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Chemical and Biological Studies of Nakiterpiosin and Nakiterpiosinone

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Abstract

Nakiterpiosin and nakiterpiosinone are two related C-nor-D-homosteroids isolated from the sponge *Terpios hoshinota* that show promise as anti-cancer agents. We have previously described the asymmetric synthesis and the revision of the relative configuration of nakiterpiosin. We now provide detailed information on the stereochemical analysis that supports our structure revision and the synthesis of the originally proposed and revised nakiterpiosin. In addition, we herein describe a refined approach for the synthesis of nakiterpiosin, the first synthesis of nakiterpiosinone, and preliminary mechanistic studies of nakiterpiosin's action in mammalian cells. Cells treated with nakiterpiosin exhibit compromised formation of the primary cilium, an organelle that functions as an assembly point for components of the Hedgehog signal transduction pathway. We provide evidence that the biological effects exhibited by nakiterpiosin are mechanistically distinct from those of well-established anti-mitotic agents such as taxol. Nakiterpiosin may be useful as an anti-cancer agent in those tumors resistant to existing anti-mitotic agents and those dependent on Hedgehog pathway responses for growth.

Introduction

Terpios is a genus of thin, encrusting sponges that harbor large amounts of symbiotic bacteria.

¹ These sponges are known to aggressively compete with corals for space by epizooism. During 1981–1985, large patches (up to 1000 m in length) of cyanobacteriosponge *Terpios hoshinota* were observed in Okinawa. They killed large populations of corals in that region and were referred to as black disease. Uemura and co-workers hypothesized that *T. hoshinota* could secrete toxic compounds and kill the covered corals. In a search for these toxins, they isolated 0.4 mg of nakiterpiosin and 0.1 mg of nakiterpiosinone from 30 kg of the sponges.

² Both compounds inhibited the growth of P388 mouse leukemia cells with IC₅₀ 10 ng/mL. The structures of these compounds were originally assigned as **3** and **4** based on NMR experiments (Figure 1). They bear a unique molecular skeleton and several unusual functional groups. To date, they are the only C-nor-D-homosteroids isolated from a marine source. We recently proposed to revise their relative configuration to that shown in **1** and **2** by analyzing the spectroscopic data of their fragments. We further synthesized **1** and **3** and confirmed that the structure of nakiterpiosin should be **1**.³ We present herein the details of our synthetic studies

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Supporting Information Available: Complete Refs. 6e and 6g, ¹H and ¹³C NMR spectra of **2**, calculation of the thermodynamic properties of **3**, details of the optimization of reaction parameters, experimental procedures, crystallographic data, and characterization data. This material is available free of charge via the Internet at <http://pubs.acs.org>.

and spectroscopic analysis of nakiterpiosin (**1**) and 6,20,25-*epi*-nakiterpiosin (**3**). In addition, we report in this article an improved synthesis of **1**, the first synthesis of nakiterpiosinone (**2**) and the results of our preliminary biological studies of **1**.

C-nor-D-Homosteroids are skeletally rearranged steroids with their C-ring contracted and D-ring expanded by one carbon. The first known, and arguably the best known, members of this family of natural products are cyclopamine (**5**) and veratramine (**6**). The discovery of these veratrum alkaloids is a rather interesting story.⁴ Ranchers in the Rocky Mountain region had long been puzzled by a mysterious birth defect in their sheep. They found that 1–20% of the lambs were born as “chattos (monkey faces)” when the mother sheep grazed in the National Forests in central Idaho during the summer. The Poisonous Plant Research Laboratory of the US Department of Agriculture was contacted in 1954 to investigate this “malformed lamb disease”. After 11 years of work, they found that ewes that grazed on corn lily (*Veratrum californicum*) on the *fourteenth* day of gestation would give birth to cyclopic lambs, while the ewes were left unaffected. They further found that **5** was responsible for the one-eye face malformation and **6** led to leg deformity.^{4a} However, it was not until 30 years later that the molecular target of **5** was identified to be Smoothened (Smo).⁵ Suppression of Hedgehog (Hh) signaling by inhibiting Smo with small molecules has since been pursued as a new strategy for cancer treatment.⁶ The semi-synthesis of **5** and **6** was first accomplished by Masamune and Johnson in 1967,^{7a–e} and recently by Giannis.^{7g} A formal synthesis was also reported by Kutney in 1975.^{7f}

Revision of the relative stereochemistry

The unique molecular structures and strong P388 growth inhibition activity of nakiterpiosin (**1**) and nakiterpiosinone (**2**) prompted us to initiate a research program to explore their laboratory synthesis⁸ and biological functions. At the onset of this project, we noticed that the C-20 and C-25 configurations of the originally proposed structures for nakiterpiosin and nakiterpiosinone (i.e., **3** and **4**)² were opposite to those of cyclopamine (**5**), veratramine (**6**) (Figure 1) and other “normal” steroids. In addition, the C-6 and C-20 configuration of **3** and **4** could not be easily rationalized based on biogenesis analysis.¹⁰ We further considered that the reported NOE data of the natural products² provided little support to the proposed stereochemistry. Most importantly, the ¹H NMR spectra of our synthetic intermediates toward **3** displayed significantly different H-6 and H-21 splitting patterns as compared to the natural products. We therefore decided to first study the relative stereochemistry of nakiterpiosin and nakiterpiosinone using model systems.

In order to probe the C-6 stereochemistry of the natural products, we synthesized a series of compounds (**7–10**)¹¹ that bear the A,B,C-ring systems of nakiterpiosin and nakiterpiosinone. We found that the $J_{H_6-H_7}$ of **7** and **9**, which bear the same C-6 configuration as nakiterpiosin and nakiterpiosinone, was very different from that of the natural products (Table 1).¹² On the other hand, we found that the corresponding C-6 epimers (**8** and **10**) and the natural products exhibited similar H-6 splitting patterns. We therefore concluded that the C-6 stereocenter of nakiterpiosin and nakiterpiosinone should be (*R*)-configured.

As to the stereochemistry of the side chain of nakiterpiosin and nakiterpiosinone, we first focused on the C-20/C-22/C-23 relative configuration and synthesized all four possible diastereomers (**11–14**)¹¹ to compare their NMR spectra with those of the natural products. We found that the $^3J_{H-H}$ coupling constants and ¹H and ¹³C chemical shifts of **11** (syn-syn), **12** (syn-anti) and **13** (anti-syn) were considerably different from those of the natural products (Figure 2). In particular, the $J_{H_{20}-H_{21}}$ of **11** (syn-syn) and **12** (syn-anti) indicated a *gauche* instead of an anti H-20/H-21 conformation. Only **14** (anti-anti) exhibited similar ¹H and ¹³C

NMR spectra compared to those of the natural products.¹³ We therefore revised the C-20 stereochemistry of nakiterpiosin and nakiterpiosinone to be (*S*).

After revising the C-20 stereochemistry, we turned our attention to the C-25 configuration of nakiterpiosin and nakiterpiosinone, synthesizing two model substrates (**15** and **16**).¹¹ We found that the coupling constants and ¹H and ¹³C NMR chemical shifts of (25*S*)-**16** (anti-anti-trans) but not (25*R*)-**15** (anti-anti-cis) matched well with those of the natural products (Table 2 and Figure 3). In particular, the *J*_{H23–H24a}, *J*_{H23–H24b} and *J*_{H23–H25} of **15** and those of the natural products were significantly different. We therefore determined that the C-25 stereocenter of nakiterpiosin and nakiterpiosinone should be (*S*)-configured.

The revised structures of nakiterpiosin and nakiterpiosinone (**1** and **2**) share the same configurations at the C-20 and C-25 positions with **5** and **6**. The C-6 and C-20 configurations are also consistent with the stereochemical outcomes of enzymatic halogenation of the steroid (Figure 4).⁹ The C-21 chlorine atoms of nakiterpiosin are likely introduced by non-heme iron halogenase through radical chlorination. It is expected that this reaction would lead to retention of the C-20 configuration. On the other hand, the C-6 bromine atom is likely introduced by vanadium-dependent bromoperoxidase through bromoetherification. It is therefore expected that the C-5,6 bromohydrin would exist in an anti stereochemical relationship.

Taken together, we proposed to revise the relative stereochemistry of nakiterpiosin and nakiterpiosinone to **1** and **2**. Indeed, we found that the ¹H and ¹³C NMR spectra of our synthetic sample of **1** agreed with those of the natural product. In contrast, those of synthetic **3** and the natural product are significantly different. We thus revised the relative stereochemistry of nakiterpiosin to be that indicated in **1** and **2**, which shares the same configuration at the C-20 and C-25 positions with cyclopamine (**5**) and veratramine (**6**).

Synthetic plans

After determining the stereochemistry of nakiterpiosin and nakiterpiosinone to be **1** and **2**, we sought to develop a general strategy to target these two steroid derivatives. Our first-generation approach³ is outlined in Figure 5. We opted to construct the central cyclopentanone ring at a late-stage for convergency reasons. We envisioned that a carbonylative cross-coupling reaction^{14–20} together with a Nazarov cyclization reaction^{21–23} could be used to effectively assemble this cyclopentanone ring. We recognized that the Nazarov cyclization of vinyl aryl ketone **17** involved a disruption of the aromaticity and therefore required significantly higher activation energy than that of the divinyl systems.²² However, we believed that a suitable set of reaction conditions could be found to realize this highly efficient plan. We also expected that **17** could be used as a versatile intermediate for synthesizing not only **1** but also **2**.

It should be noted that the carbonylative cross-coupling of **18** and **19** is also considerably challenging because of their sensitive functionalities and congested reaction sites. Compared to the recent advancement in transition metal-catalyzed cross-coupling,^{24,25} catalytic carbonylative cross-coupling is relatively under-developed.¹⁵ Suppression of the direct coupling reactions remains challenging when the more nucleophilic coupling components are used. In general, the Stille and Suzuki carbonylative coupling reactions are more successful because of the slower rate of transmetalation. We later found that the nearly neutral conditions of the Stille carbonylative reaction are crucial to the successful implementation of this strategy to the coupling of highly acid- and base-sensitive **18** and **19**.

For the synthesis of the coupling fragments, we envisioned that the electrophilic coupling component **17** could be synthesized by an intramolecular Diels–Alder reaction;^{26,27} and the nucleophilic coupling component **18** by a vinylogous Mukaiyama aldol reaction.^{28,29} It should be noted that the intramolecular Diels–Alder reaction of furan derivatives is normally exo-

selective and much less kinetically and thermodynamically favored because of the aromaticity of furan and the ring strain of the product.^{26b} The exo selectivity should lead to the desired relative stereochemistry between the oxo bridge and angular methyl group. We planned to use the C-6 stereogenic center to control the diastereoselectivity of this reaction. The use of a substituent group on the tether α to the diene to control the diastereoselectivity was first studied by Roush, Taber and Boeckman.²⁷ Since the C-6 bromine atom resides at the axial position, we anticipated that introduction of the bromine atom to the cycloaddition product with an inversion of configuration would setup the correct C-6 configuration.

In terms of the vinylogous Mukaiyama aldol reaction, we planned to use the C-20 stereocenter to control the installation of the C-22 and C-23 stereocenters. We were able to use this versatile approach to obtain a diverse array of C-20/22/23 diastereomers (**11**–**16**: syn-syn, syn-anti, anti-syn, anti-anti) for the previously described model studies.¹¹ The absolute stereochemistry of **18** and **19** were established by the Noyori reduction³⁰ and the Sharpless epoxidation³¹ reactions.

The first generation synthesis of nakiterpiosin

We previously reported our first synthetic approach to **1**.³ This sequence commenced with a Friedel–Craft acylation of furan with succinic anhydride³² and the formation of Weinreb amide **23** from acid **22**. Subsequently, a Noyori reduction³⁰ was used to set the C-6 stereochemistry. While a significant amount of dehydrated lactone by-product was formed under the conventional NEt_3 – HCOOH azeotropic conditions,^{30a,b} we found that the in-water protocol developed by Xiao^{30c–e} provided significant enhancement of the reaction rate and allowed low catalyst loading. Alcohol **24** was obtained in 91% ee without the formation of the undesired lactone. The dienophile was then introduced to **24** by addition of isopropenyl Grignard reagent, giving enone **25**, setting the stage for the Diels–Alder cyclization.

It has been shown that the conversion of the intramolecular furan Diels–Alder reaction is highly dependent on the relative basicity of the reactant and product, and the stoichiometry of the Lewis acids.^{26b} We found that, for the reaction of **25**, 2.5 equiv of Me_2AlCl provided the best yield of **26** (Table 3), indicating that the reaction was driven by the complexation of Me_2AlCl to **26**. The reaction proceeded with good stereochemical control and gave only the desired exo product with an equatorial hydroxyl group. When the C-6 hydroxyl group of **25** was protected as a TES ether, the reaction proceeded with the opposite enantiomeric preference and provided the exo product with an axial TES ether group.¹¹

Since the cycloaddition product **26** was not stable and underwent a retro-Diels–Alder reaction upon gentle heating or exposure to other Lewis acids, we dihydroxylated the olefin before the introduction of the bromine atom to prevent the retro-Diels–Alder reaction. The steric congestion around the C-6 position necessitated the use of an electron-deficient aryl sulfonate group (**27**) for 6-OH activation. Thus, treatment of **28** with lithium bromide in warm acetone allowed the introduction of the bromine atom with inversion of configuration and concomitant acetonide protection to give **29**. The use of an electron-deficient sulfonate was crucial to the success of this $\text{S}_{\text{N}}2$ reaction. 4-Toluenesulfonate (Ts) was unreactive and 4-nitrophenylsulfonate (Ns) was less reactive under these reaction conditions. The absolute and relative configurations of **29** were confirmed by X-ray analysis.¹¹ Finally, enol triflate installation completed the synthesis of the electrophilic coupling component **30**.

The synthesis of the nucleophilic coupling component started with reduction of acid **31** to aldehyde **33** followed by Horner–Wadsworth–Emmons reaction and reduction of the resulting ester to afford **35**. Asymmetric installation of the C-20 stereocenter was achieved by Sharpless epoxidation with 92% ee.³¹ The resulting alcohol was protected as a TBS ether and then subjected to the pinacol-type rearrangement using Yamamoto's conditions.³³ Previously, we

reported that aldehyde **38** was obtained with significant erosion of enantiomeric purity (71% ee).³ We have since determined that the loss of ee occurred during workup of the reaction and purification of **38** by column chromatography. Quenching the reaction with 1 N HCl aqueous solution instead of sodium fluoride afforded **38** with 90% ee. Aldehyde **38** should be used directly without purification. We have also found that while low conversions were observed when catalytic amounts of the Yamamoto's aluminum salt were used for the reaction of **37**, only 5 mol % of Cr(TPP)(OTf)^{33e} was needed to drive this reaction to completion. However, a significant amount of ketone was also obtained (aldehyde:ketone=3:1).

The previously reported problem of the subsequent vinylous Mukaiyama aldol reaction has also been solved. The bismuth(III) triflate-catalyzed reaction between purified **38** (71% ee) and 3-methyl-2-(triisopropoxy)furan yielded **39** with only 56% conversion, 11:1 dr and 60% ee.³ We recently found that two equivalents of tin(II) triflate promoted this reaction with 83% conversion. Alcohol **39** was obtained from crude **38** as the only diastereomer in 80% isolated yield and 90% ee. For large scale reactions, we found that boron trifluoride diethyl etherate was a more economical Lewis acid. Full conversion could be obtained and no loss of ee was observed. It provided **39** in comparable yields despite having a diastereoselectivity of only 4:1. Results from the survey of fourteen different Lewis acids can be found in the Supporting Information.

We previously used the Crabtree catalyst^{29b,34} to direct the hydrogenation of **39** and set the desired C-25 configuration of **40** with moderate diastereoselectivity (dr 6:1). We later found that **40** could be obtained as the only diastereomer by conjugate reduction with L-Selectride. Overall, these new protocols are much more cost-effective and provide significantly improved yield and stereoselectivity of the desired products.

To invert the C-22 configuration, we oxidized **40** with Dess–Martin periodinane, and reduced the resulting ketone with sodium borohydride. We then protected the C-22 alcohol as a TBS ether, selectively removed the C-21 TBS protecting group and oxidized the primary alcohol. Introduction of the C-21 *gem*-dichloride was achieved by reacting aldehyde **45** with Cl₂/P(OPh)₃.³⁵ The hydrazone method developed by Myers³⁶ was not compatible with the other functional groups of **45**. The relative and absolute stereochemistry of **46** was determined by single crystal X-ray analysis after TBS deprotection.¹¹ The synthesis of the nucleophilic coupling component **47** was concluded by a palladium-catalyzed stannylation of **46**. Despite of the high catalyst loading and moderate yield, this protocol continues to be the best conditions for the introduction of the trimethylstannane to **47**.

After establishing the routes to both cross-coupling fragments, our next goal was to realize the carbonylative coupling and Nazarov cyclization strategy to assemble the C-ring of **1** and **2**. We used several model systems carrying a simplified side-chain on the E-ring to study these two key reactions. We found that the carbonylative coupling of **30** and **48** could be achieved with a modified Stille's protocol to afford **49**.¹⁶ After an extensive survey of the reaction conditions, we conclude that Pd(PPh₃)₄ is the best promoter, copper(I) chloride additive³⁷ is crucial and DMSO is the ideal solvent for this reaction. The use of catalytic amounts of Pd(PPh₃)₄ gave only small amounts of the desired product. No reaction occurred when using Pd(OAc)₂ in combination with AsPh₃, P(furyl)₃, PCy₃ and P(*t*-Bu)₃ under otherwise identical conditions. The use of Pd(OAc)₂/P(OMe)₃ led mainly to decomposition. It is also worth noting that **49** was highly sensitive to both acids and bases. Addition of lithium chloride, prolonged heating and employment of base-promoted carbonylative Suzuki coupling protocols led to the elimination of the bromine atom.

The sensitive nature and aromaticity of **49** made the implementation of the Nazarov cyclization strategy challenging. Normally, the Lewis acid-promoted Nazarov cyclization of aryl vinyl

ketones requires harsh reaction conditions or activated substrates.²² Indeed, exposure of **49** to various Lewis acids only resulted in substrate decomposition. However, we found that irradiation of a solution of **49** in acetonitrile at 350 nm readily delivered the desired annulation product **50** in its enol form, which tautomerized to the ketone form as a 1:1 mixture of C-9 diastereomers upon addition of ammonium chloride. Treating this mixture with diisopropylamine in warm methanol gave the desired diastereomer **50**, the structure of which was confirmed by X-ray analysis.¹¹ The reaction can also be carried out in methanol to directly give the C-9 diastereomeric mixture of **50** with slightly lower yield. The photo-Nazarov cyclization reaction of aryl vinyl ketones was first reported by Smith and Agosta.^{23c} Subsequent mechanistic studies by Leitich and Schaffner revealed the reaction mechanism to be a thermal electrocyclozation induced by photolytic enone isomerization.^{23d,e} The mildness of the reaction conditions and the selective activation of the enone functional group was key to the success of this reaction.

To complete the synthesis of **1**, we carried out the carbonylative coupling of **30** and **47** under the optimized conditions to give **51**, which was effectively transformed to **53** upon photolysis and C-9 epimerization (Scheme 4). The previous stereochemical issues of **51** are now solved (99.5% ee and dr 10:1) because **47** could be prepared with high enantiomeric purity.

With the central C-ring in place, the remaining task was to assemble the A-ring of **1**. We first removed the acetone protecting group of **53** and cleaved the diol of **54** to afford bis-hemiacetal **55**. Selective reduction of the less hindered hemiacetal of **55** followed by TBS deprotection³⁸ concluded the synthesis of **1**. A single crystal suitable for X-ray analysis¹¹ was recently obtained by evaporating the solvent from a 30% ethyl acetate/hexanes solution of **1**, allowing unambiguous determination of its relative and absolute configuration.

To confirm our proposal for the structure revision of **1**, we compared the ¹H and ¹³C NMR spectra of our synthetic **1** with those of the natural product.² We were pleased to find that the difference in chemical shift for all peaks was within 0.03 ppm for the ¹H and 0.2 ppm for the ¹³C signals, and the splitting pattern for all ¹H signals was also consistent with those of the natural product. While the optical rotation of the natural product was not reported, the Mosher esters analysis of our synthetic **1** was consistent with the reported data of the natural product. The $\Delta\delta^{SR}$ of H-4 was found to be +0.03 ppm in the 4-Mosher ester of **1**, compared to +0.04 ppm for that of H-4 in the 4,22-di-Mosher ester of the natural product. We have also determined that nakiterpiosin (**1**) was levorotatory in methanol with $[\alpha]_D^{20} -33^\circ$ (*c* 0.067, MeOH).

The second generation synthesis of nakiterpiosin

While the sequence shown in Scheme 4 successfully delivered **1**, it required a further six steps after the convergent fragment coupling reaction. We anticipated that all the functional groups of **1** could tolerate the carbonylative coupling and photo-Nazarov cyclization reactions. We therefore decided to perform the C-ring construction on a fully elaborated system to improve the overall efficiency of the synthesis of **1** (Scheme 5).

Taking ketone **29**, we introduced the A-ring functionality using the chemistry previously described to generate **10**. We then protected the hemi-acetal and installed the triflate to give **57**. We also removed the TBS protecting group of **47** prior to the carbonylative coupling. Pleasingly, the fragment coupling and photo-Nazarov cyclization proceeded in good yields. Subsequent TES deprotection provided **1** with significantly improved efficiency. However, it was unfortunate that photolysis of des-TES-**58** resulted in significant amounts of decomposition.

Synthesis of 6,20,25-*epi*-nakiterpiosin

To unambiguously determine the relative stereochemistry of nakiterpiosin, we also synthesized its originally proposed structure (6,20,25-*epi*-nakiterpiosin, **3**) and compared the NMR spectra of **3** with the natural product. Several approaches were explored in an attempt to introduce the C-6 bromine atom. We first sought to use the C-6 bromine atom as the stereochemical controlling element for the intramolecular Diels–Alder reaction and set its equatorial configuration. However, nucleophilic substitution of the C-6 hydroxyl group of **24** under various conditions failed to deliver the desired product. The use of a chelating leaving group³⁹ did not result in retention of configuration and an S_N2 instead of S_Ni reaction occurred. Equally fruitless was the effort to replace the hydroxyl group of **26** or its derivatives with a bromine atom through radical chemistry. Eventually, we found that the desired C-6 configuration could be set through isomerization of vinyl bromide **62** (Scheme 6).

Starting from **26**, the olefin was first dihydroxylated and the resulting diol was protected as an acetonide. The C-6 hydroxyl group of **59** was then protected and the ketone group reduced to afford **60**. Subsequent TBS protection, TES deprotection and alcohol oxidation provided **61**. The vinyl bromide was then introduced by the Barton–Myers method to give **62**.^{36,40} Removal of the TBS protecting group and alcohol oxidation under Dess–Martin conditions induced olefin isomerization to yield enone **63**. Finally, conjugate reduction yielded a crystalline ketone, allowing unambiguous assignment of the C-6 configuration.¹¹ Conversion of the ketone to enol triflate furnished the electrophilic coupling component **64** (6-*epi*-**30**).

To set the syn-anti-cis configuration of the side-chain, an anti selective vinylogous aldol reaction was needed. Despite the fact that efforts to reverse the syn selectivity failed, we found that a useful amount of the desired diastereomer could be obtained with Lewis acids boron trifluoride diethyl etherate, zirconium(IV) chloride, ytterbium(III) triflate or scandium(III) triflate.¹¹ Reaction of *ent*-**38** and 3-methyl-2-(triisopropoxy)furan gave syn-anti-**65** as the minor diastereomer (syn-syn:syn-anti=4:1). The C-25 configuration was then set by catalytic hydrogenation using Adams' catalyst without debromination. Protection of the alcohol as a TBS ether gave the desired syn-anti-cis-**66**.

Following the protocols previously described for the synthesis of **47**, the *gem*-dichloromethyl and stannyl groups were then introduced to provide **68**. The relative stereochemistry of **68** was confirmed by X-ray analysis on a racemic sample after removal of the TBS protecting group.¹¹ Its carbonylative coupling with **64** and the subsequent photolysis reaction proceeded uneventfully to yield **70**. Interestingly, cleavage of the diol gave **71** as a bis-aldehyde instead of bis-acetonide. Nevertheless, reduction of **71** with Et₃SiH in the presence of BF₃•OEt₂ induced the acetal formation. After TBS deprotection, 6,20,25-*epi*-nakiterpiosin (**3**) was obtained as an equilibrium mixture of the C-4 aldehyde and hemiacetal forms. Through variable-temperature NMR experiments, we determined the Δ*H* and Δ*S* of this equilibrium to be –2.5 kcal/mol and –6.0 cal/mol respectively.¹¹ By comparing the ¹H and ¹³C NMR spectra of **1**, **3** and the natural product,² we confirmed that the structure of nakiterpiosin should be **1** instead of **3** (Tables 5, Figures 6 and Figure 7).

Synthesis of nakiterpiosinone

After completion of the synthesis of nakiterpiosin (**1**), we turned our attention to the related steroid nakiterpiosinone (**2**). Initially, we planned to selectively oxidize the C-4 hydroxyl group of diol **28** or **54** to accomplish this goal. However, we encountered considerable difficulties in utilizing this strategy to synthesize **2**. First, despite being able to selectively protect the C-3 hydroxyl group and oxidize the C-4 hydroxyl group of **28**, we were not able to perform C-3 epimerization without considerable decomposition. Next, our attempts to introduce a C-3 hydroxyl group to the more hindered β-face of **26** by Prévost–Woodward dihydroxylation⁴¹

was complicated by the Wagner-Meerwein rearrangement of the [2.2.1] bicyclic system (Scheme 8).^{42,43} Equally unsuccessful was the oxidation of diol **28** to diketone due to diol cleavage or decomposition.⁴⁴ We therefore sought to install the C-3 stereocenter by an alternative approach.

Since the β -face of the A-ring of **2** is significantly less hindered than its α -face, we decided to set the desired C-3 stereochemistry by reduction of the corresponding ketone (Scheme 9). Starting from **27**, the enol triflate was first installed and the olefin of **74** was hydroborylated with 9-BBN to afford alcohol **75** as the only regioisomer after oxidative cleavage of the borane. Subsequently, the alcohol was oxidized to the ketone and the bromine atom was introduced. The nucleophilic bromination reaction of **76** was much more difficult than **28**. Nevertheless, **77** could be obtained in good yield when reacting **76** with lithium bromide in 2-heptanone at 120 °C.

We next found α -oxidation of ketone **77** to be particularly challenging. Oxidation by the Rubottom method, Davis reagent or selenium(IV) oxide was unsuccessful.⁴⁵ However, reaction of **77** with $\text{PhI}(\text{OAc})_2$ under basic conditions gave **78** in good yield.⁴⁶ While oxidation of the C-4 ketone regioisomer of **77** may deliver the desired protected hydroxyketone directly, we found that hydroborylation of **74** with the complementary regioselectivity did not proceed with good efficiency. Deprotection of the dimethyl acetal of **78**, and subsequent reduction of the resulting ketone (**79**) gave cis-diol **80** with the desired C-3 configuration. The structure of **80** was confirmed by analyzing its acetonide derivative by X-ray. Selective protection of the C-3 hydroxyl group of **80** afforded the electrophilic coupling component **81**. Carbonylative coupling of **81** with **47** and the following photo-Nazarov cyclization proceeded smoothly to give **83**. Interestingly, ketone **83** with the desired 9*S* configuration was obtained directly as the only diastereomer. Finally, oxidation of the hydroxyl group and removal of the TES and TBS protecting groups concluded the synthesis of **2**. The ¹H NMR spectrum of **2** agreed with that of the natural product,^{2b,11} confirming the structure of nakiterpiosinone to be **2**.

Biological studies of nakiterpiosin

Uemura and co-workers initially reported that nakiterpiosin (**1**) and nakiterpiosinone (**2**) inhibited the growth of P388 mouse leukemia cells with an IC_{50} of 10 ng/mL.² However, the limited amount of material that can be isolated from the sponge has hampered further efforts to investigate the anti-cancer potential of these compounds. Having resolved the re-supply issue through total synthesis, we are now pursuing further mechanistic studies focused on the anti-cell proliferative properties associated with **1**.

The steroid skeleton of **1** is related to that of cyclopamine (**5**), a potent Hedgehog (Hh) pathway antagonist. Cyclopamine (**5**) inhibits Hh signaling by influencing the movement of Smoothened (Smo), a seven-pass transmembrane protein, in and out of the primary cilium.^{5d,e} The primary cilium is a microtubule-based assembly point organelle for Hh pathway components.⁴⁷ Indeed, **1** suppressed Hh signaling at sub-micromolar concentrations in NIH3T3 mouse fibroblasts (Figure 8A). Surprisingly, as part of our efforts to evaluate the effects of **1** on the movement of Smo with respect to the primary cilium, we observed that cell populations treated with nakiterpiosin show a loss of the primary cilium (Figure 8B).

Because the formation and retraction of the primary cilium is cell cycle- and microtubule-dependent,⁴⁸ we suspected **1** to be an anti-mitotic agent. Indeed, flow cytometry experiments suggested that **1** induces cell cycle arrest in the G2/M-phase (Figure 9). We first determined the cell growth IC_{50} of **1** in HeLa cells to be 375 nM. Subsequently, we incubated the HeLa cells with **1** at this concentration for 16 h and found the population of cells in the G2/M phase to be significantly increased as compared to the DMSO control. Since the most common mechanism for G2/M arrest is the targeting of microtubule assembly, and it was reported that

JK184, a microtubule depolymerizing agent,⁴⁹ effectively regulates Hh signaling, we next examined the effect of **1** on microtubule dynamics. However, we found that, in contrast to taxol and nocodazole, tubulin polymerization was not influenced in the presence of 5 μ M of **1** in an in vitro assay (Figure 10). Taken together, these results suggested that **1** may be useful in tumors resistant to existing anti-mitotic chemotherapeutic agents. Lastly, given the frequency of mutations in Smo that render it resistant to Hh pathway antagonists currently under clinical development,^{5c} considerable effort is being devoted to the identification of small molecules that act upstream or downstream of Smo.⁵⁰ The ability of **1** to block Hh pathway responses suggests that it may be particularly useful against Hh-dependent tumors.

Summary

Through extensive spectroscopic analysis and convergent total synthesis, we have established the stereochemistry of nakiterpiosin (**1**) and nakiterpiosinone (**2**). Our synthetic route provides **1** in 21 steps with 5% overall yield along the longest linear sequence. Our preliminary biological studies show that **1** is anti-mitotic and has functions distinct from those of other well-established anti-mitotic agents such as taxol. Nakiterpiosin may be an alternative chemotherapeutic against tumors resistant to anti-tubulin agents and dependent on Hh signaling.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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12. The chemical shift of H-6 in **7–10** and the residual H₂O are too close in CD₃OD for the accurate measurement of the coupling constants. We therefore recorded their ¹H NMR spectra in CDCl₃. We reasoned that the rigid polycyclic ring systems of **7–10** will adopt similar conformations as the natural products even in different solvents. Therefore, the coupling constants should not be significantly influenced by the NMR solvents.
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Figure 1.
The structures of C-nor-D-homosteroids 1–6.

Figure 2.
Probing the C-20/C-22/C-23 configurations of nakiterpiosin and nakiterpiosinone.

Figure 3.
Probing the C-25 configurations of nakiterpiosin and nakiterpiosinone.

Figure 4.
Biogenetic analysis of nakiterpiosin.

Figure 5.
The retrosynthetic analysis of nakiterpiosin.

Scheme 1.

Synthesis of the electrophilic coupling component **30**.

Scheme 2.

Synthesis of the nucleophilic coupling component 47.

Scheme 3.

Model studies for the carbonylative coupling/Nazarov cyclization strategy.

Scheme 4.
Completion of the synthesis of nakiterpiosin (1).

Scheme 5.
An improved synthesis of nakiterpiosin (**1**).

Scheme 6.
Synthesis of **64** (6-*epi*-**30**).

Scheme 7.
Synthesis of the 6,20,25-*epi*-nakiterpiosin (**3**).

Figure 6. Difference in the chemical shifts of the ^1H NMR spectra of **1**, **3** and the natural product.

Figure 7.
Difference in the chemical shifts of the ^{13}C NMR spectra of **1**, **3** and the natural product.

Scheme 8.
Rearrangement of the [2.2.1] bicyclic system.

Scheme 9.
Synthesis of nakiterpiosinone (2).

Figure 8.

Effects of nakiterpiosin on Hh signaling and primary cilia. (A) The Hh pathway response of Light2 cells (NIH3T3 cells with a Hh-specific firefly luciferase reporter) in the presence of nakiterpiosin. (B) NIH3T3 cells treated with nakiterpiosin showing a compromised ability to form primary cilia, as detected by anti-acetylated tubulin antibody.

Figure 9.
The DNA profiles of nakiterpiosin treated HeLa cells.

Figure 10.
The in vitro tubulin polymerization assays.

Table 1

Nakiterpiosin and Nakiterpiosinone.

itosinone

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2.8 Hz

5.5 Hz

2.8 Hz

5.6 Hz

Hz

Table 2

Studies of the C-20/C-22/C-23 configurations of nakiterpiosin and nakiterpiosinone.

	Nakiterpiosin	Nakiterpiosinone
<p>15 (anti-anti-cis)</p>			
$J_{H20-H21}$	10.3 Hz	10.1 Hz	10.2 Hz
$J_{H23H-24a}$	8.2 Hz	8.2 Hz	10.0 Hz
$J_{H23-H24b}$	3.7 Hz	3.7 Hz	5.8 Hz
$J_{H24aH-25}$	8.4 Hz	8.2 Hz	12.1 Hz
$J_{H24bH-25}$	N/A	9.2 Hz	8.8 Hz

Table 3Influence of the Lewis acid on the intramolecular Diels–Alder reaction of **25**.

Entry	Lewis Acid	Equivalent	25:26	Note
1	Me ₂ AlCl	2.5	0:100	
2	Me ₂ AlCl	1.0	30:70	
3	Me ₂ AlCl	0.1	100:0	
4	MeAlCl ₂	1.0	15:85	with decomposition
5	MeAlCl ₂	0.1	100:0	

Table 5

Selected ^1H NMR chemical shifts and coupling constants of nakiterpiosin.

	natural sample [*]	synthetic 1 [†]	synthetic 3 [‡]	natural sample [*]	synthetic 1 [†]	synthetic 3 [‡]
6	4.70 (dd) $J = 2.7, 1.4$ Hz	4.71 (dd) $J = 3.4, 2.5$ Hz	4.74 (dd) $J = 12.3, 5.1$ Hz	21	6.32 (d) $J = 10.3$ Hz	6.66 (d) $J = 3.6$ Hz
7a	2.28 (m)	2.29 (ddd) $J = 13.7, 12.7, 3.4$ Hz	2.22 (ddd) $J = 12.3, 12.0, 12.0$ Hz	22	4.41 (dd) $J = 7.9, 3.2$ Hz	4.52 (dd) $J = 10.2, 2.3$ Hz
7b	2.74 (ddd) $J = 13.4, 2.7, 1.4$ Hz	2.75 (ddd) $J = 13.7, 2.5, 2.3$ Hz	2.94 (ddd) $J = 12.0, 5.1, 2.7$ Hz	24a	1.71 (ddd) $J = 13.2, 8.2, 8.1$ Hz	2.00 (m)
8	3.58 (m)	3.55 (ddd) $J = 12.7, 9.4, 2.3$ Hz	3.03 (ddd) $J = 12.0, 9.1, 2.7$ Hz	24b	2.29 (ddd) $J = 13.2, 4.8, 3.9$ Hz	2.00 (m)

^{*} Recorded at 800 MHz as reported by Uemura et al. (Ref 2).

[†] Recorded at 600 MHz.

[‡] Recorded at 500 MHz.