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Synthesis, absolute stereochemistry and molecular design of the new antifungal and antibacterial antibiotic produced by *Streptomyces* sp.201

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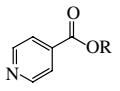
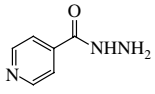
Abstract—The absolute stereochemistry of the new antifungal and antibacterial antibiotic produced by *Streptomyces* sp.201 has been established by achieving the total synthesis of the product. A series of analogues have also been synthesized by changing the side chain and their bioactivity assessed against different microbial strains. Among them, **1e** ($R = C_8H_{17}$) was found to be the most potent with MIC of 8 μg/mL against *Mycobacterium tuberculosis*, 12 μg/mL against *Escherichia coli* and 16 μg/mL against *Bacillus subtilis* 6 μg/mL against *Proteus vulgaris*. This was followed by **1b** ($R = C_5H_{11}$) with MIC of 10–20 μg/mL range and **1d** ($R = C_7H_{15}$) with MIC of 14–24 g/mL, whereas **1a** ($R = C_4H_9$) and **1f** ($R = C_{18}H_{35}$) were found to be completely inactive. Besides, **1c** ($R = C_6H_{13}$) showed certain extent of antibacterial activity in the range of 24–50 μg/mL. *Mycobacterium tuberculosis* was very sensitive to **1e** ($R = C_8H_{17}$) with MIC of 8 μg/mL. Antifungal activity of analogues **1d** ($R = C_7H_{15}$) and **1e**, ($R = C_8H_{17}$) against *Fusarium oxysporum* and *Rhizoctonia solani* were found promising with MFCs in the 15–18 μg/mL range.

1. Introduction

In our continued search for novel microbial metabolites having agricultural and pharmaceutical potential a number of *Streptomyces* strains were screened. This has resulted in characterization of several antibiotic-producing strains isolated from untapped mega biodiversity hot zone of Indo-Burma belt. We have previously reported the isolation and structure elucidation of a broad spectrum and novel antibiotic 2-methylheptyl isonicotinate **1** (R = 2-methylheptyl), a natural analogue of anti-TB drug isoniazid.[1–5] In view of its promising in vitro activity against *Mycobacterium tuberculosis* H37rv, the compound has been investigated in details. In this article we have reported the total synthesis of both the isomers of the molecule in the chirally pure form as well as the outcome of our studies on minimum structural requirement for optimum activity.



1 R = 2-methylheptyl



Isoniazid

2. Materials and methods

2.1. Test pathogens

Fusarium semitectum Berkeley and Ravenel and *Rhizoctonia solani* Kuehn were obtained through the courtesy of ICRISAT, Hyderabad, India. Pathogenic bacteria such as *M. tuberculosis* MTCC 300, *E. coli* DSMZ 1103 and *P. vulgaris* ATCC 6380, *B. subtilis* ATCC 11774 were obtained from MTCC and Gene Bank, IMTECH, Chandigarh, India.

2.2. Antimicrobial agents

Stock solution of antibiotic **1** and their derivatives **1a**–**f**, (*R*)-(+)-**1** and (*S*)-(−)-**1** were prepared in dimethyl

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sulfoxide. Further dilutions were made in phosphate buffer, pH 7.4 and with culture broth as diluent. The antimicrobial agents were tested in the final concentration range of 1–600 μg/mL.

2.3. Medium

M. tuberculosis was grown in M-33 medium (g/L): Yeast extract 2.5, Tryptone 5.0, Glucose 1.0, pH 7.0. *B. subtilis*, *E. coli* and *P. vulgaris* were grown in Nutrient broth medium (Hi-Media Laboratories, Mumbai, India). *F. semitectum* and *R. solani* were grown in M_2 agar medium (g/L): Yeast extract 5.00, NaCl 10.00, KH_2PO_4 0.10, $MgSO_4{\cdot}7H_2O$ 0.05, dextrose 10.00, agar 15.0, pH 7.1.

2.4. Antifungal and antibacterial susceptibility tests

Broth minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) were determined in the culture tube containing 4.5 mL of either nutrient broth or M-33 broth or molten M_2 agar. Final volume was adjusted to 5 mL after addition of culture media and the required antibiotic solution. Spore suspensions were prepared in Ringer's solution and adjusted to a final inoculum size of 3×10^5 colony forming units (CFU)/mL. After inoculation the culture tubes were shaked well and then incubated at 35 °C for 24 h for pathogenic bacteria, 48 h for fungi and 120 h for *M. tuberculosis*, respectively. Incubated broth showed visible turbidity in both control and the tubes with sublethal doses of antibiotic.

2.5. Determination of MIC and MFC

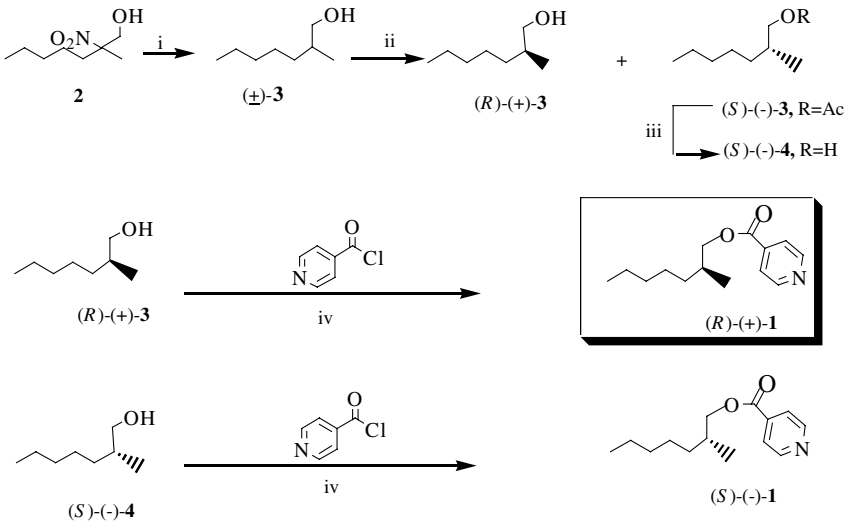
MICs of bacterial test pathogens were determined after a definite period of incubation by removing 10 μL of the contents from each tube showing no visible growth and spreading them onto agar plates of specific medium. The plates were incubated at 35 °C and growth (if any) was carefully examined under microscope. Minimum fungicidal concentration (MFCs) were determined in molten M_2 agar following the same procedure and incubated for 48 h. MFC of an antibiotic was defined as the lowest concentration at which 95% of the inoculum was killed. MICs of the bioactive molecules were defined as the lowest concentration at which there was 100% inhibition of growth compared to antibiotic free control.

3. Results and discussion

It has been already established that 2-methylheptylisonicotinate **1**, is a natural analogue of potent anti-TB drug isoniazid (INH).[1–5] The mode of action of INH also have been firmly established. New insights into the mechanism of INH have been provided by the application of genetic tools.[12] However, another interesting observation is that INH is not reported to have antifungal properties. In contrast, compound **1** has shown promising activity[2] against fungi viz *Fusarium semitectum* and *Rhizoctonia solani*. As the structural difference between **1** and INH is only in the side chain; hence, the side chain might play a vital role in exhibiting activity in certain fungi. In order to study the effect of the chain length, and to establish minimum structural requirement for optimum bioactivity, a series of analogues of **1** (R = 2-methylheptyl) were synthesized by changing the chain length, and these analogues were tested against several bacterial and fungal test pathogens. The results have been presented in Table 1. It is evident from Table 1 that the length of the chain has indeed got a major effect on bioactivity. The analogue with a carbon chain length of 8, **1e** ($R = C_8H_{17}$) showed maximum activity with MICs in the 8 μg/mL against *M. tuberculosis*, 12 μg/mL against *E. coli*, 16 μg/mL against *B. subtilis* and 6 μg/mL against *P. vulgaris*. This was followed by **1b** ($R = C_5H_{11}$) a 5-carbon chain with MICs in the 10–20 μg/mL range and **1d** ($R = C_7H_{15}$) with MICs in the 15–24 μg/mL. Interestingly, **1a** ($R = C_4H_9$) and **1f** ($R = C_{18}H_{37}$) were found to be completely inactive; although **1c** ($R = C_6H_{13}$) showed certain extent of antibacterial activity in the 24–50 μg/mL range. The antifungal activity of **1e** ($R = C_8H_{17}$), **1b** ($R = C_5H_{11}$) and **1d** ($R = C_7H_{15}$) showed consistency in their antifungal potency with MFCs in the 10–50 μg/mL, whereas **1a** ($R = C_4H_9$) and **1f** ($R = C_{18}H_{37}$) were found to be inactive with MFCs in the 500–600 μg/mL range. The fact that analogue **1e** ($R = C_8H_{17}$) having MFCs value very close to that of the natural **1** (R = 2-methylheptyl) indicates that the methyl group at position 2 of the hydrocarbon chain does not have a marked effect on the antimicrobial activity of **1**. On the other hand it is

Table 1. Antimicrobial activity of 2-methylheptylisonicotinate and its derivatives

Acid esters tested	MIC (μg/mL)				MFC (μg/mL)	
	Bacillus subtilis	*Escherichia coli*	*Mycobacterium tuberculosis*	*Proteus vulgaris*	*Fusarium semitectum*	*Rhizoctonia solani*
1a, $R = C_4H_9$	600	500	280	500	500	600
1b, $R = C_5H_{11}$	20	20	10	14	10	14
1c, $R = C_6H_{13}$	50	35	24	36	44	44
1d, $R = C_7H_{15}$	24	16	16	16	15	15
1e, $R = C_8H_{17}$	16	12	8	6	16	18
1f, $R = C_{18}H_{37}$	600	56	320	560	500	600
(*R*)-(+)-**1**	16	30	10	6	24	24
(*S*)-(−)-**1**	20	40	14	14	30	32



Scheme 1. Reagents and conditions: (i) *n*-Bu_3SnH, AIBN, dry benzene, 80 °C, 6 h; (ii) *Candida rogusa* lipase, vinyl acetate/hexane, 12 h; (iii) K_2CO_3/MeOH, 1 h; (iv) Amberlyst A-21, dry CH_3CN, MW (40%, 5 min).

further observed that the MICs and MFCs of **1e** ($R = C_8H_{17}$) are better than that of the natural molecule, which indicates that the length of the chain plays a major role on the antimicrobial activity of these types of molecules. Another important observation is that the *R*-isomer of **1** is slightly more active than the corresponding *S*-isomer indicating that the methyl group in position 2 of the hydrocarbon chain has got some effect on the bioactivity.

4. Total synthesis of the individual isomers of 2-methylheptyl isonicotinate (*R*)-(+)-1 and (*S*)-(−)-1

The racemic alcohol 2-methylheptanol was prepared starting from 2-bromoheptane. Treatment of 2-bromoheptane with $NaNO_2$ in dimethylformamide yielded the 2-nitroheptane in 60% yield as a gum, which was subjected to formylation with 30% aqueous formaldehyde solution in the presence of K_2CO_3 at rt to give nitro alcohol **2** in 72% yield. Denitration of **2** was effected by refluxing a solution of **2** in dry benzene with *n*-tributyltinhydride in the presence of azobisisobutyronitrile to give (±)-**3** in 75% yield.[7] The individual *R*- and *S*-isomers of the resulting 2-methyl-1-heptanol, (±)-**3** were resolved by lipase catalyzed transesterification with lipase from *Candida rogusa* giving (*R*)-(+)-**3** in 49% yield having $[\alpha]_D^{20}$ +13.8 (*c* 0.8, $CHCl_3$) with ee 96% and the acetate (*S*)-(−)-**3** in 46% yield,[8] with $[\alpha]_D^{20}$ −12.4 (*c* 0.8, $CHCl_3$) and ee 97%. The (*S*)-(−)-**4**, with $[\alpha]_D^{20}$ −11.3 (*c* 0.7, $CHCl_3$) and ee 95% was generated by hydrolyzing acetate (*S*)-(−)-**3** with K_2CO_3 in methanol in an inert atmosphere.[8] Esterification of isonicotinoyl chloride hydrochloride with (*R*)-(+)-**3** yielded (*R*)-(+)-**1**[16] in 52% yield having $[\alpha]_D^{20}$ +8.3 (*c* 0.6, $CHCl_3$) with ee 94% and esterification of isonicotinoyl chloride hydrochloride with alcohol (*S*)-(−)-**4** gave (*S*)-(−)-**1**[16] in 40% yield, $[\alpha]_D^{20}$ −9.5 (*c* 0.8, $CHCl_3$) and ee 95%. Both (*R*)-(+)-**1** and gave (*S*)-(−)-**1** were separately subjected for bioassay against different test organisms. Compound (*R*)-(+)-**1** resembled both in bioactivity and sign of optical rotation [α] with that of natural 2-methylheptyl isonicotinate. Hence the absolute stereochemistry of the methyl group of natural 2-methylheptyl isoniconate has been assigned as '*R*'. The synthetic steps are depicted in Scheme 1.

The alarming spread of tuberculosis especially in developing and underdeveloped countries due to HIV infection has focused the attention on the need to understand the pathogenesis of this disease. In July 2003 report, the WHO has called TB, and not AIDS, India's biggest health concern.[9–11] The report says that over 4.5 million people in India suffer from TB annually, the highest number of cases reported in the world. It is also reported that the last two decades witnessed the frequencies and types of life threatening fungal infections that have increased dramatically among the immunocompromised patients.[13–15] Moreover, secondary fungal infections that appear towards the later part of medication, among the TB patients, aggravate the situation.[6] Hence the need for novel anti-TB drug with antifungal activity is highly felt. In view of this isoniazid analogue **1** isolated by us and the analogue **1e** $R = C_8H_{17}$ having both antifungal and antibacterial activity reported here hold lot of promise towards developing new drug to fight this menace.

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16. *Selected data*: (*R*)-(+)-**1**, oil, $[\alpha]_D^{20}$ +8.3 (*c* 0.6, $CHCl_3$). IR ($CHCl_3$): 2956, 2926, 2856, 1732, 1696, 1561, 1380 and 1060 cm^{-1}. ^1H NMR (300 MHz $CDCl_3$) δ ppm: 0.88 (t, $J = 6.15$ Hz, 3H), 0.93 (d, $J = 7$ Hz, 3H), 1.25–1.42 (br, 6H), 1.45 (m, 2H), 1.48 (m, 1H), 4.22 (d, $J = 6$ Hz, 2), 7.51 (dd, $J = 4$, 6 Hz, 2H), 7.91 (dd, $J = 4$, 6 Hz, 2H), MS (*m*/*z*): 258.1 (M^++Na). (*S*)-(−)-**1**, oil, $[\alpha]_D^{20}$ −9.5 (*c* 0.8, $CHCl_3$). IR ($CHCl_3$): 2958, 2930, 2861, 1737, 1690, 1566, 1377, 1238 and 1050 cm^{-1}. ^1H NMR (300 MHz $CDCl_3$) δ ppm: 0.85 (t, $J = 6.5$ Hz, 3H), 0.92 (d, $J = 7$ Hz, 3H), 1.25–1.43 (br, 6H), 1.44 (m, 2H), 1.48 (m, 1H), 4.22 (d, $J = 6$ Hz, 2H), 7.6 (dd, $J = 4$, 6 Hz, 2H), 8.0 (dd, $J = 4$, 6 Hz, 2H), MS (*m*/*z*): 258.0 (M^++Na).