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Neuroactive diol and acyloin metabolites from cone snail-associated bacteria

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Abstract

The bacterium *Gordonia* sp. 647W.R.1a.05 was cultivated from the venom duct of the cone snail, *Conus circumcissus*. The *Gordonia* sp. organic extract modulated the action potential of mouse dorsal root ganglion neurons. Assay-guided fractionation led to the identification of the new compound circumcin A (**1**) and 11 known analogs (**2–12**). Two of these compounds, kurasoin B (**7**) and soraphinol A (**8**), were active in a human norepinephrine transporter assay with K_i values of 2575 and 867 nM, respectively. No neuroactivity had previously been reported for compounds in this structural class. *Gordonia* species have been reproducibly isolated from four different cone snail species, indicating a consistent association between these organisms.

Keywords

Natural product; symbiont; neuroassay

Symbiotic bacteria living with animals have been an excellent and still comparatively untapped source of new molecules of importance to drug discovery. Although cone snail mollusks are well defended by their shells and their arsenal of peptide toxins, they also house symbiotic bacteria that synthesize neuroactive small-molecule natural products.¹ and ² At least some of these compounds occur within the host animals themselves, implicating symbiotic bacteria in the production of molecules with potential ecological importance.

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We have been investigating bacteria cultivated from cone snails as a novel source of compounds with neuroactivity and other biological effects.³ Our primary screen involves primary cultures of mouse dorsal root ganglion (DRG) neurons.^{2, 4, 5, and 6} These cultures contain at least 12 definable cell types that express distinct receptor and channel populations. Therefore, the DRG assay casts a broad net for compounds with diverse activities that are directly relevant to pain and other conditions. Here, we report the application of this methodology to the discovery of neuroactive compounds from cone snail-associated bacteria.

Informed local and national consent and intellectual property agreements were obtained prior to performing this study. Strain 647W.R.1a.05 was cultivated from the venom duct of the mollusk *C. circumcissus*, collected in Cebu, Philippines. 16S rRNA gene sequence analysis identified the strain as *Gordonia* sp. (99% identity to *Gordonia terrae* strain KUDC1762).

Organic extracts of cone-snail associated bacteria were screened in the DRG assay to identify strain 647W.R.1a.05 as an active hit. The extract (50 μ g / mL) observably increased depolarization elicited by 25 mM KCl (Figure 2). HPLC analysis showed that the strain produced a family of metabolites, which were further purified by bioassay- and chemistry-guided fractionation, leading to the identification of 12 structurally related compounds (**1**–**12**). Compounds **2**–**12** have been previously described and were identified by comparison of NMR and MS data with literature values.

Circumcin A (**1**) was isolated as a colorless solid and assigned the molecular formula C₁₆H₁₈O₃ on the basis of HRESIMS analysis (m/z 259.1340 for [M+H]⁺) and NMR experiments (Table 1). Detailed examination of the ¹H NMR data (Table 1) revealed the presence of a *p*-hydroxyphenyl moiety (¹H 6.67 d, 2H and 7.01 d, 2H) and a phenyl moiety (¹H 7.15–7.24, 5H). The HSQC data showed additionally the presence of two methylenes (¹³C 38.3, C-7 and 39.4, C-10) and two methines (¹³C 74.0, C-8 and 73.6, C-9) in **1**, which were assigned to a 2,3-butanediol moiety on the basis of ¹H-¹H COSY correlations between H-7/H-8/H-9/H-10. HMBC correlations (Table 1) from H-7 to C-3/5 and from H-10 to C-12/16 allowed the planar structure to be established as **1** (Figure 1). Furthermore, NOESY correlations (Table 1) between H-7/8 and H-3/5 and between H-9/10 and H-12/16 confirmed the assignment. In related vicinal diols, coupling constants between methine moieties were previously determined to be 5.4 and 2.2 Hz for the *erythro* and *threo* configurations, respectively.⁷ A small coupling constant (2.2 Hz, both in CD₃OD and acetone-*d*₆) for the vicinal protons in **1** were observed, indicating a *8R*,9R** configuration.⁷

Circumcins B (**2**) and C (**3**) and 1,4-diphenyl-2,3-butanediol (**9**) have been synthesized but are not previously known as natural products.^{8 and 9} Other known compounds were first identified in bacterial fermentations, including: diolmycin A1 and A2 (**4** and **5**);⁷ kurasoin A and B (**6** and **7**);¹⁰ soraphinol A (**8**);^{11 and 12} diolmycin B2 (**10**);⁷ 4-hydroxysattabacin (**11**);¹³ and soraphinol C (**12**).¹⁴

Four pure compounds (**2**, **8**, **11** and **12**) were selected for further study in the DRG assay because they are representative of the structural motifs found in **1**–**12**. Compounds **2** and **8** showed very similar activity with that of the crude extract (Figure 2) and were responsible for its activity, while **11** and **12** were inactive at a concentration of 90 μ M (Figure 2). This indicated two aromatic rings were required; all compounds containing two aromatic rings (**1**–**10**) were further screened at the National Institute of Mental Health's Psychoactive Drug Screening Program (PDSP). Compounds were tested against 44 human receptors, transporters, and channels. Only compounds **7** and **8** exhibited activity below the 10 μ M threshold, and only in a human norepinephrine transporter (NET) assay,¹⁵ with *K_i* values of

2757 and 867 nM, respectively. Related transporters for serotonin and dopamine were not affected at the highest tested doses (10 μ M). These assays were ligand displacement assays and therefore do not provide evidence concerning function of the compounds.

The fluorometric calcium flux DRG assay allows for the simultaneous study of 100–150 neurons with single application of compounds.^{4, 5, and 6} Multiple neuronal cell types are present in each well, including a substantial fraction of nociceptors (~30–50%); each cell type has different combinations of receptors and channels and exhibits a different pharmacological profile. In the standard discovery assay, responses elicited by chemicals are normalized by pulsing with 25 mM KCl, which leads to depolarization, followed by washout, bringing cells back to baseline. Subsequently, samples of extracts or pure compounds are added to observe any direct depolarizing effects, and then samples are added in tandem with 25 mM KCl to observe any increase or decrease of the KCl induced depolarization. By following changes in intracellular Ca^{2+} over these steps, fine information about the activity of extracts is revealed, enabling discovery of new agents. Here, the DRG assay led to the direct identification of neuroactive components. Norepinephrine has been shown to mediate allodynia in DRG neurons,¹⁶ although further work would be required to connect DRG activity and any effects on individual targets.

Compounds **1–12** are closely related to each other, belonging to a family previously known from diverse bacteria. *Gordonia* was not previously reported to contain this compound family. *G. terrae* and related bacteria have been found in many different environments, and some are associated with human infections.¹⁷ We previously reported that *Gordonia* spp., including a strain identified as *G. terrae*, were consistently isolated from three different species of cone snails (*Conus rolandi*, *Conus pulicarius*, and *Conus tribblei*).² Therefore, it appears that *Gordonia* is a consistent associate of cone snails. Further investigation is required to determine if circumcins can be isolated directly from cone snail tissues, and whether *Gordonia* performs roles such as those associated with symbiosis or pathogenesis in their hosts.

Compounds **2–12** have a wide range of biological activities. Diolmycins (**4**, **5**, and **10**) were previously identified as potent anticoccidial agents^{7 and 18} but here were inactive in mammalian neurons. Likewise, 4-hydroxysattabacin (**11**)¹³ and soraphinol C (**12**)¹⁴ were previously reported as inhibiting Herpes simplex virus and as a free-radical scavenger, respectively, but were not neuroactive in our assays. Kurasoins (**6** and **7**) were previously reported as modestly inhibiting human protein farnesyltransferase (FTase),¹⁰ with IC_{50} s of 59 μ M; here **7** bound NET while **6** did not. Notably, **7** is about $20 \times$ more potent on NET than on FTase. For the remaining compounds, biological activity was not previously reported. This is the first report of neuroactivity for compounds in this class. A clear SAR for NET binding can be observed in the activity of the natural analogs, which may serve as the basis for future optimization. A potent (~5 nM)¹⁹ NET inhibitor atomoxetine (Strattera) is currently FDA-approved to treat attention deficit-hyperactivity disorder. Interestingly, it bears some structural similarity with **7** and **8**.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.
Structures of compounds 1–12.

Figure 2. Activity of crude extract and compounds 2 and 8 observed by calcium imaging of dissociated DRG neurons in culture

Each trace is the response of a single neuron. Responses from ~100 neurons were monitored individually and simultaneously in a given experimental trial. Selected traces are shown from three different experimental trials. The y-axis is a measure of relative intracellular (cytoplasmic) calcium concentration, $[Ca^{2+}]_i$, obtained by standard ratiometric calcium-imaging techniques (i.e. ratio of 340 nm/380 nm excitation while monitoring fluorescence emission at 510 nm). The x-axis is time in minutes. Increases in $[Ca^{2+}]_i$ were elicited by briefly depolarizing the neurons with 25 mM KCl, as indicated by vertical arrows. Each KCl pulse elicited an increase in $[Ca^{2+}]_i$ (by activating voltage-gated calcium channels) that is observed as a peak in each trace. After the second KCl pulse, the compound was applied to the cells for 5 minutes (horizontal bar). Both the crude extract (100 μ g/mL) (A) and the two test compounds **2** (73 μ M) and **8** (67 μ M) (B) caused an amplification of the calcium-transient elicited by a KCl pulse.

Table 1

NMR data of circumcin A (**1**) in CD₃OD-*d*₄.

No.	¹ H	¹³ C	HMBC	¹ H- ¹ H COSY	NOESY
1	-	155.4 C			
2/6	6.67 d (8.0)	114.6 CH	C-6/2, C-1, C-4	H-3/5	
3/5	7.01 d (8.0)	130.0 CH	C-1, C-5/3, C-7	H-2/6	H-7, H-8
4	-	129.6 C			
7	2.79 dd (13.8, 6.3); 2.69 dd (13.8, 8.1)	38.3 CH ₂	C-4, C-3/5, C-8, C-9	H-8	H-3/5
8	3.59 ddd (8.1, 6.3, 2.2)	74.0 CH		H-7, H-9	H-3/5
9	3.62 ddd (8.1, 5.2, 2.2)	73.6 CH		H-8, H-10	H-12/16
10	2.87 dd (13.4, 5.2), 2.78 dd (13.4, 8.1)	39.4 CH ₂	C-9, C-8, C-11, C-12/16	H-9	H-12/16
11	-	139.8 C			
12/16	7.18 d (7.7)	129.0 CH	C-10	H-13/15	
13/15	7.24 dd (7.7, 7.2)	127.8 CH		H-12/16, H-14	
14	7.15 dd (7.2, 7.2)	125.6 CH		H-12/16	