Natural Products

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Isolation and Total Synthesis of Kirkamide, an Aminocyclitol from an Obligate Leaf Nodule Symbiont**

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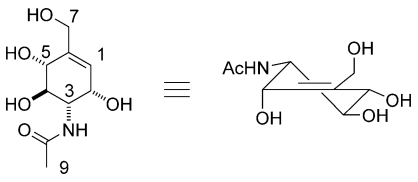
Abstract: *The new C_7N aminocyclitol kirkamide (**1**) was isolated from leaf nodules of the plant Psychotria kirkii by using a genome-driven ^1H NMR-guided fractionation approach. The structure and absolute configuration were elucidated by HRMS, NMR, and single-crystal X-ray crystallography. An enantioselective total synthesis was developed, which delivered kirkamide (**1**) on a gram scale in 11 steps and features a Ferrier carbocyclization and a Pd-mediated hydroxymethylation. We propose that kirkamide is synthesized by Candidatus Burkholderia kirkii, the obligate leaf symbiont of Psychotria kirkii. Kirkamide (**1**) was shown to be toxic to aquatic arthropods and insects, thus suggesting that bacterial secondary metabolites play a protective role in the Psychotria/Burkholderia leaf nodule symbiosis.*

In recent years, investigations of the molecular interactions of symbiotic bacteria with their hosts have transformed a number of research fields, from the human microbiome to ecology to crop protection, and these studies have revealed some of the chemical aspects of successful cooperation on a molecular level.[1,2] However, one of the main challenges in this scientific approach resides in the difficulties associated with the cultivation of the bacterial symbionts in the absence of their hosts, which hampers chemical analyses owing to the limited amount of material.[3] Therefore, many studies have focused on systems where material supply, both for genetic and chemical studies, can be secured. As a result, many symbioses between animals and prokaryotes have been investigated.[1–3]

In stark contrast, only one symbiosis has been described in higher plants for which the bacteria are vertically transmitted and which can be considered obligate. Bacterial leaf nodule symbiosis was first described in 1902, and studies over the last century have provided ample evidence that leaf nodules represent arguably the most complex and certainly the most intimate association between bacteria and higher plants, since the survival of both partners is dependent on the successful establishment of symbiosis.[4] In spite of over 100 years of research, the chemical constituents involved in this symbiosis have remained elusive. In this study, we present the structure elucidation, total synthesis, and biological evaluation of the new natural product kirkamide (**1**) from the bacterial leaf symbiont of the Rubiaceous shrub *Psychotria kirkii* (*P. kirkii*), and discuss the potential role of this C_7N aminocyclitol in the symbiosis.

Analysis of the genome of the uncultured Gram-negative bacterium *Candidatus* Burkholderia kirkii (*B. kirkii*) identified genes potentially involved in the biosynthesis of a putative C_7N aminocyclitol.[5,6] These genes have no other known homologues within the genus *Burkholderia* and have been maintained in the genome of *B. kirkii* despite rampant genome erosion. Moreover, the C_7N aminocyclitol family of compounds is known to display a wide range of biological activities.[7] Together, these facts point towards a key role of these secondary metabolites in leaf nodule symbiosis. We therefore decided to isolate and characterize this putative new C_7N aminocyclitol synthesized by the endosymbiont of *P. kirkii*.

Since aminocyclitols display a characteristic methylene proton signal at around 6 ppm in the ^1H NMR spectrum, we opted for an unusual genome-based, NMR-guided fractionation approach. In order to overcome sample limitation, we used a 1.7 mm micro-cryoprobe 600 MHz NMR spectrometer in the early stage of the discovery process. Submicrogram amounts of sample were sufficient to record a ^1H NMR spectrum that confirmed the presence of a C_7N aminocyclitol in the extract. Importantly, this signature could not be detected in crude extracts from stunted aposymbiotic *P. kirkii* plants. After multiple reversed phase (RP)-HPLC runs on a Synergi Hydro column, a mixed fraction with the appropriate ^1H NMR signal was obtained. Sucrose was identified as the contaminant and was removed by treating the crude extract under acidic conditions. The final, successful



Kirkamide (**1**)

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isolation of the new C_7N aminocyclitol kirkamide (**1**) was then possible through a combination of RP-HPLC and Cu^{II}-coated preparative thin layer chromatography, a procedure reported for the separation of saccharides.[8]

High resolution ESI-MS of kirkamide (**1**) revealed an exact mass of *m/z* 240.0844, which supports the molecular formula $C_9H_{15}NO_5Na$ for the $[M+Na]^+$ pseudomolecular ion. ^1H and ^{13}C NMR spectroscopic data ([D_6]DMSO) for kirkamide (**1**) were compared with those reported for streptol.[9] This comparison suggested a different substitution pattern on C-2, as well as the presence of an acetyl group. This group was established as an NH-acetyl fragment based on HMBC correlations between the quaternary carbonyl C-8 with the NH and H-9 proton signals. The assignment of the carbocyclic core structure was then deduced by using the ^1H-^{13}C HMBC correlations between H-5, H-7a and H-7b, and C-6, as well as between H-5 and C-1, and ^1H-^1H COSY correlations between H-1 and H-2, H-2 and H-3, H-3 and H-4, NH and H-3, H-4 and H-5, and H-5 and H-1. The relative configuration was then assigned by comparing the *J*-coupling constants between H-2 and H-3, H-3 and H-4, and H-4 and H-5 in [D_6]DMSO (Table 1) with that reported for streptol (D_2O).[9] To exclude the influence of the solvent, the recorded spectroscopic data for valienamine in [D_6]DMSO (see the Supporting Information) showed that the compounds shared the same conformation, with H-2 in an equatorial position and H-3, H-4, and H-5

Table 1: NMR Spectroscopic data (500 MHz, [D_6]DMSO) for kirkamide (**1**).

C/N no.	δc, type	δ_H (*J* in Hz)	HMBC[a]
1	121.5, CH	5.63, dq[b] (4.8, 1.5)	3, 5, 7
2	64.3, CH	3.99, m	
3	53.1, CH	3.63, ddd (10.9, 8.1, 3.9)	
NH		7.36, d (8.1)	8
4	70.4, CH	3.56, dd (10.9, 7.1)	3, 5
5	72.9, CH	3.79, d (7.1)	1, 4, 6
6	142.5, C		
7a	60.8, CH_2	4.01, d (14.8)	1, 6
7b		3.95 d (14.8)	1, 6
8	169.2, C		
9	23.0, CH_3	1.85, s	8

[a] HMBC correlations are given from proton(s) stated to the indicated carbon atom. [b] Apparent splitting pattern.

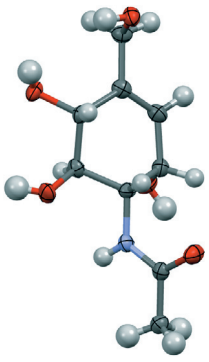
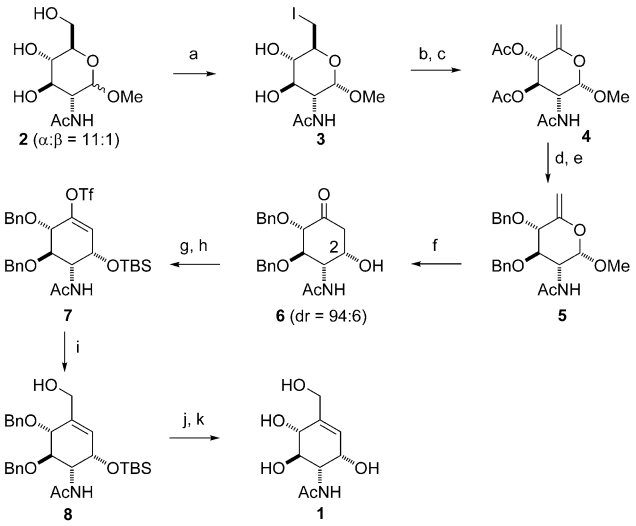


Figure 1. Single-crystal X-ray structure analysis of kirkamide (**1**).[20]

in axial positions. Finally, X-ray crystal structure analysis (Figure 1) established the constitution and configuration of kirkamide (**1**).

After the structure of kirkamide (**1**) was established, we turned to total synthesis in order to obtain large quantities of this compound for subsequent biological studies (Scheme 1). The synthesis started with the known methyl-*N*-acetyl-D-glucosamine **2**,[10] which was transformed via a Garegg–Samuelsson reaction with PPh_3, imidazole, and I_2 to give the iodide **3**.[11] A one-step procedure to reach the benzyl-protected enol ether **5** was



Scheme 1. Synthesis of kirkamide (**1**). Reaction conditions: a) Ph_3P, Imid, I_2, THF, reflux, 15 min; b) Ac_2O, pyridine, RT, 24 h; c) AgF, pyridine, RT, 48 h, exclusion of light, 50% over 3 steps; d) $NH_{3(gas)}$, MeOH, RT, 3 h; e) NaH, BnBr, DMF, 0°C, 12 h, 36% over 2 steps; f) $HgSO_4$ dioxane: aq. H_2SO_4 (5 mM) 2:1, microwave, 60°C, 15 min; g) TBSOTf, 2,6-lutidine, THF, 0°C, 12 h, 63% over 2 steps; h) Comins' reagent, NaHMDS, THF, −78°C, 5 min, 62%; i) Bu_3SnCH_2OH, Pd(PPh_3)$_4$, LiCl, dioxane, microwave, 105°C, 1 h, 85%; j) TBAF, THF, RT, 3 h, 98%; k) Na/$NH_{3(liq)}$, THF, −78°C, 30 min, 59%. Imid: imidazole, THF: tetrahydrofuran, DMF: dimethylformamide, TBS: *tert*-butyldimethylsilyl, HMDS: hexamethyldisilazane. TBAF: tetra-*n*-butylammonium fluoride.

evaluated by reacting the substrate **3** with NaH and BnBr. Unfortunately, a bicyclic byproduct was observed as the major compound. Therefore, the iodide **3** was first acetylated, followed by AgF-mediated elimination to give the desired enol ether **4** in 50% yield over 3 steps.[12] The protecting groups were exchanged from acetyl to benzyl in two steps to afford the key intermediate **5**.[12] The conversion of the exocyclic enol ether to the cyclohexanone **6** was achieved via a Ferrier carbocyclization with $HgSO_4$ as a catalyst under microwave conditions.[13,14] To our delight, excellent diastereoselectivity (94:6) at C-2 in cyclohexane **6** was obtained in the course of this reaction. Protection of this β-hydroxy ketone appeared daunting at first, since the elimination of H_2O seemed plausible, but silyl protection and triflation with Comins' reagent[15,16] proceeded smoothly to give the Stille coupling precursor **7**. The cross-coupling reaction[17] provided the primary alcohol **8**, and subsequent deprotection first with TBAF, and then under Birch conditions,[18] resulted in synthetic kirkamide (**1**). All of the synthetic steps up to the final deprotection were amenable to gram-scale preparation, which demonstrates the robustness of this route. The spectroscopic data for synthetic kirkamide (**1**) confirmed the structure of the natural product and finally enabled the assignment of all of the OH resonances in the ^1H NMR spectrum (see the Supporting Information).

We tested the cytotoxicity of pure synthetic kirkamide (**1**) by using a brine shrimp lethality assay with instar II nauplii of *Artemia*.[19] Synthetic kirkamide (**1**) displayed an median lethal concentration (LC_{50}) value at 48 h of 0.84 μg mL^{-1}, thus indicating that it is toxic to crustaceans. Since insects play

a major role as herbivores, and bacterial secondary metabolites could play a protective role for the plant, we also tested the insecticidal activity of synthetic kirkamide (**1**) against pollen beetles (*Meligethes aeneus*) collected from the wild. In our assay, pollen beetles fed with pollen tainted with 0.3 % w/w of kirkamide (**1**) reached mortality levels of up to 90 % 14 days after treatment.

In summary, kirkamide (**1**), a new natural product possessing a C_7N aminocyclitol core structure was isolated from leaf nodules of *Psychotria kirkii*. Genomic evidence strongly suggests that the *B. kirkii* symbiotic bacteria are responsible for the synthesis of kirkamide.[6] To investigate the biological activity of kirkamide (**1**), we developed a gram-scale total synthesis with a Garegg–Samuelsson reaction as a key step to efficiently provide the enol ether intermediate **5**. Through this approach, kirkamide (**1**) was synthesized via a Ferrier carbocylization and a Stille cross-coupling. Kirkamide (**1**) is toxic to aquatic arthropods and insects, thus suggesting a protective role for bacterial secondary metabolites in the *Psychotria/Burkholderia* leaf nodule symbiosis.

Keywords: aminocyclitols · natural products · ecology · symbiosis · total synthesis

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[20] CCDC 1054238 contain the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.

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