Studies of Precursor-Directed Biosynthesis with Streptomyces, 3[+]

Structural Diversity of 1-*O*-Acyl α-L-Rhamnopyranosides by Precursor-Directed Biosynthesis with *Streptomyces griseoviridis*

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Dedicated to Professor Hans Zähner on the occasion of his 70th birthday

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The ability of *Streptomyces griseoviridis* (strain Tü 3634) to glycosylate various carboxylic acids with L-rhamnose was investigated by feeding mainly heteroaromatic and aromatic carboxylic acids to growing cultures. The special application of the precursor-directed biosynthesis (PDB) gave rise to a wide variety of acyl α-L-rhamnopyranosides as novel metabolites. The experiments resulted in furanyl, pyrrolyl, thienyl, indolyl, and pyridyl derivatives (**1**–**8**), the analogues **9**–**24** were generated by feeding fluoro-, hydroxy- or aminobenzoic acids or cinnamic acids. All results are discussed with respect to the substrate specificity of the corresponding enzyme system.

Introduction

Recently we described acyl α-L-rhamnopyranosides, metabolites from *Streptomyces* in which six different carboxylic acids are linked to the anomeric oxygen of α-L-rhamnose in a glycosidic ester bond.[2] The biosynthesis of this novel family of compounds requires a rhamnosyl transferase with an unusual substrate specificity. The structure and biosynthesis of the resulting rhamnoconjugates were investigated. Since acyl hexopyranosides occur very rarely as microbial metabolites,[3] compared to *O*-glycosidically linked deoxy sugars, we decided on further investigation.

Precursor-directed biosynthesis (PDB) as efficient access to derivatives of microbial metabolites is performed by feeding precursor analogues to the fermentation broth of the producing organism, which channels those precursors into the enzymatic process, thus giving rise to the production of modified metabolites.[4] The known broad substrate specificity of enzymes involved in secondary metabolism is an important prerequisite, as it is that precursors are water soluble. The well-established and applied method in biotechnology allows peripheral changes within large molecules (e.g. penicillin V) or changes in the skeleton of molecules (e.g. manumycin analogues).[5] Within the families of the glycoside antibiotics celesticetin and paulomycin, one side chain of their sugar moieties can be esterified with exogenously added acids, involving the enzyme activity of an ester synthase and resulting in modified antibiotics.[6] Compared to methods using cell-free extracts and isolated enzymes, no extensive preparatory work is necessary, and activated precursors do not require any cofactor regeneration. Advantageously, the application of enzymes in glycosylation reactions circumvents the tedious protecting group usage needed in total synthesis. Experiments on PDB with *Streptomyces griseoviridis* (strain Tü 3634) resulted in over 20, mostly new acyl α-L-rhamnopyranosides (**1**−**24**) in yields of up to 158 mg/L. This paper describes the isolation and structure elucidation of the novel compounds, and discusses the conclusions in the light of the specificity of the supposed rhamnosyl transferase.

Precursor-Directed Biosynthesis and Isolation

Strain Tü 3634 was selected for further investigations from five available strains producing different acyl α-L-rhamnopyranosides due to reliable growth and production. Cultivation in four different media containing oatmeal (A), malt and yeast (B), or glycerol (C, and D), respectively, revealed medium A to be optimum and convenient for production.[2] Feeding of exogenous carboxylic acids as acceptors gave insight into the enzyme activity and the specificity of the rhamnose transfer reaction, and allowed us to generate new rhamnosides. In all cases, precursors were continuously added to the culture as aqueous solutions at the beginning of the production of **1**, in amounts of 7.3 mmol over a period of 10 hours. Higher concentrations (up to 50 mmol) of fed precursors resulted in scanty cell growth of the strain. Newly appearing products could be easily detected by TLC analysis due to intensive yellow to green, or rarely, red discoloration with anisaldehyde/H_2SO_4 staining reagent. Formation of the original natural products was completely suppressed in most cases, which made the

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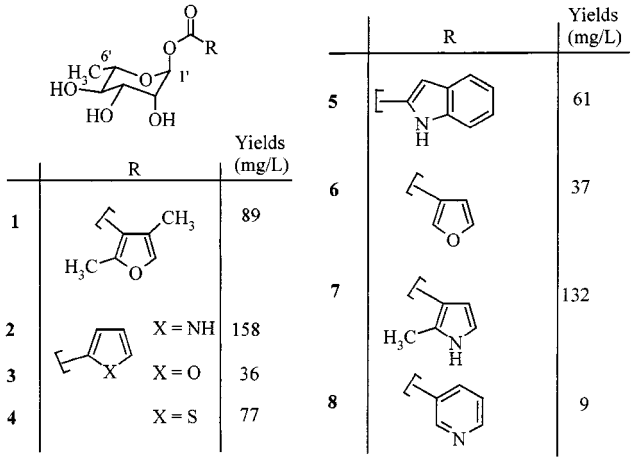
use of enzyme inhibitors preventing the biosynthesis of the natural aglycone unnecessary. For better comparison of the results, optimized standard conditions were established in a 1-L fermentor with 1000 mL of medium A for the duration of 72 hours. Parallel cultivations of strain Tü 3634 in flasks without adding precursors were used as a control for normal metabolite production. Feeding experiments with eight heteroaromatic carboxylic acids, e.g. the readily available synthetic 2,4-dimethylfuran-3-carboxylic acid[7] or pyrrole-2-carboxylic acid, resulted in the already described metabolites **1** and **2**,[2] but with much higher yields. Other "unnatural" heteroaromatic carboxylic acids gave rise to **3**−**8** (Scheme 1). Schemes 2 and 3 summarize the results of the precursor-directed biosynthesis with substituted benzoic and cinnamic acids. Described workup procedures by XAD-adsorption and column chromatography separated the surplus carboxylic acids, and yielded the new acyl α-L-rhamnopyranosides **1**−**24** from the culture filtrate as pure colourless solids or yellow oils. All products are readily soluble in methanol, and slightly soluble in chloroform. Their R_f values on TLC silica gel plates are given in Table 1.



Scheme 1. Structures and yields of the acyl α-L-rhamnopyranosides **1**−**8**

Table 1. R_f values of the acyl α-L-rhamnopyranosides **3**−**18**, **21**−**24** on silica gel TLC plates

Compound[a]	A	B
3	0.31	0.63
4	0.57	0.67
5	0.29	0.64
6	0.31	0.63
7	0.18	0.59
8	0.17	0.37
9	0.33	0.59
10	0.18	0.54
11	0.19	0.54
12	0.24	0.69
14	0.08	0.56
15	0.17	0.61
16	0.39	0.60
17	0.22	0.71
18	0.61	0.70
21	0.70	0.63
22	0.48	0.66
23	0.35	0.73
24	0.13	0.57

[a] Solvent systems: (A) $CHCl_3$/MeOH = 85:15; (B) acetic acid/1-butyl alcohol/water = 1:4:5 (upper layer).

Structures and Characterization

Heteroaromatic Carboxylic Acids as Precursors

2,4-Dimethylfuran-3-carboxylic acid led to **1** in yields (86 mg/L) 10-fold higher than from unfed cultures (8−11 mg/L). Based on this result, feeding experiments with unsubstituted pyrrole-2-carboxylic acid and its O- and S- analogues successfully generated the acyl α-L-rhamnopyranosides **2**−**4** (Scheme 1). Under these conditions, **2** could be obtained in amounts, dramatically 100 times higher than in regular cultivations of Streptomyces sp. (S 2087).[2] These findings on enhanced production of **1** and **2** upon feeding of their putative precursors indicated that the carboxylic acids are a limitation factor for production under standard conditions.[2] The lower yields in the case of the other examples (Scheme 1) exhibit significant differences in their enzyme affinity.

The pure acyl α-L-rhamnopyranosides were characterized spectroscopically and their structures were elucidated by analyzing the mass, IR, and UV spectra, as well as ^{1}H-, ^{13}C-NMR, ^{1}H-^{1}H-, and ^{1}H-^{13}C-shift correlation data. Similarly, for all isolated rhamnosides, the sugar moiety, as a distinctive structural feature, was identified as rhamnose in accordance with the data for **1**,[2] as well as by comparing mass spectra and the typical chemical shifts with literature values, and analyzing the coupling patterns of 1′-H (δ_H = 6.12) through 6′-H (δ_H = 1.27) (Table 2). NMR data of **7**, **16**, and **20** show individual deviations. The appearance of only one set of NMR signals clearly indicated that **1**−**24** are diastereomerically pure natural products. An α-L-configuration was assigned for the rhamnose moiety, by comparing data, e.g. $^1J_{C-H}$ coupling constants and optical rotation values, with that for **1** and **2**, and literature values.[2]

Table 2. Typical ^{1}H- (200, 300, or 500 MHz, CD_3OD) (*J* in Hz) and ^{13}C-NMR (50.3, 75.5, or 125.7 MHz, CD_3OD) data of the rhamnosyl moiety of **1**−**22** (except for **7**, **16**, and **20**), individual deviation smaller +/− 0.09 (^{1}H), 1.1 (^{13}C) ppm

Atom	^{1}H-NMR	^{13}C-NMR
1′	6.13 (d, 2.0, 1 H)	95.5, d
2′	3.92 (dd, 3.5, 2.0, 1 H)	71.3, d
3′	3.81 (dd, 9.5, 3.5, 1 H)	72.2, d
4′	3.48 (dd, 9.5, 9.5, 1 H)	73.4, d
5′	3.72 (dq, 9.5, 6.5, 1 H)	72.5, d
6′	1.27 (d, 6.5, 3 H)	18.1, q

The molecular formula of **3** ($C_{11}H_{14}O_7$) was derived from DCI MS by detecting a peak at *m*/*z* = 276 [M + NH_4^+] and a fragmentation peak at *m*/*z* = 164, typical for the rhamnosyl moiety. The ^{1}H-NMR spectrum (CD_3OD) revealed signals of the furan ring (δ_H = 6.63, 7.31, 7.78) and protons of the sugar moiety determined as rhamnose. In **3**, eleven corresponding signals in the ^{13}C-NMR spectrum bear the

prominent, upfield-shifted signal of the ester carbonyl (δ_C = 158.0) in the 2-position of the furan. Thus, **3** was a novel furanyl-2-carbonyl α-L-rhamnopyranoside.

Metabolite **4** represents the first sulfur containing acyl rhamnoside. The UV spectrum was dominated by the typical absorption bands of thiophene (λ_{max} = 249, 271 nm). The DCI mass spectrum confirmed the molecular mass of 274 with a peak at *m/z* = 292 [M + NH_4^+]. Coupling constants enabled the assignment of all protons in the ^{1}H-NMR spectrum for the thiophene, at δ_H = 7.85, 7.19, 7.80. ^{13}C-NMR signals were clearly attributed to structure **4**. PDB, carried out with the thioester activated β-(N-acetylcysteaminyl) thiophene-2-carbothiolate in concentrations of only 2.3 mM because of toxic effects, resulted in 23 mg/L of **4**, a 10-fold higher yield compared to 2 mg/L from feeding thiophene-2-carboxylic acid (2.3 mM). The reason for this is most likely the improved metabolic availability of the CoA-ester mimiking precursor.

Because of water insolubility of indole-2-carboxylic acid, another supplementation technique was applied. The precursor was adsorbed on Amberlite XAD-2, and then added to the growing culture. Thus the acyl α-L-rhamnopyranoside **5** (61 mg/L) was obtained, and whose structure was confirmed spectroscopically. Adding other heteroaromatic carboxylic acids with the carboxyl group in 3-position yielded **6**−**8** as single products. They show the signals of an ester linked rhamnose moiety in the ^{1}H-NMR and ^{13}C-NMR spectra, and the expected signals for the heterocycle according to the corresponding fed acid. "Tandem" feeding, i.e. the synchronous addition of two acids under standard conditions (each at 7.3 mM), by combining pyrrole-2-carboxylic acid either with furan-2-carboxylic acid or with thiophene-2-carboxylic acid, clearly showed preference for the former. This was demonstrated by up to 7-fold higher yields of **2** compared to **3** and **4**. This result was not reproducible in all cases, in some "tandem" feeding experiments, for example, only **2** was produced.

Aminobenzoic Acids as Precursors

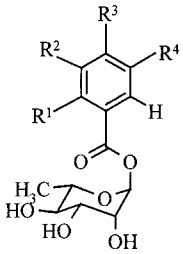
Supplementation of the culture medium with the three isomeric aminobenzoic acids resulted in the expected aminobenzoyl α-L-rhamnopyranosides **9**, **10**, and **12** (Scheme 2). The new *N*-acetyl derivatives **11**, **13**, and **15** were found in lower yields, and were unequivocally characterized by spectroscopic data. The presence of **11**, **13**, and **15** indicated a moderate *N*-acetylation activity of strain Tü 3634. For the aminobenzoyl metabolites **9**, **10**, and **12**, the DCI-MS revealed peaks at *m/z* = 301 [M + NH_4^+] and *m/z* = 284 [M + H^+], respectively, whereas the NMR spectra in the complete assignment clearly confirmed the structures with the corresponding signals and characteristic coupling patterns for both the aminobenzoyl and rhamnosyl moiety. Evidence for the *N*-acetyl group of **11**, **13**, and **15** was given by NMR signals for the methyl (δ_H = 2.10, δ_C = 23.9) and the carbonyl group (δ_C = 171.9), and from the mass spectra by the fragment *m/z* = 43. PDB with 3-amino-5-hydroxy- and 3-amino-4-hydroxybenzoic acids resulted in the formation of the acyl rhamnopyranosides **14** and **15**. The seven novel metabolites **9**−**15** turn out to be useful examples for producing a variety of unsubstituted and substituted aminobenzoyl α-L-rhamnopyranosides.

Benzoic and Cinnamic Acids as Precursors

Various derivatives of the rhamnoconjugates **19** and **20**, both belonging to the original metabolites[2] from strain Tü 3634, were produced by PDB. Studies on the tolerated distance of the carboxyl group from the phenyl ring were carried out with benzoic acid and phenylacetic acid, to give rise to **19** and **20**, respectively, and with 3-phenylpropionic acid and 4-phenylbutyric acid, which did not result in acyl α-L-rhamnopyranosides. In the latter experiments only **1** was detected in small yields (3 mg/L). Feeding cinnamic acid and ferulic acid successfully yielded the expected **21** and **22**, whereas 5-(4-aminophenyl)-2,4-pentadienoic acid and *trans,trans*-muconic acid were not accepted for rhamnosylation. The artificial metabolites whose production was achieved by the broad substrate specificity of the involved transferase are summarized in Scheme 3.

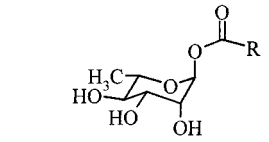
Benzoic acid, as a precursor, can be readily replaced by 4-hydroxy- or 3-fluorobenzoic acids to obtain **17** and **18**, respectively. The formation of **18** was proved by ^{19}F-NMR spectroscopy (δ_F = −112.48, *J* = 9.5, 9.5, 5.5 Hz). In an effort, to generate even larger structural diversity by feeding cyclohexane derived carboxylic acids, only the α,β-unsaturated acid gave the α-L-rhamnoside **16** in reasonable amounts (Scheme 3). Cyclohexanoic acid and coumalic acid were not accepted as precursors.

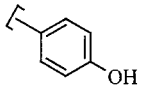
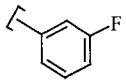
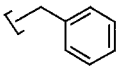
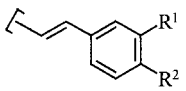
The results demonstrate that several exogenous acids are well accepted from the glycosyl transferase system of strain Tü 3634. The distance between the carboxyl group and the



	R^1	R^2	R^3	R^4	Yields (mg/L)
9	NH_2	H	H	H	35
10	H	NH_2	H	H	61
11	H	$NHCOCH_3$	H	H	12
12	H	H	NH_2	H	43
13	H	H	$NHCOCH_3$	H	6
14	H	NH_2	H	OH	11
15	H	$NHCOCH_3$	OH	H	6

Scheme 2. Structures and yields of the acyl α-L-rhamnopyranosides **9**−**15**



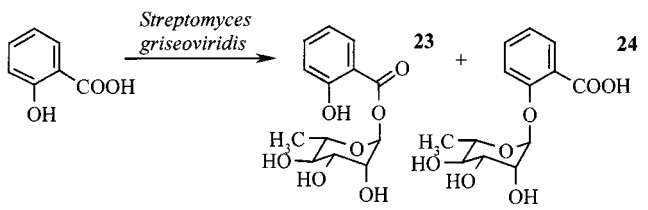
	R	Yields (mg/L)
16		29
17		2
18		35
19		35
20		15
21	 $R^1 = H$ $R^2 = H$	49
22	$R^1 = OCH_3$ $R^2 = OH$	46

Scheme 3. Structures and yields of the acyl α-L-rhamnopyranosides **16**−**22**

phenyl ring is not very variable, one methylene group or one ethylene group is accepted. 1-Cyclohexenoic acid shows that an α,β-unsaturation seems to be sufficient for rhamnosylation.

Salicylic Acid as Precursor

To investigate whether phenolic hydroxy groups are suitable as rhamnose acceptors, and how they compete with the carboxyl group, salicylic acid was supplemented to the culture medium of strain Tü 3634. The fermentation yielded two glycosylated products that were readily separated by Sephadex LH-20 gel filtration. One was the expected, novel 2-hydroxybenzoyl α-L-rhamnopyranoside (**23**, 38 mg/L) and the other was 2-*O*-(α-L-rhamnopyranosyl)salicylic acid (**24**, 8 mg/L), see Scheme 4. Compound **23** showed the molecular peak in the DCI-MS at m/z = 302 [M + NH_4^+], and a distinctive ester absorption at 1682 cm^{-1} in the IR spectrum. In the DCI-MS of **24**, the peak at m/z = 301 [M + NH_3] is indicative of the molecular mass. The very similar ^{1}H-NMR spectra of **23** and **24** exhibit identical coupling patterns but with differences in the chemical shifts (Table 3). The signals of the anomeric center provide evidence for the ester linkage in **23** (δ_H = 6.23, δ_C = 96.2) and the acetal group in **24** (δ_H = 5.55, δ_C = 100.7). The formation of two different types of rhamnosides in a ratio ester/acetal = 5:1 indicates that the specificity of the rhamnosyl transferase is not restricted to carboxyl groups. Similar effects have been observed in plant cell cultures that metabolize 4-hydroxybenzoic acid to either 4-*O*-glucosylbenzoic acid or 4-hydroxybenzoyl glucopyranoside.[8] *O*-Phenyl α-L-rhamnopyranosides were not found in the natural metabolite pattern of the strain, and therefore PDB opened up further biosynthetic pathways for designing metabolites.



Scheme 4. Rhamnosides **23**−**24** of salicylic acid by precursor-directed biosynthesis

Discussion

The precursor-directed biosynthesis (PDB), based on feeding of artificial carboxylic acids into the fermentation broth of *Streptomyces griseoviridis* Tü 3634, resulted in various new metabolites within the novel family of acyl α-L-rhamnopyranosides (**1**−**24**, Schemes 1 to 3). Oatmeal as nutrient solution (medium A) and a concentration of 7.3 mM of the precursors were established as the optimum fermentation conditions. The highest yield under comparable conditions was obtained by feeding pyrrole-2-carboxylic acid to give **2** (158 mg/L). In all other cases the yields were in the range of 2−89 mg/L, meaning that quantities of up to only 10% of the precursors were transformed into the desired product. On the other hand, without supplementation of acids, the four described metabolites **1**, **2**, **19**, and **20** could only be isolated in the range of 10 mg/L or less.[2] PDB gave rise to new acyl α-L-rhamnopyranosides, whereas the original metabolites (**1**, **19**) disappeared. The surplus of the fed acid is probably necessary to suppress the formation of the original metabolites, or to compete more successfully for rhamnosylation. The involvement of a special rhamnosyl transferase is quite likely. Its affinity to heteroaromatic and aromatic carboxylic acids is fairly selective. The more than 10-fold higher yields of rhamnoconjugates obtained with PDB compared to the original yields, e.g. of **1**, indicate that all the metabolic pathways via activated L-rhamnose and ending in the rhamnosyl transfer have a larger capacity than the pathways liberating the strain inherent carboxylic acids like 2,4-dimethylfuran-3-carboxylic acid. Whether the supply of the rhamnosyl transferase is independent of the carboxylic acid concentration or is regulated by it is still unknown.

Variations of PDB were carried out in order to influence the yields. A thioester precursor enhanced the yield of **4** drastically, and a fermentation in the presence of Amberlite XAD-2 helped to overcome the insufficient water solubility

Table 3. ^{1}H- (500 MHz, CD_3OD) (J in Hz), ^{13}C-NMR (125.7 MHz, CD_3OD), and IR (KBr) data of **23** and **24** ($\tilde{\nu}$ in cm^{-1})

Atom	**23** ^{1}H-NMR	^{13}C-NMR	**24** ^{1}H-NMR	^{13}C-NMR
1	–	113.1, s	–	125.5, s
2	–	163.1, s	–	155.5, s
3	6.75 (dd, 8.5, 1.0)	118.6, d	7.33 (dd, 8.5, 1.0)	116.8, d
4	7.26 (ddd, 8.5, 7.0, 1.5)	137.4, d	7.46 (ddd, 8.5, 7.5, 2.0)	133.6, d
5	6.57 (ddd, 8.0, 7.0, 1.0)	120.5, d	7.10 (ddd, 7.5, 7.5, 1.0)	123.3, d
6	7.77 (ddd, 8.0, 1.5)	131.0, d	7.74 (dd, 7.5, 2.0)	131.5, d
7	–	169.5, s	–	171.0, s
1′	6.15 (d, 2.0)	96.2, d	5.55 (d, 2.0)	100.7, d
2′	3.92 (dd, 3.5, 2.0)	71.1, d	4.10 (dd, 3.5, 2.0)	71.8, d
3′	3.82 (dd, 9.5, 3.5)	72.1, d	3.83 (dd, 9.5, 3.5)	72.4, d
4′	3.48 (dd, 9.5, 9.5)	73.3, d	3.47 (dd, 9.5, 9.5)	73.7, d
5′	3.74 (dq, 9.5, 6.0)	72.8, d	3.65 (dq, 9.5, 6.0)	71.2, d
6′	1.27 (d, 6.0)	18.1, q	1.23 (d, 6.0)	18.0, q
IR	3421, 2926, 1682, 1616, 1581		3422, 2930, 1699, 1650, 1604, 1518	

of indole-2-carboxylic acid, yielding **5**. A "tandem" feeding experiment confirmed the higher affinity of pyrrole-2-carboxylic acid to the enzyme compared with its oxygen and sulfur analogues by the 7-fold higher yields of **2** with up to 158 mg/L. Experiments in which strain Tü 3634 with its dominant rhamnosyl transferase activity is combined in mixed cultures with a second strain which produces pyrrole-2-carboxylic acid yielded the corresponding **2** (5 mg/L).

Benzoic acid and its derivatives gave further insight into the substrate specificity of the involved enzyme. Substituents in any position of the phenyl ring are well tolerated, in the case of amino groups an additional *N*-acetylation activity was observed to give **11**, **13**, and **15**. A hydroxy group *ortho* to the carboxyl group gives rise to an alternative *O*-rhamnosylation of secondary importance concerning the yields (**23**, **24**), which was not observed for 4-hydroxybenzoic acid. Furthermore, the distance of the carboxyl group from the phenyl ring is restricted, only one methylene or ethylene group is possible. Whether this characterises a substrate specificity due to a steric fixation of the unsaturated environment and the carboxyl group or a limitation in the transport through the membranes is still not clear. The different behaviour, however, of 1-cyclohexenoic acid (accepted) and cyclohexanoic acid (not accepted) speaks against the latter argument.

In contrast with the variability of the acyl moiety, there is a strict specificity of the rhamnosyl residue, only α-L-rhamnopyranosides have been observed. The rhamnosylation requires dTDP L-rhamnose, which is normally generated starting from dTDP D-glucose. We assume that the transferase catalyzes a nucleophilic attack of the carboxylate ion at the anomeric carbon atom. Comparison of the acidity of the fed carboxylic acids does not explain the differences in the yields.

Extending these studies to cell free systems and isolated enzymes will be the subject of future efforts.

PDB with strain Tü 3634 turned out to be a versatile and attractive approach on the background of the "One Strain/Many Compounds" concept (OSMAC-Method) for exploring chemical diversity among new microbial secondary metabolites,[9] and allows for an extension of a product pattern in a qualitative as well as quantitative way.

Experimental Section

General Remarks: See ref.[2] – ^{19}F NMR spectra: Varian I 500; Chemical shifts are expressed in δ values, as internal standards $CFCl_3/C_6F_6$ was used.

Nutrient Solutions. – Medium A: Oatmeal 20 g/L, 2.5 mL/L trace element solution, pH = 6.8 prior to sterilisation. – **Medium B:** Malt extract 10 g/L, yeast extract 4 g/L, glucose 4 g/L, pH = 7.0 prior to sterilisation. – **Medium C:** Malt extract 10 g/L, yeast extract 4 g/L, glycerol 20 g/L, $CaCO_3$ 20 mg/L, pH = 7.0 prior to sterilisation. – **Medium D:** Glycerol 30 g/L, casein peptone 2 g/L, K_2HPO_4 1 g/L, NaCl 1 g/L, $MgSO_4 \cdot 7\,H_2O$ 0.5 g/L, 5 mL/L trace element solution, pH = 7.3 prior to sterilisation. – **Trace element solution:** $CaCl_2 \cdot 2\,H_2O$ 3 g/L, Fe^{III} citrate 1 g/L, $MnSO_4$ 0.2 g/L, $ZnCl_2$ 0.1 g/L, $CuSO_4 \cdot 5\,H_2O$ 25 mg/L, $Na_2B_4O_7 \cdot 10\,H_2O$ 20 mg/L, $CoCl_2$ 4 mg/L, $Na_2MoO_4 \cdot 2\,H_2O$ 10 mg/L.

Fermentation and PDB: Strain Tü 3634 (*Streptomyces griseoviridis*) was maintained as a stock culture on agar slants (medium B) and cultivated in medium A under standard conditions in a stirred vessel (1 L working volume, 700 rpm, 28 °C, aeration 1.6 vvm, 72 h) as described.[2] Isolation and purification of the acyl α-L-rhamnosides were carried out according to established protocols from the separated culture filtrate.[2] All compounds (**1**–**24**) are readily soluble in MeOH, ethyl acetate, DMSO, slightly soluble in $CHCl_3$ and water, and insoluble in *n*-hexane. Under acidic (pH < 4) or alkaline (pH > 9) conditions the compounds are unstable, and are destroyed while heating above 40 °C. Spectroscopic and other experimental data of **1**, **2**, **19**, and **20** were identical with those already published.[2] Feeding experiments with various carboxylic acids were carried out under standard conditions (7.3 mM, 1-L fermentor) and continuous addition of precursors for 10 h by a low rate pump (7 mL/h) starting 16 h after inoculation. Precursors were dissolved in 70 mL of deionized water, adjusted to pH = 6.5 with 0.1 M NaOH, and sterilized at 120 °C for 30 min. The yields of the obtained products are given in Schemes 1–3. Amberlite XAD-2 (30 g) in 150 mL methanol was stirred with indole-2-carboxylic acid (1.16 g, 3 h) and evaporated in vacuo to yield the feeding material, which was added to the cultures in 20 portions over 10 hours.

For IR, ^{1}H- and ^{13}C-NMR data of **3**−**22** see Supporting Information, except for **7**, **16**, **23**, and **24** (see Table 2 and below). Data for **1**, **2**, **19**, and **20** were described in the previous paper.[2]

2-Furanylcarbonyl α-L-Rhamnopyranoside (3): Feeding of furan-2-carboxylic acid (0.82 g). − M.p. 120 °C. − $[\alpha]_D^{20} = -42$ ($c = 0.31$ in MeOH). − UV (MeOH): λ_{max} (log ε) = 202 nm (3.62), 253 (3.53). − DCI-MS: *m/z* (%) = 276 (26) [M + NH_4^+], 164 (100) [$C_6H_{11}O_5$+H^+]. − $C_{11}H_{14}O_7$ (258.23).

Thiophene-2-carboxylic Acid (*N*-Acetylcysteamin)thioester: DPPA (5.0 mL, 23 mmol) and triethylamine (3.5 mL, 25 mmol) were added to a mixture of thiophene-2-carboxylic acid (1.6 g, 13 mmol) and *N*-acetyl cysteamine (2.3 g, 19 mmol) in 10 mL DMF at 0 °C while stirring. After stirring for 15 h at room temp., the reaction mixture was quenched with an excess of H_2O, acidified with 2 M HCl, and extracted with $CHCl_3$. The combined organic layers were washed with saturated $NaHCO_3$ solution, dried with Na_2SO_4, and evaporated to dryness. The crude product was purified by chromatography on silica gel (CH_2Cl_2/MeOH 95:5) and Sephadex LH-20 (column: 100 × 2.5 cm, acetone) to yield 2.4 g (11 mmol, 85%) of an amorphous white solid. − M.p. 83 °C (dec.). − IR (KBr) $\tilde{\nu}$ = 3430 cm^{-1}, 3320, 1634, 1551, 1512. − UV (MeOH): λ_{max} (log ε) = 202 nm (3.87), 252 (3.87), 291 (4.01). − ^{1}H NMR (500 MHz, CD_3OD): δ =1.94 (s, 3 H, 4′-H_3), 3.19 (t, J = 6.2 Hz, 2 H, 1′-H_2), 3.49/ 3.51 (t, J = 6.2 Hz, 2 H, 2′-H_2), 7.10 (dd, J = 4.0, 5.0 Hz, 1 H, 4-H), 7.62 (dd, J = 5.0, 1.0 Hz, 1 H, 5-H), 7.79 (dd, J = 4.0, 1.0 Hz, 1 H, 3-H). − ^{13}C NMR (125.7 MHz, CD_3OD): δ = 23.2 (q, C-4′), 28.7 (t, C-1′), 39.7 (t, C-2′), 127.9 (d, C-4), 131.4 (d, C-3 o. C-5), 133.0 (d, C-3 o. C-5), 141.6 (s, C-2), 170.4 (s, C-6), 184.2 (s, C-3′). − EI MS: *m/z* (%) = 229.1 (10) [M^+], 111.0 (100) [C_5H_3OS]. − $C_9H_{11}NO_2S_2$ (229.31).

2-Thiophenylcarbonyl α-L-Rhamnopyranoside (4): Feeding of thiophene-2-carboxylic acid (2.7 mM, 0.35 g; 7.3 mM 0.94 g) or thiophene-2-carboxylic acid (*N*-acetylcysteamin)thioester (2.7 mM, 0.53 g). − M.p. 65 °C (dec.). − $[\alpha]_D^{20} = -36$ ($c = 0.15$ in MeOH). − UV (MeOH): λ_{max} (log ε) = 249 nm (3.99), 271 (3.88). − DCI-MS: *m/z* (%) = 292 (100) [M + NH_4^+]. − $C_{11}H_{14}O_6S$ (274.29).

2-Indolylcarbonyl α-L-Rhamnopyranoside (5): Feeding of indole-2-carboxylic acid (1.16 g). − M.p. 107 °C. − $[\alpha]_D^{20} = -28$ ($c = 0.20$ in MeOH). − IR (KBr): $\tilde{\nu}$ = 3410 cm^{-1}, 2925, 1704, 1621, 1529. − UV (MeOH): λ_{max} (log ε) = 218 nm (4.17), 294 (4.18). − ^{1}H NMR (500 MHz, CD_3OD): δ =1.29 (d, J = 6.0 Hz, 3 H, 6′-H_3), 3.53 (dd, J = 9.5, 9.5 Hz, 1 H, 4′-H), 3.84 (dd, J = 9.5, 6.0 Hz, 1 H, 5′-H), 3.94 (dd, J = 9.5, 3.5 Hz, 1 H, 3′-H), 3.98 (dd, J = 3.5, 2.0 Hz, 1 H, 2′-H), 6.20 (d, J = 2.0 Hz, 1 H, 1′-H), 7.07 (ddd, J = 8.0, 8.0, 0.8 Hz, 1 H, 5-H), 7.22 (d, J = 0.8 Hz, 1 H, 3-H), 7.26 (ddd, J = 8.0, 8.0, 0.8 Hz, 1 H, 6-H), 7.45 (dd, J = 8.0, 0.8 Hz, 1 H, 7-H), 7.62 (ddd, J = 8.0, 0.8, 0.8 Hz, 1 H, 4-H). − ^{13}C NMR (75.5 MHz, CD_3OD): δ = 18.1 (q, C-6′), 71.3 (d, C-2′), 72.1 (d, C-3′), 72.5 (d, C-5′), 73.5 (d, C-4′), 95.6 (d, C-1′), 110.2 (d, C-3), 113.3 (d, C-7), 121.5 (d, C-5), 123.3 (d, C-4), 126.3 (d, C-6), 127.8 (s, C-2), 128.5 (s, C-3a), 139.4 (s, C-7a), 161.4 (s, C-8). − DCI-MS: *m/z* (%) = 342 (46) [M + NH_3 + NH_4^+], 325 (100) [M + NH_4^+]. − $C_{15}H_{17}NO_6$ (307.30).

3-Furanylcarbonyl α-L-Rhamnopyranoside (6): Feeding of furan-3-carboxylic acid (0.82 g). − M.p. 165−170 °C. − $[\alpha]_D^{20} = -45$ (c = 0.20 in MeOH). − UV (MeOH): λ_{max} (log ε) = 202 nm (3.68), 238 (3.38). − DCI-MS: *m/z* (%) = 276 (63) [M + NH_4^+], 182 (40) [$C_6H_{11}O_5$ + NH_4^+ + H^+], 164 (100) [$C_6H_{11}O_5$ + H^+]. − $C_{11}H_{14}O_7$ (258.23).

2-Methyl-3-pyrrolylcarbonyl α-L-Rhamnopyranoside (7): Feeding of 2-methylpyrrole-3-carboxylic acid[10] (1.98 g). − M.p. 149 °C. − $[\alpha]_D^{20} = -50$ ($c = 0.1$ in MeOH). − UV (MeOH): λ_{max} (log ε) = 201 nm (4.15), 228 (3.82), 259 (3.73); UV (MeOH/NaOH): λ_{max} (log ε) = 203 (4.06), 227 (3.85), 258 (3.76). − (+)-ESI-MS: *m/z* (%) = 294 (20) [M + Na]$^+$, 565 (100) [2 M + Na]$^+$. − $C_{12}H_{17}NO_6$ (271.27).

3-Pyridylcarbonyl α-L-Rhamnopyranoside (8): Feeding of nicotinic acid (0.90 g). − M.p. 47 °C. − $[\alpha]_D^{20} = -18$ ($c = 0.69$ in MeOH). − UV (MeOH): λ_{max} (log ε) = 201 nm (3.66), 219 (3.85), 263 (3.29). − DCI-MS: *m/z* (%) = 287 (100) [M + NH_4^+], 270 (37) [M + H^+]. − $C_{12}H_{15}NO_6$ (269.25).

2-Aminobenzoyl α-L-Rhamnopyranoside (9): Feeding of anthranilic acid (1.00 g). − M.p. 190 °C. − $[\alpha]_D^{20} = -80$ ($c = 0.40$ in MeOH). − UV (MeOH): λ_{max} (log ε) = 221 (4.29), 248 (3.78), 342 (3.65); UV (MeOH/HCl): λ_{max} (log ε) = 202 (3.97), 222 (4.17), 342 (3.37). − DCI-MS: *m/z* (%) = 301 (20) [M + NH_4^+], 284 (100) [M + H^+]. − $C_{13}H_{17}NO_6$ (283.28).

3-Aminobenzoyl α-L-Rhamnopyranoside (10): Feeding of 3-aminobenzoic acid (1.00 g). − M.p. 71 °C. − $[\alpha]_D^{20} = -37$ ($c = 0.22$ in MeOH). − UV (MeOH): λ_{max} (log ε) = 223 nm (4.35), 324 (3.09); UV (MeOH/HCl): λ_{max} (log ε) = 227 (4.03), 273 (2.65). − DCI-MS: *m/z* (%) = 301 (100) [M + NH_4^+], 284 (80) [M + H^+]. − $C_{13}H_{17}NO_6$ (283.28).

3-(Acetylamino)benzoyl α-L-Rhamnopyranoside (11): Feeding of 3-aminobenzoic acid (1.00 g). − M.p. 115−120 °C. − $[\alpha]_D^{20} = -35$ ($c = 0.36$ in MeOH). − UV (MeOH): λ_{max} (log ε) = 225 nm (4.39), 300 (3.19). − DCI-MS: *m/z* (%) = 343 (100) [M + NH_4^+]. − $C_{15}H_{19}NO_7$ (325.32).

4-Aminobenzoyl α-L-Rhamnopyranoside (12): Feeding of 4-aminobenzoic acid (1.00 g). − M.p. 109 °C. − $[\alpha]_D^{20} = -27$ ($c = 0.21$ in MeOH). − UV (MeOH): λ_{max} (log ε) = 203 nm (4.07), 222 (3.91), 298 (4.27). − EI MS: *m/z* (%) = 283 (13) [M^+], 137 (70) [M^+ − $C_6H_{10}O_4$], 120 (100) [M^+ − $C_6H_{11}O_5$]. − DCI-MS: *m/z* (%) = 301 (100) [M + NH_4^+], 284 (60) [M + H^+]. − $C_{13}H_{17}NO_6$ (283.28).

4-(Acetylamino)benzoyl α-L-Rhamnopyranoside (13): Feeding of 4-aminobenzoic acid (1.00 g). − M.p. 118 °C. − $[\alpha]_D^{20} = -28$ (c = 0.14 in MeOH). − UV (MeOH): λ_{max} (log ε) = 202 nm (4.21), 274 (4.20). − HREI-MS: found as calcd. for $C_{15}H_{19}NO_7$: 325.1161. − EI MS: *m/z* (%) = 325 (2) [M^+], 162 (32) [M^+ − $C_6H_{11}O_5$], 43 (36) [$COCH_3$]$^+$. − $C_{15}H_{19}NO_7$ (325.32).

3-Amino-5-hydroxybenzoyl α-L-Rhamnopyranoside (14): Feeding of 3-amino-5-hydroxy benzoic acid (1.38 g). − M.p. 75 °C. − $[\alpha]_D^{20}$ = −35 ($c = 0.20$ in MeOH). − UV (MeOH): λ_{max} (log ε) = 211 nm (4.24), 229 (4.19), 330 (3.31); UV (MeOH/NaOH): λ_{max} (log ε) = 219 (4.27), 278 (3.40), 344 (3.34). − DCI-MS: *m/z* (%) = 317 (100) [M + NH_4^+], 300 (80) [M + H^+]. − $C_{13}H_{17}NO_7$ (299.28).

3-(Acetylamino)-4-hydroxy-benzoyl α-L-Rhamnopyranoside (15): Feeding of 3-amino-4-hydroxybenzoic acid (1.13 g). − M.p. 172 °C. − $[\alpha]_D^{20} = -26$ ($c = 0.1$ in MeOH). − UV (MeOH): λ_{max} (log ε) = 203 nm (4.15), 236 (4.14), 261 (3.88); UV (MeOH/NaOH): λ_{max} (log ε) = 206 nm (4.14), 247 (3.91), 307 (3.95). − HREI-MS: calcd. for $C_{13}H_{18}O_7$: 286.1052, found: 286.1058. − DCI-MS: *m/z* (%) = 169 (100) [M − $C_7H_{11}O_6$ + H^+ + NH_4^+]. − (−)-ESI-MS: *m/z* (%) = 341.7 (100) [M]$^-$; (+)-ESI-MS: *m/z* (%) = 364.3 (100) [M + Na]$^+$. − $C_{15}H_{19}O_8$ (341.32).

1-Cyclohexen-1-ylcarbonyl α-L-Rhamnopyranoside (16): Feeding of 1-cyclohexene-1-carboxylic acid (0.94 g). − M.p. 72 °C. − $[\alpha]_D^{20}$ = −46 ($c = 0.05$ in MeOH). − IR (KBr): $\tilde{\nu}$ = 3424 cm^{-1}, 2931, 1713, 1643. − UV (MeOH): λ_{max} (log ε) = 220 nm (3.91). − ^{1}H NMR

(500 MHz, CD_3OD): δ = 1.25 (d, *J* = 6.5 Hz, 3 H, 6′-H_3), 1.61 (m, 2 H, 3-H_2), 1.67 (m, 2 H, 4-H_2), 2.23 (m, 4 H, 5-H_2, 6-H_2), 3.44 (dd, *J* = 9.5, 9.5 Hz, 1 H, 4′-H), 3.64 (dd, *J* = 9.5, 6.5 Hz, 1 H, 5′-H), 3.70 (dd, *J* = 9.5, 3.0 Hz, 1 H, 3′-H), 3.83 (dd, *J* = 3.0, 2.0 Hz, 1 H, 2′-H), 5.97 (d, *J* = 2.0 Hz, 1 H, 1′-H), 7.05 (m, 1 H, 2-H). − ^{13}C NMR (125.7 MHz, CD_3OD): δ = 18.1 (q, C-6′), 22.4 (t, C-3), 23.1 (t, C-4), 25.1 (t, C-5), 26.8 (t, C-6), 71.3 (d, C-4′), 72.2 (d, C-3′), 72.3 (d, C-5′), 73.5 (d, C-2′), 95.2 d, C-1′),131.0 (s, C-1), 142.7 (d, C-2), 166.7 (s, C-7). − DCI-MS: *m/z* (%) = 290 (87) [M + NH_4^+], 181 (27) [$C_6H_{11}O_5$ + NH_4^+], 164 (100) [$C_6H_{11}O_5$ + H^+]. − $C_{13}H_{10}O_6$ (272.30).

4-Hydroxybenzoyl α-L-Rhamnopyranoside (17): Feeding of 4-hydroxybenzoic acid (1.01 g). −M.p. 158 °C. − $[\alpha]_D^{20}$ = −31 (*c* = 0.19 in MeOH). UV (MeOH): λ_{max} (log ε) = 203 nm (4.02), 209 (4.01), 259 (4.05); UV (MeOH/HCl): λ_{max} (log ε) = 203 nm (4.02), 209 (4.01), 259 (4.05); (MeOH/NaOH): λ_{max} (log ε) = 206 nm (3.73), 223 (3.79), 301 (4.22). − DCI-MS: *m/z* (%) = 302 (100) [M + NH_4^+], 181 (29) [$C_6H_{11}O_5$ + NH_4^+], 164 (90) [$C_6H_{11}O_5$ + H^+]. − $C_{13}H_{16}O_7$ (284.27).

3-Fluorbenzoyl α-L-Rhamnopyranoside (18): Feeding of 3-fluorbenzoic acid (1.02 g). − M.p. 143 °C (dec.). − $[\alpha]_D^{20}$ = −28 (*c* = 0.11, in MeOH). − UV (MeOH): λ_{max} (log ε) = 201 nm (3.91), 228 (4.02), 277 (3.19). − ^{19}F NMR (470.27 MHz, CD_3OD), ref.: $CFCl_3$/C_6F_6): δ = 112.48 (ddd, 9.5, 9.5, 5.5 Hz, 1F, 3−F). − DCI-MS: *m/z* (%) = 304 (62) [M + NH_4^+], 164 (100) [$C_6H_{11}O_5$ + H^+]. − $C_{13}FH_{15}O_6$ (286.26).

3-Phenyl-2-propenoyl α-L-Rhamnopyranoside (21): Feeding of cinnamic acid (1.08 g). − M.p. 132 °C. − $[\alpha]_D^{20}$ = −47 (*c* = 0.42 in MeOH). UV (MeOH): λ_{max} (log ε) = 205 nm (4.13), 217 (4.16), 278 (4.36). − DCI-MS: *m/z* (%) = 312 (100) [M + NH_4^+]. − $C_{15}H_{18}O_6$ (294.30).

3-(4-Hydroxy-3-methoxyphenyl)-2-propenoyl α-L-Rhamnopyranoside (22): Feeding of ferulic acid (1.42 g) − M.p. 95 °C (dec.). − $[\alpha]_D^{20}$ = −37 (*c* = 0.20 in MeOH). UV (MeOH): λ_{max} (log ε) = 204 nm (4.12), 218 (4.09), 237 (4.04), 329 (sh) (4.27); (MeOH/NaOH): λ_{max} (log ε) = 208 nm (4.04), 228 (3.89), 252 (3.90), 310 (3.58), 387 (4.41). − DCI-MS: *m/z* (%) = 358 (100) [M + NH_4^+]. − $C_{16}H_{20}O_8$ (340.33).

2-Hydroxybenzoyl α-L-Rhamnopyranoside (23): Feeding of salicylic acid (1.01 g). − M.p. 91 °C (dec.). − $[\alpha]_D^{20}$ = −36 (*c* = 0.67 in MeOH). − UV (MeOH): λ_{max} (log ε) = 209 nm (4.38), 229 (3.97), 308 (3.62); (MeOH/NaOH): λ_{max} (log ε) = 210 nm (4.18), 237 (3.85), 308 (3.49). − DCI-MS: *m/z* (%) = 302 (100) [M + NH_4^+]. − $C_{13}H_{16}O_7$ (284.27).

2-*O*-(α-L-Rhamnopyranosyl)benzoic Acid (24): Feeding of PDB with salicylic acid (1.01 g). − M.p. 121 °C (dec.). − $[\alpha]_D^{20}$ = −19 (*c* = 0.16 in MeOH). − UV (MeOH): λ_{max} (log ε) = 205 nm (4.31), 278 (3.27); UV (MeOH/NaOH): λ_{max} (log ε) = 208 nm (3.99), 283 (3.21) − DCI-MS: *m/z* (%) = 301 (10) [M^+ + NH_3], 164 (22) [$C_6H_{11}O_5$ + H^+], 155 (100) [$C_7H_5O_3$ + NH_4^+]. − $C_{13}H_{16}O_7$ (284.27).

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