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The synthesis and biological evaluation of mycobacterial *p*-hydroxybenzoic acid derivatives (*p*-HBADs)†

Jean Bourke,[a] Corinna F. Brereton,[b] Stephen V. Gordon,[c] Ed C. Lavelle*[b] and Eoin M. Scanlan*[a]

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Mycobacterium tuberculosis establishes chronic infection and causes disease through manipulation of the host's innate and adaptive immune response. The bacterial cell wall is highly complex and contains a rich variety of glycosylated compounds that are secreted during infection and have been proposed as immunomodulatory molecules. Amongst the most important of these are the *p*-hydroxybenzoic acid derivatives (*p*-HBADs). Here we report the synthesis of this important class of biomolecules and the first *in vitro* study of the immunomodulatory effects of these compounds in isolation from the host bacterium. The compounds do not have stimulatory properties but, in contrast, can inhibit the production of inflammatory cytokines, particularly interferon-γ (IFN-γ), by T-cells. This study offers a fundamental insight into the effect of these glycans on the immune response.

Introduction

Mycobacterium tuberculosis (Mtb), the causative agent of tuberculosis is one of the world's most deadly pathogens, with 1.4 million deaths in 2011 alone. The pathogen is characterized by a sophisticated and complex cell wall architecture.[1–4] Many of the lipids and glycolipids that make up the Mtb cell wall are believed to be virulence factors that play a key role in host-pathogen interactions and disease pathogenesis.[5–10] Glycosyl processing enzymes are essential for bacterial cell wall biosynthesis and many of the key components of the cell wall display complex carbohydrates.[11,12] The critical and diverse biological functions of these glycans in the life-cycle of the mycobacterium are only beginning to be understood. Phenolic glycolipids (PGLs) are a family of bacterial glycolipids with a lipid core similar to dimycocerosates of phthiocerol (DIMs).[13–17] PGLs contain an aromatic nucleus that is glycosylated by a strain-specific, mono, di, tri or tetrasaccharide (Fig. 1). The carbohydrate region is composed of the 6-deoxy sugars, L-rhamnose and L-fucose and is usually decorated with a very specific methylation pattern. These glycosylated lipids are important for mycobacterial disease pathogenesis and have been associated with an inhibition of the release of key inflammatory effector molecules by cells of the host's innate immune response.[18,19] PGL-1 has been isolated from strains of the related mycobacterium, *M. leprae*, the causative agent of leprosy. The carbohydrate region of PGL-1 binds to the α2LG1, α2LG4 and α2LG5 modules of the peripheral nerve laminin α2 chain and promotes phagocytosis of *M. leprae* by macrophages and Schwann cells.[20] The carbohydrate region of PGLs is critical to their activity. Despite the acute biological effects of these

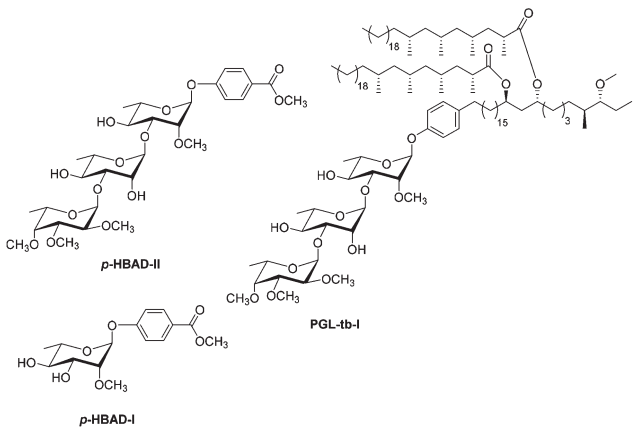

Fig. 1 Structures of *p*-HBAD-I, *p*-HBAD-II and the related PGL-tb1 from *M. tuberculosis*. The lipid core of PGL is composed of phenolphthiocerol esterified by mycocerosic acids.

[a]*School of Chemistry, Trinity Biomedical Sciences Institute, 152-160 Pearse Street, Trinity College, Dublin 2, Ireland. E-mail: eoin.scanlan@tcd.ie; Fax: +353 (1) 671 2896; Tel: +353 (1) 896 2514*
[b]*Adjuvant Research Group, School of Biochemistry, Trinity Biomedical Sciences Institute, 152-160 Pearse Street, Trinity College Dublin, Dublin 2, Ireland*
[c]*Conway Institute of Biomolecular & Biomedical Research, University College Dublin, Belfield, Dublin 4, Ireland*
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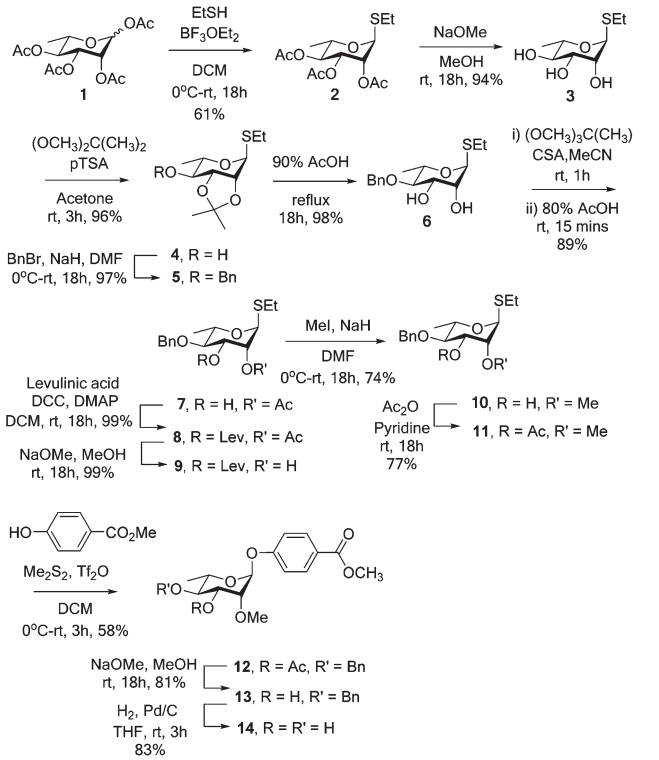
molecules, it has been found that most clinical isolates of *M. tuberculosis* do not synthesize PGLs.[19] A subclass of carbohydrate molecules related to PGLs are the *para*-hydroxybenzoic acid derivatives (*p*-HBADs).[21,22] These small glycoconjugates contain an identical glycosylated phenolic moiety to PGLs and are formed through a related biosynthetic pathway.[23–25] The *p*-HBADs are a component of the bacterial cell wall and unlike the related PGLs, all strains of *M. tuberculosis* appear to synthesize *p*-HBADs.[21] The fact that all strains of *M. tuberculosis* have retained the ability to produce such complex molecules suggests that these carbohydrates are extremely important for bacterial pathogenesis. Indeed it has been demonstrated that *M. tuberculosis* mutants defective in the synthesis of *p*-HBADs promote a stronger inflammatory response than wild type strains.[22] The fact that these compounds do not have a lipid component strongly suggests that it is the carbohydrate region, with its defined methylation pattern, that is the most important factor in modulating biological activity. The *p*-HBADs are secreted during host infection but despite their prominent role in disease infection and pathogenesis, little is known about the biological targets of these molecules.

Since publication of the Mtb genome,[26] a number of genes associated with glycosylation and subsequent methylation of the *p*-hydroxybenzoic acid aromatic nucleus have been identified.[23,24] The structural similarities between PGLs and *p*-HBADs suggest that they share a similar biosynthetic pathway originating from *p*-hydroxybenzoic acid. A chorismate pyruvate-lyase has been identified as the sole enzymatic source of *p*-hydroxybenzoic acid in *M. tuberculosis*.[21] Methylation of the 2-hydroxyl group located on C-2 of the rhamnosyl residue linked directly to the phenolic core appears to be of paramount importance for biological activity. In order to directly address the role of *p*-HBADs in Mtb pathogenesis, we set out to prepare fully synthetic *p*-HBADs with their native methylation pattern intact. A synthetic variant possessing an unnatural methylation pattern was also prepared and studied *in vitro*.

Results and discussion

The *p*-HBADs play a key role in *M. tuberculosis* disease pathogenesis and recent studies have suggested that these glycans have potent immunomodulatory activity. Data from Mtb strains defective in the synthesis of *p*-hydroxybenzoic acid derivatives suggest that *p*-HBADs influence the release of key inflammatory cytokines by macrophages.[22] It is difficult to derive definitive conclusions regarding the biological roles of specific *p*-HBADs from studies using Mtb since the bacterium secretes heterogeneous mixtures of glycoconjugates with variable ratios of *p*-HBADs. It is also challenging to probe the biological role of the unusual methylation pattern displayed on the *p*-HBADs. In order to directly study the immunomodulatory effects of the *p*-HBADs in the absence of the parent bacterium, we set out to prepare synthetic samples of *p*-HBAD-I (both methylated and unmethylated at the 2-OH position) and *p*-HBAD-II with its native methylation pattern. The immunomodulatory effects of these compounds were tested *in vitro* using mouse splenocytes and bone marrow derived macrophages.

Synthesis of the trisaccharide unit of *p*-HBAD-II has been described previously.[27,28] However, our reported strategy for regioselective protection of hydroxyl groups offers an alternative synthetic route with comparable yields to these reported syntheses. Recently, the total synthesis of PGL-tb1 has been reported but this approach also differs considerably from our reported strategy.[29] The synthesis of the PGL mycoside-B and *p*-HBAD-I have also recently been reported, highlighting the interest in accessing synthetically pure samples of these compounds for biological study.[30] The overall synthetic route to *p*-HBAD-I is outlined in Scheme 1. The regioselective methylation of the 2-OH position of L-rhamnose required a number of protecting group manipulations and a general protecting group strategy was developed that allowed regioselective access to each hydroxyl groups on rhamnose moiety. Glycosylation with methyl *p*-hydroxybenzoate was carried out at a late stage in the synthesis. Starting from per-acetylated rhamnose **1**, a Lewis acid catalysed glycosylation reaction with ethane thiol furnished thioglycoside donor **2**. Thioglycosides are widely used as glycosyl donors in carbohydrate synthesis due to their relative stability and ability to tolerate functional group modifications on the sugar.[31] Removal of the acetate protecting groups under Zemplén conditions followed by regioselective protection of the 2- and 3-OH groups with a cyclic acetal furnished partially protected donor **4**. Benzylation of the free 4-OH followed by hydrolysis of the acetal protecting group furnished

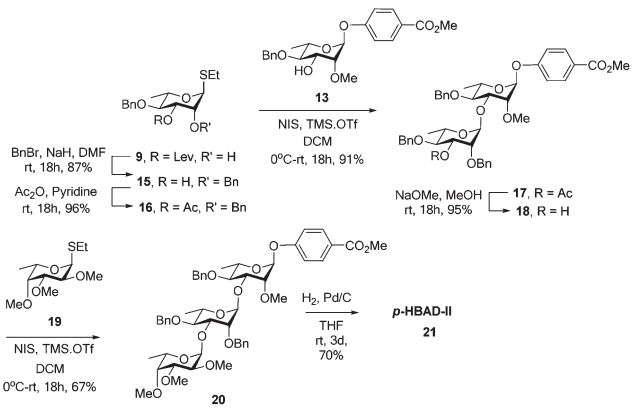


Scheme 1 Synthetic strategy for the preparation of *p*-HBAD-I **14** from peracetylated rhamnose **1**.

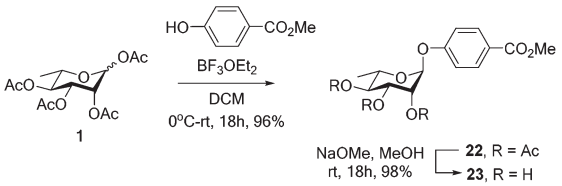
the diol **6**.[32,33] A regioselective hydrolysis of an orthoester was used to access **7**.[34] The resulting free 3-OH was protected with a levulinoyl protecting group which was selected to be orthogonal to both the acetyl and benzyl protecting groups. Following deacetylation of the 2-OH to give **9** (this compound was employed as a starting point for the synthesis of *p*-HBAD-II), the methyl group was introduced in good yield using methyl iodide as the alkylating agent. It was found that using an excess sodium hydride could remove the levulinoyl protecting group quantitatively in a one-pot system to give **10**. The free 3-OH was acetylated to give thioglycoside **11** which was employed as a glycosyl donor for the glycosylation reaction with methyl *p*-hydroxybenzoate to furnish the protected *p*-HBAD-I **12**. Deprotection of **12** in two steps, furnished the fully synthetic *p*-HBAD-I **14** in a good yield. The synthetic strategy is advantageous in that all of the synthetic steps are high yielding and the key intermediates **9** and **13** could be used directly for the preparation of the more complex oligosaccharide, *p*-HBAD-II.

The synthesis of the *p*-HBAD-II trisaccharide was based on the strategy developed for *p*-HBAD-I. A sequential glycosylation strategy employing two thioglycoside donors was used. The overall synthetic scheme is outlined in Scheme 2. Starting from the levulinoyl protected compound **9**, treatment with benzyl bromide and an excess of sodium hydride gave the partially protected glycosyl donor **15**. This compound was acetylated at the 3-OH position and then employed in a glycosylation reaction with the acceptor **13** that was prepared previously as part of the *p*-HBAD-I synthesis. The disaccharide **17** was formed in good yield with complete alpha selectivity (as determined by ^{1}H-NMR analysis), despite the absence of a participating group at the 2-OH position. The 3-OH position of the terminal rhamnose residue was deprotected and glycosylation with the per-*O*-methylated fucosyl thioglycoside donor **19**[35] furnished the protected trisaccharide **20** in good yield. Removal of the benzyl protecting groups under palladium-catalysed hydrogenation conditions furnished the fully synthetic *p*-HBAD-II with the native methylation pattern.

In order to probe the role of the methyl group on the 2-OH position of *p*-HBAD-I, the unmethylated derivative (UM-*p*-HBAD-I) was also prepared. This unmethylated *p*-HBAD was



Scheme 2 Synthetic strategy for the preparation of *p*-HBAD-II.



Scheme 3 Synthesis of unmethylated *p*-HBAD-I **23** from per-OAc-rhamnose **1**.

previously isolated from a mutant strain of *M. tuberculosis* taken from a transposon mutant library, and may be an intermediate in the biosynthesis of *p*-HBADs.[21] The per-acetylated rhamnosyl donor **1** was glycosylated with the methyl *p*-hydroxybenzoate to furnish **22** which was subsequently deprotected to furnish the fully unmethylated UM-*p*-HBAD-I **23** (Scheme 3).

Cell studies

Mouse splenocytes and Bone Marrow Derived Macrophages (BMM) were employed for the *in vitro* studies. Splenocytes comprise a mixed population of white blood cells originating in the spleen that play a key role in immune responses. Here, splenocytes were incubated with the T lymphocyte activating stimulus, anti-CD3e, to promote lymphocyte proliferation and cytokine production. Cells were also incubated with each of the *p*-HBADs at various concentrations alone or together with anti-CD3e antibody to assess if the molecules could induce, inhibit or modulate cytokine secretion. Cytokine production by splenocytes and macrophages was quantified by enzyme-linked immunosorbent assay (ELISA). Flow cytometry was used to examine intracellular cytokines produced by CD4 and CD8 cells and to assess cell proliferation by intracellular fluorescent label carboxyfluorescein diacetate succinimidyl ester (CFSE) incorporation (Fig. 2).

p-HBADs were tested at concentrations of 20 μM, 100 μM and 500 μM for their ability to induce cytokines or to modulate anti-CD3e (0.4 μg mL^{-1}) induced cytokine secretion by splenocytes. *p*-HBAD-I, *p*-HBAD-II and UM-*p*-HBAD-I inhibited the secretion of IFN-γ, IL-17 and IL-10 by anti-CD3e-stimulated splenocytes, particularly at a concentration of 500 μM. This reduction was significant at the higher concentration of 500 μM but was still observed at the lower concentrations of the *p*-HBADs. In the absence of anti-CD3e there was no enhancement in cytokine secretion indicating that the *p*-HBADs themselves do not have immunostimulatory properties. The effect of *p*-HBADs on intracellular IFN-γ was examined by flow cytometry (Fig. 3). *p*-HBAD-I, *p*-HBAD-II or UM-*p*-HBAD-I alone did not induce CD4$^+$ T cell proliferation or intracellular IFN-γ production. However, *p*-HBAD-I, *p*-HBAD-II and UM-*p*-HBAD-I suppressed intracellular IFN-γ production by activated CD4$^+$ T cells.

From the above data we can conclude that in the presence of anti-CD3, the three *p*-HBADs inhibit IFN-γ production but not the proliferation of T_h cells. Following from this we can conclude that the *p*-HBADs examined antagonize the effect of the anti-CD3 stimulus on IFN-γ production but enhance its effect on T_h cell proliferation. Thus we can put forth the

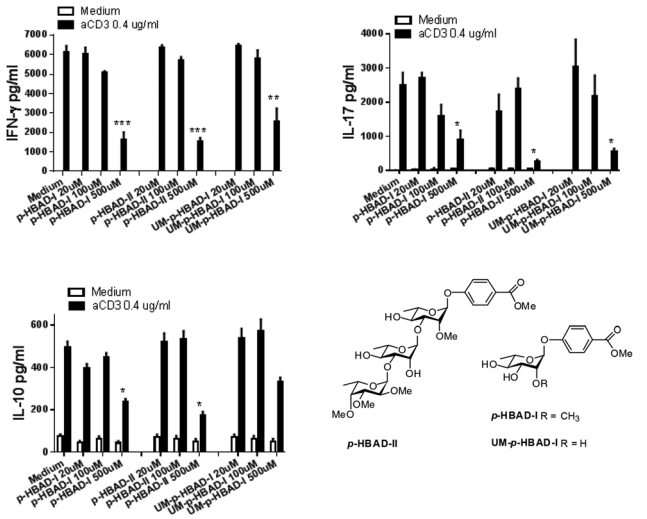


Fig. 2 *p*-HBADs inhibit the secretion of IFN-γ by splenocytes. Cells were incubated with medium, *p*-HBADs alone, anti-CD3e or *p*-HBADs with anti-CD3e. Supernatants were collected after 72 h and IFN-γ, IL-17 and IL-10 concentrations were determined by ELISA.

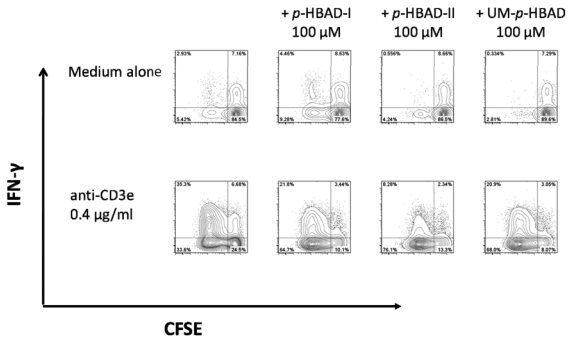


Fig. 3 Flow cytometry data. *p*-HBADs suppress IFN-γ production by $CD4^+$ T cells. Splenocytes were incubated with medium, *p*-HBADs alone or in the presence of anti-CD3e. Cells were incubated for 72 h and T cell proliferation and IFN-γ production was assessed.

postulate that *p*-HBADs inhibit the production of proinflammatory cytokines (due to the knock-on effect of decreased IFN-γ production) but do not adversely affect T_h cell proliferation. The *p*-HBADs appear to have similar effects on both CD3 + $CD4^+$ and CD3 + $CD4^-$ cells. Given the central importance of IFN-γ production in protective immunity to Mtb[36,37] these results indicate that *p*-HBADS may help the bacterium evade the antibacterial effects of this key cytokine.

Macrophages play a vital role in Mtb pathogenesis and are often the first line of defence in the host response to Mtb infection. Macrophages are also key antigen presenting cells that can induce antigen specific $CD4^+$ T cell responses which have been implicated in protective immunity against TB.[38] In addition, recent studies with Mtb have determined that some cell wall glycolipids can directly inhibit $CD4^+$ T cell activation.[39–42] The effects of the *p*-HBADs on macrophages therefore provide a valid *in vitro* measure of the immunomodulatory effects of *p*-HBADs in the context of Mtb pathogenesis.

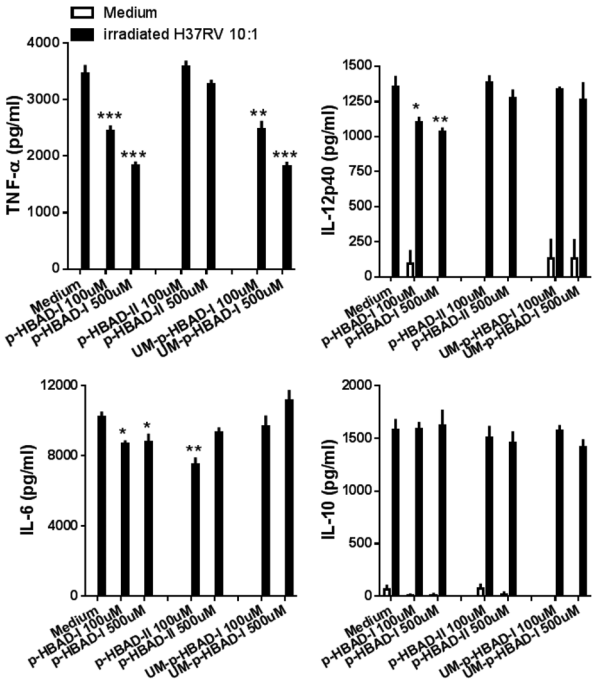


Fig. 4 *p*-HBAD-I and UM-*p*-HBAD-I suppress pro-inflammatory cytokine production by BMDMs stimulated with irradiated *M. tuberculosis* H37Rv. BMDMs were incubated with medium or *p*-HBADs alone (100 or 500 μM) in the presence or absence of irradiated H37Rv (10 : 1). Supernatants were collected after 24 h and TNF-α, IL-12p40, IL-6 and IL-10 concentrations were determined by ELISA.

BMM were incubated with irradiated H37Rv in the presence or absence of the *p*-HBADs, with the compounds added directly to the cell supernatant. The results of the macrophage experiments are presented in Fig. 4. Cells stimulated with irradiated H37Rv produced the pro-inflammatory cytokines TNF-α, IL-12p40 and IL-6, as well as the anti-inflammatory cytokine IL-10. Stimulation in the presence of *p*-HBAD-I significantly suppressed the production of TNF-α and IL-12p40 in response to irradiated *M. Tuberculosis* H37RV, but had a reduced effect on IL-6 production and no effect on IL-10 production. Similarly, co-incubation of Bone-marrow derived macrophages (BMM) with UM-*p*-HBAD-I significantly reduced the production of TNF-α compared to cells stimulated with H37Rv alone, and also had no effect on IL-10 production induced by the irradiated bacteria. In contrast to the results obtained from the splenocyte studies, *p*-HBAD-II did not appear to affect pro-inflammatory cytokine production by BMM co-stimulated with irradiated H37Rv. This data shows that *p*-HBAD-I and UM-*p*-HBAD-I suppress TNF-α production but exert no effect on the production of the cytokines IL-10 or IL-6.

Conclusions

For the first time a number of synthetic *p*-HBADs have been prepared and their immunomodulatory effects have been studied in isolation from the parent bacterium. It has

been determined that these small molecules have the ability to suppress host immune response *in vitro* but that they are not immunostimulatory in themselves. In particular, an inhibition of key immunomodulatory cytokines, IFN-γ and IL-17 by anti-CD3e-stimulated splenocytes was observed, albeit at a high concentration of the corresponding *p*-HBAD. This research has important implications for developing an understanding of Mtb pathogenesis and for developing new treatments for TB infection.

Experimental

General experimental methods

For NMR spectra, 400 MHz spectrometer was employed for ^{1}H (400.13 MHz) and ^{13}C (100.61 MHz) spectra, 600 MHz spectrometer was employed for ^{1}H (600.13 MHz) and ^{13}C (150.90 MHz) spectra. Resonances δ, are in ppm units downfield from an internal reference in $CDCl_3$ (δ_H = 7.26 ppm, δ_C = 77.0 ppm), MeOH (δ_H = 3.31 ppm, δ_C = 49.0 ppm). Optical rotations are quoted in deg cm^3 g^{-1} dm^{-1}. For oligosaccharides the notation a, b, c.... refers to the monosaccharide from the reducing end. Mass spectrometry analysis was performed with Maldi-quadrupole time-of-flight (Q-Tof) mass spectrometer equipped with Z-spray electrospray ionization (ESI). Silica gel (200 mesh) was used for column chromatography. Analytical thin-layer chromatography was performed using silica gel (pre-coated sheets, 0.2 mm thick, 20 cm × 20 cm) and visualized by UV irradiation or molybdenum staining. DCM, MeOH, THF and toluene were dried over flame dried 3 Å or 4 Å sieves. Dimethylformamide (DMF), triethylamine (Et_3N) and trifluoroacetic acid (TFA) were used dry from sure/seal bottles. Other reagents were purchased from an industrial supplier.

Compound 2[43]

Peracetylated rhamnose **1** (4.8 g, 14.7 mmol) was dissolved in anhydrous dichloromethane (40 mL) at 0 °C under N_2. Ethane thiol (1.2 mL, 19.0 mmol) was added followed by $BF_3{\cdot}OEt_2$ (9.1 mL, 73.5 mmol) in increments. The stirred solution was allowed to warm to room temperature and stirred for 18 h to give a red solution which was quenched by treatment with saturated sodium bicarbonate solution (*ca.* 20 mL) and solid $NaHCO_3$. The organic layer was filtered and concentrated. Purification by column chromatography (hexane–EtOAc 80 : 20) furnished **2** as a colourless oil with an overall yield of 82% (4.01 g) and 61% for the α anomer (3.01 g). Data for α anomer: ^{1}H-NMR (400 MHz, $CDCl_3$, δ): 5.36 (1H, dd, $J_{2,1}$ = 1.6 Hz, $J_{2,3}$ = 3.4 Hz, H2), 5.26 (1H, dd, $J_{3,2}$ = 3.4 Hz, $J_{3,4}$ = 10.1 Hz, H3), 5.22 (1H, d, J = 1.5 Hz, H1), 5.12 (1H, app t, H4), 4.26 (1H, dq, $J_{5,6}$ = 6.2 Hz, $J_{5,4}$ = 9.7 Hz, H5), 2.67 (2H, m, CH_2), 2.13, 2.08, 2.01 (3H, s, CH_3), 1.32 (3H, t, J = 7.5 Hz, CH_2CH_3), 1.26 (3H, d, $J_{6,5}$ = 6.1 Hz, H6); ^{13}C-NMR ($CDCl_3$, δ): 170.1, 170.0, 169.9 (C═O), 82.0 (C1), 71.5 (C2), 71.3 (C4), 69.5 (C3), 66.7 (C5), 25.4 (CH_2), 21.0, 20.8, 20.7 (CH_3), 17.4 (C6), 14.9 (CH_2CH_3); HRMS-ESI (*m*/*z*): $[M + Na]^+$ calculated for $C_{14}H_{22}O_7SNa$ 357.0984; found 357.0973.

Compound 3[43]

Thioglycoside **2** (650 mg, 1.95 mmol) was dissolved in methanol with catalytic sodium methoxide, quenched with DOWEX after 3 hours, filtered and concentrated to give **3** in 94% yield (410 mg). ^{1}H-NMR (400 MHz, $CDCl_3$, δ): 5.29 (1H, s, H1), 4.07 (1H, m, H2), 4.05 (1H, m, H5), 3.77 (1H, dd, $J_{3,2}$ = 2.9 Hz, $J_{3,4}$ = 9.4 Hz, H3), 3.53 (1H, app t, H4), 2.65 (2H, m, CH_2), 1.36 (3H, d, $J_{6,5}$ = 6.2 Hz, H6), 1.32 (3H, t, J = 7.5 Hz, CH_3). ^{13}C-NMR ($CDCl_3$, δ): 84.2 (C1), 73.5 (C4), 72.6 (C2), 72.3 (C3), 68.5 (C5), 25.2 (CH_2) 17.5 (C6), 14.9 (CH_3). HRMS-ESI (*m*/*z*): $[M + Na]^+$ calculated for $C_8H_{16}O_4NaS$ 231.0667; found 231.0676.

Compound 4[43]

3 (1.56 g, 7.50 mmol) was dissolved in anhydrous acetone (20 mL), 2,2-dimethoxy propane (3.67 mL, 30 mmol) was added along with catalytic *para*-toluenesulfonic acid. After 3 h the reaction was quenched by the addition of sat. aq. $NaHCO_3$ (*ca.* 15 mL). The mixture was filtered and concentrated and extracted with DCM. The organic layer was dried with $MgSO_4$, filtered and concentrated to give **4** in 96% yield (1.85 g). ^{1}H-NMR (400 MHz, $CDCl_3$, δ): 5.19 (1H, s, H1), 4.17 (1H, d, $J_{2,3}$ = 5.5 Hz, H2), 4.04 (1H, dd, $J_{3,2}$ = 5.5 Hz, $J_{3,4}$ = 7.6 Hz, H3), 3.44 (1H, m, H4), 2.61 (2H, m, CH_2), 1.53, 1.34 (3H, s, CH_3), 1.30 (3H, app t, CH_2CH_3), 1.29 (3H, d, $J_{6,5}$ = 6.3 Hz, H6). ^{13}C-NMR ($CDCl_3$, δ): 109.0 (Cq), 79.4 (C1), 78.3 (C3), 76.8 (C2), 75.4 (C4), 66.0 (C5), 28.2, 26.4(CH_3), 24.4 (CH_2), 17.2 (C6), 14.6 (CH_3). HRMS-ESI (*m*/*z*): $[M + Na]^+$ calculated for $C_{11}H_{20}O_4NaS$ 271.0980; found 271.0991.

Compound 5[27]

4 (14.46 g, 58.3 mmol) was dissolved in DMF (5 mL) under N_2; NaH (2.80 g, 70.0 mmol) and BnBr (9.0 mL, 75.8 mmol) were added. After 18 h the reaction was quenched with *ca.* 2 mL MeOH, concentrated, redissolved in diethyl ether (50 mL), washed with water (2 × 40 mL) and saturated $NaHCO_3$ (2 × 40 mL), dried with $MgSO_4$, and concentrated. Purification by column chromatography (hexane–EtOAc 80 : 20) furnished **5** as a colourless solid in 97% yield (19.0 g). ^{1}H-NMR (400 MHz, $CDCl_3$, δ): 7.36 (5H, m, Ar-H), 5.50 (1H, s, H1), 4.91 (1H, d, J = 11.6 Hz, CH_2), 4.74 (2H, dd, J = 11.4 Hz, J = 21.4 Hz, CH_2), 4.63 (1H, d, J = 11.6 Hz, CH_2), 4.24 (1H, dd, $J_{3,2}$ = 5.4 Hz, $J_{3,4}$ = 7.2 Hz, H3), 4.18 (1H, app d, J = 5.4 Hz, H2), 4.03 (1H, m, H5), 3.30 (1H, $J_{4.3}$ = 7.2 Hz, $J_{4.5}$ = 9.8 Hz, H4), 2.60 (2H, m, CH_2CH_3), 1.52, 1.36 (3H, s, CH_3), 1.29 (3H, t, J = 7.3 Hz, CH_2CH_3), 1.28 (3H, d, $J_{6,5}$ = 6.1 Hz, H6). ^{13}C-NMR ($CDCl_3$, δ): 138.3 (Cq), 128.7, 128.3, 128.1, 127.9, 127.6 (Ar–C), 109.4 (Cq), 82.0 (C4), 79.5 (C1), 78.4 (C3), 77.0 (C2), 75.0, 73.1 (CH_2), 65.3 (C5), 28.1, 26.5 (CH_3), 24.4 (CH_2), 17.9 (C6), 17.7 (CH_2CH_3). HRMS-ESI (*m*/*z*): $[M + Na]^+$ calculated for $C_{18}H_{26}O_4NaS$ 361.1450; found 361.1436. IR: 2895, 1369, 1248, 1093 cm^{-1}.

Compound 6[27,44]

5 (585 mg, 1.73 mmol) was dissolved in 9 : 1 acetic acid–H_2O (5 mL) and reacted for 18 h at 50 °C then concentrated. Purification by column chromatography (hexane–EtOAc 60 : 40) gave **6** as a colourless solid in 98% yield (515 mg). $[\alpha]_D^{20}$ −181.8 (*c* 0.11, $CHCl_3$). ^{1}H-NMR (400 MHz, $CDCl_3$, δ): 7.40 (5H, m, Ar-H), 5.27 (1H, d, $J_{1,2}$ = 1.3 Hz, H1), 4.77 (2H, dd, J = 11.4 Hz, J = 19.8 Hz, CH_2), 4.13 (1H, m, H5), 4.06 (1H, dd, $J_{2,3}$ = 3.4 Hz, $J_{2,1}$ = 1.4 Hz, H2), 3.91 (1H, dd, $J_{3,2}$ = 3.4 Hz, $J_{3,4}$ = 9.1 Hz, H3), 3.41 (1H, app t, H4), 2.65 (2H, m, CH_2CH$_3$), 1.39 (3H, $J_{6,5}$ = 6.3 Hz, H6), 1.32 (3H, t, J = 7.4 Hz, CH_2CH_3). ^{13}C-NMR ($CDCl_3$, δ): 138.2 (Cq), 128.7, 128.1, 127.9 (Ar–C), 83.6 (C1), 82.0 (C4), 75.0 (CH_2), 72.6 (C2), 71.9 (C3), 67.7 (C5), 25.1 (*C*H_2CH$_3$), 18.0 (C6), 14.9 (CH_2*C*H$_3$). HRMS-ESI (*m*/*z*): $[M - H]^-$ calculated for $C_{15}H_{21}O_4S$ 297.1161; found 297.1151. IR: 3289, 2929, 1749, 1453, 1082 cm^{-1}.

Compound 7[44,45]

6 (420 mg, 1.41 mmol) was dissolved in acetonitrile (15 mL) with trimethylorthoacetate (0.36 mL, 2.82 mmol) and camphorsulfonic acid (catalytic) under N_2. The mixture was allowed to react for 45 min then 5 mL 4 : 1 acetic acid–H_2O was added. After 15 min it was diluted with DCM, washed with H_2O and aq. saturated $NaHCO_3$ solution, dried with $MgSO_4$ and concentrated. Purification by column chromatography (hexane–EtOAc 60 : 40) gave **7** as a colourless oil in 89% yield (427 mg). ^{1}H-NMR (400 MHz, $CDCl_3$, δ): 7.36 (5H, m, Ar–H) 5.23 (1H, s, H1), 5.23 (1H, m, H2), 4.85, 4.75 (1H, d, J = 11.0 Hz, CH_2), 4.14 (1H, m, H5), 4.09 (1H, m, H3), 3.42 (1H, app t, H4), 2.65 (2H, m, CH_2CH_3), 2.19 (3H, s, CH_3), 1.39 (3H, d, $J_{6.5}$ = 6.2 Hz, H6), 1.31(3H, t, J = 7.4 Hz, CH_2CH_3). ^{13}C-NMR ($CDCl_3$, δ): 170.8 (C═O) 138.1 (Cq), 128.6, 128.0, 127.9 (Bn), 82.1 (C1), 82.0 (C4), 75.2 (CH_2), 74.6 (C2), 70.8 (C3) 68.0 (C5), 25.6 (*C*H_2CH$_3$), 21.2 (CH_3), 18.0 (C6), 15.0 (CH_2CH_3). HRMS-ESI (*m*/*z*): $[M + Na]^+$ calculated for $C_{17}H_{24}O_5SNa$ 363.1242; found 363.1241. IR: 3457, 2931, 1740, 1373, 1231 cm^{-1}.

Compound 8

7 (12.5 g, 36.7 mmol) was dissolved in DCM (30 mL), and levulinic acid (17.25 g, 146.24 mmol), DCC (30.3 g, 146.24 mmol), TEA (catalytic 2 mL) and DMAP (catalytic) were added. After 18 h the mixture was filtered through cotton wool, washed with aq. sat. $NaHCO_3$ (25 mL), aq. sat. NaCl (25 mL), H_2O (25 mL), dried with $MgSO_4$, filtered and concentrated. Purification by column chromatography (hexane–EtOAc 70 : 30) gave the product **8** as a colourless oil in 99% yield (16.0 g). $[\alpha]_D^{20}$ −163 (*c* 0.136, $CHCl_3$). ^{1}H-NMR (400 MHz, $CDCl_3$, δ): 7.34 (5H, m, Ar–H), 5.36 (1H, dd, $J_{2,1}$ = 1.6 Hz, $J_{2.3}$ = 3.2 Hz, H2), 5.27 (1H, dd, $J_{3.2}$ = 3.2 Hz, $J_{3.4}$ = 9.7 Hz, H3), 5.18 (1H, app s, H1), 4.76, 4.66 (1H, d, J = 11.3 Hz, CH_2), 4.20 (1H, m, H5), 3.57 (1H, app t, H4), 2.68 (4H, m, CH_2), 2.51 (2H, m, CH_2), 2.19, 2.18 (3H, s, CH_3), 1.37 (3H, d, $J_{6.5}$ = 6.3 Hz, H6), 1.30 (3H, t, J = 7.3 Hz, CH_2CH_3). ^{13}C-NMR ($CDCl_3$, δ): 206.1, 171.5, 169.9 (C═O), 137.9 (Cq), 128.3, 127.62, 127.59 (Ar–C), 81.1 (C1), 78.8 (C4), 74.8 (CH2), 72.3 (C3), 71.8 (C2), 68.1 (C5), 37.7 (CH_2), 29.6 (CH_3), 27.7, 25.2 (CH_2), 20.8 (CH_3), 17.7 (C6), 14.7 (CH_2CH_3). HRMS-ESI (*m*/*z*): $[M + Na]^+$ calculated for $C_{22}H_{30}O_7NaS$ 461.1610; found 461.1625. IR: 2931, 1743, 1368, 1231, 1096 cm^{-1}.

Compound 9

8 (16.00 g, 36.49 mmol) was dissolved in MeOH (50 mL) and a catalytic amount of NaOMe was added. After stirring at rt for 18 h the reaction was quenched with a catalytic amount of DOWEX, filtered and concentrated to give **9** as a white solid in 99% yield (14.33 g). $[\alpha]_D^{20}$ −156.8 (*c* 0.0051, $CHCl_3$). ^{1}H-NMR (400 MHz, $CDCl_3$, δ): 7.39 (5H, m, Ar–H), 5.27 (1H, d, $J_{1,2}$ = 1.4 Hz, H1), 4.77 (2H, dd, J = 11.4 Hz, J = 15.6 Hz, CH_2), 4.13 (1H, m, H5), 4.05 (1H, dd, $J_{2,1}$ = 1.4 Hz, $J_{2,3}$ = 3.4 Hz, H2), 3.91 (1H, dd, $J_{3,2}$ = 3.4 Hz, $J_{3,4}$ = 9.1 Hz, H3), 3.41 (1H, app t, J = 9.3 Hz, H4), 2.79 (2H, t, J = 6.6 Hz, CH_2), 2.63 (2H, m, CH_2CH$_3$), 2.61 (2H, t, J = 6.6 Hz, CH_2), 2.22 (3H, s, CH_3), 1.38 (3H, d, $J_{6,5}$ = 6.3 Hz, H6), 1.31 (3H, t, J = 7.4 Hz, CH_2CH_3). ^{13}C-NMR ($CDCl_3$, δ): 206.7, 173.3 (C═O), 138.2 (Cq), 128.7, 128.0, 127.9 (Ar–C), 83.6 (C1), 82.0 (C4), 75.0 (CH_2Bn), 72.6 (C2), 71.9 (C3), 67.7 (C5), 38.0 (CH_2), 29.9 (CH_3), 27.7 (CH_2), 25.1 (CH_2), 18.0 (C6), 14.9 (CH_2CH_3). HRMS-ESI (*m*/*z*): $[M + Na]^+$ calculate for $C_{20}H_{28}O_6NaS$ 419.1499; found 419.1490. IR: 3290, 2923, 1453, 1081 cm^{-1}.

Compound 10[44]

9 (250 mg, 0.63 mmol) was dissolved in DMF (4 mL) under N_2, NaH (35 mg, 0.88 mmol) and MeI (0.047 mL, 0.76 mmol) were added. After 18 h the reaction was quenched with *ca.* 2 mL MeOH, concentrated, redissolved in diethyl ether (20 mL), washed with water (2 × 15 mL) and sat. $NaHCO_3$ (2 × 15 mL), dried with $MgSO_4$, and concentrated. Purification by column chromatography (hexane–EtOAc 80 : 20) gave **10** as a yellow oil in 74% yield (146 mg) with the dimethylated compound, also a yellow oil, as a side product. $[\alpha]_D^{20}$ −220.5 (*c* 0.0069, $CHCl_3$). ^{1}H-NMR (400 MHz, $CDCl_3$, δ): 7.38 (5H, m, Ar-H), 5.41 (1H, d, $J_{1,2}$ = 1.2 Hz, H1), 4.93, 4.70 (1H, d, J = 11.2 Hz, CH_2), 4.07 (1H, m, H5), 3.91 (1H, dd, $J_{3,2}$ = 3.7 Hz, $J_{3,4}$ = 9.3 Hz, H3), 3.61 (1H, dd, $J_{2,1}$ = 1.2 Hz, $J_{2,3}$ = 3.7 Hz, H2), 3.51 (3H, s, CH_3), 3.33 (1H, app t, H4), 2.65 (2H, m, CH_2CH$_3$), 1.34 (3H, d, $J_{6,5}$ = 6.3 Hz, H6), 1.32 (3H, t, J = 7.4 Hz, CH_2CH_3).^{13}C-NMR ($CDCl_3$, δ): 138.5 (Cq), 128.4, 128.0, 127.7 (Ar–C), 82.5 (C4), 82.3 (C2), 80.1 (C1), 75.1 (CH_2Bn), 72.1 (C3), 67.6 (C5), 58.1 (CH_3), 25.2 (*C*H_2CH$_3$), 17.9 (C6), 15.0 (CH_2*C*H$_3$). HRMS-ESI (*m*/*z*): $[M + Na]^+$ calculated for $C_{16}H_{24}O_4NaS$ 335.1293; found 335.1292. IR: 3469, 2930, 1454, 1090 cm^{-1}.

Compound 11[44]

10 (580 mg, 1.86 mmol) was dissolved in pyridine (5 mL) and Ac_2O (5 mL) under N_2. After 18 h the mixture was quenched with iced water, diluted with EtOAc, washed with water (20 mL), dilute aq. $CuSO_4$ (6 × 20 mL), washed with water (20 mL), dried with $MgSO_4$, filtered and concentrated. Purification by column chromatography (hexane–EtOAc 80 : 20) gave **11** as a colourless oil 77% yield (518 mg). $[\alpha]_D^{20}$ −143.9

(*c* 0.0532, $CHCl_3$). ^{1}H-NMR (400 MHz, $CDCl_3$, δ): δ 7.34 (5H, m, Ar–H), 5.34 (1H, d, $J_{1,2}$ = 1.8 Hz, H1), 5.19 (1H, dd, $J_{3,2}$ = 3.3 Hz, $J_{3,4}$ = 9.5 Hz, H3), 4.70 (2H, dd, J = 11.4 Hz, J = 33.7 Hz, CH_2), 4.15 (1H, m, H5), 3.78 (1H, dd, $J_{2,1}$ = 1.8 Hz, $J_{2,3}$ = 3.3 Hz, H2), 3.60 (1H, app t, H4), 3.47 (3H, s, OCH_3), 2.65 (2H, m, CH_2CH_3), 2.08 (3H, s, CH_3), 1.35 (3H, d, $J_{6,5}$ = 6.2 Hz, H6), 1.32 (3H, t, J = 7.5 Hz, CH_2CH_3). ^{13}C-NMR ($CDCl_3$, δ): 170.2 (C═O), 138.3 (Cq), 128.4, 127.7, 127.6 (Ar–C), 80.9 (C1), 80.2 (C2), 79.4 (C4), 75.0 (CH_2), 74.1 (C3), 68.1 (C5), 58.6 (OCH_3), 25.3 (CH_2), 21.1 (CH_3), 17.9 (C6), 14.9 (CH_2CH_3). HRMS-ESI (*m*/*z*): [M + Na]$^+$ calculated for $C_{18}H_{26}O_5NaS$ 377.1399; found 377.1399. IR: 2932, 1737, 1369, 1233, 1084 cm^{-1}.

Compound 12

Trifluoromethanesulfonic anhydride (0.328 mL, 1.49 mmol) was added to a solution of dimethyl disulfide (0.195 mL, 2.3 mmol) in anhydrous DCM (1 mL) at 0 °C and reacted for 30 min at 0 °C to give a dark yellow solution. **11** (520 mg, 1.29 mmol) was dissolved in anhydrous DCM (7 mL) under N_2 at 0 °C along with methyl 4-hydroxybenzoate (296 mg, 1.94 mmol). 1.5 eq. of the activating Me_2S_2–Tf_2O solution was added. The mixture was reacted at 0 °C for 1 h, quenched with *ca.* 1 mL TEA, diluted with DCM (15 mL), washed with 1 M HCl (20 mL), sat. aq. $NaHCO_3$ (20 mL), H_2O (20 mL), dried with $MgSO_4$, filtered and concentrated. Purification by column chromatography (hexane–EtOAc 70 : 30) gave **12** as a pale yellow oil in 58% yield (330 mg). $[\alpha]_D^{18}$ −92.8 (*c* 0.0070, $CHCl_3$). ^{1}H-NMR (400 MHz, $CDCl_3$, δ): 8.02 (2H, m, Ar–H), 7.34 (5H, m, Ar-H), 7.14 (2H, m, Ar), 5.61 (1H, d, $J_{1,2}$ = 1.9 Hz, H1), 5.44 (1H, dd, $J_{3,2}$ = 3.4 Hz, $J_{3,4}$ = 9.4 Hz, H3), 4.72 (2H, dd, J = 11.2 Hz, J = 33.2 Hz, CH_2), 3.91 (3H, s, OCH_3), 3.90 (1H, m, H2), 3.83 (1H, m, H5), 3.65 (1H, app t, H3), 3.55 (3H, s, OCH_3), 2.13 (3H, s, CH_3), 1.30 (3H, d, $J_{6,5}$ = 6.2 Hz, H6). ^{13}C-NMR ($CDCl_3$, δ): 170.4 (C═O), 169.7, 159.8, 138.0 (Cq), 131.6, 128.5, 127.8, 127.6 (Ar–C), 124.1 (Cq), 115.9 (Ar–C), 95.2 (C1), 78.9 (C4), 78.3 (C2), 75.1 (CH_2), 73.5 (C3), 68.8 (C5), 59.6, 52.0 (OCH_3), 21.2 (CH_3), 17.0 (C6). HRMS-ESI (*m*/*z*): [M + Na]$^+$ calculated for $C_{24}H_{28}O_8Na$ 467.1682; found 467.1689. IR: 2936, 1718, 1606, 1279, 1233 cm^{-1}.

Compound 13

12 (97.3 mg, 0.219 mmol) was dissolved in 7 mL MeOH along with a catalytic amount of NaOMe and allowed to react for 18 h. The reaction was quenched with a catalytic amount of DOWEX, filtered and concentrated to give **13** as a pale yellow oil in 81% yield (71.3 mg). $[\alpha]_D^{18.6}$ −79.3 (*c* 0.097, $CHCl_3$). ^{1}H-NMR (400 MHz, $CDCl_3$, δ): 8.01 (2H, m, Ar), 7.34 (5H, m, Ar–H), 7.10 (2H, m, Ar–H), 5.65 (1H, d, $J_{1,2}$ = 1.6 Hz, H1), 4.94, 4.64 (1H, d, J = 10.9 Hz, CH_2), 3.90 (3H, s, CH_3), 3.82(1H, dd, $J_{2,1}$ = 1.6 Hz, $J_{2,3}$ = 3.3 Hz, H2), 3.80 (1H, dd, $J_{3,2}$ = 3.3 Hz, $J_{3,4}$ = 9.1 Hz, H3), 3.69 (1H, m, H5), 3.61 (3H, s, CH_3), 3.53 (1H, app t, H4), 1.27 (3H, d, $J_{6,5}$ = 6.1 Hz, H6). ^{13}C-NMR ($CDCl_3$, δ): 210.8, 166.6 (C═O), 159.8 (Cq), 131.5, 128.3, 127.9, 127.6 (Ar–C), 123.9 (Cq), 115.7 (Ar), 94.9 (C1), 81.2 (C3), 80.1 (C4), 77.0 (C2), 75.3 (CH2), 68.8 (C5), 59.4, 57.9, 51.8 (CH_3), 17.8 (C6). HRMS-ESI (*m*/*z*): [M + Na]$^+$ calculated for $C_{22}H_{26}O_7Na$ 425.1576; found 425.1565. IR: 2931, 1716, 1605, 1278, 1098 cm^{-1}.

Compound 14

13 (60.8 mg, 0.151 mmol) was dissolved in THF and a catalytic amount of palladium on carbon added. The mixture was first degassed by bubbling N_2 through the solution for 1 hour then saturated with H_2 by bubbling H_2 through the mixture for 30 min. This saturation was repeated periodically over 2 days and the reaction kept under H_2 throughout. When TLC analysis indicated the consumption of the starting material N_2 was again bubbled through before exposing the mixture to air. The palladium was filtered off and the resulting solution concentrated. Purification by column chromatography (acetone–toluene 20 : 80) furnished **14** as a colourless solid in 83% yield (39.4 mg). $[\alpha]_D^{22}$ −86.9 (*c* 0.0184, $CHCl_3$).^{1}H-NMR (400 MHz, $CDCl_3$, δ): 8.01, 7.11 (2H, d, J = 8.7 Hz, Ar–H), 5.67 (1H, d, $J_{1,2}$ = 1.4 Hz, H1), 3.94 (1H, dd, $J_{3,2}$ = 3.7 Hz, $J_{3,4}$ = 9.6 Hz, H3), 3.90 (3H, s, CO_2CH_3), 3.70 (1H, dd, $J_{2,1}$ = 1.4 Hz, $J_{2,3}$ = 3.7 Hz, H2), 3.67 (1H, m, H5), 3.56 (3H, s, OCH_3–), 3.47 (1H, app t, J = 9.6 Hz, H4), 1.27 (3H, d, $J_{6,5}$ = 6.5 Hz, H6). ^{13}C-NMR ($CDCl_3$, δ): 166.7 (C═O), 159.9 (Cq), 131.6 (Ar–C), 124.2 (Cq), 115.8 (Ar–C), 94.3 (C1), 79.9 (C2), 73.8 (C4), 71.3 (C3), 68.9 (C5), 59.3, 52.0 (OCH_3), 17.6 (C6). HRMS-ESI (*m*/*z*): [M − H]$^-$ calculated for $C_{15}H_{19}O_7$ 311.1131; found 311.1134. IR: 3426, 2933, 1718, 1281, 1106 cm^{-1}.

Compound 15[46]

9 (1.00 g, 2.52 mmol) was dissolved in DMF (10 mL) with NaH (121 mg, 3.03 mmol) and BnBr (0.375 mL, 3.03 mmol) under N_2. After 18 h the reaction was quenched with *ca.* 2 mL MeOH, concentrated, diluted with diethyl ether (75 mL), washed with water (2 × 50 mL) and sat. $NaHCO_3$ (2 × 50 mL), dried with $MgSO_4$, filtered, and concentrated. Purification by column chromatography (hexane–EtOAc 80 : 20) gave the product as a colourless oil in 87% yield (849 mg). $[\alpha]_D^{20}$ −139 (*c* 0.1, $CHCl_3$). ^{1}H-NMR (400 MHz, $CDCl_3$, δ): 7.39 (10H, m, Ar-H), 5.40 (1H, app s, H1), 4.95 (1H, d, J = 11.2 Hz, CH_2), 4.81 (1H, d, J = 11.7 Hz, CH_2), 4.70 (1H, d, J = 11.2 Hz, CH_2), 4.60 (1H, d, J = 11.7 Hz, CH_2), 4.10 (1H, m, H5), 3.97 (1H, dd, $J_{3,2}$ = 3.7 Hz, $J_{3,4}$ = 9.1 Hz, H3), 3.88 (1H, dd, $J_{2,1}$ = 1.4 Hz, $J_{2,3}$ = 3.7 Hz, H2), 3.41 (1H, app t, H4), 2.63 (2H, m, CH_2CH_3), 1.38 (3H, d, $J_{6,5}$ = 6.3 Hz, H6), 1.30 (3H, t, J = 7.4 Hz, CH_3). ^{13}C-NMR ($CDCl_3$, δ): 138.6, 137.6 (Cq), 128.7, 128.4, 128.2, 128.1, 128.0, 127.7 (Ar–C), 82.6 (C4), 81.0 (C1), 80.3 (C2), 75.1, 72.5 (CH_2), 72.2 (C3), 67.7 (C5), 25.2 (CH_2CH_3), 18. (C6), 15.0 (CH_2CH_3). HRMS-ESI (*m*/*z*): [M + Na]$^+$ calculated for $C_{22}H_{28}O_4NaS$ 411.1606; found 411.1599. IR: 3472, 2929, 1716, 1454, 1091 cm^{-1}.

Compound 16[46]

15 (803 mg, 2.07 mmol) was dissolved in pyridine (5 mL) and Ac_2O (5 mL) under N_2. After 18 h the mixture was quenched with iced water, diluted with EtOAc, washed with water (20 mL), dil. aq. $CuSO_4$ (6 × 20 mL), washed with water (20 mL), dried with $MgSO_4$, filtered and concentrated. Purification by column chromatography (hexane–EtOAc 90 : 10) gave

16 as pale yellow oil 96% yield (859 mg). $[\alpha]_D^{20}$ −73.3 (*c* 0.105, $CHCl_3$). ^{1}H-NMR (400 MHz, $CDCl_3$, δ): 7.36 (10H, m, Bn), 5.30 (1H, d, $J_{1,2}$ = 1.7 Hz, H1), 5.18 (1H, dd, $J_{3,2}$ = 3.4 Hz, $J_{3,4}$ = 9.5 Hz, H3), 4.72 (2H, dd, J = 11.7 Hz, J = 30.1 Hz, CH_2), 4.71 (1H, d, J = 12.6 Hz, CH_2), 4.57 (1H, d, J = 12.2 Hz, CH_2), 4.17 (1H, m, H5), 4.00 (1H, dd, $J_{2,1}$ = 1.7 Hz, $J_{2,3}$ = 3.4 Hz, H2), 3.70 (1H, app t, H4), 2.62 (2H, m CH_2), 1.99 (3H, s, CH_3), 1.38 (3H, d, $J_{6,5}$ = 6.2 Hz, H6), 1.29 (3H, t, J = 7.4 Hz, CH_2CH_3). ^{13}C-NMR ($CDCl_3$, δ): 170.1 (C═O), 138.3, 137.8 (Cq), 128.5, 128.4, 127.96, 127.95, 127.7, 127.6 (Ar–C), 81.7 (C1), 79.4 (C4), 77.7 (C2), 74.9 (CH_2), 74.0 (C3), 72.6 (CH_2), 68.2 (C5), 25.3 (CH_2), 21.1 (CH_3), 18.0 (C6), 15.0 (CH_2CH_3). HRMS-ESI (*m*/*z*): $[M + Na]^+$ calculated for $C_{24}H_{30}O_5NaS$ 453.1712; found 435.1714. IR: 2930, 1737, 1454, 1292, 1080 cm^{-1}.

Compound 17

13 (118 mg, 0.293 mmol) was dissolved in anhydrous DCM (5 mL) under N_2. **16** (152 mg, 0.352 mmol) was added followed by NIS (106 mg, 0.352 mmol) and a catalytic amount (*ca.* 10 μL) of TMS·OTf at 0 °C. After 18 h at rt the mixture was quenched with *ca.* 1 mL of TEA, washed with sat. $NaHCO_3$ solution (15 mL), dried with $MgSO_4$, filtered and concentrated. Purification by column chromatography (hexane–EtOAc 80 : 20) gave **17** as a pale yellow oil in 95% yield (214 mg). $[\alpha]_D^{20}$ −34.9 (*c* 0.103, $CHCl_3$). ^{1}H-NMR (600 MHz, $CDCl_3$, δ): 8.03 (2H, m, Ar–H), 7.33 (15H, m, Ar–H), 7.12 (2H, m, Ar–H), 5.62 (1H, d, $J_{1,2}$ = 1.6 Hz, H1b), 5.32 (1H, dd, $J_{3,2}$ = 3.1 Hz, $J_{3,4}$ = 9.5 Hz, H3a), 5.17 (1H, d, $J_{1,2}$ = 1.5 Hz, H1a), 4.87 (1H, d, J = 11.5 Hz, CH_2), 4.77 (1H, d, J = 11.3 Hz, CH_2), 4.69 (2H, d, J = 11.7 Hz, CH_2), 4.47, 4.32 (1H, d, J = 12.1 Hz, CH_2), 4.25 (1H, dd, $J_{3,2}$ = 3.1 Hz, $J_{3,4}$ = 9.5 Hz, H3b), 4.06 (1H, m, H5b), 3.94 (1H, dd, $J_{2,1}$ = 1.9 Hz, $J_{2,3}$ = 1.5 Hz, H2a), 3.92 (3H, s, CO_2CH_3), 3.80 (1H, dd, $J_{2,1}$ = 1.9 Hz, $J_{2,3}$ = 3.0 Hz, H2b), 3.75 (1H, m, H5b), 3.71 (1H, app t, H4a), 3.62 (1H, app t, H4b), 3.60 (3H, s, OCH_3), 1.98 (3H, s, CH_3), 1.41 (3H, $J_{6.5}$ = 6.3 Hz, H6a), 1.27 (3H, d, $J_{6,5}$ = 6.2 Hz, H6b). ^{13}C-NMR ($CDCl_3$, δ): 170, 166.5 (C═O), 159.8 (Cq), 138.3, 138.1, 137.8 (Ar–C), 137.1 (Cq), 128.3, 128.24, 128.23, 128.12, 128.08, 128.0, 127.9, 127.85, 127.82, 127.6, 127.59, 127.55, 127.54, 127.51, 127.4, 115.8 (Ar–C), 100.0 (C1b), 94.7 (C1a), 79.9 (C2b), 79.7(C4b), 79.0 (C3b), 78.9 (C4a), 76.9 (C2a), 74.9, 74.8 (CH_2), 73.4 (C3a), 72.8 (CH_2), 69.1 (C5b), 68.4(C5a), 59.0 (OCH_3), 51.8 (CO_2CH_3), 20.9 (CH_3), 18.1 (C6a), 17.8 (C6b). HRMS-ESI (*m*/*z*): $[M + Na]^+$ calculated for $C_{44}H_{50}O_{12}Na$ 793.3200; found 793.3180. IR 2932, 1718, 1605, 1234, 1098 cm^{-1}.

Compound 18

17 (91.7 mg, 0.126 mmol) was dissolved in 10 mL MeOH, a catalytic amount of NaOMe was added and the mixture stirred at rt for 48 h. The reaction was quenched with a catalytic amount of DOWEX, filtered and concentrated to give the product **18** as a pale yellow oil in 95% yield (69.0 mg). $[\alpha]_D^{20}$ −50.9 (*c* 0.159, $CHCl_3$). ^{1}H-NMR (400 MHz, $CDCl_3$, δ): 8.04 (2H, m, Ar), 7.30 (15H, m, Ar–H), 7.14 (2H, m, Ar–H), 5.63 (1H, app s, H1b), 5.26 (1H, app s, H1a), 4.95(1H, d, J = 10.6 Hz, CH_2), 4.83 (1H, J = 11.1 Hz, CH_2), 4.73 (2H, m, CH_2), 4.45 (1H, d, J = 11.9 Hz, CH_2), 4.29 (1H, dd, $J_{3,2}$ = 3.0 Hz, $J_{3,4}$ = 9.5 Hz, H3b), 4.24 (1H, d, J = 11.9 Hz, CH_2), 4.06 (1H, dd, $J_{3,2}$ = 3.6 Hz, $J_{3,4}$ = 9.3 Hz, H3a), 3.95 (1H, m, H5a), 3.93 (3H, s, OCH_3), 3.78 (3H, m, H2a, H2b, H5b), 3.61 (1H, app t, H4b), 3.59 (3H, s, OCH_3) 3.40 (1H, app t, H4a), 1.42 (3H, d, $J_{6,5}$ = 6.3 Hz, H6a), 1.29 (3H, d, $J_{6,5}$ = 5.8 Hz, H6b). ^{13}C-NMR ($CDCl_3$, δ): 166.7(C═O), 159.9, 138.5, 138.3, 137.7 (Cq), 131.7, 128.5, 128.46, 128.4, 128.0, 127.9, 127.8, 127.7, 127.6, 127.0 (Ar–C), 124.1 (Cq), 115.9 (Ar–C), 99.4 (C1a). 94.9 (C1b), 82.1 (C4a), 80.3 (C4b), 80.1(C2b), 79.2 (C2a), 78.4 (C3b), 75.1, 75.0, 72.7 (CH_2), 71.6 (C3a), 69.3 (C5b), 68.0 (C5a), 59.2, 52.0 (OCH_3), 18.2 (C6b), 18.0 (C6a). HRMS-ESI (*m*/*z*): $[M + Na]^+$ calculated for $C_{43}H_{52}O_{10}Na$ 751.3458; found 751.3466. IR: 3376, 2926, 1717, 1606, 1279 cm^{-1}.

Compound 20

19 (91.7 mg, 0.1258) was dissolved in anhydrous DCM (5 mL) under N_2. 12 (28 mg, 0.112 mmol) was added followed by NIS (40.0 mg, 0.1792 mmol) and a catalytic amount (*ca.* 10 μL) of TMS·OTf at 0 °C. After 18 h at rt the mixture was quenched with *ca.* 1 mL of TEA, washed with sat. $NaHCO_3$ solution (15 mL), dried with $MgSO_4$, filtered and concentrated. Purification by column chromatography (hexane–EtOAc 80 : 20) furnished **20** as a pale yellow oil in 67% yield (69.0 mg). $[\alpha]_D^{20}$ −82.0 (*c* 0.1, $CHCl_3$). ^{1}H-NMR (600 MHz, $CDCl_3$, δ): 8.02 (2H, m, Ar), 7.34 (15H, m, Ar–H), 7.12 (2H, m, Ar), 5.63 (1H, d, $J_{1,2}$ = 1.4 Hz, H1c), 5.26 (1H, d, $J_{1,2}$ = 1.3 Hz, H1b), 5.24 (1H, d, $J_{1,2}$ = 1.5 Hz, H1a), 5.21 (1H, d, J = 11.3 Hz, CH_2), 4.89, 4.70 (1H, d, J = 11.6 Hz, CH_2), 4.64 (1H, d, J = 11.3 Hz, CH_2), 4.59, 4.28 (1H, d, J = 12.2 Hz, CH_2), 4.27 (1H, dd, $J_{2,3}$ = 3.2 Hz, $J_{3,4}$ = 9.7 Hz, H3c), 4.12 (1H, dd, $J_{2,3}$ = 3.2 Hz, $J_{3,4}$ = 9.5 Hz, H3b), 3.98 (1H, m, H5b), 3.91 (3H, s, OCH_3), 3.85 (1H, dd, $J_{2,1}$ = 1.8 Hz, $J_{2,3}$ = 3.2 Hz, H2b), 3.81 (1H, dd, $J_{2,1}$ = 1.8 Hz, $J_{2,3}$ = 3.2 Hz, H2c), 3.76 (1H, m, H5c), 3.71 (1H, m, H5a), 3.66 (1H, app t, J = 9.4 Hz, H4b), 3.60 (1H, app t, H4c), 3.59 (3H, s, OCH_3), 3.58 (2H, m, H2a, H3a), 3.56, 3.53, 3.36 (3H, s, OCH_3), 3.24 (1H, app s, H4a), 1.37 (3H, d, $J_{6,5}$ = 6.3 Hz, H6b), 1.25 (3H, d, $J_{6,5}$ = 6.3 Hz, H6c), 1.01 (3H, d, $J_{6,5}$ = 6.3 Hz, H6a). ^{13}C-NMR ($CDCl_3$, δ): 166.7 (C═O), 156.0, 139.1, 138.4, 138.3 (Cq), 131.6, 128.4, 128.3, 128.2, 127.7, 127.5, 127.4, 127.3, 227.2, 127.1 (Ar–C), 124.1 (Cq), 115.8 (Ar–C), 99.6 (H1c), 99.4 (H1a), 94.7 (H1), 80.4 (H2c), 80.1 (H2), 79.77 (H4), 79.75 (H4b), 79.4 (H3, H3b),79.2 (H4c), 78.9 (H2b), 77.9 (H3c), 75.0, 74.7, 71.5 (CH_2), 69.2 (H5), 68.9 (H5b), 67.4 (H5c), 61.7, 59.2, 59.0, 58.0, 52.0 (OCH_3), 18.2 (H6b), 17.9 (H6), 16.7 (H6c). HRMS-ESI (*m*/*z*): $[M + Na]^+$ calculated for $C_{51}H_{64}O_{15}Na$ 939.4143; found 939.4139. IR: 2917, 1750, 1606, 1509, 1220 cm^{-1}.

Compound 21

20 (118 mg, 0.129 mmol) was dissolved in THF and a catalytic amount of palladium on carbon added. The mixture was degassed under vacuum and then stirred vigorously under a hydrogen atmosphere until TLC analysis indicated the consumption of the starting material. A N_2 atmosphere was introduced before exposing the mixture to air. The palladium was filtered off and the resulting solution concentrated. Purification by column chromatography (40 : 60 acetone–toluene)

furnished **21** as a pale yellow, amorphous solid in 70% yield (58.4 mg). $[\alpha]_D^{20}$ −89.3 (*c* 0.103, $CHCl_3$). ^{1}H-NMR (600 MHz, $CDCl_3$, δ): 8.03, 7.14 (2H, m, Ar–H), 5.65 (1H, d, $J_{1,2}$ = 1.6 Hz, H1c), 5.165 (1H, d, $J_{1,2}$ = 3.3 Hz, H1a), 5.162 (1H, d, $J_{1,2}$ = 1.4 Hz, H1b), 4.13 (1H, dd, $J_{2,1}$ = 1.4 Hz, H2b), 4.08 (1H, m, H4a), 4.05 (1H, dd, $J_{3,2}$ = 3.3 Hz, $J_{3,4}$ = 9.0 Hz, H3c), 3.96 (1H, m, H5b), 3.92 (3H, s, CO_2Me), 3.82 (1H, dd, $J_{3,2}$ = 3.3 Hz, $J_{3,4}$ = 9.6 Hz, H3b), 3.80 (1H, dd, $J_{2,1}$ = 1.7 Hz, $J_{2,3}$ = 3.3 Hz, H2c), 3.73 (1H, m, H5c), 3.72 (1H, m, H4c), 3.69 (1H, dd, $J_{2,1}$ = 3.3 Hz, $J_{2,3}$ = 9.9 Hz, H2a), 3.68 (1H, m, H4b), 3.66 (1H, m, H4a), 3.63, 3.61, 3.55, 3.54 (3H, s, OCH_3), 3.50 (1H, app d, J = 2.5 Hz, H3a), 1.40 (3H, d, $J_{6,5}$ = 6.4 Hz, H6b), 1.30 (3H, d, $J_{6,5}$ = 5.6 Hz, H6c), 1.30 (3H, d, $J_{6,5}$ = H6a). ^{13}C-NMR ($CDCl_3$, δ): 166.8 (C═O), 160.0 (Cq), 131.6 (Ar–C), 124.2 (Cq), 115.9 (Ar–C), 102.2 (C1b), 100.9 (C1a), 94.5 (C1c), 83.2 (C3b), 81.0 (C4a), 79.8 (C3c), 79.0 (C3a), 78.8 (C2 s), 71.6 (C4b), 71.5 (C5c), 71.2 (C2b), 69.5 (C4c), 68.7 (C5b), 67.6 (C4a), 61.9, 60.3, 58.7, 57.7 (OCH_3), 52.0 (CO_2CH_3), 17.9 (C6b), 17.7 (C6c), 16.6 (C6a). HRMS-ESI (*m*/*z*): $[M + Na]^+$ calculated for $C_{30}H_{46}O_{15}Na$ 669.2734 found 669.2763. ν_{max}(L) 3443, 2928, 1717, 1283, 1091 cm^{-1}.

Compound 22

1 (2.00 g, 6.04 mmol) was dissolved in anhydrous DCM (20 mL) at 0 °C under N_2. Methyl-4-hydroxybenzoate (1.20 g, 7.85 mmol) was added followed by $BF_3{\cdot}OEt_2$ (3.73 mL, 30.2 mmol) in increments. After 18 h the mixture was quenched by treatment with sat. $NaHCO_3$ solution (*ca*. 50 mL) and solid $NaHCO_3$. The organic layer was filtered, dried with $MgSO_4$, filtered and concentrated. Purification by column chromatography (hexane–EtOAc 80 : 20) gave **22** as a colourless solid in 96% yield (2.459 g). $[\alpha]_D^{20}$ −88.8 (*c* 0.072, $CHCl_3$). ^{1}H-NMR (400 MHz, $CDCl_3$, δ): 8.02, 7.13 (2H, m, Ar–H), 5.55 (1H, d, $J_{1,2}$ = 1.8 Hz, H1), 5.52 (1H, dd, $J_{3,2}$ = 3.5 Hz, $J_{3,4}$ = 10.1 Hz, H3), 5.45 (1H, dd, $J_{2,1}$ = 1.8 Hz, $J_{2,3}$ = 3.5 Hz, H2), 5.18 (1H, app t, H4), 3.95 (1H, m, H5), 3.91(3H, s, OCH_3), 2.22, 2.07, 2.05 (3H, s, CH_3), 1.21 (3H, d, $J_{6,5}$ = 6.2 Hz, H6). ^{13}C-NMR ($CDCl_3$, δ): 170.0, 166.6, 159.3 (C═O), 131.6 (Ar), 124.5 (Cq), 115.8 (Ar), 95.3 (C1), 90.7 (Cq), 70.8 (H4), 69.5 (C2), 68.8 (C3), 67.5 (C5), 52.0 (OCH3), 20.9, 20.8, 20.7 (CH_3), 17.4 (C6). HRMS-ESI (*m*/*z*): $[M + Na]^+$ calculated for $C_{20}H_{24}O_{10}Na$ 447.1267; found 447.1259. IR: 2986, 1746, 1606, 1369, 1215 cm^{-1}.

Compound 23

22 (1.07 g, 2.51 mmol) was dissolved in MeOH (15 mL) with a catalytic amount of NaOMe. After 18 h the reaction was quenched with a catalytic amount of DOWEX, filtered and concentrated to give **23** as a colourless solid in 98% yield (734 mg). $[\alpha]_D^{20}$ −117.6 (*c* 0.142, MeOH). ^{1}H-NMR (400 MHz, CD_3OD, δ): 7.96, 7.13 (2H, m, Ar–H), 5.52 (1H, d, $J_{1,2}$ = 1.2 Hz, H1), 4.04 (1H, dd, $J_{2,1}$ = 1.2 Hz, $J_{2,3}$ = 3.3 Hz, H2), 3.86 (3H, s, CH_3), 3.83 (1H, dd, $J_{3,2}$ = 3.3 Hz, $J_{3,4}$ = 9.5 Hz, H3), 3.57 (1H, m, H5), 3.46 (1H, app t, H4), 1.21 (3H, d, $J_{6,5}$ = 6.1 Hz, H6). ^{13}C-NMR (CD_3OD, δ): 167.2 (C═O), 160.9 (Cq), 131.6 (Ar–C), 124.0 (Cq), 116.1 (Ar–C), 98.7 (C1), 72.7 (C4), 71.1 (C3), 70.8 (C2), 70.0 (C5), 51.5 (OCH_3), 17.0 (C6). HRMS-ESI (*m*/*z*): $[M + Cl]^-$ calculated for $C_{14}H_{18}O_7Cl$ 333.0741; found 333.0750. IR: 3365, 2937, 1700, 106, 1436, 1238 cm^{-1}.

Mice. C3H/HeJ mice were purchased from Harlan UK. All mice were maintained according to EU regulations and experiments were performed under licence from the Department of Health and Children and with approval from the Trinity College Dublin Bioresources Ethics Committee.

Spleen cells. Cells were isolated from the spleens of C3H/HeJ mice. 1.5×10^6 cells per mL were activated on 96-well flat-bottomed tissue-culture plates (Greiner) that had been coated with 0.4 μg anti-CD3e (BD Pharmingen) for 3 h at 37 °C, and then washed 3 times with sterile PBS. Spleen cells were cultured in medium alone or with *p*-HBad I, *p*-HBad II or UM-*p*-HBad (100 μM). Supernatants were collected after 4 d and IFNγ (BD Pharmingen), IL-17 and IL-10 (R&D Systems) concentrations were determined by ELISA. Spleen cells were stimulated for 5 h with PMA (0.1 μg mL^{-1}) and ionomycin (0.5 μg mL^{-1}) and for the final 4 h with brefeldin A (10 μg mL^{-1}). Cells were washed, and blocked with Fcγ blocker (BD Pharmingen 1 μg mL^{-1}) before staining with aqua Live/Dead stain (Invitrogen). Extracellular staining for surface CD3, CD4 and CD8 (eBioscience) was carried out and then spleen cells were fixed and permeabilized (Intracellular Fixation and Permeabilization kit (eBioscience)) and stained intracellularly for IFNγ (eBioscience). Flow cytometric analysis was performed using a BD LSRFortessa cell analyser (BD Biosciences).

Bone-marrow derived macrophages (BMDMs). Bone marrow-derived macrophages (BMDMs) were prepared by culturing bone marrow cells from C3H/HeJ mice in medium supplemented with 15% macrophage colony-stimulating factor (M-CSF). M-CSF was collected in the supernatant of L929 cells in culture. On day 4, fresh medium containing 20% MCSF was added to the cell cultures. On day 7 cells were removed from the culture flasks and re-suspended in fresh medium containing 15% M-CSF. BMDMs were cultured with medium alone or irradiated H37Rv (10 : 1), with or without *p*-HBad I, *p*-HBad II or UM-*p*-HBad (1, 10 or 100 μM). Supernatants were recovered after 24 h and IL-12p40 (BD Pharmingen) and TNF-α (R&D Systems) concentrations were determined by ELISA.

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