Onchidin B: A New Cyclodepsipeptide from the Mollusc *Onchidium* sp.

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Onchidin B: A New Cyclodepsipeptide from the Mollusc Onchidium sp.

Rogelio Fernández, Jaime Rodríguez, Emilio Quinñoá, Ricardo Riguera, Luis Muñoz, Myriam Fernández-Suárez, and Cécile Debitus.

Abstract: Onchidin B (4) is a cyclic depsipeptide isolated from the pulmonate mollusc Onchidium sp. Its structure was determined by extensive 2D-NMR, FABMS, tandem FABMS/MS, selective hydrolysis, and synthesis. It contains four α-amino acids [two units of N-methyl valine (MeVal), two units of proline (Pro)], four α-hydroxy acids [two 2-hydroxyisovaleric acids (Hiv), two 2-hydroxy-3-methylpentanoic acid moieties (Hmp)] and two units of the new β-hydroxy acid: 3-hydroxy-2-methylcyclohept-7-ynoic acid (Hymo)]. Selective hydrolysis and direct comparison by chiral GC-MS with authentic samples of the α-amino and α-hydroxy acids allowed us the assignment of the entire absolute stereochemistry of onchidin B. In this way, the α-hydroxy acids were found to be (S)-Hiv and (S,S)-Hmp, and the α-amino acids (R)-proline, (S)-proline, and (R)-MeVal. In order to establish the absolute configuration of the new β-hydroxy acid, Hymo, its four possible stereoisomers were stereoselectively synthesized using chiral N-propionyl oxazolidinones and hex-5-ynal as starting material. Comparison by HPLC-MS of the synthetic samples with the natural Hymo (all derivatized as esters of (→)-(R)-α-methoxy-α-(9-amylocacetic acid), affirmed its absolute stereochemistry as (2R,3R). Thus, onchidin B (4) is cyclo [(R)-MeVal-(R,R)-Hymo-(S)-Pro-(S,S)-Hmp-(S)-Hiv-(R)-MeVal-(R,R)-Hymo-(R)-Pro-(S,S)-Hmp-(S)-Hiv]-H. It is formed by a head-to-tail linkage of two halves, each one built by five units and identical sequence. The lack of symmetry of onchidin B is thus due to the presence of one (S)-Pro unit in one half and one (R)-Pro unit in the other. The structural similarity between onchidin B (4) and onchidin (2), both isolated from the same organism, and between the β-hydroxy acid Hymo (5) and the β-amino acid Amo (3) found in onchidin is noticed.

Introduction

Natural products derived from marine organisms have become an increasingly important source of biologically active compounds. Some of the most interesting ones are the cyclic depsipeptides. This class of metabolites frequently offer an unrivaled chemical diversity incorporating new amino and/or hydroxy acids. From a chemical point of view, this is one of the reasons why these compounds have attracted the effort of researchers resulting on the report of a fair number of new structures. Most of the depsipeptides from marine organisms have been isolated from sponges; striking examples are the geodinamolides, arenastatin, and the antifungal and cytotoxic jasplakinolide (jaspamide). On the other hand, only three molluscs have been found to build by five units and identical sequence. The lack of symmetry of onchidin B is thus due to the presence of one (S)-Pro unit in one half and one (R)-Pro unit in the other. The structural similarity between onchidin B (4) and onchidin (2), both isolated from the same organism, and between the β-hydroxy acid Hymo (5) and the β-amino acid Amo (3) found in onchidin is noticed.

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isolated along the last ten years, more than a dozen dolastatins from Dolabella auricularia. Particularly important from the pharmacological point of view is the highly cytotoxic dolastatin 10, the most potent antineoplastic agent known to date. It belongs to the selected group of marine natural products that are in clinical or preclinical stage as antitumoral compounds. Other marine molluscs that were reported to produce cyclic depsipeptides are Elysia rufescens and Onchidium sp., collected in New Caledonia, from which kahalalide F (1) and onchidin (2) were isolated, respectively. Onchidin constitutes the only example reported to date of a cyclic, symmetric depsipeptide from a mollusc. Its structure is composed of two identical halves: cyclo [(S)-N-methylvaline-(2S,3S)-3-amino-2-methylcyclohept-7-ynoic acid-(8-hydroxyisovaleric acid)-(S)-valine-(3-hydroxyisovaleric acid)-(S)-hydroxyisovaleric acid]-linked together in a head-to-tail way so onchidin has a C2 axis of symmetry and, as a result, produces only half the expected NMR signals. In addition to known α-amino and α-hydroxy acids, onchidin (2) incorporates two identical units of the new β-amino acid, 3-amino-2-methylcyclohept-7-ynoic acid (3, Amo).

We reported some time ago several proplyopropionate pyrones named onchitrols. Those metabolites showed moderate antitumor activity and were isolated from the low polarity cytotoxic extracts of Onchidium sp. From this marine organism, we now

wish to report the bioassay guided isolation from the cytotoxic CCl₄ extracts (97% inhibition to Kb cells at 10 μg/mL) of another cyclic depsipeptide, which we have named onchidin B (4) (Chart 3), and that shares with onchidin (2) a great number of structural features.

Isolation and Structural Analysis

*Onchidium* sp. (Pulmonata, order: Stylommatophora, family: Onchiidiidae) was collected at Chesterfield archipelago in the intertidal zone of New Caledonia. Isolation and Structural Analysis

*Kb* (human epidermoid carcinoma cells) bioactivity-guided separation of *Onchidium* sp. (family: Stylommatophora, order: Pulmonata, suborder: Helicinidae) was performed at Mouillage isles (45° 00' S, 166° 08' E) on the basis of its HRFABMS peak (m/z 1153.6999 [M-H]⁻, Δ + 9.9 mnu). The edited DEFT and HMBC spectra showed a molecular ion *(m/z)* at 1153, whose molecular formula C₆₂H₁₉₆N₄O₁₆ (17 unsaturations) was deduced on the basis of its HRFABMS peak (m/z 1153.6999 [M-H]⁻, Δ + 9.9 mnu). The edited DEFT and HMBC spectra showed a molecular ion *(m/z)* at 1153, whose molecular formula C₆₂H₁₉₆N₄O₁₆ (17 unsaturations) was deduced on the basis of its HRFABMS peak (m/z 1153.6999 [M-H]⁻, Δ + 9.9 mnu). The edited DEFT and HMBC spectra showed a molecular ion *(m/z)* at 1153, whose molecular formula C₆₂H₁₉₆N₄O₁₆ (17 unsaturations) was deduced on the basis of its HRFABMS peak (m/z 1153.6999 [M-H]⁻, Δ + 9.9 mnu). The edited DEFT and HMBC spectra showed a molecular ion *(m/z)* at 1153, whose molecular formula C₆₂H₁₉₆N₄O₁₆ (17 unsaturations) was deduced on the basis of its HRFABMS peak (m/z 1153.6999 [M-H]⁻, Δ + 9.9 mnu). The edited DEFT and HMBC spectra showed a molecular ion *(m/z)* at 1153, whose molecular formula C₆₂H₁₉₆N₄O₁₆ (17 unsaturations) was deduced on the basis of its HRFABMS peak (m/z 1153.6999 [M-H]⁻, Δ + 9.9 mnu). The edited DEFT and HMBC spectra showed a molecular ion *(m/z)* at 1153, whose molecular formula C₆₂H₁₉₆N₄O₁₆ (17 unsaturations) was deduced on the basis of its HRFABMS peak (m/z 1153.6999 [M-H]⁻, Δ + 9.9 mnu). The edited DEFT and HMBC spectra showed a molecular ion *(m/z)* at 1153, whose molecular formula C₆₂H₁₉₆N₄O₁₆ (17 unsaturations) was deduced on the basis of its HRFABMS peak (m/z 1153.6999 [M-H]⁻, Δ + 9.9 mnu). The edited DEFT and HMBC spectra showed a molecular ion *(m/z)* at 1153, whose molecular formula C₆₂H₁₉₆N₄O₁₆ (17 unsaturations) was deduced on the basis of its HRFABMS peak (m/z 1153.6999 [M-H]⁻, Δ + 9.9 mnu). The edited DEFT and HMBC spectra showed a molecular ion *(m/z)* at 1153, whose molecular formula C₆₂H₁₉₆N₄O₁₆ (17 unsaturations) was deduced on the basis of its HRFABMS peak (m/z 1153.6999 [M-H]⁻, Δ + 9.9 mnu). The edited DEFT and HMBC spectra showed a molecular ion *(m/z)* at 1153, whose molecular formula C₆₂H₁₉₆N₄O₁₆ (17 unsaturations) was deduced on the basis of its HRFABMS peak (m/z 1156.6999 [M-H]⁻, Δ + 9.9 mnu). The edited DEFT and HMBC spectra showed a molecular ion *(m/z)* at 1153, whose molecular formula C₆₂H₁₉₆N₄O₁₆ (17 unsaturations) was deduced on the basis of its HRFABMS peak (m/z 1153.6999 [M-H]⁻, Δ + 9.9 mnu). The edited DEFT and HMBC spectra showed a molecular ion *(m/z)* at 1153, whose molecular formula C₆₂H₁₉₆N₄O₁₆ (17 unsaturations) was deduced on the basis of its HRFABMS peak (m/z 1153.6999 [M-H]⁻, Δ + 9.9 mnu). The edited DEFT and HMBC spectra showed a molecular ion *(m/z)* at 1153, whose molecular formula C₆₂H₁₉₆N₄O₁₆ (17 unsaturation...
protonated carbons. The chemical shifts obtained for the methyl group showed COSY correlations in each case, an a-methine consistent with the presence of oxygen substituents. Thus, these HMQC and a methylene group connected to another methine bonded to two pairs of methyl groups in the COSY and TOCSY spectra for the 2-hydroxyisovaleric acids (Hiv). Thus, the a-methine protons H-27 and H-57 showed COSY correlations to 2.15 (H-3) and this last signal to Me-4 and Me-5 (1.01 and 0.88, respectively). The same information was deduced from the following COSY correlation set: 5.14 (H-32, MeVal 2)/2.19 (H-33)/1.10-0.96 (Me-34, Me-35). One bond heteronuclear correlations allowed the identification of a-carbons, [δ 59.6 (MeVal 1, C-2) and 60.8 (MeVal 2, C-32)] and ß-carbons [δ 28.2 (MeVal 1, C-3) and 29.3 (MeVal 2, C-33)] for both residues. HMBC correlations between the N-methyl signal at 3.21 and the a-carbon at 59.6 (MeVal 1, C-2) and 60.8 (MeVal 1, C-32) and ß-carbons are notably downfield from those of the valines, allowing the unambiguous assignment of the signals for the a-carbons obtained from 13C-lH long range correlations. This connectivity pattern is exactly the same as we have already found in the ß-amino acid Amo (3), present in onchidin (2), but the chemical shift of the ß-carbons are notably downfield from those of Amo. All these data led us to identify these two spin systems (4e) as belonging to a new ß-hydroxy acid, 3-hydroxy-2-methyloct-7-ynoic acid, Hymo (5), that is reported for the first time in this work.

Relevant information pertaining to the determination of the bonding sequence was carried out using 13C-1H long range correlation experiments (HMBC), assigning the connectivities from carbonyl carbons to the adjacent amino or hydroxy substituted Cα-H protons. In this way, the presence of the structural segment Hiv 2-MeVal 1-Hymo 1 was established by the following correlations (Figure 2): CO (C-56, Hiv 2)/H-57 (Hiv 2); CO (C-1, MeVal 1)/H-57 (Hiv 2) and H-2 (MeVal 1); CO (C-6, Hymo 1)/H-2 (MeVal 1), NMe (MeVal 1), and Me-§ (Hymo 1). Similarly, two other skeletal segments were deduced from the correlations shown next: CO (C-29, Hmp 1)/H-16 (Pro 1) and H21 (Hmp 1) (segment Pro 1-Hmp 1), CO (C-31, MeVal 2)/H-27 (Hiv 1) and H-32 (MeVal 2); CO (C-36 Hymo 2)/H-32 (MeVal 2), NMe (MeVal 2), and Me-§ (Hymo 2) (segment Hiv 1-MeVal 2-Hymo 2).10

Two spin systems belong to 2-hydroxy-3-methylpentanoic acid units, Hmp (4d).

The remaining spin systems have the following connectivity pattern according to COSY, HMOC, and TOCSY analysis: each unit possessed a methine group [1H/13C NMR (δ 3.10/40.0 (C-7, Hymo 1) and 3.15/39.2 (C-37, Hymo 2)] connected to a methyl group [δ 1.07/15.0 (C-8, Hymo 1) and 1.13/14.4 (C-38, Hymo 2)] as well as to an oxygen-bearing methine [δ 5.34/75.3 (C-9, Hymo 1) and 4.90/78.2 (C-29, Hymo 2)] which is bonded to a chain formed by three methylenes, the last one being connected to a terminal acetylene [δ 1.91/68.6 (C-14, Hymo 1) and 1.94/68.9 (C-44, Hymo 2)]. Further HMBC experiments reaffirmed the presence of the alkyne group: correlations from the signal at 2.19 ppm (H-12, Hymo 1) to carbons at 68.6 (C-14, Hymo 1) and 83.9 ppm (C-13, Hymo 1) and from the proton at 2.22 (H-42, Hymo 2) to carbons at 68.9 (C-44, Hymo 2) and 82.8 ppm (C-43, Hymo 2). This connectivity pattern is exactly the same as we have already found in the ß-amino acid Amo (3), present in onchidin (2), but the chemical shift of the ß-carbons are notably downfield from those of Amo. All these data led us to identify these two spin systems (4e) as belonging to a new ß-hydroxy acid, 3-hydroxy-2-methyloct-7-ynoic acid, Hymo (5), that is reported for the first time in this work.
Stereochemistry of Onchidin B

Information about the absolute stereochemistry of onchidin B and additional evidence of its structure was achieved by GC-MS analysis of its components. Hydrolysis of 4 with 6 M HCl (110 °C, 24 h) followed by derivatization and GC-MS analysis on a chiral column (Chirasil-Val) revealed the presence of (S)-Hiv, (S,S)-Hmp, (R)-MeVal, (S)-Pro, and (R)-Pro in a 2:2:1:1 ratio.

Furthermore, smooth basic hydrolysis (1 M NaOH/MeOH) of 4 cleaves the ester bonds and affords, after treatment with diazomethane, the expected three dipeptides (Figure 4): (S,S)-Hmp-(S)-Pro-OMe (6), (S,S)-Hmp-(R)-Pro-OMe (7), and Hymo-(R)-MeVal-OMe (8). 6 and 7 were identified by comparison (GC-MS) with authentic samples, unambiguously confirming the presence of both (S)-Pro and (R)-Pro in onchidin B (4). A third component (8) from the mixture was identified by EIMS proving the location of Hymo next to MeVal as previously suggested by NMR.

At this stage, the only remaining point to be solved was the absolute configuration of the two asymmetric centers of the 3-hydroxy-2-methyl-7-ynoic acid (5) were synthesized in a diastereoselective mode. The synthetic strategy relies on the well-known reaction developed by Evans et al.11 between the enolborinate of optically pure N-propionyl oxazolidinones and aldehydes to obtain enantiomerically pure aldols and on the Mitsunobu reaction12 which allows the inversion of configuration at the hydroxylated carbon of the aldol, yielding its epimer.

Reaction of N-propionyl oxazolidinone 9a, derived from (S)-phenylalaninol,13 with hex-5-ynal (prepared from hex-5-yn-1-ol by a Swern oxidation)14 gives the aldol 10 with configuration (2S, 3R), whereas the reaction of oxazolidinone 9b, obtained from (1S,2R)-norephedrine, with the same aldehyde provides aldol 11 with configuration (2R,3S).

Compounds 10 and 11 were obtained optically pure when the reactions were carried out with freshly opened bottles of dibutylborontriflate, since no other diastereomers were detected by NMR. Standard hydrolysis15 of 10 and 11, followed by diazomethane treatment, afforded β-hydroxy methyl esters 12b (2S,3R) and 13b (2R,3S), respectively.

In order to prepare the other two stereoisomers (2R,3R and 2S,3S), the inversion of one of the chiral centers of both 10 and 11 was carried out with freshly opened bottles of dibutylborontriflate, since no other diastereomers were detected by NMR. Standard hydrolysis15 of 10 and 11, followed by diazomethane treatment, afforded β-hydroxy methyl esters 12b (2S,3R) and 13b (2R,3S), respectively.

Onchidin B was hydrolyzed with 6 M HCl at 110 °C for 24 h, and the mixture containing the free Hymo was esterified first with diazomethane and then with (−)-(R)-α-methoxy-α-(9-anthryl)acetic acid. HPLC-MS of that mixture showed a peak with identical tR, UV, and MS as isomer (2R,3R)-3-O-[(-)-(R)-α-methoxy-α-(9-anthryl)acetyl]-2-methyloct-7-ynoic methyl ester. Therefore, the absolute configuration of the natural Hymo (5) is (2R,3R) and the structure of onchidin B (4) is cyclo [(R)-MeVal-(R,R)-Hymo-(S)-Pro-(S, S)-€hp-(S)-Hiv].

Finally, in order to assign the (R) and (S) stereochemistries to the prolines, characteristic 13C chemical shifts differenced and additional NOEs were considered. The linkage between Pro 1 and Hmp 1 is probably trans, as suggested by the value obtained for $\Delta\delta_{pp} = 5.3$ ppm for C-17 and C-18, while the bond between Pro 2 and Hmp 2 is probably cis in accordance with the value $\Delta\delta_{pp} = 7.8$ ppm for C-47 and C-48. A ROESY correlation between H-21 (Hmp 1) and H-19 (Pro 1) confirms the trans assignment for Pro 1. These considerations, together with the NOE observed between H-21 (Hmp 1) and H-16 (Pro 1), suggest that both hydrogens lie on the same side, so C-16 and 11 was needed. Since the configuration at position 2 could not be fully inverted easily, we decided to concentrate our efforts on the inversion of the configuration at carbon 3 making use of the Mitsunobu reaction. When β-hydroxy methyl ester 12b was submitted to Mitsunobu conditions, no successful results were obtained. Nevertheless, a smooth inversion took place when aldols 10 and 11 were treated in similar conditions and p-nitrobenzoate esters 14 and 15 were obtained in good yields. Simultaneous hydrolysis of the benzoate ester and oxazolidinone moieties, followed by diazomethane treatment, afforded the desired (2S,3S)-β-hydroxy methyl ester 16 and its (2R,3R) isomer 17, in good yields.

Direct comparison of the methyl esters of the four synthetic stereoisomers with the methyl ester of Hymo, the natural component of onchidin B (4), by chiral GC and HPLC was not successful in spite of our efforts to find experimental conditions suitable for a good separation of the four isomers. This problem was finally overcome by HPLC-MS analysis of the 3-O-{(−)-(R)-α-methoxy-α-(9-anthryl)acetyl}-2-methyloct-7-ynoic methyl esters (18–21). These derivatives were prepared by standard treatment of the hydroxy esters with (−)-(R)-α-methoxy-α-(9-anthryl)acetic acid, and were chosen because they provide four beautifully separated peaks in the HPLC chromatogram. In addition, they present strong and characteristic UV absorption and give clean CIMS fragmentations that enabled a safe comparison by coinjection with the natural Hymo.

Thus, reaction of the four synthetic β-hydroxy esters with (−)-(R)-α-methoxy-α-(9-anthryl)acetic acid, in the presence of DCC and DMAP, afforded the corresponding derivatives that showed the following retention times in HPLC-MS with the mass detector fixed at m/z 378: (S,R) 28.09 min, (R,R) 30.93 min, (R,S) 41.74 min, and (S,S) 46.54 min.


and C-21 must have identical configuration and Pro 1 should be (S). Furthermore, the lack of NOE between H-46 (Pro 2) and H-51 (Hmp 2), in spite of the cis stereochemistry, suggests that both protons do not lie on the same side of the molecule, and so the absolute configuration of Pro 2 should be (R).

Ochonidin B (4) is then a cyclic depsipeptide formed by the head-to-tail bonding of two chains (MeVal-Hymo-Pro-Hmp-Hiv) with identical sequence. In spite of this common sequence, ochonidin B (4) has no C2 symmetry and shows signals for all the 62 carbons in the NMR spectra. This lack of coincidence of NMR signals is related to the presence of the two enantiomers of proline that renders the two halves of the molecule different.

Similarly, other natural products isolated from this organism, but, in that case, the repeating amino and hydroxy acids have the same configuration and so compound (2) has C2 symmetry and presents only half the expected proton and carbon resonances.

A further point of interest is the presence in ochonidin B (4) and ochonidin (2) of the obviously related acetylenic \( \beta \)-hydroxy and \( \beta \)-amino acids, [Hymo (5) and Amo (3)], respectively. A \( \beta \)-hydroxy acid with the same skeleton but an additional methyl group at C-2 has recently been described by P. Scheuer as a component of the cyclopentapeptide kilolide isolated from the mollusc Phalipus speciosus.\(^{21}\) Another related molecule is the \( \beta \)-hydroxy acid, (2R,3S)-7,7-dichloro-3-hydroxy-2-methyltannic acid, isolated from the marine sea hare Dolabella auricularia\(^{16}\) that can be viewed as the dichlorinated derivative of a Hymo stereoisomer.

**Experimental Section**

**General Methods.** IR and UV spectra were obtained on MIDAC Prospect FTIR and 8452 A spectrophotometers, respectively, and optical rotations on a Jasco DIP-370 digital polarimeter. NMR spectra were recorded on Bruker AMX 500, Bruker AMX 300, or Bruker WM 250 spectrometers using CDCl3 as solvent and internal standard. The H-1\(^{1}\) COSY spectrum was acquired with 4K \( \times \) 354 matrix and eight scans per increment. The TOCSY spectrum was acquired with 4K \( \times \) 300 matrix and 16 scans per increment. The ROESY spectrum was acquired with 2K \( \times \) 200 matrix and 32 scans per increment. The TOCSY and ROESY experiments were recorded using a mixing time of 0.070 and 0.280 ms, respectively. The HMQC\(^{22}\) and HMBC\(^{23}\) sequences were performed with 2K \( \times \) 204 matrix and 64 scans per increment. FABMS were obtained on a Kratos MS-50 (employing Xe atoms at 7–9 KeV; glycerol and 2-hydroxyethyphosphite + NaCl matrix) and FINIGAN 4000 (NBA matrix). Collisions induced tandem mass spectra (MS/MS) in the FAB mode were obtained on a four sector tandem mass spectrometer VG 70-SE-4F. EIMS was obtained on a Hewlett-Packard HP59970 spectrometer.

HPLC separations were performed on a Waters Model 600A using reversed-phase \( \mu \)-Bondapack C18 (7.8 mm I.D. \( \times \) 30 cm) and \( \mu \)-porasil (7.8 mm I.D. \( \times \) 30 cm). Gas chromatography analyses were performed using a Hewlett-Packard spectrometer with a Chirasil-Val III capillary column (50 m \( \times \) 0.25 mm).

Cytotoxic activity was assessed in vitro using KB cells.\(^{24}\)

(1,5,2R)-Norephedrine, (5)-phenylalanine, hex-5-yn-1-ol, and dibutyrylmonophosphate were purchased from Aldrich Chemical Company and were used without further purification. All solvents were distilled under argon utilizing standard laboratory procedures. Flash column chromatography was performed using Kieselgel 60 230–400 mesh SiO\(_2\) gel into glass columns. Synthetic products were purified by HPLC before optical rotation measurements. Synthetic yields were not optimized.

**Extraction and Isolation.** Animal Collection. *Onchidium* sp. is a Pulmonata, order: Stylonematophora, family: Ochoniidae, collected in Fiji (1988 from Chetouille Ilipangena in the intertidal zone on "Ile Longue" (Mouilage isles, 450 NW of New Caledonia), sample reference MG 332. Each animal weight was 300 g approximately. A voucher specimen is in deposit at the Museum National d'Histoire Naturelle in Paris, and it has been identified by Dr. Philippe Bouchet.

**Extraction and Partitioning.** Three kilograms of mollusc were freeze-dried (600 g dry weight) and then extracted with methanol (4 \( \times \) 2 L). The methanol extract was decanted off and concentrated in vacuum. The viscous concentrate was partitioned between 400 mL of 10% aqueous methanol and hexanes (2 \( \times \) 400 mL). The methanolic portion was made 20% aqueous and extracted with CCl\(_4\) (2 \( \times \) 400 mL). Then the methanolic portion was made 40% aqueous and extracted with CH\(_2\)Cl\(_2\) (3 \( \times \) 400 mL). The organic layers were concentrated in vacuum to yield 2.9 g of hexane extract (15% inhibition to KB cells at 10 \( \mu \)g/mL), 1.2 g of CCl\(_4\) extract (97% inhibition to KB cells at 10 \( \mu \)g/mL), and 0.7 g of CH\(_2\)Cl\(_2\) extract (99% inhibition to KB cells at 10 \( \mu \)g/mL).

The CCl\(_4\) extract was subjected to flash silica gel column chromatography (3 \( \times \) 20 cm, silica gel 60, Merck 70–230 mesh) stepwise elution from 100% dichloromethane to 100% methanol giving five fractions: F1 (inactive), F2 (cytotoxic to KB cells at 10 \( \mu \)g/mL: 93% inhibition), F3 (inactive), F4 (cytotoxic to KB cells at 10 \( \mu \)g/mL: 95% inhibition), and F5 (inactive).

**Isolation of Ochonidin B.** Fraction F2 was purified on a reversed-phase \( \mu \)-Bondapack C18 HPLC column (30 cm \( \times \) 7.8 mm, 397: MeOH: \( \mathrm{H}_2\)O: flow rate: 2.0 mL/min) affording 5 mg (17 \( \times \) 2% from the CCl\(_4\) extract) of ochonidin B (4), \( \alpha \)-127.7 min [\( \alpha \)]\(_{20}^{D}\) = 220.1 (c 0.1, CHCl\(_3\)); FTIR (KBr) 3280, 2980, 2940, 2880, 1747, 1650, 1640 cm\(^{-1}\); HRFABMS calculated for C\(_{23}\)H\(_{32}\)N\(_2\)O\(_4\)Na\(_2\) m/z 1153.6899 [M + H\(^{+}\)]\(^{2}\), found 1153.6999, \( \Delta + 9.9 \) ppm; HRFABMS calculated for C\(_{25}\)H\(_{34}\)Na\(_2\)O\(_4\) m/z 577.3488 [M + H\(^{+}\)]\(^{2}\), found 577.3479, \( \Delta - 0.9 \) ppm; (+) FABMS (NBA matrix): m/z (relative intensity): 1153 [M + H\(^{+}\)]\(^{2}\) (1), 757 [M + H\(^{+}\)]\(^{2}\) (7), 577 (100); (+) FABMS (2-hydroxyethyl disulfide matrix): m/z (relative intensity): 1175 [M + Na\(^{+}\)]\(^{2}\) (1), 1153 [M + H\(^{+}\)]\(^{2}\) (5), 577 (100).

\( ^{1}H \) and \( ^{13}C \) NMR see Table 1.

**Amino Acid Analysis by Chiral GC-MS.** Ochonidin B (4) (1 mg) was subjected to hydrolysis with 6 M HCl (0.5 mL) in a sealed tube at 110 °C for 24 h. The excess HCl was removed by passing a stream of N\(_2\), and the residue was dried under vacuum. The hydrolysates were diluted with water (1 mL), and the hydroxy acids were extracted with diethyl ether (3 \( \times \) 1 mL).

The ethereal solution was dried under vacuum, and the residue was dissolved in 1 mL of ether and treated with diazomethane for 30 min. The excess of reagent was removed with a stream of dry N\(_2\). Capillary
under reduced pressure, yielding a viscous yellow oil. The crude was washed with 5% NaHCO₃ (40 mL) under reduced pressure, the residue was redissolved in water (40 mL), and brine (40 mL), dried over anhydrous MgSO₄, and concentrated.

The water solution was dried under vacuum, and the resulting residue was added to a solution of 2 "clmin to 200 °C) to show peaks at 267 (1), 238 (20), 230 (20), 201 (4), 144 (9), 138 (19), 129 (20), 121 (20). HREIMS: calcd for C₁₀H₁₆O₃: 184.0944; found 184.0937. FTIR (CHCl₃) 3453, 3292, 2943, 1728, 1448, 1295, 1181 cm⁻¹.

The crude was dissolved in ether (1 mL) and evaporated in a stream of nitrogen. Capillary GC-MS analysis were carried out using a Chirasil-Val III capillary column; flow rate 1 mL/min. Standards (S) and (R)-MeVal were also derivatized by the same procedure.

Retention times (min) were as follows: 30.12 and 35.65 for (S)- and (R)-N-methyl valine, respectively.

Capillary GC-MS analysis were carried out using a Chirasil-Venax II column (50 m x 0.25 mm; He carrier gas; flow rate 1 mL/min; column temperature: 50 °C, 60 min isothermal) to show peaks at τₙ 13.59 and 33.50 min. Standards (S) and (R)-Hiv and (S), (R), (S), (R)- and (S), (R), (S), (R)-MeVal were also converted to the methyl derivaties by the same procedure. Retention times (minutes) were as follows: 15.51 ([S], Hiv), 16.18 ([R], Hiv), 31.10 ([S],[R]-Hiv), 32.50 ([S],[R]-Hiv), 33.69 ([S],[S]-Hiv), and 35.25 ([R],[R]-Hiv).

The water solution was dried under vacuum, and the resulting residue was divided into two equal portions. One of them was dissolved in dichloromethane (0.5 mL) and N-isopropyl isocyanate (1 mL) was added in a screw-capped vial. After 60 min at 100 °C the excess of reagent was removed with a steady stream of dry N₂. Capillary GC-MS analysis were carried out using the same column Chirasil-Venax III column (50 m x 0.25 mm; He carrier gas; flow rate 1 mL/min; column temperature 50 °C, 45 min isothermal, then programmed an increase of 5 °C/min to 200 °C) to show peak at τₙ 33.11 min. Standards (S) and (R)-MeVal were also derivatized by the same procedure. Retention times (min) were as follows: 30.12 and 35.65 for (S)- and (R)-N-methyl valine, respectively.

The second portion was dissolved in n-BuOH (0.5 mL) and HCl (3 M, 0.5 mL) and heated in a sealed tube at 100 °C for 30 min. The product was evaporated, dissolved in trifluoracetic anhydride (0.5 mL) and CH₂Cl₂ (0.5 mL), reacted at a sealed tube at 150 °C for 10 min and evaporated in a stream of nitrogen. Capillary GC-MS analysis were carried out using the same column Chirasil-Venax III column (50 m x 0.25 mm; He carrier gas; flow rate 1 mL/min; column temperature 50 °C, 30 min isothermal then programmed an increase of 2 °C/min to 200 °C) to show peak at τₙ 60.24 and 60.42 min. Standards (S) and (R)-Pro were also derivatized by the same procedure. Retention times (min) were as follows: 60.36 and 60.55 for (R)- and (S)-proline, respectively.

Alkaline Hydrolysis of Onchidin B. Onchidin B (4) (0.5 mg) in MeOH (0.5 mL) was treated under an argon atmosphere with NaOH (1 M, 0.5 mL) at 0 °C. After 1 h the reaction mixture was diluted with CH₂Cl₂ (2 mL) and water (1 mL). The aqueous phase was acidified to pH 2 with 1 M HCl and extracted with CH₂Cl₂ (2 x 2 mL). The CH₂Cl₂ extract was dried with anhydrous Na₂SO₄ and concentrated in vacuum. The residue dissolved in ether (1 mL) was treated with excess CH₂N₂ at room temperature for 30 min, and the excess of reagent was removed with a steady stream of dry N₂. Capillary GC-MS analysis were carried out using a Chirasil-Venax III column (50 m x 0.25 mm; He gas carrier; flow rate 1 mL/min; column temperature 150 °C, 45 min isothermal then programmed at 5 °C/min to 200 °C) to show peaks at τₙ 62.86 min ([S],[S]-Hiv), ([R],-[R]-Hiv), ([S],[R]-Hiv), and ([R],[S]-Hiv). HREIMS: calcd for C₁₀H₁₄N₂O₄: 182.0839; found 182.0837. FTIR (CHCl₃) 3453, 3292, 2943, 2341, 1785, 1448, 1295, 1181, 972 cm⁻¹.

The aldol reaction of the aldol 11 was obtained as a colorless viscous oil (3.4 g, 80% yield). [α]₉ = +14.3 (c 0.4, CHCl₃). (+)FABMS (glycerol matrix) m/z (relative intensity): 185 [M + H]⁺ (89), 167 (15), 153 (38), 135 (37), 127 (57), 115 (78). (−)FABMS (glycerol matrix) m/z (relative intensity): 184.1099. FTIR (CHCl₃) 3453, 3292, 2943, 1749, 1456, 1408, 1210, 1078, 958 cm⁻¹. ¹H NMR (300.13 MHz, CDCl₃): δ 1.20 (d, J = 7.2 Hz, 3 H), 1.52~1.79 (m, 4 H, 1.96 (t, J = 6.9 Hz, 3 H), 1.25 (d, J = 6.9 Hz, 3 H), 1.54~1.79 (m, 14 H, 1.96 (t, J = 2.3 Hz, 1 H), 2.22~2.27 (m, 2 H), 2.78 (s, broad, 1 H), 3.78 (m, 1 H), 4.00 (m, 1 H), 4.80 (m, 1 H), 5.69 (d, J = 7.3 Hz, 1 H), 7.26~7.46 (m, 5 H). ¹³C NMR (75.45 MHz, CDCl₃): δ 10.6 (c), 14.7 (c), 18.6 (t), 25.3 (t), 33.1 (t), 42.6 (t), 55.1 (d), 69.0 (s), 71.4 (d), 79.3 (d), 84.5 (d), 126.0 (d), 129.1 (d), 129.2 (d), 133.4 (s), 150.3 (s), 177.7 (s).

Methyl (2S,3R)-3-[3'-Hydroxy-2'-methyl-7'-cyano]-4-phenyl-4-methyl-2-oxazolidinone (11). Using the method described for the preparation of (4S,2'S,3'R)-3-(3'-hydroxy-2'-methyl-7'-cyano)-4-phenyl-2-methyl-2-oxazolidinone (10), N-propionyl onchidin 9b (3.875 g, 16.63 mmol) was treated with 1 M dibutyrobromitrifluate (18.3 mL, 18.3 mmol) and triethylamine (3 mL, 21.62 mmol) and the resulting enolboronate was allowed to react with hex-5-ynyl (2.1 g). After workup and purification the aldol 11 was obtained as a white solid (mp 97~100 °C, 4.1 g, 75% yield). [α]₉ = +8.3 (c 1.6, CHCl₃). EIMS m/z (relative intensity): 262 (M⁺, 9), 234 (15), 233 (100), 178 (20), 134 (36), 188 (42), 170 (67), 91 (21), 57 (25). HREIMS: calcd for C₁₀H₁₅NO₄: 182.0996; found 182.1000. standards were also derivatized by the same procedure.

Retention times (min) were as follows: 60.36 and 60.55 for (R)- and (S)-proline, respectively.

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p-nitrobenzoic acid was treated with LiOH (30%, 129.2 mg) in benzene (1 mL). After workup and purification, the solvent was removed under reduced pressure. The resulting crude was sequentially purified by flash chromatography (EtOAc:hexanes 1:7), yielding the nitrobenzoate ester as a colorless viscous oil (500 mg, 86%). 

**Method 2 (2R,3S)-3-(2-Methyl-4-[(4-nitrobenzoyloxy)-2-mercaptophenylazo]-2-phenyl-1,4-phenylazo-2-oxazolidine (14).** A solution of diethylazido-carboxylate (1.48 g, 8.47 mmol) in benzene (5 mL) was added to a solution of aldo 10 (400 mg, 1.21 mmol), triphenylphosphine (2.22 g, 8.47 mmol), and p-nitrobenzoic acid (1.42 g, 8.47 mmol) in benzene (40 ml) at room temperature. The resulting solution was stirred for 17 h at room temperature. The solvent was removed under reduced pressure, and the crude reaction was purified by flash chromatography (EtOAc:hexanes 1:7), yielding the nitrobenzoate ester as a colorless viscous oil (774 mg, 74% yield). 

**Method 3 (2R,3R)-3-(2-Methyl-4-[(4-nitrobenzoyloxy)-2-mercaptophenylazo]-2-mercaptophenylazo-2-oxazolidine (15).** Aldol 11 (270 mg, 0.82 mmol) was treated with triphenylphosphine (1.5 g, 5.74 mmol), p-nitrobenzoic acid (960 mg, 5.74 mmol), and diethylazido-carboxylate (0.91 mL, 5.74 mmol) in the manner described for the preparation of nitrobenzoate ester 14. After workup and purification ester 15 was obtained as an oil together with a small amount of another product which could not be separated by HPLC (530 mg, 90% yield). 

**Method 4 (2R,3S)-3-O-[(2R)-a-methoxy-a-(9-anthryl)acetamide]-2-methyl-7-yl-nicotine Ester 18.** The esters 18–21 were prepared by treatment of the corresponding β-hydroxy ester (3 mg) with (-)-(R)-α-methoxy-a-(9-anthryl)acetamide (5 mg) in the presence of DCC (4 mg) and DMAP (catalytic) in CHCl3 at room temperature. After 1 h the solvent was evaporated, and the crude was sequentially purified by flash chromatography (CH2Cl2 and HPLC (n-propanol, 30 cm × 7.8 mm; Aq-Ethanol 7:3; flow rate: 2.0 ml/min).

**Method 5 (2R,3S)-3-O-[(2R)-α-methoxy-a-(9-anthryl)acetamide]-2-methyl-7-yl-nicotine Ester (19).** 

HREIMS calcd for C27H28O5 478.1740 (M'), found 478.1745. FTIR (KBr): 3291, 2948, 1780, 1720, 1604, 1528, 1493, 1473, 1350, 1274, 1214, 1110, 1020 cm⁻¹. 

**Method 6 (2R,3S)-3-O-[(2R)-α-methoxy-a-(9-anthryl)acetamide]-2-methyl-7-yl-nicotine Ester (20).**
Determination of Absolute Configuration of Hymo (S). Onchidin B (0.5 mg) was submitted to hydrolysis with 6 M HCl (0.5 A) in a sealed tube at 110 °C for 24 h. The excess HCl was removed by passing a stream of N2, and the residue was dried under vacuum. The hydrolyzates were diluted with water (1 mL), and the hydroxy acids were extracted with diethyl ether (3 x 1 mL). The ethereal solution was dried under vacuum, and the residue was dissolved in 1 mL of ether and treated with diazomethane for 30 min. The excess of reagent was removed with a stream of dry N2. The hydroxy methyl ester was treated with (-)-(R)-a-methoxy-a-(9-anthryl)acetic acid (0.5 mg), DCC (0.5 mg), and DMAP (catalytic) in CH2Cl2 at room temperature. After 12 h the solvent was evaporated, and the residue was subjected to HPLC-MS (u-porasil, 30 cm x 7.8 mm; AcOEt:hexane 7:93; flow rate: 2.0 mL/min) to show peak at t~ 30.69 min.

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Supporting Information Available: 1H NMR, 13C NMR, COSY, TOCSY, HMQ, ROESY spectra, and expansions of HMBC spectra, MS (FABMS and FABMS/MS) spectra of 4 (12 pages). See any current masthead page for ordering and Internet access instructions.

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