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Synthesis and antimycobacterial activity of bornylamine derived amido-alcohols

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Dedicated to Acad. Dimiter Ivanov on the occasion of his 120th birth anniversary

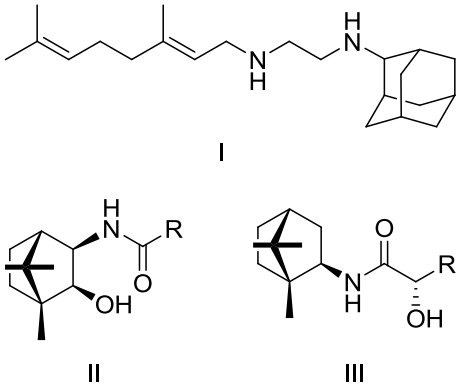
Three novel amido-alcohols were synthesized on the base of the camphane scaffold. Natural amino acids were transformed into their α-hydroxy analogues with retention of configuration, and attached to bornylamine. The compounds were evaluated for their in vitro activity against *Mycobacterium tuberculosis* H37Rv. The activity shifts from micromolar to nanomolar inhibitory concentrations depending on the α-hydroxy acid moiety. The valine derived compound demonstrates activity 25 times higher than the referent ethambutol. The amido-alcohols with camphane scaffolds emerge as promising new class of antimycobacterial agents.

Key words: camphane, α-hydroxy acids, *M. tuberculosis* H37Rv, cytotoxicity

INTRODUCTION

Tuberculosis (TB) still remains a growing problem in the context of diagnosis and treatment of multidrug-resistant TB [1]. The unacceptable large number of TB deaths necessitates the search for new antimycobacterial agents with novel structures and mode of action. SQ 109 (Fig. 1. **I**), emerged as capable second line drug with promising antimycobacterial potencies and pharmacokinetic properties [2]. It is very likely that its highly lipophilic adamantane structure helps the penetration into the bacterial wall and thus is decisive for the activity [3].

Inspired by the analogy of the camphane scaffold as compact lipophilic moiety to the adamantyl fragment in SQ 109, we have previously studied camphor derived structures as novel antimycobacterial agents [4,5]. A practical synthesis of a small number of new amido-alcohols and amido-diols was accomplished on the base of 3-*exo*-aminoisoborneol (Fig. 1. **II**) [4]. These were screened for antimycobacterial activity against two MTB strains (H37Rv and MDR strain 43) and some of the compounds show activity much higher than the referent ethambutol. Additionally, we expanded the study towards amido-alcohols derived from isobornylamine and α-hydroxy acids (Fig. 1. **III**) [5]. Thereby, we had the opportunity to investigate how the difference in the camphor derived fragment influences the activity. The latter shifted from micromolar to nanomolar inhibitory concentrations depending on the α-hydroxy acid moiety. Noteworthy, two of the structures possess very high activity in combination with low levels of cytotoxicity.



I II III

Fig. 1. Structures of SQ109 (**I**), 3-*exo*-aminoisoborneol (**II**) and isobornylamine (**III**) based amido-alcohols.

Encouraged by these observations, we expanded the approach to the synthesis of bornylamine analogues where the asymmetric center neighboring the nitrogen in the targeted structures is with the opposite (*S*)-configuration compared to the isobornylamine and 3-*exo*-aminoisoborneol structures. This afforded the chance to investigate the role of chirality on the structure-activity relationship.

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Thus, we pursue the exploration of the camphane based structures as novel class of anti-TB compounds.

EXPERIMENTAL

General

Reagents were commercial grade and used without further purification. Thin layer chromatography (TLC) was performed on aluminum sheets pre-coated with Merck Kieselgel 60 F_{254} 0.25 mm (Merck). Flash column chromatography was carried out using Silica Gel 60 230-400 mesh (Fluka). Commercially available solvents for reactions, TLC and column chromatography were used after distillation (and were dried when needed). Melting points of the compounds were determined using "Electrothermal" MEL-TEMP apparatus (uncorrected). Optical rotations ($[\alpha]_D^{20}$) were measured on Perkin–Elmer 241 polarimeter. The NMR spectra were recorded on a Bruker Avance II+ 600 spectrometer (600.13 MHz for ^{1}H, 150.92 MHz for ^{13}C NMR) in $CDCl_3$ with TMS as internal standards for chemical shifts (δ, ppm). ^{1}H and ^{13}C NMR data are reported as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, br = broad, m = multiplet), coupling constants (Hz), integration, identification. The assignment of the ^{1}H and ^{13}C NMR spectra was made on the basis of DEPT, COSY, HSQC, HMBC and NOESY experiments. Elemental analyses were performed by Microanalytical Service Laboratory of Faculty of Pharmacy, Medical University of Sofia, using Vario EL3 CHNS(O). Dimethyl sulfoxide (DMSO) for testing of bioactivities was commercial (spectroscopic grade) and was used without distillation.

*General Procedure for the Preparation of the Amides (**4a**-**c**).*

1-Hydroxybenzotriazole (HOBt) (1.1 equiv) and the respective α-hydroxy acid (1 equiv) were suspended in dichloromethane, and the mixture was stirred for 5 min. Then, *N*-[3-(dimethylamino)-propyl]-*N*-ethylcarbodiimide (EDC) (1.1 equiv) was added, followed by bornylamine (1 equiv). Stirring was continued at room temperature until the starting material was completely consumed (TLC). The mixture was quenched with water, extracted with CH_2Cl_2, washed with 2M HCl, sat. aq. $NaHCO_3$ and brine. The organic phase was dried over Na_2SO_4, and concentrated under vacuum. The residue was purified by flash column chromatography on silica gel.

(S)-2-Hydroxy-2-phenyl-N-((1R,2S,4R)-1,7,7-trimethylbicyclo[2.2.1]heptan-2-yl)acetamide ***4a***. Yield: 54%; waxy solid. $[\alpha]^{20}_D$ = +41.3 (c 0.492, $CHCl_3$). ^{1}H NMR 7.41-7.38 (m, 4H, arom.), 7.35-7.33 (m, 1H, arom.), 5.99 (d, $J_{H,H}$ = 8.8 Hz, 1H, NH), 5.01 (d, $J_{H,H}$ = 2.6 Hz, 1H, *CH*OH), 4.23-4.18 (m, 1H, 2-H$_{exo}$), 3.90 (d, $J_{H,H}$ = 3.2 Hz, 1H, OH), 2.33-2.28 (m, 1H, 3-H$_{exo}$), 1.73-1.68 (m, 1H, 5-H$_{exo}$), 1.64 (t, $J_{H,H}$ = 4.5 Hz, 1H, 4-H), 1.27-1.21 (m, 1H, 5-H$_{endo}$), 1.10-1.05 (m, 2H, 6-H$_{exo}$, 6-H$_{endo}$), 0.91 (s, 3H, 8-H), 0.83 (s, 3H, 9-H), 0.76 (dd, $J_{H,H}$ = 13.4, 4.5 Hz, 1H, 3-H$_{endo}$), 0.69 (s, 3H, 10-H) ppm. ^{13}C NMR 172.16 (CO), 139.83 (1 arom. C), 128.87 (2 arom. CH), 128.62 (1 arom. CH), 126.73 (2 arom. CH), 73.98 (*CH*OH), 53.92 (2-C), 49.64 (1-C), 48.09 (7-C), 44.77 (4-C), 37.33 (3-C), 28.21 (6-C), 27.48 (5-C), 19.68 (9-C), 18.55 (8-C), 13.48 (10-C) ppm. $C_{18}H_{22}NO_2$ (287.40): calcd. C 75.22, H 8.77, N 4.87, found C 75.18, H 8.56, N 5.12.

(2S,3S)-2-Hydroxy-3-methyl-N-((1R,2S,4R)-1,7,7-trimethylbicyclo[2.2.1]heptan-2-yl)pentanamide ***4b***. Yield: 60%; white crystals; m.p. 97-101°C. $[\alpha]^{20}_D$ = -20.5 (c 0.538, $CHCl_3$). ^{1}H NMR 6.44 (d, $J_{H,H}$ = 8.8 Hz, 1H, NH), 4.28-4.23 (m, 1H, 2-H$_{exo}$), 4.02 (dd, $J_{H,H}$ = 5.2, 3.3 Hz, 1H, *CH*OH), 2.96 (d, $J_{H,H}$ = 5.3 Hz, 1H, OH), 2.39-2.33 (m, 1H, 3-H$_{exo}$), 1.90-1.83 (m, 1H, CH_3*CH*), 1.81-1.75 (m, 1H, 5-H$_{exo}$), 1.68 (t, $J_{H,H}$ = 4.5 Hz, 1H, 4-H), 1.46-1.51 (m, 1H, 6-H$_{exo}$), 1.44-1.37 (m, 2H, 6-H$_{endo}$, CH_3*CH*$_2$), 1.24-1.17 (m, 2H, 5-H$_{endo}$, CH_3*CH*$_2$), 1.01 (d, $J_{H,H}$ = 7.0 Hz, 3H, *CH*$_3$CH), 0.96 (s, 3H, 8-H), 0.90 (t, $J_{H,H}$ = 7.5 Hz, 3H, *CH*$_3$CH$_2$), 0.88 (s, 3H, 9-H), 0.82 (s, 3H, 10-H), 0.81 (dd, $J_{H,H}$ = 13.3, 4.5 Hz, 1H, 3-H$_{endo}$) ppm. ^{13}C NMR 173.08 (CO), 76.20 (*CH*OH), 53.42 (2-C), 49.44 (1-C), 48.13 (7-C), 44.85 (4-C), 39.06 (CH_3*CH*), 37.57 (3-C), 28.32 (5-C), 27.94 (6-C), 23.08 (CH_3*CH*$_2$), 19.78 (9-C), 18.61 (8-C), 15.63 (*CH*$_3$CH), 13.67 (10-C), 11.91 (*CH*$_3$CH$_2$) ppm. $C_{16}H_{29}NO_2$ (267.41): calcd. C 71.86, H 10.93, N 5.24, found C 71.93, H 10.64, N 5.58.

(S)-2-Hydroxy-3-methyl-N-((1R,2S,4R)-1,7,7-trimethylbicyclo[2.2.1]heptan-2-yl)butanamide ***4c***. Yield: 48%; white crystals; m.p. 88-91°C. $[\alpha]^{20}_D$ = -25.2 (c 0.445, $CHCl_3$) ^{1}H NMR 6.44 (d, $J_{H,H}$ = 6.4 Hz, 1H, NH), 4.26-4.22 (m, 1H, 2-H$_{exo}$), 3.97 (dd, $J_{H,H}$ = 5.2, 3.1 Hz, 1H, *CH*OH), 3.05 (brs, 1H, OH), 2.38-2.35 (m, 1H, 3-H$_{endo}$), 2.15-2.10 (m, 1H, $(CH_3)_2$*CH*), 1.80-1.74 (m, 1H, 5-H$_{exo}$), 1.67 (t, $J_{H,H}$ = 4.6 Hz, 1H, 4-H), 1.46-1.50 (m, 1H, 6-H$_{endo}$), 1.41-1.36 (m, 1H, 6-H$_{exo}$), 1.20-1.16 (m, 1H, 5-H$_{endo}$), 1.03 (d, $J_{H,H}$ = 6.9 Hz, 3H, (*CH*$_3$)$_2$CH), 0.94 (s, 3H, 8-H), 0.87 (s, 3H, 9-H), 0.86 (d, $J_{H,H}$ = 6.9 Hz, 3H,

(CH_3)$_2$CH), 0.81 (s, 3H, 10-H), 0.80 (dd, $J_{H,H}$ = 13.4, 4.6 Hz, 1H, 3-H$_{endo}$) ppm. ^{13}C NMR 173.22 (CO), 76.13 (*CH*OH), 53.46 (2-C), 49.47 (1-C), 48.18 (7-C), 44.89 (4-C), 37.63 (3-C), 32.18 ((CH$_3$)$_2$*CH*), 28.36 (5-C), 28.00 (6-C), 19.82 (9-C), 19.23 ((CH_3)$_2$CH), 18.66 (8-C), 15.44 ((CH_3)$_2$CH), 13.72 (10-C) ppm. $C_{15}H_{27}NO_2$ (253.38): calcd. C 71.10, H 10.74, N 5.53, found C 71.44, H 10.97, N 5.63.

Antimycobacterial activity

The antimycobacterial activity was determined through the proportional method of Canetti towards reference strain *M. Tuberculosis* H37Rv and multi-drug resistant strain 43 (resistant to Rifampin and Isoniazid), recovered from Bulgarian adult HIV-negative pulmonary TB patient, who was permanent resident of the country. This method, recommended by the WHO, is the most commonly used one worldwide for exploration of sensitivity/ resistance of tuberculosis strains towards chemotherapeutics [6-10]. It allows precise determination of the proportion of resistant mutants to a certain drug.

A sterile suspension/solution of each tested compound was added to Löwenstein-Jensen egg based medium before its coagulation (30 min at 85°C). Each compound was tested at five concentrations – 5 mg/ml, 2 mg/ml, 0.2 mg/ml, 0.1 mg/ml and 0.05 mg/ml in DMSO. Tubes with Löwenstein-Jensen medium (5 ml) containing tested compounds and those without them (controls) were inoculated with 0.2 ml suspension of *M. tuberculosis* H37Rv (10^5 cells/ml) and incubated for 45 days at 37°C. The ratio between the number of colonies of *M. tuberculosis* grown in medium containing compounds and the number of colonies in control medium were calculated and expressed as percentage of inhibition. The MIC is defined as the minimum concentration of compound required to inhibit bacterial growth completely (0% growth). The MIC values are calculated and given as μM.

Cytotoxicity

The human embryonal kidney cell line 293T cells were obtained from the German Collection of Microorganisms and Cell Cultures. Cells were kept in controlled environment e RPMI-1640 medium, supplemented with 10% heat-inactivated fetal calf serum and 2mM L-glutamine, at 37ºC in a 'Heraeus' incubator with 5% CO_2 humidified atmosphere.

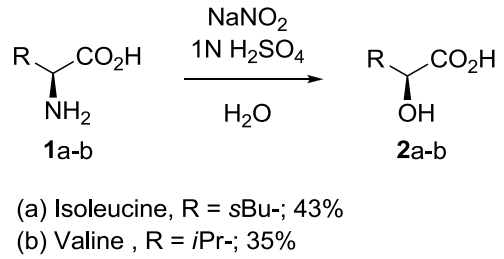
The cytotoxicity of the newly synthesized compounds was assessed using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]-dye reduction assay as described by Mossman with some modifications [11,12]. In brief, exponentially growing cells were seeded in 96-well microplates (100 μl/well) at a density of 3.5 - 105 cell/ml and allowed to grow for 24 h prior the exposure to the studied compounds. Stock solutions of the tested compounds were freshly prepared in DMSO and thereafter were subset to serial dilutions with growth medium in order to obtain the desired final concentrations. At the final dilutions the solvent concentration never exceeded 0.5%. Cells were exposed to the tested agents for 72 h, whereby for each concentration a set of at least 8 separate wells was used. After the exposure period MTT solution (10 mg/ml in phosphate-buffered saline) aliquots (100 μl/well) were added to each well. The plates were further incubated for 4 h at 37ºC and the MTT-formazan crystals formed were dissolved through addition of 110 ml of 5% HCOOH in 2-propanol. The MTT-formazan absorption of the samples was measured by a multimode microplate reader DTX 880 (Beckman Coulter) at 580 nm. Cell survival fractions were calculated as percentage of the untreated control. The experi-mental data were fitted to sigmoidal concentration-response curves and the correspond-ding IC_{50} values (concentrations causing 50% reduction of cellular survival *vs* the untreated control) via non-linear regression (GraphPad Prizm software for PC).

RESULTS AND DISCUSSION

Chemistry

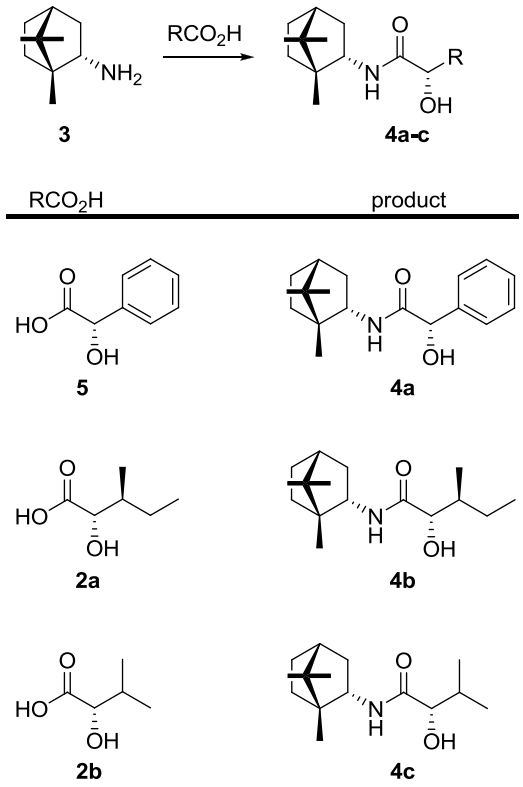
Since peptides and peptide-related structures have a wide variety of physiological and pharmacological actions, the concept of peptidomimetics was aimed by the design of α-amino acids derived amido-alcohols. A series of chiral α-hydroxy acids as starting building blocks was prepared from their corresponding natural amino acid analogues. Ingold first developed the transformation of L-phenylalanine to its α-hydroxy acid analogue with retention of configuration [13]. The reaction proceeded via initial deamination followed by nucleophilic attack of the neighbouring carboxyl group to heterocyclic intermediate and final nucleophilic attack of water. The product had the same configuration due to two consecutive Walden inversions. Applying the procedure to isoleucine **1a**

and valine **1b** afforded the corresponding α-hydroxy analogues **2a** and **2b** (Scheme 1).



Scheme 1. Synthesis of α-hydroxy acids.

Readily available bornylamine **3** was selected as the camphane starting compound [14]. The targeted structures were aimed by simple and effective synthetic transformations (Scheme 2).



Scheme 2. Synthesis of bornylamine based amido-alcohols.

The amide linkage between the α-hydroxy acids and bornylamine **3** was accomplished by procedures developed for peptide synthesis. The reaction was optimized for the commercially available mandelic acid **5** in the presence of *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDCI) and 1-hydroxybenzotriazole hydrate (HOBT) as coupling reagents to yield **4a**. Following the same protocol for α-hydroxy acids **2a-b** we synthesized the corresponding amido-alcohols **4b-c** (Scheme 2). The products were obtained in good yields and excellent purity after flash column chromatography.

All compounds were identified by elementary analysis, ^{1}H NMR and ^{13}C NMR. The spectral analyses were in accordance with the assigned structures.

Antimycobacterial activity

The synthesized compounds were evaluated for their in vitro activity against *M. tuberculosis* H37Rv using the method of Canetti (Table 1). The mandelic acid derived amido-alcohol **4a** exhibited activity against *M. tuberculosis* H37Rv with MIC of 6.96 μM, which is comparable with the one of the reference compound ethambutol. In the case of the isoleucine derived amido-alcohol **4b** the activity dropped to MIC of 18.70 μM. The best result was observed with the valine derived amido-alcohol **4c**, which exhibited MIC of 0.20 μM (Table 1). Interestingly, a slight variation of the alkyl side chain, a switch of the isobutyl group to isopropyl, greatly increased the activity.

No correlation could be assigned between the configuration of the stereogenic center at the nitrogen and the activity. In both cases: compounds with (*R*)-configuration derived from 3-*exo*-aminoisoborneol [4] and isobornylamine [5], and compounds with (*S*)-configuration derived from bornylamine; we detected either nanomolar or micromolar activities. Therefore, SAR dependence on the chirality in camphane based structures should be handled with care.

Cytotoxicity

The cytotoxicity of the presented compounds was assessed against the human embryonal kidney cell line 293T in order to examine the selectivity of the antiproliferative effects. Evident from the IC_{50} values (Table 1), the compounds were characterized with low **4a** to moderate **4b**, **4c** cytotoxicity against the human cells. The results are in favour of structure **4c**. The latter exhibits high antimycobacterial activity and moderate cytotoxicity, which is represented by its excellent selectivity index: 217.15. Considering that compounds **4b** and **4c** are practically equi-toxic against human cells while displaying prominent selective inhibition of H37RV it could be concluded that they modulate a distinct target peculiar for mycobacteria.

Table 1. In vitro screening data for antimycobacterial activity and cytotoxicity of the synthesized compounds.

Compound	Anti-MTB[a] MIC (μM)	Cytotoxicity[b] IC_{50} (μM)	SI[c] IC_{50}/MIC
4a	6.96	124.40	17.87
4b	18.70	43.86	2.34
4c	0.20	43.43	217.15
ETM.2HCl[d]	7.20	-	-

[a]Antimycobacterial activity towards reference strain of *Mycobacterium tuberculosis* H37Rv; [b]In vitro cytotoxicity towards human embryonal kidney cell line 293T; [c]Selectivity index; [d]EMB.2HCl – ethambutol dihydrochloride (reference compound).

The analogy between the bulky lipophilic camphane fragment and the adamantyl fragment in the molecule of SQ109, a drug currently in clinical trials for the treatment of tuberculosis, made us speculate that they share the same target [3]. Further docking studies are due in a short time.

CONCLUSION

In summary, three new amido-alcohols were synthesized by condensation of bornylamine with α-hydroxy acids. The compounds were screened for their antimycobacterial activity against *M. tuberculosis* H37Rv. The activity shifts from micromolar to nanomolar inhibitory concentrations depending on the α-hydroxy acid moiety. The valine derived compound shows 25 times higher activity than the classical anti-TB drug ethambutol and moderate level of cytotoxic activity against a human embryonal kidney cell line 293T. The mandelic acid derived amido-alcohol demonstrates activity comparable with ethambutol and low level of cytotoxicity. Further evaluation of camphane based structures as potential anti-TB agents is in progress and will be reported in due lines.

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СИНТЕЗ И АНТИМИКОБАКТЕРИАЛНА АКТИВНОСТ НА АМИДОАЛКОХОЛИ ПРОИЗВОДНИ НА БОРНИЛАМИН

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(Резюме)

Синтезирани са три нови амидо-алкохоли с камфанов скелет. Природни амино-киселини бяха трансформирани в техните α-хидрокси аналози със запазване на конфигурацията и в последствие кондензирани с борниламин. Веществата бяха изследвани за тяхната *in vitro* активност срещу щама *Mycobacterium tuberculosis* H37Rv. Активността варира от микромоларни до наномоларни инхибиращи концентрации в зависимост от α-хидрокси киселинния остатък. Валиновото производно показва активност 25 пъти по-висока от референта етамбутол. Амидоалкохолите с камфанов скелет се проявяват като обещаващ нов клас антимикобактериални агенти.