Trisoxazole Macrolides from *Hexabranchus* Nudibranchs and Other Marine Invertebrates

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Abstract. Trisoxazole macrolides are cytotoxic and antifungal metabolites initially isolated from the egg-ribbons of the *Hexabranchus* nudibranch and later found in other marine invertebrates. They possess a characteristic macrolide portion, in which three contiguous oxazole units are integrated, and a side-chain with an *N*-methyl-vinylformamide terminus. The planar structures of the first members of this group, ulapualides and kabiramide C, were determined by interpretation of spectral data in conjunction with chemical degradation. Following these studies, the structures of approximately 35 congeners have been reported, including mycalolides from a marine sponge *Mycale* sp. The absolute stereochemistry of mycalolides was determined by chemical methods. Trisoxazole macrolides depolymerize F-actin and form a 1:1 complex with G-actin, thereby exhibiting potent toxicity toward eukaryotic cells. X-ray crystallography established the mode of binding of some of the members to G-actin and their absolute stereochemistry.

11.1 Introduction

The observations that shell-less gastropods are immune to predation by their surrounding carnivores led to a hypothesis that they are chemically defended. Succeeding studies disclosed diverse protection strategies: acid secretion, displaying cnidocytes obtained from the cnidarian prey, and protection by repugnant organic compounds which are acquired from their prey, such as algae or sponges, or biosynthesized de novo (Faulkner and Ghiselin 1983; Faulkner 1988; Avila 1995; Cimino and Ghiselin 1999; Cimino et al. 1999, 2001). Dorid nudibranchs procure defensive chemicals from sponges on which they prey. *Hexabranchus sanguineus* is a dorid nudibranch distributed commonly in the Indo-Pacific area and well known for its large size (up to 60 cm), brilliant color, and swimming behavior (Gohar and Soliman 1963; Thompson 1972; Francis 1980; Wägele and Willan 2000; Valdés 2002). More conspicuous in the reef are their bright red egg-ribbons, prominently deposited on rocks or coral debris.

In the antimicrobial screening of marine invertebrates, we found that the extract of the egg-ribbons deposited by *Hexabranchus* and collected at Kabira Bay on Ishigaki Island in the Ryukyu Islands exhibited prominent antifungal but no antibacterial activity; and we started to study the active

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constituents (Matsunaga et al. 1986). Simultaneously, studies on the biologically active constituents from the *Hexabranchus* nudibranch and its egg-ribbons from Oahu and those from the Kwajalein Atoll were in progress (Roesner and Scheuer 1986; Kernan et al. 1988). Also at the same time, *Halichondria* sponges from Kwajalein and Palau were shown to contain metabolites found in *H. sanguineus* (Kernan and Faulkner 1987; Andersen et al. 2004), which was followed by the isolation of further congeners from sponges (Fusetani et al. 1989). The biologically active constituents contained in *H. sanguineus* were a series of trisoxazole macrolides with an unprecedented structure with no counterpart in the natural products from terrestrial organisms.

11.2 Isolation and Structure Elucidation of Trisoxazole Macrolides from Marine Invertebrates

At the time of the discovery, the structures of trisoxazole macrolides were unique, especially the presence of three contiguous oxazoles in a macrolide ring and the side-chain terminating with *N*-methyl-vinylformamide. In order to confirm this novel structure, we had to detect small ^{1}H–^{13}C long-range couplings through time-consuming ^{13}C NMR spectroscopy (Matsunaga et al. 1986) and Professor Scheuer's group had to isolate a small amount of an oxidation product (Roesner and Scheuer 1986). The side-chain portions of this class of molecules were similar to the one in tolytoxin, which had been isolated from a marine blue-green alga and for which only a partial structure was reported in a review (Moore 1981). Because of the similarity, a close relationship between trisoxazole macrolides and the tolytoxin class of metabolites was speculated (Ishibashi et al. 1986). After the structure elucidation of ulapualides and kabiramides, important NMR techniques such as inverse-detection and pulse field gradient were introduced. These techniques and other advances in instrumental analysis significantly decreased the sample size and time-frame required for structure elucidation and made the structure elucidation of complex molecules less laborious.

In this section, the structure elucidation of trisoxazole macrolides is summarized.

11.2.1 Ulapualides

Ulapualides were isolated from the egg-ribbons of *H. sanguineus* collected at Pupukea, Oahu (Roesner and Scheuer 1986). The extract of the egg-ribbons was subjected to solvent partitioning, followed by reversed-phase

HPLC and silica HPLC to furnish ulapualides A (**1**) and B (**2**; Fig. 11.1). The structural study was mainly conducted on ulapualide B. The molecular formula was determined by high-resolution fast atom bombardment mass spectrometry, together with elemental analysis. Partial structures V, W, X, and Y were assigned on the basis of 2D-NMR data such as COSY, CH-COSY, and long-range CH-COSY together with difference decoupling experiments in ^{1}H NMR. Restricted rotation about the amide bond in the *N*-methyl-vinylformamide moiety gave rise to doubled ^{1}H and ^{13}C NMR signals in the vicinity of this group. The most difficult part of the structure elucidation was the assignment of the trisoxazole moiety, whose presence was suggested by the ^{13}C NMR and ^{15}N NMR data (δ_N231.9, 222.5, 213.7). Because the orientation of the central oxazole ring was not assignable by spectroscopic methods, ulapualide B was subjected to ozonolysis, followed by a reductive work-up and acetylation. An aromatic compound (**3**; Fig. 11.1), analyzed for $C_{10}H_9N_3O_5$ and structurally assigned on the basis of ^{1}H NMR data, was isolated from a complex mixture of reaction products. The structure implied that the compound arose from cleavage of ring C and the Δ^{19}-olefin. Compound **3** was converted into a nonsymmetrical bis-oxazole (**4**). If the orientation of the central oxazole ring is reversed, the product should be symmetrical. Because **4** exhibited a pair of oxazole protons, the orientation of the central oxazole unit was determined as shown. Ulapualide A was a C30-ketone analogue.

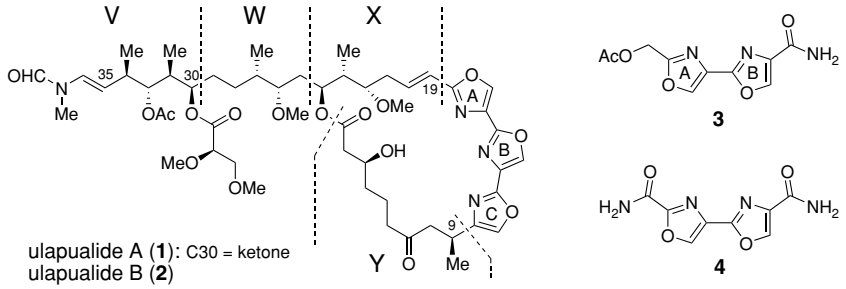


Fig. 11.1. Structures of ulapualides and the degradation products

The stereochemistry of all the stereogenic centers in ulapualide A (**1**) was predicted by hypothesizing that ulapualide A should form a complex with metals and that the compound possessing the stereochemistry of the natural product should form the lowest energy chelate (Maddock et al. 1993). The proposed structure later turned out to be incorrect by a total synthesis (Chattopadhyay and Pattenden 2000). Although it is of interest, the biological activity of the synthetic compound was not reported. In this connection, the cation-binding property of dihydrohalichondramide (vide infra) was shown not to be associated with its antifungal activity: a trisoxazole fragment which exhibited the same degree of cation-binding

property as that of dihydrohalichondramide was not antifungal (James et al. 1993).

The absolute stereochemistry of the C-19 to C-35 portion of ulapualide B (**2**) was shown to be identical with that of mycalolide B, by converting to the same degradation product (vide infra; Matsunaga et al. 1999) and the total stereochemistry of ulapualide A was solved by X-ray crystallography of a complex with actin (Allingham et al. 2004). Interestingly, the side-chain beyond C-31 was disordered in the crystal structure, demonstrating a weak contact of this portion with actin. This is in agreement with a highly specific binding between actin and swinholide A which differs significantly in the structure of the relevant portion (Bubb et al. 1995; Klenchin et at. 2005).

11.2.2 Kabiramides

Kabiramide C (**5**; Fig. 11.2) was isolated from the egg-ribbons deposited at Kabira Bay on Ishigaki Island by an unidentified nudibranch (Matsunaga et al. 1986), which was later identified as *Hexabranchus* sp. (Matsunaga et al. 1989). The major HPLC peak of the antifungal fraction of the extract of the egg-ribbons was named kabiramide C. Kabiramide C (**5**) was very abundant in the egg-ribbons and isolated in a yield of 0.25% wet weight. Several NMR signals were doubled, as was the case with ulapualides. Interpretation of the COSY spectrum together with the CH-COSY spectrum, which assisted the assignments of the crowded aliphatic region,

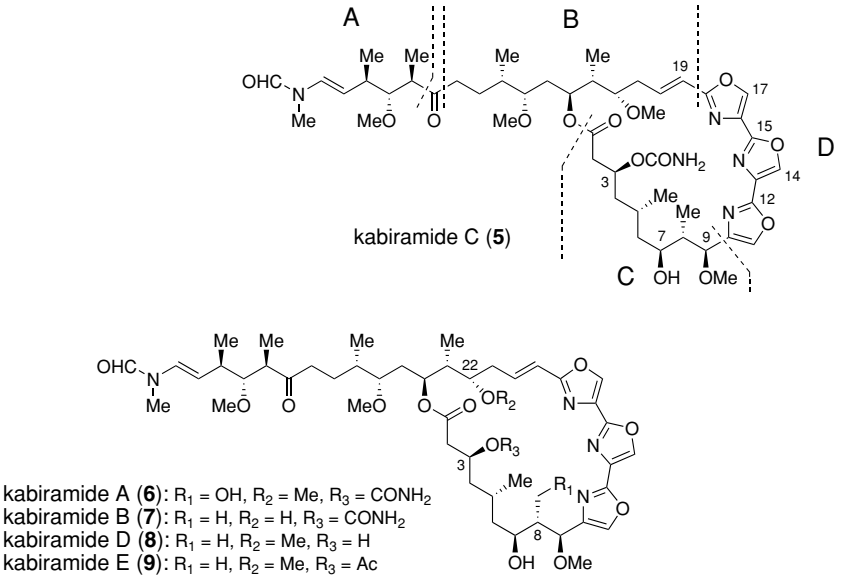


Fig. 11.2. Structures of kabiramides

allowed us to assign partial structures A–C, in which four methoxy groups were placed on the basis of NOE difference experiments. The location of a free hydroxyl group at C-7 was confirmed by a 1.3-ppm shift of the oxymethine proton in the acetate. Units A and B were joined through a doubled ketone (δ 214.0, 214.1) on the basis of NOE difference experiments and chemical shift arguments. A connection between the partial structures B and C was accomplished by a long-range selective proton decoupling (LSPD) experiment. One proton was irradiated overnight and a ^{13}C NMR spectrum without broad-band decoupling was acquired. We had to rely on such time-consuming experiments before the era of inverse-detected 2D-NMR experiments. After the assignment of these units, ten sp carbons, three low-field singlet aromatic protons, and a broad exchangeable signal integrating for 2H remained unassigned. It took some time before we recognized the presence of three iterative units by inspecting the distribution of carbon chemical shifts. At the time of the structure elucidation, heteroaromatic rings such as oxazole and thiazole were not familiar among marine natural products, except for peptides from ascidians (Ireland and Scheuer 1980). Being encouraged by the precedence of two contiguous thiazoles in bleomycins, antitumor nonribosomal peptides of microbial origin (Takita et al. 1978), we presumed the presence of three contiguous oxazole rings (partial structure D), which was unprecedented, and a carbamate ester (δ_C 157.3).

In order to confirm the idea, we again relied on LSPD experiments, which gave not only coupling partners but also magnitudes of coupling constants. We determined long-range ^{1}H–^{13}C coupling constants within five-membered rings, which matched well with those reported for oxazoles. Unfortunately, long-range couplings across oxazole rings were not obvious from the LSPD experiments, because they were very small. However, careful inspection of carbon signals indicated a clear narrowing due to perturbations of long-range couplings: irradiation of H-14 and H-17 sharpened C-12 and C-15, respectively. This observation allowed the determination of the orientation of the central oxazole ring. By placing the carbamate ester at C-3 on the basis of an LSPD experiment, we completed the structure elucidation of kabiramide C.

Kabiramides A, B, D, E (**6**, **7**, **8**, **9**, respectively; Fig. 11.2) were isolated as minor metabolites from the egg-ribbons collected at Ishigaki Island (Matsunaga et al. 1989). Their structures were determined by comparing the NMR data with those of kabiramide C. In kabiramide A, the C-8 methyl group was oxidized to a hydroxymethyl group; kabiramide B had a hydroxyl group at C-22 instead of a methoxy group in kabiramide C; kabiramide D was the decarbamoyl derivative of kabiramide C; and kabiramide E was the C-3 acetate of kabiramide D. Kabiramides B and C were succeedingly isolated from a sponge *Halichondria* sp., strongly suggesting the sponge origin of kabiramides (Kernan et al. 1988). It is not

known whether kabiramides A, D, and E are sponge metabolites or are converted within the body of the nudibranch.

Due to the difficulty in preparing crystals suitable for X-ray crystallography and conformational heterogeneity (cf. Sect. 11.2.5), the stereochemistry of kabiramides remained unknown for almost two decades. However, by immersing a solution of kabiramide C in preformed crystals of actin, the relative and absolute stereochemistry of kabiramide C was determined by X-ray crystallography of the complex (Klenchin et al. 2003).

11.2.3 Halichondramide and Congeners

The discovery of halichondramide (**10**; Fig. 11.3) from a marine sponge *Halichondria* sp. indicated that the trisoxazole macrolides are originally produced by sponges (Kernan and Faulkner 1987). The structure of halichondramide was determined by interpretation of NMR data, including COSY, CH-COSY, and long-range CH-COSY (COLOC), which was popular before the introduction of the HMBC technique, and comparison of the NMR data with those of kabiramide C.

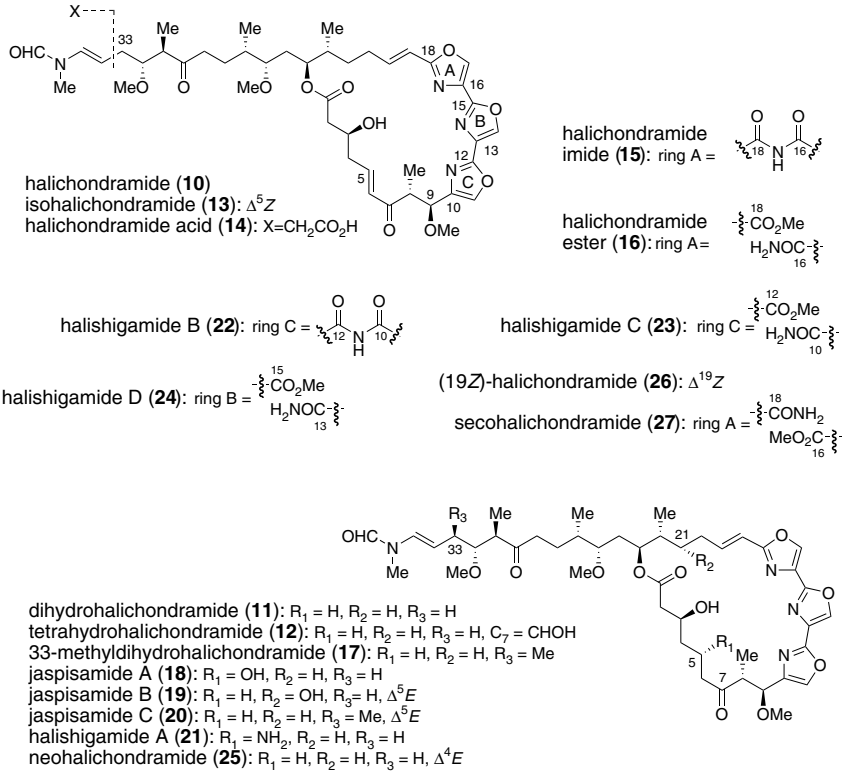
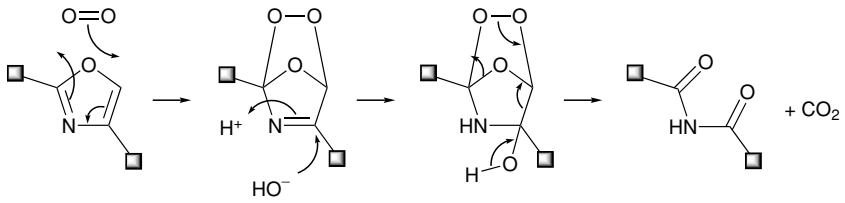


Fig. 11.3. Structures of halichondramide and its congeners

The isolation of halichondramide and its congeners (Fig. 11.3) from *H. sanguineus* which fed on *Halichondria* sp. was reported by Kernan et al. (1988). Dihydrohalichondramide (**11**) was the reduction product of halichondramide at the Δ^5-olefin; and tetrahydrohalichondramide (**12**) was the compound further reduced at the C7-ketone. Tetrahydrohalichondramide was produced by reduction of dihydrohalichondramide with Li(*O*-*t*-Bu)$_3$BH. Isohalichondramide (**13**) was the 5*Z*-isomer of halichondramide and was slowly converted to halichondramide in $CDCl_3$ solution. Halichondramide acid (**14**) had the carboxylic acid group instead of the *N*-methyl-vinylformamide group at the end of the side-chain. Jones' oxidation of halichondramide afforded halichondramide acid through the terminal aldehyde generated by acid-catalyzed removal of the *N*-methylformamide group. The NMR signals of halichondramide acid were no more doubled, due to the lack of the terminal amide moiety. Halichondramide imide (**15**) had an imide group [δ_H9.86 (brs, 1H)] instead of the oxazole proton (H-17) and exhibited infrared absorption at 1,760 cm^{-1}, which was ascribed to the absorption of an imide group. The location of the imide group was determined by an NOE difference experiment. The imide was probably produced by air oxidation of the oxazole ring (Wasserman 1970), followed by hydration and generation of carbon dioxide (Scheme 11.1). Halichondramide ester (**16**) was the methanolysis product of the imide, which was confirmed by a chemical transformation from the imide to the ester. Given the susceptibility of oxazole rings toward oxidation, compounds **15** and **16** may be artifacts. Dihydrohalichondramide (**11**) and a new congener, 33-methyldihydrohalichondramide (**17**), were isolated from the egg-ribbons collected at Hachijo Island, which also contained ulapualides A and B (Matsunaga et al. 1989).



Scheme 11.1. Opening of ring A of halichondramide and formation of imide functional group

Jaspisamides are minor cytotoxic constituents of a marine sponge *Jaspis* sp. collected at Ishigaki Island, halichondramide being the major constituent of the sponge (Kobayashi et al. 1993). Jaspisamide A (**18**) is a hydration product of halichondramide at C-5, jaspisamide B is 21-hydroxyhalichondramide (**19**), and jaspisamide C is 33-methylhalichondramide (**20**). Hydroxylation at C-21 and methylation at C-33 are

both observed in ulapualides and kabiramides. Halishigamides are also minor constituents of a marine sponge *Halichondria* sp. collected at Ishigaki Island (Kobayashi et al. 1997). Halishigamide A (**21**) is the ammonia adduct of halichondramide at C-5. The presence of an amino group was confirmed by a positive ninhydrin reaction and the preparation of an *N*-Boc derivative. Halishigamide B (**22**), in which ring C was converted to an imide, is isomeric to halichondramide imide (**15**). The location of the imide group was shown by the HMBC and NOESY correlations. Halishigamide C (**23**) is a methanolysis product of halishigamide B. The locations of the methyl ester and amide groups were demonstrated by a decoupled HMBC experiment. Halishigamide D (**24**) with oxidized ring B was isomeric to halishigamide C. The locations of the methyl ester and amide groups in **24** were determined on the basis of decoupled-HMBC experiment in which several four-bond H–C couplings were observed. Neohalichondramide (**25**), (19*Z*)-halichondramide (**26**), and secohalichondramide (**27**) were isolated from the marine sponge *Chondrosia corticata* collected at Guam, the predominant trisoxazole constituent of the sponge being halichondramide (**10**; Shin et al. 2004). Dihydrohalichondramide (**11**), jaspisamide A (**18**), and halishigamide D (**24**) were also found in the sponge. Neohalichondramide was the Δ^4-isomer of halichondramide, whereas secohalichondramide was the positional isomer of halichondramide ester. Compound **26** was the first 19*Z*-isomer in this class of metabolites.

The absolute stereochemistry of halichondramide was determined by X-ray crystallography of a complex with actin (Klenchin et al. 2003). Actin-catalyzed hydration of halichondramide to jaspisamide A was observed during crystallization.

11.2.4 Mycalolides

Mycalolides A–C (Fig. 11.4) were isolated from a marine sponge *Mycale* sp. (Fusetani et al. 1989). Their structures were assigned by analysis of 2D-NMR data. Mycalolide A (**28**) is a hybrid of halichondramide (C-1 to C-9 portion) and ulapualide A (C-19 to C-35 portion), whereas mycalolide B (**29**) is a hybrid of halichondramide and ulapualide B. Mycalolide C (**30**) is the 38-*O*-des-methyl derivative of mycalolide B.

Mycalolide C and two related macrolides, mycalolides D (**31**) and E (**32**; Fig. 11.4), were isolated from the stony coral *Tubastrea faulkneri* collected at the Great Barrier Reef (Rashid et al. 1995). The structure of mycalolide D, the imide derivative of a putative dehydroulapualide B, was assigned on the basis of HMBC data. Mycalolide E is the ring C oxidation product of a positional isomer of a putative dihydroulapualide A.

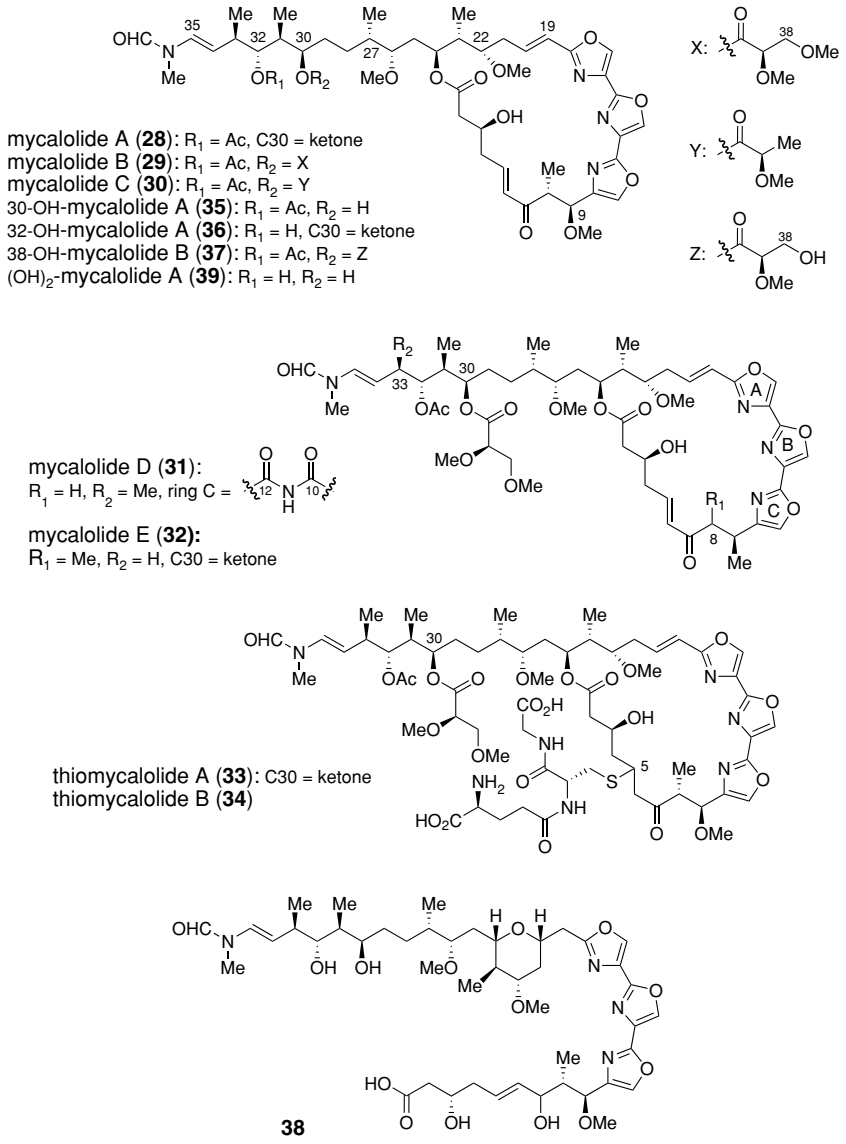


Fig. 11.4. Structures of mycalolides and their degradation products

A polar fraction of the *Mycale* sp. that contained mycalolides A–C showed potent cytotoxic activity. From the fraction were isolated thiomycalolides A, B (**33**, **34**; Fig. 11.4; Matsunaga et al. 1998a). Thiomycalolide B is a glutathione adduct of mycalolide B, whereas thiomycalolide A is that of mycalolide A. Thiomycalolide A was prepared from the reaction between mycalolide A and glutathione.

Three congeners of mycalolides (**35**–**37**; Fig. 11.4), all of which had a hydroxyl group near the terminus of the side-chain, were isolated from *M. magellanica* collected in Suruga Bay (Matsunaga et al. 1998b). Their structures were determined by interpretation of spectral data. In 30-hydroxymycalolide A (**35**), the C30-ketone of mycalolide A was reduced to a secondary alcohol. This was confirmed by converting **28** and **35** to a common reduction product. 32-Hydroxymycalolide A (**36**) was the deacetylation product of mycalolide A, which was confirmed by converting **28** and **36** to the same diacetate. 38-Hydroxymycalolide B (**37**) possessed a hydroxyl group at C-38 instead of a methoxy group in mycalolide B. This was substantiated by converting **29** and **37** to the same tetrahydropyran (**38**) by saponification of the $NaBH_4$ reduction products. Additionally, mycalolide C and 30-hydroxymycalolide A were also converted to **38**. From these experiments, it was demonstrated that the stereochemistries of all stereogenic centers in mycalolides from *Mycale* spp. were identical.

Additionally, 30,32-dihydroxymycalolide A (**39**) was isolated as a minor constituent of *M. izuensis* (Phuwaraisirisan et al. 2002).

11.2.5 Stereochemistry of Mycalolides

Initially, it was envisaged that the stereochemistry of mycalolides could be determined by a combination of conformational analysis and the modified Mosher ester analysis in the ^{1}H NMR spectroscopy. However, it was not possible to correlate the stereochemistry of C-7 with that of C-3 and the assignment of relative stereochemistry of the C-22 to C-27 portion was difficult due to the presence of more than one conformer in this region. Although it is still possible to propose the most probable structure by considering coupling constants as weighted averages of several conformers and NOESY cross-peaks as the sum of each conformer, such analysis carries a high potential of misassignments. Therefore, we elected to carry out chemical degradations in order to prepare conformationally stabilized derivatives and to obtain fragments for direct comparison with authentic samples (Fig.11.5). The degradation started with the cleavage of Δ^{19}- and Δ^{34}-olefinic bonds with RuO_4, followed by methanolysis to remove the esters. The resulting dicarboxylic acid was dehydrated with DCC to form the bis-δ-lactone (**40**). The two δ-lactone rings were both in a boat-like conformation and the relative stereochemistry within the rings was firmly assigned by interpretation of NMR data. Relative stereochemistry at C-26 was correlated to that of C-24 with high credibility because the C-24 to C-26 portion adopted a single conformation with an extended carbon chain. However, a coupling constant of 3.7 Hz between H-26 and H-27 and numerous NOESY cross-peaks observed from these protons suggested

that this section of the molecule adopted two or more conformations not only in mycalolides but also in **40**. The relative stereochemistry of C-27 with respect to that of C-26 in mycalolides remained ambiguous, even after analysis of the ^{1}H NMR data of two model compounds (**41**, **42**). Therefore, it appeared necessary to make a direct comparison between a fragment obtained by chemical degradation and synthetic compounds with defined stereochemistry. Before preparing synthetic fragments, we had to reduce the number of possible isomers.

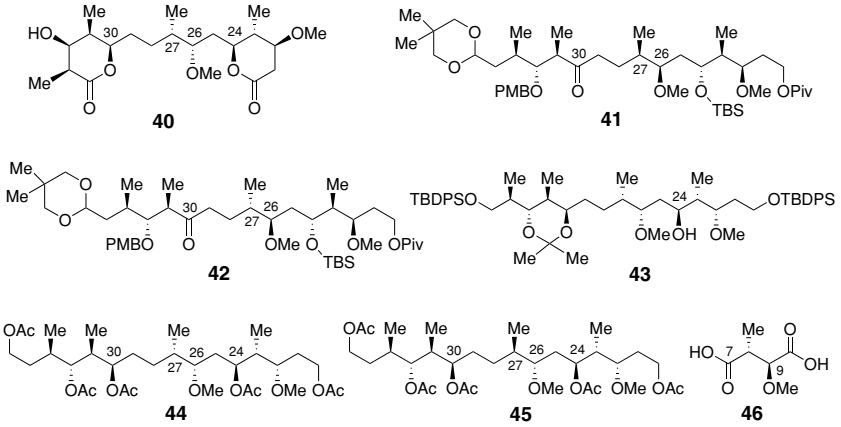


Fig. 11.5. Structures of degradation products and synthetic fragments of mycalolides

It is possible to determine the stereochemistry of compounds containing many hydroxyl groups by preparing a per-MTPA ester and applying the modified Mosher method (Kobayashi et al. 1994). In order to carry out the analysis, it is necessary to elucidate the conformation of the molecule and estimate the additive effects of spatially close MTPA groups, which are associated with the possibility of misassignment. Therefore, we intended to avoid the introduction of MTPA groups in proximate hydroxyl groups. Fortunately, there were natural mycalolides whose hydroxyl groups were differentially unmasked. We used them for the modified Mosher analysis (Ohtani et al. 1991). We used 30-hydroxymycalolide A and 32-hydroxymycalolide A for the analysis of C-30 and C-32, respectively. In order to liberate a hydroxyl group at C-24, we prepared acetonide **43** (Fig. 11.5) by a standard sequence of chemical reactions. By application of the Mosher ester analysis to these compounds, we demonstrated the 24*S*,30*R*,32*R*-stereochemistry. Additionally, the 3*S*-stereochemistry was simultaneously defined by the Mosher ester analysis. With these assignments in hand, there were only two possibilities left for the stereochemistry of the C-19 to C-35 portion, differing in the configuration at C-27. Then, two C-27 epimers of the C-20/C-35 pentaacetate fragment (**44**, **45**) were synthesized and compared

with the one prepared from 38-hydroxymycalolide B. The ^{1}H and ^{13}C NMR data of the synthetic fragment with 26,27-*syn* relationship coincided with those prepared from the natural product.

Although there was a strong NOE between H-5 and H-8, it was not possible to establish the spatial relationship between H-3 and H-5, because H-5 coupled equally to methylene protons on C-4. We took advantage of the presence of oxidatively cleavable functional groups besides the C-8/C-9 unit. On oxidation with RuO_4, 38-hydroxymycalolide B afforded the expected dicarboxylic acid (**46**), whose relative stereochemistry was assigned on the basis of the ^{1}H NMR data. Compound **46** was converted to the bis-*p*-bromophenacyl ester and its retention time was compared with those of synthetic standards in HPLC analysis using a chiral stationary phase (Chiralcel OJ), which showed the 8*R*,9*S*-stereochemistry. The last stereogenic centers to be assigned were those in the pendant esters at C-30: 2,3-*O*,*O*′-dimethylglycerate in mycalolide B, *O*-methyllactate in mycalolide C, and 2-*O*-methylglycerate in 38-hydroxymycalolide. Each of the three mycalolides were saponified and the product was transformed to the *p*-bromophenacyl ester. After brief purification by silica gel chromatography, each product was analyzed by HPLC with Chiralcel AD or OJ and the retention time was compared with those of synthetic enantiomers. The stereochemistry at C-37 was assigned as *R* in each case, finalizing the assignment of the relative and absolute stereochemistry of mycalolides (Matsunaga et al. 1999).

The proposed stereochemistry of mycalolide A was confirmed by a total synthesis (Panek and Liu 2000). The stereochemistry of scytophycins, e.g., scytophycin B (**47**), and aplyronines, e.g., aplyronine A (**48**; Fig. 11.6), had been determined before we assigned the stereochemistry of mycalolides (Yeung and Paterson 2002). As for the side-chain, these metabolites shared the same stereochemistry for all stereogenic centers. A notable structural similarity in the side-chain was observed in mycalolides as well. Because initial analysis of the NMR data suggested that the relative stereochemistry of the C-22 to C-26 and C-30 to C-33 portions in trisoxazole macrolides was identical to that in scytophycins, it was tempting to propose that the stereochemistry of the C-22 to C-33 portion for both classes of compounds was identical. Contrary to our expectations, mycalolides and scytophycins were enantiomeric in the C-22 to C-26 portion and identical in the C-27 to C-33 portion. Because mycalolides, aplyronines, and scytophycins share the same cellular target (Patterson et al. 1993; Saito et al. 1994, 1996), the biochemical importance of the terminal portion of the side-chain was emphasized by the structure elucidation of mycalolides (Matsunaga et al. 1999).

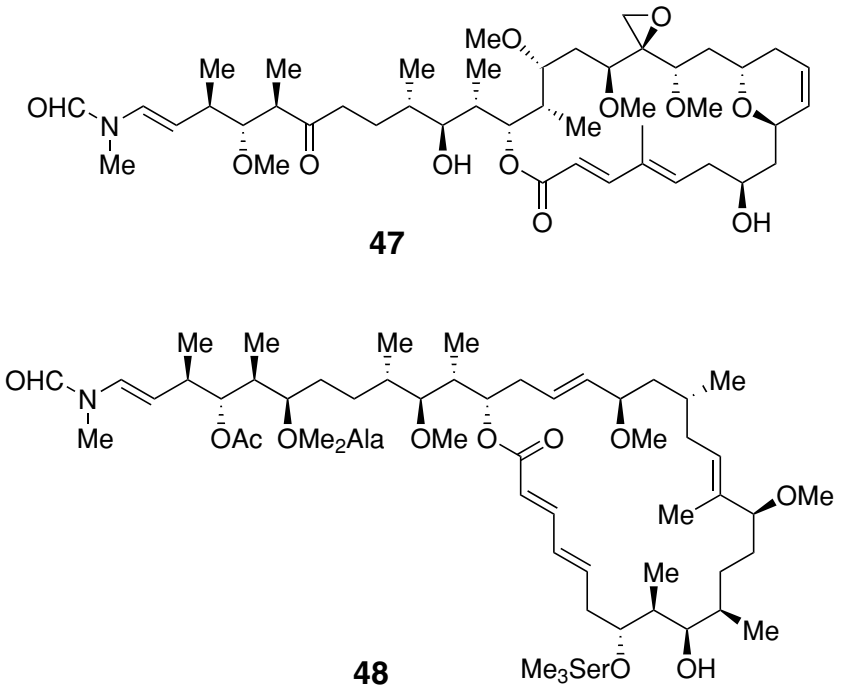


Fig. 11.6. Structures of scytophycin B and aplyronine A

11.3
Cellular Target of Trisoxazole Macrolides

Trisoxazole macrolides exhibited potent antifungal and cytotoxic activities but did not show antibacterial activity. Therefore, they were expected to perturb cellular components present specifically in eukaryotes. The effects of mycalolide B on smooth muscle contraction were examined (Hori et al. 1993). The contraction was inhibited by mycalolide B, but the effects were dependent neither on the concentration of Ca^{2+} nor on the phosphorylation state of the myosin light chain, suggesting that one or more of the contractile elements were affected. Mycalolide B inhibited the Mg^{2+}-ATPase activity of actomyosin, but cytochalasin D, the well-known actin-perturbing agent, did not. Therefore, the possibility of mycalolide B affecting actin was excluded and mycalolide B was proposed to be an inhibitor of actin–myosin interaction. Meanwhile, based on microscopic observations of KB and A10 cells and using purified actin, the biological activity of tolytoxin was ascribed to the disruption of microfilament organization and the depolymerization of actin (Patterson et al. 1993). Being inspired by this

finding, a further examination of the effects of mycalolide B on purified actin was carried out, which disclosed that mycalolide B severed F-actin and formed a 1:1 complex with G-actin (Saito et al. 1994).

It was shown by the structure–activity relationship study on aplyronines, another actin depolymerizer, that the vinylformamide portion of the side-chain was not essential for activity (Suenaga et al. 1997). Therefore, we envisaged attaching biotin to the terminus of mycalolide B and kabiramide D in order to observe the cellular behavior of the compounds. The *N*-methyl-vinylformamide group was removed with acid to generate an aldehyde to which biocytin was attached by reductive amination (Wada et al. 1998). Many intracellular proteins in cultured rat 3Y1 fibroblast cells bound to biocytinylated mycalolide B (**49**), whereas only actin was detected as the cellular protein that bound to biocytinylated kabiramide D (**50**; Fig. 11.7). The promiscuity of mycalolide B was ascribed to the presence of the enone group, which was susceptible to Michael addition. Biocytinylated kabiramide D bound specifically to stress fibers in the formaldehyde-fixed 3Y1 cells. The binding specificities of other actin-binding molecules were examined using biocytinylated kabiramide D. Purified actin pretreated with either kabiramide D or misakinolide A did not bind to biocytinylated kabiramide D, whereas actin pretreated with either cytochalasin D or latrunculin A bound to biocytinylated kabiramide D. Therefore, the actin-binding site of misakinolide A was identical with or partially overlapped with that of kabiramide D, but those of cytochalasin D and latrunculin A were different. X-ray crystallography of a complex between latrunculin A and actin showed that the binding site of latrunculin was different from that of the trisoxazole macrolides (Morton et al. 2000).

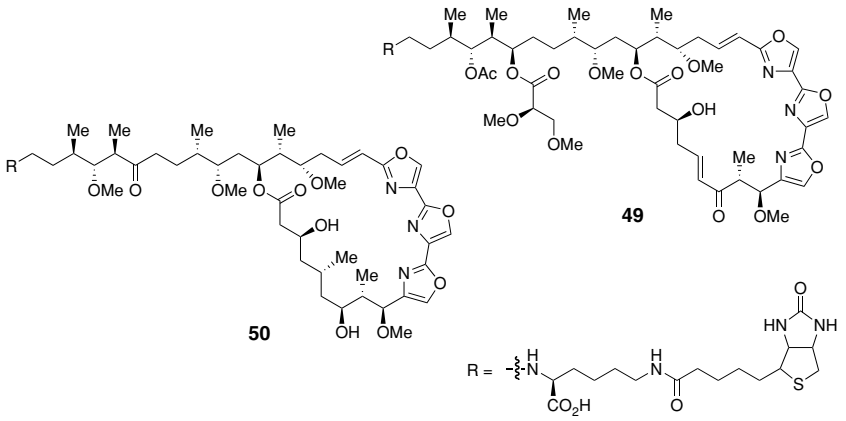


Fig. 11.7. Structures of biocytinylated mycalolide B and kabiramide D

11.4
Modes of Binding of Trisoxazole Macrolides to Actin

The mode of binding of kabiramide C to G-actin was studied by using prodan-actin as a fluorescent probe. The binding of kabiramide C to prodan-G-actin mediated a red-shift in the emission and decreased the quantum yield of the probe. By an examination of the time-course, the binding was shown to consist of two steps. The initial fast step was speculated to be the binding of the macrocyclic portion and the slow step to be that of the side-chain. Fractionation of F-actin treated with substoichiometric amounts of kabiramide C showed that the severing action of kabiramide C was slow (Tanaka et al. 2003). X-ray crystallography of a complex between actin and either kabiramide C, halichondramide, or ulapualides A demonstrated that the side-chain portion of these macrolides is inserted into the hydrophobic cavity which associates with the hydrophobic stretch of another actin molecule in forming F-actin (Klenchin et al. 2003; Allingham et al. 2004).

11.5
Structure–Activity Relationships of Trisoxazole Macrolides

Due to their effects on actin cytoskeletons, trisoxazole macrolides exhibit potent cytotoxic and antifungal activities (Wada et al. 1998; Spector et al. 1999). There is a clear relationship between the chemical structure and cytotoxicity among trisoxazole macrolides (Table 11.1). Compounds with the intact trisoxazole portion possess potent activity. However, when one oxazole ring is oxidized and converted to either imide or methyl ester, the activity of the compound is significantly reduced. Given the close association of the side-chain, as shown by the crystal structures of the complex, we should point out the importance of the side-chain in binding. This was confirmed by the potent actin-depolymerization activity of a synthetic C-22/C-35 fragment of mycalolide B as well as the corresponding portion of aplyronine A (Suenaga et al. 2004). It is of interest to examine whether these fragments possess cytotoxicity or not. Also, the actin-depolymerization activity of the imide and ester derivatives should be examined in order to understand the reason for their diminished cytotoxicity. The low cytotoxicity of the imide and ester derivatives may be attributable to their reluctance to penetrate the cellular membrane.

Table 11.1. Cytotoxicity of trisoxazole macrolides

compound	cytotoxicity (μg/mL)	examined cells
ulapualide A (**1**)	0.01-0.03	L1210
ulapualide B (**2**)	0.01-0.03	L1210
kabiramide C (**5**)	0.01	L1210
kabiramide A (**6**)	0.03	L1210
kabiramide B (**7**)	0.03	L1210
kabiramide D (**8**)	0.02	L1210
kabiramide E (**9**)	0.02	L1210
halichondramide (**10**)	4	sea urchin
dihydrohalichondramide (**11**)	0.03	L1210
tetrahydrohalichondramide (**12**)	1	sea urchin
isohalichondramide (**13**)	4	sea urchin
halichondramide acid (**14**)	>>4	sea urchin
halichondramide imide (**15**)	>>4	sea urchin
halichondramide ester (**16**)	>>4	sea urchin
33-methyldihydrohalichondramide (**17**)	0.05	L1210
jaspisamide A (**18**)	<0.001	L1210
jaspisamide B (**19**)	<0.001	L1210
jaspisamide C (**20**)	<0.001	L1210
halishigamide A (**21**)	0.004	L1210
halishigamide B (**22**)	4.4	L1210
halishigamide C (**23**)	5.2	L1210
halishigamide D (**24**)	1.1	L1210
neohalichondramide (**25**)	0.38	K562
(19*Z*)-halichondramide (**26**)	0.90	K562
secohalichondramide (**27**)	0.90	K562
mycalolide A (**28**)	>500	B-16
mycalolide B (**29**)	<0.001	B-16
mycalolide C (**30**)	<0.001	B-16
mycalolide D (**31**)	<0.001	average[b]
mycalolide E (**32**)	0.6	–
thiomycalolide A (**33**)	NT[a]	P388
thiomycalolide B (**34**)	0.02	P388
30-OH-mycalolide A (**35**)	0.02	L1210
32-OH-mycalolide A (**36**)	0.01	L1210
38-OH-mycalolide B (**37**)	0.02	L1210
$(OH)_2$-mycalolide A (**39**)	0.003	HeLa

For references, see text
[a] Not tested.
[b] Average value for 60 cancer cell lines.

11.6 Biological Significance of Trisoxazole Macrolides in the Nudibranch *H. sanguineus*

The defensive role of the trisoxazole macrolides in *H. sanguineus* was suggested by fish-feeding experiments and anatomical study (Pawlik et al. 1988). Trisoxazole macrolides were the most potent fish-feeding inhibitors studied by the Faulkner team (Pawlik et al. 1988). The nudibranchs were separated into four parts and the macrolide content of each part was determined. This experiment showed that trisoxazole macrolides were concentrated in the dorsal mantle, which is exposed to

potential predators, but only small amounts of macrolides were present in the foot tissue. High concentrations of macrolides were detected in a mixture of digestive gland and gonad, because the macrolides came from the diet and a significant amount of the macrolides should be delivered to the egg-ribbons. Egg-ribbons, which were prominently deposited, contained tenfold or greater concentrations of the macrolides than those in the tissues of the nudibranch (Roesner and Scheuer 1986; Pawlik et al. 1988). The aposematic coloration of the animal from the Red Sea was due to a rare carotenoid hurghadin, which was localized in the mantle (Guo et al. 1998).

H. sanguineus fed selectively on the sponge *Halichondria* sp. that contained halichondramide (Pawlik et al. 1988). Because they accumulated dihydrohalichondramide and tetrahydrohalichondramide, which were minor constituents of the sponge, it was speculated that halichondramide was reduced by the nudibranch. Given the susceptibility of halichondramide to nucleophilic attacks at the enone portion, the reduction should take place immediately after the uptake of the molecule. *H. sanguineus* collected in the Red Sea was found to contain swinholide A, another actin-binding molecule (Carmely and Kashman, personal communication) and kabiramide C (Guo et al. 1998).

11.7 Conclusions

H. sanguineus is unique among criptobranch dorids in a morphological sense (Wägele and Willan 2000; Valdés 2002). This uniqueness reflects its peculiar chemistry: it is the only nudibranch that is known to use trisoxazole macrolides for chemical defense. Sponges containing trisoxazole macrolides are widely distributed, because *H. sanguineus* from various geographical areas contain the macrolides. However, no other nudibranchs have so far been reported to contain this class of metabolites. Therefore, it is likely that other nudibranchs are not immune to the trisoxazole macrolides. The mechanism of the tolerance of *H. sanguineus* toward trisoxazole macrolides is an interesting question.

The distribution of trisoxazole macrolides in sponges and hard coral poses a question about the true producer of this class of metabolites. In natural products chemistry, when one class of compounds is found in taxonomically remote organisms, the involvement of symbiotic microorganisms in their production is suspected and, in some cases, verified (Yotsu et al. 1987; Piel et al. 2004). At the time of the discovery of trisoxazole macrolides, the mixed biosynthetic pathway of polyketide and nonribosomal peptide was not common, which led to a proposal of the biosynthesis of oxazole rings through Beckman rearrangement (Ishibashi

et al. 1986). Nowadays, many natural products are known to be biosynthesized by the mixed pathway (Shen et al. 2005) and so this is also likely the case with trisoxazole macrolides. The true producer of this class of metabolites and their mechanism of biosynthesis will be clarified in the future.

Actin is involved in several disease states; and compounds interacting with actin are anticipated to be candidates for anticancer agents (Yeung and Paterson 2002). Because actin has integral functions in eukaryotic cells, its perturbation is often associated with lethality. With knowledge of the precise modes of binding of several natural products to actin (Morton et al. 2000; Klenchin et al. 2003, 2005), the creation of therapeutic compounds that bind actin may be actualized.

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