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Chemical Synthesis of a Glycolipid Library by a Solid-Phase Strategy Allows Elucidation of the Structural Specificity of Immunostimulation by Rhamnolipids**

Jörg Bauer,[a, b] Klaus Brandenburg,[c] Ulrich Zähringer,[c] and Jörg Rademann*[a, d]

Abstract: The first synthesis of a glycolipid library by hydrophobically assisted switching phase (HASP) synthesis is described. HASP synthesis enables flexible switching between solution-phase steps and solid-supported reactions conducted with molecules attached to a hydrophobic silica support. A library of glycolipids derived from the lead compound **1**—a strongly immunostimulatory rhamnolipid—with variations in the carbohydrate part, the lipid components, and the stereochemistry of the 3-hydroxy fatty acids was designed and synthesized. The enantioselective synthesis of the 3-hydroxy fatty acid building blocks was achieved by employing asymmetric hydrogenation of 3-oxo fatty acids. Glycolipids were prepared by this approach without any intermediary isolation steps, mostly in excellent yields. Final deprotection to the carboxylic acids was accomplished by enzymatic ester cleavage. All prepared rhamnolipids were tested for their immunostimulatory properties against human monocyte cells by assaying the secretion of the cytokine tumor necrosis factor α (TNFα) into the medium. The observed structure–activity relationships of rhamnolipids indicate a specific, recognition-based mode of action, with small structural variations in the rhamnolipids resulting in strong effects on the immunostimulatory activities of the rhamnolipids at low micromolar concentrations.

Keywords: combinatorial chemistry · glycolipids · innate immunity · rhamnolipids · solid-phase synthesis

Introduction

Glycolipids are structurally heterogeneous membrane components found in all species from bacteria to man. Bacterial glycolipids, thanks to their enormous structural diversity, serve as efficient agonists (pathogen-associated molecular patterns, PAMPs)[1] for the receptors of the innate immune system (pathogen recognition receptors, PRRs).[2] The immunostimulatory effects of glycolipids can lead to septic shock[3] and are mediated in a very specific way through the toll-like receptors TLR-2 and TLR-4.[4] Strong immunostimulatory properties were recently discovered in the rhamnolipid RL-2,$2_{14,14}$ (**1**), an L-rhamnose-containing glycolipid, triggering the excretion of cytokines[5a] and antibacterial peptides[5b] in human cells. The activation was independent of the known glycolipid receptors TLR-2 and TLR-4,[5a] suggesting a relevant alternative antibacterial strategy of the innate immune system, such as the activation of natural killer T cells by α-galactosylceramide.[2a]

For investigation of the biological structure–activity relationships of rhamnolipids, a synthetic rhamnolipid library featuring systematic variation of the lipid and of the carbohydrate parts was an essential tool. Carbohydrate-based li-

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braries are difficult to obtain, however, and suitable methods to achieve them require further development.[6] The synthesis of glycolipids is even more demanding, due to the amphiphilic character hampering all synthesis and isolation steps.[7] No example of a solid-supported library synthesis of rhamnolipids (or any other glycolipids) has been reported so far, and only a single report has described the preparation of a rhamnolipid in standard solution synthesis.[8]

Therefore, we embarked on the development of a synthetic strategy that would be broadly applicable for the preparation of glycolipid libraries. Recently, we found that oligosaccharides can be synthesized efficiently in a solid-supported manner by employing a specifically designed dilipid anchoring molecule for quantitative and reversible retention on a hydrophobic solid phase.[9] These initial findings inspired the idea that hydrophobically assisted switching phase (HASP) synthesis might be an ideal basis for the preparation of glycolipid libraries, which we have investigated in this contribution.

Results and Discussion

The lead rhamnolipid RL-2,2$_{14,14}$-COOH **1** (Figure 1), isolated from *Burkholderia pseudomallei* and *Pseudomonas aeruginosa* (together with the shorter-fatty-acid compound RL-2,2$_{12,12}$-COOH, **30**), is based on two β-(*R*)-hydroxymyristic acid components [(14:0(3-OH)] connected through an ester linkage.[5a,10] We designed a rhamnolipid library representing variations in the amphiphilic character of such molecules by introducing specific modifications both in the fatty acid part and in the sugar component. This was achieved particularly through variation in the number, lengths, and stereochemistry of the β-hydroxylated carboxylic acids and in the number of sugars, and also through reduction of the free carboxylate to an uncharged alcohol moiety.

Achiral β-ketoesters **3**$_n$ of different lengths were generated by C-acylation of Meldrum's acid (**2**, Scheme 1).[11] Asymmetric reduction of the ketones **3** in the presence of a chiral ruthenium 2,2′-bis(diphenylphosphino)-1,1′-binaphthyl (BINAP) catalyst yielded the enantiomerically pure β-hydroxy esters (*R*)-**4**$_n$ and (*S*)-**4**$_n$.[12] These secondary alcohols were obtained in high yields and with optical purities, as determined by NMR spectroscopy and chiral GC separation of the corresponding Mosher's ester derivatives **5**$_n$ (Figure 2).[13] Subsequent saponification and silylation furnished the corresponding 3-*O*-triethylsilyl-substituted carboxylic acids **6**$_n$ and **7**$_n$ as optically pure key building blocks (Scheme 1). For the synthesis of rhamnolipid alcohols, the terminally protected 1,3-diol building block **9** was required. This could be accessed via the 3-(2-methoxyethoxy)methyl-protected acetate **8**.

After the esterification of **6**$_i$ and **4**$_j$, C_{18} reversed-phase silica support (RP-18) was added to the reaction mixture, the solvents were evaporated, and all monolipid starting materials were easily and quantitatively removed by filtration with MeOH/water (Scheme 2). Subsequently, the silylated esters were deprotected with dilute trifluoroacetic acid (TFA) while attached to the solid support. After washing and filtration, $MgSO_4$ was added and the diastereomerically pure 3-hydroxy dilipid compounds **12**$_{i,j}$ were desorbed quantitatively with CH_2Cl_2. The pure products obtained from the previous solid-phase step were glycosylated with an excess (1.3 equiv) of rhamnose donor **13** in CH_2Cl_2.[9] The progress of the reaction was monitored by TLC and MALDI-MS. Workup of **14**$_{i,j}$ (and **17**$_{i,j}$ and **20**$_{i,j}$) was performed by a phase switch from solution phase back to the RP-18 solid support. Removal of the temporary phenoxyacetate (POAc) protecting group at the 2-position of rhamnose was effected on the solid support, as was the final removal of the butane-2,3-dione (BDA) protective group, yielding **16**$_{i,j}$ (and **19**$_{i,j}$ and **22**$_{i,j}$). For the construction of higher glycosylated rhamnolipid methyl esters, compounds **15**$_{i,j}$ (**18**$_{i,j}$) were glycosylated in a second (third) HASP reaction cycle, yielding pure products without the need for a single purification step (Figure 3).

Cleavage of the methyl ester group was most successful under enzymatic conditions. Results of a primary screen of a set of commercially available lipases (Table 1) and a secondary screen for the optimal solvent conditions (data not shown) led us to use the solid-supported lipase of *Candida antarctica*, which furnished the rhamnolipid acids in good to excellent yields and with exceptionally broad substrate toler-

Abstract in German: *Die erste Synthese einer Glycolipidbibliothek unter Verwendung der hydrophob unterstützten phasenwechselsynthese—hydrophobically assisted switching phase (HASP) synthesis—wird beschrieben. Das HASP-Synthesekonzept ermöglicht den flexiblen Wechsel zwischen Reaktionsschritten in Lösung und an der Festphase, bei denen die Moleküle an einem hydrophoben Kieselgelträger immobilisiert sind. Abgeleitet von der Leitverbindung **1**, einem stark immunstimulierendem Rhamnolipid, wurde eine Glycolipidbibliothek entworfen und synthetisiert, die Variationen im Kohlenhydratteil, den Lipidmuster und der Konfiguration der 3-Hydroxyfettsäuren enthielt. Die enantioselektive Synthese von 3-Hydroxyfettsäuren wurde durch die asymmetrische Hydrierung von 3-Oxofettsäuren realisiert. Mit diesem Ansatz wurden Glycolipide in exzellenten Ausbeuten hergestellt, ohne dass eine einzige Isolierung von Zwischenprodukten notwendig war. Die abschließende Entschützung zu den Carbonsäuren wurde durch enzymatische Esterspaltung erreicht. Alle hergestellten Rhamnolipide wurden auf ihre immunstimulierende Wirkung gegenüber menschlichen Monozyten getestet, indem die Ausschüttung des Cytokines Tumornekrosefaktor α (TNFα) in das Zellmedium gemessen wurde. Die beobachteten Struktur–Aktivitätsbeziehungen von Rhamnolipiden deuten auf einen spezifischen, Erkennungs-basierten Wirkmechanismus hin. Geringfügige strukturelle Variationen der Rhamnolipide führten zu einem starken Effekt auf die immunstimulierende Aktivität der Rhamnolipide bei Konzentrationen im unteren mikromolaren Bereich.*

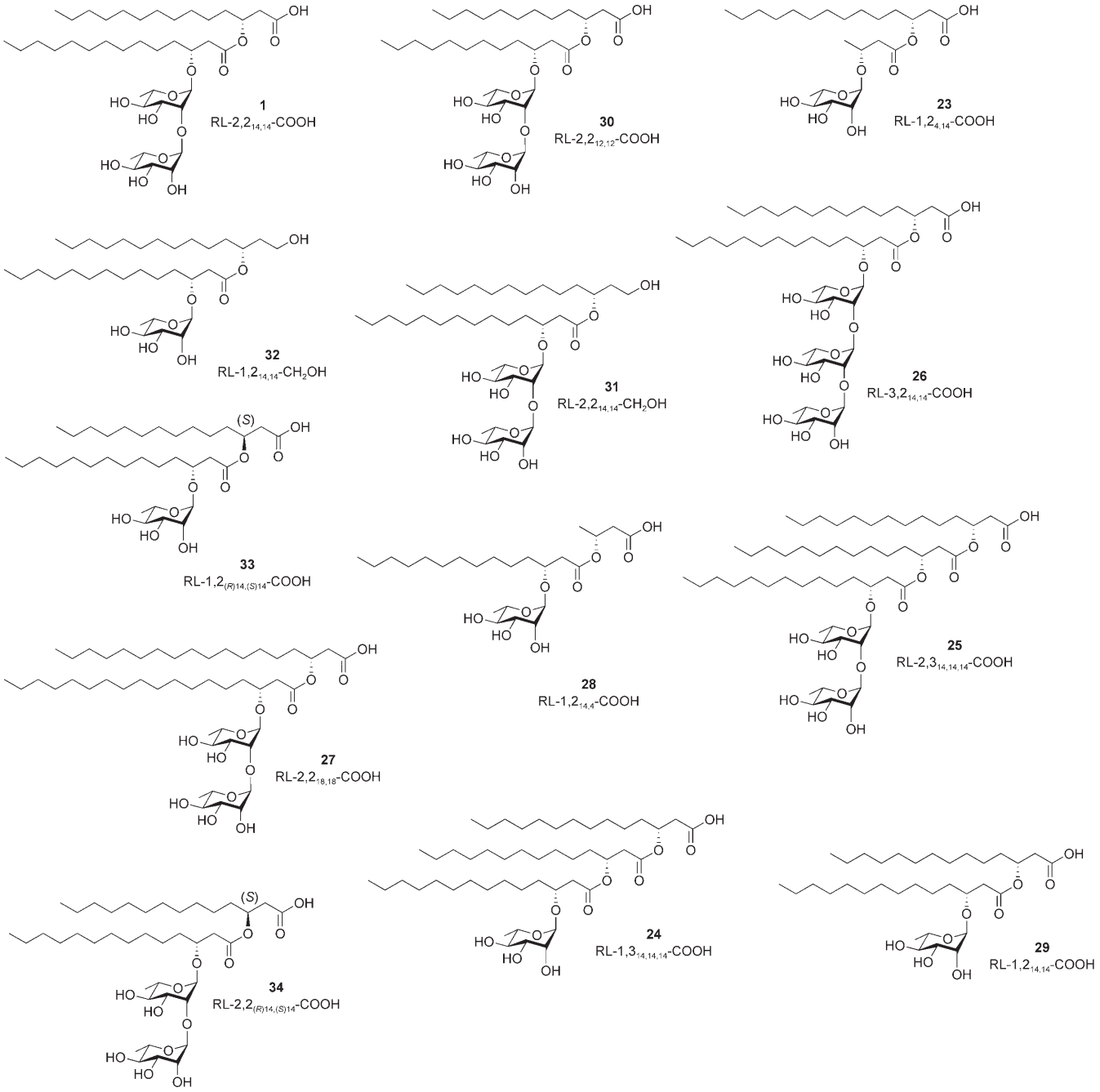
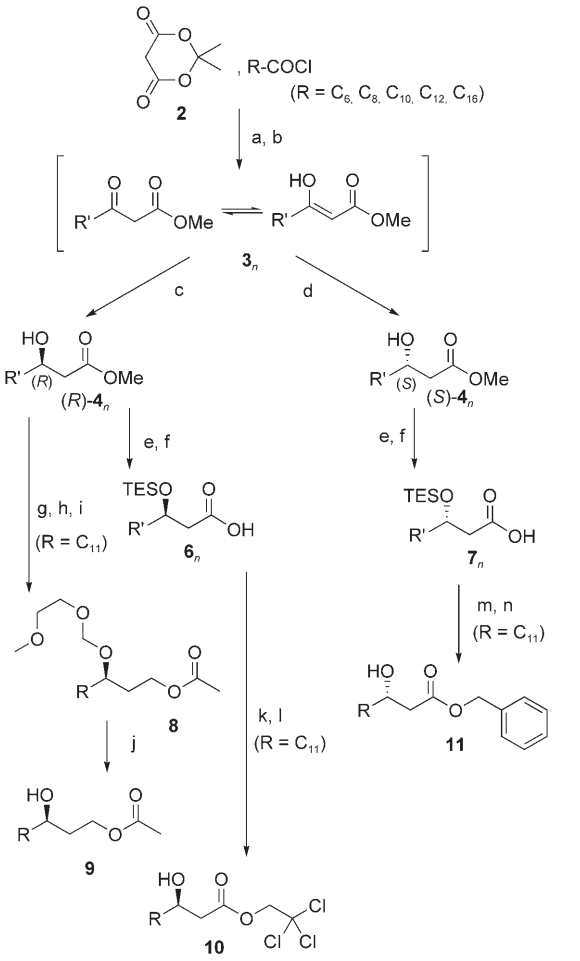


Figure 1. Rhamnolipid library obtained by HASP synthesis.

ance (Table 2). All the (*R*,*R*′)-configured rhamnolipid methyl esters $\mathbf{16}_{i,j}$, $\mathbf{19}_{i,j}$, and $\mathbf{22}_{i,j}$ could be hydrolyzed to the corresponding diastereomeric RL-acids, however, the (3*S*)-configured fatty acid methyl esters resisted successful substrate recognition by the enzyme. Therefore, lipid esters amenable to chemical deprotection were synthesized and employed for HASP construction of the corresponding RL esters. Because the trichloroethyl ester **10** underwent transesterification upon treatment with $MeNH_2$ in MeOH, the benzyl ester **11** was employed to access (*R*,*S*′)-configured rhamnolipid acids (Scheme 3). Excellent yields of both (*R*,*S*′)-RL acids, formed by employing the benzyl ester **11**, and rhamnolipid alcohols, synthesized from the acetate **10**, were accomplished through analogous HASP cycles (Table 2).

To assay the immunostimulation produced by the individual rhamnolipids (Figure 1), human mononuclear cells were treated with a rhamnolipid preparation and the amount of the cytokine tumor necrosis factor (TNFα) secreted into the medium was determined by conducting sandwich-ELISA[5a] (Figure 4).



Scheme 1. Synthesis of enantiopure β-hydroxy lipid building blocks from Meldrum's acid (**2**). a) CH_2Cl_2, pyridine, 1 h (0 °C), 77.9 %; b) MeOH, 3 h, reflux; c) 0.1 mol % (*R*)-BINAP-Ru^{II}, MeOH, 5 bar H_2, 5 h, 100 °C, 95.6 %, optical purity 98.3 % er determined by ^{1}H NMR as %dr (diastereomeric ratio) of the corresponding (*R*,*R*)-Mosher's ester $\mathbf{5}_{14}$; d) identical to (c), but with (*S*)-BINAP; e) LiOH, MeOH/H_2O (4:1), 12 h, 97.6 %; f) triethylsilyl chloride, pyridine, 2 h, 60 °C, 85.6 %; g) 2-methoxyethoxymethyl chloride, DIPEA, CH_2Cl_2, 20 h, 93 %; h) $LiAlH_4$, THF, 2 h, 0 °C, 99 %; i) Ac_2O, cat. DMAP, pyridine, 12 h, 92 %; j) dry $ZnBr_2$, CH_2Cl_2, 16 h, 87 %; k) trichloroethanol, EDC, cat. DMAP, CH_2Cl_2, 12 h; l) TFA, 10 min, 62 % (2 steps); m) benzyl alcohol, EDC, cat. DMAP, CH_2Cl_2, 12 h, 81 %; n) TFA, CH_2Cl_2, 10 min, 98 %. Yields and purities given refer to the corresponding myristic acid derivatives (R = C_{11}).

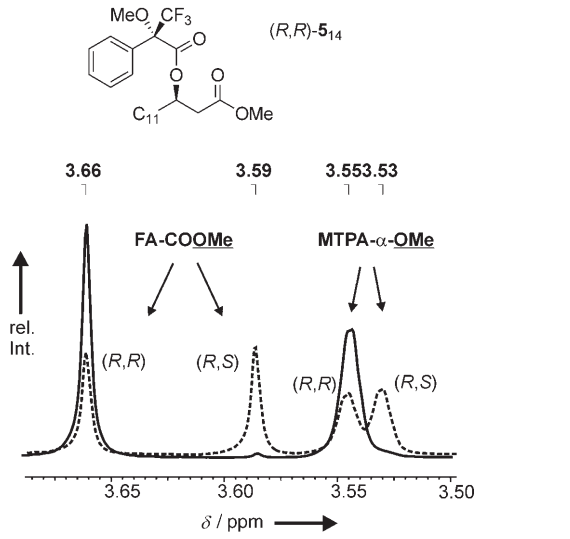


Figure 2. ^{1}H NMR (400 MHz, $CDCl_3$, OMe area, $\delta = 3.7$–3.5 ppm) of MTPA derivative $\mathbf{5}_{14}$. The MTPA ester obtained from racemic methyl 3-hydroxymyristate (*rac*-$\mathbf{4}_{14}$) shows signals for both the (*R*,*S*) and the (*R*,*R*) diastereomers (dotted line). The corresponding Mosher's ester derivative (*R*,*R*)-$\mathbf{5}_{14}$ obtained by asymmetric (*R*)-BINAP-$RuCl_2$-catalyzed hydrogenation shows only one set of signals, allowing the determination of optical purities by integration (98.3 % dr in (*R*,*R*)-$\mathbf{5}_{14}$, corresponding to 98.3 % er in (*R*)-$\mathbf{4}_{14}$).

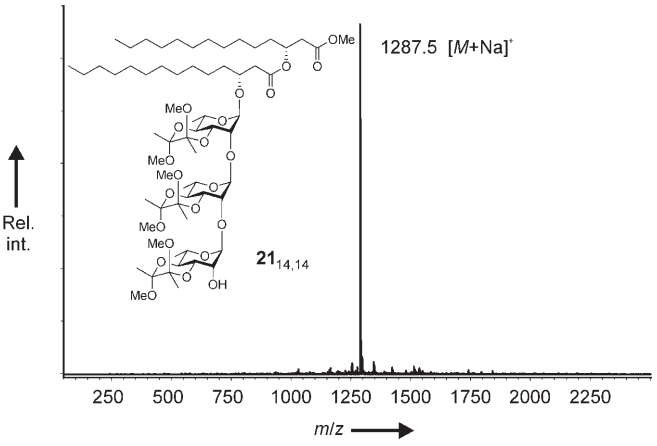
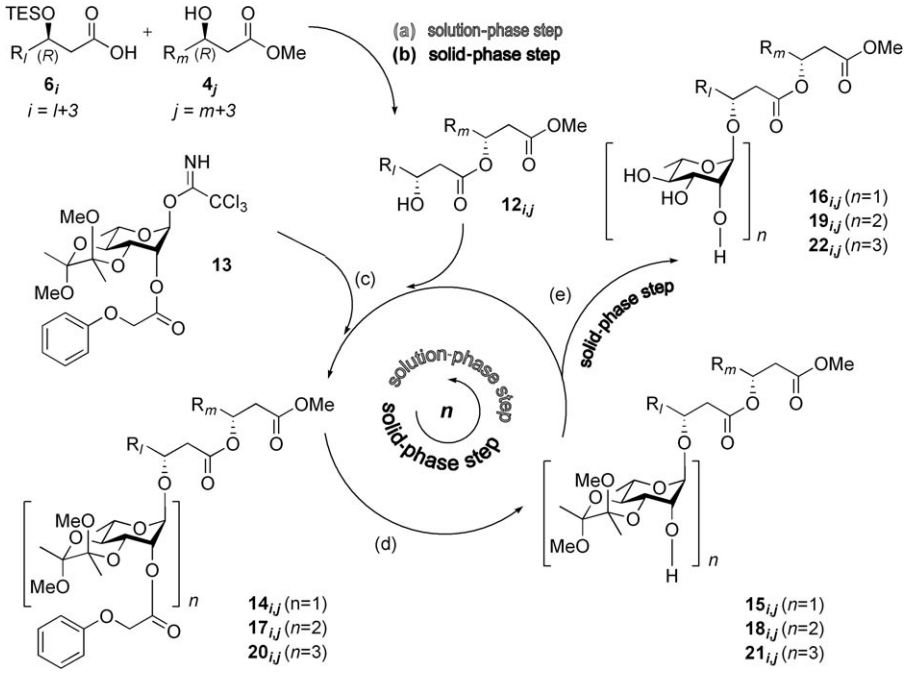


Figure 3. ESI-MS of crude BDA-protected rhamnolipid RL-3,$2_{14,14}$-COOMe $\mathbf{21}_{14,14}$ after three glycosylation/deprotection cycles, demonstrating the high purity of glycolipids obtained by HASP without the need for intermediary purification steps.

Comparison of the library of rhamnolipids indicated a strong influence of glycolipid structure on the proinflammatory activity in human mononuclear cells, as determined by TNFα secretion. Lead structure **1** and rhamnolipids **23**, **26**, and **29** were active in human macrophages in a dose-dependent manner, expressing their half-maximal activities at a low-μM concentration range of the stimulus (1 μg mL^{-1} = 1.3 μM for compound **1**). The number of rhamnoses and the length of the second fatty acid were not critical for bioactivity, but the biological activity dropped significantly as the lengths of both fatty acids were either increased (**27**) or decreased (**30**). Dramatic attenuation in bioactivity was also observed if the negative charge was removed (**31**, **32**), or if a single enantiomeric center in the fatty acid was changed (**33**, **34**).

Conclusion

We have demonstrated the preparation of a novel glycolipid library by using the HASP synthesis concept. Our strategy allowed flexible combinations of solid-phase reactions with



Scheme 2. HASP synthesis of rhamnolipid methyl esters on RP-18 silica as polymer support. a) EDC, cat. DMAP, CH_2Cl_2, 12 h, addition of RP-support, evaporation, washing steps (MeOH/H_2O 80:20), 97.5 %. b) 5 % TFA, MeOH/H_2O (80:20), washing steps (MeOH/H_2O 80:20), $MgSO_4$, desorption (CH_2Cl_2), 98 %. c) 1.3 equiv donor **13**, 0.05 equiv of TMSOTf, CH_2Cl_2, 30 min, neutralization (DIPEA), evaporation, washing steps (MeOH/H_2O 80:20), 94 %. d) 40 equiv aqueous $MeNH_2$, MeOH/THF (1:1), 1 h, washing steps (MeOH/H_2O 80:20), 93 %. e) TFA/H_2O (90:10), 3 × 10 min, washing steps (MeOH/H_2O 80:20), $MgSO_4$, desorption (CH_2Cl_2), 98 %. Yields given refer to the respective myristic acid derivatives ($n = 1$, R = C_{11}). For the purpose of fully quantifying all HASP steps, the products of solid-supported steps were desorbed, characterized, and readsorbed.

Table 1. Screening of commercially available hydrolases for their activity against RL-2,$2_{14,14}$-COOMe **19**$_{14,14}$. Data show the results for the OMe-hydrolysis of 3 μmol (2.3 mg) RL-2,$2_{14,14}$-COOMe **19**$_{14,14}$ on employment of 3 mg enzyme in ACN/Na_iPO_4 buffer (8 mL, pH 7.0, 1:3 v/v) or *n*-hexane/Na_iPO_4 buffer (5 mL, 3:1 v/v) at RT for 72 h. Hydrolytic activity was evaluated by performing MALDI-MS of the reaction mixture: (-o-) = no turnover, (+) = turnover < 1/3, (++) = 1/3 < turnover < 2/3; (+++) = turnover > 2/3.

Name/source of the hydrolase		ACN/buffer	*n*-hexane/buffer
Alcaligines sp.	ASL	-o-	-o-
Aspergillus niger	ANL	-o-	-o-
Candida antarctica, fract. A	CAL-A	-o-	+
Candida antarctica, fract. B	CAL-B	+	+
Candida antarctica (immobilized)	CAL-B	+++	-o-
Candida eugosa	CRL	-o-	+++
Bovine pancreas chymotrypsine	CHYM	-o-	-o-
Horse liver	HLE	-o-	-o-
Rhizomucor miehei	RML	-o-	++
Rhizomucor miehei (Lipozym)	RML	-o-	+
Pig liver (E-1)	PLE-E1	-o-	-o-
Pig liver (E-2)	PLE-E2	-o-	-o-
Pig liver	PLE	-o-	-o-
Porcine pancreas	PPL	-o-	-o-
Rhizopus arrhizus	RAL	-o-	++
Thermomyces lanuginosa	TLL	+	+

solution-phase couplings through quantitative and reversible phase switching in a commercial parallel synthesizer. This work should facilitate access to libraries and enable structural and biological studies of hydrophobic molecules that have not yet been attainable so far.

The biological results strongly suggest a specific—that is, recognition-based—mode of monocyte activation by rhamnolipids. The library is currently the subject of further investigations, including the biophysical profiling of the synthesized glycolipids, the structural characterization of active aggregates, and the tracing of rhamnolipids within target cells.

Experimental Section

General remarks: Hydrophobically assisted synthesis was conducted with bulk reversed-phase Grom-Sil ODS-4 HE silica gel (50 μ, 120 Å). All chemicals were purchased from Sigma, Aldrich, or Fluka and were used without further purification. Reactions were carried out in commercially available dry solvents unless otherwise stated. Column chromatography was performed by using Kieselgel 60 silica gel (Merck, 0.04–0.063 mm). TLC analysis was carried out by using Merck silica gel 60 F_{254} plates and Merck RP-18 F_{254} plates, detection of compounds was achieved by measuring UV absorption (254 nm) and by charring after spraying with 5 % H_2SO_4 or with ammonium molybdate and ceric ammonium sulfate in 10 % aqueous H_2SO_4. ^1H and ^{13}C NMR spectra were recorded by using a Bruker AMX2 600 MHz, a Bruker Avance 400 MHz, or a Bruker AC 250 MHz spectrometer. ^1H and ^{13}C chemical shifts are given in ppm relative to the solvent signal as internal standard. Assignments were based on homonuclear decoupling experiments and homo- and heteronuclear correlation. Mass spectra were measured by using a Bruker Autoflex MALDI-TOF instrument with α-cyano-4-hydroxycinnamic acid (CHCA) as matrix. HRESI-MS was performed by using a 4.7 Tesla APEX II spectrometer (Bruker Daltonics, Bremen). Optical rotations were measured by using a Perkin–Elmer 341 polarimeter.

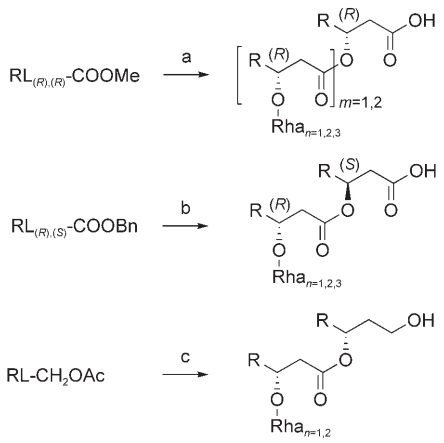
Methyl (*R*)-3-hydroxymyristate (4$_{14}$): Methyl 3-oxomyristate (**3**$_{14}$, 7.50 g, 29.25 mmol) was dissolved in dry MeOH (30 mL) under argon and placed in a hydrogenation autoclave. The freshly prepared (*R*)-BINAP-$RuCl_2$ hydrogenation catalyst (29.3 μmol, 0.1 mol %) was added, the autoclave was sealed and flushed with hydrogen, and a H_2 pressure of 5 bar was provided. After the reaction temperature of 100 °C had been reached (by heating in a silicon oil bath), the reaction mixture was stirred for 5 h. The crude product was concentrated and purified by column chromatography (hexane/ethyl acetate 5:1) to yield **4**$_{14}$ as a white solid (7.22 g, 95.6 %).

Enantiomeric purity: 98.3 % er (enantiomeric ratio, determined via the (*R*,*R*)-α-methoxy-α-(trifluoromethyl)phenylacetic acid (MTPA)-ester derivative **5**$_{14}$); $[\alpha]_D^{20} = -13.9°$ ($c = 1.0$ in $CHCl_3$); $R_f = 0.2$ (hexane/ethyl ace-

Table 2. Summary of building blocks used and yields [%] of each HASP step in the synthesis of the focused rhamnolipid library.

Building blocks	Desilylated lipid ester[a]	Glycosylation[e]	POAc removal	BDA removal	Removal of terminal OMe/OBn/OAc	Final RL ([mg])	
$\mathbf{6}_4$–$\mathbf{4}_{14}$	87.5	88.1	76.9	92.7	44.6[h]	$1,2_{4,14}$-COOH, **23**	(39.1)
$\mathbf{6}_{14}$–$\mathbf{6}_{14}$–$\mathbf{4}_{14}$	95.7[b]	84.9	97.6	94.0	68.4[h]	$1,3_{14,14,14}$-COOH, **24**	(140.2)
$\mathbf{6}_{14}$–$\mathbf{6}_{14}$–$\mathbf{4}_{14}$	[c]	83.5	96.7	71.5	73.0[h]	$2,3_{14,14,14}$-COOH, **25**	(32.6)
$\mathbf{6}_{14}$–$\mathbf{4}_{14}$	[c]	91.6	[f]	70.7[g]	85.6[h]	$3,2_{14,14}$-COOH, **26**	(89.3)
$\mathbf{6}_{18}$–$\mathbf{4}_{18}$	99.2	95.1	[f]	93.5[g]	31.4[h]	$2,2_{18,18}$-COOH, **27**	(44.4)
$\mathbf{6}_{14}$–$\mathbf{4}_4$	73.6	86.7	70.5	95.2	46.6[h]	$1,2_{14,4}$-COOH, **28**	(32.7)
$\mathbf{6}_{14}$–$\mathbf{4}_{14}$	98.2[d]	93.8	86.1	96.9	57.0[h]	$1,2_{14,14}$-COOH, **29**	(84.4)
$\mathbf{6}_{12}$–$\mathbf{4}_{12}$	93.5	94.8	94.9	81.7	64.8[h]	$2,2_{12,12}$-COOH, **30**	(75.4)
$\mathbf{6}_{14}$–$\mathbf{4}_{14}$	[c]	98.3	92.5	98.7	83.4[h]	$2,2_{14,14}$-COOH, **1**	(85.7)
$\mathbf{6}_{14}$–**9**	[c]	98.7	[f]	92.5[g]	70.2[i]	$2,2_{14,14}$-CH_2OH, **31**	(26.7)
$\mathbf{6}_{14}$–**9**	86.7	94.8	89.1	96.3	72.1[i]	$1,2_{14,14}$-CH_2OH, **32**	(52.4)
$\mathbf{6}_{14}$–**11**	99.5	87.2	85.0	98.8	75.4[j]	$1,2_{(R)14,(S)14}$-COOH, **33**	(41.3)
$\mathbf{6}_{14}$–**11**	[c]	97.1	[f]	93.4[g]	81.0[j]	$2,2_{(R)14,(S)14}$-COOH, **34**	(55.7)

[a] Combined yield for esterification and TES-deprotection. [b] Combined yield for the route of two esterifications and two TES-desilylations. [c] As for the corresponding monoglycosylated lipid. [d] Isolated yield for the desilylation step. [e] Yields refer to the last glycosylation step of the final RL compound. [f] Not isolated for quantification. [g] Combined yields for POAc and BDA removal. [h] Yields of the enzymatic OMe ester hydrolysis after chromatographic purification. [i] Yields of the OAc ester hydrolysis after chromatographic purification. [j] Yields of the hydrogenolytic OBn ester deprotection after chromatographic purification.



Scheme 3. General synthesis of RL acids and alcohols from RL esters. a) Terminal (*R*)-configured RL methyl ester, lipase from *Candida antarctica*, Na_2HPO_4 buffer, pH 7.0, 20% DMSO, 40°C, 72 h. b) Pd/C, H_2, THF/HOAc, 5 h. c) Cat. LiOH, pH 8.5, THF/MeOH/H_2O.

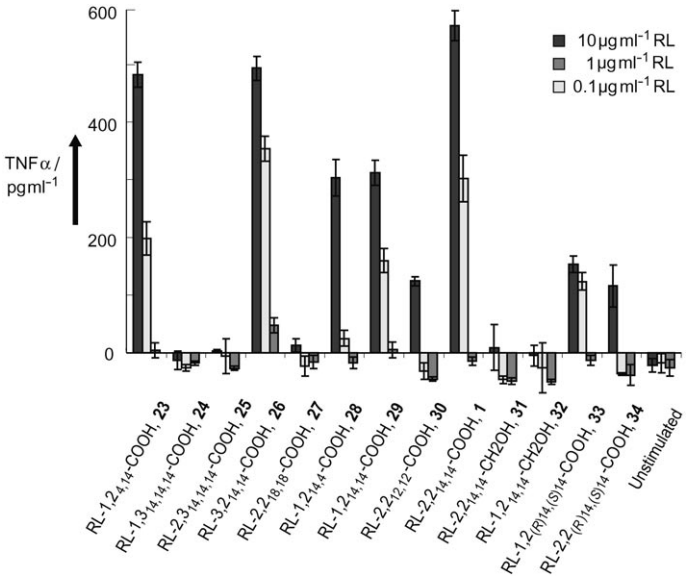


Figure 4. TNFα responses of human mononuclear cells (MNCs) after stimulation with various synthetic rhamnolipids.

tate 5:1); ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 3.98$ (m, 1H; C*H*-OH), 3.69 (s, 3H; OMe-CH_3), 2.87 (br, 1H; O*H*), 2.52–2.36 (m, 2H; CH-CH_2-CO), 1.52–1.24 (m, 20H; CH_2), 0.86 ppm (t, 3H; CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 173.5$, 68.0, 51.7, 41.1, 36.5, 31.9, 29.6, 29.5, 29.4, 29.3, 25.5, 22.7, 14.1 ppm.

(*R*)-3-*O*-Triethylsilyl myristic acid ($\mathbf{6}_{14}$): A solution of (*R*)-3-hydroxymyristic acid (2.5 g, 10.23 mmol) in pyridine (60 mL) was heated to 60°C, and triethylsilyl chloride (1.80 mL, 10.74 mmol) was added dropwise from a dropping funnel. The reaction mixture was allowed to stir for 2 h and was then concentrated, and the residue was dissolved in $CHCl_3$ (200 mL), washed with an aqueous solution of $NaHCO_3$ (3×200 mL, 5%), dried with sodium sulfate, and concentrated. The crude product was concentrated and purified by column chromatography (hexane/ethyl acetate 3:1, 1% triethylamine (TEA)) to yield $\mathbf{6}_{14}$ as a white and waxy solid (3.14 g, 85.6%).

$[\alpha]_D^{20} = -3.1°$ ($c = 1.0$ in $CHCl_3$); $R_f = 0.4$ (hexane/ethyl acetate 3:1); ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 4.07$ (m, 1H; C*H*-OH), 2.59–2.44 (m, 2H; α-CH_2), 1.54–1.24 (m, 20H; CH_2), 0.98–0.92 (m, 12H; CH_3, TES-CH_3), 0.61 ppm (q, 6H; TES-CH_2); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta =$ 175.4, 69.4, 41.8, 37.3, 31.9, 29.6, 29.6, 29.5, 29.5, 29.3, 25.3, 22.7, 14.1, 6.8, 4.8 ppm; HRMS (FT-ICR-MS, ICR = ion cyclotron resonance): *m*/*z* calcd for $C_{20}H_{42}O_3SiNa$ $[M+Na]^+$: 381.27954; found: 381.27959, $\Delta m/z =$ 0.13 ppm.

Benzyl (*S*)-3-hydroxymyristate (11): Benzyl alcohol (435 µL, 4.2 mmol) was added to a solution of (*S*)-3-*O*-TES-myristic acid (TES = triethylsilyl) $\mathbf{7}_{14}$ (502 mg, 1.40 mmol) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDC) (805 mg, 4.20 mmol) in dry CH_2Cl_2 (5 mL). A catalytic amount of 4-dimethylaminopyridine (DMAP) (10 mg) was added and the reaction mixture was allowed to stir for 12 h at RT. After removal of the solvents by concentration, the product was obtained by column chromatography (hexane/ethyl acetate 30:1) as a clear and viscous oil (511 mg, 81%).

$R_f = 0.3$ (hexane/ethyl acetate 30:1); $[\alpha]_D^{20} = +10.3°$ ($c = 1.0$ in $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 7.36$–7.31 (m, 5H; Ar-*H*), 5.11 (s, 2H; Bn-CH_2), 4.16 (m, 1H; C*H*-OSi), 2.57–2.42 (m, 2H; α-CH_2), 1.53–1.25 (m, 20H; CH_2), 0.96–0.87 (m, 12H; CH_3, TES-CH_3), 0.59 ppm (q, 6H; TES-CH_2); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 171.6$, 135.9, 128.5, 128.2, 128.1, 69.4, 66.1, 42.8, 37.7, 31.9, 29.6, 29.5, 29.3, 25.1, 22.7, 14.1, 6.8,

4.9 ppm; HRMS (FT-ICR-MS): m/z calcd for $C_{27}H_{48}O_3SiNa$ $[M+Na]^+$: 471.32649; found: 471.32456, $\Delta m/z = 0.15$ ppm.

Trifluoroacetic acid (2.5 mL) was added to a solution of benzyl (*S*)-3-*O*-TES-hydroxymyristate (505 mg, 1.05 mmol), prepared as described above, in dry CH_2Cl_2 (50 mL). The mixture was stirred for 10 min and then washed with water (3×50 mL), and the combined organic layers were collected, dried with sodium sulfate, and concentrated. Purification by column chromatography (hexane/ethyl acetate 8:1) yielded **11** as a colorless wax (357 mg, 98%).

$R_f = 0.25$ (hexane/ethyl acetate 8:1); $[\alpha]_D^{20} = +12.9°$ ($c = 1.0$ in $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 7.36$–7.32 (m, 5H; Ar-*H*), 5.15 (s, 2H; Bn-CH_2), 4.01 (m, 1H; C*H*-OH), 2.77 (br, 1H; O*H*), 2.55–2.45 (m, 2H; α-CH_2), 1.49–1.26 (m, 20H; CH_2), 0.87 ppm (t, 3H; CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 172.9$, 135.6, 128.6, 128.4, 128.2, 68.0, 66.5, 41.3, 36.5, 31.9, 29.6, 29.6, 29.5, 29.5, 29.4, 29.3, 25.4, 22.7, 14.1 ppm; HRMS (FT-ICR-MS): m/z calcd for $C_{21}H_{34}O_3Na$ $[M+Na]^+$: 357.24002; found: 357.23980, $\Delta m/z = 0.6$ ppm.

(*R*)-3-MEM-*O*-myristyl acetate (MEM = methoxyethoxymethyl) (8): (*R*)-3-MEM-*O*-myristyl alcohol (2.25 g, 7.06 mmol) was dissolved in pyridine (50 mL), and acetic anhydride (6.67 mL, 70.6 mmol) was added slowly. The reaction mixture was stirred with a catalytic amount of DMAP (15 mg) for 12 h. After completion of the reaction, the mixture was concentrated and the residue was dissolved in $CHCl_3$ (100 mL), washed with an aqueous solution of $KHSO_4$ (3×100 mL, 5%), concentrated, and purified by column chromatography (hexane/ethyl acetate 4:1) to give **8** as a clear oil (2.34 g, 92%).

$[\alpha]_D^{20} = -16.1°$ ($c = 1.0$ in $CHCl_3$); $R_f = 0.2$ (hexane/ethyl acetate 4:1); ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 4.72$ (2d, 2H; O-CH_2-O (AB-system)), 4.12 (m, 2H; CH_2-OAc), 3.70–3.66 (m, 3H; C*H*-O-MEM, O-CH_2-CH_2), 3.53 (m, 2H; O-CH_2-CH_2), 3.37 (s, 3H; OCH_3), 2.02 (s, 3H; OAc), 1.79 (m, 2H; CH_2-CH_2-OAc), 1.54–1.24 (m, 20H; CH_2), 0.86 ppm (t, 3H; CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 171.1$, 94.4, 74.5, 71.7, 67.1, 61.4, 59.0, 34.4, 33.2, 31.9, 29.7, 29.6, 29.6, 29.6, 29.3, 25.0, 22.7, 21.0, 14.1 ppm; HRMS (FT-ICR-MS): m/z calcd for $C_{20}H_{40}O_5Na$: 383.27680 $[M+Na]^+$; found: 383.27654, $\Delta m/z = 0.7$ ppm.

(*R*)-3-Hydroxymyristyl acetate (9): Powdered anhydrous zinc bromide (7.18 g, 31.9 mmol) was added under argon to a solution of (*R*)-3-MEM-*O*-myristyl acetate **8** (2.30 g, 6.38 mmol) in dry CH_2Cl_2 (50 mL). The suspension was agitated for 16 h at RT. After the reaction was complete, $CHCl_3$ (100 mL) was added, the mixture was washed with water (3× 200 mL), and the combined organic layers were dried with sodium sulfate and concentrated. The crude product was purified by column chromatography (hexane/ethyl acetate 2:1) to give **9** as a colorless, waxy solid (1.51 g, 87%).

$[\alpha]_D^{20} = -2.7°$ ($c = 1.0$ in $CHCl_3$); $R_f = 0.3$ (hexane/ethyl acetate 2:1); ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 4.25$–4.05 (m, 2H; CH_2-OAc), 3.58 (m, 1H; C*H*-OH), 2.54 (br, 1H; O*H*), 1.97 (s, 3H; OAc), 1.77–1.54 (m, 2H; CH_2-CH_2-OAc), 1.37–1.18 (m, 20H; CH_2), 0.80 ppm (t, 3H; CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 171.3$, 68.4, 67.1, 37.4, 36.1, 31.8, (29.5), (29.4), 29.2, 25.5, 22.5, 20.8, 13.9 ppm; HRMS (FT-ICR-MS): m/z calcd for $C_{16}H_{32}O_3Na$ $[M+Na]^+$: 295.22437; found: 295.22418, $\Delta m/z = 0.6$ ppm.

HASP synthesis of methyl (*R*)-3-*O*-[(*R*)-3′-*O*-TES-myristyl]myristate: **General procedure for parallel HASP synthesis**: EDC (230 mg, 1.2 mmol) and a catalytic amount of DMAP (15 mg) were added to a solution of methyl (*R*)-3-hydroxymyristate (**4**$_{14}$, 103.4 mg, 0.4 mmol) and (*R*)-3-*O*-triethylsilyl myristic acid (**6**$_{14}$, 286.9 mg, 0.8 mmol) in dry CH_2Cl_2 (3 mL). The reaction mixture was shaken for 12 h at RT on a standard Büchi Syncore workstation for parallel synthesis. After completion of the reaction step, reversed-phase silica gel (1.5 g, Grom-Sil ODS-4 HE, 50 μ, 120 Å) was added to the reaction vessel. To adsorb the compounds, solvents were removed by evaporation. The dry reversed-phase silica gel on which the reaction mixture was adsorbed was then washed three times with methanol/water (80:20, 8 mL) under vigorous shaking for 3 min. After each washing step, the solvents were filtered on the Syncore workstation under a gentle stream of compressed air. After the third washing/filtration step, anhydrous $MgSO_4$ (equivalent volume to RP-silica) and distilled CH_2Cl_2 (10 mL) were added, and the suspension was vigorously shaken for 20 min and then filtered off. For complete product desorption, CH_2Cl_2 (10 mL) was added and the suspension was shaken for 3 min and filtered twice more. The filtrates containing the desorbed compound were collected and concentrated to give a clear and colorless oil (233.6 mg, 97.5%). The RP-silica-bound TES-dilipids were desorbed for analytical purposes only and usually remained immobilized for further solid-supported chemical transformation.

^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 5.18$ (m, 1H; C*H*-O), 4.07 (m, 1H; C*H*-OSi), 3.65, (s, 3H; OMe), 2.62–2.40 (m, 4H; α-CH_2, α′-CH_2), 1.58–1.24 (m, 40H; CH_2), 0.93 (t, 9H; CH_3, TES-CH_3), 0.86 (t, 6H; CH_3), 0.58 ppm (q, 6H; TES-CH_2); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 171.0$, 170.8, 70.5, 69.1, 51.7, 42.9, 38.9, 37.5, 33.9, 31.9, 29.7, 29.6, 29.6, 29.5, 29.5, 29.4, 29.3, 25.1, 22.7, 14.1, 6.8, 4.9 ppm.

HASP synthesis of methyl (*R*)-3-*O*-[(*R*)-3′-hydroxymyristyl]myristate ($12_{14,14}$): TES-dilipid ester prepared as described above (239.6 mg, 0.40 mmol), still immobilized on RP-silica from the previous step, was suspended in methanol/water (3.8 mL, 80/20 v/v). Trifluoroacetic acid (200 μL) was added and the suspension was shaken vigorously for 20 min. After completion of the reaction, the workup by HASP filtration and HASP release of products was performed as described above to yield **12**$_{14,14}$ as a colorless, clear oil (190.4 mg, 98.2%).

^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 5.25$ (m, 1H; C*H*-O-C=O), 3.98 (m, 1H; C*H*-OH), 3.66, (s, 3H; OMe), 2.81 (br, 1H; O*H*), 2.57–2.34 (m, 4H; α-CH_2, α′-CH_2), 1.61–1.24 (m, 40H; CH_2), 0.87 ppm (t, 6H; CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 172.5$, 171.1, 70.9, 68.3, 51.9, 41.8, 39.0, 36.5, 34.0, 31.9, 29.6, 29.6, 29.6, 29.5, 29.5, 29.4, 29.3, 29.3, 25.5, 25.1, 22.7, 14.1 ppm; HRMS (FT-ICR-MS): m/z calcd for $C_{29}H_{56}O_5Na$ $[M+Na]^+$: 507.40200; found: 507.40241, $\Delta m/z = 0.8$ ppm.

HASP synthesis of 1-*O*-{methyl (*R*)-3-*O*-[(*R*)-3′-*O*-myristyl]myristate}-2-*O*-phenoxyacetyl-[3,4-*O*-(2,3-dimethoxybutane-2,3-diyl)]-(1→2)-α-L-rhamnopyranoside ($14_{14,14}$): Dilipid ester methyl (*R*)-3-*O*-[(*R*)-3′-hydroxymyristyl]myristate **12**$_{14,14}$ (104.2 mg, 0.215 mmol) and donor 3,4-*O*-(2,3-dimethoxybutane-2,3-diyl)-2-*O*-phenoxyacetyl-α-L-rhamnopyranosyl trichloroacetimidate (**13**, 155.6 mg, 0.28 mmol) were dissolved in dry CH_2Cl_2 (3 mL) under argon.[9] A freshly prepared trimethylsilyl trifluoromethanesulfonate (TMSOTf) solution (0.05 equiv) in dry CH_2Cl_2 (215 μL, 0.05 M) was added and the reaction mixture was shaken for 30 min. After completion, the solution was neutralized with N,N-diisopropylethylamine (DIPEA) (10 μL), and HASP filtration and HASP release of products were conducted as described above to yield **14**$_{14,14}$ as a colorless, clear oil (177.3 mg, 93.8%).

^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 7.29$–6.91 (m, 5H; Ar*H*), 5.14 (m, 1H; C*H*-O-C=O), 5.09 (d, $^3J_{1,2} < 1$ Hz, $^3J_{2,3} = 2.8$ Hz, 1H; H-2), 4.80 (d, $^3J_{1,2} <$ 1 Hz, 1H; H-1), 4.65 (s, 2H; POAc-CH_2), 3.97 (dd, $^3J_{2,3} = 2.8$ Hz, $^3J_{3,4} =$ 10.1 Hz, 1H; H-3), 3.96 (m, 1H; C*H*-O-Rha), 3.80 (m, 1H; H-5), 3.60, (s, 3H; OMe), 3.54 (t, $^3J_{4,5} = 10.1$ Hz, 1H; H-4), 3.17 (s, 3H; BDA-OMe), 3.15 (s, 3H; BDA-OMe), 2.60–2.34 (m, 4H; α-CH_2, α′-CH_2), 1.54–1.18 (m, 49H; CH_2, H-6, BDA-Me), 0.81 ppm (t, 6H; CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 170.8$, 170.4, 168.5, 157.9, 129.5, 121.6, 114.8, 100.0, 99.7, 97.2, 75.4, 71.7, 70.8, 68.6, 67.0, 66.1, 65.2, 51.7, 48.0, 47.6, 40.3, 38.9, 33.9, 31.9, 31.8, 29.6, 29.6, 29.6, 29.5, 29.5, 29.5, 29.3, 29.3, 25.1, 24.8, 22.7, 17.7, 17.6, 16.5, 14.1 ppm; HRMS (FT-ICR-MS): m/z calcd for $C_{49}H_{82}O_{13}Na$ $[M+Na]^+$: 901.56476; found: 901.46425, $\Delta m/z = 0.57$ ppm.

HASP synthesis of 1-*O*-{methyl (*R*)-3-*O*-[(*R*)-3′-*O*-myristyl]myristate}-3,4-*O*-(2,3-dimethoxybutane-2,3-diyl)-2-*O*-{α-L-rhamnopyranosyl-[3′,4′-*O*-(2,3-dimethoxybutane-2,3-diyl)]-2′-*O*-phenoxyacetyl}-(1→2)-α-L-rhamnopyranoside ($17_{14,14}$): The second glycosylation, at the 2-hydroxy position of rhamnolipid BDA-HO-RL-1,2$_{14}$-COOMe **15**$_{14,14}$ (160.0 mg, 0.215 mmol), was performed analogously to the glycosylation step described above, but with two equivalents of donor **13** (239.2 mg, 0.43 mmol) to yield **17**$_{14,14}$ as a highly viscous oil (240.45 mg, 98.3%).

^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 7.35$–6.78 (m, 5H; Ar*H*), 5.43 (dd, $^3J_{1,2} <$ 1 Hz, $^3J_{2,3} = 2.8$ Hz, 1H; H-2^A), 5.18 (m, 1H; C*H*-O-C=O), 5.10 (d, $^3J_{1,2} <$ 1 Hz, 1H; H-1^A), 4.77 (d, $^3J_{1,2} < 1$ Hz, 1H; H-1^B), 4.67 (s, 2H; POAc-CH_2), 3.96 (dd, $^3J_{2,3} = 2.8$ Hz, $^3J_{3,4} = 10.1$ Hz, 1H; H-3^A), 4.03–3.59 (m, 10H; H-2^B, H-3^B, H-4^B, H-5^B, H-4^A, H-5^A, C*H*-O-Rha, OMe), 3.26–3.16 (4×s, 12H; BDA-OMe), 2.63–2.35 (m, 4H; α-CH_2, α′-CH_2), 1.58–1.18 (m, 58H; CH_2, H-6^A, H-6^B, BDA-Me), 0.85 ppm (t, 6H; CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 170.8$, 170.4, 167.7, 158.0, 129.4, 121.5, 114.7,

100.0, 99.8, 99.7, 99.4, 99.0, 98.8, 75.6, 74.7, 71.0, 70.7, 68.7, 68.5, 68.4, 67.3, 67.1, 66.4, 65.2, 51.8, 47.9, 47.8, 47.6, 47.4, 40.4, 38.9, 33.9, 33.7, 31.9, 29.7, 29.6, 29.6, 29.5, 29.3, 25.1, 24.9, 22.7, 17.8, 17.8, 17.7, 17.7, 17.6, 17.5, 14.1 ppm; ESI-MS: m/z: 1161.3 $[M+Na]^+$.

HASP synthesis of 1-*O*-{methyl (*R*)-3-*O*-[(*R*)-3′-*O*-myristyl]myristate}-3,4-*O*-(2,3-dimethoxybutane-2,3-diyl)-2-*O*-{α-L-rhamnopyranosyl-[3′,4′-*O*-(2,3-dimethoxybutane-2,3-diyl)]}-(1→2)-α-L-rhamnopyranoside ($18_{14,14}$): Fully protected rhamnolipid BDA-POAc-RL-2,2_{14}-COOMe $\mathbf{17}_{14,14}$ (224.0 mg, 0.197 mmol), still immobilized on the RP-support from the previous HASP step, was suspended in methanol/THF (4 mL, 1:1 v/v). Methylamine (684 μL, 40 equiv of a 40 % (11.5 M) aqueous solution) was added and the suspension was shaken for 1 h. After completion of the reaction, water was added (12 mL) and the reaction mixture was filtered off. Subsequent HASP washing steps and HASP release of products were performed as described above to yield $\mathbf{18}_{14,14}$ as a colorless, highly viscous oil (182.8 mg, 92.5 %).

^{1}H NMR (400 MHz, $CDCl_3$): δ = 5.19 (m, 1H; C*H*-O-C=O), 5.09 (d, $^3J_{1,2}$ < 1 Hz, 1H; H-1^A), 4.77 (d, $^3J_{1,2}$ < 1 Hz, 1H; H-1^B), 4.07 (dd, $^3J_{1,2}$ < 1 Hz, $^3J_{2,3}$ = 2.8 Hz, 1H; H-2^A), 3.99–3.96 (m, 2H; C*H*-O-Rha, H-3^A), 3.87–3.83 (m, 3H; H-5^A, H-2^B, H-3^B), 3.77–3.64, (m, 5H; H-5^B, H-4^A, OMe), 3.56 (t, $^3J_{4,5}$ = 10.1 Hz, 1H; H-4^B), 3.28–3.17 (4×s, 12H; BDA-OMe), 2.64–2.36 (m, 4H; α-CH_2, α′-CH_2), 2.08 (br, 1H; O*H*), 1.59–1.17 (m, 58H; CH_2, H-6^A, H-6^B, BDA-Me), 0.86 ppm (t, 6H; CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 170.8, 170.4, 101.0, 100.0, 99.7, 99.5, 99.4, 99.0, 75.2, 74.6, 70.6, 69.6, 68.6, 68.4, 68.3, 67.9, 67.1, 66.8, 51.7, 47.8, 47.7, 47.6, 47.4, 40.4, 38.9, 33.9, 33.6, 31.9, 29.6, 29.6, 29.6, 29.5, 29.5, 29.3, 25.1, 24.8, 22.6, 17.8, 17.7, 17.6, 17.6, 16.5, 16.6, 14.1 ppm; ESI-MS: m/z: 1027.7 $[M+Na]^+$.

HASP synthesis of 1-*O*-{methyl-(*R*)-3-*O*-[(*R*)-3′-*O*-myristyl]myristate}-2-*O*-α-L-rhamnopyranosyl-(1→2)-α-L-rhamnopyranoside ($19_{14,14}$): BDA-acetal protected rhamnolipid methyl ester BDA-RL-2,2_{14}-COOMe $\mathbf{18}_{14,14}$ (144.6 mg, 0.144 mmol), still immobilized on the RP-support from the previous HASP step, was suspended in trifluoroacetic acid/water (4 mL, 90:10 v/v). The suspension was agitated for 10 min, water (5 mL) was added, and the reaction mixture was filtered off. For completion of the reaction, the BDA removal was performed three times as described. Subsequent HASP washing steps and HASP release of products were performed as described above to yield $\mathbf{19}_{14,14}$ as a colorless, highly viscous oil (109.6 mg, 98.1 %).

MALDI-TOF-MS: m/z: 799.1 $[M+Na]^+$.

Rhamnolipid RL-2,$2_{14,14}$-COOH (1): The fully deprotected rhamnolipid methyl ester RL-2,$2_{14,14}$-COOMe $\mathbf{19}_{14,14}$ (104.6 mg, 0.135 mmol) and lipase from *Candida antarctica* (270 mg, 2 g mmol^{-1}; CAL-B, Fluka, >2 U mg^{-1}) were suspended in DMSO/phosphate buffer (5 mL, 1:4 v/v, phosphate buffer prepared as a 0.1 M solution from Na_2HPO_4 and NaH_2PO_4 and adjusted to pH 7). Hydrolysis was conducted for 72 h at 40 °C by shaking the reaction vessel at 300 rpm. After completion of hydrolysis, the mixture was concentrated and purified by column chromatography (60 mL SiO_2, $CHCl_3$/MeOH/HOAc 30:3:1). Solvents were removed and the pure rhamnolipid **1** was obtained after three lyophilization steps from *tert*-butanol/water (4:1 v/v)as a colorless, fluffy solid (85.7 mg, 83.4 %).

^{1}H NMR (600 MHz, CD_3COOD): δ = 5.32 (m, 1H; C*H*^{3}-O), 5.04 (d, $^3J_{1,2}$ < 1 Hz, 1H; H-1^A), 5.00 (d, $^3J_{1,2}$ < 1 Hz, 1H; H-1^B), 4.13 (m, 2H; C*H*$^{3'}$-O-Rha, H-2^B), 3.91–3.86 (m, 3H; H-3^A, H-3^B, H-2^A), 3.81 (m, 1H; H-5^A), 3.75 (m, 1H; H-5^B), 3.57 (t, $^3J_{4,5}$ = 9.3 Hz, 1H; H-4^A), 3.50 (t, $^3J_{4,5}$ = 9.3 Hz, 1H; H-4^B), 2.64–2.50 (m, 4H; α-CH_2, α′-CH_2), 1.65–1.24 (m, 46H; CH_2, H-6^A, H-6^B), 0.87 ppm (t, 6H; CH_3); ^{13}C NMR (150 MHz, CD_3COOD): δ = 104.6, 99.5, 81.8, 76.4, 75.8, 75.4, 73.8, 73.7, 73.5, 73.2, 71.4, 71.1, 42.5, 41.6, 36.5, 35.4, 34.4, 32.2, 32.1, 32.1, 32.0, 32.0, 31.9, 31.8, 27.5, 27.2, 25.1, 19.4, 19.3, 16.0 ppm; MALDI-TOF-MS: m/z: 785.4 $[M+Na]^+$, 807.5 $[M-H+2Na]^+$; HRMS (FT-ICR-MS): m/z calcd for $C_{40}H_{74}O_{13}Na$ $[M+Na]^+$: 785.50216; found: 785.50155, $\Delta m/z$ = 1.20 ppm.

Rhamnolipid RL-1,$2_{(R)14,(S)14}$-COOH (33): The (3′*R*,3*S*)-configured rhamnolipid benzyl ester RL-1,$2_{(R)14,(S)14}$-COOBn **11** (0.089 mmol, 62.8 mg) was dissolved in THF (20 mL). Acetic acid (1 mL) was added. After addition of palladium-charcoal (10 % Pd/C, 30 mg), the reaction mixture was vigorously shaken at RT in a standard hydrogenation apparatus at 4 bar H_2 for 5 h. The mixture was filtered through a Celite pad, the filtrate was concentrated under reduced pressure, and the residue was purified by column chromatography (60 mL SiO_2, $CHCl_3$/MeOH/HOAc 40:1:1). Solvents were removed and the pure rhamnolipid was obtained after three lyophilization steps from *tert*-butanol/water (4:1 v/v) as a colorless, fluffy solid (41.3 mg, 75.4 %).

^{1}H NMR (600 MHz, CD_3COOD): δ = 5.27 (m, 1H; C*H*^{3}-O), 4.98 (d, $^3J_{1,2}$ = 1.0 Hz, 1H; H-1), 4.09 (m, 1H; C*H*$^{3'}$-O-Rha), 3.96 (dd, $^3J_{1,2}$ = 1.0 Hz, $^3J_{2,3}$ = 3.1 Hz, 1H; H-2), 3.83 (dd, $^3J_{2,3}$ = 3.1 Hz, $^3J_{3,4}$ = 9.3 Hz, 1H; H-3), 3.79 (m, 1H; H-5), 3.56 (t, $^3J_{4,5}$ = 9.3 Hz, 1H; H-4), 2.68–2.54 (m, 4H; α-CH_2, α′-CH_2), 1.69–1.26 (m, 43H; CH_2, H-6), 0.89 ppm (t, 6H; CH_3); ^{13}C NMR (150 MHz, CD_3COOD): δ = 176.8, 172.5, 100.1, 75.9, 73.9, 72.5, 72.4, 72.2, 69.8, 41.3, 39.5, 34.8, 34.4, 32.9, 30.7, 30.6, 30.6, 30.5, 30.4, 30.3, 26.1, 25.9, 23.6, 27.7, 14.4 ppm; MALDI-TOF-MS: m/z: 639.9 $[M+Na]^+$, 661.6 $[M-H+2Na]^+$; HRMS (FT-ICR-MS): m/z calcd for $C_{34}H_{64}O_9Na$ $[M+Na]^+$: 639.44425; found: 639.44499, $\Delta m/z$ = 1.20 ppm.

Rhamnolipid alcohol RL-2,$2_{14,14}$-CH_2OH (31): A mixture of THF/H_2O/MeOH (8 mL, 70:20:10 v/v) was prepared and adjusted to pH 8.5 with a solution of lithium hydroxide (0.1 M). The rhamnolipid alcohol acetate RL-2,2-CH_2OAc (0.051 mmol, 40.2 mg), prepared through analogous HASP steps starting from lipid acetate **9**, was added and the reaction mixture was allowed to stir at RT for 2 h. After completion of the reaction, the mixture was neutralized with a solution of dilute aqueous $KHSO_4$, the solution was concentrated under reduced pressure, and the crude product was purified by column chromatography (60 mL SiO_2, $CHCl_3$/MeOH/HOAc 25:2:1). Solvents were removed and the pure rhamnolipid was obtained after three lyophilization steps from *tert*-butanol/water (4:1 v/v) as a colorless, fluffy solid (26.7 mg, 70.2 %).

^{1}H NMR (600 MHz, CD_3COOD): δ = 5.04 (d, $^3J_{1,2}$ < 1 Hz, 1H; H-1^A), 5.01 (d, $^3J_{1,2}$ < 1 Hz, 1H; H-1^B), 4.30–4.16 (m, 2H; CH_2-OH), 4.14–4.11 (m, 2H; C*H*$^{3'}$-O-Rha, H-2^B), 3.89–3.65 (m, 6H; C*H*-CH_2OH, H-2^A, H-3^A, H-3^B, H-5^A, H-5^B), 3.57 (t, $^3J_{4,5}$ = 9.3 Hz, 1H; H-4^A), 3.51 (t, $^3J_{4,5}$ = 9.3 Hz, 1H; H-4^B), 2.59–2.52 (m, 4H; α-CH_2, α′-CH_2), 1.89–1.69 (m, 2H; CH_2-CH_2OH), 1.61–1.24 (m, 46H; CH_2, H-6^A, H-6^B), 0.86 ppm (t, 6H; CH_3); ^{13}C NMR (150 MHz, CD_3COOD, selected signals): δ = 102.7, 97.6, 79.6, 74.9, 73.5, 73.3, 71.6, 71.1, 69.3, 69.3, 69.1, 69.0, 65.5, 52.0, 41.0 ppm; MALDI-TOF-MS: m/z: 771.4 $[M+Na]^+$; HRMS (FT-ICR-MS): m/z calcd for $C_{40}H_{76}O_{12}Na$ $[M+Na]^+$: 771.52290; found: 771.52263, $\Delta m/z$ = 0.35 ppm.

Stimulation of human mononuclear cells (MNCs): MNCs were isolated from heparinized (20 IE mL^{-1}) blood taken from healthy donors and processed directly by mixing with an equal volume of Hank's balanced solution and centrifugation in a Ficoll density gradient for 40 min (21 °C, 500 g). The interphase layer of mononuclear cells was collected and washed twice in Hank's medium and once in RPMI 1640 containing L-glutamine (2 mM), penicillin (100 U mL^{-1}), and streptomycin (100 μg mL^{-1}). The cell number was equilibrated at 5×10^6 N mL^{-1}. For stimulation, 200 μL of MNCs per well (5×10^6 cells mL^{-1}) were transferred into 96-well culture plates. The RL-stimuli were serially diluted in HEPES buffer (pH 7.2), added to the cultures at 20 μL per well, and the cultures were incubated for 4 h at 37 °C under 5 % CO_2. Cell-free supernatants were collected after centrifugation of the culture plates for 10 min at 400 *g* and then stored at −20 °C until determination of the cytokine content. Immunological determination of TNFα in the cell supernatant was performed in a sandwich-ELISA as described previously:[14] 96-well plates (Greiner, Solingen, Germany) were coated with a monoclonal (mouse) anti-human TNFα antibody (clone 16 from Intex AG, Switzerland). Cell-culture supernatants and the standard (recombinant human TNFα, Intex) were diluted with buffer. After exposure to appropriately diluted test samples and serial dilutions of standard rTNFα, the plates were exposed to peroxidase-conjugated (sheep) anti-mouse IgG antibody. Subsequently, the color reaction was started by addition of tetramethylbenzidine/H_2O_2 in alcoholic solution and stopped after 5 to 15 min by addition of 1 N sulfuric acid. In the color reaction, the substrate was cleaved enzymatically, the product was measured photometrically by using an ELISA reader at a wavelength of 450 nm, and the values were correlated to the standard. TNFα was determined in duplicate at three different dilutions and the values were averaged.

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