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Original article

2,3-Dideoxy hex-2-enopyranosid-4-uloses as promising new anti-tubercular agents: Design, synthesis, biological evaluation and SAR studies

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ABSTRACT

The alarming resurgence of tuberculosis (TB) underlines the urgent need for development of new and potent anti-TB drugs. Towards this goal we herein report the design and synthesis of 2,3-dideoxy hex-2-enopyranosid-4-uloses as promising new anti-tubercular agents. These easily accessible, small molecules were found to exhibit *in vitro* activity against *Mycobacterium tuberculosis* H37Rv in a MIC range of 0.78 μg/mL to 25 μg/mL. A detailed SAR study on these hex-2-enopyranosid-4-uloses led to the identification of compound **5g** (**S007**–**724**) which on the basis of low MIC (0.78 μg/mL-*M. tuberculosis* H37Rv; 1.56 μg/mL-MDR, SDR strains of *M. tuberculosis*; 0.78 μg/mL-inhibition of intracellular replication of *M. tuberculosis*) and SI value of 13.5 has been identified as a promising lead molecule.

1. Introduction

Tuberculosis (TB), a chronic bacterial infection is a leading cause of mortality worldwide from a single infectious agent [1–3]. The emergence of multi-drug resistant TB (MDR-TB) and of late extensively drug resistant TB (XDR-TB) along with the problem of co-infection with HIV has made the threat from TB very serious raising the spectre that TB may become incurable once again [4–6]. The presently used anti-TB drugs were introduced more than 40 years ago [7] when the problem of XDR-TB, MDR-TB and co-infection with HIV did not exist. Thus, to effectively combat the menace of TB in its present manifestation there is an urgent need for development of new anti-TB agents that have unique mechanisms of action from presently used drugs with improved properties such as enhanced activity against MDR and XDR strains, effectiveness against latent TB, shortened duration of therapy, reduced toxicity and rapid mycobactericidal mechanism of action [5,7,8]. This paradigm shift in chemotherapeutic strategies has drawn attention towards the pathogens' outer cell wall and membranes as an attractive target for exploring new drugs [9,10]. Mycobacterial cell wall possesses in its structure complex polysaccharides lipoarabinomannan and arabinogalactan [11,12]. The inhibition of the glycosyltransferases involved in their biosynthesis could lead to the disturbance of the cell wall biosynthesis [13,14]. Sugar-derived molecules are considered good candidates for this type of drugs which are thought to act by interfering with the cell wall biosynthesis of *Mycobacterium tuberculosis* [13–19].

Deoxysugars, a distinct class of carbohydrates has received special attention during the past decades due to their unique properties [20]. Moreover, current efforts are directed mainly towards the development of small molecules as drugs which are in many ways more attractive than large molecules such as antibodies and proteins. They are more stable, more active, can be easily formulated, have a broader array of dosage forms, have greater potential for bio-availability and their synthesis can be easily developed

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[21,22]. Since TB is primarily a disease of the poor [1] so cost of treatment is a major issue in the eradication of TB [23]. Thus smaller the drug molecule, easier would be its synthesis and the lesser its cost. A major stumbling block in the development of carbohydrate based drugs has been their rapid breakdown in the bloodstream by glycosidases before they can show their therapeutic effect. This pharmacokinetic problem can be overcome by using modified non-natural sugars which may not be recognized by the body's normal complement of glycosidases [24,25]. In light of the above discussion we have been focusing our efforts towards the synthesis of modified deoxy sugar based anti-tubercular agents [18,19,26–28]. In continuation of our efforts we herein report the synthesis of a new series of 2,3-dideoxy hex-2-enopyranosid-4-uloses and their derivatives, which are easily accessible small molecules exhibiting better activity profile and lesser toxicity than our previously reported anti-tubercular agents [19].

2. Chemistry

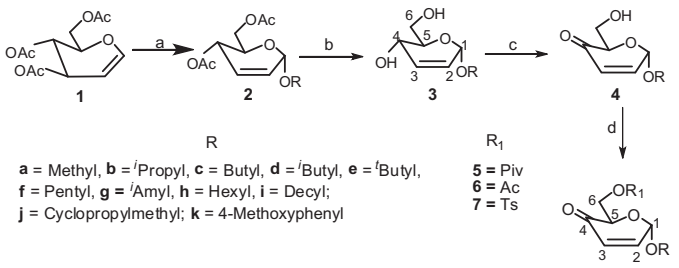
The synthesis were initiated with 3,4,6-tri-*O*-acetyl-D-glucal **1**, easily obtained from D-glucose. Compound **1** was subjected to Ferrier rearrangement [29] with different alcohols in the presence of iodine to furnish 2,3-dideoxy glucopyranoside derivative **2**. It was deacetylated in the presence of sodium methoxide to obtain dihydroxy derivative **3** followed by allylic oxidation with MnO_2 to obtain hydroxyl enuloside **4**. Pivaloyl, acetyl and tosyl protection of C-6 hydroxy group in **4** yielded C-6 protected enulosides **5**, **6** and **7** respectively (Scheme 1) in good yields [30,31].

In order to study the structure activity relationship of compounds **5**, **6** and **7,** their C-3 alkylaryl branched derivatives **5am**, **5dm**, **5gm**, **5go**, **5im**, **6gn**, **6go**, **6in and 7bn** were synthesized by our earlier reported synthetic protocol involving a highly diastereoselective Morita–Baylis–Hillman reaction in the presence of $TiCl_4$/TBAI in DCM at −78 °C to −30 °C (Scheme 2) [30,31].

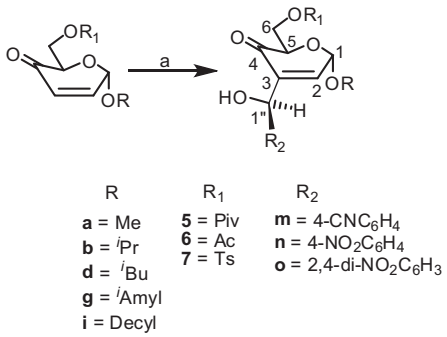
Further the enulosides **5b** and **5g** (**S007-724**) were treated with 37% aqueous soln. of HCHO in THF in the presence of DMAP to obtain their C-3 hydroxymethyl branched enulosides **8** and **9** (Scheme 3) [31].

3. Biological evaluation

All the compounds were evaluated for their *in vitro* anti-tubercular activity by BACTEC radiometric method using *M. tuberculosis* H37Rv as test strain for direct determination of the minimum inhibitory concentration (MIC). The assay grows *M. tuberculosis* in 7H12 medium containing ^{14}C labeled palmitic acid as substrate and $^{14}CO_2$ is produced. The amount of $^{14}CO_2$ detected reflects the rate and amount of growth occurring in the vial and is expressed in term of the "Growth Index" (GI). On addition of an anti-tubercular drug to the medium, suppression of growth of the test organism



Scheme 1. Reagents and conditions: (a) ROH, THF, I_2, 3 h-5 h, N_2 atm., RT; (b) NaOMe, MeOH, 1 h-2 h, RT; (c) MnO_2, $CHCl_3$, 20 h-40 h, RT; (d) $(CH_3)_3CCOCl$/$(CH_3CO)_2O$/TsCl, Pyridine/DCM, 6 h-48 h, −30 °C–5 °C.



Scheme 2. Reagents and conditions: (a) $TiCl_4$, TBAI, R_2CHO, DCM, 5 h–8 h, −78 °C → −30 °C.

M. tuberculosis could be detected by either a decline or very small increase of the daily GI output as compared to the control. Streptomycin (6 μg/mL) and Rifampin (2 μg/mL) were used as positive control. The MIC of compounds ranged from 0.78 μg/mL to 25 μg/mL.

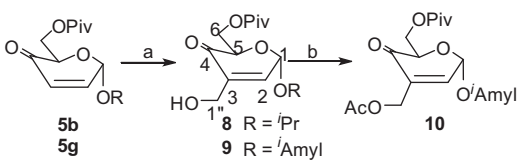
4. Result and discussion

4.1. Structure activity relationship

The present work was designed on the basis of the reports of Georgiadis et al. that substituted-2*H*-pyran-3(6*H*)-ones, which are structurally very similar to 2,3-dideoxy hex-2-enopyranosid-4-uloses **I** (Fig. 1), exhibit activity against gram positive bacteria [32,33] as well as our previous report on the anti-tubercular activity of C-3-alkyl and -alkylaryl 2,3-dideoxy hex-2-enopyranosides [19]. The above reports led us to the idea that simple 2,3-dideoxy hex-2-enopyranosid-4-uloses **I** may also exhibit anti-tubercular activity.

To test our hypothesis, **5b**, the precursor of our previously reported most active C-3 branched 2,3-dideoxy hex-2-enopyranosid-4-ulose **5bm** (Fig. 2) [19], was evaluated for its *in vitro* anti-tubercular activity. We found that the compound **5b** exhibited one fold better anti-tubercular activity at MIC of 3.125 μg/mL compared to that of **5bm** (MIC-6.25 μg/mL) [34–36]. This result prompted us to synthesize a series of 2,3-dideoxy hex-2-enopyranosid-4-uloses (Scheme 1) and carry out a detailed SAR study in order to delineate the structural features associated with highest activity and least toxicity.

First enulosides **4b**, **6b** and **7b** having same substituent at anomeric position (O^iPropyl) but different substituents at C-6, were evaluated for their anti-tubercular activity. It was found that **4b** having an unprotected hydroxyl group at C-6 showed a moderate MIC of 25 μg/mL while **6b** and **7b** having an acetyl and tosyl group respectively at C-6 exhibited a good MIC value of 3.125 μg/mL that was same as that reported for **5b** having a pivaloyl group at C-6.A similar trend was also observed in the case of other enulosides bearing different alkoxy groups at C-1. These results clearly indicated that the activity of any enuloside was enhanced when an acyl



Scheme 3. Reagents and conditions: (a) 37% aq. HCHO, DMAP, THF, −5 °C, 12 h–16 h; (b) $(CH_3CO)_2O$, Pyridine, 0 °C → 5 °C, 12 h.

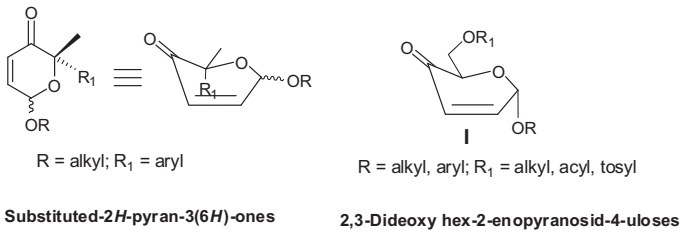


Fig. 1. Substituted-2*H*-pyran-3(6*H*)-ones which are structurally very similar to 2,3-dideoxy hex-2-enopyranosid-4-uloses **I**, exhibit activity against gram positive bacteria.

or tosyl group was introduced at C-6. Next the role of the group attached to C-1 (anomeric carbon) of the enulosides was scrutinized. It is well documented that biological activity of alcohols, including anti-bacterial activity showed regularities of increase and decrease depending on the structure of the alkyl substituent [37–41]. On the whole lengthening of the carbon chain causes an increase in pharmacological effects, but further lengthening brings about a parabolic decrease in activity [37–42]. Branching raises activity and so does transition from primary to tertiary alkyl alcohols through secondary in an isomeric series [41]. Similar type of effect has also been seen in case of anti-tubercular activity as was evident from the SAR studies leading to the discovery of ethambutol [43]. In the light of these reports we decided to explore the effect of the alkoxy substituent at C-1 on anti-tubercular activity of the enuloside. A series of enulosides having different alkoxy substituents (OMethyl, O^{i}Propyl, OButyl, O^{i}Butyl, O^{t}Butyl, OPentyl, O^{i}Amyl, OHexyl, ODecyl) at C-1 were synthesized (Scheme 1) and evaluated for their anti-tubercular activity (Table 1) for a comparative study of their anti-tubercular activities with their precursor C-3 unsubstituted enulosides.

It was found that enulosides having the same substituent at C-6 showed a general increase in activity on moving from one carbon chain (OMethyl) to five carbon chain (OPentyl and O^{i}Amyl) at C-1. Further lengthening of the carbon chain led to a decrease in activity and it was lowest for the enuloside bearing a ten carbon ODecyl substituent at C-1 (Fig. 3, Table 1). The enulosides (**5g** and **6g**) having an O^{i}Amyl substituent at C-1 were found to be the most active.

To further explore the effect of the substituent at C-1 on anti-tubercular activity, compounds **4j** and **5j** having a cyclopropylmethoxy [44,45] and compounds **4k** and **5k** having a 4-methoxyphenyl group [46,47] respectively at C-1 were synthesized (Scheme 1). However, evaluation of their anti-tubercular activity revealed that the MIC's of the most active cyclopropylmethoxy containing enuloside **5j** and 4-methoxyphenyl containing enuloside **5k** were 3.125 μg/mL and 6.25 μg/mL respectively, which were much higher than the MIC of the alicyclic O^{i}Amyl substituent containing enuloside **5g** (Table 1).

We next synthesized a series of C-3 alkylaryl 2,3-dideoxy hex-2-enopyranosid-4-ulose derivatives (Scheme 2) for a comparative study of their anti-tubercular activities with their precursor enulosides (Table 2).

It was found that the anti-tubercular activities of the C-3 alkylaryl 2,3-dideoxy hex-2-enopyranosid-4-uloses were generally one

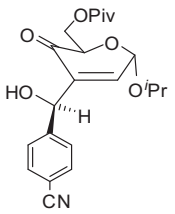


Fig. 2. Structure of compound **5bm**.

Table 1
In vitro activity of the synthesized 2,3-dideoxy hex-2-enopyranosides and 2,3-dideoxy hex-2-enopyranosid-4-uloses against *M. tuberculosis* H37Rv.

Entry	Compd. Code	R	R_1	MIC (μg/mL)
1	**3g**	*iso*-Amyl	H	Inactive[a]
2	**3i**	Decyl	H	Inactive
3	**4a**	Methyl	H	25
4	**4b**	*iso*-Propyl	H	25
5	**4d**	*iso*-Butyl	H	12.5
6	**4e**	*tert*-Butyl	H	25
7	**4g**	*iso*-Amyl	H	6.25
8	**4i**	Decyl	H	12.5
9	**4j**	Cyclopropylmethyl	H	25
10	**4k**	4-Methoxyphenyl	H	Inactive at 25 μg/mL
11	**5a**	Methyl	Pivaloyl	6.25
12	**5b**	*iso*-Propyl	Pivaloyl	3.125
13	**5c**	*n*-Butyl	Pivaloyl	3.125
14	**5d**	*iso*-Butyl	Pivaloyl	3.125
15	**5e**	*tert*-Butyl	Pivaloyl	6.25
16	**5f**	*n*-Pentyl	Pivaloyl	1.56
17	**5g**	*iso*-Amyl	Pivaloyl	0.78
18	**5h**	*n*-Hexyl	Pivaloyl	3.125
19	**5i**	Decyl	Pivaloyl	12.5
20	**5j**	Cyclopropylmethyl	Pivaloyl	3.125
21	**5k**	4-Methoxyphenyl	Pivaloyl	6.25
22	**6a**	Methyl	Acetyl	6.25
23	**6b**	*iso*-Propyl	Acetyl	3.125
24	**6e**	*tert*-Butyl	Acetyl	6.25
25	**6g**	*iso*-Amyl	Acetyl	0.78
26	**6i**	Decyl	Acetyl	6.25
27	**7b**	*iso*-Propyl	Tosyl	3.125
Rifampin				2
Streptomycin				6

[a] Inactive at a MIC of 50 μg/mL unless otherwise indicated.

to three folds lower than that of their corresponding precursor enulosides (Table 2). Interestingly, a similar correlation between anti-tubercular activity and chain length of the OAlkyl substituent at C-1, as noticed in the case of the precursor hex-2-enopyranosid-4-uloses was also observed here (Table 2).

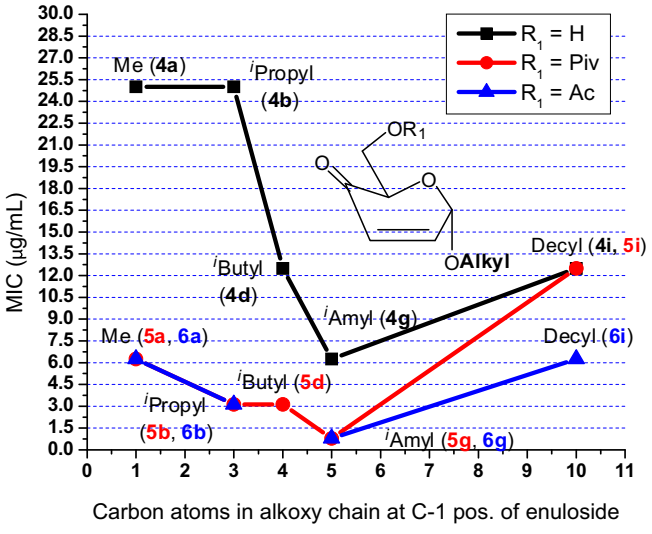
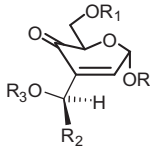


Fig. 3. Correlation of anti-tubercular activity of 2,3-dideoxy hex-2-enopyranosid-4-uloses with length of alkoxy chain at C-1 position.

Table 2
In vitro activity of the synthesized C-3 branched 2,3-dideoxy hex-2-enopyranosid-4-uloses against *M. tuberculosis* H37Rv.



Entry	Compd. Code	R	R_1	R_2	R_3	MIC (μg/mL)
1	**5am**	Methyl	Pivaloyl	4-cyanophenyl	H	12.5
2	**5 dm**	*iso*-Butyl	Pivaloyl	4-cyanophenyl	H	6.25
3	**5gm**	*iso*-Amyl	Pivaloyl	4-cyanophenyl	H	6.25
4	**5go**	*iso*-Amyl	Pivaloyl	2,4-dinitrophenyl	H	6.25
5	**5im**	Decyl	Pivaloyl	4-cyanophenyl	H	12.5
6	**6gn**	*iso*-Amyl	Acetyl	4-nitrophenyl	H	3.125
7	**6go**	*iso*-Amyl	Acetyl	2,4-dinitrophenyl	H	6.25
8	**6in**	Decyl	Acetyl	4-nitrophenyl	H	25
9	**7bn**	*iso*-Propyl	Tosyl	4-nitrophenyl	H	6.25
10	**8**	*iso*-Propyl	Pivaloyl	H	H	25
11	**9**	*iso*-Amyl	Pivaloyl	H	H	25
12	**10**	*iso*-Amyl	Pivaloyl	H	Acetyl	Inactive
Rifampin						2
Streptomycin						6

To further elucidate the role of branching at C-3 position of these enulosides, C-3 hydroxymethyl branched derivatives **8** and **9** of the highly active hex-2-enopyranosid-4-uloses **5b** and **5g** respectively (Scheme 3) were synthesized [31]. A sharp decrease in anti-tubercular activity was observed in case of both the C-3 hydroxymethyl branched derivatives **8** and **9** (Table 2).

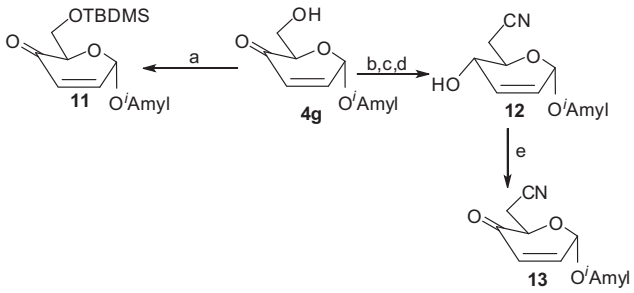
Acetylation of the hydroxymethyl group in **9** resulted in C-3 acetoxymethyl derivative **10** which was found to be inactive at the concentration of 50 μg/mL (Scheme 3, Table 2). This result was contrary to the results obtained in the case of acetylation of the C-5 hydroxymethyl group in enulosides **4** (Scheme 1, Table 1, compounds of series **6**).

The above results clearly showed that introduction of any substituent at C-3 position of the enulosides **5a**, **5d**, **5g**, **5i**, **6g**, **6i** and **7b** invariably led to a decrease in the anti-tubercular activity though the quantum of decrease may be related to the nature of the C-3 substituent.

Once the correlation between anti-tubercular activity and the substituents at C-1 and C-3 of the enulosides was established, we carried further modifications at C-6 position of the enulosides to understand more precisely the SAR associated with the substituent at C-6. When an OTBDMS (ether) group was introduced at C-6 position of **4g** (the most active 6-OH containing enuloside) to obtain compound **11** (Scheme 4), the anti-tubercular activity dropped to 12.5 μg/mL (Table 3). Likewise when **4g** was converted into 2,3,6-trideoxy hex-2-enopyranosid-4-ulose **13** (Scheme 4), activity got reduced to 12.5 μg/mL (Table 3).

From the above study it became clear that the C-6 substituent had a prominent role in modulating the anti-tubercular activity in the title compounds with the enulosides having an acyl group at C-6 exhibiting the best anti-tubercular activity (Table 1, Table 3).

Finally we probed the role of C-4 keto group and the double bond between C-2 and C-3 towards the anti-tubercular activity of these enulosides (Scheme 5). For this study we chose the most active hex-2-enopyranosid-4-ulose **5g**. Reduction of the C-4 keto group yielded its 4-hydroxy derivative **14** which was found to be inactive (Scheme 5). When the double bond between C-2 and C-3 in **5g** was hydrogenated, the resulting saturated compound **15** was found to be inactive (Scheme 5). Likewise compound **17**, the



Scheme 4. Synthesis of enulosides having different substituent at C-6. Reagents and conditions: (a) TBDMSCl, Et_3N, DCM, 0 °C → 5 °C, 40 h; (b) TsCl, Pyridine, DCM, −30 °C → 5 °C, 24 h; (c) $NaBH_4$, $CeCl_3 \cdot 7H_2O$, EtOH, 0 °C → 5 °C, 50 min; (d) NaCN, DMF, 100 °C-110 °C, 4 h; (e) MnO_2, $CHCl_3$, 16 h, RT.

hydrogenated derivative of **5b** was also found to be inactive (Scheme 5). From the above observations it can be argued that the presence of the C-4 keto group and the 2,3-double bond in the pyran ring (α, β-unsaturated keto system) were essential for anti-tubercular activity of these compounds [48–52].

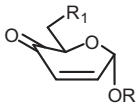
The SAR study suggested that the anti-tubercular activity of these hex-2-enopyranosid-4-uloses may be resulting from the presence of Michael acceptors in the form of α, β-unsaturated keto system in their structure, as is well documented in literature that the biological activities [48–51], including anti-bacterial activity [48] of a number of α, β-unsaturated carbonyl structural element containing compounds seem to originate from their reaction with nucleophiles by a Michael-type addition [48–51]. In addition the SAR study also seems to suggest a role for the conformation of the enone moiety in the anti-tubercular activity of these enulosides [52].

The above argument was supported by the observation that when enuloside **5g** was subjected to epoxidation (Scheme 5), the resulting epoxy derivative **16** was found to exhibit moderate anti-activity at MIC of 12.5 μg/mL unlike in the case of hydrogenation of **5g** wherein the resulting saturated derivative **15** became inactive. This result could be rationalized in terms of similar conformation of the enone moiety in **5g** and **16** [52].

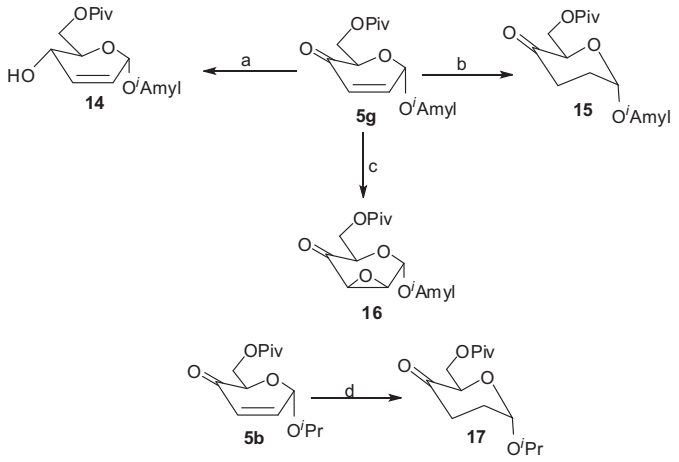
4.2. In vitro toxicity evaluation

The most active compounds **5b**, **5c**, **5d**, **5f**, **5g**, **5h**, **5j**, **6b**, **6g**, **7b** and **6gn** were tested for their cytotoxicity (Table 4) in an *in vitro* model for toxicity with Vero monkey kidney cells originally obtained from ATCC (ATCC CCL-81) using Resazurin assay as described in the experimental section below [53,54]. The data from this toxicity testing and MIC values were used to calculate a selectivity index (SI), the ratio of CC_{50}: MIC (Table 4), an important

Table 3
Variation of anti-tubercular activity of enulosides with the nature of C-6 substituent.



Entry	Compd. Code	R	R_1	MIC (μg/mL)
1	**4g**	*iso*-Amyl	OH	6.25
2	**11**	*iso*-Amyl	OTBDMS	12.5
3	**13**	*iso*-Amyl	CN	12.5
Entry 17, Table 1	**5g**	*iso*-Amyl	OPivaloyl	0.78
Entry 25, Table 1	**6g**	*iso*-Amyl	OAcetyl	0.78
Rifampin				2
Streptomycin				6



Scheme 5. Reagents and conditions: (a) $NaBH_4$, $CeCl_3 \cdot 7H_2O$, EtOH, 0 °C → 5 °C, 70 min; (b) H_2, Pd/C, RT, normal pressure, 80 min; (c) 30% aq. H_2O_2, 1M NaOH soln., MeOH, RT, 30 min, −10 °C → 0 °C; (d) H_2, Pd/C, RT, normal pressure, 90 min.

Table 4
Calculation of Selectivity Index (SI) for the most active compounds (MIC ≤ 3.125 μg/mL).

Compound	MIC (μg/mL)	MW	MIC (μM)	CC_{50} (μM)	SI
5b	3.125	270	11.5	12.5	1.09
5c	3.125	284	11	42.46	3.85
5d	3.125	284	11	28.16	2.56
5f	1.56	298	5.23	7.38	1.41
5g	**0.78**	**298**	**2.65**	**36**	**13.50**
5h	3.125	312	10.01	4.80	0.48
5j	3.125	282	11	77.16	6.96
6b	3.125	228	13.7	104.4	7.60
6g	0.78	256	3	19.8	6.60
7b	3.125	340	9.19	13.4	1.40
6gn	3.125	407	7.6	14.3	1.80

selection criteria for moving the compounds forward into advanced *in vitro* and *in vivo* studies.

4.3. Detailed biological study on compound **5g** (S007-724)

The compound **5g** was identified for further study on the basis of MIC of 0.78 μg/mL and selectivity index (SI) value of 13.50 (Table 1, Table 4).

4.3.1. Evaluation of in vitro activity against MDR, SDR and clinical strains of M. tuberculosis

The compound **5g** was found to exhibit *in vitro* activity against MDR (Multi Drug Resistant), SDR (Single Drug Resistant) and clinical strains of *M. tuberculosis* at a MIC of 0.78−1.56 μg/mL as determined by BACTEC radiometric detection method (Table 5).

4.3.2. Determination of in vitro efficacy of **5g** in macrophages

The compound **5g** was tested for *in vitro* efficacy in murine macrophage cell line J774A.1 at 1× and 2× MIC concentrations as described in experimental section below. Lysates from 0 and 6 days of control and test samples were inoculated in BACTEC vials and growth index (GI) was monitored over 8 day period [55]. As shown in Fig. 4, the GI rose to 225 in control without the compound, while at 1× and 2× MIC concentration, GI did not rise beyond 10 over the same period (Fig. 4). The result clearly demonstrated effective inhibition of intracellular replication of *M. tuberculosis* within mouse macrophages at 1× and 2× MIC concentrations.

Table 5
In vitro activity of **5g** against SDR, MDR and clinical strains of *M. tuberculosis*.

M. tuberculosis strain	Susceptibility or resistance to anti-tubercular drugs	MIC (μg/mL)
433 (SDR)	INH^r	1.56 μg/mL
TB-2506/06 (SDR)	EMB^r	1.56 μg/mL
2643 (MDR)	Rif^r INH^r	1.56 μg/mL
TB-1678/05 (MDR)	Rif^r INH^r	1.56 μg/mL
2280	Drug sensitive	0.78 μg/mL

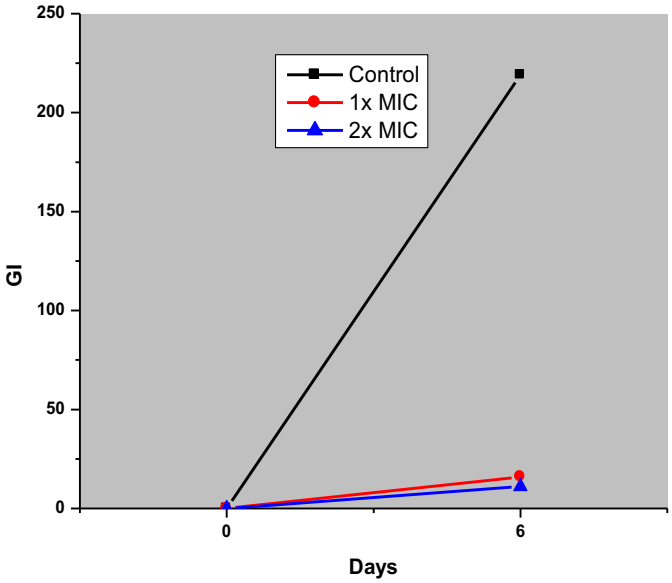


Fig. 4. Graph showing intracellular activity of **5g** against *M. tuberculosis* in terms of Growth Index (GI).

5. Conclusion

In summary, we have reported the design and synthesis of 2,3-dideoxy hex-2-enopyranosid-4-uloses as potentially useful, new anti-tubercular agents. These small molecules have shown promising *in vitro* anti-tubercular activity against virulent H37Rv strain of *M. tuberculosis* in a MIC range of 25 μg/mL to 0.78 μg/mL. A detailed SAR study was carried out to understand the structural basis of anti-tubercular activity of the title compounds and guide the future development of even more potent 2,3-dideoxy hex-2-enopyranosid-4-uloses based anti-tubercular entities. It was revealed that the substituents at C-1 and C-6 were playing a pivotal role in modulating the activity of these molecules. It was also found that the presence of α, β-unsaturated carbonyl system was essential for anti-tubercular activity of these compounds. One of the enuloside **5g**, on the basis of low MIC (0.78 μg/mL-*M. tuberculosis* H37Rv; 1.56 μg/mL-MDR, SDR strains of *M. tuberculosis*; 0.78 μg/mL-inhibition of intracellular replication of *M. tuberculosis*) and SI value of 13.5 has been identified as a promising lead molecule.

These results clearly points to the potential usefulness of the title 2,3-dideoxy hex-2-enopyranosid-4-uloses as lead structures for the development of cost-effective and potent new anti-tubercular drugs.

6. Materials and methods-biology

6.1. Bacterial strains

M. tuberculosis H37Rv and clinical strains of *M. tuberculosis* isolated from TB patients were employed to determine anti-tubercular activity of compounds. Clinical strains obtained from Dr. Sarman

Singh, All India Institute of Medical Sciences, New Delhi were characterized biochemically and by 16S rRNA sequencing [56].

6.2. BACTEC radiometric susceptibility assay

H37Rv strain of *M. tuberculosis* was grown in BACTEC 12 B vial until the GI reached around 300. Test compounds were dissolved in DMSO. Lyophilized Rifampin, Streptomycin and Isoniazid (Becton Dickinson) were reconstituted as per instructions in manual in deionized water and 0.1 mL was added into 12B medium. The final concentration of streptomycin, isoniazid, ethambutol and rifampin were 6, 0.4, 7.5 and 2 μg/mL respectively. Test compounds were added at varied concentrations in 0.1 mL into 12B medium. 0.1 mL of the bacterial suspension (GI ~300) was added into each of the BACTEC 12B vial containing the drug. The control vial contained dilute *M. tuberculosis* suspension 1:100 prepared by transferring 0.1 mL of the suspension into 9.9 mL of special diluting fluid and inoculating 0.1 mL of this dilution into the control 12B vial. The vials were incubated at 37 °C ± 1 °C and read every day at the same time of day on BACTEC instrument until a GI of 30 or more is achieved in the control vial.

The difference in the GI values from the previous day and designated as ΔGI was determined for all samples and compared with ΔGI of the control vial. If the GI was less in the drug vial than the control, the population was susceptible; if more, it was resistant. MIC of a compound was defined as the lowest concentration which gave negative ΔGI value [57,58].

6.3. Cytotoxity studies

The compounds for cytotoxicity were tested in an *in vitro* model for toxicity with Vero monkey kidney cells using Resazurin assay [53,54]. The Vero cells (ATCC CCL-81) were seeded overnight at 1×10^4–3×10^6 cells per well in 96-well plates at 37 °C in RPMI supplemented with 10% heat-inactivated fetal bovine serum and 5% CO_2. Cells were exposed to dilutions of experimental and control drugs in triplicate for 24 h with each compound in a range of concentrations from 100 to 1.56 μg/mL. Rifampin was run as a control at the same concentrations. Each well had 100 μL of the test material in serially descending concentrations. After 72 h of incubation, 10 μL of resazurin indicator solution (0.1%) was added and incubation was continued for 4–5 h. The color change was assessed visually. Any color change from purple to pink or colorless was recorded as positive. Fluorescence of each sample was measured with excitation wavelength at 530 nm and emission wavelength at 590 nm using the BMG Polar Star Galaxy. CC_{50} values (50% inhibitory concentrations) were calculated by plotting fluorescence values using Microsoft excel template.

6.4. In vitro efficacy of compounds in macrophages

The murine macrophage J774A.1 cells were infected with *M. tuberculosis* H37Rv cells at MOI 1:20, for 6 h at 37 °C and 5% CO_2. For infection, *M. tuberculosis* cells grown in Middlebrook 7H9 (Difco) supplemented with BSA, dextrose, catalase at 37 °C with 5% CO_2 were harvested by centrifugation at 2500 × g for 10 min and washed twice with serum free RPMI. The cells were finally resuspended in RPMI supplemented with 5% heat-inactivated FBS and used for infection. The infected J774A.1 cells were seeded in triplicate overnight at 5×10^5 cells/well in 24 well plates at 37 °C and 5% CO_2 in presence of gentamicin (50 μg/mL) to kill extracellular mycobacteria. Cells were washed three times in incomplete RPMI and replaced with fresh complete RPMI medium containing test compound at 1× and 2× MIC and incubated at 37 °C and 5% CO_2. Control well contained murine macrophage cells infected with *M. tuberculosis* without drug. The medium was replaced every day at same time with complete RPMI containing 1× and 2× MIC test compound. Macrophage cells were lysed by the addition of 100 μL of 0.05% SDS. 100 μL lysate was sampled from three wells on day 0 and day 6 for control, test compounds at 1× MIC and 2 × MIC. The lysate was inoculated in BACTEC vials and growth was monitored. The protocol for macrophage infection has been described earlier [54,55].

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Appendix. Supplementary data

Supplementary data related to this article can be found online at doi:10.1016/j.ejmech.2011.03.002.

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