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Short communication

Synthesis of isonicotinoylhydrazones from anacardic acid and their in vitro activity against *Mycobacterium smegmatis*

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Abstract

Isonicotinoylhydrazones were synthesized from a natural product anacardic acid, a major constituent of cashew nut shell liquid. The unsaturated side chain in anacardic acid and its 5-nitro derivative were converted into $C_{8'}$-aldehydes by oxidative cleavage. $C_{8'}$-aldehydes are then coupled with isoniazid (an anti-TB drug) to obtain *N*-isonicotinoyl-*N*′-8-[(2′-carbohydroxy-3′-hydroxy) phenyl] octanal hydrazone (**5**) and *N*-isonicotinoyl-*N*′-8-[(2′-carbohydroxy-3′-hydroxy-6-nitro) phenyl] octanal hydrazone (**6**). These isonicotinoylhydrazones of anacardic aldehydes showed potent antimycobacterial activity against *Mycobacterium smegmatis* mc^2155. The synergistic studies of **5** and **6** with isoniazid showed more inhibitory activities than isoniazid alone. Compounds **5** and **6** also showed activity against *Mycobacterium tuberculosis* $H_{37}Rv$.

Keywords: Anacardic acid; Cashew nut shell liquid; Isonicotinoylhydrazone; Isoniazid; *Mycobacterium smegmatis* mc^2155; *Mycobacterium tuberculosis* $H_{37}Rv$

1. Introduction

Worldwide spread of HIV infection, which results in weakening of the immune system of infected individuals, and the development of drug-resistant strains of *Mycobacterium tuberculosis*, have contributed to a significant TB increase in recent years [1–3]. In fact combination of two or more drugs is now being used in order to overcome the resistance caused by usage of a single drug [4]. However, the present anti-TB regimen is rather complex and lengthy. Hence there is a need to develop new drugs having short treatment regimen unlike older drugs. Various isonicotinoylhydrazones have been synthesized because of the development of isoniazid-resistant *M. tuberculosis* strains [5–8]. In our continuing efforts to make drug analogues [9–10] from a natural product anacardic acid, isolated from cashew nut shell liquid (CNSL), we have synthesized novel isonicotinoylhydrazones.

Anacardic acid (**1**) present in cashew nut shell liquid is a salicylic acid derivative with a non-isoprenoid alk(en)yl side chain (**1**, Fig. 1) [11] and has been implicated in a wide variety of biological activities [12–19]. However, the antimycobacterial activities of anacardic acid or its derivatives have not been reported. Antimycobacterial activity of isonicotinoylhydrazones (**5** and **6**) was tested against *Mycobacterium smegmatis* mc^2155 and their synergistic effects studied in combination with isoniazid (INH).

2. Results and discussion

The ene mixture of 5-nitroanacardic acid (**2**) has been prepared from ene mixture of anacardic acid (**1**) by treating it

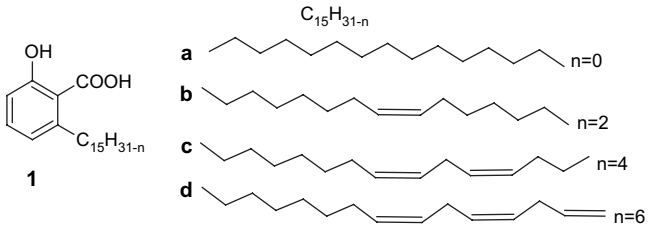

Fig. 1. Structure of anacardic acid.

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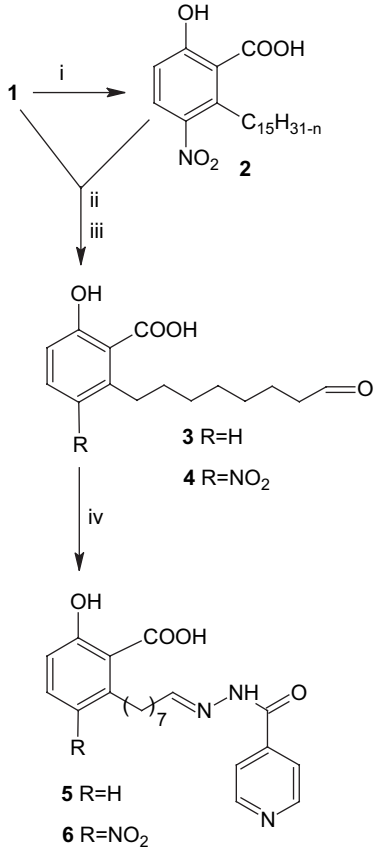
with HNO_3 in acetic acid. During nitration, the formation of 5-nitroanacardic acid was found to be the major with traces of 3-nitroanacardic acid. Compound **2** was purified by silica gel column chromatography. $C_{8'}$-aldehydes (**3** and **4**) have been prepared from **1** and **2**, respectively, by oxidative cleavage of their side chains [20,21]. These $C_{8'}$-aldehydes were coupled with INH to get isonicotinoylhydrazones **5** and **6** (Scheme 1) whose structures were established by ^{1}H NMR, ^{13}C NMR, and mass spectrometry.

The in vitro antimycobacterial activity of **5** and **6** was tested against *M. smegmatis* mc^{2}155 by broth dilution method and their minimum inhibitory concentration (MIC) values were determined. The results are shown in Table 1. Both the isonicotinoylhydrazones **5** and **6** have showed potent activity against this strain with MIC of 4 μg/ml and 5 μg/ml, respectively. These results indicate that isonicotinoylhydrazones derived from anacardic acid have shown activity against *M. smegmatis* while anacardic acid (**1**) and its nitro analogue (**2**) did not show any. This indication necessitates to make different hydrazone derivatives of anacardic acid, which is abundantly present in CNSL, a by-product of cashew nut industry and to study their activity.

Isonicotinoylhydrazones **5** and **6** were further evaluated for synergistic studies with INH. The synergistic studies were done for different concentrations of **5** and **6** in combination with 1 μg/ml and 0.5 μg/ml of INH. Simultaneously, inhibition was recorded for **5**, **6** and INH alone. The graphs were plotted



Scheme 1. Reagents: (i) HNO_3/CH_3COOH, 65 °C; (ii) HCO_3H, 40 °C, aq. HCOOH; (iii) $NaIO_4$; (iv) isoniazid, methanol, 80 °C.

Table 1
MIC values against *M. smegmatis* mc^{2}155

	Compound				
	1	**2**	**5**	**6**	INH
MIC (μg/ml)	Inactive[a]	Inactive[a]	4	5	1.5

[a] Inactive at 100 μg/ml.

with the values obtained in synergistic studies (Fig. 2 for **5** and Fig. 3 for **6**). A test using 1 part of INH (1 μg/ml) and 0.5 part of **5** (0.5 μg/ml) showed 93% inhibition (Fig. 2a) whereas INH alone at 1 μg/ml showed only 36% inhibition (Fig. 2d) and **5** alone at 0.5 μg/ml showed no inhibition (Fig. 2c) under similar experimental conditions. The inhibition was steadily increased with increasing concentration of **5** up to 1.5 μg/ml in combination with 1 μg/ml of INH. But further increasing concentration of **5** did not show any increase in % inhibition (see Fig. 2). In another combination, 0.5 μg/ml of INH and 0.5 μg/ml of **5** showed only 37% inhibition (Fig. 2b), and again further increase in the concentration of **5** did not show any significant increase in % inhibition.

Similarly, in the synergistic studies of **6** with INH; a test containing 1 part of INH (1 μg/ml) and 0.5 part of **6** (0.5 μg/ml) showed 59% inhibition (Fig. 3a), whereas **6** alone did not show any inhibition (Fig. 3c). And in another test when keeping INH concentration constant at 0.5 μg/ml and varying the concentration of **6** did not show significant increase in % inhibition (Fig. 3b).

Fractional inhibitory concentration (FIC) indices for compound **5** and INH and compound **6** and INH were determined [22]. FIC indices for **5** in combination with INH were found to be 0.4280 and 0.8090 and for **6** in combination with INH were found to be 0.4280 and 0.8804. In all the cases the FIC values were found to be <1 confirming the synergism. Further the *P* values were found to be significant ($P < 0.05$) corroborating the above finding. From the above synergistic studies, it can be concluded that compounds **5** and **6** work well in combination with INH. Particularly, **5** showed significant synergism with INH.

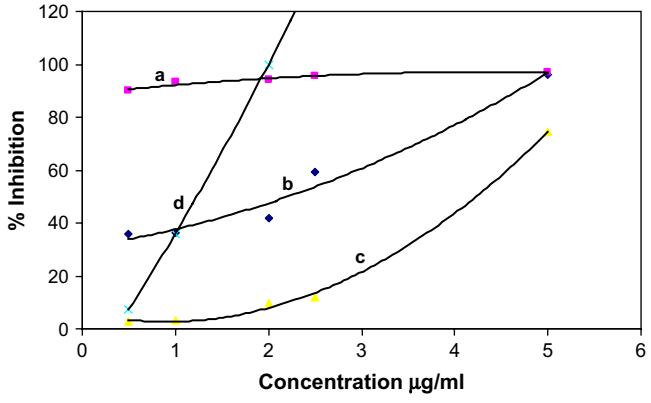


Fig. 2. Synergy between compound **5** and INH: (**a**) INH 1 μg/ml and varying concentrations of **5** (FIC = 0.4280); (**b**) INH 0.5 μg/ml and varying concentrations of **5** (FIC = 0.809); (**c**) only **5**; (**d**) only INH. The results of these experiments have been found to be significant. *$P < 0.05$.

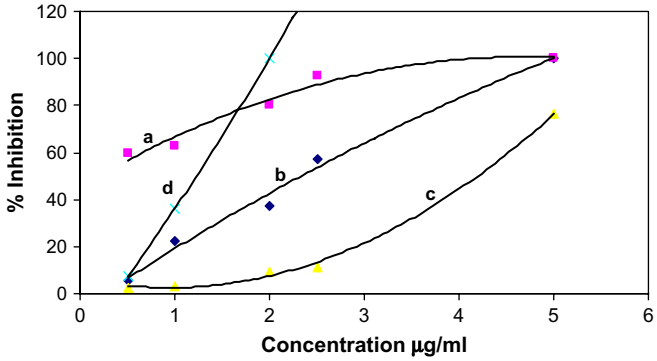


Fig. 3. Synergy between compound **6** and INH: (**a**) INH 1 µg/ml and varying concentrations of **6** (FIC = 0.4280); (**b**) INH 0.5 µg/ml and varying concentrations of **6** (FIC = 0.8804); (**c**) only **6**; (**d**) only INH. The results of these experiments have been found to be significant. $*P < 0.05$.

Compounds **5** and **6** were subjected to inhibitory activity against pathogenic strain *M. tuberculosis* $H_{37}Rv$. Both the compounds showed complete inhibition at 10 µg/ml concentration and no inhibition at 0.5 µg/ml, thereby indicating that MIC values should fall between these two values. However, the inhibitory activity of these compounds is less than INH (0.2 µg/ml).

3. Conclusions

In summary, novel isonicotinoylhydrazones have been synthesized from anacardic acid for the first time and characterized. We explored the antimycobacterial activity for these compounds against *M. smegmatis* mc^2155 and MIC values reported. Though compounds **5** and **6** showed lesser inhibitory activity compared to INH, they showed significant higher activity when treated synergistically with INH. Synergistic studies of compounds **5** and **6** using pathogenic strain *M. tuberculosis* $H_{37}Rv$ may be worth undertaking.

4. Experimental

4.1. General methods/instruments

Thin-layer chromatography (TLC) was performed on E. Merck AL silica gel 60 F_{254} plates and visualized under UV light. Column chromatography was conducted using E. Merck silica gel (100–200 mesh). ^1H NMR and ^{13}C NMR spectra were recorded in $CDCl_3$/DMSO-d_6 at 200 MHz on a Bruker AG spectrometer. All the chemical shift values are reported in δ units and the down field from tetramethylsilane as internal standard. IR spectra were recorded with Thermo Nicolet FT-IR (100) spectrometer. Mass spectra were recorded using GCMS-QP2010S (Direct probe) and on Q-TOF micro™ AMPS MAX 10/6A system. Melting points are uncorrected and were determined with a melting point apparatus (Acro Steels Pvt. Ltd.). Mycobacterial assays were performed using *M. smegmatis* mc^2155 by Youman–Karlson broth medium.

Anacardic acid **1** has been isolated from natural CNSL by an established procedure in our laboratory [23].

4.2. Preparation of 5-nitroanacardic acid (ene mix.) (**2**) [24]

Anacardic acid (ene mix.) **1** (10 g, 28 mmol) was dissolved in glacial acetic acid (60 ml) and kept stirring at 65 °C. To this, 70% nitric acid (2.86 g, 1.83 ml, 32 mmol) was added and stirring continued for 15 min. The reaction mixture was allowed to attain room temperature and then poured into 200 ml of ice water. The reaction mass was extracted with ethyl acetate (70 ml). The organic layer was washed with water, dried over anhydrous sodium sulfate and concentrated under reduced pressure to get the crude product (10 g). The product was purified by column chromatography over silica gel using hexane–ethyl acetate (9:1 v/v) as eluent to get a pale yellow colored viscous liquid (10.7 g). IR (KBr): 3340, 2926, 2855, 1705, 1600, 1520, 1467, 1270, 1220, 1130, 898, 834, 740 cm^{-1}.

4.3. Preparation of 8-[(2-carbohydroxy-3-hydroxy) phenyl] octanal (**3**)

To a stirred solution of freshly prepared performic acid (70 ml, by mixing 30 ml of hydrogen peroxide and 40 ml of formic acid at room temperature) at 40 °C, anacardic acid (ene mix.) **1** (20 g, 58 mmol) was added drop wise for 30 min by maintaining the temperature at <50 °C. The reaction mixture was allowed to attain room temperature and distilled water (20 ml) was added and stirring continued for another 10 min. The reaction mixture was poured into ice water and extracted with ethyl acetate (150 ml). Organic layer was washed with water, dried over anhydrous sodium sulfate and concentrated under reduced pressure to get a mixture of anacardic acid diols (25 g). This was dissolved in THF–water (75 ml, 1:1 v/v) and sodium metaperiodate (22 g, 103 mmol) and triethylbenzylammoniumchloride were added (500 mg, 2.2 mmol) and stirring continued for 30 min at room temperature. After completion of the reaction, the reaction solution was poured into 500 ml of water and extracted with ethyl acetate. The organic layer was washed with water, dried over anhydrous sodium sulfate and evaporated. The product was purified by column chromatography over silica gel using hexane–ethyl acetate (1.5:8.5 v/v) as eluent to get reddish viscous liquid (4.9 g). IR (KBr): 3390, 2930, 2856, 1720, 1658, 1606, 1451, 1298, 1244, 1212, 1167, 1119, 820 cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$): δ 1.33 (bs, 6H), 1.58 (m, 4H), 2.43 (t, 2H, $J = 6.6$ Hz), 2.92 (t, 2H, $J = 6.2$ Hz), 6.73 (d, 1H, $J = 7.2$ Hz), 6.84 (d, 1H, $J = 7.8$ Hz), 7.30 (dd, 1H, $J = 7.2$ and 7.8 Hz), 9.75 (bs, 1H,) ppm; GCMS (DI) (*m/z*): (% relative intensity) 264 (M^+ 35), 246 (37), 200 (40), 185 (42), 175 (34), 162 (28), 147 (100), 134 (72), 118 (34), 105 (75), 97 (22), 77 (46), 71 (39), 66 (32), 41 (74).

4.4. Preparation of 8-[(2-carbohydroxy-3-hydroxy-6-nitro) phenyl] octanal (**4**)

In a procedure similar to that for the synthesis of **3**, compound **2** (14 g, 36 mmol) was treated first with freshly

prepared performic acid (40 ml) and secondly with sodium metaperiodate (14 g, 65 mmol) and triethylbenzylammonium-chloride (250 mg, 1.1 mmol) to get reddish viscous liquid (3.45 g). IR (KBr): 3460, 2935, 2854, 1718, 1661, 1601, 1528, 1454, 1283, 1129, 751 cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$): δ 1.25 (bs, 6H), 1.56 (m, 4H), 2.34 (m, 2H), 2.89 (t, 2H, $J = 7.4$ Hz), 6.34 (d, 1H, $J = 8.2$ Hz), 6.82 (d, 1H, $J = 7.8$ Hz), 9.77 (bs, 1H) ppm.

4.5. Preparation of N-isonicotinoyl-N′-8-[(2′-carbohydroxy-3′-hydroxy) phenyl] octanal hydrazone (5)

Compound **3** (1 g, 3.78 mmol) was dissolved in methanol (5 ml) to which isoniazid (518 mg, 3.78 mmol) was added and heated to 70 °C for 1 h. The reaction mixture was poured into water and extracted with ethyl acetate. The organic layer was washed with water, dried over anhydrous sodium sulfate and evaporated. The product was purified by column chromatography over silica gel using ethyl acetate–methanol (9.5:0.5 v/v) as eluent to get white solid (0.52 g). M.p. 148–150 °C; IR (KBr): 3442, 3219, 2928, 1662, 1458, 1221, 1054, 839 cm^{-1}; ^1H NMR (200 MHz, DMSO-d_6): δ 1.3 (bs, 6H), 1.49 (m, 4H), 2.25 (m, 2H), 2.65 (t, 2H, $J = 7.2$ Hz), 3.33 (bs, 1H), 6.63 (d, 1H, $J = 6.6$ Hz), 6.66 (d, 1H, $J = 7.4$ Hz), 7.11 (dd, 1H, $J = 6.6$ Hz and 7.4 Hz), 7.75 (m, 3H), 8.74 (d, 2H, $J = 5.4$ Hz), 11.69 (s, 1H) ppm; ^{13}C NMR (200 MHz, DMSO-d_6): δ 26.80, 29.44, 29.78, 31.95, 32.85, 34.58, 114.49, 120.82, 121.14, 122.35, 131.29, 141.56, 142.96, 151.09, 154.80, 157.91, 162.09, 171.46 ppm; TOF-MS (*m/z*): Calcd for $C_{21}H_{25}N_3O_4$ $(M + Na)^+ = 406.1736$, found = 406.1716.

4.6. Preparation of N-isonicotinoyl-N′-8-[(2′-carbohydroxy-3′-hydroxy-6-nitro) phenyl] octanal hydrazone (6)

In a procedure similar to that for the preparation of **5**, compound **4** (1.5 g, 4.85 mmol) was coupled with INH (665 mg, 4.85 mmol) in methanol to get red colored semi-solid (0.54 g). IR (KBr): 3421, 3220, 2922, 1681, 1654, 1560, 1413, 1344, 1130, 1020, 928 cm^{-1}; ^1H NMR (200 MHz, DMSO-d_6): δ 1.31 (bs, 6H), 1.49 (m, 4H), 2.26 (q, 2H), 3.07 (d, 2H, $J = 7.4$ Hz), 6.32 (d, 1H, $J = 8.4$ Hz), 7.67 (dd, 1H, $J = 8.4$ Hz and 1.0 Hz), 7.77 (m, 3H), 8.74 (d, 2H, $J = 5$ Hz), 11.69 (s, 1H) ppm; ^{13}C NMR (200 MHz, DMSO-d_6): δ 21.91, 26.86, 29.58, 30.08, 31.92, 32.84, 36.01, 116.46, 122.35, 127.60, 128.67, 140.91, 141.54, 151.09, 154.29, 162.13, 170.28 ppm; TOF-MS (*m/z*): Calcd for $C_{21}H_{24}N_4O_6$ $(M + Na)^+ = 451.1587$, found = 451.1590.

4.7. Procedure for bioassay

MIC values for compounds **5** and **6** were determined against *M. smegmatis* mc^2155 cells which were grown to saturation in Youman–Karlson (YK) broth medium [25] at 37 °C. Antimycobacterial activity of the compounds was assayed by the broth dilution method. Stock solutions (10 μg/ml) of the compounds were prepared in methanol and serially diluted from 0.5 μg/ml to 5.0 μg/ml using YK culture broth with 0.5 ml as the final volume. To all the serially diluted solutions, 0.5 ml of suitably diluted inoculum was added (10^6 cfu) and incubated at 37 °C for 24 h. The lowest concentration of the compound that inhibited growth of the organism was taken as minimum inhibitory concentration.

For evaluating the inhibitory activity of compounds **5** and **6** in combination with INH, two sets of solutions with concentrations of 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5 and 5.0 μg/ml were prepared. To one set 0.5 μg/ml and to another set 1 μg/ml of INH was added. The treatments were incubated at 37 °C for 24 h and percentage inhibition was determined by measuring OD at 600 nm.

M. tuberculosis $H_{37}Rv$ cells were grown in Middle Brook (MB) 7H9 broth medium in an atmosphere of carbon dioxide, oxygen and nitrogen. Compounds **5** and **6** with known concentrations (10 μg/ml and 0.5 μg/ml) in DMSO were added to the MB/Bact process bottles containing 10 ml of broth and incubated at 37 °C up to 6 weeks. INH and DMSO were taken in separate bottles as controls. The sensor present in each bottle detected the presence of carbon dioxide which formed a basis for the measure of microbial growth.

4.8. Supplementary data

^1H NMR, ^{13}C NMR, mass spectra, details of determination of FIC and *P* values of **5** and **6** are available free of charge via the Internet at http://www.sciencedirect.org.

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