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Spirodionic Acid, a Novel Metabolite from *Streptomyces* sp., Part 1: Structure Elucidation and Diels–Alder-Type Biosynthesis**

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Dedicated to Professor Lutz F. Tietze on the occasion of his 65th birthday

Abstract: Spirodionic acid (**1**), a novel microbial metabolite with a spiro[4.5]decene skeleton, the 6-ethyl-2*H*-pyrone **5**, dihydrosarkomycin (**6**), and other metabolites were isolated from the strain *Streptomyces* sp. Tü 6077. Structural elucidation was accomplished by NMR spectroscopic and mass-spectrometric studies, and the biosyntheses of compounds **1**, **5**, and **6** were investigated by feeding experiments with ^{13}C-labeled precursors. All results indicate a biogenetic sequence with metabolite **5** and sarkomycin (**7**) as precursors in the formation of spirocyclus **1** through an intermolecular Diels–Alder-type reaction.

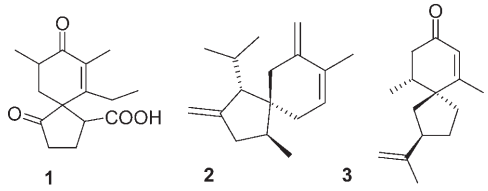
Keywords: biosynthesis · natural products · spiro compounds · streptomyces · structure elucidation

Introduction

Actinomycetes are prolific producers of natural products with an enormous diversity of bioactive secondary metabolites. The genus *Streptomyces* sp., a subgroup of Actinomycetes, is especially interesting as 7300 out of a total number of 12500 bacterial natural products originate from this genus.[1] Various biosynthetic pools and pathways deliver compounds that are sugar, amino acid, or polyketide derived or are hybrids of different biogenetic sources. Additionally, individual strain-specific biosynthetic reactions further decorate the initial metabolite skeletons to create even more miscellaneous chemical structures. Macrolides, aminoglykosides, or cyclopeptides are typical for *Streptomyces*, but many structural principles of the numerous different chemical classes are unique or show only some similarities with rare substances from plants, other microorganisms, or synthetic chemistry.[2]

Spiro[4.5]decanes occur as subunits of highly functionalized, complex terpenoid compounds from diverse sources.[3] In contrast, natural discrete spiro[4.5]decane skeletons are limited so far to a distinct group and are usually only found in plant species. The acorane (e.g., **2**),[4] spirovetivane (e.g., **3**),[5] and alaskene types differ in the placement of substituents and oxidation patterns and have inspired the development of analytical and synthetic methods for decades (Scheme 1).[6]

Although little biological-activity data has been reported for these compounds, studies have revealed that acoranes



Scheme 1. Structures of naturally occurring spiro[4.5]decane skeletons **1**–**3**.

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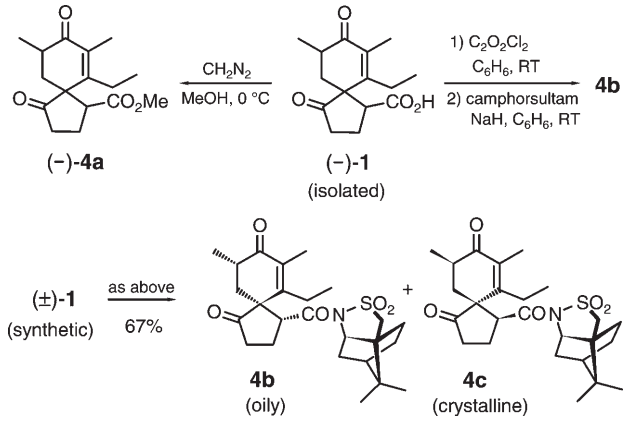
act as germination inhibitors[4b] and solavetivone (**3**) and lubimin[7] represent antifungal phytoalexins, stress metabolites that are only produced by fungi-infected potato tubers.[8] These plant-derived spiro[4.5]decanes are sesquiterpene products biosynthesized from farnesyl pyrophosphate, which cyclizes presumably followed by a 1,2-Wagner–Meerwein rearrangement.[6,9]

Herein, we report on spirodionic acid (**1**), the first microbial spiro[4.5]decane with an unique bicarbonyl structure isolated from strain *Streptomyces* sp. Tü 6077. We describe the results of the analysis of the metabolite pattern and of labeled feeding experiments that establish a Diels–Alder-type biosynthesis of **1**.

Results and Discussion

Isolation and chemical characterization: Fermentations of *Streptomyces* sp. (strain Tü 6077) were initially performed in a 2-L fermenter or 300-mL shaking flasks for 72 h using a complex medium (1% starch, 1% glucose, 1% glycerol, 0.25% cornsteep powder, 0.5% peptone). The culture filtrate was adsorbed on XAD-16 resin and elution with methanol yielded the crude extract. The metabolite pattern was analyzed by the database-assisted physicochemical HPLC–diode array detection (DAD) screening method.[10] In this way, the immunosuppressive siderophore desferrioxamine, 3-indolecarboxylic acid, griseolutein A, and griseoluteic acid were readily identified.[1,10,11] However, an apparently unknown secondary metabolite aroused our interest. The pure colorless solid **1** was isolated from the crude extract of a scale-up cultivation (10 L), which was extracted with ethyl acetate and purified by gel filtration on Sephadex LH-20/MeOH to yield 1.8 mg L^{-1}. By TLC analysis, **1** was detectable under UV light (254 nm) and gave a slight pink coloration with the anisaldehyde/H_2SO_4 staining reagent. The EI mass spectrum of **1** showed an ion peak at *m/z* 264, and the molecular formula $C_{15}H_{20}O_4$ was established from high-resolution EI-MS analysis (*m/z* 264.1360 [M^+]). The characteristic IR absorption peaks suggested hydroxy (3436 cm^{-1}) and carbonyl (1738, 1664 cm^{-1}) groups. The UV spectra showed an absorption maximum at 253 nm, which was slightly shifted on addition of NaOH (251 nm) or HCl (255 nm). The constitution of **1** was fully resolved from ^1H, ^{13}C, and 2D NMR spectroscopic experiments and from chemical derivatization. The ^1H NMR spectrum ([D_5]pyridine) exhibited signals of nineteen protons, three signals suggest methyl groups ($\delta_H = 1.11$, 1.26, and 1.95 ppm) appearing as a doublet, triplet, and singlet, respectively. There were four well separated signals at $\delta_H = 1.73$, 2.05, 2.92, and 3.56 ppm and a multiplet area at $\delta_H = 2.25$–2.65 ppm. The ^{13}C NMR spectrum was consistent with two keto groups ($\delta_C = 200.0$ and 218.4 ppm), a carboxylic acid unit ($\delta_C = 175.8$ ppm), a fully substituted double bond ($\delta_C = 135.0$ and 155.8 ppm), and a quaternary carbon atom ($\delta_C = 59.9$ ppm).[12] Additionally, HSQC and APT NMR spectroscopic data provided the assignment of four methylene ($\delta_C = 23.3$, 26.4, 34.5, and 37.8 ppm) and two methine ($\delta_C = 36.2$ and 49.0 ppm) groups, which were mapped by $^1H,^1H$-COSY correlation and HMBC correlations to two fragments. Methylation of **1** with diazomethane afforded ester **4a**, which confirmed the presence of the carboxylic acid group (Scheme 2). A two-ring



Scheme 2. Derivatization of isolated and synthetic spirodionic acid (**1**).

skeleton of metabolite **1** corresponds with the degree of unsaturation and, in combination with the other data, determines a spiro carbon center and thus unequivocally the constitution as 6-ethyl-7,9-dimethyl-4,8-dioxospiro[4.5]deca-6-ene-1-carboxylic acid, which was named spirodionic acid (**1**). 1D ROESY and 2D NOESY experiments failed to elucidate the relative configuration at the spiro carbon atom, but indicated the proximity of the 1-H proton to the C–C double bond and showed NOE interactions of the 3-H_2 methylene protons with 12-H_2 and 9-H; thus in summary, the assigned structure **1** is supported. Remarkably, the C-9 methyl group in the α'-position to the enone turned out to be epimerically stable as no isomerization occurred even on prolonged standing in [D_5]pyridine. To elucidate the absolute configuration of the three stereocenters of **1**, various derivatives of the natural product were semi-synthetically prepared,[13] but attempts to obtain crystals suitable for an X-ray analysis failed.

Studies towards the total synthesis of stereoisomers with the same constitution as **1** were carried out by von Zezschwitz and co-workers.[14] The racemic natural product was obtained in eight steps from cyclopent-2-enone in 10% overall yield. For a resolution of the enantiomers, the racemic mixture was transformed using oxalyl chloride into the respective acid chlorides, which were treated with the sodium salt of (1*S*,5*R*,7*R*)-10,10-dimethyl-3λ^6-thia-4-azatricyclo[5.2.1.$0^{1,5}$]decane 3,3-dioxide (camphorsultam).[15] The diastereomeric camphorsultam amides **4b**,**c** were thus obtained in 67% yield and could be readily separated by trituration as one diastereomer was an oil and the other a crystalline solid. Slow crystallization of the latter from $CHCl_3$ furnished crystals suitable for an X-ray structure analysis, which proved this diastereomer to be **4c** with an 1*S*,5*S*,9*R* configuration of the spirocycle (Figure 1).[16] In ad-

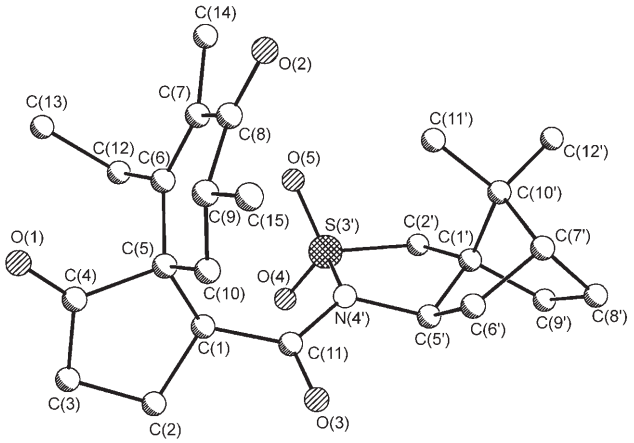
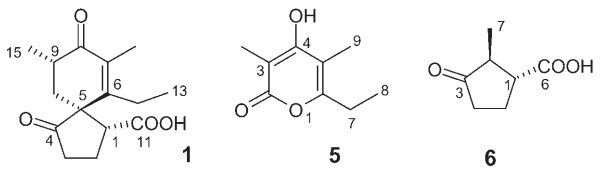


Figure 1. Crystal structure of camphorsultam amide **4c** with an atomic labeling scheme.[16]

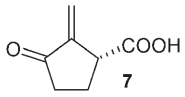
dition, the isolated metabolite **1** was converted into a single, diastereomerically pure camphorsultam amide, which was compared with the synthetic samples **4b**,**c** by ^{13}C NMR spectroscopic analysis and HPLC analysis (Daicel Chiralpak IB column, hexane/isopropanol). Both experiments, with the three single compounds and with mixed samples, unambiguously proved the identity of the amide from the isolated natural product with the oily synthetic diastereomer **4b**. In conclusion, spirodionic acid (**1**) is (1*R*,5*R*,9*S*)-6-ethyl-7,9-dimethyl-4,8-dioxospiro[4.5]deca-6-ene-1-carboxylic acid, and thus resembles a new class of spiro compounds with an unprecedented substitution pattern.[1,6] Spirodionic acid (**1**) does not display any antimicrobial or cytotoxic activity in common-cell assays.

In the course of the biosynthetic studies (see below), the isolation procedure of **1** by column chromatography and preparative HPLC furnished two further compounds, **5** and **6**, in yields of 2 and 14 mg L^{-1}, respectively. Compounds **5** and **6** were identified from their characteristic NMR spectroscopic data and mass-spectrometric analysis (Scheme 3).

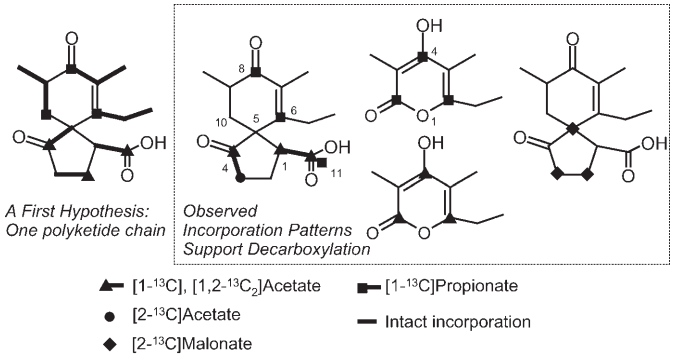


Scheme 3. Metabolites from strain *Streptomyces* Tü 6077.

Metabolite **5** is 6-ethyl-4-hydroxy-3,5-dimethylpyran-2-one ($C_9H_{12}O_3$), which was first isolated from the filamentous fungi *Emericella heterothallica* and is known from studies of *Streptomyces* polyketide synthases.[17] From its physical and spectroscopic data, **6** is (1*R*,2*S*)-2-methyl-3-oxocyclopentane-1-carboxylic acid ($C_7H_{10}O_3$), named dihydrosarkomycin.[18] Compound **6** is a known metabolite from *Streptomyces erythrochromogenes* sp. and was isolated together with the antitumor substance sarkomycin (**7**), a rather unstable analogue (as a result of the exocyclic C–C double bond), which was used in the 1960s in Japan as a prescription drug against cancer.[19]



Biosynthesis: While most known natural spiro[4.5]decanes are plant-derived sesquiterpenoids,[6] as *Streptomyces* products they are more likely to be derived from polyketide biosynthetic pathways. Thus, our first working hypothesis was that a polyketide chain from three propionate units is extended with three acetate units (Scheme 4).[13] Therefore,



Scheme 4. First hypothesis and observed labeling patterns of metabolites **1** and **5** from ^{13}C-acetate, malonate, and propionate feeding experiments.

biosynthetic investigations were performed with the effective method of isotopically labeled precursors. Here, a prerequisite is the reliable production of the metabolites of interest. We thus systematically altered the cultivation parameters to afford a maximum production of **1**: Additives in the nutrient media (medium S) were HEPES buffer (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), sodium bromide, sodium acetate, sodium propionate, XAD-16 resin, glycerol, or α-ketoglutarate. The final simplified and optimized conditions were cultivation in a 1-L stirred vessel bioreactor (72 h, 500 rpm, 4.0 vvm, 28 °C) with a starch/glycerol/casein peptone medium (medium S). In all feeding experiments, **1** was routinely purified by column chromatography on Sephadex LH-20 and RP-18 columns and by semipreparative HPLC to afford the pure compound **1** in amounts of up to 15 mg L^{-1}.

Tracer experiments were carried out by adding the sodium salts of [1-^{13}C]acetate, [2-^{13}C]acetate, [1,2-^{13}C$_2$]acetate, [1-^{13}C]propionate, [2-^{13}C]malonate, and [U-^{13}C$_3$]glycerol, respectively, to the growing cultures of strain *Streptomyces* sp. Tü 6077 (12 h of continuous feeding starting 24 h after inoculation). Purified **1** was analyzed by ^{13}C NMR spectroscopic analysis and specific enrichments and ^{13}C,^{13}C coupling constants were determined (Table 1).[13]

Feeding [1-^{13}C]acetate resulted in a high specific enrichment only of C-1, C-4, and C-11 of **1** (Scheme 4). Consequently, feeding experiments with [2-^{13}C]acetate and

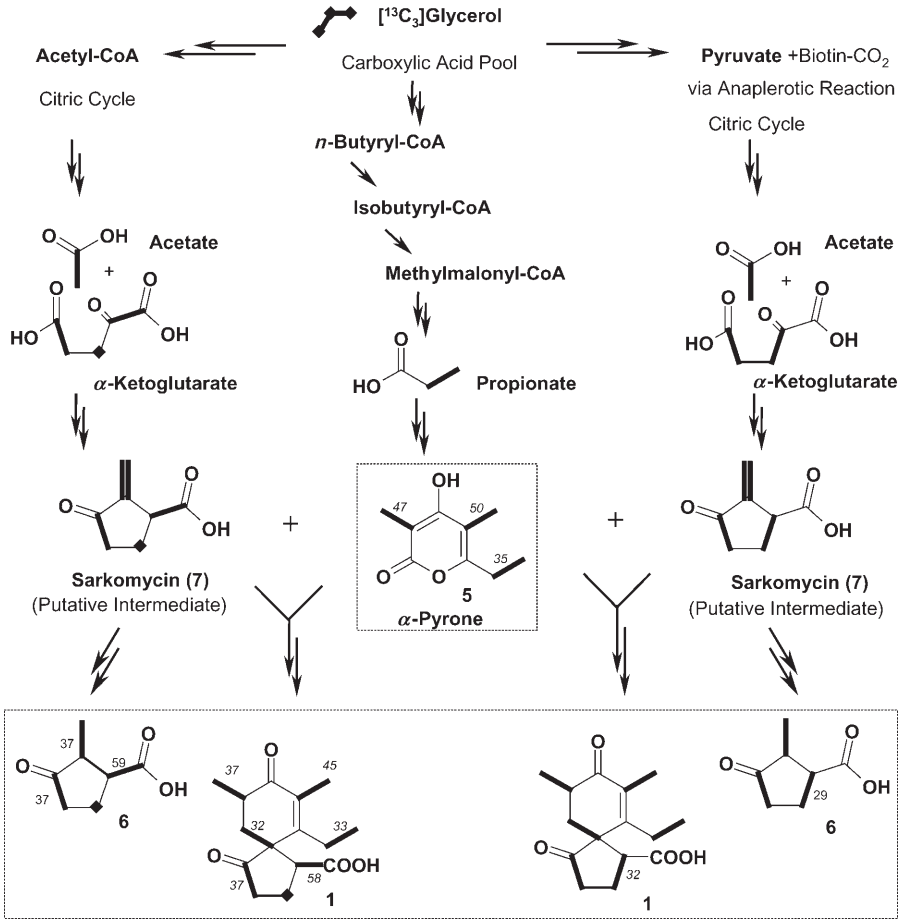
Table 1. ^{13}C NMR spectroscopic analysis of enriched metabolites **1**, **5**, and **6** after feeding of [U-$^{13}C_3$]glycerol.

Carbon	δ_C[a] (**1**) [ppm]	J(C,C) (**1**) [Hz]	Carbon	δ_C[c] (**5**) [ppm]	J(C,C) (**5**) [Hz]	Carbon	δ_C[b] (**6**) [ppm]	J(C,C) (**6**) [Hz]
C-1	49.0	32, 58	C-2	165.7	–	C-1	49.9	29, 59
C-2	23.3	32	C-3	98.3	n.d.	C-2	47.9	37
C-3	37.8	37	C-4	166.1	–	C-3	217.9	37
C-4	218.4	37	C-5	106.9	n.d.	C-4	37.2	37
C-5	59.9	32	C-6	159.2	–	C-6	177.0	59
C-6	155.8	–	C-7	24.4	35	C-5	25.2	29
C-7	135.0	45	C-8	11.7	35	C-7	13.4	37
C-8	200.0	–	C-9	10.0	50			
C-9	36.2	37	C-10	10.2	47			
C-10	34.5	32						
C-11	175.8	58						
C-12	26.4	33						
C-13	13.0	33						
C-14	12.4	45						
C-15	16.0	37						

[a] ^{13}C NMR spectrum: 150.8 MHz, [D_5]pyridine. [b] ^{13}C NMR spectrum: 125.7 MHz, [D_6]acetone. [c] ^{13}C NMR spectrum: 150.8 MHz, [D_4]methanol. n.d. = not determined.

[1,2-$^{13}C_2$]acetate were performed and supported the results because of two pairs of coupling signals (C-3/C-4, C-1/C-11) and a clearly enhanced signal for C-3, but only a weak one for C-1. Repeated experiments gave comparable results and feeding of [2-^{13}C]malonate furnished a poor yield of **1**, but supported some of the results from the acetate feeding with strong incorporation into C-2, C-3, and C-5.[13] To demonstrate the origin of the cyclohexenone moiety, [1-^{13}C]propionate was also fed, thus resulting in particular high signal enrichments of C-6, C-8, and C-11. Weak, but significant enrichments were observed for C-4, C-5, and C-7 as a result of intensive propionate metabolism. The α-pyrone metabolite **5** was also isolated from the respective [1-^{13}C]acetate and [1-^{13}C]propionate feeding experiments and gave the expected moderate incorporations from acetate and very strong enrichments for the heads of the three propionate units (C-2, C-4, and C-6). Thus, the cyclohexenone methylene C-10 in metabolite **1** showed no enrichment from all feeding experiments. All the results consistently indicated an uncommon biosynthetic pathway and clearly ruled out a single hexaketide chain from propionate and acetate as the precursors.[13] Therefore, the first hypothesis for the biosynthesis of the microbial spiro[4.5]decene **1** had to be reconsidered.

An additional feeding experiment with [U-$^{13}C_3$]glycerol was carried out under specially adapted fermentation conditions: As **1** is only produced in glycerol-rich media, two cultivations of strain Tü 6077 were simultaneously started, one in the usual medium S and one in a glycerol-free medium S. The cells were harvested by centrifugation after 24 h of incubation, and the cells from the first vessel were transferred, whilst maintaining sterility, to the glycerol-free medium. Feeding of labeled [U-$^{13}C_3$]glycerol led to highly enriched **1**, **5**, and **6** and their ^{13}C NMR spectra showed the significant and informative coupling patterns indicated in Scheme 5. Intact C_2 units for C-3/C-4, C-1/C-11,

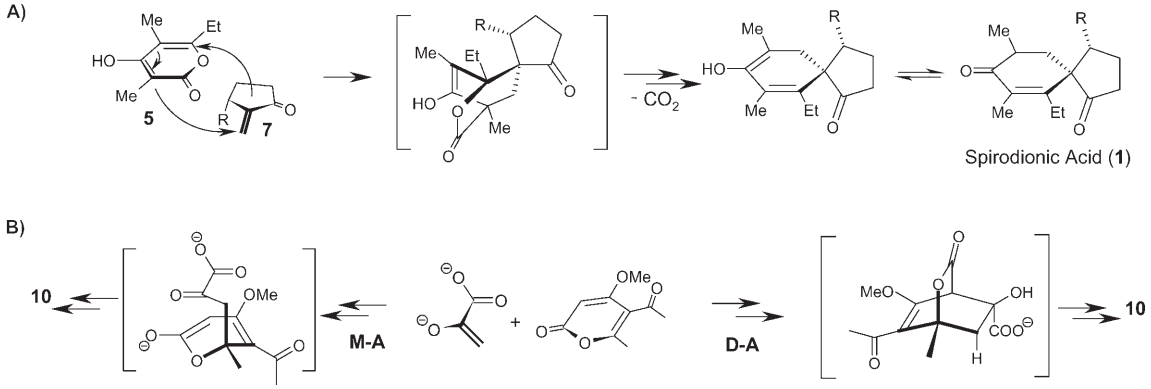


Scheme 5. Proposed biosynthesis of **1** and metabolites **5** and **6** on the basis of observed labeling patterns (dashed boxes). ^{13}C,^{13}C coupling constants are given (in Hz), diamonds mark significant single enrichments.

C-5/C-10, C-9/C-15, C-7/C-14, and C-12/C-13 of **1** correspond with the known metabolization of glycerol via glyceraldehyde 3-phosphate and phosphoenol pyruvate into acetyl coenzyme A (CoA).[20] The fully labeled acetyl CoA in turn gives rise to *n*-butyryl CoA, which fills the methylmalonyl CoA pool via isobutyryl, finally resulting in significantly labeled [2,3-$^{13}C_2$]propionate units in the metabolites of strain *Streptomyces* Tü 6077.[20] It should be stated that no statistical scrambling of the ^{13}C-label from glycerol by other metabolic pathways was observed. Therefore, [U-$^{13}C_3$]glycerol significantly labels acetate (at both C-1 and C-2) and propionate (at both C-2 and C-3) precursors in the biosynthesis of **1**, **5**, and **6**. An additional pair of coupling signals (C-1/C-2) points to the anaplerotic production of oxaloacetate from pyruvate by carboxylation from biotin–CO_2, which is known for bacteria.[21] Therefore, the labeling patterns indicate that the cyclopentanone unit of **1** is formed from α-ketoglutarate (α-KGL) of the citric cycle, which is fed from two different metabolic sources, namely, acetyl CoA and biotin-dependent anabolism (in a ratio of 3:2).[20] Dihydrosarkomycin (**6**) resembles the same unusual coupling pattern as C-1/C-2/C-3/C-4/C-11 of **1** with an extra acetate unit (Scheme 5), and thus derives from α-KGL and acetate as precursors. As shown before,[17] the 2*H*-pyrone **5** is biosynthesized from three propionate units, underlined by the [1-^{13}C]propionate and [U-$^{13}C_3$]glycerol labeling patterns, which are again similar to the cyclohexenone unit of **1** (Scheme 5).[13]

In conclusion, the striking structural and biosynthetic relationship of the isolated compounds **1**, **5**, and **6** indicates that the biosynthesis of spirodionic acid (**1**) proceeds through an intermolecular reaction and not from a single polyketide chain. Our results suggest that the dienophile sarkomycin (**7**) and the diene 2*H*-pyrone **5** react in a Diels–Alder-type cyclization[22] with subsequent decarboxylation to afford the spiro[4.5]decene structure.

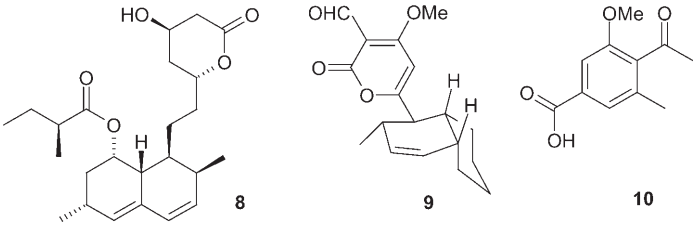
The feeding experiments reveal the character of a biosynthetic Diels–Alder reaction: 1) While **1** exhibits remarkably high enrichments of C-6 and C-8 from [1-^{13}C]propionate, C-10 is not labeled as decarboxylation of C-2 of the pyrone **5** occurs. Indeed, the isolated α-pyrone **5** still shows the high enrichment for all three propionate units; therefore, diene **5** is the reasonable precursor of the cyclohexenone moiety in **1**. Admittedly, the opened chain form of pyrone **5** represents an alternative precursor and the biosynthesis of **1** would then proceed through Diels–Alder-type cyclization followed by decarboxylation and an additional dehydration. 2) Moreover [$^{13}C_3$]glycerol gives strong labeling of C-5/C-10 of **1** (and C-2/C-7 of **6**) as a result of the incorporation of an intact unit, which forms the *exo*-methylene group of the proposed sarkomycin precursor (**7**) and the methyl group of isolated dihydrosarkomycin (**6**). 3) The five-membered rings of **1** and **6** mirror the origin of ketoglutarate from the two metabolic pathways in the same ratios. That is, pyruvate, through the anaplerotic reaction with biotin–CO_2, and acetyl CoA enter the citric acid cycle and both serve the ^{13}C-labeled α-ketoglutarate formation. 4) The absolute configuration of C-1 of **1** and the metabolite **6** is the same. 5) The relative configuration of C-1 and C-5 of spirodionic acid results from the selectivity of the Diels–Alder-type reaction. Thus, the diene approaches the dienophile on the face opposite to the carboxy moiety at C-1 of the cyclopentanone so that the reaction occurs on the α-C *si* face of **7**. In addition, the dienic system in the pyrone **5** is oriented away from the carbonyl moiety of the enone (Scheme 6). However, one has to consider that **7** represents a Michael acceptor. Thus, a ionic mechanism is a second option besides a concerted [4+2] cycloaddition and would comprise a pyrone enolate as the nucleophile. In any case, we assume a true enzymatic process, as the specific reaction would otherwise presumably not occur at room temperature under physiological conditions. Additionally, the instability and facile dimerization[19c] of **7** and the isolation of **1** as a single stereoisomer with respect to newly formed stereogenic centers C-5 and C-9 make a nonenzymatic reaction unlikely. As ^{13}C-labeled acetate does not label the C-5/C-10-unit of **1**, whereas [2-^{13}C]malonate does so, the direct precursor of the *exo*-methylene group of **7** and thus the methyl group of **6** is still not clear. However, it is known that the same extender units can be labeled very differently within one secondary metabolite as in the case of acetate in aurodox or kirromycin.[23]



Scheme 6. Proposed biosynthetic pathways: A) Intermolecular Diels–Alder reaction for the bacterial **1** deduced from isolated diene and dienophile compounds of strain Tü 6077. B) Tandem Michael–aldol reaction (M–A) deduced from quantum mechanical and molecular mechanical (QM/MM) simulations[27] and inverse-electron-demand Diels–Alder reaction (D–A) deduced from X-ray analysis[26] for macrophomic acid (**10**).

So the C-5/C-10-unit of **1** might stem either from a C_2 unit from the carboxylic acid pool or a glycerol-derived unit with the loss of a carbon atom.

Diels–Alder cycloaddition reactions in nature are known, but until recently the existence of an enzymatic catalytic process remained elusive and is still a hot case at present.[24–27] The first reported biomolecule with catalytic Diels–Alder properties was a monoclonal antibody in 1989, followed by RNA-based Diels–Alderase activities.[24,25] The isolation of enzymatically active proteins has established the Diels–Alder reaction as a viable biosynthetic principle. Hitherto, only three purified enzymes, all from fungal producers, have been reported as naturally occuring Diels–Alderases to mediate the biosyntheses of lovastatin (**8**) and solanapyrone (**9**) in intramolecular reactions and macrophomic acid (**10**) in intermolecular reactions (Scheme 7).[25] Evidence for the "true Diels–Alderase activity" was gained



Scheme 7. Fungal metabolites from putative Diels–Alderase-mediated biosyntheses.

through experiments in vivo by heterologous expression (for **8**), cell-free reactions (for **8**–**10**), or analysis of an X-ray structure (for **10**).[27] These Diels–Alderases presumably catalyze several subsequent distinct chemical reactions, such as oxidations, decarboxylations, dehydrations, and C–C bond formation (Scheme 6). Recently, theoretical studies on the X-ray analysis of macrophomate synthase suggests that a tandem Michael–aldol mechanism is more plausible in this system as a result of a more stable transition state relative to that of a concerted [4+2] cycloaddition (Scheme 6).[28] However, it cannot be deduced whether a concerted Diels–Alder or stepwise Micheal–aldol reaction takes place in the biosynthesis of **10**, even with the full data of a sophisticated X-ray crystallographic structure of the enzyme and its substrate.[27,28] To date, the outcome of the race for evidence of the enzymatic catalysis of a truly concerted Diels–Alder reaction in nature cannot be foreseen on the basis of studies carried out todate.

Conclusion

With the isolation and structure elucidation of the spiro-[4.5]decene spirodionic acid (**1**) from *Streptomyces* sp. Tü 6077 featuring an unprecedented carbon skeleton and substitution pattern, we have established a new microbial compound class. Our optimized cultivation (tenfold titer improvement of **1**) and sound isolation procedures enabled us to identify 6-ethyl-4-hydroxy-3,5-dimethylpyran-2-one (**5**) and dihydrosarkomycin (**6**) from strain Tü 6077, in addition to the enantiomerically pure title compound **1**. The highly interesting biosynthetic origin of **1** was probed with stable-isotope labeling experiments. This excluded a single polyketide chain, but pointed to an intermolecular reaction of two precursors. Furthermore, the isolation of both a diene **5** and a dienophile, the trapped metabolite **6**, from the same cultivation of the producer strain Tü 6077 strongly suggests that an enzyme-catalyzed selective Diels–Alder-type reaction is involved in the biosynthesis of **1**. However, a Michael-type mechanism cannot be excluded. Further evidence was sort from a feeding experiment with the diene analogue 4-hydroxy-3,5,6-trimethylpyran-2-one, but no respective cycloadduct was found. Therefore, we are working on the syntheses of more sophisticated ^{13}C-labeled precursors, such as sarkomycin (**7**) and the pyrone **5**, or its open form, which might give more insight into the detailed mechanism. In addition, study is in progress to design homologous probes to target the putative PKS, then to clone and sequence genes responsible for the biosynthesis of **1**.

Altogether, the new low-molecular-weight microbial metabolite spirodionic acid (**1**) and the components **5** and **6** add to the discussion of the role of complex and powerful Diels—Alder-type catalysts in biosynthesis.

Experimental Section

General: ^1H, ^{13}C, and 2D NMR spectra were recorded on a Varian Inova-600 (600 and 150.8 MHz, respectively), Varian Mercury-300, or Varian Unity-300 (300 and 75.5 MHz, respectively) spectrometer. Chemical shifts are reported as δ values (ppm) with the residual proponated solvent as the internal reference. The ESI mass spectra were recorded on a Finnigan MAT 95 A spectrometer (200 eV). The IR spectra were recorded on a Perkin-Elmer Model 1600 spectrometer (KBr discs). The UV spectra were recorded on a Varian Model Cary 3E spectrophotometer. The optical rotations were recorded on a Perkin-Elmer 241 machine. The R_f values were determined on silica TLC plates (Merck 60 F_{254}, 0.2 mm, 20×20 cm), and the evaluation length was 10 cm. Column chromatography was carried out on silica gel 60 (0.04–0.063 mm, Machery & Nagel), Sephadex LH-20 (Pharmacia), and Lobar RP-18 (Merck). TLC analysis was performed on silica gel plates (Merck 60 F_{254}, 0.25 mm). Staining reagents were anisaldehyde/sulfuric acid: anisaldehyde (1.0 mL) in methanol (85 mL) with conc. sulfuric acid (5 mL) and acetic acid (10 mL). Fermentation was carried out in Braun Biostat B and E fermentors (2 L, 500 rpm, aeration: 1.6 vvm; 10 L, 200 rpm, aeration: 1.0 vvm; Braun, Melsungen, Germany). HPLC analyses were performed using a Jasco Nucleosil-100 C-8 5 μm (250×8 mm) column or a Daicel Chiralpak IB column, a Jasco pump PU-2080 Plus, and a UV detector MD-2010 Plus. All the chemicals used were of analytical grade. Nutrient solutions: Medium S: starch (10 gL^{-1}), glycerol (4 gL^{-1}), casein peptone (4 gL^{-1}), yeast extract (0.5 gL^{-1}), meat extract (0.5 gL^{-1}), liver extract (0.5 gL^{-1}), NaCl (1 gL^{-1}) adjusted to pH 7.0 prior to sterilization; complex medium (SGG): glucose (10 gL^{-1}), glycerol (10 gL^{-1}), starch (10 gL^{-1}), corn steep powder (2.5 gL^{-1}), casein peptone (5 gL^{-1}), yeast extract (2 gL^{-1}), NaCl (1 gL^{-1}), $CaCO_3$ (3 gL^{-1}) adjusted to pH 7.3 prior to sterilization; medium SM: soy flour (20 gL^{-1}), mannite (20 gL^{-1}) adjusted to pH 7.0 prior to sterilization. Labeled precursors: sodium [1-^{13}C]acetate (99% ^{13}C; Campro Scientific), sodium [2-^{13}C]acetate (99% ^{13}C; Deutero), sodium [1,2-^{13}C]acetate (99% ^{13}C; Cambridge Isotope Lab.), sodium [1-^{13}C]propionate (99% ^{13}C; Cambridge Isotope Lab.), disodium [2-^{13}C]malonate (98.9% ^{13}C; Chemotrade), [U-$^{13}C_3$]glycerol (99% ^{13}C; Chemotrade).

Fermentation: Strain Tü 6077 (*Streptomyces* sp.) was incubated for 5 days at 28 °C and maintained on agar plates (medium SM). A 1-cm^2 piece of agar from 5-day-old cultures was used to inoculate 100 mL of medium SGG in 300-mL flasks (with three spoilers, 180 rpm, 28 °C, 72 h). Production in medium SGG was carried out in a 2- or 10-L fermentor (28 °C, 72 h) or 300-mL Erlenmeyer flasks (with three spoilers, 180 rpm, 28 °C, 72 h) using 1 % as inoculum of the preculture. Under optimized conditions, a 1-cm^2 piece of agar from 5-day-old cultures was used to inoculate 100 mL of medium S in 1-L Erlenmeyer flasks. These cultures were incubated on an angular shaker (120 rpm, 28 °C) for 72 h. To isolate **1**, a 100-mL aliquot of the culture broth was used to inoculate a stirred vessel (2-L working volume, medium S, 500 rpm, 28 °C, aeration: 4 vvm). Initially, the pH value rose from 6.8 to 7.3, between 24 and 50 h of fermentation the pH value was about 5.7, and after 50 h of fermentation the pH value was 8.2. After 10 h of incubation, a 0.5-gL^{-1} aliquot of α-KGL as a potential precursor was fed over 1 h. The culture broth was harvested after 72 h.

Feeding experiments: Feeding experiments with the ^{13}C-labeled compounds were carried out under the fermentation conditions described above. In general, the precursors were administered to the fermentation as sterile aqueous solutions and continuous feeding with a low-rate pump was carried out within 24–36 h of fermentation. The following amounts were added: sodium [1-^{13}C]acetate: 653 mgL^{-1} (8.0 mmolL^{-1}); sodium [1,2-$^{13}C_2$]acetate: 634 mgL^{-1} (7.7 mmolL^{-1}); sodium [2-^{13}C]acetate: 706 mgL^{-1} (8.6 mmolL^{-1}); sodium [1-^{13}C]propionate: 701 mgL^{-1} (7.3 mmolL^{-1}); disodium [2-^{13}C]malonate: 1000 mgL^{-1} (6.8 mmolL^{-1})]; [U-$^{13}C_3$]glycerol: 505 mgL^{-1} (5.5 mmolL^{-1}). The feeding experiment with [U-$^{13}C_3$]glycerol was conducted by using the "separated-cell experiment" method: The strain was cultivated in parallel in glycerol-rich and glycerol-free media in two vessels. Both fermentations were harvested by centrifugation (4 °C, 4000 rpm, 1 min) after 24 h of cultivation. The cells grown in the glycerol medium were transferred under sterile conditions into the vessel containing the glycerol-free medium. The cultivation of this fermentor was continued and ^{13}C-labeled glycerol was fed until 36 h.

Isolation and purification: The culture broth was adjusted to pH 4 and separated by filtration from the mycelia, which was discarded. The culture filtrate was lyophilized and extracted with ethyl acetate (2× 500 mL). The solvent was removed by evaporation. Prepurification was achieved by performing gel permeation chromatography on a Sephadex LH-20 column (100×2.5 cm), and two fractions were obtained by using methanol as the eluent. One fraction was purified by column chromatography on reversed-phase silica gel (50×2.5 cm, RP-18; MeOH/H_2O 6:4) and silica gel (30×2 cm, $CHCl_3$/MeOH 85:15), thus yielding 14 mg of **6**. The other fraction containing **1** was subjected to reversed-phase chromatography on silica gel (Lobar column B, RP-18; MeOH/H_2O 6:4). Final purification was performed by preparative HPLC on a Jasco Nucleosil-100 C-8 (5 μm, 250×8 mm) column with CH_3CN/H_2O 26:74 (2.5 mLmin^{-1}, 0.005 % HCOOH), thus yielding **1** (1–15 mgL^{-1}) and **5** (0.9–2 mgL^{-1}) as colorless solids.

(1*R*,5*R*,9*S*)-6-Ethyl-7,9-dimethyl-4,8-dioxospiro[4.5]deca-6-ene-1-carboxylic acid (1): The title compound was isolated as described above as a colorless solid. R_f=0.48 ($CHCl_3$/MeOH 85:15); m.p. >305 °C; $[\alpha]_D^{20}$=−4.78 (*c*=0.33, MeOH); UV (MeOH): λ_{max} (ε)=253 (3.82) nm; ^{1}H NMR ([D$_5$]pyridine, 35 °C, 600 MHz): δ=1.11 (d, ^3J(H,H)=6.5 Hz, 3H, 15-H$_3$), 1.26 (t, ^3J(H,H)=8.0 Hz, 3H, 13-H$_3$), 1.73 (t, ^3J(H,H)=14.0, 14.0 Hz, 1H, 10-H$_a$), 1.95 (s, 3H, 14-H$_3$), 2.05 (dq, ^3J(H,H)=15.0, 8.0 Hz, 1H, 12-H$_a$), 2.30 (m, 1H, 2-H$_a$), 2.36 (m, 1H, 12-H$_b$), 2.47 (m, 1H, 3-H$_a$), 2.50 (m, 1H, 10-H$_b$), 2.58 (m, 1H, 2-H$_b$), 2.61 (m, 1H, 3-H$_b$), 2.92 (ddq, ^3J(H,H)=14.0, 7.0, 6.5 Hz, 1H, 9-H), 3.56 (dd, ^3J(H,H)=11.0, 6.5 Hz, 1H, 1-H) ppm; ^{13}C NMR ([D$_5$]pyridine, 35 °C, 150.8 MHz): δ=12.4 (q, C-14), 13.0 (q, C-13), 16.0 (q, C-15), 23.3 (t, C-2), 26.4 (t, C-12), 34.5 (t, C-10), 36.2 (d, C-9), 37.8 (t, C-3), 49.0 (d, C-1), 59.9 (s, C-5), 135.0 (s, C-7), 155.8 (s, C-6), 175.8 (s, C-11), 200.0 (s, C-8), 218.4 (s, C-4) ppm; IR (KBr): $\tilde{\nu}$=3436, 1738, 1664, 1457, 1379 cm^{-1}; MS (EI, 70 eV): *m/z* (%): 264 (27) $[M]^+$, 222 (98) $[M-C_2H_2O]^+$, 177 (100) $[M-C_4H_7O_2]^+$, HR-MS (EI): *m/z*: calcd for $C_{15}H_{20}O_4$: 264.1362; found 264.1360 $[M]^+$.

6-Ethyl-4-hydroxy-3,5-dimethylpyran-2-one (5): Colorless solid; R_f=0.43 ($CHCl_3$/MeOH 85:15); UV (MeOH): λ_{max} (ε)=290 (0.93), 205 (2.25); UV (MeOH/NaOH): λ_{max} (ε)=288 (1.05), 213 (2.87), 208 (2.45), 204 (1.56) nm; ^{1}H NMR ([D$_5$]pyridine, 35 °C, 300 MHz): δ=1.09 (t, ^3J(H,H)=8 Hz, 3H, 8-H$_3$), 1.97 (s, 3H, 10-H$_3$), 2.26 (s, 3H, 9-H$_3$), 2.41 (q, ^3J(H,H)=8 Hz, 2H, 7-H$_2$), 3.60 (s, 1H, OH) ppm; ^{13}C NMR ([D$_5$]pyridine, 35 °C, 75.5 MHz): δ=10.0 (q, C-9), 10.2 (q, C-10), 11.7 (q, C-8), 24.4 (t, C-7), 98.3 (s, C-3), 106.9 (s, C-5), 159.2 (s, C-6), 165.7 (s, C-2), 166.1 (s, C-4) ppm; IR (KBr): $\tilde{\nu}$=3421 (br), 2940, 2881, 1683, 1658 cm^{-1}; MS (EI, 70 eV): *m/z* (%): 168 (80) $[M]^+$, 140 (64) $[M-CO]^+$, 125 (100) $[M-C_2H_3O]^+$; MS (ESI positive mode): *m/z* (%): 169 (32) $[M+H]^+$, 191 (28) $[M+Na]^+$; MS (ESI negative mode): *m/z* (%): 167 (100) $[M-H]^-$; HR-MS (EI): *m/z*: calcd for $C_9H_{12}O_3$: 168.0786; found 168.0785 $[M]^+$.

(1*R*,2*S*)-2-Methyl-3-oxocyclopentanecarboxylic acid (6): Colorless, crystalline solid. R_f=0.42 ($CHCl_3$/MeOH 85:15), $[\alpha]_D^{20}$=+64 (*c*=0.08, MeOH), ^{1}H NMR ([D$_6$]acetone, 300 MHz): δ=1.04 (d, ^3J(H,H)=7 Hz, 3H, 7-H$_3$), 1.93 (m$_c$, 1H, 5-H$_a$), 2.15 (m$_c$, 1H, 4-H$_a$), 2.25 (m$_c$, 1H, 5-H$_b$), 2.33 (m$_c$, 2H, 2-H, 4-H$_b$), 2.65 (m$_c$, 1H, 1-H) ppm; ^{13}C NMR ([D$_6$]acetone, 75.5 MHz): δ=13.4 (q, C-7), 25.2 (t, C-5), 37.2 (t, C-4), 47.9 (d, C-2), 49.9 (d, C-1), 177.0 (s, C-6), 217.9 (s, C-3) ppm; IR (KBr): $\tilde{\nu}$=3448, 2974, 1733, 1183 cm^{-1}; MS (EI, 70 eV): *m/z* (%): 142 (100) $[M]^+$, 114 (66) $[M-CO]^+$, 97 (50) $[M-COOH]^+$; HR-MS (EI): *m/z*: calcd for $C_7H_{10}O_3$: 142.0630; found 142.0629 $[M]^+$.

Methyl 6-ethyl-7,9-dimethyl-4,8-dioxospiro[4.5]deca-6-ene-1-carboxylate (4a): At 0 °C, a solution of diazomethane in diethyl ether was carefully added to a solution of **1** (3 mg) in methanol (5 mL), until the solution kept a yellow color. The solvent was removed in vacuo and the residue was purified by column chromatography on silica gel (column: 28× 1.5 cm, *c*-Hex/EtOAc 1:2) and Sephadex LH-20 (column: 40×1.5 cm, acetone) to furnish 1.2 mg of the methyl ester **4a** as a colorless solid. R_f= 0.51 (*c*-Hex/EtOAc 1:2); $[\alpha]_D^{20}$=−5.6 (*c*=0.625, MeOH); ^{1}H NMR ([D$_6$]acetone, 600 MHz): δ=0.96 (d, ^3J(H,H)=7 Hz, 3H, 15-H$_3$), 1.13 (t, ^3J(H,H)=8 Hz, 3H, 13-H$_3$), 1.42 [m$_c$, 1H, 10-H$_a$], 1.79 (s, 3H, 14-H$_3$), 1.94 (dd, ^3J(H,H)=13, 8 Hz, 1H, 12-H$_a$), 1.99 (m, 1H, 9-H), 2.20 (dd, ^3J(H,H)=14, 5 Hz, 1H, 10-H$_b$), 2.24–2.28 (m, 3H, 2-H$_2$, 12-H$_b$), 2.36 (m, 1H, 3-H$_a$), 2.48 (m$_c$, 1H, 3-H$_b$), 3.46 (dd, ^3J(H,H)=11, 6.5 Hz, 1H, 1-H), 3.62 (s, 3H, 16-H$_3$) ppm; ^{13}C NMR ([D$_6$]acetone, 150.8 MHz): δ=12.2 (q, C-14), 13.0 (q, C-13), 15.8 (q, C-15), 23.1 (t, C-2), 26.3 (t, C-12), 34.4 (t, C-10), 36.2 (d, C-9), 37.7 (t, C-3), 48.2 (d, C-1), 52.3 (q, C-16), 60.2 (s, C-5), 135.5 (s, C-7), 155.5 (s, C-6), 173.7 (s, C-11), 199.5 (s, C-8), 217.7 (s, C-4) ppm; IR (KBr): $\tilde{\nu}$=3459, 2930, 1736, 1670, 1457, 1382 cm^{-1}; MS (EI, 70 eV): *m/z* (%): 278 (46) $[M]^+$, 250 (39) $[M-CO]^+$.

Resolution of synthetic *rac*-spirodionic acid (1): Oxalyl chloride (42.6 μL, 482 μmol) was added to a solution of *rac*-6-ethyl-7,9-dimethyl-4,8-dioxospiro[4.5]dec-6-ene-1-carboxylic acid[14] (24.0 mg, 91.0 μmmol) in benzene (1 mL), and the reaction mixture was stirred for 3 h at room temperature. The excess of oxalyl chloride and the solvent were removed in vacuo and the acid chloride was redissolved in benzene (1 mL). A solution of the sodium salt of (−)-camphorsultam was added to this mixture, which was prepared by stirring a mixture of the (−)-camphorsultam[15] (25.7 mg, 119 μmol) and sodium hydride (3.46 mg, 144 μmol) in benzene (0.5 mL) for 1 h at room temperature. The reaction mixture was stirred for 2 h at room temperature and poured onto diethyl ether (20 mL) and hydrochloric acid (1 M, 10 mL). The resulting phases were separated and the aqueous phase was extracted with diethyl ether (2× 10 mL). After drying over $MgSO_4$ and removal of the solvent in vacuo, 62 mg of crude product was obtained, which was purified by column chromatography on silica gel (10 g, hexane/EtOAc 5:1), thus yielding a mixture (28 mg, 67 %) of the two diastereomeric camphorsultam amides. Repeated trituration of the crude product with methanol furnished 10 mg (22 %) of pure crystalline diastereomer **4c** which could be separated by filtration. From the mother liquors, 12 mg (26 %) of the oily diastereomer **4b** were isolated. (1*S*,5*S*,9*R*)-4-[(1*S*,5*R*,7*R*)-10,10-Dimethyl-3,3-dioxo-3λ^6-thia-4-azatricyclo[5.2.1.0^{1,5}]decane-4-carbonyl]-6-ethyl-7,9-dimethylspiro-[4.5]dec-6-ene-1,8-dione (**4c**): R_f=0.38 (Hex/EtOAc 3:1); ^{1}H NMR (300 MHz, $CDCl_3$): δ=0.91 (s, 3H, C(CH$_3$)$_2$), 0.94 (s, 3H, C(CH$_3$)$_2$), 1.05 (t, ^3J(H,H)=7.5 Hz, 3H, 13-H$_3$), 1.08 (d, ^3J(H,H)=7.5 Hz, 3H, 15-H$_3$), 1.25–1.45 (m, 2H, 8′-H$_2$), 1.63–2.07 (m, 7H, 10-H$_2$, 6′-H$_2$, 7′-H, 9′-H$_2$), 1.76 (s, 3H, 15-H$_3$), 2.12–2.69 (m, 8H, 2-H$_2$, 3-H$_2$, 4-H, 9-H, 12-H$_2$), 3.47

(AB, ^2J(H,H) = 15.1 Hz, 2H, 2′-H_2), 3.91 (m_c, 1H, 5′-H) ppm; ^{13}C NMR (75.6 MHz, $CDCl_3$): δ = 11.7 (q, C-14), 12.3 (q, C-13), 16.0 (q, C-15), 19.7 (q, C(CH_3)$_2$), 20.1 (q, C(CH_3)$_2$), 23.7 (t, C-3), 26.3 (t, C-12), 26.6 (t, C-9′), 32.9 (t, C-8′), 34.1 (t, C-10), 36.1 (d, C-9), 37.7 (t, C-6′), 37.8 (t, C-2), 44.6 (d, C-7′), 47.6 (s, C-1′), 47.9 (s, C-10′), 49.2 (s, C-4), 53.1 (t, C-2′), 59.8 (s, C-5), 65.5 (d, C-5′), 135.0 (s, C-7), 155.7 (s, C-6), 172.7 (s, C-11), 199.6 (s, C-8), 217.2 (s, C-1) ppm. (1*R*,5*R*,9*S*)-4-[(1*S*,5*R*,7*R*)-10,10-Dimethyl-3,3-dioxo-3λ^6-thia-4-azatricyclo[5.2.1.0^{1,5}]decane-4-carbonyl]-6-ethyl-7,9-dimethylspiro[4.5]dec-6-ene-1,8-dione (**4b**): R_f = 0.38 (Hex/EtOAc 3:1); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 0.95 (s, 3H, C(CH_3)$_2$), 1.02 (t, ^3J(H,H) = 7.3 Hz, 3H, 13-H_3), 1.06 (d, ^3J(H,H) = 6.6 Hz, 3H, 15-H_3), 1.12 (s, 3H, C-(CH_3)$_2$), 1.18–1.43 (m, 2H, 8′-H_2), 1.71–2.08 (m, 7H, 10-H_2, 6′-H_2, 7′-H, 9′-H_2), 1.77 (s, 3H, 14-H_3), 2.14–2.49 (m, 7H, 2-H_2, 3-H_2, 9-H, 12-H_3), 2.61 (m_c, 1H, 4-H), 3.42 (AB, ^2J(H,H) = 13.8 Hz, 2H, 2′-H_2), 3.80–4.00 (m, 1H, 5′-H) ppm; ^{13}C NMR (75.6 MHz, $CDCl_3$): δ = 11.9 (q, C-14), 12.5 (q, C-13), 15.9 (q, C-15), 19.8 (q, C(CH_3)$_2$), 20.8 (q, C(CH_3)$_2$), 26.0 (t, C-3), 26.2 (t, C-12), 26.3 (t, C-9′), 32.6 (t, C-8′), 34.2 (t, C-10), 36.3 (d, C-9), 37.9 (t, C-6′), 38.3 (t, C-2), 44.4 (d, C-7′), 47.8 (s, C-1′), 48.2 (s, C-10′), 48.3 (d, C-4), 53.0 (t, C-2′), 58.9 (s, C-5), 65.4 (d, C-5′), 135.1 (s, C-7), 154.9 (s, C-6), 172.2 (s, C-11), 200.6 (s, C-8), 217.6 (s, C-1) ppm.

X-ray crystal structure analysis of 4c: Formula: $C_{25}H_{35}NO_5S$, M_w = 461.59, orthorhombic, space group: P_{21212}, Z = 4, a = 1568.0(2), b = 1970.6(3), c = 773.47(8) pm, V = 2.390(5) nm^3, ρ_{calcd} = 1.283 Mg m^{-3}, crystal dimensions: 0.1 × 0.2 × 0.1 mm, unique reflections: 4067. Stoe two-circle diffractometer with area counter; the structure was determined by using SHELXS and refined by using the least-squares method (SHELXL-97[29]). All non-hydrogen atoms were refined anisotropically, the hydrogen atoms were included in calculated positions and refined by using a riding model. R values: R_1 = 0.0671 (for $I > 2\sigma(I)$), wR_2 = 0.1333 (for all data) with 290 parameters and 0 restraints, maximum and minimum residual electron density 392 and −246 e nm^{-3}.

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