cl	Et	OH
7	Cl	H	Bu	OH
8	Cl	H	Et	OMe
9	Cl	Cl	Pr	OMe

Taxonomy of Producing Organism

The producing strain No. X10/78/978 was isolated from soil collected in Kenya in 1988. It has been deposited at the National Collections of Industrial and Marine Bacteria under accession number 40808.

The physiological characteristics of strain X10/78/978 are shown in Table 1. The substrate mycelium was well developed and extensively branched. In general, the organism produced a Vellum coloured substrate mycelium and a white aerial mycelium. The aerial mycelium formed *Rectiflexibiles* spore chains that were sympodial and greater than 20 spores in length.

The spore mass colour was Light Aircraft Grey. No diffusible pigment was produced on any of the media. Analysis of the cell wall hydrolysate revealed that the cell wall contained LL-diaminopimelic acid.

Based on the chemical and physiological characteristics described above, strain X10/78/978 was identified as *Streptomyces rimosus* according to WILLIAMS *et al.*[9] The strain does, however, show some atypical characteristics for this species, such as *Rectiflexibilis* spore chains and a red pigment in the substrate mycelium.

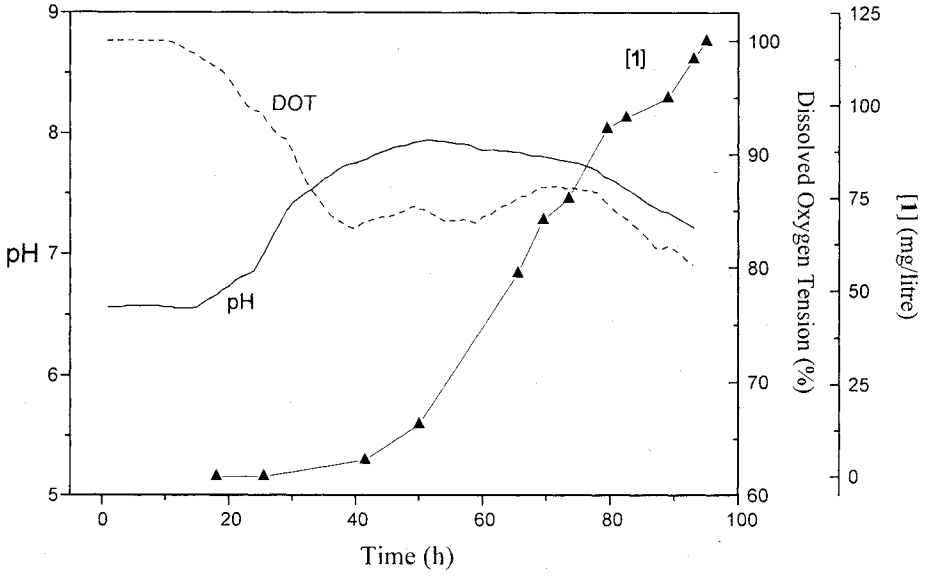
Fermentation

The original fermentation of the organism described above resulted in the isolation of a series of chlorinated compounds (**1**, **2**, **3**, **4**, **7**, **8** and **9**). The profile of a typical fermentation at 75 litre scale, showing pH, dissolved oxygen tension (DOT), and accumulation of compound **1** is shown in Fig. 2. Fermentations were also carried out in the presence of sodium halides in order to see whether incorporation of an alternative halide in the place of chlorine could be achieved. The brominated compound **5** was isolated from the fermentation in production medium supplemented with NaBr and, although no iodinated

Table 1. Physiological characteristics of strain X10/78/978.

Character	Score
Spore mass grey	+
Spore mass red	-
Spore mass green	-
Spore surface smooth	+
Spore surface rugose	-
Spore chain biverticillate	-
Spore chain *Spirales*	-
Spore chain *Retinaculiaperti*	-
Spore chain *Rectiflexibiles*	+
Substrate pigment yellow-brown	+
Substrate pigment red	+
Melanin production	-
Fragmentation	-
Pectin hydrolysis	+
Lecithinase	+
NO_3 reduction	+
H_2S produced	-
Growth at 45°C	-
Resistance to:	
Rifampicin	+
Neomycin	+
Antibiosis against:	
Aspergillus niger	+
Streptomyces murinus	+
Bacillus subtilis	+
Growth with (w/v):	
Na Azide (0.01%)	+
NaCl (7%)	+
Phenol (0.1%)	-
Degradation of:	
Arbutin	+
Xanthine	+
Allantoin	+
Utilisation of:	
Adonitol	+
Cellobiose	+
Rhamnose	+
Raffinose	+
Inositol	+
Mannitol	+
Xylose	+
L-Hydroxyproline	+
L-Histidine	+
DL-Aminobutyric acid	+
Inulin	-
Fructose	+

Fig. 2. Changes in pH, dissolved oxygen tension (DOT) and titre of compound **1** during 75 litre fermentation of *Streptomyces rimosus* X10/78/978.



compounds were isolated, compound **6** was isolated from a fermentation supplemented with NaI.

Structure Elucidation

The major metabolite present in fermentations of *Streptomyces rimosus* X10/78/978 was **1**. Its physico-chemical properties are summarised in Table 2. The compound is soluble in organic solvents such as methanol, DMSO and chloroform but insoluble in water. The ^{1}H and ^{13}C NMR spectra of **1** exhibited 14 carbons and 12 proton signals respectively and are summarised in Tables 3 and 4. DEPT spectra revealed the presence of one CH_3, two CH_2, three CH and eight quaternary carbons. The ^{1}H NMR spectrum indicated that the CH_3 and two CH_2 carbons were linked as a propyl group. An HMQC experiment established the one bond connectivities of the proton and carbon atoms. Two proton signals at δ 11.20 and δ 9.78 did not correlate to carbons, suggesting that they were hydroxyl protons. The carbon skeleton of **1** was established in two portions by an INADEQUATE NMR experiment and these were linked together by inspection of the correlations observed in an HMBC long range ^{1}H-^{13}C correlation NMR experiment to define the structure of **1** as 2-chloro-6,8-dihydroxy-7-propyl-9*H*-pyrrolo [2,1-b] [1,3] benzoxazine-9-one. This work is not reported in detail as the structure of **1** was subsequently published as streptopyrrole, a metabolite of *Streptomyces armeniacus*.[8] The structure of **1** was confirmed unambiguously by X-ray crystallography and the X-ray crystal structure of **1** is shown in Fig. 3.

The physico-chemical properties, ^{1}H NMR and ^{13}C NMR spectra (where recorded) of compounds **2**~**9** are summarised in Tables 2~4 respectively. The structures of these compounds were determined by comparison of these data with the spectroscopic and physico-chemical data of compound **1**, streptopyrrole. The significant differences are outlined in the following paragraphs.

2-Chloro-6,8-dihydroxy-7-ethyl-9*H*-pyrrolo [2,1-b] [1,3] benzoxazine-9-one, **2**

Mass spectral data indicated a molecular weight of 279 and a molecular formula of $C_{13}H_{10}NO_4Cl$, suggesting the loss of a CH_2 unit. The ^{1}H and ^{13}C NMR signals were essentially unchanged.

1,2-Dichloro-6,8-dihydroxy-7-propyl-9*H*-pyrrolo [2,1-b] [1,3] benzoxazine-9-one, **3** and 1,2-dichloro-6,8-dihydroxy-7-ethyl-9*H*-pyrrolo [2,1-b] [1,3] benzoxazine-9-one, **6**

Mass spectral data for **3** yielded a molecular weight of 327 and a molecular formula of $C_{14}H_{11}NO_4Cl_2$, indicating the replacement of a proton by a second chlorine atom. Inspection of the ^{1}H and ^{13}C NMR data suggested that H-3 was still present and was now a singlet while the proton signal for H-1 was missing. The chemical shift on C-1

Table 2. Physico-chemical properties of compounds **1**~**9**.

Compound	**1**	**2**	**3**
Appearance	White powder	White powder	White powder
EI-MS (*m/z*)	293/295 (M^+) 264/266 ($[M-C_2H_5]^+$)	279/281 (M^+) 264/266 ($[M-CH_3]^+$)	327/329/331 (M^+) 298/300/302 ($[M-C_2H_5]^+$) 264/266 ($[M-C_2H_5Cl]^+$)
Molecular formula	$C_{14}H_{12}NO_4Cl$	$C_{13}H_{10}NO_4Cl$	$C_{14}H_{11}NO_4Cl_2$
HREI-MS			
Found:	293.0478	279.0301	327.0067
Calculated:	293.0454	279.0298	327.0065
UV: λ_{max} (H_2O-acetonitrile) nm	254, 293, 336	245, 293, 336	247, 295, 342
IR: ν_{max} (KBr) cm^{-1}	3343, 3149, 2965, 2930, 2868, 1643, 1584, 1512, 1464, 1405, 1296, 1226, 1192, 1118, 1101, 785, 757, 704, 624, 580	3267, 2966, 2933, 2874, 1641, 1594, 1506, 1468, 1401, 1296, 1195, 1122, 1095, 756, 619, 581	3430, 3215, 2962, 2929, 2868, 1639, 1589, 1510, 1458, 1412, 1386, 1292, 1225, 1197, 1128, 776, 745, 626, 582

Compound	**4**	**5**	**6**
Appearance	White powder	White powder	White powder
EI-MS (*m/z*)	307/309 (M^+) 278/280 ($[M-C_2H_5]^+$)	337/339 (M^+) 308/310 ($[M-C_2H_5]^+$)	313/315/317 (M^+)
Molecular formula	$C_{15}H_{14}NO_4Cl$	$C_{14}H_{12}NO_4Br$	$C_{13}H_9NO_4Cl_2$
HREI-MS			
Found:	307.0600	336.9957	—
Calculated:	307.0611	336.9950	—
UV: λ_{max} (H_2O-acetonitrile) nm	248, 291, 341	243, 295, 333	245, 293, 336
IR: ν_{max} (KBr) cm^{-1}	3143, 2964, 2930, 2869, 1664, 1640, 1597, 1501, 1459, 1411, 1300, 1208, 1138, 1101, 810, 790, 740	—	—

Compound	**7**	**8**	**9**
Appearance	White powder	White powder	White powder
EI-MS (*m/z*)	307/309 (M^+) 264/266 ($[M-C_3H_7]^+$)	293/295 (M^+) 278/280 ($[M-CH_3]^+$) 248/250 ($[M-C_2H_5O]^+$)	341/343/345 (M^+) 312/314/316 ($[M-C_2H_5]$) 282/284/286 ($[M-C_3H_7O]^+$)
Molecular formula	$C_{15}H_{14}NO_4Cl$	$C_{14}H_{12}ClNO_4$	$C_{15}H_{13}NO_4Cl_2$
HREI-MS			
Found:	307.0600	293.0448	341.0214
Calculated:	307.0611	293.0454	341.0222
UV: λ_{max} (H_2O-acetonitrile) nm	240, 295, 340	248, 291, 341	252, 291, 348
IR: ν_{max} (KBr) cm^{-1}	3260, 2956, 2929, 2864, 1641, 1585, 1504, 1469, 1425, 1298, 1195, 1127, 761	—	—

Fig. 3. Crystal structure of compound **1**, which co-crystallised with acetone.

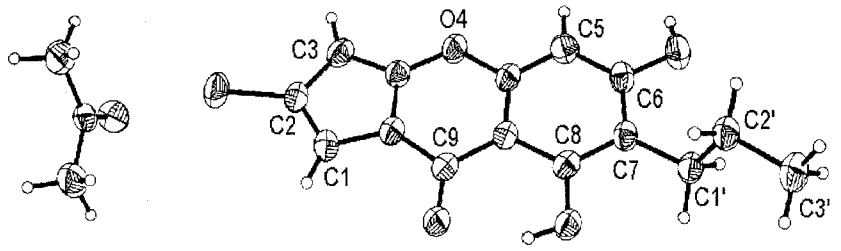


Table 3. ^{1}H NMR assignments for compounds **1** to **9**.

Position	δ_H/ppm in d_6-acetone[a]; coupling constants expressed in Hz are given in parentheses				
	1	**2**	**3**	**4**[b]	**5**
1	7.20, *d* (2.0)	7.21, *d* (1.7)	—	7.16, *d* (2.0)	7.26, *d* (2.0)
3	5.95 *d* (2.0)	5.94, *d* (1.6)	6.00, *s*	5.82, *d* (2.0)	5.96, *d* (2.0)
5	6.41, *s*	6.44, *s*	6.40, *s*	6.33, *s*	6.41, *s*
6-OH	9.78, *s*	n.o.	n.o.	—	n.o.
6-OCH_3	—	—	—	3.89, *s*	—
8-OH	11.20, *s*	11.2, *bs*	11.2, *bs*	11.05, *s*	n.o.
1′	2.62, *t* (7.5)	2.68, q (7.4)	2.65, *t* (7.6)	2.62, *t* (7.6)	2.65, *t* (7.6)
2′	1.60, *sext* (7.5)	1.12, *t* (7.4)	1.57, *sext* (7.5)	1.52, *sext* (7.3)	1.54, *sext* (7.5)
3′	0.95, *t* (7.4)	—	0.95, *t* (7.4)	0.93, *t* (7.4)	0.96, *t* (7.4)
4′	—	—	—	—	—
	6	**7**	**8**[b]	**9**[b]	
1	—	7.20, *d* (1.5)	7.18, *d* (2.0)	—	
3	6.02, *s*	5.95, *d* (1.5)	5.83, *d* (2.1)	5.84, *s*	
5	6.41, *s*	6.43, *s*	6.34, *s*	6.28, *s*	
6-OH	n.o.	n.o.	—	—	
6-OCH_3	—	—	3.91, *s*	3.88, *s*	
8-OH	n.o.	11.20, *bs*	11.06, *s*	11.05, *s*	
1′	2.65, q (7.5)	2.65, *t* (7.5)	2.67, q (7.5)	2.62, *t* (7.5)	
2′	1.12, *t* (7.4)	1.55, m	1.10, *t* (7.5)	1.53, *sext* (7.5)	
3′	—	1.40, m	—	0.93, *t* (7.4)	
4′	—	0.95, *t* (7.5)	—	—	

[a] Referenced to acetone signal at 2.05 ppm referenced to external TMS
[b] Measured in $CDCl_3$; referenced to $CHCl_3$ signal at 7.26 ppm referenced to external TMS
n.o. = Not observed

was unchanged. This is consistent with a 1,2-dichloro derivative. Similarly **6** was determined to be a 1,2-dichlorinated analogue with a 7-ethyl side chain.

2-Bromo-6,8-dihydroxy-7-propyl-9*H*-pyrrolo [2,1-b] [1,3] benzoxazine-9-one, **5**

This compound was isolated from a fermentation conducted in media containing sodium bromide. Its molecular formula $C_{14}H_{12}NO_4Br$ indicated replacement of the chlorine atom in **1** by a bromine atom. The ^{1}H and ^{13}C

Table 4. ^{13}C NMR assignments of compounds **1**~**7**.

Position	δC/ppm in d_6-acetone[a]					
	1	**2**	**3**	**4**[b]	**5**	**7**
1	104.8	104.9	104.4	104.1	107.3	104.1
2	118.7	118.7	116.6	118.4	n.o.	118.5
3	90.8	90.8	90.0	89.6≠	92.6	90.5
3a	142.0	142.1	141.4	140.8	142.7	140.7
4a	154.2	154.4	154.0	153.8	154.6	153.2
5	94.3	94.5	94.4	90.4≠	94.8	93.8
6	164.4	164.6	166.2	164.8*	167	161.2
6-O-CH_3	-	-	-	55.9	-	-
7	112.9	114.5	113.2	113.6	113.2	111.7
8	160.8	160.6	161.0	160.0*	160.5	160.2
8a	93.4	93.5	93.2	93.4	n.o.	93.6
9	159.3	159.4	160.5	158.4*	159.3	158.4
1′	24.8	16.2	24.9	24.0	24.8	30.7
2′	22.5	13.5	22.6	21.9	22.5	22.6
3′	14.3	-	14.3	13.9	14.3	21.8
4′	-	-	-	-	-	13.8

a) Referenced to acetone signal at 29.8 ppm referenced to external TMS
b) Measured in $CDCl_3$; referenced to $CHCl_3$ signal at 76.9 ppm referenced to external TMS
*, ≠ These assignments are interchangeable
n.o. = Not observed

NMR spectra were essentially identical with those of **1** indicating halogenation at C-2.

7-Butyl-2-chloro-6,8-dihydroxy-9*H*-pyrrolo [2,1-b] [1,3] benzoxazine-9-one, **7**

Mass spectral data indicated a molecular weight of 307 and molecular formula $C_{15}H_{14}NO_4Cl$, with one extra CH_2 group compared to **1**. Inspection of the ^1H NMR spectrum showed that this was explained by a 7-butyl side chain.

2-Chloro-8-hydroxy-6-methoxy-7-propyl-9*H*-pyrrolo [2,1-b] [1,3] benzoxazine-9-one, **4**; 2-Chloro-7-ethyl-8-hydroxy-6-methoxy-9*H*-pyrrolo [2,1-b] [1,3] benzoxazine-9-one, **8**; 1,2-Dichloro-8-hydroxy-6-methoxy-7-propyl-9*H*-pyrrolo [2,1-b] [1,3] benzoxazine-9-one, **9**

The ^1H NMR spectra for these three compounds all contained methyl singlet signals with similar chemical shifts suggestive of methoxyl groups. The hydrogen-bonded 8-OH signal was still present in all three spectra indicating that these were likely to be 6-*O*-methyl derivatives. The remaining spectroscopic data for all three compounds was used to determine that **4**, **8** and **9** were 6-*O*-methyl derivatives of **1**, **2** and **3** respectively.

Biological Activity

The histidine kinase used in this assay, NRIIc, is a fusion protein consisting of maltose binding protein at the amino terminus and NRII at the carboxy terminus. Construction of the expression plasmid encoding this protein, and the methods of production and isolation of the purified protein, have been described by KAMBEROV *et al.*[10] Members of the isolated series of compounds were found to exhibit potent inhibition of autophosphorylation of this histidine kinase. The IC_{50} values for the compounds tested against the histidine kinase are shown in Table 5 and ranged from 9 to >340 μM.

Since histidine kinase is an antibacterial target, the purified compounds were also tested for whole cell antimicrobial activity against a range of bacteria and fungi using the broth dilution method performed in microtitre plates. The results are shown in Tables 6a and 6b. Compound **1** was the most widely tested (Table 6a) and exhibited growth inhibitory activity primarily against

Gram-positive bacteria. The MICs of 0.2~0.78 μg/ml were recorded for compound **1** against *Bacillus subtilis* NCIMB 8054 and three strains of *Staphylococcus aureus*, including the drug resistant strains ATCC 27659 and ATCC 33591. Compounds **2**, **3**, **6** and **7** (Table 6b) also showed activities in the MIC range of 0.78~12.5 μg/ml against these bacteria. There was no growth inhibition of the Gram-negative bacteria *Escherichia coli* NCIMB 12210, *Pseudomonas aeruginosa* NCIMB 8295, *Klebsiella pneumoniae* NCIMB 9111 or *Serratia marcescens* NCIMB 8889, although activity against *Proteus vulgaris* NCIMB 4175 was observed (MICs measured in the range 1.56~25 μg/ml). For the fungal strains on the test panel, compounds **1**, **2**, **3**, **6** and **7** exhibited activity against the yeast *Cryptococcus neoformans* NCPF 3379, and compound **1** also showed growth inhibitory activity against some strains of *Candida*.

Table 5. IC_{50} Values for inhibition of histidine kinase by streptopyrroles.

Compound	IC_{50} (μM)
1	20
2	50
3	13
5	71
6	80
7	9
8	>340

Compound **1** was also tested for toxicity towards a number of human cancer cell lines (A2780 ovarian cancer, A549 lung carcinoma, HT29/219 colon carcinoma, K562 chronic myelogenous leukaemia, MCF7 breast adenocarcinoma and SKMEL28 melanoma). The compound caused total inhibition of cell proliferation at concentrations in the range 12.7~71.7 μM, depending on the cell line, during a 48 hour exposure period (data not shown).

Discussion

In the present study we have isolated a series of halogenated compounds from fermentations of an actinomycete that we have identified as a strain of *Streptomyces rimosus*. They were discovered during a

Table 6a. Antimicrobial activity of compound **1**.

Strain No.	MIC (μg/ml)
	1
Staphylococcus aureus NCIMB 6571	0.78
Staphylococcus aureus ATCC 27659	0.78
Staphylococcus aureus ATCC 33591	0.2
Bacillus subtilis NCIMB 8054	0.78
Proteus vulgaris NCIMB 4175	1.56
Klebsiella pneumoniae NCIMB 9111	>50
Escherichia coli NCIMB 12210	>50
Pseudomonas aeruginosa NCIMB 8295	>50
Serratia marcescens NCIMB 8889	>50
Cryptococcus neoformans NCPF 3379	0.78
Candida albicans NCPF 3121	>50
Candida albicans ATCC 15404	>50
Candida guilliermondii NCPF 3099	6.25
Candida kefyr NCPF 3263	12.5
Candida parapsilosis NCPF 3207	12.5
Candida stellatoidea NCPF 3108	12.5
Candida tropicalis NCPF 3112	>50

Table 6b. Antimicrobial activity of compounds **2**, **3**, **4**, **6**, **7** and **8**.

Strain No.	MIC (μg/ml)					
	2	**3**	**4**	**6**	**7**	**8**
Staphylococcus aureus NCIMB 6571	1.56	1.56	NT	12.5	1.56	NT
Staphylococcus aureus ATCC 27659	1.56	1.56	NT	12.5	1.56	NT
Staphylococcus aureus ATCC 33591	0.78	0.78	>50	3.12	1.56	>50
Bacillus subtilis NCIMB 8054	1.56	1.56	>50	12.5	1.56	>50
Proteus vulgaris NCIMB 4175	3.12	>50	NT	25	12.5	NT
Klebsiella pneumoniae NCIMB 9111	>50	>50	NT	>50	>50	NT
Escherichia coli NCIMB 12210	>50	>50	>50	>50	>50	>50
Pseudomonas aeruginosa NCIMB 8295	>50	>50	NT	NT	>50	NT
Serratia marcescens NCIMB 8889	>50	>50	NT	>50	>50	NT
Cryptococcus neoformans NCPF 3379	1.56	12.5	NT	25	6.25	NT

NT=Not tested

screening programme for inhibitors of the NRIIc histidine kinase from *E. coli* and were also found to exhibit potent whole cell antibacterial and more modest antifungal activities. The major component of the fermentation, **1**, was published as a *Streptomyces armeniacus* metabolite, streptopyrrole, subsequent to these studies.[8] Compounds **2**~**9** have not been reported previously. Of these, compounds **2**, **3**, **4**, **7**, **8** and **9** were found as minor components produced under the original fermentation conditions. Supplementation of the production medium with NaBr was found to lead to the incorporation of bromine into compound **1** in place of chlorine to yield compound **5**. Compound **6** was isolated as a minor component from a fermentation in the presence of NaI, although no iodinated products were found.

The discovery of a series of compounds such as this enables initial inferences on structure activity relationships to be drawn. Increasing the length of the side chain at R_3 from ethyl (**2**) through propyl (**1**) to butyl (**7**) results in an increase in the potency of inhibition of the autophosphorylation of the NRIIc histidine kinase, a pattern which is also seen as the chain length at R_3 increases from ethyl (**6**) to propyl (**3**) for the dichlorinated members of the series. Substitution of the Cl in **1** for bromine (**5**) led to a decrease in activity, with the IC_{50} increasing from 20 to 71 μM. Where tested, derivatives where the 6-hydroxy group is methylated (**4**, **8** and **9**), showed no detectable inhibition of histidine kinase or antimicrobial activity. These compounds were of very low polarity and had poor solubility in both aqueous and organic solvents.

In contrast to our findings, the original streptopyrrole publication[8] reports only 'weak' growth inhibitory activity towards a broad range of micro-organisms, although this may reflect the differences in methodology used. More detailed information has been published for the pyralomicins,[11] which are structural relatives of the streptopyrroles. The MIC values reported for the pyralomicins are in a similar range to those of streptopyrrole, but against a different group of bacteria. They were reported to show activity against *Eschericha coli*, for example, but little or none against strains of *Bacillus subtilis* tested.

A series of compounds with both antibacterial and histidine protein kinase inhibition activity has previously been reported by BARRETT *et al.*[7] The compounds are hydrophobic tyramine derivatives and are synthetic in origin. The authors report a direct correlation between the potency of antimicrobial and effects of the histidine kinase inhibition, and hypothesise that the mechanism of antibacterial action may possibly be due to inhibition of one or more two-component signal transduction systems. Investigations into the mechanism of action of the streptopyrroles will be the subject of a future publication (OLSON *et al.*, in preparation).

Experimental

Taxonomy

The morphological observations were made on oatmeal agar, Emerson's agar and glucose yeast extract agar, after 14 days cultivation at 28°C, using a light microscope. Cultural and physiological characteristics were determined by the methods of WILLIAMS *et al.*[9] The colour names used for morphological observations were taken from the British Standards Institution colour chart.[12] Diaminopimelic acid determination was carried out according to the method of STANECK and ROBERTS.[13]

Fermentation for Production of Compounds **1**, **2**, **3**, **4**, **7**, **8** and **9**

A 1.5 ml cryovial containing 1ml of macerated vegetative mycelium suspended in a 10% (v/v) solution of glycerol was retrieved from storage at −135°C. This was then inoculated into a 250 ml baffled Erlenmeyer flask containing 40 ml of seed medium. The seed medium contained D-glucose 1%, glycerol 1.5%, soya bean peptone 1.5%, NaCl 0.3%, $CaCO_3$ 0.1%, malt extract 0.5%, yeast extract 0.5%, Tween 80 0.1%, Antifoam A 0.1%, Junlon PW110 0.1% in deionised water, adjusted to pH 7.0. This pre-culture was incubated at 28°C, 240 rpm for 2 days before being transferred to 2 litres of seed medium in a 3 litre fermentation vessel. The fermenter was agitated at 500 rpm, with aeration at 0.5 vvm and temperature controlled to 28°C for 3 days.

For the production stage, the contents of the 3 litre fermenter were transferred into 50 litre of production medium in a 75 litre fermenter. The production medium contained soybean flour 1.77%, mannitol 2%, Antifoam A 0.1% in deionised water, adjusted to pH 7.0. The fermentation was carried out for 5 days at 28°C, with aeration at 0.5 vvm and stirring at 350 rpm.

Fermentation for Production of Compounds **5** and **6**

A preculture was produced as described above and 40 ml of this culture was used to inoculate 2 litres of seed medium in a 3 litre fermenter. The fermenter was agitated at 500 rpm, aerated at 0.5 vvm and the temperature controlled at 28°C for 3 days. After this period, 560 ml of the resultant culture was used to inoculate a 20 litre fermentation vessel containing 14 litres of production medium (as above) supplemented with 0.5 g/litre of either NaBr or NaI. The fermenter was stirred at 350 rpm, aerated at 0.5 vvm and the temperature controlled at 28°C for 5 days, after which the fermentation was terminated.

Purification of Compounds **1**, **2**, **3**, **4**, **7**, **8** and **9**

The material from a 75 litre fermenter was harvested by filtration using a Schenk Niro 430 filter press. The retained biomass was extracted with 30 litres of recirculating methanol for 24 hours. The methanolic extract was harvested *via* filtration through the filter press and evaporated to an aqueous concentrate using a thin film evaporator.

The aqueous concentrate (5 litres) was then back extracted with 2×7 litres of ethyl acetate. The solvent extracts were pooled and concentrated *in vacuo* to a gum. This was purified by normal phase chromatography using a Biotage Flash 75 chromatography system and a Flash 75 KP-Sil silica (32~62 μM 60 Å) column (i.d. 7.5×30 cm length) with an isocratic mobile phase (ethyl acetate: hexane (2 : 8), 200 ml/minute flow rate). 1 litre fractions were collected and analysed by thin layer chromatography on silica gel plates [Merck 5554 plates, 20 cm×20 cm, 0.2 mm thickness of silica gel $60F_{254}$] using the same mobile phase as the developing solvent.

Fractions rich in compound **1** (R_f 0.50) were pooled, concentrated to dryness *in vacuo* and subjected to further purification by preparative reversed phase HPLC using a Beckman 350 HPLC with a Shandon Hyper Prep HS BOS C_{18} (100 Å 12 μm) column (i.d. 10×30 cm length) and an isocratic mobile phase (80% acetonitrile: water, flow rate 170 ml/minute). The eluate was monitored at 242 nm. The peak collected between 22 and 32 minutes was evaporated to dryness to yield compound **1** (4.5 g).

Flash chromatography fractions rich in compound **1** obtained by purification of the 75 litre fermentation of *S. rimosus* were also found to contain a number of minor components related to compound **1** and with similar chromatographic properties. After the preparative reversed phase HPLC step for compound **1** the HPLC fractions eluting immediately before and after those containing compound **1** were combined and concentrated to dryness. The material thus obtained was further purified by preparative reversed phase HPLC using a Waters NovaPak C_{18} (100 Å 5 μm) column (2×(i.d. 4×10 cm length)) and a linear acetonitrile: water gradient starting at 70% acetonitrile and increasing to 100% acetonitrile over 30 minutes, flow rate 55 ml/minute. The column eluate was monitored at 252 nm. The peaks collected at 5, 9.5, 10, 15, 20 and 23 minutes were concentrated to dryness *in vacuo* to yield compound **2** (50 mg), compound **3** (4 mg), compound **7** (200 mg), compound **8** (1.5 mg), compound **4** (10 mg) and compound **9** (1 mg), respectively.

Purification of Compounds **5** and **6**

The biomass from 20 litre fermentations in the presence of sodium bromide and sodium iodide were harvested, extracted with methanol and concentrated, collected by centrifugation, lyophilised and then extracted with 4 litres of methanol. The methanolic extract from the NaBr fermentation was purified by reversed phase HPLC on a Waters NovaPak C_{18} (6 μm, 60 Å) column (2×(i.d. 40×100 mm length)) eluted isocratically (70% acetonitrile: water, 50 ml/minute, monitoring at 250 nm). The fractions eluting after 12 minutes were combined and concentrated *in vacuo* to yield a brown solid (20 mg) which was further purified by preparative thin layer chromatography (Merck 5717, silica gel plates, 20×20 cm, 2 mm thickness of Kiesel gel $60F_{250}$, developed with ethyl acetate : hexane (1 : 1)).

The material in the main band (Rf=0.60) was finally purified by reversed phase HPLC on a Waters NovaPak C_{18} (6 μm, 60 Å) column (i.d. 8×100 mm length) using an isocratic mobile phase (80% methanol : water, 2 ml/minute). The peak eluting after 19 minutes was concentrated *in vacuo* to yield compound **5** (1.4 mg).

The fermentation in the presence of sodium iodide did not produce any detectable iodinated analogues of compound **1**. The methanol concentrated extract was purified by normal phase chromatography using a Biotage Flash 75 system with KP-Sil silica (32~62 μm, 60 Å) column (i.d. 7.5×30 cm length) eluted isocratically (60% hexane : ethyl acetate, 200 ml/minute). 1 litre fractions were collected and analysed by thin layer chromatography using the same mobile phase as the developing solvent. Compound **6** (Rf=0.77~0.96) rich fractions were pooled, evaporated to dryness *in vacuo* and further purified by reversed phase HPLC on a Beckman 350 system with a Shandon Hyper prep HS BOS C_{18} (12 μm, 100 Å) column (i.d. 10×300 cm length) under isocratic conditions (75% acetonitrile : water, 200 ml/minute, monitoring at 290 nm). The peak collected between 24~25 minutes was evaporated to dryness to yield compound **6** (7.9 mg).

Determination of Physico-chemical Properties

UV/visible spectra were measured on a Perkin-Elmer Lambda 17 UV/visible spectrometer. IR spectra were recorded in KBr on a Nicolet 5PC FTIR spectrometer. Low resolution EI-MS and DCI-MS were obtained on a Finnigan Mat 95 mass spectrometer. Electrospray MS (ESI-MS) were obtained on a Finnigan SSQ710C instrument interfaced to a Waters 600-MS HPLC system. High resolution EI-MS and ESI-MS were obtained on a Finnigan Mat 95 mass spectrometer. ^1H and ^{13}C NMR spectra were recorded at 308K on a Bruker ACF400 spectrometer at 400 MHz and 100 MHz respectively. All chemical shifts (δ) are quoted in ppm and are referenced to external TMS (0 ppm). Standard techniques were used to obtain the DEPT, COSY-45, HMQC, and HMBC spectra. In HMQC experiments the $^1J_{CH}$ was optimised for 145 Hz. In HMBC experiments the long range coupling constant $^{3\sim5}J_{CH}$ was optimised for 5 Hz. A 2D INADEQUATE experiment was performed using a data matrix of 4K points by 96 points, optimised for $^1J_{CC}$ of 45 Hz, with an experiment time of 28.8 hours. The concentration of **1** used in the experiment was approximately 1 M in 0.5 ml d_6-acetone.

Crystal Structure Analysis of **1**

The molecular structure of **1** is shown in Fig. 3. Compound **1** was recrystallised by slow cooling in acetone solution. The crystal and molecular structure were determined from a colourless block crystal of 0.3×0.4×0.5 mm^3. Diffraction data were obtained using graphite monochromated Mo-Kα radiation (λ=0.71073 Å) on a Bruker SMART-CCD detector diffractometer equipped with a Cryostream N_2 flow cooling device.[14] To prevent solvent loss, the sample was rapidly coated with perfluoro-polyether (Riedel-de-Häen RS3000) and cooled in the stream of cold nitrogen gas to 150K. Series of narrow ω-scans (0.3°) were performed at several ϕ-settings in such a way as to cover a hemisphere of data to a maximum resolution of 0.84 Å. Raw frame data were integrated using the SAINT program.[15] The structures were solved using Direct Methods and refined by full-matrix least squares on F^2 using SHELXTL.[16]

The crystal data are summarized as follows: Empirical Formula: $C_{17}H_{18}NO_5Cl$. FW: 351.77. Crystal system: triclinic. Lattice parameters: a=7.9947(1) Å, b=9.4276(3) Å, c=11.4359(3) Å, α=82.623°(1), β=83.393°(1), γ=85.427°(1). V=847.27(4) $Å^3$. Space group: P-1 (no 2). Z=2. Dcalc: 1.379 g/cm^3. Linear absorption coefficient: 0.25 mm^{-1}. Of the 4146 reflections collected, 2393 were unique. The number of parameters refined was 289. All non-hydrogen atoms were refined with anisotropic displacement parameters. Hydrogen atoms were located from Difference Fourier maps and their coordinates and isotropic displacement parameters refined. Final R1 value was 4.13% for 2145 reflections with Fobs>4σ(Fobs), wR2 for all data was 11.78% and the goodness-of-fit was 1.051.

Histidine Kinase Assay

Assays were performed in 96 well polypropylene microtitre plates. In each experimental well, 20 μl of a 3 μM solution of enzyme made up in assay buffer (3 M AEBSF [4-(2-aminoethyl) benzenesulphonyl fluoride], 50 mM Tris,

50 mM KCl, 10 mM $MgCl_2$, pH8.0) was mixed with 5 μl of a known concentration solution of test compound in methanol. A 25 μl aliquot of ATP solution (0.46 pM ([^{33}P]-ATP, 70 μM ATP in assay buffer) was then added and the reaction left to proceed at room temperature for 15 minutes before being terminated by the addition of 100 μl EDTA solution (50 mM, pH 8.0). The reaction mixture was then transferred to the corresponding well in a 96-well high protein binding 0.45 μm filter plate (Millipore) and the liquid removed by filtration under vacuum. The enzyme retained on the filter was washed with 6×1.5 ml wash buffer (10 mM Tris, 10 mM EDTA, pH 8.0) per well. After drying of the filters at 65°C for 1 hour, 20 μl of Microsint 0 (Packard) was dispensed into each well and the radioactivity measured using a Canberra Packard Top Count liquid scintillation counter. Total binding was determined by replacing the sample with assay buffer (50 mM Tris, 50 mM KCl, 10 mM $MgCl_2$, pH 8.0). Non specific binding was determined by replacement of the enzyme with assay buffer.

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