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Rare 1,2'-binaphthyls Produced by *Nodulisporium hinnuleum* Smith (ATCC 36102)

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Abstract: In the course of processing extracts from fermentations with *Nodulisporium hinnuleum* Smith (ATCC 36102) to obtain demethoxyvirdin, we noticed that this fungus produced several other secondary metabolites as well. In an effort to identify these components, four new, related natural products, designated hinnulin A-D (**1-4**) were isolated and characterized. Structure elucidation of the hinnulins, primarily by NMR spectroscopy, revealed that these belong to the rare class of 1,2'-binaphthyl natural products.

Keywords: *Nodulisporium hinnuleum;* binaphthyl; fungal metabolite; oxygen reactive compounds.

1. Introduction

In our search for natural products with potential application in human or animal health, we investigated the extract of the fungus *Nodulisporium hinnuleum* Smith (ATCC 36102), a known producer of demethoxyvirdin[1,2]. With an interest in finding demethoxyvirdin-related metabolites, we also inspected other metabolites present in the culture extract. Four new compounds, initially thought to be related to demethoxyvirdin because of their similar molecular weights (e.g.: 322 vs. 348), were isolated and characterized. However, in depth analysis revealed that these "minor" components were unique natural products and not related to demethoxyvirdin. The structures of these new metabolites, named hinnulin A-D (**1-4**), were to some extend deduced from their UV spectra which implied the presence of an unaltered 1,8-dihydroxynaphthalene moiety, and then were

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completely elucidated by NMR spectroscopy. Here, we wish to present the structures of these hinnulins and establish them as new members of the 1,2'-binaphthyl class of natural product.

2. Materials and Methods

2.1. General

LC-MS data were obtained on a Thermoquest LCQ Deca instrument equipped with an Agilent 1100 LC system including a diode array detector and a YMC ODS-A column (0.2 x 10 cm, 3μ); flow 0.3 mL/min (Solvent A: 0.025% formic acid in H_2O; solvent B: 0.025% formic acid in MeCN; gradient: 5% B to 95% B into A over 20 min, then holding for 10 min. A total scan UV chromatogram is acquired over a scan range from 190 to 500 nm. UV spectra are acquired on the fly over the same range with scan steps of 2 nm. After emerging from the UV flow cell, the effluent stream goes into the ion trap mass spectrometer. The mass spectrometer is fitted with an electrospray ionization (ESI) probe and is operated in alternating positive-ion and negative-ion full scan (100 - 2000 mass units) mode. The capillary voltages are set at 29 for positive and at -10 for negative ion detection, respectively. The capillary temperature is set to 200°C and nitrogen is used as the sheath and auxiliary gas.

NMR spectra were recorded on a Bruker Avance DPX-400 or 500 MHz NMR spectrometer at 400 (500) and 100 (125) MHz for ^{1}H and ^{13}C, respectively, using a 3 mm broadband probe. Chemical shifts are given in *ppm* relative to the solvent signals of acetone-d_6 (δ_H 2.04, δ_C 29.81), or MeOH-d_3 (MeOH-d_4) (δ_H 3.30, δ_C 49.00), respectively.

All solvents were obtained from J. T. Baker, Inc., and were of the highest commercially available purity.

2.2. Fermentation, isolation and purification

A seed culture of *Nodulisporium hinnuleum* Smith (ATCC 36102), derived from frozen vegetative mycelia in a cryogenic vial, was plated on Bennet's Agar (Yeast extract 1.0g/L, Beef extract 1.0 g/L, NZ Amine Type A 2 g/L, Glucose 10 g/L, Agar 20 g/L, in distilled water, pH 7.3) and allowed to grow for 7 days and examined for culture purity. A two stage seed was utilized by excising 2 x 1mm squares of fungal growth and adding to 10 mL of seed medium (Dextrose (Glucose) 10.0g/L, Yeast Extract (YE) 5.0g/L, Agar 0.4g/L, Soluble Starch 20.0g/L, Calcium Carbonate ($CaCO_3$) 1.0g/L, NZ Amine (Type A) 5.0g/L, pH6.8) to a 25x150 seed culture tube containing two 6mm glass beads. This first stage seed was grown for 3 days at 22°C, 200rpm, 2"throw and transferred in its entirety to a 250 mL Erlenmeyer flask containing 40 mL of seed medium. The second stage culture was then allowed to grow an additional 4 days at 22°C, 200rpm, 2"throw. The second stage seed was used in it's entirely (50 mL) to inoculate 2.8L Fernbach culture flasks (5.0%) containing 1.0L of production medium. (Potato Starch 4.0g/L, Dextrose 20.0g/L). After 8 days, approximately 192 hours, the culture was harvested. A pool of ten 1-L Erlenmeyer flasks was processed as a batch. The whole broth was centrifuged (3000 rpm/20 min.). The supernatant was discarded, while the wet pellet was collected and then freeze-dried. To extract the metabolites, the dry pellet was slurried with 400 mL CH_2Cl_2 and filtered, and concentrated. This first extract containing 1.8g oily material was enriched in triglycerides and demethoxyviridin was used for the isolation of demethoxyviridins. The remaining pellet was further extracted with 400 mL acetone yielding a yellow-brown extract containing the bulk of the hinnulins. The spent pellet was then discarded, while the acetone extract was concentrated yielding 740 mg of oily brown residue. This was applied to a prepared open silica gel column (3 x 30 cm, 40μ, Grace Co) packed in methylene chloride to separate the components. The column was developed in

steps of 225 mL acetone/methylene chloride mixtures. The first step contained 2% acetone increasing to 25% acetone in step eight. Step nine contained 50% acetone, step ten 75%, and step eleven consisted of 100% acetone. Finally, step twelve consisted of 100% methanol. Hinnulin B (**2**) together with Hinnulin D elutes in fraction 3 (~ 5% acetone) followed by **3** (~ 10% acetone), whereas **1** is only effectively eluted from the silica gel column when methanol is added. Although the hinnulins were readily separated from each other on silica gel, additional chromatography on Sephadex LH-20 allows an easy purification of each of the hinnulins since aromatic compounds are highly retained. Therefore, methanolic solutions of each of the **1**, **2** or **3** containing fractions from the silica gel column was loaded onto a prepared Sephadex LH-20 column (2.0 x 42 cm) and developed with MeOH. Fractions of ca. 30 mL each were collected at regular intervals and analyzed by LCMS for content as described above. The fractions were dried by centrifugal evaporation (speed-vac) yielding 14.7 mg of **1** in fractions 8 to 10 as a red solid after evaporation of the solvent. Separately, 14.6 mg of **2** was obtained as a yellowish-red solid after evaporation of MeOH in LH-20 fractions 8 to10, while 6 mg of **3** was obtained as a yellow solid in a final run on Sephadex LH-20 (fractions 9 and 10).

2.3. Characteristics of hinnulins

***1',4,5,6'-tetrahydroxy-1,2'-binaphthyl-5',8'-dione (1) (*Hinnulin A):** UV (MeCN/H_2O) λ_{max}: 234, 300, 326, 340, 434 nm; ^{1}H NMR(acetone-d_6): (see Table 1); ^{13}C NMR(acetone-d_6) (see Table 1); HRMS (ESI negative mode): *m/z* 347.05610 [$C_{20}H_{11}O_6$], (347.05611 calc'd for $C_{20}H_{11}O_6$ $^{(1-)}$ Δ = -0.00001 *m/z*); MS (ESI-pos.) $[M+H]^+$= *m/z* 349.1 (3%); MS(ESI-neg.) $[M-H]^-$= *m/z* 347.4 (100%).

1',4,5,6'-tetrahydroxy-6',7'-dihydro-1,2'-binaphthyl-5',8'-dione (2) (Hinnulin B): UV (MeCN/H_2O) λ_{max}: 234, 314, 328, 340, 360 nm; ^{1}H NMR(acetone-d_6): (see Table 1), ^{13}C NMR(acetone-d_6): (see Table 1); HRMS (ESI negative mode): *m/z* 349.07162 [$C_{20}H_{13}O_6$], (349.07176 calc'd for $C_{20}H_{13}O_6$ $^{(1-)}$ Δ = -0.00014 *mu*); MS (ESI-pos.) $[M+H]^+$= *m/z* 351.1 (5%); MS(ESI-neg.) $[M-H]^-$= *m/z* 349.4 (100%).

***1',4,5,5',6'-pentahydroxy-6',7'-dihydro-1,2'-binaphthyl-8'(5'H)-one (3) (*Hinnulin C):** UV (MeCN/H_2O) λ_{max}: 232, 272 (sh), 328 (sh), 340 nm; ^{1}H NMR (acetone-d_6): (see Table 1); ^{13}C NMR(acetone-d_6): (see Table 1); HRMS (ESI negative mode): *m/z* 351.08758 [$C_{20}H_{15}O_6$], (351.08741 calc'd for $C_{20}H_{15}O_6$ $^{(1-)}$ Δ = -0.00017 *mu*) ; MS (ESI-pos.) $[M+H]^+$= *m/z* 353.2 (12%); MS(ESI-neg.) $[M-H]^-$= *m/z* 351.4 (100%).

1,2'-binaphthyl-1',4,5,8'-tetraol (4) (Hinnulin D): UV (MeCN/H_2O) λ_{max}: 232, 316, 328, 342 nm; HRMS (ESI negative mode): *m/z* 317.08185 [$C_{20}H_{13}O_4$], (317.08193 calc'd for $C_{20}H_{13}O_4$ $^{(1-)}$ Δ = -0.00008 *mu*); MS (ESI-pos.) $[M+H]^+$= *m/z* 319.2 (8%); MS(ESI-neg.) $[M-H]^-$= *m/z* 317.5 (100%).

3. Results and Discussion

3.1. Isolation and purification

The hinnulins were obtained in side fractions during the isolation of demethoxyviridin which was prepared in an adaptation of a procedure described by Aldridge[1] that avoids the use of protic solvents. Basically, the fungal pellet was collected from all fermentation flasks, freeze-dried, and then macerated while extracting with methylene chloride. Filtration provided a clear extract which contained the bulk of the demethoxyviridin produced. The retained pellet was further extracted with acetone yielding a yellow-brown extract enriched in hinnulins. This extract (its LC-MS analysis is shown in the supporting information as figure S1) was concentrated, and applied to a silica gel column to separate the components. The column was developed by a step gradient of increasing amounts of acetone mixed into methylene chloride. Hinnulin B (**2**)**,** together with the minor component hinnulin D

(**4**) (~ 5% acetone), elutes first followed by hinnulin C (**3**) (~ 10% acetone), whereas hinnulin A (**1**) is only effectively eluted from the silica gel column when methanol is added. Although the hinnulins were readily separated from each other on silica gel, the respective fractions still contained other components such as hinnuliquinone[3,4,5], demethoxyviridin, or demethoxyviridiol[1]. It was therefore required to further purify each individual hinnulin fraction by Sephadex LH-20 chromatography since highly aromatic compounds are well retained on this resin, and, for this reason, are usually obtained in pure form. Due to the propensity of the hinnulins to react with (air) oxygen, and/or to slowly polymerize by forming insoluble, black products, it was virtually impossible to maintain any hinnulin component in pure form for an extended period of time neither as a solid, nor in solution. A similar reactivity has already been described for 1,8-dihydroxynaphthalene and its derivatives[6-9].

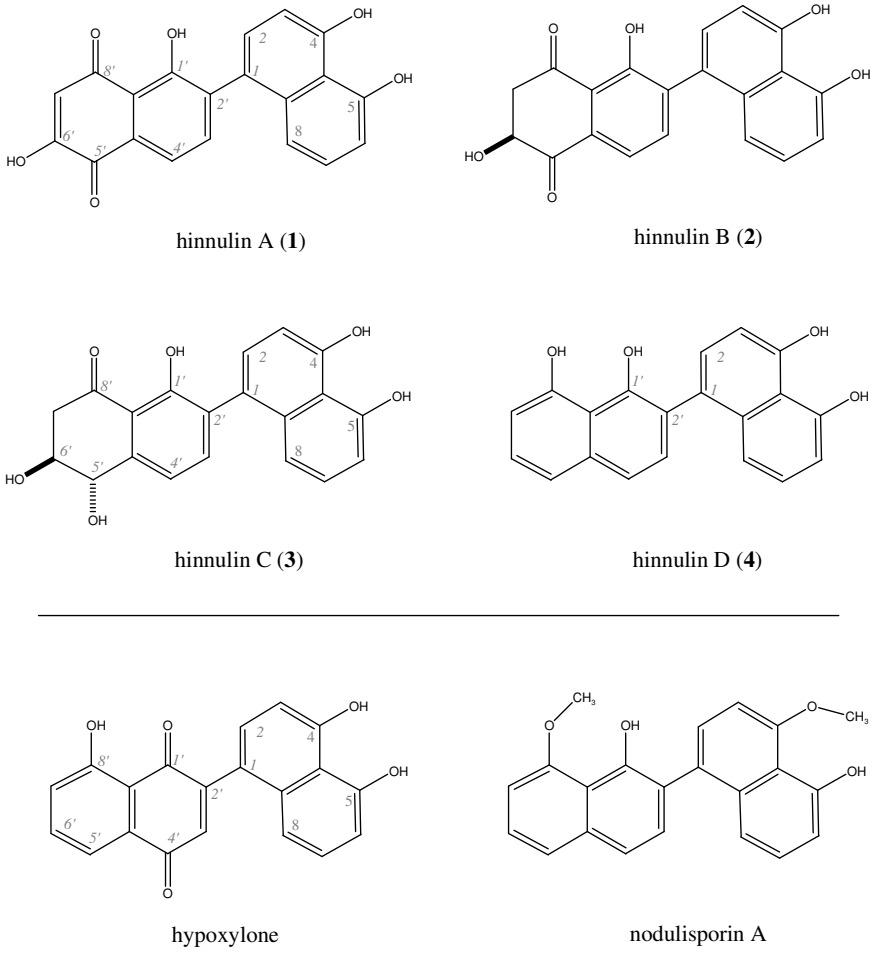

Figure 1. Structures of hinnulins **1-4** in comparison with known 1,2'-binaphthyl natural products.

3.2. Structure elucidation

The hinnulins are identified mass-spectrometrically by their molecular ions ($[M+H]^+$ or $[M-H]^-$ ranging around *m/z* 350) in the LC-MS chromatograms and their characteristic UV spectra (see supplemental figures S1 and S2). The structure determination is based on the interpretation of spectroscopic data, especially NMR data that include ^1H, ^{13}C, APT, COSY, ROESY, HSQC, and HMBC, revealing that the skeleton of all hinnulins is comprised of 20, mostly aromatic, carbon atoms. As suggested by the UV curves, the ^{13}C chemical shift values and heteronuclear NMR correlations, they are composed of two structurally similar subunits, one of which is the 1,8-dihydroxynaphthalene moiety as indicated by characteristic absorptions at 328 and 340 nm in the UV spectra (see supplemental figure S2). In NMR correlations, this moiety has a characteristic set of 10 aromatic carbons, five of which carry one hydrogen atom each, while two of the five non-protonated carbon atoms, resonating at ~156 ppm, bear hydroxy groups (compare figure 1). The 1,8-naphthalenediol[10] composition is confirmed by predictable ^1H and ^{13}C chemical shifts that correlate in the expected manner in HSQC, HMBC and other spectra. The NMR correlations also reveal that position 1 of the 1,8-naphthalenediol subunit is always the attachment point of the other subunit for each hinnulin. Therefore, the structural differences between the hinnulins are due to modifications of only the second subunit but not the point of attachment as this reportedly is the difference between nodulisporin A and B[11] (A is shown figure 1).

Table 1. NMR Spectroscopic Data (400 MHz, acetone-d_6) for Hinnulins A (**1**), B (**2**), and C (**3**).

		Hinnulin A (**1**)		Hinnulin B (**2**)		Hinnulin C (**3**)	
Position	mult	δ_C	δ_H (mult., *J* = Hz)	δ_C	δ_H (mult., *J* = Hz)	δ_C	δ_H (mult., *J* = Hz)
1	s	127.4	-	126.7	-	126.7	-
2	d	129.4	7.19 (d, 7.5)	129.5	7.24 (d, 7.5)	129.4	7.18 (d, 7.5)
3	d	109.1	6.84 (d, 7.5)	109.3	6.89 (d, 7.5)	109.2	6.85 (d, 7.5)
4	s	155.9	-	155.7	-	155.3	-
5	s	155.8	-	155.4	-	155.0	-
6	d	109.6	6.78 (d, 7.8)	109.9	6.83 (d, 7.8)	109.6	6.79 (d, 7.8)
7	d	127.7	7.16 (dd, 8.2, 7.8)	127.4	7.22 (dd, 8.2, 7.8)	127.5	7.19 (dd, 8.2, 7.8)
8	d	118.6	7.00 (d, 8.2)	118.5	6.95 (d, 8.2)	118.8	6.95 (d, 8.2)
4a	s	116.0	-	115.7	-	115.7	-
8a	s	136.0	-	135.9	-	136.3	-
1'	s	160.3		160.5	-	160.8	-
2'	s	138.8	-	137.1	-	129.8	-
3'	d	135.8	7.38 (d, 7.5)	139.9	7.73 (d, 7.5)	139.7	7.50 (d, 7.5)
4'	d	118.6	7.56 (d, 7.5)	118.5	7.64 (d, 7.5)	119.1	7.29 (d, 7.5)
5'	s	187.6	-	197.5	-	73.3	4.78 (d)
6'	s	171.4	-	72.5	5.00 (m)	71.6	4.21 (m)
7'	d (t)	108.8	5.81 (s)	46.7	3.30, 3.40	44.7	3.13, 2.81
8'	s	191.1	-	203.2	-	204.7	-
4a'	s	131.8	-	134.7	-	145.6	-
8a'	s	116.7	-	119.3	-	116.5	-
6'-OH	-	-	n.d.	-	4.97 (d)	-	n.d.
1'-OH	-	-	14.34 (s)	-	12.46 (s)	-	12.64 (s)
4,5-OH	-	-	n.d.	-	10.3 (br s)	-	n.d.

n.d. = not detected

Structures **1**, **2** and **3** are readily assembled from NMR correlations using COSY, HSQC and HMBC pulse programs (see figure S3 in the supporting information). In **1** the 1,2'-linkage of the naphthalenediol subunit to the second subunit is apparent from characteristic HMBC crosspeaks. Proton H-2 [δ_H 7.19] not only correlates with C-4 [δ_C 155.9] and C-8a [δ_C 136.0] of subunit 1, but also with C-1' [δ_C 138.8] of subunit 2. In turn, H-3' [δ_H 7.38] of the second subunit correlates with C-1' [δ_C 160.3] and C-4a' [δ_C 131.8] of its own subunit as well as with C-1 [δ_C 127.4] of subunit 1. The connection of the two rings comprising the quinoid substructure 2 is apparent from correlations involving H-7' [δ_H 5.81]. This proton can only be located in the 7'-position and not in the 6'-position as it shows strong HMBC's to C-8a' [δ_C 116.7] and C-6' [δ_C 171.4], but not to C-4a' [δ_C 131.8] or C-8' [δ_C ~190] as would be expected for an aromatic proton at C-6'.

Similarly, the linkages in structure **2** are corroborated by the following observed correlations: proton H-2 [δ_H 7.24] not only correlates with C-4 [δ_C 155.7] and C-8a [δ_C 135.9] of substructure 1, but also with C-2' [δ_C 137.1] of subunit 2. In turn, H-3' [δ_H 7.73] of the second subunit not only correlates with C-1' [δ_C 160.5] and C-4a' [δ_C 134.7] of its own subunit, but also with C-1 [δ_C 126.7] of substructure 1. Hinnulin B (**2**) contains one hydroxymethine and one methylene group within subunit 2 which could be placed in the 6'-position and the 7'-position respectively. The placing of the methylene group is directed by a key correlation between the equatorial proton H-7' [δ_H 3.40] of this methylene group with C-8a' [δ_C 119.3] of subunit 2. This correlation is only feasible if the methylene group is located in position 7, since placement into position 6 would make this an unlikely 4-bond correlation (compare figure S3a). All further correlations within subunit 2 are consistent with this configuration which is also matching structure **1** assuming that **1** is derived from **2** via oxidation (see figure 2).

The correlations of the NMR data for structure **3** are very similar to those observed for structure **2** in that the 1,2'-linkage between the subunits is supported by a number of 3-bond correlations. Here, proton H-2 [δ_H 7.18] correlates with C-4 [δ_C 155.3] and C-8a [δ_C 136.3] of subunit 1, and also with C-2' [δ_C 129.8] of subunit 2. Conversely, H-3' [δ_H 7.50] of the second subunit not only correlates with C-1' [δ_C 160.8] and C-4a' [δ_C 145.6] of its own subunit, but also with C-1 [δ_C 126.7] of subunit 1. Further analysis of NMR correlations pertaining to the modifications at substructure 2 suggests that hinnulin C (**3**) is a reduced derivative of hinnulin B (**2**) in that it features two hydroxymethine groups at the 5'- and 6'- positions.

This substitution pattern suggest that the original naphthalenediol moiety is initially oxidized at C-5' and C-6' and then rearranged further to yield **3** as shown in figure 2. The relative configuration in this area is difficult to determine however. The relevant HMBC correlations reveal that H-5' [δ_H 4.78] is in a plane with the aromatic portion of this subunit, which, therefore, is in an equatorial position. Its coupling is unexpectedly complex as it appears in the ^{1}H-NMR spectrum as a broadened "triplet" that, perhaps, could also be interpreted as two overlapping "doublets". Consistent with the COSY data, only one doublet would have been expected here, as was noted previously for the similarly configured diepoxin δ[10]. The apparent coupling constant for this H-5' "doublet" is ~ 6 Hz suggesting that the 5'–hydroxy and 6'-hydroxy groups are positioned in a *trans* configuration. However, two "doublets" for H-5' are more readily explained if the conformation of the cyclohexenone ring can assume two distinct, but slowly interchanging forms, as then, on average, two signals for the same proton- and carbon atoms can be observed. Indeed, all signals, especially the ^{13}C-signals, assignable to this cyclohexenone subunit are doubled in both the ^{1}H- and the ^{13}C-spectrum, which would suggest the coexistence of two conformers. Although this leaves ambiguity in determining the relative configuration of hinnulin C (**3**), its substitution pattern can still be established with certainty based on the observed NMR correlations.

Structures **1**, **2** and **3** support the proposal that the hinnulins are generated from a single precursor, likely hinnulin D (**4**), via one oxidation step that places oxygen atoms at C-5' and C-6' as outlined in figure 2. Thus, the hinnulins are formed by a different oxidation

pathway than hypoxylone[12] (see figure 1) although both may be produced via the same precursor **4**. In this situation however, the presumably enzyme-guided oxidation of an aromatic region would have to occur at a different region of the molecule. Since the NMR data for hypoxylone[12] clearly indicate that the two subunits are linked between the quinone portion of the second subunit and the (first) naphthalenediol unit, oxygenation would have to occur at C-3' and C-4' close to where the two naphthalenediol subunit are linked.

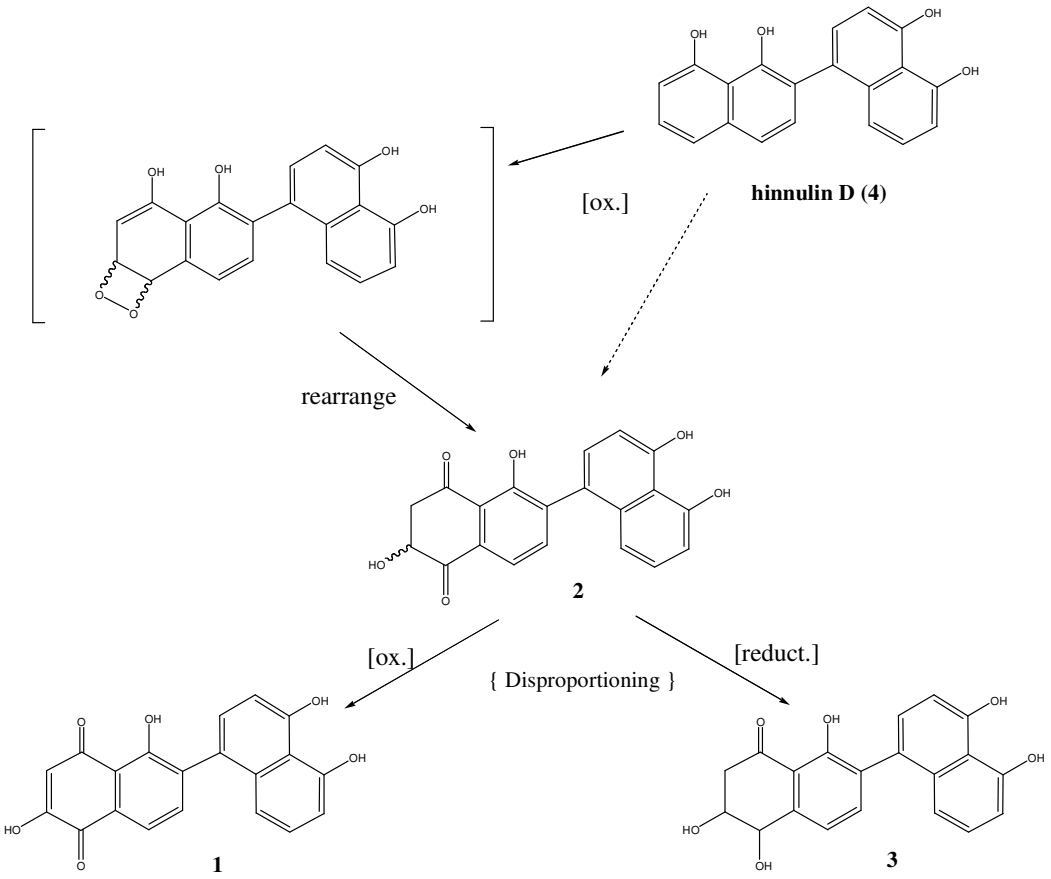

Figure 2. Proposed biosynthetic scheme to generate **hinnulins A-C (1-3)** from **hinnulin D**.

4. Conclusion

Several non-viridin-related metabolites, such as hinnuliquinone[3,4,5], and the hinnulins A-D (**1-4**), were isolated from cultures of the fungus *Nodulisporium hinnuleum* Smith (ATCC 36102). Since the hinnulins were unknown compounds, their chemical structures were determined revealing that they are new members of the 1,2'-binaphthyl class, of which nodulisporin A[11] and hypoxylone[12] have been reported previously. Compounds of this class have been reported to display herbicidal, antifungal or antibacterial activity[11] which is probably linked to the propensity of naphthalenediol containing compounds to be sensitive towards oxygen forming radical containing species. This is also reflected in the fact that, in solid form, they noticeably deteriorate and eventually turn into a dark-brown solid material that is insoluble in common solvents. Judging from the dark-

brown color, a similar substance may be a formed naturally during fungal melanine production. Of the three binaphthyl-comprising natural product classes, the 1,1'-binaphthyl metabolites such as daldinol[11,13] are the most frequently encountered examples, but fungal metabolites of the 2,2'-binaphthyl class are also known[14], and nodulisporin B[11] is a member of this class.

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Supporting Information

Supporting information for this article is available at http://www.acgpubs.org/RNP

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