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Actinoallolides A−E, New Anti-trypanosomal Macrolides, Produced by an Endophytic Actinomycete, *Actinoallomurus fulvus* MK10-036

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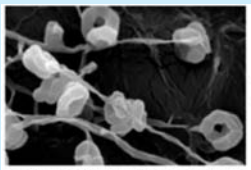
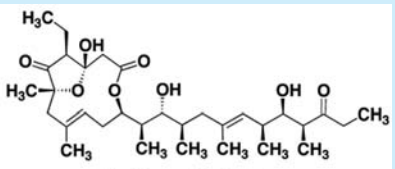
Supporting Information

ABSTRACT: Five new anti-trypanosomal macrolides, actinoallolides A−E (**1**−**5**), were discovered from the cultured broth of *Actinoallomurus fulvus* MK10-036. The structures of the actinoallolides, including absolute stereochemistry, were elucidated by a combination of spectroscopic analyses and a series of chemical derivatization studies. Actinoallolide A showed the most potent and selective in vitro anti-trypanosomal activity without cytotoxicity. A new class of promising lead compounds was identified for the development of drugs for both sleeping sickness and Chagas disease.



Actinoallomurus fulvus MK10-036



Actinoallolide A

Actinomycetes have been rich sources of new bioactive compounds, including commercially significant compounds, such as antibiotics, enzyme inhibitors, and pharmacologically active agents.[1] We investigated endophytic actinomycetes and reported several bioactive compounds, such as spoxazomicins[2] and trehangelins.[3]

Human African Trypanosomiasis (HAT, also known as sleeping sickness) and American Trypanosomiasis (also known as Chagas disease) are caused by the parasitic flagellated protozoa, *Trypanosoma brucei* and *T. cruzi*, respectively. Both parasitic infectious diseases are recognized as tropical neglected diseases that have historically caused significant and widespread morbidity and mortality. The World Health Organization estimated that HAT caused around 18 000 deaths and Chagas disease caused 7806 deaths in 2012.[4] Even though cases of HAT are in steep decline and the disease may possibly be eliminated in the near future, significant problems remain with drugs commonly used for treatment. All drugs for HAT (suramin, pentamidine, eflornithine, and melarsoprol) are toxic, expensive, difficult to administer, have difficulty crossing the blood/brain barrier, and parasite resistance to them is increasing. For the 8 million people infected with Chagas disease parasites, available therapeutic drugs (benznidazole and nifurtimox) are also unsatisfactory due to their limited efficacy, particularly in the chronic phase, with frequent side effects that can lead to treatment discontinuation. Therefore, there is an urgent need for new anti-trypanosomal drugs that are more effective, safer, affordable, easier to use, and which, ideally, have a novel mode of action.

As a result of our further investigation of the metabolic profiles of endophytic actinomycetes by TLC or LC-MS analysis (we named this methodology "Physicochemical Screening"), we have discovered five new unique macrolide antibiotics, actinoallolides A−E (**1**−**5**) (Figure 1), produced by *Actinoallomurus fulvus* MK10-036 (Figure S1) isolated from the roots of *Capsicum frutescens* collected in Thailand. Furthermore, we found that they all exhibit in vitro anti-trypanosomal activity.

The EtOAc extract of a 10 day cultured broth of *Actinoallomurus fulvus* MK10-036 was subjected to column chromatography and HPLC purification to afford **1**−**5** (Scheme S1).

The physico-chemical properties of actinoallolides are summarized in Table S1. The compounds are readily soluble in MeOH and $CHCl_3$ but insoluble in water and *n*-hexane. The IR absorption at 1630−1760 and 3420 cm^{-1} suggested the presence of carbonyl and hydroxyl groups. Similarity in physico-chemical properties among the actinoallolides strongly suggested that they are structurally related.

Actinoallolide A (**1**) was obtained as a major constituent from the cultured broth of MK10-036. The molecular formula of **1** was elucidated by HR-FAB-MS to be $C_{32}H_{52}O_8$, requiring seven degrees of unsaturation. The ^1H and ^{13}C NMR spectral data in DMSO-d_6 and $CDCl_3$ of **1** are listed in Table S2. The ^1H NMR and COSY spectrum in $CDCl_3$ of **1** displayed obvious

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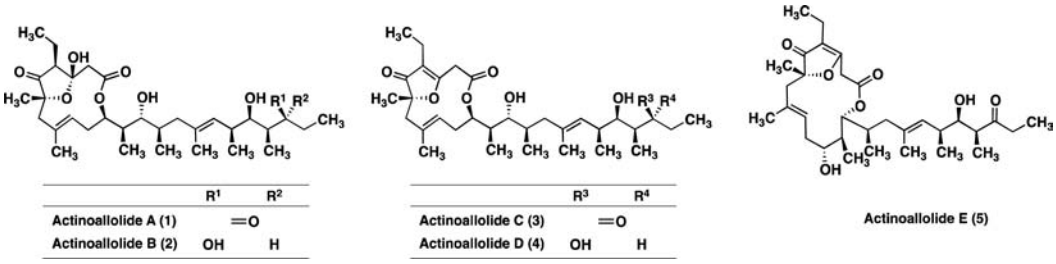

Figure 1. Structures of actinoallolides A–E (**1**–**5**).

signals for four secondary methyl groups [δ_H 0.88, 1.01, 1.05, and 1.07], three tertiary methyl groups [δ_H 1.34, 1.47, and 1.63] including two olefinic methyl ones, two ethyl groups [δ_H 1.14 (3H, t), 1.61, and 1.84 and δ_H 1.04 (3H, t), 2.45, and 2.50], two sp^2 methines [δ_H 4.88 and 5.13], eight sp^3 methines [δ_H 1.78, 1.81, 2.45, 2.67, 2.83, 3.26, 3.64, and 5.40] including three O-bearing ones, and four methylenes [δ_H 2.91 and 2.77, δ_H 2.30 and 2.35, δ_H 2.34 and 2.49, and δ_H 1.79 and 2.13]. The ^{13}C NMR and HSQC spectra indicated 32 carbons, which were classified into two ketone carbonyl carbons [δ_C 217.3 and 217.6], one ester carbonyl carbons [δ_C 170.2], two sp^2 quaternary carbons [δ_C 131.7 and 133.7], two sp^2 methine carbons [δ_C 126.5 and 128.8], eight sp^3 methine carbons (including three O-bearing ones), one acetal sp^3 quaternary carbon [δ_C 102.3], one oxygenated sp^3 quaternary carbon [δ_C 82.2], six sp^3 methylene carbons, and nine methyl carbons. The presence of three carbonyl groups and two olefin groups accounted for five of seven sites of unsaturation, suggesting the existence of two rings in **1**.

In the $^1H-^1H$ COSY experiments of **1** in $CDCl_3$ and DMSO-d_6, five segments I–V were observed (Figure 2), in

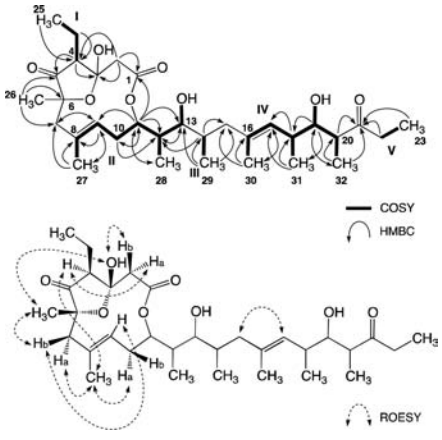

Figure 2. Key correlations in $^1H-^1H$ COSY, HMBC, and ROESY of actinoallolide A (**1**).

which two ^4J long-range spin systems between 9-H/27-H_3 and 17-H/30-H_3 and ^3J spin systems between 13-H/13-OH and 19-H/19-OH were also observed. The HMBC correlations from 23-H_3 and 22-H_2 of segment V to C-21 and from 32-H_3 and 20-H of segment IV to C-21 indicated the connection between segments V and IV via the ketone carbonyl group at C-21. The HMBC correlations from 29-H_3 and 14-H of segment III to C-15 and from 30-H_3 of segment IV to C-15 revealed the linkage between segments III and IV via a methylene at C-15. The 12-membered macrolide ring was established by HMBC correlations from 4-H of segment I to C-3 and C-5, from 2-H_2 to C-1, C-3, and C-4, from 26-H_3 to C-5, C-6, and C-7, from 27-H_3 of segment II to C-7, from 7-H_2 to C-9, and from 11-H of segment II to C-1. Then, HMBC correlations from 29-H_3 of segment III to C-13 and from H-14 of segment III to C-12 suggested a connection between segments II and III to form a side chain linked to a 12-membered macrolide ring. The geometry of the two double bonds were determined to be 8*E* and 16*E* on the basis of the ROESY correlations between 7b-H and 9-H, between 27-H_3 and 10a-H, and between 15-H_2 and 17-H. Finally, the hemiacetal carbon at C-3 (δ_C 102.3) and oxygenated carbon C-6 (δ_C 82.2) were connected via an oxygen atom to form a tetrahydrofuran ring fused with a macrolide ring, based on the remaining one degree of unsaturation and their ^{13}C chemical shifts. Thus, the planar structure of **1** was elucidated as a new type of 12-membered macrolide, and **1** was designated as actinoallolide A (Figure 1).

The relative configuration of the macrolide ring moiety of **1** was elucidated by the ROESY in DMSO-d_6. The ROESY correlations between 2b-H (δ_H 2.88) and 3-OH, between 3-OH and 26-H_3, between 26-H_3 and 7b-H (δ_H 2.18), and between 7b-H (δ_H 2.18) and 9-H confirmed that 3-OH and 26-H_3 were on the β-face of the molecule, and the observed ROESY correlations between 2a-H (δ_H 2.43) and 4-H, between 4-H and 27-H_3, and between 27-H_3 and 7a-H (δ_H 2.11) confirmed that 4-H was on the α-face of the molecule (Figure 2).

The absolute configuration of **1** was elucidated by single-crystal X-ray crystallographic analysis. In the process of the acylation of the hydroxyl group at C-13 with α-methoxy-α-trifluoromethylphenylacetyl (MTPA) chloride, we obtained the crystals of the 13-*O*-(*R*)-MTPA ester of **1** from a mixed solution of CH_2Cl_2/*n*-hexane (1:4). The ORTEP drawing is shown in Figures S3–6. Therefore, the absolute configuration of **1** was revealed as 3*S*, 4*S*, 6*R*, 11*R*, 12*R*, 13*R*, 14*R*, 18*S*, 19*R*, and 20*S*.

The molecular formulas of **3** and **5** were elucidated by HR-MS to be both $C_{32}H_{50}O_7$, requiring eight degrees of unsaturation. The ^1H and ^{13}C NMR spectral data in $CDCl_3$ of **3** and **5** are listed in Table S4, which were very close to those of **1**. The analysis of 2D NMR spectra revealed that the planar structure of **3** was a dehydrated analogue **1** between 3-OH and 4-H and designated as actinoallolide C (Figures 1 and S4). In the comparison of the chemical shifts between **3** and **5**, those of 11-H (δ_H 5.27 in **3**, δ_H 3.26 in **5**) and 13-H (δ_H 3.27 in **3**, δ_H 4.97 in **5**) were different. Moreover, the HMBC correlations from 13-H to C-1 and the other interpretation of 2D NMR spectra of **5** revealed that the planar structure of **5** was a 14-membered macrolide, which was cyclized between C-1 and C-13 in **3** instead of C-1 and C-11 to form an ester bond and designated as actinoallolide E (Figures 1 and S4).

The absolute configurations of **3** and **5** were determined by conversion of **1** to **3** and **5** (Supporting Information S5-4).

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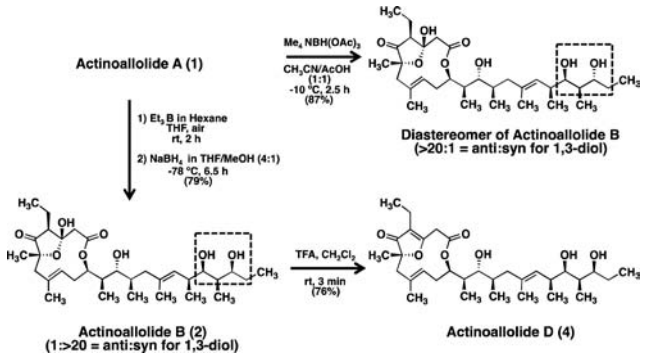
Compound **1** was immediately converted to **3** and **5** by treatment with trifluoroacetic acid (TFA). Thus, the absolute configurations of **3** and **5** were elucidated as 3*S*, 6*R*, 11*R*, 12*R*, 13*R*, 14*R*, 18*S*, 19*R*, 20*S* and 3*S*, 6*R*, 11*R*, 12*R*, 13*R*, 14*R*, 18*S*, 19*R*, 20*S*, respectively.

The molecular formula of **2** was elucidated by HR-ESI-MS to be $C_{32}H_{54}O_8$, requiring six degrees of unsaturation. The ^1H and ^{13}C NMR spectral data in $CDCl_3$ of **2** are listed in Table S4, which were very close to those of **1**. However, one oxygenated sp^3 methine carbon (δ_C 79.3) was observed in **2** instead of a ketone carbonyl carbon (δ_C 217.3) of **1** at C-21. Further analysis of 2D NMR spectra (Figure S4) revealed the planar structure of **3** as shown in Figure 1 and designated as actinoallolide B. The absolute configuration of **2** was elucidated by chemical conversion of **1** to **2** (Scheme 1, Supporting Information S5-5). The reduction by $Me_4NBH(OAc)_3$ of **1** mainly produced the epimer of **2** at C-21 (>20:1 = anti/syn for 1,3-diol at C-19 and C-21).[5] Reduction by triethylborane followed by $NaBH_4$ treatment produced the stereoisomer identical to **2**, mainly (1:>20 = anti/syn for 1,3-diol at C-19 and C-21).[6,7] The configuration of 1,3-*syn*-diol at C-19 and C-21 in **2** was confirmed by NOE experiments of an isopropylidene acetal derivative (Scheme S5-2). Thus, the absolute configuration of **2** was elucidated as 3*S*, 4*S*, 6*R*, 11*R*, 12*R*, 13*R*, 14*R*, 18*S*, 19*R*, 20*R*, and 21*S*.

Scheme 1. Conversion of Actinoallolide A (1) to Actinoallolide B (2), C-21 Epimer of 2, and Actinoallolide D (4)



The molecular formula of **4** was elucidated by HR-FAB-MS to be $C_{32}H_{52}O_7$, requiring seven degrees of unsaturation. The ^1H and ^{13}C NMR spectral data in $CDCl_3$ of **4** are listed in Table S4, which were very close to those of **2**. However, two sp^2 olefinic carbons (δ_C 177.4 and 119.0) were observed in **4** instead of a sp^3 methine carbon (δ_C 53.4) at C-4 and an acetal quaternary carbon (δ_C 102.3) at C-3 of **2**. Further analysis of 2D NMR spectra (Figure S4) revealed the planar structure of **4** as a hydrate analogue of **2** as shown in Figure 1 and designated as actinoallolide D. The absolute configuration of **4** was elucidated by chemical conversion of **2** to **4** (Scheme 1). Compound **2** was immediately converted to **4** by treatment with TFA. Thus, the absolute configuration of **4** was elucidated as 6*R*, 11*R*, 12*R*, 13*R*, 14*R*, 18*S*, 19*R*, 20*R*, and 21*S*.

As shown in Table 1, **1** showed the most potent and selective in vitro anti-trypanosomal activity against *T. b.b.* GUTat 3.1 strain (causative agent of Nagana disease in animals) with an IC_{50} value of 0.0049 μg mL^{-1}, without cytotoxicity against MRC-5 cells (IC_{50} > 100 μg mL^{-1}). The selectivity index (>20 408) was over 5-fold better than that of the commonly used therapeutic drug, pentamidine (IC_{50} = 0.0016 μg mL^{-1}) and over 300-fold higher than that of suramin and eflornithine. Moreover, **1** displayed in vitro anti-trypanosomal activity against *T. b. rhodesiense* STIB900 strain (causative agent of HAT), with an IC_{50} value of 0.086 μg mL^{-1} (Table 2). In addition, **1** showed in vitro anti-trypanosomal activity against *T. cruzi* Tulahuen C4C8 strain (causative agent of Chagas disease), with an IC_{50} value of 0.226 μg mL^{-1}, which was similar to that of commonly used therapeutic drug, benznidazole (IC_{50} = 0.418 μg mL^{-1}) (Table 2). Actinoallolides B–E (**2**–**5**) showed moderate anti-trypanosomal activity against the *T. b.b.* GUTat 3.1 strain (IC_{50} values of 0.11–1.01 μg mL^{-1}), which we believe is due to a ketone group at C-21 and an acetal group at C-3 of **1**. This observation should play an important role in the study of anti-trypanosomal activity.

Table 1. In Vitro Anti-trypanosomal Activity against *Trypansoma brucei brucei* GUTat3.1 and Cytotoxicity in MRC-5 Cells of Actinoallolide A-E (1-5)

	IC_{50} (μg mL^{-1})		
	anti-trypanosomal activity (*T. b. brucei*)	cytotoxicity	selectivity index
1	0.0049	>100	>20408
2	1.01	51.8	51.3
3	0.11	32.4	295
4	0.77	16.5	21.4
5	0.13	4.7	36.2
pentamidine[a]	0.0016	5.7	3614
suramin[a]	1.58	>100	>63
eflornithine[a]	2.27	>100	>44

[a]Commonly used therapeutic drugs for HAT.

Table 2. In Vitro Anti-trypanosomal Activity against *Trypansoma brucei rhodensiense* STIB900 and *T. cruzi* Tulahuen C4C8 of Actinoallolide A (1)

	IC_{50} (μg mL^{-1}) anti-trypanosomal activity	
	T. b. rhod.	*T. cruzi*
1	0.086	0.226
melarsoprol[a]	0.002	not tested
benznidazole[a]	not tested	0.418

[a]Commonly used therapeutic drugs for HAT or Chagas disease.

Moreover, all actinoallolides showed no antibacterial activity, even at 10 μg using a paper disc test against Gram-positive and -negative bacteria, as well as no bioactivity against fungi and yeast.

So far, some 12-membered macrolide antibiotics have been reported to have various biological activities. Methymycin is an antibacterial.[8] NK30424-A and -B displayed inhibitory activity against lipopolysaccharide-induced tumor necrosis factor alpha production in murine peritoneal macrophages.[9] Pladienolides demonstrated inhibitory activity against vascular endothelial growth factor expression and antitumor activity.[10] However, to our knowledge, anti-trypanosomal 12-membered macrolides have not been reported until now, the actinoallolides being the first example.

HAT and Chagas disease combined cause 26 000 deaths annually and are a major disease burden among impoverished communities throughout Africa and Latin America. Especially, Chagas disease has recently spread internationally, with cases

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increasingly being reported in the United States, Canada, Europe, and Western Pacific countries. Treatment options for Chagas disease are minimal, only two nitro-heterocyclic drugs. The adverse side effects of these drugs have limited their use, so safe and efficacious new treatment options are urgently required.[11,12] Actinoallolide A exhibited activity against *T. cruzi* similar to that of benznidazole. Therefore, actinoallolides represent a promising new class of lead compounds for the development of novel drugs for use in combatting Chagas disease.

In conclusion, this paper describes the discovery of actinoallolides that possess potent in vitro anti-trypanosomal activity, indicating that further investigation should be undertaken to evaluate the possibility of actinoallolides and their synthetic analogues being used to develop new anti-trypanosomals. Investigation of the mode of action of these compounds is also currently underway.

■ ASSOCIATED CONTENT

Supporting Information

Experimental details and additional figures, tables, and schemes as well as a crystallographic data file (CIF) for 13-*O*-(*R*)-MTPA ester of **1**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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