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Original article

Synthesis, structure and biological activity of pseudopeptidic macrolides based on an amino alcohol

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Abstract

A series of new pseudopeptidic macrolides **2a–f** based on an amino alcohol were synthesized and evaluated for in vitro antibacterial and antifungal activities. The structure–activity relationships of these compounds were studied and the results showed that compounds **2a** and **2d** exhibited moderate antibacterial activity against *Staphylococcus aureus*, *Bacillus subtilis* and *Escherichia coli*, whereas compound **2e** showed potent antifungal activity against all the fungal species tested, showing a promising broad-spectrum antifungal activity. All the compounds have been studied in vitro for the hemolytic activity as a measure of their cytotoxicity, showing that these compounds have low lytic properties.

Keywords: Pseudopeptidic macrolides; Amino alcohol; Antibacterial activity; Antifungal activity; Hemolytic activity

1. Introduction

Deep mycosis we routinely encounter include candidiasis, cryptococcosis and aspergillosis [1]. With the increase in incidence of deep mycosis in recent years [2–4], there has been an increasing emphasis on the importance of antifungal chemotherapy. Antifungal regimens against mycoses were recommended according to the causative pathogen, since these fungi showed different patterns of antifungal susceptibilities. However, in those who were treated with potent antifungals, there exist some cases with treatment failure because of severe adverse reactions or antifungal resistance. Various structural classes of compounds, such as macrolides, peptides, nucleosides, polyenes, heterocycles, etc., exhibited antifungal activity [5]. However, there were only a few drugs available for treating systemic antifungal infection, e.g., amphotericin B and the azoles such as fluconazole. Thus the development of broad-spectrum antifungal agents with a potent anti-aspergillus activity and good safety is needed [6,7].

In the past decade, there has been considerable interest in polyamido-polyester compounds, which are widely found in nature and constitute an extensive range of natural products with diverse biological activity [8–10]. The successful design, synthesis, and use of chiral macrocyclic ligands capable of the selective recognition of other species are of great interest to workers in catalysis [11], separations [12,13], enzyme mimics [14], and other areas involving chiral molecular recognition [15]. Recently, new prototype antifungal agents, chiral macrolides exhibited antifungal activities against *Sporothrix schenckii*, *Microsporum gypseum*, *Aspergillus niger* and *Chaetomium globosum* [16].

In order to inhibit deep mycosis, such as candidiasis, cryptococcosis and aspergillosis better, we synthesized new chiral pseudopeptidic macrolides based on a chiral amino alcohol, (1*R*, 2*S*)-1-amino-2,3-dihydro-1*H*-inden-2-ol, which is the intermediate of crixivan-inhibitor of HIV protease. In this paper, we report herein our structural finding and in vitro antibacterial, antifungal and hemolytic activity of the small family of chiral pseudopeptidic macrolides.

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2. Chemistry

Six pseudopeptidic macrolides based on (1*R*, 2*S*)-1-amino-2,3-dihydro-1*H*-inden-2-ol were synthesized, which include some possible combinations of pyridine, benzene and thiophene **2a–f** (Scheme 1). 2,6-Isophthalic acid, 2,6-pyridine dicarboxylic acid or 2,5-thiophene dicarboxylic acid undergo reaction with $SOCl_2$ to form the corresponding dicarbonyl dichlorides. Condensation of 2,6-isophthalic dicarbonyl dichloride or 2,6-pyridine dicarbonyl dichloride with the chiral amino alcohol, yielded pseudopeptidic alcohols **1a** and **1b** [17]. In the subsequent steps, 2,6-isophthalic dicarbonyl dichloride, 2,6-pyridine dicarbonyl dichloride or 2,5-thiophene dicarbonyl dichloride undergo reaction with pseudopeptidic alcohols **1a** and **1b** to form the pseudopeptidic macrolides **2a–f** in 18–63% yield. All new compounds gave satisfactory IR, ^{1}H and ^{13}C NMR, ESI-MS, and elemental data.

3. Results and discussion

3.1. Crystal and molecular structure

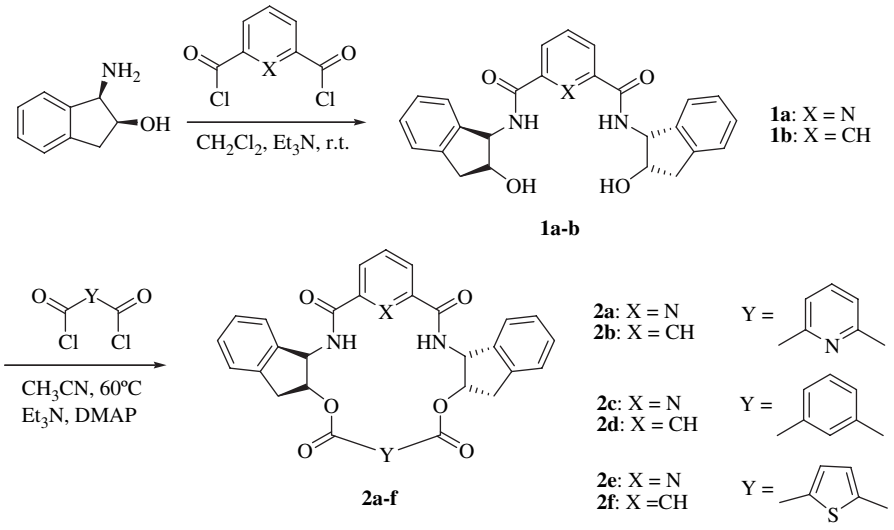
An X-ray diffraction analysis was performed to confirm the molecular structure of the products of the described process. The crystal data and structure refinement parameters for compound **2a** are given in Table 1. The structure of compound **2a** is presented in Fig. 1. Compound **2a** crystallizes in the monoclinic space group $P2_1$. It has butterfly-shaped molecular structure with a guest water molecule residing in the cavity of the macrolide **2a**. It is not C_2-symmetry in the crystalline state, N(1)–H(1) of the carbamoyl moieties are involved in H-bonding between the pyridyl nitrogen [N(1)···N(2), 2.672(3) Å], however, it interacts with its guest water molecule through four H-bonding interactions: (i) O(7) H-bonds to carbamoyl N–H groups with N(1)···O(7) and N(3)···O(7) distances of 2.919(3) and 2.952(3) Å, respectively; (ii) the water hydrogen H-bond to the macrolide through the carbonyl ester oxygen where heavy atom O(7)···O(5) distance is 3.015(3) Å; (iii) the water hydrogen H-bond to N atom of pyridine ring with O(7)···N(4) distances of 3.070 (3) Å.

Table 1
Crystal data and structure refinement of pseudopeptidic macrolide **2a**

Complex	**2a**
Empirical formula	$C_{32}H_{26}N_4O_7$
Formula weight	578.57
Crystal system	Monoclinic
Space group	$P2_1$
Unit cell dimensions	$a = 11.2202$ (19) Å, $\alpha = 90°$ $b = 10.2220$ (17) Å, $\beta = 95.851$ (3)° $c = 12.165$ (2) Å, $\gamma = 90°$
Volume	1388.0 (4) Å^3
Z	2
Density (calculated)	1.384 mg/m^3
Absorption coefficient	0.099 mm^{-1}
$F(000)$	604
Crystal size	$0.24 \times 0.18 \times 0.12$ mm^3
Crystal description	Colorless block
θ Range for data collection	2.61–25.74°
Index ranges	$-14 \le h \le 11$, $-12 \le k \le 12$, $-10 \le l \le 15$
Reflections collected	7917
Independent reflections	3008 [$R_{int} = 0.0258$]
Completeness to θ	99.5%
Refinement method	Full matrix least-squares on F^2
Data/restraints/parameters	3008/1/402
Goodness-of-fit on F^2	1.55
Final R indices [$I > 2\ \sigma(I)$]	$R1 = 0.0331$, $\omega R2 = 0.0678$
R indices (all data)	$R1 = 0.0504$, $\omega R2 = 0.0754$
Largest diff. peak and hole	0.113 and −0.134 eÅ^{-3}

3.2. Antibacterial studies

The newly synthesized compounds **2a–f** were screened for their antibacterial activity in vitro against two representative Gram-positive organisms viz. *Staphylococcus aureus* (ATCC 25923) and *Bacillus subtilis* (ATCC 6633) and two Gram-negative organisms viz., *Escherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 27853) bacterial strains by the two-fold serial microdilution method. Chloramphenicol



Scheme 1.

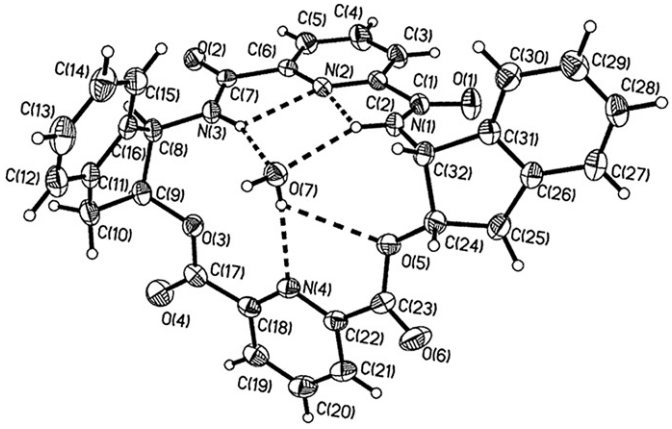


Fig. 1. The ORTEP diagram of compound **2a**.

was chosen as a standard in antibacterial activity measurements.

The minimum inhibitory concentration (MIC) values of compounds **2a**–**f** are presented in Table 2. Compound **2c**'s antibacterial activity (MIC = 4 μg/ml) against *B. subtilis* showed better results than chloramphenicol against the bacteria. Compounds **2a** and **2d** had the same activity against *S. aureus* (MIC = 4 μg/ml) and *B. subtilis* (MIC = 8 μg/ml), respectively, when compared with chloramphenicol. Compound **2c** was as active against *P. aeruginosa* as chloramphenicol, and the other compounds did not exhibit in vitro antibacterial activity against *P. aeruginosa*. In general, all the synthesized compounds showed a good activity profile versus Gram-positive bacteria. Correlating the structure of the macrocycles to their antibacterial activity revealed that the most active compound contains two pyridine or two benzene moieties in the macrocyle. The best compound **2d** included two benzene moieties which completely inhibited the growth of *S. aureus*, *B. subtilis* and *E. coli*.

3.3. Antifungal activity

The in vitro antifungal activity of the synthesized novel pseudopeptidic macrolides **2a**–**f** was studied against the fungal strains viz., *Candida albicans* (ATCC 10231), *Cryptococcus neoformans* (SYM 523), *Rhodothece glutinis* (SYM 2209), *Saccharomyces cerevisiae* (SYM 3513), *A. niger* (ATCC 16404), *Aspergillus flavus* (SYM 223) and *Rhodothece nigricans* (SYM 2417) according to both the recommendations of the NCCLS [18,19]. Fluconazole and amphotericin B were used as standard drugs whose MIC values are provided in Table 3.

Table 2
In vitro antibacterial activity of pseudopeptidic macrolides **2a**–**f**

Compound	MIC[a] (μg/ml)			
	Gram positive		Gram negative	
	S. aureus ATCC 25923	*B. subtilis* ATCC 6633	*E. coli* ATCC 25922	*P. aeruginosa* ATCC 27853
2a	4	8	32	NA[c]
2b	16	NA[c]	32	NA[c]
2c	32	4	NA[c]	32
2d	4	8	16	NA[c]
2e	8	NA[c]	NA[c]	NA[c]
2f	16	NA[c]	16	NA[c]
Chlor[b]	4	8	8	32

[a] Minimum inhibitory concentration.
[b] Chloramphenicol.
[c] Not active (>64 μg/ml).

Comparison of activity of compounds **2a**–**f** referred to in Table 3 with antifungal drug fluconazole showed that compound **2e** had better activity against all the pathogenic fungi (MIC range from 1 to 8 μg/ml). Compounds **2d**–**f** had the same activity against *C. albicans* and *R. nigricans* (MIC = 1 μg/ml), when compared with amphotericin B. Compound **2a** possessed higher activity against *C. albicans*, however, it was only moderately active against the other strains of fungi. In addition compounds **2a**–**f** when compared with fluconazole which was not active against *R. glutinis*, were found to exhibit high activity against fungi. Moreover, it is a pleasure that all the compounds were moderately active against *A. niger* and *A. flavus*, except that compound **2a** was inactive against *A. flavus*. Correlating the structure of the macrocycles to their antifungal activity revealed that the most active compound contains a pyridine and a thiophene moiety in the macrocycle. The best compound **2e** completely inhibited the growth of *C. albicans*, *Cr. neoformans*, *R. glutinis*, *S. cerevisiae*, *A. niger*, *A. flavus* and *R. nigricans*. The enhanced antifungal activity of this compound was attributed to the presence of both N and S heterocyclic subunits in the chiral macrolide.

3.4. Hemolytic activity

Since the pseudopeptidic macrolides proved to be broad-spectrum agents, showing a strong inhibition of the growth of fungi and bacteria tested, we therefore determined the hemolytic activity in vitro of all the compounds as a measure of their cytotoxicity.

As shown in Table 4, results demonstrated that compound **2e** showing a strong inhibition of the growth of all the fungi tested did not lyse 0.5% of erythrocytes until the concentration was 250 μg/ml, even at the concentration of 4000 μg/ml, which is 100-fold greater than the MICs, it only lysed 14% of erythrocytes. Moreover, **2a** and **2d** with a strong antibacterial activity did not lyse erythrocytes at the concentration of 1000 μg/ml. It is clear that the pseudopeptidic macrolides have low lytic properties.

4. Conclusion

We have synthesized a series of chiral pseudopeptidic macrolides **2a**–**f** based on (1*R*, 2*S*)-1-amino-2,3-dihydro-1*H*-inden-2-ol. A single crystal structure of **2a** was determined. Amongst them the promising compound **2e** has shown significant in vitro antifungal activity against *C. albicans* and *R. nigricans*, and all the compounds have shown moderate activity against *A. niger* and *A. flavus* except compound **2a**. However, compounds **2a** and **2d** exhibited marked antibacterial activity

Table 3
In vitro antifungal activity of pseudopeptidic macrolides **2a–f**

Compound	MIC[a] (μg/ml)						
	C. albicans ATCC 10231	*C. Neoformans* SYM 523	*R. glutinis* SYM 2209	*S. cerevisiae* SYM 3513	*A. niger* ATCC 16404	*A. flavus* SYM 223	*R. nigricans* SYM 2417
2a	1	16	16	64	4	NA[d]	8
2b	4	16	8	32	8	2	4
2c	4	8	8	16	4	8	4
2d	1	4	4	32	8	2	1
2e	1	4	4	8	4	1	1
2f	1	4	4	16	32	8	1
AMPH[b]	1	0.5	2	0.5	1	0.5	1
FLCZ[c]	16	32	NA[d]	32	64	64	8

[a] Minimum inhibitory concentration.
[b] Amphotericin B.
[c] Fluconazole.
[d] Not active (>64 μg/ml).

in vitro against *S. aureus*, *B. subtilis* and *E. coli*. All the compounds tested, **2a–f** have low lytic properties. The results suggest that the pseudopeptidic macrolides based on the chiral amino alcohol would be potent broad-spectrum antifungal agents with low toxicity.

5. Experimental protocols

5.1. Chemistry

All the reagents and solvents employed were of the best grade available and were used without further purification. The elemental analyses for the compounds were carried out using the YANACO CHN CORDER MT-3 analyzer. Melting points were determined using an electrothermal apparatus and were uncorrected. Mass spectra were obtained using a Thermofinnigan TRACE-DSQ electrospray ionization (ESI) mass spectrometer. The ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AC-P300 instrument in $CDCl_3$, the following abbreviations were used to designate chemical shift mutiplicities: s = singlet, d = doublet, t = triplet, m = multiplet, br = broad. The IR spectra (in KBr pellets) were recorded on a Bio-Rad FTS-135 spectrophotometer.

5.2. General procedure for the synthesis of the pseudopeptidic macrolides (*2*)

A dry flask was charged with compound **1** (1.2 mmol) and MeCN (40 ml). On heating the system to 60 °C and stirring magnetically, Et_3N (2 ml) and DMAP (20 mg) were added. To the above system, the corresponding dicarbonyl dichloride (0.5 g) in MeCN (20 ml) was added dropwise over a period of 1 h. The reaction solution was stirred for another 10 h. White solid, $Et_3N{\cdot}HCl$ was formed, and was separated. The remaining solvent was evaporated under reduced pressure. The residue was purified by chromatography on silica gel.

Table 4
Hemolytic activity of chiral pseudopeptidic macrolides **2a–f**

Compound	Percentage lysis of human erythrocytes (%)				
	4000 μg/ml	2000 μg/ml	1000 μg/ml	500 μg/ml	250 μg/ml
2a	2	1	0	0	0
2b	7	4	2	0	0
2c	9	6	3	0	0
2d	2	0.5	0	0	0
2e	14	6	4	1	0.5
2f	9	4	1	0	0

5.2.1. Compound *2a*

Yield: 20%; m.p. = 188–190 °C; $[\alpha]_D^{20} = +100.8$ (*c* 0.4, CH_2Cl_2); anal. calcd. for $C_{32}H_{24}N_4O_6$: C 68.57, H 4.28, N 10.00; found: C 68.60, H 4.26, N 10.05; ESI-MS *m/z* 561.12 $[M+H]^+$; ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 8.74–8.72 (d, $J = 9.0$ Hz, 2H), 8.47–8.45 (d, $J = 7.8$ Hz, 2H), 8.08–8.03 (m, 3H), 7.93–7.87 (m, 1H), 7.31–7.26 (m, 8H), 6.02–5.97 (m, 2H), 5.79–5.77 (d, $J = 2.4$ Hz, 2H), 3.50–3.30 (m, 4H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 165.7, 164.8, 149.4, 148.9, 140.1, 139.6, 138.9, 131.2, 129.1, 128.6, 127.9, 126.0, 125.3, 124.2, 79.3, 56.5, 38.0; IR (KBr): ν (cm^{-1}): 3319.5, 3250.0, 2960.4, 1723.2, 1676.5, 1538.6, 1442.3, 1377.1, 1331.3, 1245.2, 1172.5, 1134.8, 1091.2, 1029.7, 998.9, 847.8, 794.9, 756.2.

5.2.2. Compound *2b*

Yield: 63%; m.p. > 300 °C; $[\alpha]_D^{20}$ +114.2 (*c* 0.7, CH_2Cl_2); anal. calcd. for $C_{33}H_{25}N_3O_6$: C 70.84, H 4.47, N 7.51; found C 70.85, H 4.46, N 7.55; ESI-MS *m/z* 560.16 $[M+H]^+$; ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 7.92–7.73 (m, 6H), 7.47–7.42 (t, $J = 7.5$ Hz, 1H), 7.39–7.20 (m, 8H), 6.98–6.97 (d, $J = 8.4$ Hz, 2H), 5.96–5.92 (m, 4H), 3.44–3.22 (m, 4H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 168.1, 164.4, 148.5, 140.1, 139.3, 138.4, 135.5, 131.2, 130.8, 129.3, 128.7, 127.7, 125.2, 124.2, 123.6, 77.9, 56.9, 37.6; IR (KBr): ν (cm^{-1}): 3325.6, 3258.2, 3064.5, 2960.4, 1723.4, 1658.7, 1530.4, 1441.3, 1387.3, 1342.1, 1254.6, 1172.5, 1134.8, 1089.2, 1031.7, 996.9, 840.8, 795.9, 754.2.

5.2.3. Compound *2c*

Yield: 31%; m.p. > 300 °C; $[\alpha]_D^{20}$ +43.6 (*c* 0.4, CH_2Cl_2); anal. calcd. for $C_{33}H_{25}N_3O_6$: C 70.84, H 4.47, N 7.51; found

C 70.86, H 4.43, N 7.56; ESI-MS *m/z* 558.19 $[M-H]^-$; ^1H NMR ($CDCl_3$, 300 MHz) δ: 8.43–8.40 (d, $J=7.8$ Hz, 2H), 8.20–8.04 (m, 6H), 7.55–7.48 (m, 1H), 7.32–7.27 (m, 8H), 5.94–5.89 (m, 2H), 5.70–5.66 (m, 2H), 3.55–3.33 (m, 4H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 166.3, 164.5, 149.7, 140.0, 139.7, 139.2, 134.5, 131.1, 129.2, 128.9, 128.2, 127.5, 126.3, 125.4, 124.1, 78.3, 56.5, 38.4; IR (KBr); ν (cm^{-1}): 3319.5, 3250.0, 3096.4 2922.7, 2863.3, 1723.4, 1657.1, 1536.4, 1496.7, 1439.9, 1339.1, 1292.6, 1247.2, 1179.8, 1149.0, 1099.8, 1038.8, 993.9, 840.1, 764.7, 748.9.

5.2.4. Compound **2d**

Yield: 18%; m.p. > 300 °C; $[\alpha]_D^{20}$ +111.7 (*c* 0.6, CH_2Cl_2); anal. calcd. for $C_{34}H_{26}N_2O_6$: C 73.12, H 4.66, N 5.02; found C 73.14, H 4.67, N 5.00; ESI-MS *m/z* 559.09 $[M+H]^+$; ^1H NMR ($CDCl_3$, 300 MHz) δ: 8.47 (s, 1H), 7.92–7.89 (d, $J=7.5$ Hz, 2H), 7.83–7.80 (d, $J=7.5$ Hz, 3H), 7.70 (s, 2H), 7.32–7.27 (m, 8H), 6.59–6.56 (d, $J=8.7$ Hz, 2H), 6.00–5.97 (m, 2H), 5.86–5.84 (m, 2H), 3.43–3.19 (m, 4H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 168.0, 165.5, 139.9, 139.6, 135.7, 133.7, 131.2, 130.5, 129.4, 127.1, 128.9, 128.5, 127.9, 125.4, 124.4, 123.7, 77.7, 57.1, 37.2; IR (KBr); ν (cm^{-1}): 3339.5, 3270.5, 3044.9, 2959.1, 1718.7, 1647.0, 1529.4, 1475.9, 1352.1, 1235.7, 1132.7, 1096.6, 1076.5, 1038.6, 909.1, 823.7, 734.9, 652.3.

5.2.5. Compound **2e**

Yield: 26%; m.p. > 300 °C; $[\alpha]_D^{20}$ +170.8 (*c* 0.4, CH_2Cl_2); anal. calcd. for $C_{31}H_{23}N_3O_6S$: C 65.84, H 4.06, N 7.40; found C 65.83, H 4.03, N 7.45; ESI-MS *m/z* 566.09 $[M+H]^+$; ^1H NMR ($CDCl_3$, 300 MHz) δ: 8.08–8.05 (d, $J=7.5$ Hz, 2H), 7.98–7.93 (m, 1H), 7.73 (s, 2H), 7.70–7.67 (d, 2H), 7.46–7.30 (m, 8H), 5.96–5.91 (m, 2H), 5.54–5.52 (d, $J=5.3$ Hz, 2H), 3.50–3.30 (m, 4H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 164.8, 161.1, 151.1, 139.8, 139.3, 138.8, 138.1, 134.5, 133.6, 129.5, 128.0, 125.4, 124.6, 77.7, 55.7, 37.6; IR (KBr): ν (cm^{-1}) = 3418.1, 3255.4, 3046.1, 2922.7, 2878.4, 1723.4, 1657.2, 1536.5, 1496.7, 1459.1, 1439.9, 1339.9, 1247.2, 1208.9, 1099.8, 840.1, 748.9.

5.2.6. Compound **2f**

Yield: 35%; m.p. > 300 °C; $[\alpha]_D^{20}$ +166.5 (*c* 0.4, CH_2Cl_2); anal. calcd. for $C_{32}H_{24}N_2O_6S$: C 68.08, H 4.26, N 4.96; found C 68.06, H 4.29, N 5.01; ESI-MS *m/z* 565.15 $[M+H]^+$; ^1H NMR ($CDCl_3$, 300 MHz) δ; 8.44 (s, 1H), 7.96–7.94 (d, $J=7.8$ Hz, 2H), 7.78 (s, 2H), 7.55–7.30 (m, 9H), 6.68–6.65 (d, $J=9$ Hz, 2H), 6.00–5.90 (m, 2H), 5.64–5.59 (m, 2H), 3.65–3.14 (m, 4H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 166.7, 161.1, 139.7, 139.2, 137.6, 135.0, 134.5, 130.2, 129.7, 129.6, 128.2, 126.4, 125.6, 125.4, 77.7, 55.5, 37.4; IR (KBr): ν (cm^{-1}) = 3456.9, 3250.0, 3054.3, 2973.6, 1722.7, 1650.3, 1538.5, 1500.3, 1459.0, 1336.9, 1247.2, 1208.0, 1134.3, 1096.7, 1030.4, 960.1, 880.1, 836.8, 823.6, 763.7, 756.0.

5.3. Crystal data collection and processing of compound **2a**

Crystals of compound **2a** are monoclinic with space group $P2_1$. The intensity data for the X-ray crystallographic determination of **2a** were collected at 294(2) K on a Bruker SMART CCD area-detector diffractometer with Mo Kα, $\lambda = 0.71073$ Å. Corrections were made for Lorentz and Polarization factors as well as for absorption (numerical). Structures were solved with direct method using SHELX-97 and were refined by full matrix least-squares method on F^2 with SHELXL-97. Non-hydrogen atoms were refined with anisotropy thermal parameters. All hydrogen atoms were geometrically fixed and allowed to refine using a riding model. The crystal data and structure refinement parameters for compound **2a** are given in Table 1.

5.4. Biological activity

5.4.1. Antibacterial studies

The in vitro antibacterial susceptibility test was done in nutrient broth (NB) by a two-fold serial microdilution method, using 96-well microplates (flat bottom). Bacterial cell suspensions were obtained after being incubated overnight. The tested compounds were initially dissolved in dimethyl sulfoxide (DMSO) at a concentration of 6.4 mg/ml and further diluted with sterile growth medium. Serial two-fold dilutions (64–0.125 μg/ml, 200 μl per well) were dispensed to each well. After inoculation (5 μl per well) was done by use of an automatic inoculator, plates were gently but thoroughly shaken and were incubated at 37 °C for 24 h. The inocula size was $1-1.5\times10^6$ cells/ml for antibacterial assay. The minimum inhibitory concentration (MIC) of each tested compound was defined as the lowest concentration exhibiting no visibly detectable bacteria growth. Chloramphenicol (Amresco 0230) was used as reference antibacterial agent. Suitable solvent control (DMSO), positive growth control, and standard drug control were also run simultaneously. All assays were performed in triplicate.

5.4.2. Antifungal studies

In vitro antifungal activity of the compounds was measured by standard broth microdilution methods of the US National Committee for Clinical Laboratory Standards for yeasts [18] and filamentous fungi [19]. RPMI 1640 (Gibco 31800022) which had been buffered to pH 7.0 with 0.165 M morpholinopropanesulfonic acid (Sigma) was used as the assay medium. Freshly grown fungi on slopes of saturated dextrose agar (logarithmic phase) were suspended with physiological saline, and the cell concentration was adjusted to concentration of 10^4 cells/ml. The test compounds were initially dissolved in dimethyl sulfoxide (DMSO) at a concentration of 6.4 mg/ml and further diluted with sterile growth medium. The final concentrations of the compounds ranged from 0.13 to 64 μg/ml. Compounds' solutions (100 μl) were added to each well of a 96-well plate (Costar 3599). After inoculation (100 μl per well, 5×10^3 cells/ml), the 96-well plates were incubated at

30 °C for 48 h or 72 h, and the minimum inhibitory concentration (MIC) of each tested complex was defined as the lowest concentration exhibiting no visible growth compared with the control wells. Fluconazole (Pfizer) and amphotericin B (Amresco 0414) were used as reference antifungal agents. A set of wells containing only inoculated medium was kept as negative control and likewise solvent controls were also done simultaneously. All assays were performed in triplicate.

5.4.3. Hemolytic studies

Human blood from healthy volunteers was collected in 10 ml Vacutainer tubes containing sodium heparin as anticoagulant. The cells were washed three times with calcium- and magnesium-free phosphate-buffered saline (PBS) and centrifuged at 2000g for 10 min. The third supernatant liquid was clear and colorless. Then 0.1 ml erythrocyte suspension diluted with PBS (erythrocytes concentration around 1.0×10^9 cells/ml) was mixed with 0.1 ml of test substances at a series of concentrations (1−4000 μg/ml). The mixtures were incubated at 37 °C for 1 h. After incubation, tubes were centrifuged at 2000g for 10 min. The supernatants were transferred into 96-well polystyrene plates (Costar 3590, incorporated) and the optical density was measured at 540 nm using MTP120 microplate reader (Colona Electric, Japan). The values for 0% and 100% lysis were determined by incubating erythrocytes with PBS, and 0.1% (v/v) Triton X-100 (Amresco 0694), respectively. Assays were carried out in triplicate and the results were confirmed in three independent experiments.

6. Supplementary data

Crystallographic data (excluding structure factors) for the structure analysis of compound **2a** has been deposited with the Cambridge Crystallographic Data Centre as supplementary publication no. CCDC 299687. Copies of the data can be obtained free of charge on application to CCDC, 12 Union Road, Cambridge CB21EZ, UK (fax: +44 1223 336 033; e-mail: deposit@ccdc.cam.ac.uk).

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