Mixed Acetate-Glycerol Biosynthesis and Formation of Benzoate Directly from Shikimate in *Streptomyces* sp.

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Dedicated to Prof. Dr. Axel Zeeck on the occasion of his 65th birthday

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Biosynthetic studies of γ-butyrolactones and 3-furanylcarbonyl α-L-rhamnopyranosides reveal a new mixed biosynthetic pathway which provides different types of secondary metabolites. The new γ-butyrolactone **1** is produced by *Streptomyces* sp. (strain GT 61150) together with (propenyl-3-furanyl)carbonyl α-L-rhamnopyranoside (**2**). Their structures were elucidated by chemical and spectroscopic methods. The biosynthesis of **1** and **2** was established by feeding ^{13}C-labelled acetate and glycerol to *Streptomyces* sp. and resulted in a complete labelling pattern of **1** and **2**. Benzoyl α-L-rhamnopyranoside (**3**) is a rare compound of the rhamnoside family and is produced by *Streptomyces griseoviridis* (strain Tü 3634). [1,7-$^{13}C_2$]Shikimic acid was fed to prove the hypothesis that the benzoyl unit is derived directly from shikimate and not from the plant-like pathway with phenylalanine as an intermediate. The results exemplify a novel bacterial benzoate biosynthesis.

Introduction

Acyl α-L-rhamnopyranosides are a rare family of secondary metabolites that have been isolated from selected *Streptomyces* strains. They are considered to be energy-rich bacterial rhamnoconjugates that are synthesized by a unique rhamnosyl transferase.[1,2] Some products, for example, **2**, **3** and **4** demonstrate inhibitory activity towards 3α-hydroxysteroid dehydrogenase (3α-HDS), a useful target for anti-inflammatory and anti-phlogistic drugs.[3]

Our previous studies of rhamnoside-producing strains revealed a series of novel furanylcarbonyl- and butyrolactone-containing metabolites. The detailed analysis of the metabolite pattern of the *Streptomyces* sp. strain (strain GT 61150) recently led to novel (propenyl-3-furanyl)carbonyl α-L-rhamnopyranoside (**2**) and rhamnosyl lactones.[3] Their structures were confirmed by spectroscopic analysis, especially by 2D NMR techniques. By applying a chemical screening method several rhamnosides have been isolated from *Streptomyces griseoviridis* (Tü 3634), for example, (2,4-dimethyl-3-furanyl)carbonyl α-L-rhamnopyranoside (**5**) and butyrolactones **6** and **7** along with several other rhamnosides like **8**.[4] The autoregulators of streptomycetes, for example, the A-factor or virginiae butanolides, which are essential for the differentiation and the production of antibiotics, are found to have similar structures. However, none of these known metabolites is linked to a deoxysugar moiety.[5] Hitherto, we postulated that in some cases the C_7 or C_9 furans and lactones from strain GT 61150 and Tü 3634 can be biosynthesized from two or three acetates and one glycerol unit followed by rhamnosylation,[3,4] which implies that the carbon skeleton can be biosynthesized from both the acetate and the carbohydrate pool.

Unsubstituted benzoyl units as found in **3** are very rare in bacterial secondary metabolites[6] and despite the production of the benzoyl rhamnoside **3** from strain Tü 3634 they have only been found in aestivophoenin A,[7] enterocin, wailupemycin,[8] all isolated from *Streptomyces*, and in the myxobacterial soraphen,[9] thiangazole and crocacin.[10] In contrast, the occurrence of benzoic acid metabolites is quite common in plants and fungi and are constituents of the important cancer drug taxol[11] and the fungicide strobilurin.[12] Eucaryotic benzoate biosynthesis proceeds via phenylalanine derived from the shikimate pathway. Recently, a plant-like pathway was established for the synthesis of the benzoyl starter unit of enterocin.[8] However, the labelling pattern of our earlier ^{13}C-glycerol-feeding experiments suggested that phenylalanine could not be a precursor of the benzoyl residue of **3**, therefore ruling out the prephenate route for this biosynthetic pathway.

Herein, the extended metabolic diversity of secondary metabolites in *Streptomyces* generated by a new mixed acet-

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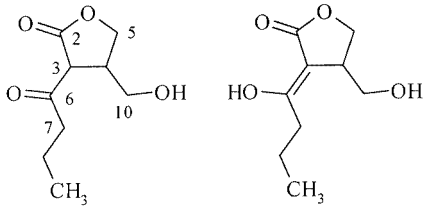
ate-glycerol and direct shikimate-benzoyl biosynthetic pathway are discussed.

Results and Discussion

Culture Conditions and Isolation

The initial fermentations of *Streptomyces* sp. (strain GT 61150) with standard culture media yielded 1.5 mg·L^{-1} of the (propenyl-3-furanyl)carbonyl α-L-rhamnopyranoside (**2**). As a prerequisite for biosynthetic studies, the production rate of **1** and **2** needed to be increased, which was achieved by the addition of glycerol (500 mg·L^{-1}) to the standard oatmeal medium A. Under these conditions the production of **2** started 72 h after incubation and reached a maximum after 120 h, as analyzed by HPLC. The butyrolactone **1** (Scheme 1) started to be produced after 24 h and was detected over 144 h . The yields of **1** and **2** were reproducible and were in the range of 9−12 mg·L^{-1} (**1**) and 2−4 mg·L^{-1} (**2**). The chemical and spectroscopic properties of **2** were identical to those given in the literature.[3]



1 **1a**

Scheme 1

3-Butyryl-4-(hydroxymethyl)-4,5-dihydrofuran-2(3*H*)-one (**1**) is a novel natural compound. It is a colourless oil less polar than **2**. It gave an intensive yellow colour reaction with anisaldehyde/H_2SO_4 as a staining reagent and was spectroscopically characterized.

3-Butyryl-4-(hydroxymethyl)-4,5-dihydrofuran-2(3H)-one (1)

The molecular formula of **1** ($C_9H_{14}O_4$, M_R = 186.21) was determined from the high resolution ESI ([M + H]$^+$ = 187.09657) and DCI ([M + NH_4]$^+$ = 204 and [2M + NH_4]$^+$ = 390) mass spectra. The UV absorption maximum (MeOH) at 238 nm characterized the cyclic chromophore. The IR spectrum exhibits a strong band at 1767 cm^{-1} and a band at 1716 cm^{-1}, which indicate a butyrolactone and a keto group, respectively.

A partial triple set of NMR signals (CD_3OD) made the analysis of the NMR spectra of **1** challenging. HPLC analysis revealed a broad signal using RP-C_{18}-encapped columns. For one component, the ^{1}H, ^{1}H−^{1}H COSY and the HSQC NMR spectra established the presence of a propyl and an isobutyryl unit as two molecular fragments and two quaternary C atoms were deduced from the HSQC NMR spectrum. The presence of a keto (δ_C = 204.0 ppm) and an ester group (δ_C = 173.5 ppm) was deduced from the ^{13}C NMR spectrum. HMBC correlations confirmed the parent structure to be 3-butyryl-4-(hydroxymethyl)-4,5-dihydrofuran-2(3*H*)-one (**1**) as the main component in [D_6]acetone and CD_3OD. In addition, the existence of the enol tautomer **1a** was confirmed by the presence of a clear triplet for the C-3 signal in **1** (δ_C = 55.9 ppm, solvent CD_3OD) due to deuterium exchange (Table 1). We also assume the presence of the hemiketal form of **1** as a result of very small signals at δ_C = 107.0 ppm (solvent CD_3OD or [D_6]acetone) in the ^{13}C NMR spectrum.[13] Compound **1** has the 3,4-disubstituted γ-butyrolactone skeleton characteristic of the known autoregulators and, with the 6-keto group, belongs to the A-factor type.[14]

Table 1. ^{1}H and ^{13}C NMR signals and HMBC correlations of 3-butyryl-4-(hydroxymethyl)-4,5-dihydrofuran-2(3*H*)-one (**1**)

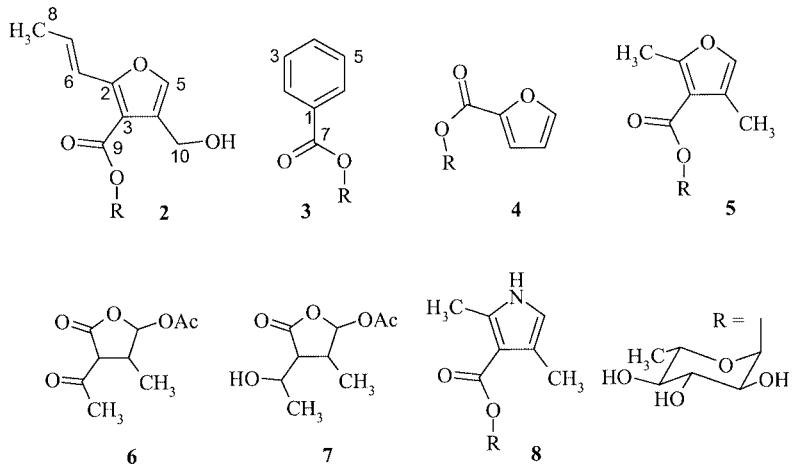
C atom[a]	δ_H [ppm]	δ_C [ppm]	$\delta_C \rightarrow \delta_H$ [ppm]
2	−	173.5	−
3	3.84 (d, 7.0)	55.6	3.15, 3.68, 4.15, 4.44
4	3.15 (m)	41.1	3.68, 4.15, 4.44
5_A	4.15 (dd, 7.0, 9.0)	70.0	3.15, 3.68
5_B	4.44 (dd, 7.0, 8.5)	70.0	3.15, 3.68
6	−	204.0	−
7_A	2.68 (dt, 7.5, 7.5)	44.9	0.92, 1.62
7_B	2.89 (dt, 7.5, 7.5)	44.9	0.92, 1.62
8	1.62 (ddq, 7.5, 7.5, 7.5)	17.2	0.92, 2.68, 2.89
9	0.92 (t, 7.5)	13.7	1.62
10	3.68 (dd, 2.0, 5.0)	63.0	3.15, 4.15, 4.44

[a] ^{1}H and ^{13}C NMR spectra were recorded at 500 and 125.7 MHz, respectively, in [D_6]acetone.

Biosynthetic Studies

Mixed Acetate-Glycerol Biosynthesis: A working hypothesis for the biosynthesis of the acyl rhamnoside **2** (Scheme 2) is envisaged to involve a mixed acetate-glycerol pathway for the synthesis of the aglycon moiety of **2**, as has been shown for the biosynthesis of **5**, **6**, acetomycin and acaterin, while the L-rhamnose group is derived from the usual carbohydrate pathway.[15−17] We were interested in whether this is a common biosynthetic scheme with which to generate the chemical structural diversity of furanyl- and lactone-containing metabolites like **1** and **2** produced by single *Streptomyces* strains.

Consequently, we carried out tracer experiments with *Streptomyces* sp. (GT 61150) by adding ^{13}C-labelled sodium [1-^{13}C]acetate, [1,2-$^{13}C_2$]acetate or [U-$^{13}C_3$]glycerol to growing cultures of the strain over 48 h (continuous feeding), starting 84 h after inoculation. After 144 h, **1** and **2** were efficiently isolated from the culture filtrate as pure compounds, and analyzed by ^{13}C NMR spectroscopy. Specific incorporations were calculated according to Scott et al.[18] NMR signals were analyzed as first-order derivatives or by spin simulation. The results of the ^{13}C NMR spectral analysis of **2** produced in the tracer experiments are presented in Table 2.



Scheme 2

Table 2. ^{13}C NMR analysis of enriched aglycon of **1** and **2** after feeding of individual precursors ($^{13}C-^{13}C$ coupling constants)

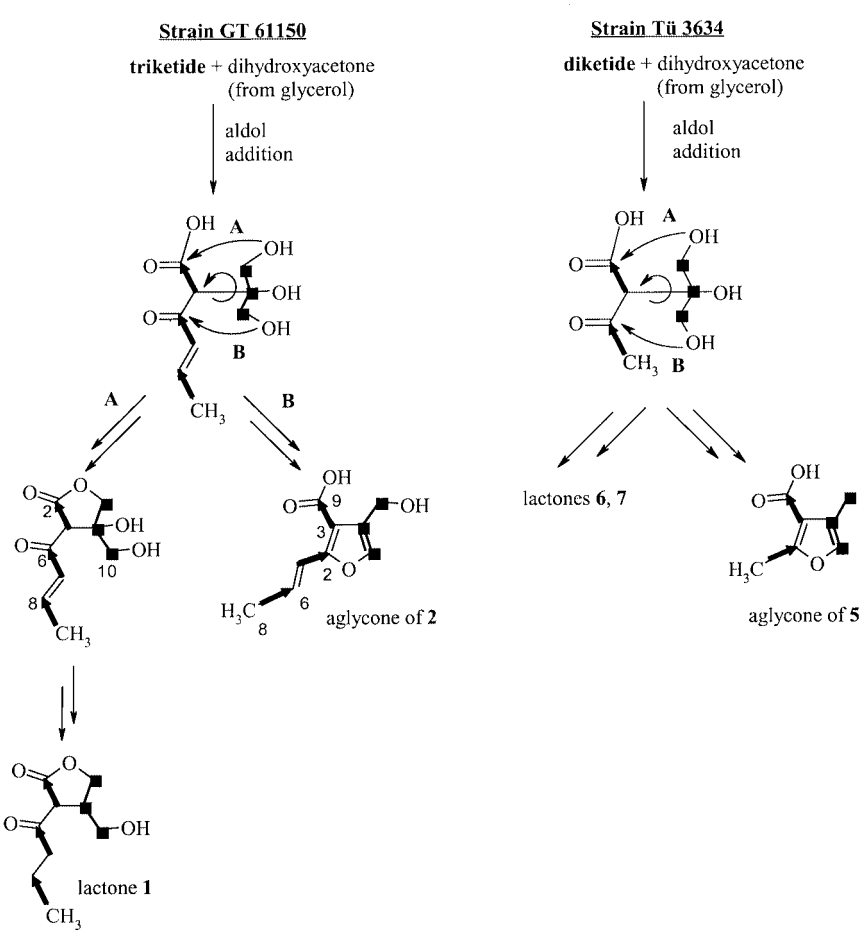
C atom	δ_C [ppm] of **1**	$J_{(C,C)}$ [Hz] of **1** [1,2-$^{13}C_2$]Acetate	[U-$^{13}C_3$]Glycerol	δ_C [ppm] of **2**	[1-^{13}C]Acetate specific incorporations	$J_{(C,C)}$ [Hz] of **2** [1,2-$^{13}C_2$]Acetate	[U-$^{13}C_3$]Glycerol
2	173.5	48	48, 2[a]	160.15	1.03	68	68, 60
3	55.6	48, 38, 12	48, 36, 12	111.06	–	86	86, 60, 52[a]
4	–	–	32, 40	128.24	–	–	53, 72, 52,[a] 3.5[a]
5	–	–	32	140.75	–	–	72, 4
6	204.0	42, 38, 2	42, 36, 2[a]	120.28	–	68, 72	68, 70
7	44.9	42, 36, 12	42, 32, 12, 6	133.29	1.66	44, 72	44,70
8	17.2	34, 36, 2	34, 32	18.91	–	44	44
9	13.7	34	34, 6	163.41	1.65	86	86, 3.5[a]
10	–	–	40	57.22	–	–	53, 4

[a] As estimated by spin simulation.

Feeding the growing cultures with [1-^{13}C]acetate resulted in enhanced ^{13}C NMR signals for only the C-7, C-2 and C-9 atoms of the 3-furanylcarbonyl residue of **2**, while feeding the cultures with [1,2-$^{13}C_2$]acetate gave three pairs of signals with distinct coupling constants that have been assigned to the C-8/C-7, C-6/C-2 and C-3/C-9 atoms. Thus, a triketide precursor contributes to the C_6 chain (C-8/C-7/C-6/C-2/C-3/C-9). [U-$^{13}C_3$]Glycerol was found to be incorporated intact at C-5/C-4/C-10 as indicated by the $^1J_{(C,C)}$ and $^2J_{(C,C)}$ coupling constants. Because of scrambling from glycerol into acetate, intact C_2 units were also detected in the triketide chain. Thus, the assembly of the aglycon requires a triketide (C_6) and a C_3 precursor derived from glycerol. Compound **1** was also isolated from these experiments, and incorporation of labelled acetate and glycerol was observed. [1-^{13}C]Acetate and [1,2-$^{13}C_2$]acetate feedings resulted in three pairs of signals that have been assigned by their coupling constants to the C-9/C-8, C-7/C-6 and C-3/C-2 atoms. This again gives evidence for a triketide precursor. The incorporation of intact [U-$^{13}C_3$]glycerol into **1** at C-5/C-4/C-10 is shown by the $^1J_{(C,C)}$ and $^2J_{(C,C)}$ coupling constants and also intact C_2 units were detected in the triketide.

Thus, the assembly of the butyrolactone requires C_6 + C_3 condensation. This finding is consistent with the assumption that **1** and **2** are two different structures based on one biosynthetic route since both are derived from a C_6 triketide (acetate pool) and a C_3 glycerol unit (carbohydrate pool). Therefore, we conclude for this mixed biosynthesis that a C_6-β-ketoacyl-CoA couples with a dihydroxyacetone-type C_3 unit by an aldol-like condensation to form an intermediate that either condensates to the butyrolactone as in **1** or the 3-furancarbonyl structure as in **2** (Scheme 3). In almost the same manner the proposed biosynthetic pathway for the aglycon of the 2,4-dimethyl-3-furanylcarbonyl rhamnoside (**5**) and the corresponding lactones **6** and **7** starts from a diketide and a glycerol unit (C_4 + C_3 pathway) (Scheme 3).[4]

Benzoate Directly from Shikimate: The biosynthesis of the benzoyl unit of **3** is not completely understood and this question can be addressed efficiently by labelled feeding experiments. In our earlier feeding experiments with strain *Streptomyces griseoviridis* (Tü 3634), no incorporation of labelled acetate, methionine or glucose was observed in the benzoyl aglycon of **3**.[4] Whereas incorporation of [2-^{13}C]glycerol labelled C-1 and C-3/C-5 (chemically equivalent nuclei), the [U-$^{13}C_3$]glycerol gave a very complex labelling pattern with two sets of jointly transferred ^{13}C atoms at C-4/C-5/C-6 and C-7/C-1/C-2.[4] These labelling signatures illustrate the shikimate pathway from erythrose-4-phosphate (E4P) and phosphoenolpyruvate (PEP) for the bio-



Scheme 3. Scheme for the mixed acetate-glycerol biosynthetic pathway to **1**, **2**, **5**, **6** and **7**

synthetic formation of the benzoyl moiety of **3**.[19] We postulated that the unsubstituted benzoyl ring originates directly from shikimate or chorismate and not via phenylalanine[11] because the [U-$^{13}C_3$]glycerol feeding experiment showed an intact three-carbon unit for at C-7/C-1/C-2. To address this hypothesis, we fed [1,7-$^{13}C_2$]shikimic acid to the growing cultures of strain Tü 3634 over a period of 9 hours under the same conditions as used in previous work.[4] The culture was harvested after 60 hours and 2 mg·L^{-1}of **3** was isolated. The ^{13}C NMR spectrum revealed besides the regular signals only one pair of doublets that have been assigned by their coupling constant (76 Hz) to C-7/C-1 (Figure 1). This labelling pattern underlines the fact that the adjacent atoms C-7/C-1 of shikimate are transferred to the final product without separation.

The experimental data prove without doubt that the benzoyl moiety of the α-L-rhamnopyranoside **3** is generated by strain Tü 3634 de novo from a shikimate intermediate, prior to the prephenate level and not via cinnamic acid. The hypothetical mechanism of benzoate formation comprises the dephosphorylation of shikimic acid 3-phosphate and dehydration steps. Similar to our results, feeding experiments with root cultures of *Swertia chirata* indicated that the 3-hydroxybenzoate of amarogentin is formed from an early shikimate pathway intermediate and not via cinnamic acid.[20] These findings are in contrast to the general plant-like biotransformation of shikimate and chorismate to benzoate via prephenate, phenylalanine and cinnamate, in which the carboxylic carbon atom of shikimate (C-7) is lost in the latter steps (Scheme 4). Recently, a plant-like biosynthesis of the benzoic acid starter unit of enterocin in the bacterium *Streptomyces maritimus* and of soraphen A in *Sorangium cellulosum* was verified by feeding experiments with labelled intermediates.[8,9]

Conclusions

In the work described herein, the mixed acetate-glycerol biosynthetic pathway was discovered to be a useful biosynthetic route in streptomycetes. It provides access to different furan and lactone derivatives with metabolic diversity. The diversity arises from the aldol-like condensation of ketides of various chain lengths and glycerol-derived C_3 units in "a biosynthesis with a flip". The ketides are presumably derived from a process that involves typical polyketide synthases (PKS). We now conclude that the formation of furan- and lactone-containing metabolites **1**, **2** and **5**−**7** from *Streptomyces* sp. strains GT 61150 and Tü 3634, respectively, follow the same biosynthetic route (Scheme 3). C_4 di-

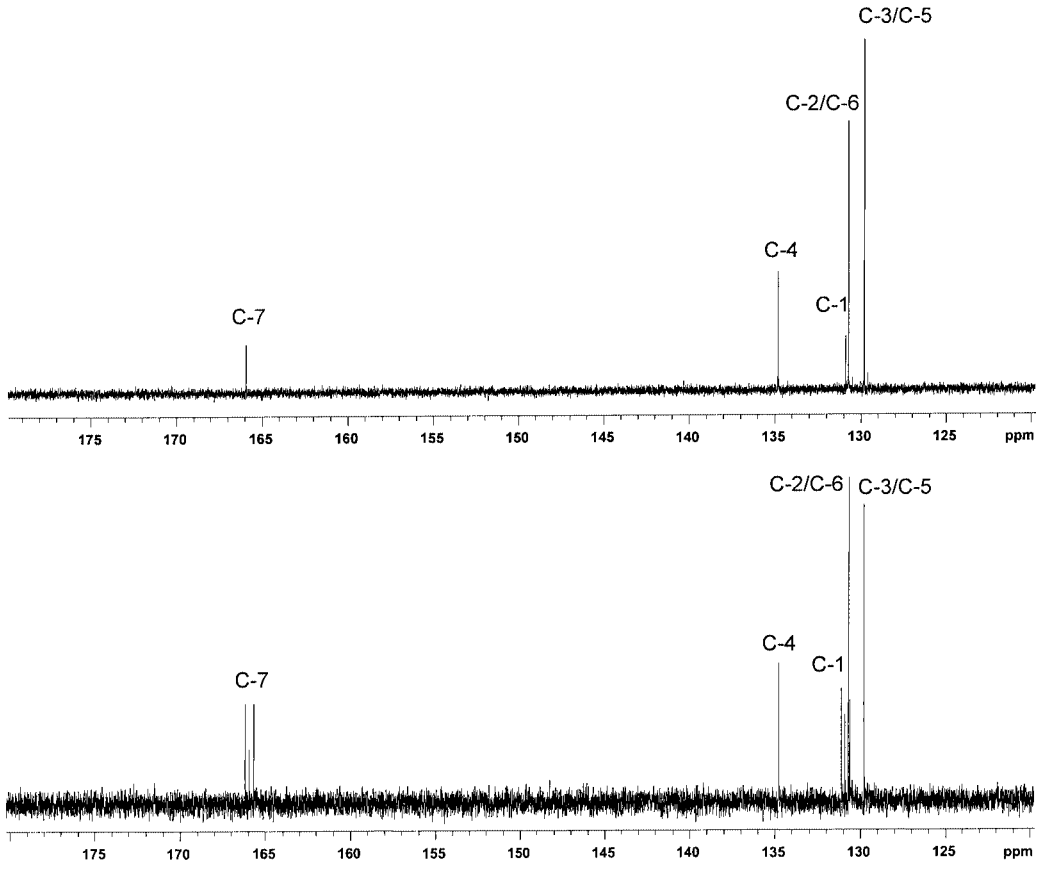
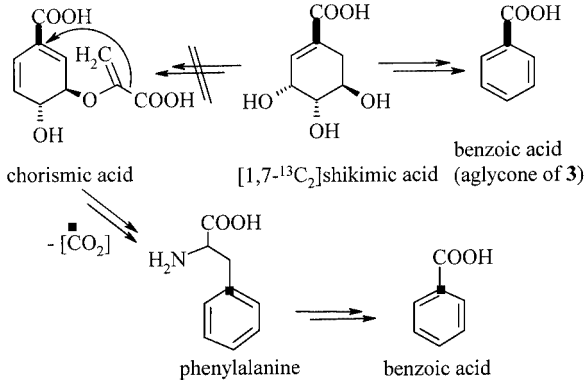


Figure 1. Section of the ^{13}C NMR spectrum of **3** (bottom: experiment with [1,7-$^{13}C_2$]shikimic acid; top: unlabelled shikimic acid)



Scheme 4. Labelling pattern and biosynthetic pathway to benzoate, the aglycon of **3**, ruling out the plant-like pathway via phenylalanine

ketides and C_6 triketides condensate with a C_3 glycerol unit, then the biosynthetic pathway subdivides by rotation of the ketides before ring closure. While the nitrogen analogue of **5**, pyrrole **8**, was formed by adding $(NH_4)_2HPO_4$ to the growing culture of Tü 3634, probably by a new pyrrole biosynthetic pathway, no such analogue could be isolated from strain GT 61150. It remains to be established to which type of PKS the enzymes producing the ketide precursors belong. Furthermore, the aldol-condensing enzyme and the *O*-acyl rhamnosyl transferase activity need to be characterized. In summary, it is envisaged that the examples discussed in this paper are produced by a new biosynthetic pathway that is based on a mixed acetate-glycerol biosynthetic pathway that subdivides during the ring closure of a common intermediate. By creating such multifunctional intermediates nature enhances the structural diversity of the resulting secondary metabolites. The compounds described herein were detected by a chemical screening method; some of them belong to the family of acyl rhamnosides (**2**, **5** and **8**) and **1**, **6** and **7** have structures similar to those of the A-factor microbial hormones. The new lactones and furans can be regarded as a variation of the widely occurring butyrolactones derived from *Streptomyces* but they are produced in much higher yields (up to 10 mg·L^{-1}). Despite their α-HDS inhibitory activity no further biological effects have yet been observed for the metabolites described herein.

Another important result of this study is the evidence for the direct conversion of shikimate to the benzoate moiety of compound **3**, an alternative microbial route to benzoate that has not yet been described in the literature. It supports our earlier hypothesis based on the results of [U-$^{13}C_3$]glycerol feeding experiments but is clearly in contrast with the common plant-like benzoate biosynthesis which is found in plants and bacteria like *Streptomyces* or *Sorangium*.[9,16] The formation of benzoate directly from shikimate marks a new biosynthetic pathway which might be responsible for the formation of more metabolites that have not yet been investigated. Our approach reveals that the different ben-

zoyl biosynthetic pathways can be explored efficiently by feeding experiments and analysis of the resulting isotope labeling patterns.

In summary, the results described herein again show the high flexibility of microbial biosynthetic pathways for the formation of secondary metabolites, which are distinguished by their remarkable structural diversity.

Experimental Section

General Remarks: ^1H, ^{13}C and 2D NMR spectra were recorded with Varian Inova 500 and 600 spectrometers. Chemical shifts are expressed in δ values (ppm), with the solvent as internal reference. The multiplicities of the ^{13}C NMR signals were assigned by using the attached proton test (APT). ESI-MS: Finnigan LCQ spectrometer. HR-ESI-MS: Bruker FTMS-7 APEX® IV 70e FT-ICR spectrometer. DCI-MS: Finnigan MAT 95A spectrometer, 200 eV, reaction gas: NH_3. IR spectra: Perkin−Elmer Model 1600 spectrometer (KBr discs). UV spectra: Varian Model Cary 3E spectrophotometer. Optical rotations: Perkin−Elmer 241 apparatus. R_f values were determined on 20 × 20 cm plates; the evaluation length was 10 cm. Column chromatography: Silica gel 60 (0.04−0.063 mm, Machery&Nagel), Sephadex LH-20 (Pharmacia), Lobar RP-18 (Merck). TLC: silica gel 60 F_{254} plates (Merck, 0.25 mm). Staining reagents: anisaldehyde/sulfuric acid: 1.0 mL of anisaldehyde in 85 mL methanol, 5 mL of concd. sulfuric acid and 10 mL of acetic acid. Fermentation: Braun BS4 (180 or 250 rpm, 28 °C), Biostat M (1 L) Braun (Melsungen, Germany). NMR assignments were made according to the position numbering shown in Scheme 1 and Scheme 2.

Nutrient Solutions: Medium A: oatmeal (20 g·L^{-1}), trace element solution (2.5 mL·L^{-1}), glycerol (500 mg·L^{-1}), pH = 7.8 prior to sterilization. Medium B: malt extract (10 g·L^{-1}), yeast extract (4 g·L^{-1}), glucose (4 g·L^{-1}), $CaCO_3$ (30 mg·L^{-1}), agar (20 g·L^{-1}), pH = 7.0 prior to sterilization. Medium C: oatmeal (20 g·L^{-1}), trace element solution (2.5 mL·L^{-1}), pH = 6.8 prior to sterilization. Trace element solution: see ref.[4]

Labelled Precursors: Sodium [1-^{13}C]acetate (99% ^{13}C): Campro Scientific; sodium [1,2-$^{13}C_2$]acetate (99% ^{13}C): Chemotrade; [U-$^{13}C_3$]glycerol (99% ^{13}C): Cambridge; [1,7-$^{13}C_2$]shikimic acid (99% ^{13}C) was synthesized as described in the literature.[21]

Fermentation. Method a: Strain GT 61150 (*Streptomyces* sp.) was cultivated on medium A for 4 days at 28 °C and maintained in 50% glycerol at −20 °C. The glycerol-containing storage mixtures (2 mL) were used to inoculate 100 mL of medium A in 300 mL Erlenmeyer flasks (with three flow spoilers). These cultures were incubated on a rotary shaker (180 rpm, 28 °C) for 96 h as standard conditions. To isolate **1** and **2**, the culture broth (100 mL) was used to inoculate a 1 L fermentor (medium A, 500 rpm, 28 °C, aeration 1.6 vvm, 6 d). Compounds **1** and **2** could be detected after 24 and 72 h respectively by HPLC analysis.

Method b: Strain Tü 3634 (*Streptomyces griseoviridis*); see ref.[4] From the eleventh hour of incubation, DMSO (5 mL·L^{-1}) was added and vitamin C (1.29 g·L^{-1}) was fed over 10 h to enhance the production of **3**. Acyl rhamnosides could be detected after 67 h and the culture broth was harvested after 96 h.

Feeding Experiments: Feeding experiments were carried out under the conditions described above. In general, precursors were added to the fermentation as sterile aqueous solutions adjusted to pH = 6.5. Continuous feeding with a low rate pump was carried out with the ^{13}C-labelled precursors with strain GT 61150 and **1** and **2** were isolated 84 h after inoculation: sodium [1-^{13}C]acetate (520 mg·L^{-1}, 6.2 mmol·L^{-1}), sodium [1,2-$^{13}C_2$]acetate (630 mg·L^{-1}, 7.7 mmol·L^{-1}) and [U-$^{13}C_3$]glycerol (750 mg·L^{-1}, 7.9 mmol·L^{-1}). To isolate **3**, [1,7-$^{13}C_2$]shikimic acid (150 mg·L^{-1}, 0.86 mmol·L^{-1}) was added from the twelfth hour of incubation of strain Tü 3634.

Isolation and Purification. Method a: Similar procedures were applied to **1** and **2**. The culture broths of strain GT 61150 were separated from the mycelium by filtration and the mycelium was discarded. The solutions obtained were acidified to pH = 5.0 with 0.5 M hydrochloric acid and extracted three times with equal volumes of ethyl acetate. The combined organic phases were concentrated to dryness and yielded the crude extracts (130 mg·L^{-1}). To isolate **1** and **2**, the crude material was subjected to chromatography on silica gel (column: 40 × 1.5 cm, $CHCl_3$/MeOH, 85:15) and the main fractions (detection by TLC) were further purified on Sephadex LH-20 (column: 20 × 0.5 cm, MeOH) to yield 9−12 mg·L^{-1} of **1** and 1−4 mg·L^{-1} of **2**.

Method b: The culture broth of strain Tü 3634 (*Streptomyces griseoviridis*) was separated from the mycelium by filtration and the mycelium was discarded. The orange solution obtained was passed through an Amberlite XAD-2 column and impurities were washed out with deionized water. The metabolites were eluted with methanol. Evaporation of the solvent yielded crude extracts (1.6 g·L^{-1}). To isolate the product, the crude extract (1 g, methanol soluble) was subjected to chromatography on Sephadex LH-20 (column: 100 × 2.5 cm, MeOH) and the main fractions (detection by TLC) were further purified on RP-18 (column: Lobar B, 0.5 bar) to yield 3 mg·L^{-1} of pure **3**.

3-Butyryl-4-(hydroxymethyl)-4,5-dihydrofuran-2(3*H*)-one (1): Colourless oil, R_f = 0.46 ($CHCl_3$/MeOH, 85:15). $[\alpha]_D^{20}$ = 30 (*c* = 0.05, MeOH). IR (KBr): $\tilde{\nu}$ = 1767, 1715, 1462, 1383 cm^{-1}. UV (MeOH): $\lambda_{max.}$ (log ε) = 238 (0.12) nm. HR-ESI-MS: calcd. for $C_9H_{15}O_4$: 187.09649; found: 187.09657. DCI-MS: *m/z* (%) = 204 (82) $[M + NH_4]^+$, 221 (10) $[M + NH_3 + NH_4]^+$, 390 (100) $[2M + NH_4]^+$. ^1H NMR (500 MHz, CD_3OD): δ = 0.94 (t, J = 7.5 Hz, 3 H, 9-H), 1.64 (ddq, J = 7.5, 7.5, 7.5 Hz, 2 H, 8-H), 2.69 (dd, ^2J = 7.5, ^3J = 7.5 Hz, 1 H, 7-H_a), 2.90 (dd, ^2J = 7.5, ^3J = 7.5 Hz, 1 H, 7-H_b), 3.12 (m, 1 H, 4-H), 3.60 (d, J = 5.0 Hz, 2 H, 10-H), 4.14 (dd, ^2J = 7.0, ^3J = 9.0 Hz, 1 H, 5-H_a), 4.44 (dd, ^2J = 7.0, ^3J = 8.5 Hz, 1 H, 5-H_b) ppm. ^{13}C NMR (125.7 MHz, CD_3OD): δ = 13.8 (q, C-9), 17.6 (t, C-8), 41.5 (d, C-4), 45.4 (t, C-7), 55.6 (m, $^1J_{(C/D)}$ = 20 Hz, C-3), 62.4 (t, C-10), 70.9 (t, C-5), 175.1 (s, C-2), 205.1 (s, C-6) ppm. ^1H NMR (500 MHz, [D_6]acetone): δ = 0.92 (t, J = 7.5 Hz, 3 H, 9-H), 1.62 (ddq, J = 7.5, 7.5, 7.5 Hz, 2 H, 8-H), 2.68 (dd, ^2J = 7.5, ^3J = 7.5 Hz, 1 H, 7-H_a), 2.89 (dd, ^2J = 7.5, ^3J = 7.5 Hz, 1 H, 7-H_b), 3.15 (m, 1 H, 4-H), 3.68 (dd, ^4J = 2.0, ^3J = 5.0 Hz, 2 H, 10-H), 3.84 (d, J = 7.0 Hz, 1 H, 3-H), 4.15 (dd, ^2J = 7.0, ^3J = 9.0 Hz, 1 H, 5-H_a), 4.44 (dd, ^2J = 7.0, ^3J = 8.5 Hz, 1 H, 5-H_b) ppm. ^{13}C NMR (125.7 MHz, [D_6]acetone): δ = 13.7 (q, C-9), 17.2 (t, C-8), 41.1 (d, C-4), 45.0 (t, C-7), 55.6 (d, C-3), 62.1 (t, C-10), 70.0 (t, C-5), 173.5 (s, C-2), 204.0 (s, C-6) ppm. $C_9H_{14}O_4$ (186.21).

[2-(1-Propen-1-yl)-4-hydroxymethyl]furan-3-yl]carbonyl α-L-Rhamnopyranoside (2): See ref.[3]

Benzoyl α-L-Rhamnopyranoside (3): See ref.[4]

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