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Using a kinase screen to investigate the constituents of the sponge *Stelletta clavosa* obtained from diverse habitats

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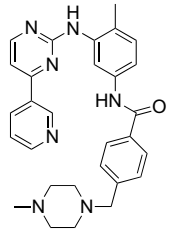
Abstract—Fourteen collections of the marine sponge *Stelletta clavosa* have been obtained from diverse Indo-Pacific locations in order to conduct a comparison of their major constituents. The dichloromethane extract of one collection (no. 00369) exhibited activity in a c-Raf-1 kinase assay. Bioactivity-directed isolation resulted in the known porphyrin analogs pyropheophorbide *a* (**2**) and purpurin 18 methyl ester (**3**). Further spectroscopic screening of the various sponge extracts resulted in the isolation of four swinholide polyketides, a carotenoid, and three diketopiperazines. Pyropheophorbide *a* (**2**) exhibited the best IC_{50} among the porphyrin type compounds (IC_{50} <0.31 μg/mL). This prompted further screening of **2** against a panel of 85 kinases.

1. Introduction

Screening Indo-Pacific sponges for kinase activity has been previously utilized to identify unique bioactive secondary metabolites.[1] Recently, kinase enzymes have attracted renewed attention as important tools to guide new discoveries in bioorganic and medicinal chemistry.[2] In part, this is evident from the dramatic rise in discovery of small organic molecules exhibiting a specific interaction with new kinase enzyme targets.[3] A recent milestone in such kinase research has been the FDA approval of the poly-aromatic alkaloid Gleevec (**1**), discovered by Novartis, and now available as an effective clinical agent to treat chronic myelia leukemia (CML) and gastrointestinal stromal tumors (GIST). The effectiveness of Gleevec is thought to be due to its selective inhibition of Bcr-Abl, c-kit, and platelet-derived growth factor receptor kinases. This example constitutes an important proof of concept to show the therapeutic value derived from the specific interaction of a small molecule with an individual kinase enzyme.



Gleevec (**1**)

We sought to extend the concepts illustrated above through the study of the kinase active constituents envisioned to be present in the extracts of the Indo-Pacific sponge *Stelletta clavosa* (syn. *Myriastra clavosa*[4]). This sponge, while not entirely cosmopolitan, is often abundant in selected habitats visited by our laboratory. In fact, during the last 14 years we have obtained 14 collections of *S. clavosa* from the diverse Indo-Pacific sites summarized in Table 1. Not unexpectedly, others have also targeted this specimen for chemical examination resulting in reports of dimeric macrolide glycosides,[5] polymethoxydienes,[6] and the phosphorus containing clavosines.[7] Interestingly, all of these studies were conducted on sponges obtained from regions very distant from those of our expedition sites. Of further surprise, as our work progressed, none of these aforementioned chemotypes were observed. Some of our isolation work was guided by a kinase bioassay while another component was directed by the spectroscopic properties

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Table 1. Overview of *Stelletta clavosa* collections, site locations, and representative compounds observed

Collection no.	Collection site	Compound types[a]
89020	Soloman Islands	PS, C
90119	Papua New Guinea	PS, S, C
91141	Papua New Guinea, Milne Bay	PS, S, C
91149	Papua New Guinea, Milne Bay	PS, C
94036	Soloman Islands, Gizo	PS, C
94525	Indonesia, Sangihe Is.	PS, C
95004	Papua New Guinea, Wahoo and Milne Bay	PS, S, C
95585	Indonesia, Togian Is.	PS, C
96587	Indonesia, N. Sulawesi	PS, C
98172	Papua New Guinea, Wewak	PS, S, C, Z, D
00369	Papua New Guinea, Rabaul	PS, C, Z
01315	Papua New Guinea, Wewak	PS, C
02128	Papua New Guinea, Milne Bay	PS, C, Z, D
02156	Papua New Guinea, Milne Bay	PS, C, D

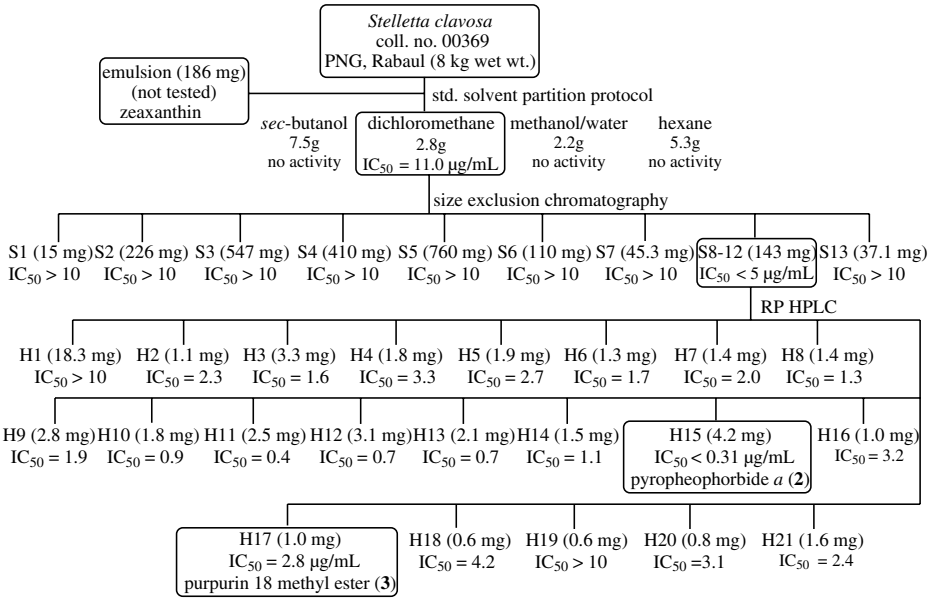
[a] PS = preswinholides; S = swinholides; C = chlorophyll derived compounds ≈**2**, **3**; Z = zeaxanthin; D = diketopiperazines.

obtained for chromatographic fractions. Reported herein is the isolation of two porphyrin compounds detected through kinase bioassay-directed isolation, with the most potent compound tested for further biological activity against a panel of 85 kinase enzyme assays. Also outlined are the spectroscopic-guided isolation of swinholide polyketides, a single carotenoid, and three diketopiperazines.

2. Results and discussion

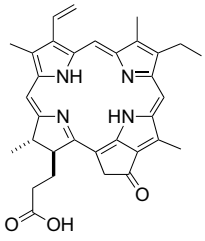
A preliminary survey of various crude extracts, obtained by a Kupchan style partition[8] of the MeOH extracts from the 14 *Stelletta clavosa* collections, constituted the starting point for this project. As illustrated in Scheme 1, one specimen (coll. no. 00369) was selected for further evaluation because its dichloromethane fraction showed promising properties in the c-Raf-1 kinase enzyme assay (IC_{50} = 11 μg/mL). A successive series of chromatographic steps beginning with size exclusion chromatography (Sephadex LH-20, 100% methanol) led to localization of the activity in several dark green fractions suspected to contain chlorophyll analogs. Chlorophyll *a* was tested in the c-Raf-1 assay and the inactivity ($IC_{50} \gg 10$ μg/mL), along with NMR data for the active fractions, indicated different molecular structures were responsible for the bioactivity. Subsequently, the dark green fractions (S8-12) were then pooled and subjected to reversed-phase (RP) HPLC, which afforded two dark pigmented compounds in pure form accompanied by 19 semipure fractions (Scheme 1).

Initial dereplication[9] by NMR and MS commenced on several of the fractions and revealed a large number of structural possibilities. Focusing on the pure fractions H15 and H17 shown in Scheme 1 and narrowing the possible hits to compounds containing an extensive chromophore of the type found in a porphyrin was useful. The structure of the pure compound from fraction

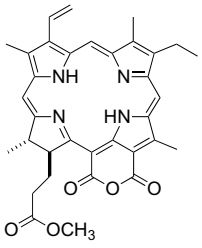


Scheme 1. The isolation and IC_{50}'s (μg/mL) in the c-Raf-1 kinase assay of the porphyrin analogs.

H15 was assigned as pyropheophorbide *a* (**2**)[10] supported by the molecular formula of $C_{33}H_{34}N_4O_3$ established from HRESI-TOFMS (*m/z* 535.2723 $[M+H]^+$). The 19 units unsaturation units required by this formula was consistent with a modified porphyrin ring comprised of 17 sp^2 carbons with the core ring system (six rings required) substituted with two carbonyl containing residues, carboxylic acid (δ_C 196.4) and ketone (δ 171.6), a vinyl group (δ_H 6.29 and δ 6.18), four methyls (δ_H 3.15, δ 3.36, δ 3.59, 3.81), and an ethyl residue (δ_H 1.59 t, $J = 7.5$ Hz and δ 3.59 q, $J = 7.5$ Hz). Evaluating the dereplication hits with these constraints revealed **2** as having physical properties identical to those we observed. A similar approach was used to define the structure of the pure compound isolated in fraction H17. A molecular formula of $C_{34}H_{34}N_4O_5$ was established from HRESI-TOFMS (*m/z* 579.2633 $[M+H]^+$). In comparison to **2** it was clear that the second compound possessed one additional degree of unsaturation, an additional carbonyl group, and a methyl ester. Using these and a similar set of search seeds discussed above for the dereplication pinpointed purpurin 18 methyl ester (**3**)[11] as the compound isolated.



pyropheophorbide *a* (**2**)



purpurin 18 methyl ester (**3**)

The pure compounds and fractions were each subjected to the kinase assay and the resulting data is shown in Scheme 1. Interestingly, only two of the fractions were less active than the parent fraction S8-12 (IC_{50} <5 μg/mL) while most displayed activity (IC_{50} 1–3 μg/mL) on par with that of the parent. Only slightly different IC_{50} values were observed for **2** and **3**, consistent with their overall similar structures. In view of this circumstance efforts to establish the structures of the compounds in the other fractions were abandoned, because very little new SAR information would be obtained. The final task in this segment of the project was to shuttle **2**, the most abundant of the active samples, into a secondary screen that utilized a panel of 85 kinase enzymes covering a diverse range of biochemical pathways. The results shown in Figure 1 revealed, relative to the control: (a) excellent selectivity (>80% inhibition) against the three enzymes Aurora-A, PRAK, and Syk; (b) 62% of the kinases were moderately inhibited, and (c) enhanced activity was observed for 10 kinases. Significantly, one of the most inhibited enzymes was PRAK (p38 regulated activated kinase), which has shown connection to the downstream signal of the p38 pathway in regulating cytoskeletal organization.[12] An 82% inhibition was observed against Syk (serine/threonine kinase), which has been implicated as a tumor suppressor in human breast carcinomas.[13] A similar 82% inhibition was observed against Aurora-A (a member of the aurora/Ip11p family), which is known to be overexpressed in both solid tumor cancers as well as leukemia.[14] These observations provide an interesting contrast to the selectivity observed for Gleevec (**1**), which exhibits 20% or greater inhibition against only 9% of the kinases of Figure 1, none of which overlap with those discussed above for **2**.

Another very different compound type was encountered as an offshoot of the isolation work on coll. no. 00369 (see Scheme 1). During the Kupchan style partitioning, an orange emulsion was often observed between the hexane and methanol–water layers. The emulsion layer was scrutinized further by drying and re-suspension in organic solvent. Surprisingly, the ^1H (5 methyl singlets) and ^{13}C NMR (20 signals including an oxygenated methine at δ_c 65.2) spectra indicated a pure compound had been obtained. These data along with the molecular formula of $C_{40}H_{56}O_2$ established from HRFAB (*m/z* 568.4236 $[M+H]^+$) indicated this material to be a diterpene with C_2 symmetry. The structure was quickly

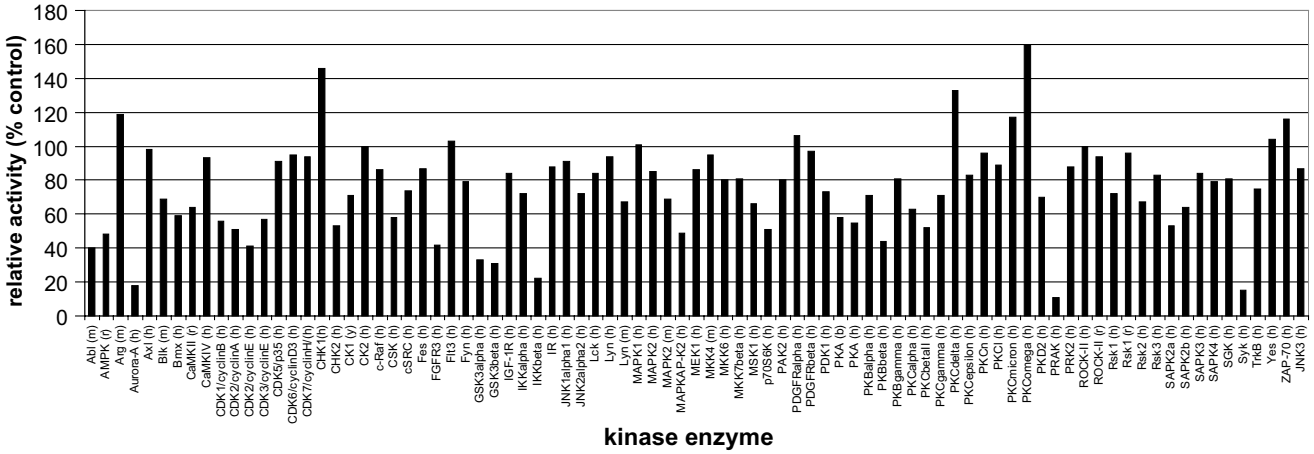
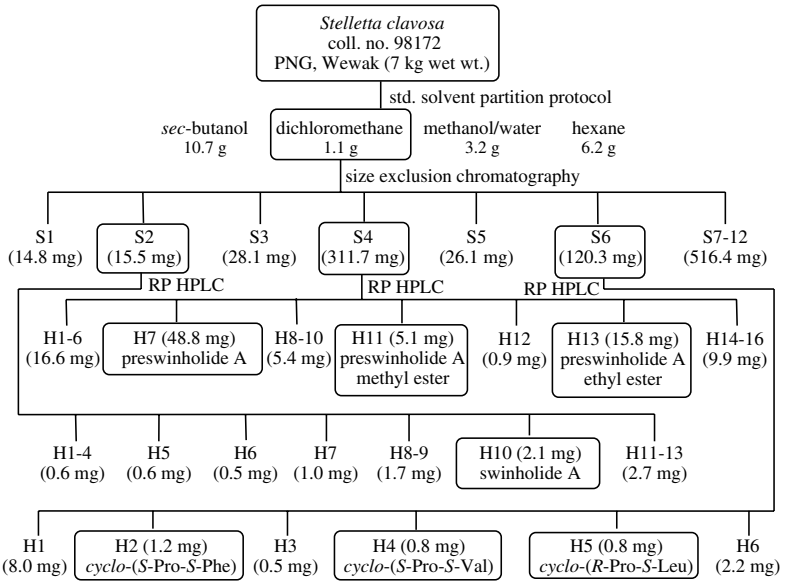


Figure 1. Relative activity for **2** against 85 kinases.



Scheme 2. The isolation of swinholide and diketopiperazine type compounds.

established as 3*R*,3′*R*-zeaxanthin.[15] The occurrence of carotenoids and chlorophyll derivatives is often observed during phytochemistry research and also known for selected bacterial microorganisms.[16] Such a situation is unknown in sponge natural products chemistry. Furthermore, 3*R*,3′*R*-zeaxanthin was found in abundance (>100 mg) and as the sole carotenoid.

The second phase of this study involved an investigation of a different collection of *S. clavosa* (coll. no. 98172) obtained from the Wewak region of Papua New Guinea. As shown in Table 1, this sponge was selected for intensive isolation work because the spectroscopic properties indicated it to be unusually diverse in constituents. A modified Kupchan style partition was then used to obtain five extracts, of which four are shown in Scheme 2. The dichloromethane fraction displayed a rich array of resonances and was selected for the further isolation efforts as outlined in Scheme 2. Especially noteworthy in this fraction were ^{1}H NMR peaks in the δ_H 7.1–δ 7.3 region and ESI MS *m*/*z* ions in the 712–750 and 1380–1410 amu regions. The compounds responsible for these data were purified during a series of chromatographic procedures beginning with a size exclusion column, which afforded seven fractions.

The next stage of purification consisted of reversed-phase HPLC on fraction S2. This yielded swinholide A[17] (2.1 mg, $C_{78}H_{132}O_{20}$), which was indicated by ESIMS signals at *m*/*z* 1411.9 $[M+Na]^+$, 1388.2, $[M-H]^-$, and 717.4 $[M+2Na]^{++}$. Distinguishing among the three swinholide analogs of this molecular formula, swinholide A, swinholide F, and isoswinholide A, was confidently accomplished by employing 1D NMR data.[18]

Reversed-phase HPLC on fraction S4 afforded preswinholide A[19] (48.8 mg, $C_{39}H_{68}O_{11}$ ESIMS *m*/*z* 713.4 $[M+H]^+$, 735.4 $[M+Na]^+$), preswinholide A methyl ester[20] (5.1 mg, $C_{40}H_{70}O_{11}$ ESIMS *m*/*z* 727.4 $[M+H]^+$) and preswinholide A ethyl ester[21] (15.8 mg $C_{41}H_{72}O_{11}$ ESIMS *m*/*z* 741.4 $[M+H]^+$). These structures were confirmed by comparing their spectral data to the published literature. The swinholides were first isolated from the sponge *Theonella swinhoei* in 1985[22] and subsequently found from other sponges including *Psammocinia* sp.,[23] and *Lamellomorpha strongylata*.[24] While the swinholides are well known to bind two monomers of actin,[25] none of the analogs isolated here were active in the kinase primary screen.

The later eluting fraction S6 contained ^{1}H NMR spectral peaks characteristic of small peptides. Reversed-phase HPLC was used to separated three compounds which were determined to be the following diketopiperazines: *cyclo*-(*S*-Pro-*S*-Phe) $[\alpha]_D$ −48, *cyclo*-(*S*-Pro-*S*-Val) $[\alpha]_D$ −140, and *cyclo*-(*R*-Pro-*S*-Leu) $[\alpha]_D$ +35. Formulas for each of $C_{14}H_{16}N_2O_2$ (*m*/*z* 245.1290 $[M+H]^+$), $C_{10}H_{16}N_2O_2$ (*m*/*z* 197.1279 $[M+H]^+$), and $C_{11}H_{18}N_2O_2$ (*m*/*z* 211.1439 $[M+H]^+$) were established from HRESI-TOFMS. The absolute stereochemistry of these diketopiperazines was determined by comparing trends in optical rotation data.[26] The potential of the diketopiperazines to be kinase inhibitors was not investigated as none of the parent fractions were active in the kinase primary screen.

3. Conclusions

The results reported above demonstrate that c-Raf-1 kinase assay is a useful discovery tool to guide the fractionation of complex mixtures of diverse small biomolecules. In addition we have shown the value of utilizing the c-Raf-1 kinase screen as a filter to select compounds for a more detailed secondary screen to evaluate for selectivity. Thus, the discovery of kinase selectivity for pyropheophorbide *a* (**2**) greatly adds to the dimension of biological properties known for compounds with a

highly modified porphyrin core. Our data complements the vast literature pertaining to the biological activity of porphyrin-containing compounds. For example, the FDA has approved Photofrin, a mixture of porphyrin oligomers, for use to treat certain lung cancers.[27] Less complex porphyrin-containing compounds are now being investigated as photosensitizers in photodynamic therapy (PDT) to treat solid tumors.[28] Porphyrin derivatives have also been reported to possess antioxidative properties[11] as well as photo-independent cytotoxic activity against a variety of cancer cells.[29] We believe that the results presented herein will stimulate additional attention on **2** congeners as molecular tools or therapeutic leads. The discovery of a broad array of chemotypes from our collections of *Stelletta clavosa*, including porphyrin derivatives, swinholides, zeaxanthin, and diketopiperazines, provides an interesting contrast to the previous findings involving this sponge. Further examination of this organism with regard to determining the role of the associated microbial community in producing secondary metabolites is warranted. For example, such investigations would provide further insights about the role of nonsponge cells in the production of swinholides gained through cell separation experiments using the sponge *Theonella swinhoei*.[30]

4. Experimental

4.1. General experimental procedures

Optical rotations were obtained on a JASCO DIP-370 digital polarimeter. UV/vis measurements were recorded on a HP 8453 diode array spectrometer. The NMR spectra were recorded on a Varian UNITY INOVA-500 spectrometer, operating at 500 and 125.7 MHz for ^{1}H and ^{13}C, respectively. High-resolution mass measurements were obtained on a bench-top Mariner ESI-TOF mass spectrometer. HPLC was performed with columns of 5 μm ODS. Chemicals for the c-Raf-1 kinase ELISA were purchased from Sigma–Aldrich unless otherwise indicated.

4.2. Biological material, collection, and identification

Specimens of *S. clavosa* were collected from 14 different sites throughout various Indo-Pacific regions including the Solomon Islands (coll. nos. 89020 and 94036, Gizo), Indonesia (coll. nos. 94525, Sangihe Is.; 95585, Togian Is.; and 96587, N. Sulawesi), and Papua New Guinea (coll. nos. 90119, 91141, 91149, 02128, and 02156, Milne Bay; 95004, Wahoo and Milne Bay; 98172, and 01315, Wewak; 00369, Rabaul). These sponges were identified as *S. clavosa* (Family: Stellettidae, Order: Astrophorida) by M. C. Diaz (UCSC, IMS). The voucher samples and the underwater photographs of the sponges are available from the Crews laboratory.

4.3. Extraction and isolation

The extractions and compound isolations of the two most representative specimens, coll. nos. 98172 and 00369, are detailed as follows.

4.4. Coll. no. 00369

The sponges (8.0 kg wet wt.) were soaked in MeOH (3 L × 3). The resulting residue was partitioned between H_2O and CH_2Cl_2. The CH_2Cl_2 portion was dried and partitioned between hexanes and MeOH–H_2O (9:1). A metallic red-orange emulsion was present between the hexane and MeOH–H_2O. This was isolated by drainage of the bottom layer and decanting of the top layer to afford 3*R*,3′*R*-zeaxanthin (132 mg) in greater than 95% purity. The MeOH–H_2O (9:1) portion was adjusted to 1:1 by addition of H_2O and subsequently extracted with CH_2Cl_2 three times to give the crude extract (3.1 g). The crude extract (2.8 g) was applied to a size exclusion column (Sephadex LH-20) using 100% MeOH as the eluent to afford 13 fractions (Scheme 1). S8-12 were combined and purified by HPLC using MeOH–H_2O (85:15 gradient up to 100:0) to afford **2** (4.2 mg) and **3** (1.0 mg).

4.5. Coll. no. 98172

The sponges (7 kg wet wt.) were soaked in MeOH three times. The resulting residue was partitioned between H_2O and CH_2Cl_2. The CH_2Cl_2 portion was dried and partitioned between hexanes and MeOH–H_2O (9:1). The MeOH–H_2O (9:1) was adjusted to 1:1 by addition of H_2O and subsequently extracted with CH_2Cl_2 three times to give the crude extract (7.2 g). The crude extract (1.1 g) was applied to a size exclusion column (Sephadex LH-20) using MeOH–CH_2Cl_2 (1:1) as the eluent to afford six fractions (Scheme 2). S2 was purified by HPLC using MeOH–H_2O (85:15 gradient up to 100:0) to afford swinholide A (2.1 mg). S4 was purified HPLC using MeOH–H_2O (65:35 gradient up to 100:0) to afford preswinholide A (48.8 mg), preswinholide A methyl ester (5.1 mg), and preswinholide A ethyl ester (15.8 mg). S6 was purified HPLC using MeCN–H_2O (15:85 gradient up to 100:0) to afford *cyclo*-(*S*-Pro-*S*-Phe) (1.2 mg), *cyclo*-(*S*-Pro-*S*-Val) (0.8 mg), and *cyclo*-(*R*-Pro-*S*-Leu) (0.8 mg).

4.6. Compound identification

Pyropheophorbide *a* (**2**),[10] purpurin 18 methyl ester (**3**),[11] zeaxanthin,[15] swinholide A,[17] preswinholide A,[19] preswinholide A methyl ester,[20] preswinholide A ethyl ester[21] *cyclo*-(*S*-Pro-*S*-Phe),[26] *cyclo*-(*S*-Pro-*S*-Val),[26] and *cyclo*-(*R*-Pro-*S*-Leu)[26] were identified by comparison of their spectral data to published data.

4.7. Pyropheophorbide *a* (2)

Dark solid; $[\alpha]_D$ +67 (*c* 0.4, $CHCl_3$, 25 °C); UV (MeOH) λ_{max} 273 nm (ε 36,100), 327 (ε 21,500), 409 (ε 11,500), 665 (ε 4500); HRESIMS 535.2723 $[M+H]^+$ = $C_{33}H_{35}N_4O_3$ (Δ +1.9 mmu of calcd).

4.8. Purpurin 18 methyl ester (3)

Dark purple-red solid, $[\alpha]_D$ −228 (*c* 0.09, $CHCl_3$, 25 °C); UV (MeOH) λ_{max} 358 nm (ε 13,800), 403 (ε 31,600), 503 (ε 2600), 545 (ε 2800), 664 (ε 6400); HRESIMS 579.2633 $[M+H]^+$ = $C_{34}H_{35}N_4O_5$ (Δ +3.1 mmu of calcd).

4.9. Zeaxanthin

Yellow solid; $[\alpha]_D$ −36 (*c* 0.29, $CHCl_3$, 25 °C); UV (EtOH) λ_{max} 454 (ε 5100), 482 (ε 2300); HRFABMS 568.4236 $[M+H]^+ = C_{40}H_{57}O_2$ (Δ −11.7 mmu of calcd).

4.10. Swinholide A

White solid; $[\alpha]_D$ +5 (*c* 0.09, MeOH, 25 °C); UV (MeOH) λ_{max} 269 (ε 9000); ESIMS 1411.9 $[M+Na]^+$, 1388.2 $[M-H]^-$.

4.11. Preswinholide A

White solid; $[\alpha]_D$ −32 (*c* 0.14, MeOH, 25 °C); UV (MeOH) λ_{max} 269 (ε 7000); ESIMS 713.4 $[M+H]^+$, 735.4 $[M+Na]^+$, 1447.9 $[2M+Na]^+$.

4.12. Preswinholide A methyl ester

White solid; $[\alpha]_D$ −34 (*c* 0.1, MeOH, 25 °C); UV (MeOH) λ_{max} 269 (ε 7000); ESIMS 727.4 $[M+H]^+$, 749.4 $[M+Na]^+$, 1475.9 $[2M+Na]^+$.

4.13. Preswinholide A ethyl ester

White solid; $[\alpha]_D$ −38 (*c* 0.09, MeOH, 25 °C); UV (MeOH) λ_{max} 269 (ε 7000); ESIMS 741.4 $[M+H]^+$.

4.14. *cyclo*-(*S*-Pro-*S*-Phe)

White solid; $[\alpha]_D$ −48 (*c* 0.16, EtOH, 25 °C); HRESIMS 245.1290 $[M+H]^+ = C_{14}H_{17}N_2O_2$ (Δ +0.6 mmu of calcd).

4.15. *cyclo*-(*S*-Pro-*S*-Val)

White solid; $[\alpha]_D$ −140 (*c* 0.11, EtOH, 25 °C); HRESIMS 197.1279 $[M+H]^+ = C_{10}H_{17}N_2O_2$ (Δ −0.6 mmu of calcd).

4.16. *cyclo*-(*R*-Pro-*S*-Leu)

White solid; $[\alpha]_D$ +35 (*c* 0.11, EtOH, 25 °C); HRESIMS 211.1439 $[M+H]^+ = C_{14}H_{17}N_2O_2$ (Δ −0.2 mmu of calcd).

4.17. c-Raf-1 kinase ELISA

Nunc Maxisorp Immuno plates (Nunc No 439454) were coated with IκBα (diluted in DPBS) overnight at 4 °C. Nonspecific binding (NSB) control wells received DPBS alone. Coated plates were washed × 4 with TBST (25 mM Tris, pH 8.0, 150 mM NaCl, and 0.05% Tween-20) and 150 μL of Superblock (Pierce No 37515) or blocking buffer (5% BSA, 50 mM Tris, pH 8.0, 150 mM NaCl, and 0.1% Tween-20) was added to each well. Nonspecific sites were blocked for at least 3 h at rt and plates were washed × 4 with TBST. c-Raf-1 kinase, diluted in assay buffer (50 mM Hepes, 10 mM $MgCl_2$, 10 mM $MnCl_2$, 0.5% BSA, 1 mM DTT, 0.01 mM ATP) and test compounds were added to wells (to a final volume of 50 μL per well, 10 μg/mL of extract or 10 μM for pure compounds). The reaction proceeded for $2\frac{1}{2}$ h at rt and was stopped by washing × 7 with TBST. Anti-IκBα [$pSpS^{32/36}$] phosphospecific antibody (Biosource No 44–726), diluted in blocking buffer, was added to wells (70 μL/well) and plates were incubated overnight at 4 °C. Plates were washed × 7 with TBST and goat anti-rabbit IgG conjugated with alkaline phosphatase (Pierce No 31340), diluted in blocking buffer, was added to wells (70 L per well). Following a 2 h incubation at room temperature, plates were washed × 7 with TBST. Attophos substrate (JBL Scientific No 1670A), dissolved to 6 mg/mL in Attophos buffer (2.4 M diethanolamine, 5.5 mM $MgCl_2$, pH 8.8), was added to wells (100 μL/well). Plates were read in a CytofluorII plate reader (PerSeptive Biosystems) with an excitation wavelength of 450 nm and emission wavelength of 580 nm.

4.18. Aurora-A (h)

In a final reaction volume of 25 μL, Aurora-A (h) (5–10 mU) is incubated with 8 mM MOPS pH 7.0, 0.2 mM EDTA, 200 M LRRASLG (Kemptide), 10 mM MgAcetate and [γ-33P-ATP] (specific activity approx. 500 cpm/pmol,concentration as required). The reaction is initiated by the addition of the MgATP mix. After incubation for 40 min at room temperature, the reaction is stopped by the addition of 5 μL of a 3% phosphoric acid solution. 10 μL of the reaction is then spotted onto a P30 filtermat and washed three times for 5 min in 50 mM phosphoric acid and once in methanol prior to drying and scintillation counting.

4.19. PRAK (h)

In a final reaction volume of 25 μL, PRAK (h) (5–10 mU) is incubated with 50 mM Na-β-glycerophosphate pH 7.5, 0.1 mM EGTA, 30 μM KKLRRTLSVA, 10 mM MgAcetate and [γ-33P-ATP] (specific activity approx. 500 cpm/pmol, concentration as required). The reaction is initiated by the addition of the MgATP mix. After incubation for 40 min at room temperature, the reaction is stopped by the addition of 5 μL of a 3% phosphoric acid solution. 10 μL of the reaction is then spotted onto a P30 filtermat and washed three times for 5 min in 50 mM phosphoric acid and once in methanol prior to drying and scintillation counting.

4.20. Syk (h)

In a final reaction volume of 25 μL, Syk (h) (5–10 mU) is incubated with 50 mM Tris pH 7.5, 0.1 mM EGTA, 0.1 mM Na_3VO_4, 0.1% β-mercaptoethanol, 0.1 mg/mL poly(Glu, Tyr) 4:1, 10 mM MgAcetate and [γ-33P-ATP] (specific activity approx. 500 cpm/pmol, concentration as required). The reaction is initiated by the addition of the MgATP mix. After incubation for 40 min at room temperature, the reaction is stopped by the addition of 5 μL of a 3% phosphoric acid solution. 10 μL of the reaction is then spotted onto a Filtermat A and washed three times for 5 min in 75 mM phosphoric acid and once in methanol prior to drying and scintillation counting.

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