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Asterriquinones Produced by *Aspergillus candidus* Inhibit Binding of the Grb-2 Adapter to Phosphorylated EGF Receptor Tyrosine Kinase

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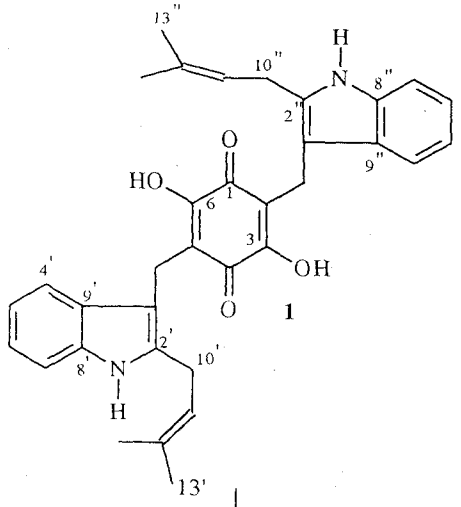
Five new asterriquinone analogs (**2**~**4**, **6**, **7**), together with previously identified neoasterriquinone (**1**) and isoasterriquinone (**5**), were isolated from a fermentation broth of the fungus *Aspergillus candidus* and purified by HSCCC (high speed counter current chromatography) followed by HPLC. The structures were determined by 1D and 2D NMR and MS/MS techniques. All seven showed inhibitory activity against the binding of a recombinant protein containing the SH2 protein domain of Grb-2 to the tyrosine phosphorylated form of the EGF receptor tyrosine kinase. Some of these asterriquinones exhibited specific inhibition of Grb-2 binding compared to Grb-7 and PLC-γ.

In this present study, we sought to identify non-peptidic inhibitors of the Grb-2 adapter protein. Grb-2 has been shown to bind to various tyrosine phosphorylated substrates in the cell through interactions that are mediated by interaction of the single SH2 (*src* homology region-2) domain of Grb-2 with tyrosine phosphorylated substrates[1]. In this regard, receptor tyrosine kinase, Shc, and other proteins have been shown to bind to Grb2 following activation of signal transduction following receptor stimulation[2]. In human cancers, Grb2 is viewed as an attractive therapeutic target since it has been shown to bridge the activation of receptor tyrosine kinase and adapters (*e.g.*, Shc) to the $p21^{ras}$ signal transduction pathway. Grb-2 has been shown to tether the tyrosine kinase through its SH2 interaction and connect to $p21^{ras}$ through interactions between its two Grb-2 SH3 (*src* homology region-3) domains and the SOS or guanine-nucleotide exchange protein[3]. Recently, a Grb-2 specific peptide that blocks SH2 binding has been shown to be an effective anti-tumor agent in animal studies.

Extracts derived from natural products have been instrumental in the identification of inhibitors of kinases involved in signal transduction including staurosporine, erbstatin, lavendustin A, genistein, halenaquinone, and penazetidine A[4~6]. In the area of tyrosine kinases, staurosporine has been useful to design synthetic tyrosine kinase inhibitors[7]. Similarly, the identification of inhibitors that block the binding of SH2 proteins to tyrosine phosphorylated proteins has been the focus of much attention[8,9]. Most recent efforts have led to the design of peptidomimetic ligands using tyrosine phosphorylated peptide substrates and structural information deduced from co-crystallographic analysis with various SH2 containing proteins[10,11]. However, few studies have identified non-peptidic SH2 ligands with good cell permeability and other pharmacologic features[12].

In order to identify Grb-2 inhibitors, we have developed an ELISA-based assay that utilizes the tyrosine phosphorylated form of the EGF receptor tyrosine kinase. Following immunolocalization of the receptor, the binding of a protein containing the Grb-2 SH2 domain and the GST (glutathione *S*-transferase) protein



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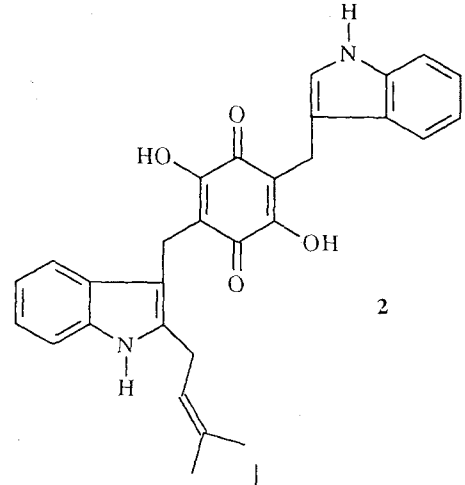

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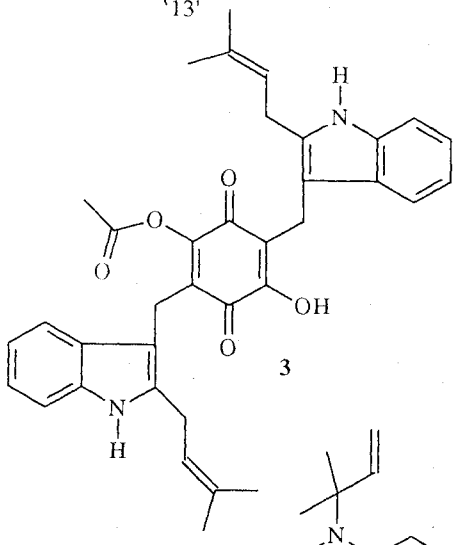

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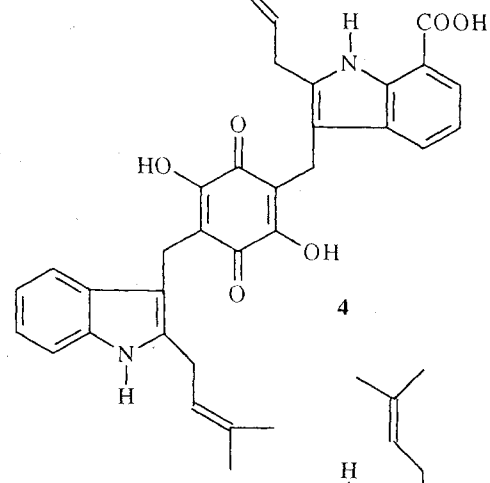

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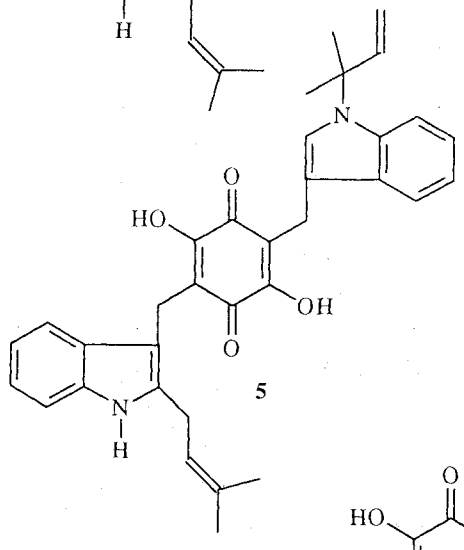

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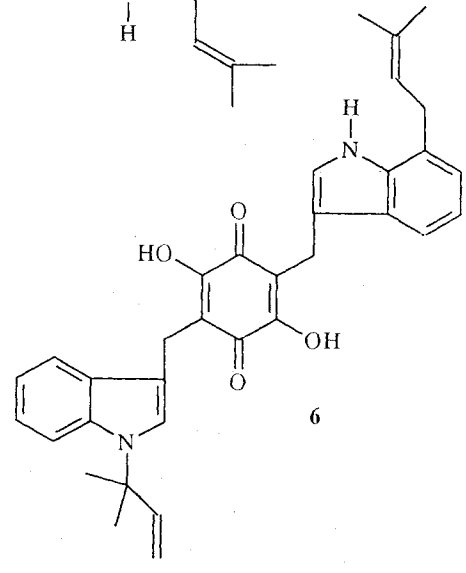

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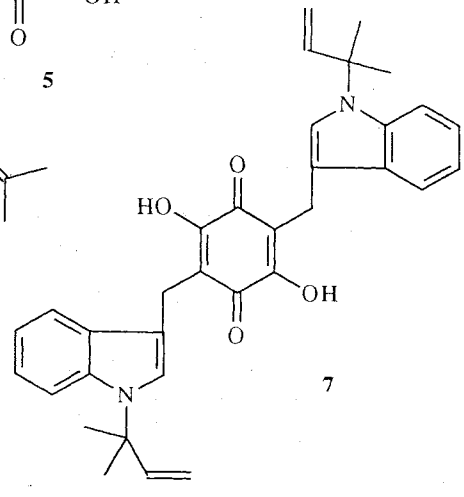

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could be measured using an anti-GST antibody detection system that utilizes HRP (horse-radish peroxidase). This assay was optimized for use in 96-well microtiter plate formats and used to process ethyl acetate extracts from microbial sources. As a result of these efforts, a single fungal (*Aspergillus candidus*) extract was discovered which exhibited inhibitory activity using the Grb-2 binding assay. Subsequently, seven structurally-related tryptophan-derived benzoquinone pigments (**1**~**7**) were isolated using bioassay-guided isolation procedures. Presented in this report are the details of the isolation and structure elucidation of these compounds along with some of their biological activities.

Materials and Methods

General Experimental Procedures

Spectral Analysis

^{1}H and ^{13}C NMR spectra were recorded at 300 K on a Bruker AC 300 spectrometer operating at 300 MHz and 75 MHz, respectively. The samples for NMR characterization were dissolved in chloroform-*d* at a concentration of approximately 5~10 mg/ml. Mass and MS/MS spectra were obtained on a PE Sciex API III triple-quadruple mass spectrometer interfaced with a Sciex Ion-Spray probe. Exact mass measurements were performed on a VG 70SEQ spectrometer at high resolution (HRFAB) using PEG300/thiogly as an internal standard. UV spectra were run on a Perkin-Elmer Lambda 6 spectrometer. IR spectra were recorded on a Perkin-Elmer FTIR 1600 spectrometer. Optical rotations were measured with a Perkin-Elmer 243B polarimeter in $CHCl_3$.

Microorganism

The fungal strain #592 was isolated from a soil sample obtained from Las Vegas, Nevada. Colonies on yeast-malt extract agar containing trace elements attained a diameter of approximately 3.5 cm in 8 days of dark incubation at 25°C and 70% relative humidity. Colonies were typically round, voluminous with a filamentous margin. Conidia were pale tannish brown, mycelium white, reverse brownish orange, yellow soluble pigment and clear exudate. The fungus was identified as *Aspergillus candidus*.

Fermentation

The fermentation procedure was a two-step process in which the suspension of spores and mycelium was inoculated into a 250 ml Erlenmeyer flask containing 30 ml of a nutrient seed medium composed of: glucose 20 g, Pharmamedia (Trader's Protein) 15 g, ammonium sulfate 3 g, zinc sulfate 0.03 g, calcium carbonate 4 g and yeast extract (Difco) 5 g, per liter final volume. After inoculation, the flasks were incubated with agitation at 250 rpm, at 28°C and 70% relative humidity for two days. After incubation, a two ml aliquot of the seed culture was used to inoculate flasks (250 ml Erlenmyer flask with 30 ml medium) of a production medium having a composition of: mannitol 60 g, soybean meal 12.5 g, citric acid 2.5 g, yeast extract (Difco) 0.5 g, per liter final volume. After inoculation, the flasks of production medium were incubated with agitation at 28°C for six days. The agitation rate was 250 rpm. After incubation, the fermentation flasks were harvested and the fermentation mixture from each flask was pooled into a single vessel for extraction.

Extraction and Isolation

The pooled fermentation mixture (40 liter) was homogenized and then extracted with 3 equal volumes of EtOAc. The combined extracts were evaporated under reduced pressure to dryness to yield an oily residue (22,169 mg). This oily residue was fractionated by dual mode high-speed countercurrent chromatography (PC Inc., Potomac, MD, U.S.A.). Solvent and full chromatographic conditions used were previously described.[7] The activity was concentrated into peaks eluting at 21~33 minutes (fractions 7~21). The active fractions were pooled and evaporated under reduced pressure to dryness (1,244 mg).

Final purification was achieved using semi-preparative HPLC. The chromatography system consisted of a Waters HPLC equipped with a Waters 600 system controller and a Waters 996 photodiode array detector. Separation was achieved on two semi-preparative C_{18}-cartridges connected in series (25 × 100 mm, Novapak 6 micron). The mobile phase was pumped as a binary system at a rate of 10 ml/minute and consisted of a 35 minutes linear gradient, starting with 45% H_2O (0.05 TFA), 55% CH_3CN to 10% H_2O (0.05 TFA), 90% CH_3CN in 30 minutes and ending with 100% CH_3CN in 5 minutes. Seven active fractions were identified and they were concentrated under reduced pressure to dryness; fractions 1, 3 and 4 afforded three pure compounds: asterriquinone SU5228 (**2**), neoasterriquinone SU5220 (**1**) and asterriquinone SU5500 (**3**) respectively. Fractions 2, 5, 6 and 7 still contained impurities therefore, they

were purified further by semi-preparative HPLC using modified conditions. Asterriquinone SU5503 (**4**) and asterriquinone SU5501 (**6**) were purified from fractions 2 and 5, respectively, using a linear gradient, starting with 40% H_2O (0.05 TFA), 60% CH_3CN and ending with 100% CH_3CN in 30 minutes. While isoasterriquinone SU5220 (**5**) and asterriquinone SU5504 (**7**) were purified from fractions 6 and 7, respectively, using a starting solvent system 35% H_2O (0.05 TFA), 65% CH_3CN and ending with 100% CH_3CN in 30 minutes.

Preparation of Cell Lysates and GST Fusion Proteins

Cellular lysates were prepared from confluent A431 (ATCC # CRL-1555) cells grown in Dulbecco's Modified Eagle's Medium (BRL-Gibco) containing 10% fetal calf serum. Cells were lysed using HNTG buffer (20 mM HEPES pH 7.5, 150 mM NaCl, 0.1% Triton X-100, 10% glycerol). A protein containing a fusion of GST to the SH2 domain of Grb-2 (SH2-Grb-2GST) was prepared in bacteria[1]. Large-scale cultures were grown, induced with 0.25 μM isopropyl β-D-thiogalactopyranoside, and the GST fusion proteins purified using glutathione-agarose beads (Pharmacia) as previously described[13].

Grb-2 Binding Assay

EGF receptor was derived from A431 cell lysates and was localized to each well of a 96-well microtiter polystyrene plate following coating of an anti-EGF receptor monoclonal antibody. After antibody coating, the plates were blocked for 30 minutes with 5% nonfat dry milk in phosphate-buffered saline (PBS: 150 μM NaCl, 16 μM Na_2HPO_4, 4 μM NaH_2PO_4, pH 7.4), washed three times with de-ionized water, and then once with TBST (50 μM Tris-HCl, pH 7.2, 150 μM NaCl, 0.1% Triton X-100). 7.0 μg of A431 cell lysate was added per well. After 30 minutes incubation, the plate was washed three times with water and once with TBST buffer. The immuno-immobilized EGF receptor was phosphorylated for 5 minutes using TBST buffer containing 3 μM ATP and 5 mM $MnCl_2$. Following receptor phosphorylation, EDTA (pH 8.0) was added to a final concentration of 20 mM. The phosphorylated receptor was washed three times with water and once with TBST. Using a separate polypropylene microtiter plate, SH2-Grb-2-GST protein was incubated with compounds resuspended in TBST buffer containing 1% DMSO into which the compounds were diluted. The SH2-Grb-2-GST protein that was pre-incubated with compounds were then added to the phosphorylated EGF receptor and incubated for 30 minutes at room temperature. The receptor complexes in the 96-well microtiter plate were washed three times with water, once with TBST buffer, and then incubated 30 minutes at room temperature with an affinity-purified rabbit anti-glutathione-*S*-transferase (anti-GST) polyclonal antibody (AMRAD Corporation, Australia, Product #90001605). After three washes with de-ionized water followed by one wash with TBST, goat anti-rabbit IgG peroxidase conjugated antibody (Biosource International, Camarillo, CA, Product #6430) was added and incubated for 30 minutes. After washing five times with water, the development of peroxidase activity was performed using 100 μM ABTS buffer (0.5 mg/ml 2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonic acid, 250 μM Na_2HPO_4/100 μM citrate pH 4.0, 0.004% H_2O_2). Microtiter plates were then read at 410 nm for absorbance using a Dynatech MR7000 ELISA plate reader. Control plates utilized a tyrosine phosphorylated peptide CDPS(pY)VNVNQ to block Grb-2-SH2 binding and use of non-phosphorylated EGF receptor to ensure that the binding was phosphorylation-dependent.

Grb-2 Immunoblotting Assay

Protein A agarose (Boehringer Mannheim, Product #1134515) was pre-incubated with 1 μg of anti-EGF receptor monoclonal antibodies for 20 minutes at room temperature followed by washing 3 times in HNTG buffer. The washed beads were then incubated for 90 minutes at 4°C with 100 μl of lysate derived from NIH 3T3 mouse fibroblasts overexpressing the human EGF receptor. The beads were washed three times with HNTG buffer after which ATP (30 μM final concentration) and $MnCl_2$ (5 mM final concentration) were added and incubated for 20 minutes at room temperature. The incubated receptor complexes were washed three times with HNTG buffer and resuspended in 150 μl of HNTG buffer with protease inhibitors (leupeptin, PMSF, aprotinin). Pre-incubated GST fusion proteins containing SH2 domains derived from Grb-2, Grb-7, and PLC-γ were prepared by incubation of proteins for 2 hours at room temperature in the presence of compounds at a final concentration of 100 μM following resuspension of compounds in DMSO. The final concentrations of fusion proteins were 0.1 μg/reaction for Grb-2 and 0.5 μg/reaction for Grb-7 and PLC-γ. After incubation of the fusion proteins with receptor for 90 minutes at 4°C, the beads were washed three times in HNTG buffer, boiled in sample buffer, and analyzed by 10% PAGE followed by visualization of tyrosine phosphorylated proteins using commercially-available antibodies, exposure, and de-

velopment of autoradiographic film.

Neoasterriquinone SU5220 (**1**): Purple powder (97.3 mg); UV λ_{max} 260, 279, 290 and 492 nm; IR ν_{max} 3404, 3316, 2912, 1644 cm^{-1}; ^1H NMR 7.31 (m, H-4′ and 4″), 7.13 (m, H-5′ and 5″), 7.13 (m, H-6′ and 6″), 7.31 (m, H-7′ and 7″), 3.45 (m, H-10′ and 10″), 5.40 (m, H-11′ and H-11″), 1.75 (s, H-13′ and 13″), 1.81 (s, H-14′ and 14″), 8.10 (s, NH), 8.05 (br s, OH); ^{13}C NMR (Table 1); Ion-spray MS (positive): *m/z* 507 ($M^+ + H$); HRFAB-MS: calcd. for $C_{32}H_{31}N_2O_4$ ($M^+ + H$) 507.2122; found: *m/z* 507.2291.

Asterriquinoe SU5228 (**2**): Purple powder (37 mg), mp 150~154; UV λ_{max} 260, 285, 290 and 489 nm; IR ν_{max} 3415, 3320, 2912, 1634 cm^{-1}; ^1H NMR 7.48 (d, J=1 Hz, H-2″), 7.39 (d, J=10 Hz, H-4′ and H-7′), 7.32 (d, J=10 Hz, H-4″), 7.07 (t, J=10 Hz, H-5′), 6.98 (t, J=10 Hz, H-5″), 6.95 (t, J=10 Hz, H-6′), 6.88 (t, J=10 Hz, H-6″), 7.17 (d, J=10 Hz, H-7″), 3.44 (m, H-10′), 5.39 (m, H-11′), 1.80 (s, H-13′), 1.73 (s, H-14′), 10.96 (s, NH), 11.35 (s, NH), 10.62 (br s, OH); ^{13}C NMR (Table 1); Ion-spray MS (positive): *m/z* 439 ($M^+ + H$); HRFAB-MS: calcd. for $C_{27}H_{22}N_2O_4$ ($M^+ + H$) 439.2289; found: *m/z* 439.2291.

Asterriquinone SU5500 (**3**): Purple powder (2.6 mg); UV λ_{max} 225, 285, 292 and 487 nm; IR ν_{max} 3404, 3316, 2912, 1764, 1644, 1610 cm^{-1}; ^1H NMR 7.28 (m, H-4′ and 4″), 7.09 (m, H-5′ and 5″), 7.09 (m, H-6′ and 6″), 7.28 (m, H-7′ and 7″), 3.44 (m, H-10′ and 10″), 5.39 (m, H-11′ and H-11″), 1.75 (s, H-13′ and 13″), 1.81 (s, H-14′ and 14″), 2.66 (s, *Me*CO), 8.17 (br s, NH), 8.24 (br s, OH); ^{13}C NMR (Table 1); Ion-spray MS (positive): *m/z* 549 ($M^+ + H$); HRFAB-MS: calcd. for $C_{34}H_{33}N_2O_5$ ($M^+ + H$) 549.2211; found: *m/z* 549.2181.

Asterriquinone SU5503 (**4**): Purple powder (4 mg); UV λ_{max} 260, 282, 294 and 479 nm; IR ν_{max} 3414, 3306, 2912, 1641 cm^{-1}; ^1H NMR 7.10 (m, H-5′, 6′), 7.16 (m, H-5″), 7.31 (d, J=10 Hz, H-4″), 7.40 (d, J=10 Hz, H-6″), 7.46 (d, J=10 Hz, H-7′, 4′), 3.47 (m, H-10′ and 10″), 5.36 (m), 1.71 (s), 1.82 (s), 8.17 (bs), 8.29 (bs); ^{13}C NMR (Table 1) Ion-spray MS (positive): *m/z* 551 ($M^+ + H$).

Isoasterriquinone SU5220 (**5**): Purple powder (6 mg); UV λ_{max} 225, 283, 289 and 480 nm; IR ν_{max} 3420, 3318, 2912, 1634 cm^{-1}; ^1H NMR 7.72 (s), 7.57 (dd, J=9.0, 2.3 Hz), 7.62 (dd, J=8.97, 3.3 Hz), 7.15 (m), 7.31 (t, J=8.97 Hz), 7.15 (m), 7.31 (t, J=8.97 Hz), 7.09 (d, J=8.7 Hz), 7.11 (d, J=8.97 Hz), 3.44 (m), 5.39 (m), 1.73 (bs), 1.80 (bs), 6.20 (dd, J=17.43, 10.72 Hz), 5.25 (d, J=10.71 Hz), 5.22 (d, J=17.42 Hz), 1.82(s), 1.82(s), 8.17 (s), 8.0 (s); ^{13}C NMR (Table 1); Ion-spray MS (positive): *m/z* 507 ($M^+ + H$).

Asterriquinone SU5501 (**6**): Purple powder (6 mg); UV λ_{max} 260, 285, 290 and 489 nm; IR ν_{max} 3404, 3316, 2912, 1644 cm^{-1}; ^1H NMR 7.72 (s, H-2″), 7.57 (d, J=8.4 Hz, H-7′, 4′), 7. 51 (d, J=8.2 Hz, H-6″), 7.32 (d, J=8.2 Hz, H-4″), 7.15 (m, H-5′, 6′), 6.88 (d, J=8.2 Hz, H-5″), 6.19 (dd, J=17.43, 10.82 Hz, H-12′), 5.51 (m, H-11″), 5.24 (d, J=17.42 Hz, H-12′), 5.27 (d, J=10.81 Hz, H-11′), 3.49 (m H-10″), 1.75 (s, H-14″), 1.81 (s, H-13″), 1.80 (s, H-13′), 8.0 (s, NH); ^{13}C NMR (Table 1); Ion-spray MS (positive): *m/z* 507 ($M^+ + H$).

Asterriquinone SU5504 (**7**): Purple powder (3.5 mg); UV λ_{max} 225, 292 and 489 nm; IR ν_{max} 3404, 3316, 2912, 1644, 1612 cm^{-1}; ^1H NMR 7.64 (s), 7.75 (s), 7.56 (m), 7.14 (m), 7.56 (m), 6.20 (dd, J=17.41, 10.71 Hz), 5.27 (d, J=10.71 Hz), 5.25 (d, J=17.42 Hz), 1.81 (s), 1.8 (s), 3.82 (s); ^{13}C NMR (Table 1); Ion-spray MS (positive): *m/z* 521 ($M^+ + H$); HRFAB-MS: calcd. for $C_{33}H_{32}N_2O_4$ ($M^+ + H$) 521.2241; found: *m/z* 521.2291.

Results and Discussion

Neoasterriquinone SU5220 (**1**) was obtained as a deep purple powder. The UV spectrum of **1** was characteristic for tryptophan-derived purple benzoquinone pigments; λ_{max} 260, 285, 290 and 489 nm[14]. The IR spectrum (KBr) showed a phenolic group absorption at 3404 cm^{-1} and a quinone group absorption at 1644 cm^{-1}. The ^1H NMR spectrum revealed the presence of a 3,3-dimethyl allyl group [δ 1.75 (s, 3H), 1.81 (s, 3H), 5.40 (s, 1H) and 3.45 (s, 2H)], four aromatic protons [δ 7.31 (m, 2H), 713 (m, 2H)], a indole NH proton [δ 8.10 (bs, 1H)], and one phenolic proton [δ 8.05 (s, 1H)]. The mass spectrum (Ion-Spray, positive mode) afforded a molecular ion at *m/z* 507 ($M^+ + H$). High-resolution mass spectrometry established the molecular formula as $C_{32}H_{31}N_2O_4$ (found: *m/z* 507.2291, $M^+ + H$, calcd.: 507.2122). Therefore, it was concluded that structure of **1** was a symmetrical dimer. It is interesting to note that the ^{13}C NMR spectrum (Table 1) showed resonances for only thirteen carbons instead of sixteen carbons (since **1** is a symmetrical dimer). The resonances due to the quinone carbons and phenolic carbons were missing in the ^{13}C NMR spectrum. This is perhaps due to the rapid interconversion of two equivalent tautomeric forms of the 2,5-dihydroxyquinone ring system[15]. These physical and spectroscopic properties were reminiscent of the indolyl benzoquinone pigments generally known as cochliodinols and asterriquinones[16,17]. Finally, the compound **1** was identified as the known compound neoasterriquinone

by comparing the spectroscopic properties of **1** with that of reported for neoasterriquinone[18,19].

All asterriquinones possess two common structural features: a) bis-indole benzoquinone ring systems and b) one or more dimethyl allyl side chains. Therefore, in this study we have noticed that as expected, they exhibited similar UV absorptions and common fragmentation pathways in their API (atmospheric pressure ionization)/MS/MS spectra. The daughter ion MS/MS spectra of the molecular ions of asterriquinones afforded abundant ions due to cleavage of dimethyl allyl side chain (68 mass units) from the bis-indole benzoquinone substructure. For example, the daughter ion MS/MS spectrum of **1** afforded two major fragments from the molecular ion at *m/z* 439 and at *m/z* 371 resulting from the sequential losses of dimethyl allyl side chains from the molecules. Therefore, in this study we have used MS/MS technique in conjunction with UV absorption data for initial structure elucidation studies.

Asterriquinone SU5228 (**2**) was isolated as a purple powder. The molecular formula was determined to be $C_{27}H_{22}N_2O_4$ on the basis of the HR-FAB measurement (found: *m/z* 439.2291 ($M^+ + H$), calcd. 439.2289). The UV and IR spectra were the same as for **1**, suggesting a common chromophore. The molecular weight of **2** is 68 mass units lower than the molecular weight of **1** and the MS/MS spectrum of **2** afforded only one major fragment $[M-68]^+$ from the molecular ion *m/z* 439, suggesting that compound **2** may possess only one dimethyl allyl side chain. The nature of the dimethyl allyl group was determined to be a 3,3-dimethyl allyl moiety from its ^1H NMR spectrum which showed four signals at δ 1.73 (bs, 3H), 1.80 (bs, 3H), 3.44 (m) and 5.39 (m). Integration of these peaks also supported the earlier conclusion that **2** contained only one 3,3-dimethyl allyl group. The rest of the ^1H NMR spectrum was very similar to **1** suggesting that compound **2** is similar to **1** except that **2** lacks one of the two 3,3-dimethyl allyl groups. After comparing the spectroscopic data of **2** with the data for the known asterriquinones, it was clear that **2** was a new member of asterriquinone family. The connectivity of proton and carbon atoms was confirmed by the HMQC spectrum. The position of the 3,3-dimethyl allyl side chain was proposed by the following observations: the ^1H NMR spectrum showed signals for eight aromatic protons which form two contiguous spin systems as observed in the ^1H-^1H COSY spectrum. This suggested that the six membered ring of the indole moiety was unsubstituted. Furthermore, the ^1H-^1H COSY spectrum of **2** showed a coupling between one downfield signal at δ 7.48 (d, $J=2$ Hz, 1H) and one of the NH protons at δ 11.35 while the second indole proton at δ 10.96 did not show any coupling. Thus, it was suggested that one of the two C-2 positions was unsubstituted. These results have been further confirmed by the HMBC correlations of the methylene protons in the 3,3-dimethyl allyl side chain at C-10′ (δ 3.47, m) with carbons C-2′ (δ 138.8) and C-3′ (δ 102.2); the HMBC spectrum also displayed cross peaks from the NH (H-1′) to C-10′, C-2′ and C-8′.

Asterriquinone SU5500 (**3**), also isolated as a purple powder, showed the highest molecular peak at *m/z* 549 ($M^+ + H$) in the ion-spray (positive) mass spectrum. High-resolution mass spectrometry established the molecular formula as $C_{34}H_{33}N_2O_5$ (found: *m/z* 549.2181, $M^+ + H$, calcd. 549.2211); the difference in molecular weight between **1** and **3** was 42 mass units (= C_2H_2O). Therefore, it is plausible that **3** has an acetate group. The MS/MS spectrum of the molecular ion of **3** showed 3 major fragments at *m/z* 507, 439 and 371 corresponding to sequential losses of a C_2H_2O unit (42 mass units) and two dimethyl allyl groups. The loss of the C_2H_2O unit from the molecule suggested the presence of an enolacetate[19] group in the molecules. The hypothesis of an acetate group in **3** was further confirmed by NMR data, which showed a methyl peak at δ 2.66 (δ 20.8) and an acetate carbonyl carbon at δ 170.3. The remaining spectroscopic properties of compound **3** were very similar to the spectroscopic properties for **1**. Therefore, the structure of **3** was proposed as shown.

Asterriquinone SU5503 (**4**) was obtained as a reddish-purple powder. It has a molecular weight of 550 [(*m/z* 551 ($M^+ + H$)]; the difference in molecular weight between neoasterriquinone and asterriquinone SU5503 was 44 mass units (= CO_2). Most of the spectroscopic properties for **4** were similar to those of **1**. Therefore, it was plausible that asterriquinone SU5503 (**4**) has an additional COOH group. This was confirmed by the MS/MS spectrum which showed a major fragment at *m/z* 507 ($M^+ + H - 44$) due to loss of CO_2. In addition, the MS/MS spectrum also afforded two peaks at *m/z* 439 and 371 due to sequential loss of the two dimethyl allyl group. The ^1H and ^{13}C NMR spectra of **4** and **1** were very similar except an additional carbonyl carbon signal was present at δ 163.8 in the ^{13}C NMR spectrum of **4**. These data strongly support the presence of a carboxylic acid group in the molecule. Integration of the aromatic region showed the presence of only seven protons which indicated the existence of a substitution in one of the benzene rings. The ^1H-^1H COSY spectrum showed a contiguous spin system between protons at C-4″ (δ 7.31),

Table 1. ^{13}C NMR Chemical shifts of asteriquinones **1**~**7**.

Carbon No.	1	2	3	4	5	6	7
2 or 5			150.1				155.5
3	119.7	119.5	120.1	116.9	no[a]	120.1	120.0
6	120.0	120.3	120.1	117.2	no[a]	120.1	120.3
2′	138.4	138.8	136.4	no[a]	128.7	138.8	128.8
2″	138.4	128.8	136.1	no[a]	128.7	128.7	128.8
3′	100.5	102.2	100.9	97.5	102.6	101.0	103.3
3″		105.2	101.6	95.9	102.6	102.7	103.5
4′	121.6	122.0	120.9	122.8	122.3	122.0	121.9
4″		122.3	120.6	135.0	122.4	122.4	121.9
5′	119.6	119.3	120.0	117.0	121.4	120.0	121.5
5″		119.5	120.0	130.2	121.6	120.0	121.5
6′	120.1	120.3	120.1	116.0	119.9	120.4	120.4
6″		120.7	120.5	130.6	120.1	120.4	120.3
7′	110.7	111.8	111.01	114.8	111.3	111.5	114.4
7″		111.6	111.01	130.5	111.5	128.4	114.2
8′	127.7	127.3	128.0	no[a]	128.3	128.2	127.1
8″		128.2	128.0	no[a]	128.3	126.4	128.2
9′	138.2	136.6	136.4	no[a]	138.8	135.8	136.8
9″		132.0	135.7	no[a]	138.8	135.6	137.2
10′	26.8	27.3	27.2	26.3	28.36	27.2	60.1
10″			27.3	26.3	60.1	60.1	60.1
11′	121.6	121.8	122.0	122.8	122.4	144.2	144.1
11″			122.3	122.8	144.2	121.6	144.3
12′	135.7	136.3	135.5	135.0	135.8	136.7	114.5
12″			135.5	135.0	114.8	114.5	114.5
13′	17.9	18.5	18.4	17.9	18.6	18.4	28.5
13″			18.3	18.0	28.4	28.4	28.5
14′	25.8	26.4	26.2	26.7	26.2	26.2	26.2
14″			26.1	26.7	26.2	28.4	26.2
*Me*CO			20.8				
Me*CO*			170.3				
*CO*OH				163.8			
O*Me*							60.2

[a] Not observed.

C-5″ (δ 7.16), C-6″ (δ 7.40) suggesting that the substitution position was to be at C-7″. In addition, the ^{13}C NMR chemical shift argument especially of C-4″-C-7″ (Table 1) also favored placement of the carboxylic group at C-7″ position.

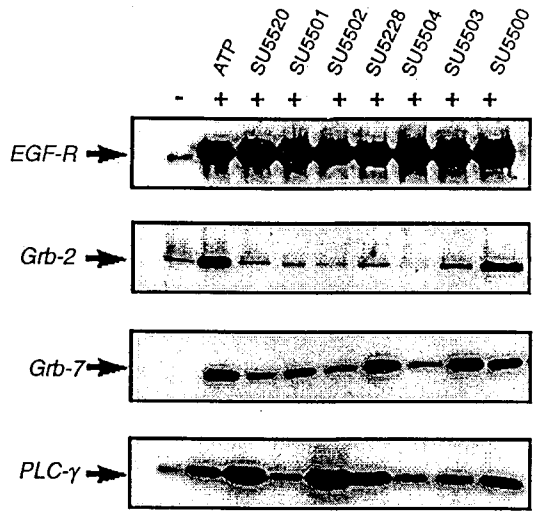
Isoasterriquinone SU5502 (**5**) was obtained as purple needles. Most of the spectroscopic properties of **5** were very similar to those noted for **1**, suggesting similarities in both structures. However, the ^1H NMR spectrum indicated the presence of two different types of dimethyl allyl groups; a 1,1-dimethyl-2-propenyl group [δ 1.82 (s, 6H), 5.22 (d, J=17.42 Hz), 5.25 (d, J=10.71 Hz) and 6.20 (dd, J=17.43, 10.72 Hz)], and a 3,3-dimethyl allyl group [δ 1.73 (bs, 3H), 1.80 (bs, 3H), 3.44 (m) and 5.39 (m)]. Subsequently, **5** was identified as the known compound isoasterriquinone by comparing its spectroscopic properties with those reported in the literature[20].

The molecular formula of asterriquinone SU5501 (**6**) and most of the spectroscopic properties were very similar to that of **5**. Just like **5**, **6** also had two types of dimethyl allyl groups (*i.e.*, a 1,1- and a 3,3-dimethyl allyl group). However, after careful examination of the NMR spectra it was clear that the substitution position of the 3,3-dimethyl allyl group was different. The one of the methine carbon at C-7″ which was readily identified by its characteristic shift of ~111 ppm was not present, but instead a new quaternary carbon at δ 128.4 appears. The methylene protons in the C-10″ position (δ 3.49) show long-range correlation to C-6″ (δ 120.1), C-7″ (δ 128.4) and C-8″ (δ 126.4). Based on these data, the dimethyl

Table 2. Inhibition of Grb-2 binding activity to tyrosine phosphorylated EGF receptor (IC_{50}).

Compound No.	Inhibition at μM
1	2.5
2	4.91
3	59.49
4	9.74
5	4.48
6	4.16
7	8.37

Fig. 1. Asterriquinones exhibited SH2 domain-specific inhibitory activity of Grab-2 binding to phosphorylated EGF receptor compared to Grb-7.



allyl group was connected at the C-7″.

Asterriquinone SU5504 (**7**) was obtained as a deep purple powder and high-resolution mass spectrometry established the molecular formula to be $C_{33}H_{32}N_2O_4$ (found: *m/z* 521.2291, $M^+ + H$, calcd. 521.2241). The ^1H NMR spectrum revealed the presence of two 1,1-dimethyl allyl groups [δ 1.81 (s, 6H), 1.80 (s, 6H), 5.25 (d, $J = 17.42$ Hz), 5.27 (d, $J = 10.71$ Hz), and 6.20 (dd, $J = 17.41$, 10.71 Hz)]. The presence of these side chains was also confirmed by characteristic fragments [*m/z* 453 ($M^+ + H$-68) and 385 ($M^+ + H$-2 × 68)] in the MS/MS spectrum. The spectroscopic properties of compound **7** were reminiscent of the spectroscopic properties of asterriquinone[20]. However, the difference in molecular weight between the latter and **7** was 14 mass units. The ^1H NMR spectrum of **7** showed an additional peak at δ 3.82 (s, 3H) and the ^{13}C NMR spectrum showed a peak at δ 60.2 suggesting the presence of a methoxy group in the molecule. The remaining spectroscopic properties of compound **7** were identical to the spectroscopic properties for asterriquinone[21]. Therefore, the structure of **7** was proposed as a *O*-methyl asterriquinone.

Biological Properties

The asterriquinones described above were tested for their ability to block the binding of Grb-2 to the EGF receptor kinase. As shown in Table 2, various asterriquinones inhibited the association of the Grb-2 fusion protein with phosphorylated EGF receptor and exhibited IC_{50} values less than 10 μM. However, **3** was found to be much less inhibitory than the other compounds of the series suggesting that nature of the substituents on the quinone nucleus may be critical for the activity. A comparison of **1**, **7**, and **3** revealed that both the hydroxyl and methyl ether substituents resulted in good inhibitory activity compared to the addition of carbonyl in the acetate substituent on the quinone nucleus of **3**. This is in contrast to various symmetrical and unsymmetrical isoprene substituents on the indoles which were shown to be quite tolerable to retain inhibitory activity. It is of interest that the addition of a carboxylate to one of the indoles in **4** did not alter the activity. This suggests that the generalized lipophilicity of the bis-indoles may impart binding in a hydrophobic domain associated with the Grb-2-SH2 domain ligand interface.

In order to extend these observations, the chemical series was used to inhibit the binding of Grb-2 to activated EGF receptor. As shown in Figure 1, activated EGF receptor resulted in substantial tyrosine phosphorylation which allowed for binding of recombinant fusion proteins containing SH2 domains derived from Grb-2, Grb-7 and PLC-γ. In this case, all of the asterriquinones except for **3** inhibited the binding of Grb-2 to the phosphorylated receptor. Of this series, **4** appeared to be the least potent compound compared to **1**, **5**, **6**, and **7**. In the case of Grb-7 binding, **4**, and **7** showed the greatest potency followed by **1** and **6**. In contrast, **1**, **3**, and **4** did not block Grb-7 binding to the EGF receptor. For PLC-γ, **6** and **7** exhibited some inhibitory activity whereas **1**, **2**, **3**, **4**, and **5** did not inhibit the binding of PLC-γ to the receptor. These experiments strongly suggest that indole

substituents may influence the relative specificity of these compounds to block the Grb-2-SH2 interaction, when these three protein domains were compared.

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