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Spirostomin, Defense Toxin of the Ciliate Spirostomum teres: Isolation, Structure Elucidation, and Synthesis

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The defense toxins, spirostomins A and B, have been isolated as a diastereomeric mixture from the ciliate microorganism Spirostomum teres. The structure of spirostomin was elucidated through a number of NMR experiments which allowed assigning the unprecedented spiro[2,5-dimethyl-5,6,7,8-tetrahydropthalalene-1,4-dione]-8,6′-(pyran-2,5′-dione)] skeleton to this natural compound. The total syntheses of the racemic spirostomins confirmed their structure and relative configurations.

The extrusive organelles (pigment granules)3 of the heterotrich ciliate Stentor coeruleus contain a blue pigment stentorin,2 while that of Blepharisma japonicum has red pigment blepharismins.3 These pigments have been studied as photoreceptors.4 Moreover, we recently showed that they constitute a chemical defense system against predators.5,6 We also isolated the defense toxin climacostol from the heterotrich ciliate Climacostomum virens, which has colorless extrusive organelles (cortical granules)7 as does Spirostomum teres. We found they had the function of chemical defense against raptorial ciliate Dileptus margaritifer.8 The toxic substances, spirostomins A (1) and B (2), were isolated as a 5:1 mixture of diastereomers, and their structures were assigned as spiro[2,5-dimethyl-5,6,7,8-tetrahydropthalalene-1,4-dione]-8,6′-(pyran-2,5′-dione)] that is an unprecedented carbon skeleton. Described herein is the isolation, characterization, and syntheses of spirostomins.

Whole cells of Spirostomum teres were dipped in aqueous 70% EtOH. After removal of the cells by filtration and concentration of the filtrate, the residue was partitioned between ethyl acetate and water. A biologically active fraction, based on lethal toxicity against the ciliate Paramecium caudatum, was collected from the organic layer and purified by preparative TLC. The spirostomins were found as a 5:1 mixture of diastereomers. Further attempts to purify the fraction containing them were not successful due to their instability and low concentration. Lethal dose 50% (LD50) of isolated spirostomins for P. caudatum was 0.25 μg mL−1.

The molecular formula of spirostomin was estimated to be C18H18O8 as determined by EI-HRMS: m/z [M]+ found 286.0836, calcld 286.0841. This data also suggested that the degree of unsaturation was 10. The 1H NMR spectrum of the major isomer 1 in CDCl3 exhibited signals due to three sp2 methine protons at δ 6.94 (d, J = 10.2 Hz), 6.89 (d, J = 10.2 Hz), and 6.64 (q, br, J = 1.5 Hz), olefinic and aliphatic methyl protons at δ 1.99 (d, J = 1.5 Hz) and 1.22 (d, J = 7.1 Hz), respectively, and one methine proton at δ 3.10 (multi). The 16 carbon signals observed in the 13C NMR spectrum were characterized by a DEPT experiment, which suggested that 1 had three sp2 quaternary carbons, three sp2 methines, one oxygenated quaternary carbon, one methine, two methylys, two methylenes, three keto carbonyls, and one ester/lactone carbonyl. These data accounted for 7 out of the 10 degrees of unsaturation, suggesting that 1 was a tricyclic compound. The 1H-1H COSY, 1H-13C HMBC, and 1H-13C HMBC spectra of 1 indicated the presence of three structural fragments A, B, and C, as depicted in Figure 1. HMBC correlations from H-3′ to C-2′ (ester carbonyl, δc 159.6) and C-5′ (keto carbonyl, δc 194.2) and from H-4′ to C-2′, and from H-3 and H-2Me to C-1 (keto carbonyl, δc 185.4) connect the A and B to the A′ and B′ fragments, respectively. HMBC correlations from H-5, H-6, and H-5Me to C-4a (quaternary, δc 151.2), from H-6 and H-7 to C-8 (quaternary, δc 83.2), and from H-5 and H-7 to C-8a (quaternary, δc 138.1), suggested that fragment C′ was a 6-member ring containing fragment C.

![Figure 1 Structural fragments for spirostomin](image_url)

A spiro structure consisting of fragments A′ and C′ was deduced from HMBC correlations between H-7 and C-5′ as well as from H-4′ to C-8. NOESY correlations also confirmed a through space interaction between H-7 and H-4′. The tricyclic structure of 1 suggested the presence of bicyclic quinone that was formed from a carbonyl carbon (δc 186.0) and fragments B′ and C′. Moreover, the UV spectrum of 1 (λmax 257 nm) was in closer agreement with that of a para-quinone rather than an ortho-quinone. The synths were
then assembled to reveal the planar structure of spirostomin (Figure 2).

![Figure 2](image)

**Figure 2** $^1$H NMR and $^{13}$C NMR assignment, and HMBC correlation for spirostomin

**Scheme 1.** Retrosynthesis of spirostomin

The retrosynthetic analysis of spirostomin is depicted in Scheme 1. Pyran ring would be constructed by nucleophilic attack on the carbonyl group with furyllithium and subsequent oxidation. This approach can be used to build the ring while retaining the stereochemistry of the quaternary carbon. This process also allowed the relative configuration of spirostomin to be assigned from the stereochemistry of the intermediate furan adduct. α–Tetralone derivative 5, which had no oxygen functional group at C-8, was selected as a starting material because nucleophilic attack on the benzylic carbonyl carbon was sensitive to the steric effect by the substituent at peri position.

**Scheme 2.** Selectivity of furan adducts

α–Tetralone derivative 6 was treated with 2-furyllithium prepared from furan and $n$-butyllithium, which yielded the corresponding furan adduct 7 as a 4:1 mixture of diastereomers (Scheme 2).

In the $^1$H NMR spectrum of 7, the signals observed at δ 7.01 and 6.60 ppm were assigned to H-8 of the major and minor products, respectively. The higher magnetic field signal for the H-8 proton of the minor component can be attributed to the magnetic anisotropy associated with the equatorially bonded furan ring.

In the major product, furan ring and methyl group at the benzylic position are anti to each other. We postulate that this resulted from a favorable axial attack to the carbonyl carbon on the opposite face of the pseudoaxial methyl group. We attempted to separate furan adducts to confirm the stereochemistry. However, not only were the diastereomers of 7 unable to be separated by silica gel column chromatography or preparative TLC, but dehydrated product 8 was also afforded.

We hypothesized that the pseudoaxial methyl group at the remote benzylic position was affected by the steric hinderance of the methoxy group. As such, we expected that an enhancement in selectivity could be achieved during the addition reaction by simply converting the phenolic hydroxyl protecting group from methoxy to the bulkier tert-butyldimethylsilyl group. This conversion provided stabilization to the furan adducts due to the reduced electron-donating nature of the silyloxy group at C-5. α–Tetralone derivative 9 was afforded by demethylation of 6 with aq HBr and acetic acid and subsequent silylation (69% in 2 steps) The addition was then repeated on 9, resulting in the formation of the furan adducts 10 and 11 in a 10:1 diastereomeric ratio, which were separable by column chromatography. The relative configurations of 10 and 11 were determined by the analysis of their $^1$H NMR and 2D-NOESY correlations. In the major product 10, the furan ring and methyl group at the benzylic position were found to be in the anti orientation, as evidenced by the NOESY correlation of H-2a with H-4a and OH and of H-3a with H-3'. On the other hand, in the minor product 11, the NOESY correlation of H-2a with H-4a and H-3' indicated a syn orientation between the furan ring and the methyl group (Figure 3).

![Figure 3](image)

**Figure 3** Selected NOESY correlations and conformation of furan adducts 10 and 11
The Achmatowicz reaction of $^{12}$ of 10 with mCPBA and subsequent oxidation of the resulting hemiacetal 12 by using equivalent amount of Jones reagent gave the spiro pyranodione 13. Deprotection of 13 with HF-pyridine afforded phenolic compound 14. Finally, oxidation of 14 with Fremy’s salt$^{13,14}$ furnished 15 and ortho quinone as a byproduct. Interestingly, we found the one-pot synthesis of 15 from 12 without ortho quinone when Jones reagent was used in excess (Scheme 3).

Scheme 3 Synthesis of compounds 13 and 15

This method was also applied for the synthesis of spirostomin 17 beginning with the minor product 11 (Scheme 4). The fact that conversion of the furan adducts 10 and 11 yielded stereospecifically 15 and 17, respectively, suggested that epimerization of the benzylidene position did not occur under these reaction conditions. The spectral characteristics of synthetic 15$^5$ and 17$^6$ were consistent with those observed for the natural spirostomin B and A, respectively. The relative configurations of spirostomin B and A were therefore determined as [5$^R$, 8$^R$] and [5$^R$, 8$^S$], respectively.

In conclusion, we accomplished the isolation, characterization, and structural elucidation of spirostomin. The relative configurations of two stereoenic centers were confirmed by chemical syntheses of 15 and 17. Stereoselective synthesis of spirostomin A and determination of the absolute configurations of spirostomin$^{17}$ will be reported in due course.

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References and Notes


6. F. Buonanno, G. Guella, C. Strim, C. Ortenzi, *Hydrobiologia* 2012, 684, 97-107. (This article partly described about spirostomin, but the structure was incorrectly drawn.)


14. Spectra data of 15: $^1$H NMR (600 MHz, CDCl$_3$) $\delta$ 1.27 (3H, d, J = 7.0 Hz), 1.69 (1H, mult), 1.99 (3H, d, J = 1.5 Hz), 2.01 (1H, mult), 2.05 (1H, mult), 2.30 (1H, mult), 2.90 (1H, mult), 6.62 (1H, q, J = 1.5 Hz), 6.83 (1H, d, J = 10.1 Hz), 6.95 (1H, d, J = 10.1 Hz); $^{13}$C NMR (150 MHz, CDCl$_3$) $\delta$ 15.5, 19.6, 25.0, 27.1, 31.7, 83.9, 134.1, 134.4, 137.1, 138.3, 145.3, 151.6, 159.9, 185.5, 186.3, 192.8; HR-EIMS m/z 286.0838 (calcd for C$_9$H$_{14}$O$_3$, 286.0841).

15. Spectra data of 17: $^1$H NMR (600 MHz, CDCl$_3$) $\delta$ 1.22 (3H, d, J = 7.1 Hz), 1.70 (1H, mult), 1.99 (3H, d, J = 1.5 Hz), 2.13 (1H, mult), 2.15 (2H, mult), 3.10 (1H, mult), 6.64 (1H, d, J = 1.5 Hz), 6.89 (1H, d, J = 10.2 Hz), 6.94 (1H, d, J = 10.2 Hz); $^{13}$C NMR (150 MHz, CDCl$_3$) $\delta$ 15.6, 18.7, 23.5, 26.0, 31.9, 83.2, 134.1, 134.2, 137.9, 138.1, 145.3, 151.2, 159.6, 185.4, 186.0, 194.2; HR-EIMS m/z 286.0842 (calcd for C$_9$H$_{14}$O$_3$, 286.0841).

The defense toxins, spirostomins A and B, have been isolated as a diastereomeric mixture from a ciliate *Spirostomum teres*. The structure of spirostomin was elucidated as spiro[(2,5-dimethyl-5,6,7,8-tetrahydronaphthalene-1,4-dione)-8,6’-(pyrane-2,5’-dione)] and was found to contain an unprecedented carbon skeleton through NMR spectral analyses. The total syntheses of the racemic spirostomins confirmed their structure and relative configurations.

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