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ScienceDirect
Bioorganic & Medicinal Chemistry 15 (2007) 3579–3586
Bioorganic & Medicinal Chemistry

Inhibition of mycobacterial arylamine *N*-acetyltransferase contributes to anti-mycobacterial activity of *Warburgia salutaris*

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Received 18 December 2006; revised 2 February 2007; accepted 8 February 2007
Available online 11 February 2007

Abstract—In this study, we show that extracts and a purified compound of *Warburgia salutaris* exhibit anti-mycobacterial activity against *Mycobacterium tuberculosis* H37Rv and *Mycobacterium bovis* BCG Pasteur. The extracts did not inhibit growth of *Escherichia coli* and were not toxic to cultured mammalian macrophage cells at the concentrations at which anti-mycobacterial activity was observed. The extract and pure compound inhibited pure recombinant arylamine *N*-acetyltransferase (NAT), an enzyme involved in mycobacterial cell wall lipid synthesis. Moreover, neither extract nor pure compound inhibited growth of a strain of *M. bovis* BCG in which *nat* has been deleted suggesting that NAT may indeed be a target within the mycobacterial cell. The purified compound is a novel drimane sesquiterpenoid lactone, 11α-hydroxycinnamosmolide. These studies show that *W. salutaris* is a useful source of anti-tubercular compounds for further analysis and supports the hypothesis of a link between NAT inhibition and anti-mycobacterial activity.

1. Introduction

The global emergence of drug resistance in tuberculosis has exacerbated the healthcare burden resulting from one-third of the global population being infected and more than two million associated deaths per year.[1] Although tuberculosis is treatable, few alternative drugs are available in cases where drug resistance is a problem. Currently available second line drugs such as *p*-aminosalicylate, kanamycin, ethionamide and fluoroquinolones are either less effective or toxic.[2] There is a great need for the development of novel agents that target pathways in the tubercle bacilli that have never been challenged for TB chemotherapy in order to avoid cross-resistance with existing drugs.

When compared to *Escherichia coli*, the genome of the causative agent of tuberculosis, *Mycobacterium tuberculosis*, encodes for 200 more enzymes involved in lipid metabolism[3] suggesting the importance of these pathways in this organism. One recently identified enzyme that is required for cell wall lipid biosynthesis is the arylamine *N*-acetyltransferase,[4] NAT. The interest in NAT was fuelled by the realization of its involvement in the polymorphic inactivation of isoniazid in humans.[5,6] Variants of NAT were found in *Mycobacterium bovis* BCG and *M. tuberculosis*[7] having 60% amino acid sequence identity to the human NAT2 isozyme.[8] *M. tuberculosis* NAT was initially investigated as a possible factor contributing to isoniazid resistance in mycobacteria.[6,7,9] Interestingly, knockout studies in which *nat* was deleted from *M. bovis* BCG resulted in altered cell wall morphology, absence of several cell wall lipids, slow growth and increased susceptibility to intracellular killing of the bacilli by macrophages.[4] The *nat* gene is encoded as the last gene in an operon and other enzymes translated from this operon also appear to be

Keywords: *Warburgia salutaris*; Tuberculosis; *N*-acetyltransferase; Terpenoids.
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doi:10.1016/j.bmc.2007.02.011

involved in lipid metabolism.[10] An inhibitor of one of these enzymes induces a phenotype in treated *M. bovis* BCG similar to that of the *nat* knockout strain, *M. bovis* BCG Δ*nat*.[10] Synthetic NAT inhibitors have been shown to also inhibit the growth of mycobacteria and elicit a phenotype in *M. bovis* BCG similar to that of the knockout strain (unpublished Data). Parallel to these studies, the 3-D structures of related NATs from *Salmonella typhimurium*,[11] *Mycobacterium smegmatis*,[8] *Pseudomonas aeruginosa*,[12] and *Mesorhizobium loti*[13] have been solved by X-ray crystallography. Novel hit compounds that could elicit a phenotype in *M. bovis* BCG similar to that of the *nat* knockout strains are also being sought from natural products.

Natural products isolated mostly from terrestrial and marine plants have played a major role in the discovery of drugs against infectious diseases; almost 75% of approved anti-infective drugs are derived from natural products.[14] Approximately 10% of the world's terrestrial plants, some being used medicinally, are found in South Africa.[15] However, few of these plants have been investigated for anti-TB activity, yet TB is one of South Africa's biggest healthcare problems. One of these plants is *Warburgia salutaris* (Bertol. F.) Chiov (Canellaceae), commonly used traditionally for the treatment of colds, coughs, flu, fever,[16] chest infections productive of purulent sputum,[17] and as an emetic to clear patches in the lungs.[18] This flowering tree came into our attention during a field survey of South African medicinal plants used by traditional healers to treat conditions or symptoms associated with respiratory ailments. *W. salutaris* grows in the Mpumalanga Province of South Africa.[15] There are two other known plants that belong to the genus *Warburgia*; *Warburgia stuhlmanii* Engl. and *Warburgia ugandensis* Sprague;[15] endemic in East Africa.

In this paper, we report on the investigation of extracts of *W. salutaris* and their effect on the growth of mycobacteria and on the ultra-structural cell morphology. Furthermore, from these active extracts we isolated and characterized a novel anti-mycobacterial compound. We have also investigated the effect of these extracts and purified compound on the activity of pure recombinant mycobacterial NAT.

2. Results

2.1. Effect of *W. salutaris* extracts on bacterial growth and cell morphology

Initially the crude extract of *W. salutaris* was tested at a concentration of 200 μg/mL to determine its effect on the growth of *M. tuberculosis* $H_{37}Rv$ using the radiometric Bactec 460 TB system. The crude extract inhibited growth of *M. tuberculosis* $H_{37}Rv$ completely over the 11 days test period (data not shown). As expected, growth of *M. tuberculosis* $H_{37}Rv$ was completely inhibited when treated with isoniazid at 0.05 μg/mL (MIC) over the same time period (data not shown). *M. bovis* BCG growth was also inhibited by the crude extract in a concentration dependent manner (Fig. 1a). The highest concentration tested (100 μg/mL) inhibited *M. bovis* BCG growth completely (Fig. 1a) over the 10 days test period. In contrast, treatment of *E. coli* with crude extract of *W. salutaris* at 100 μg/mL did not affect the growth; the growth pattern of treated cells was indistinguishable from that of untreated control cells (Fig. 1b). Interestingly, the intermediate concentration (50 μg/mL) tested on the growth of *M. bovis* BCG (Fig. 1a) resulted in the extension of the lag phase of growth. This extension of the lag phase of growth has been observed previously when *nat* is deleted in *M. bovis* BCG.[4]

A partially purified component of the crude extract of *W. salutaris*, Fr 30, when tested against the growth of *M. bovis* BCG on Middlebrook agar at concentrations

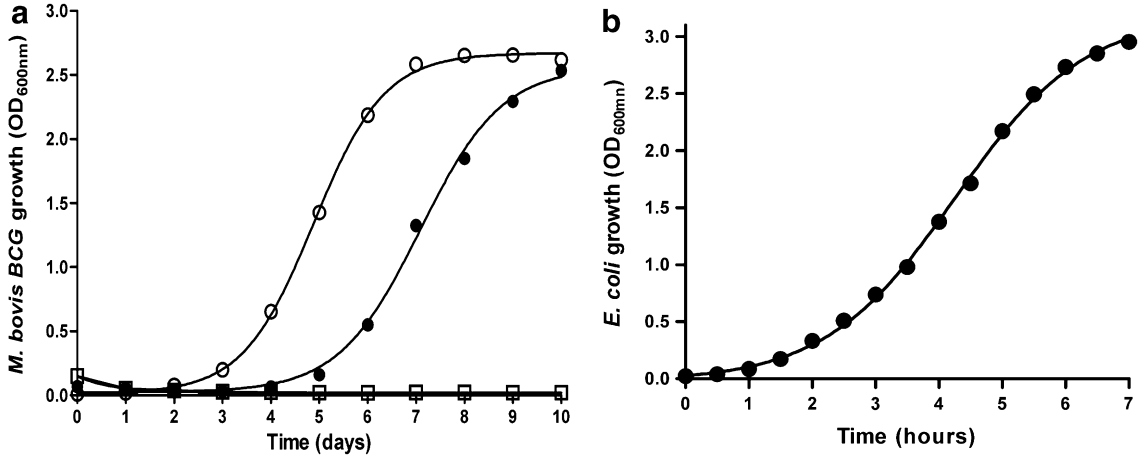

Figure 1. Effects of *Warburgia salutaris* extracts on the growth of *M. bovis* BCG and *E. coli*. (a) *M. bovis* BCG grown at 37 °C (with shaking at 2 rpm) in Middlebrook 7H9 broth supplemented with 10% ADC (albumin, dextrose, catalase) in the presence of crude extract of *W. salutaris* at 100 μg/mL (□) and 50 μg/mL (●), respectively, while broth with solvent alone was added in the controls (○). Optical density values measured at 600 nm are plotted on the *y*-axis *versus* time (days) on the *x*-axis. (b) *E. coli* cultures grown at 37 °C (with shaking at 180 rpm) in LB broth in presence of crude extract of *W. salutaris* (100 μg/mL) or LB broth with solvent alone (controls). Optical density readings measured at 600 nm are plotted on the *y*-axis versus time (h) on the *x*-axis. Both growth curves are superimposed and only one is shown for clarity.

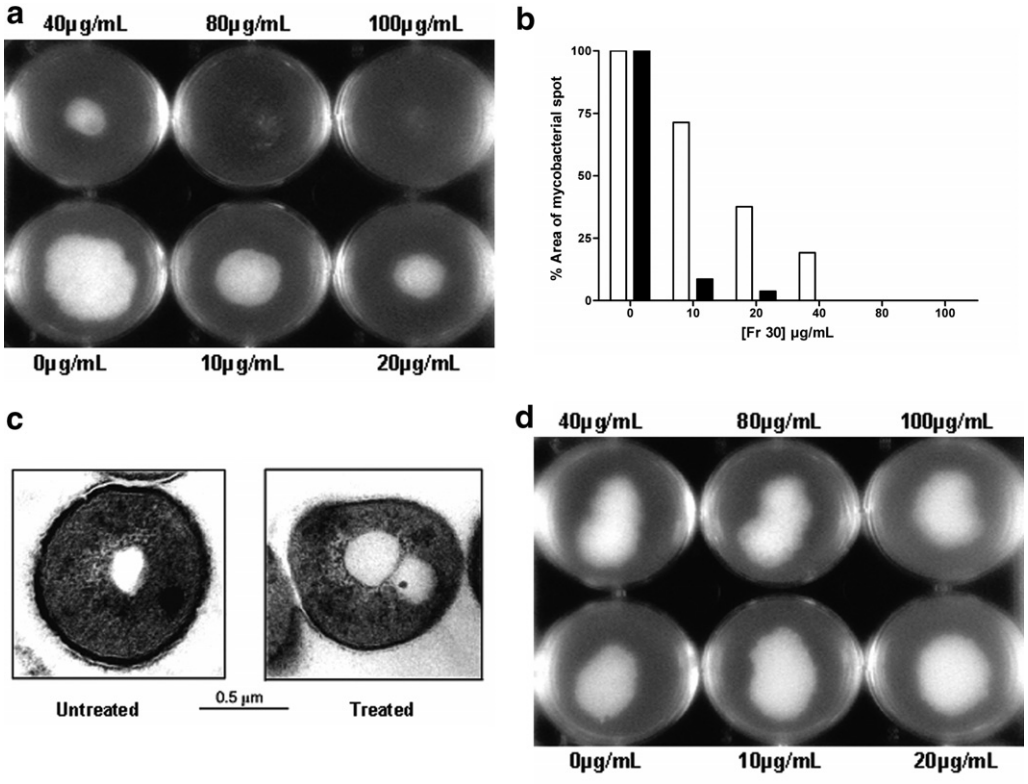

Figure 2. Effect of partially purified extract (Fr 30) of *Warburgia salutaris* on the growth of mycobacteria. (a) Approximately 500 *M. bovis* BCG cells were spotted on solidified Middlebrook 7H10 agar containing different concentrations of Fr 30 (10, 20, 40, 80 and 100 µg/mL) in a 6-well plate. Pictures of cultures that grew as spots were taken after 21 days of incubation at 37 °C using a Bio-Rad Gel-Doc 2000 system. (b) *M. tuberculosis* H37Rv was grown in the presence of similar concentrations of Fr 30 as in (a) above. A comparison of effects of Fr 30 on *M. bovis* BCG (open bars) versus *M. tuberculosis* H37Rv (closed bars) is illustrated on the bar graph. (c) *M. bovis* BCG grown at 37 °C (with shaking at 2 rpm) in Middlebrook 7H9 broth supplemented with 10% ADC (albumin, dextrose, catalase) in the presence of crude extract of *W. salutaris* (50 µg/mL, treated) or broth with solvent alone (untreated) before the morphology of the cells was analysed by transmission electron microscopy as previously described (10). (d) *M. bovis* Δ*nat* (4) was also treated with different concentrations of Fr 30 over a 21-day period exactly as described in (a) above before photographs were taken.

ranging between 10 and 100 µg/mL, also inhibited the growth in a dose-dependent manner (Fig. 2a). Preliminary investigation showed that Fr 30 exhibited increased antimycobacterial activity when tested against *M. tuberculosis* H37Rv growth on middlebrook agar over a similar concentration range (Fig. 2b). Furthermore, treatment of *M. bovis* BCG with Fr 30 (50 µg/mL) resulted in an impaired outer cell wall structure as shown by TEM (Fig. 2c). Fr 30 had little effect on the growth of the *nat* knockout strain of *M. bovis* BCG (Fig. 2d).

2.2. Effect of *W. salutaris* extracts on recombinant NAT activity

NAT catalyses the hydrolysis of AcCoA in the presence of an arylamine or hydrazine substrate resulting in production of free thiol, CoA, which can be measured spectroscophotometrically.[19] Pure recombinant NAT was prepared for the enzymatic assays and isoniazid was used as a substrate. The initial velocity of the enzymatic activity was measured in the linear range over 10 min. Both the crude extract of *W. salutaris* and Fr 30 inhibited the acetylation reaction. The crude extract was added to the final concentrations 0.2, 0.5 and 1.0 mg/mL versus five different substrate concentrations ranging between 0.2 and 1 mM prior to the addition of NAT in the reaction vessel, resulting in reduction of reaction rates compared to controls without crude extract (Fig. 3a). The Lineweaver–Burk plots indicated that the inhibition observed with both the crude extract (Fig. 3b) and Fr 30 (data not shown) appeared to be competitive in nature.

2.3. Isolation, purification and characterization of a novel compound

Fr 30 was subjected to further purification using HPLC. A range of individual peaks were identified in Fr 30 based on their absorbance at 220 nm. The compounds that eluted at the beginning of the chromatogram could not be sufficiently separated for preparative-scale isolation, resulting in mixtures of compounds in too low yields for further purification, characterization or biological testing. Two major analytes, designated compounds **1** and **2**, were successfully isolated using preparative HPLC.

Whilst the peak containing compound **2** appeared to be a single peak in both analytical and preparative HPLC chromatograms, when subjected to LC-MS, the chromatogram showed it gave a multitude of peaks (Fig. S1B). The ^{1}H NMR and high-resolution mass

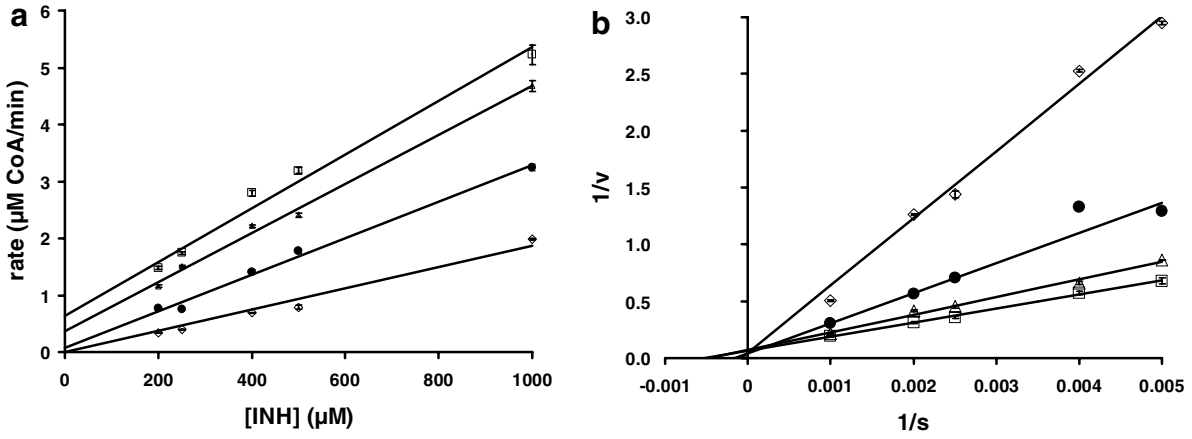

Figure 3. Effect of extracts of *Warburgia salutaris* on NAT activity. (a) Pure recombinant NAT from *M. smegmatis* (1 µg/100 µL) was incubated at 37 °C for 10 min with substrate, isoniazid (200, 250, 400, 500 and 1000 µM), in the presence of different concentrations of crude extract [0.0 (□), 0.2 (△), 0.5 (●) and 1.0 mg/mL (◊)]. The reaction was started by addition of acetylCoA (400 µM) and stopped with a solution containing DTNB before measurement of optical densities at 405 nm and determination of reaction rate. The reaction rates are plotted on the *y*-axis versus substrate concentrations on the *x*-axis. (b) The Lineweaver–Burk plot shows reciprocals of reaction rates (min/µM) plotted versus reciprocals of substrate concentrations (l/µM).

spectroscopy data for this peak gave a profile identical to that expected of the already known drimane sesquiterpenoid dialdehyde, cinnamodial[20] (Fig. 4a). Thus, these results suggest that the compound has been degraded by the LC-MS procedure.

Compound **1** was found to be a single pure species by mass spectrometry (Fig. S1A). To identify the chemical structure of compound **1**, infrared spectroscopy, mass spectrometry and extensive nuclear magnetic resonance spectroscopy were used (see Supplementary data, including Figs. S2–S10). The successful identification of compound **1** was also supported by data of related compounds from *W. salutaris*[21] and other Canellaceae plants from which sesquiterpenoids have been isolated, such as *Cinnamosma fragans* Baillon.[22,23] High-resolution mass spectrometry together with 1D and 2D NMR spectra enabled the identification of compound **1** as the novel drimane sesquiterpene, 11α-hydroxycinnamosmolide (Fig. 4b and c). Complete unambiguous proton and carbon assignments are reported in the Section 5 and structure elucidation is discussed in the Supplementary material.

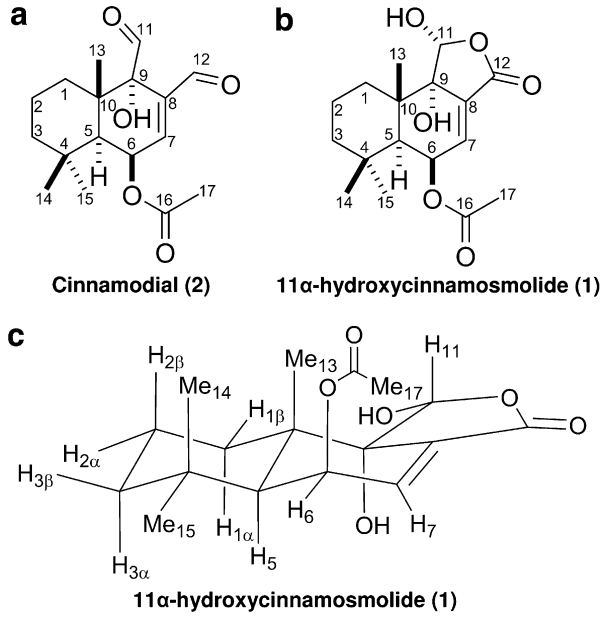

Figure 4. (a) Depicts the absolute and relative stereochemistry of compound **2**, which has been identified as cinnamodial. (b) and (c) show the relative stereochemistry of compound **1** isolated from Fr 30.

Compound **1** inhibited the growth of *M. bovis* BCG wild-type in a dose-dependent fashion (Fig. 5a), while it had little effect of the growth of *M. bovis* BCG *nat* deleted mutant (Fig. 5b). Compound **1** also inhibited recombinant NAT activity but gave 35% inhibition of acetylation at 0.5 mg/mL compared to 55% inhibition by Fr 30 at the same concentration (data not shown).

2.4. Cytotoxicity of *W. salutaris* extracts

To prove that *W. salutaris* extracts possess genuine anti-mycobacterial activity and that they are not toxic to mammalian cells, we tested the extracts against the mouse macrophage cell line RAW 264.7 at the lowest anti-mycobacterial concentration (50 µg/mL). There was no significant difference in the number of cells that grew in the presence of either DMSO, INH (a known anti-tubercular drug), crude extract or partially purified extract (Fr 30) from which compound **1** was isolated (Fig. 6). These results show that the extracts of *W. salutaris* are not toxic to a mammalian cell line at the concentrations at which anti-mycobacterial activity was observed.

3. Discussion

We have demonstrated that extracts of the bark of *W. salutaris* exhibited two important biological activities. The extracts inhibited the growth of mycobacteria

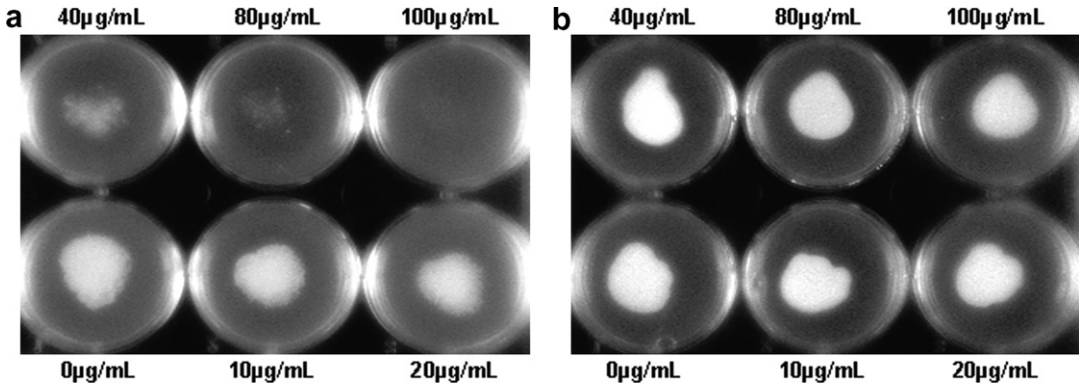

Figure 5. Effects of novel compound **1** on *M. bovis* BCG growth. Approximately 500 *M. bovis* BCG wild-type (a) and *M. bovis* Δ*nat* (b) cells were spotted on solidified Middlebrook 7H10 agar containing different concentrations of compound **1** (10, 20, 40, 80 and 100 µg/mL) in 6-well plates. Pictures of cultures that grew as spots were taken after 21 days of incubation at 37 °C using a Bio-Rad Gel-Doc 2000 system.

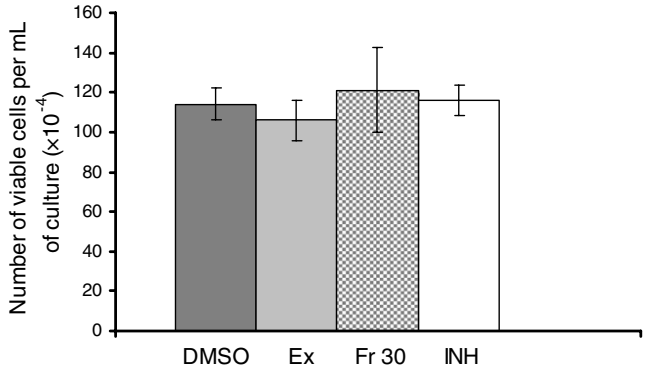

Figure 6. Effect of crude extract and Fr 30 of *Warburgia salutaris* on the viability of mammalian cells. Mouse macrophage cell line RAW 264.7 was grown for 48 h in RPMI 1640 medium either in presence of 0.1% DMSO, 10 µg/mL INH, 50 µg/mL crude extract (Ex) or 50 µg/mL Fr 30. An inverted phase contrast microscope (Olympus, Tokyo, Japan) was used at 40× magnification to count the RAW cells. Viable RAW cells were determined by Trypan blue exclusion method out of total number of cells counted per field on a haemocytometer and expressed as growth/mL shown on the *y*-axis. Results (±SD) are shown as the average of four determinations where at least 200 individual cells were counted in each case.

and also inhibited recombinant mycobacterial NAT. The partially purified component that contained most of these two activities, Fr 30, inhibited mycobacterial growth in a dose-dependent fashion but did not inhibit the *nat* knockout strain of *M. bovis* BCG, suggesting that NAT may be the target within mycobacterial cells. Furthermore, Fr 30 altered the outer cell wall structure of *M. bovis* BCG, a phenomenon that was observed with the *nat* knockout strain. Preliminary investigations of the effects of *W. salutaris* extracts and other NAT inhibitors on the synthesis of cell wall lipids suggested that these agents may act at a range of different lipid synthetic steps.

One of the major components in Fr 30, compound **1**, is a novel drimane sesquiterpenoid lactone (11α-hydroxycinnamosmolide). Though less potent, compared to Fr 30, compound **1** exhibited significant dose-dependent inhibition of *M. bovis* BCG growth and recombinant NAT activity inhibition. Just like Fr 30, compound **1** had little effect on the *nat* knockout strain of *M. bovis* BCG suggesting that NAT may be a target within mycobacterial cells. Terpenoids are abundant lipophilic secondary metabolites that originate from mevalonate and isopentenyl pyrophosphate.[24] They have a range of potential therapeutic activities including anti-fungal, anti-bacterial, and anti-cancer.[24] Novel anti-mycobacterial coloratane sesquiterpenoids have been isolated from a dichloromethane extract of the stem bark of *Warburgia ugandensis*.[25] Sesquiterpenoid lactones of the germacranolide, guaianolide and eudesmanolide are known to be anti-tubercular with MICs ranging from 2 to 128 µg/mL.[26] Structure–activity analysis revealed that the presence of an exocyclic α-methylene-γ-lactone ring, as it is in dehydrocostuslactone (MIC = 2 µg/mL), is crucial for potent anti-tubercular activity.[26] As a result of low yields of these *W. salutaris* natural products (compound **1**, 0.015% w/w) and the environmental issues related to the extinction of this plant in the wild, a chemical synthesis programme has been started to further investigate the relationship between NAT inhibitory and anti-tubercular activities of *Warburgia* compounds and their cytotoxicity against mammalian cells.

We have shown that extracts of *W. salutaris* are not toxic to a mammalian cell line, meaning that they are safe at the concentrations at which they showed anti-mycobacterial activity. They can thus be investigated further to establish their in vivo activity against *M. tuberculosis* with minimal risk to experimental animals. Due to low yields of compound **1**, cytotoxicity of this novel compound against this mammalian cell line could not be established. However, one could argue based on the above-mentioned result that compound **1** is also not toxic to mammalian cells.

The ongoing identification of the other compounds present in Fr 30 using a hyphenated HPLC-UV-SPE-NMR/MS technique will be reported on in due course. The structural knowledge of the other components in Fr 30 is intended to guide future organic syntheses, to ensure that the chemical diversity that this extract possesses is recorded and exploited for future structure–activity relationship studies.

4. Conclusions

Warburgia salutaris contains anti-mycobacterial sesquiterpenoids including a novel drimane sesquiterpenoid lactone, 11α-hydroxycinnamosmolide, that seems to act by inhibiting arylamine *N*-acetyltransferase. Extracts of *W. salutaris* need to be investigated further against drug-sensitive and drug-resistant strains of the aetiological agent of tuberculosis, *M. tuberculosis*, as a valuable source of novel antitubercular drugs. Since *W. salutaris* is going extinct in the wild, further work on this plant might have medicinal and environmental importance.

5. Experimental

5.1. Collection and preparation of plant material

The bark of *W. salutaris* was harvested at the Silver Glen Nursery in Durban, June 2002, just after the flowering season. A voucher specimen has been deposited at the South African National Botanical Institute's herbarium in Durban under the name N. Crouch 947, NH. The bark was dried in air away from sunlight for 7 days before it was ground into a fine powder (1 kg). A dichloromethane extract was prepared from the ground powder. The dichloromethane was removed by rotary evaporation at 40 °C followed by drying to completion under inert nitrogen gas resulting in 10 g of dried crude extract.

5.2. Test for anti-bacterial activity

5.2.1. *M. bovis* BCG Pasteur. Mid-log phase (OD 1.0) cultures of *M. bovis* BCG Pasteur were diluted 100-fold in 100 mL of Middlebrook 7H9 broth (MB broth) supplemented with 10 mL of aseptically added ADC (Albumin, Dextrose, Catalase; Difco) enrichment. Test agents were added to the diluted cultures before incubation at 37 °C with constant mixing (two revolutions per minute) for 10 days. Optical density readings were taken every 24 h at a wavelength of 600 nm using a Hitachi U-2001 spectrophotometer.

5.2.2. *E. coli* JM 109. A 100-fold dilution of mid-log phase cultures (OD 1.9) of *E. coli* JM 109 was prepared in 100 mL of Luria–Bertani (LB) broth and incubated with constant agitation (180 revolutions per minute) at 37 °C for a period of 7 h after test agents were added. Optical density measurements were taken at a wavelength of 600 nm every hour using a Hitachi U-2001 spectrophotometer.

Alternatively, test agents were tested on the growth of mycobacteria using the spot culture method on Middlebrook 7H10 agar (MB agar) as described in the following text.

5.3. MB agar spot culture method for testing of anti-mycobacterial activity

Extracts of *W. salutaris* and purified compound were prepared in DMSO at a range of concentrations up to 100 mg/mL. The stock solutions (5 μL) were added into different wells in 6-well plates with 5 μL DMSO alone added to control wells (0.1% v/v). Molten MB agar (5 mL) containing OADC (Oleic acid–Albumin–Dextrose–Catalase, Difco) was poured immediately into wells of the 6-well plates containing the extracts with thorough mixing. Once solidified, MB agar containing test agents was inoculated with 5 μL of a 10^5 dilution of a mid-log phase culture (OD 1.0) of *M. bovis* BCG Pasteur or *M. tuberculosis* H37Rv, containing approximately 500 cells. The cells were allowed to soak onto MB agar before plates were covered, sealed with parafilm, inverted and incubated at 37 °C for 21–28 days. The resulting circular spot cultures were photographed using a BioRad Gel-Doc 2000 system.

5.4. Investigation of effects of extracts on cell morphology

Mycobacterium bovis BCG cultures (OD 1.0), treated and untreated with test agents, were harvested by centrifugation (2000 revolutions per minute, 4 °C, 10 min) and subjected to transmission electron microscopy as described previously.[27]

5.5. Expression and enzymatic assay for pure recombinant NAT protein

The open-reading frame of the gene for *M. smegmatis* *N*-acetyltransferase (MSNAT) plus an *N*-terminal hexa-histidine tag was expressed in *E. coli* BL21(DE3)-*pLysS*[7] and purified using Ni–NTA agarose.[11] The hexa-his-tag was removed by thrombin cleavage[28] and the purity of the recombinant protein was confirmed by SDS–PAGE. An MSNAT enzymatic assay used to identify ligands and inhibitors has been developed. [19] It exploits the NAT catalysed hydrolysis of acetylCoA in the presence of substrate (isoniazid in this case) to produce free CoA. Experimental conditions were adjusted such that the reaction rates were in the linear range over the time course used to determine the rate. Pure recombinant MSNAT (1 μg/100 μL reaction volume) was incubated at 37 °C with isoniazid for 10 min in the presence or absence of different concentrations of extracts. The reaction was started at 37 °C by addition of acetylCoA as described previously.[19] The amount of acetylCoA hydrolysed was determined as the complex between the released thiol and 5,5-dithiobis(2-nitrobenzoic acid) [DTNB] by measurement of optical densities at 405 nm using a microplate reader (Anthos 2020) after the addition of DTNB.[19]

5.6. Purification of *W. salutaris* extract

The crude dichloromethane extract of *W. salutaris* bark (2 g) was dissolved in 100 mL of 100% acetonitrile before it was filtered through a Whatman 0.45 μm filter paper to exclude insoluble waxy material. Double distilled water (400 mL) was added to yield a milky solution. Fractionation of 50 mL of this solution was carried out on pre-conditioned endcapped isolute C-18 reversed phase columns (70 mL, 10 mg silica bed embedded). The solution was allowed to drain through the column at a flow rate of 1 mL/min to release unbound material, Fr 20. Bound material was eluted at the same flow rate with

increasing concentrations of HPLC grade acetonitrile in water, in the ratios of 30:70, 60:40 and 100:0 yielding 3 fractions Fr 30, Fr 60 and Fr 100, respectively. Analysis and purification of Fr 30 was carried out at room temperature by analytical [150 × 4.6 mm C_{18} Waters XTerra column (5 μm, 100 Å)] and preparative [150 × 19 mm C_{18} Waters XTerra column (5 μm, 122 Å)] HPLC, respectively, using Gilson 306 pumps, a Gilson 881C dynamic mixer, a Gilson 806 manometric module with automated sample injection with a Gilson 215 liquid handler, configured with a Gilson 819 valve actuator. A stepwise gradient starting from 40% to 100% acetonitrile in water, 0.1% trifluoroacetic acid (v/v) was employed using a flow rate of 1 mL/min for analytical and 15 mL/min for preparative HPLC, respectively. The chromatograms were monitored at 220, 254 and 280 nm using a Gilson 170 diode array detector.

5.7. Cytotoxicity testing

Mouse macrophage cell line RAW 264.7 was grown for 48 h in RPMI 1640 medium either in presence of 0.1% DMSO alone or in presence of antitubercular extracts of *W. salutaris* dissolved in 0.1% DMSO or isoniazid. Viable RAW cells were determined by Trypan blue exclusion method out of total number of cells counted per field.

5.8. Structural determination

The purified compound was characterized by high-resolution mass spectrometry, specific rotation, IR spectrometry, UV spectrometry and ^{1}H, ^{13}C, HSQC, COSY, HMBC and NOESY NMR experiments. The ^{1}H NMR experiments were recorded in methanol-d_4 on a Bruker DRX 500 MHz instrument, while the ^{13}C NMR on a Bruker 62.5 MHz instrument. Optical rotation was measured on a Perkin-Elmer 241 polarimeter, using a path length of 10 cm. The infrared spectrum was recorded as a KBr disc using a Bruker Tensor 27 FT-IR spectrophotometer. The UV spectrum was recorded using a Perkin-Elmer Lambda 25 UV/vis spectrometer. Low-resolution mass spectra (*m*/*z*) were recorded on either a VG Masslab 20-250 instrument (CI^+, NH_3) or a VG Autospec instrument (CI^+, NH_3). Accurate mass GCToF measurements (CI^+) were run on a Micromass GCT instrument fitted with a Scientific Glass Instruments BPX5 column (15 m × 0.25 mm), using amyl acetate as a lock mass or on a Waters 2790-Micromass LCT electrospray ionisation mass spectrometer. Full experimental details can be found in the Supplementary information.

5.8.1. Structural data for compound 1. $[\alpha]_{20}^{D} = -308$ 10^{-1} degcm^{-1} g^{-1} ($c = 0.306$, acetonitrile); ^{1}H NMR (500 MHz, d_4-MeOH): δ 6.67 (d, $J = 2.5$, 1H, H-7), 5.84 (br s, 1H, H-11), 5.77 (t, $J = 4.5$), 1H, H-6), 2.11 (d, $J = 4.0$, 1H, H-5), 2.08 (s, 1H, H-17), 2.03 (m, 1H, H-1α), 1.71 (m, 1H, H-2β), 1.52 (m, 1H, H-2α), 1.43 (d, $J = 12.5$, 1H, H-3β), 1.30 (d, $J = 12.5$, 1H, H-3α), 1.35 (m, 1H, H-1β), 1.20 (s, 3H, 14-CH_3), 1.19 (s, 3H, 13-CH_3), 1.02 (s, 3H, 15-CH_3); ^{13}C NMR (62.5 MHz, d_4-MeOH): 171.8 (C-16), 169.2 (C-12), 135.3 (C-8), 134.9 (C-7), 100.5 (C-11), 77.0 (C-9), 67.7 (C-6), 46.7 (C-5), 45.9 (C-3), 40.2 (C-10), 34.7 (C-4), 33.5 (C-15), 33.1 (C-1), 25.1 (C-14), 21.5 (C-17), 19.9 (C-13), 19.1 (C-2); IR (KBr): 1746 cm^{-1} (Lactonic carbonyl); UV/vis: λ_{max} 213 nm; HRMS (*m*/*z*): $[M+NH_4]^+$ calcd for $C_{17}H_{24}O_6$, 342.1916; found, 342.19.14.

Acknowledgments

Our greatest appreciation goes to Traditional Doctor George Rhesha of Queenstown, South Africa, for supplying information of medicinal usage of *W. salutaris*, to Dr. Neil Crouch of the South African National Botanical Institute for supplying an authenticated specimen for research, to Dr Neil Oldham of the MS unit at Oxford University Chemistry Research Laboratory for running the LC-MS analysis of compounds **1** and **2**. My highest thanks go to Dr. Cailean Clarkson for her assistance in the proof reading and editing of this paper. We thank the South African Medical Research Council, Lord Sainsbury of Candover on behalf of The Sainsbury/Linbury Trust and Oxford Isis Innovation for financial support.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2007.02.011.

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