Antitumor Activity of Quinazolinone Alkaloids Inspired by Marine Natural Products

Solida Long, Diana I. S. P. Resende, Anake Kijjoa, Artur M. S. Silva, André Pina, Tamara Fernández-Marcelo, M. Helena Vasconcelos, Emília Sousa, and Madalena M. M. Pinto

Abstract: Many fungal quinazolinone metabolites, which contain the methyl-indole pyrazino[1,2-b]quinazoline-3,6-dione core, have been found to possess promising antitumor activity. The purpose of this work was to synthesize the enantiomeric pairs of two members of this quinazolinone family, to explore their potential as antitumor and their ability to revert multidrug resistance. The marine natural product fiscalin B (4c), and antienantiomers (4b, 5b, and 5c) were synthesized via a one-pot approach, while the syn enantiomers (4a, 4d, 5a, and 5d) were synthetized by a multi-step procedure. These strategies used anthranilic acid (i), chiral N-protected α-amino acids (ii), and tryptophan methyl esters (iii) to form the core ring of pyrazino[2,1-b]quinazoline-3,6-dione scaffold. Four enantiomeric pairs, with different enantiomeric purities, were obtained with overall yields ranging from 7 to 40%. Compounds 4a–d and 5a–d were evaluated for their growth inhibitory effect against two tumor cell lines. Differences between enantiomeric pairs were noted and 5a–d displayed GI50 values ranging from 31 to 52 µM, which are lower than those of 4a–d. Nevertheless, no effect on P-glycoprotein (P-gp) modulation was observed for all compounds. This study disclosed new data for fiscalin B (4c), as well as for its analogues for a future development of novel anticancer drug leads.

Keywords: antitumor; enantiomers; fiscalins; quinazolinones; synthesis

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1. Introduction

During the past ten years, great attention has been focused on drug development from Marine Natural Products (MNPs) as well as on their synthetic and semi-synthetic analogues. While terrestrial sources such as higher plants and microorganisms have reached the limelight, the marine environment increasingly becomes the newest and untapped resource of bioactive compounds [1]. A number of quinazolinone derivatives have played important roles in medicinal chemistry due to their broad spectrum of biological properties such as antibacterial, antifungal, anticonvulsant, anti-inflammatory, anti-HIV, anticancer, and analgesic activities [2,3]. One subclass of quinazolinone-derived Natural Products are the indolylmethyl pyrazinoquinazoline alkaloids (1) (Figure 1), characterized by a fused piperazine ring system linked to an indole moiety. So far, approximately 80 secondary metabolites of this subclass, covering structurally diverse compounds, have been isolated from fungi, mainly of marine origin. These include (i) compounds containing only a substituted piperazine ring such as glyantrypine (2), isolated from the culture broth of the mangrove-derived fungus *Cladosporium* sp. PJX-41 [4], fumiquinazoline F (3), isolated from the marine-derived fungus *Aspergillus fumigatus* strain H1-04 and *Aspergillus* sp. [5,6], and fiscalin B (4), isolated from the culture of the fungal strain *Dichotomomyces eijii* which was recovered from sediments of the Brazilian coast [7]; (ii) compounds with a more complex structure containing several rings such as fumiquinazoline K (6), isolated from the Mediterranean sponge-derived fungus *Aspergillus* sp., the soft coral (*Simulatrix* sp.)-associated fungus *Aspergillus fumigatus* KMM 4631 [8], and a gorgonian-associated fungus [9]; (iii) *spiro* compounds such as fumiquinazoline C (7), isolated from the marine-derived fungus *Aspergillus fumigatus* strain H1-04, and N-formyllapatin A (8), isolated from the marine-derived fungus *Penicillium adametziodes* (AS-53) [10]; (iv) compounds with complex 3-indolyl groups such as cladoquinazoline (9) and epi-cladoquinazoline (10), isolated from the mangrove-derived fungus *Cladosporium* sp. PJX-41 [4], neofiscalin A (11) from *Neosartorya siamensis* KUFC 6349, which was isolated from a forest soil [11], as well as from *N. siamensis* KUFC 6349, isolated from the sea fan *Rumphella* sp. [12], and fumiquinazoline S (12), isolated from a solid-substrate culture of *Aspergillus* sp., collected from a marine-submerged wood [13]; or (v) compounds with indole glucosides such as fumigatoside A (13), isolated from *Aspergillus fumigatus* which was derived from the jellyfish *Nemopilema nomurai* [14].

Marine alkaloids containing indolymethyl pyrazinoquinazoline ring system can be considered conformationally constrained peptidomimetics exhibiting very interesting biological properties [15]. For instance, glyantrypine (2) is an antibacterial agent, active against *Vibrio harveyi* [16]; fumiquinazolines are antitumor compounds with moderate cytotoxicity [17]; fiscalins are substance P inhibitors and anticancer agents [7,18]; cladoquinazolines (9 and 10) are active against influenza A virus (H1N1); fumiquinazoline S (12) exhibits a weak inhibition against Na⁺/K⁺-ATPase, and N-acetylardeemin (14) is a potent inhibitor of multidrug resistant (MDR) tumor cells [4,13,19,20]. Moreover, the pyrazino[2,1-b]quinazoline ring system has been already ascribed as essential for the above-mentioned activities, and its enantioselective effects were observed for their antibacterial activity. For example, neofiscalin A (11) showed potent antibacterial activity against Gram-negative bacteria [12,21], whereas fiscalin C (15), epi-neofiscalin A (16), and epi-fiscalin C (17) were inactive in the same study. Interestingly, fiscalin C (15) displayed a synergistic effect against methicillin-resistant *Staphylococcus aureus* (MRSA) when combined with oxacillin [11,22]. Concerning antitumor activity, studies on quinazolinone compounds have mainly focused on natural or synthetic compounds with different substituents at the stereogenic C-1 and C-4; however, there is no report on analogues with different configurations at C-1 and C-4.

Therefore, the aim of this study was to synthesize the diastereomers of fiscalin B (4c), i.e., 4a–d, and their homologues 5a–d, to further explore their potential as growth inhibitors of tumor cells, their ability to revert MDR by inhibiting P-gp activity, as well as to perform the SAR study.
2. Results and Discussion

2.1. Synthesis of Pyrazinoquinazoline Alkaloids

The pyrazino[2,1-b]quinazoline-3,6-dione ring system (1) is the core structure of fumiquinazoline-derived group of alkaloids. There are two main methods to synthesize compounds containing this scaffold (i) the Eguchi-aza Wittig approach that consists of a selective acylation of diketopiperazines with o-azidobenzoyl chloride, followed by dehydrative cyclization [23]; and (ii) the Mazurkiewicz–Ganesan approach, consisting of coupling of linear tripeptides followed by the isomerization of 4-imino-4\(^H\)-3,1-benzoxazines to obtain the corresponding quinazolin-4-ones [24,25].

In 2005, Liu et al. [26] reported a highly effective and environmentally friendly approach using a microwave-assisted multicomponent one-pot one step polycondensation of amino acids for the total syntheses of glyantrypine (2), fumiquinazoline F (3), and fiscalin B (4). With this procedure, the authors reported that the addition of a \(N\)-protected \(\alpha\)-amino acid (ii) to anthranilic acid (i), under a conventional heating condition at 55 °C with triphenylphosphite, (PhO)\(_3\)P, generated the intermediate benoxazin-4-one, followed by the addition of tryptophan (Trp) ester (iii), and then submitted to
microwave irradiation at 220 °C for 1.5 min, to furnish the desired final products. Inspired by this simple and highly efficient methodology, we were able to prepare 4 and 5 (Scheme 1, method A). Attempts to obtain the syn enantiomers by this one-pot approach failed since only vestigial amounts could be detected due to the isomerization to the antienantiomers. The antienantiomers 4b/4c and 5b/5c were then obtained, starting from enantiomerically pure amino acids, from which no syn enantiomer were isolated (Entry 1–8, Table 1). Therefore, the syn enantiomers 4a/d and 5a/d were synthesized by the Mazurkiewicz–Ganesan method [25] (Scheme 1, method B) with some modifications. First, coupling of i with iii, using 1,1,3,3-tetramethylaminium tetrafluoroborate (TBTU) in basic conditions, afforded the dipeptide (iv). Coupling of iv with N-protected α-amino acids chloride (v) [27] in a two-phase Schotten–Baumann condition yielded the tripeptides vi. The intermediates vii were obtained by adding the dehydrating agent, triphenylphosphine (Ph3P), to convert β-keto amides (vi) to the oxazoles (vii), followed by Fmoc-deprotection by 20% piperidine to afford 4a/d and 5a/d with a moderate yields of 21–40%. After purification by chromatographic techniques, the purity of 4a–d and 5a–d, as determined by a reversed-phase HPLC (C18, MeOH:H2O; 60:40 or CH3CN:H2O; 50:50) was found to be higher than 90% (Supplementary Materials, Figures S1–S8). The enantiomers ratio was determined by the chiral HPLC equipped with amylose tris-3,5-dimethylphenylcarbamate column, using hexane: ethanol (80:20) as a mobile phase (Table 1, Supplementary Materials, Figures S9–S16).

Table 1. Enantiomers and diastereomers of the pyrazinoquinazolinone alkaloids 4 and 5.

| Entry | R  | Tryptophan | % Anti Compound | % Syn Compound | [α]D b | Enantiomeric Ratio c | Purity (%) d |
|-------|----|------------|-----------------|----------------|--------|----------------------|-------------|
| 1     | L-v | L-tryptophan | 10 (4b)         | -              | +63.8  | 40:60                | 87          |
| 2     | D-v | L-tryptophan | 14 (4b)         | -              | +65.2  | 39:61                | 89          |
| 3     | L-v | D-tryptophan | 8 (4c)          | -              | −248.1 | 62:37                | 90          |
| 4     | D-v | D-tryptophan | 10 (4c)         | -              | −100.0 | 63:37                | 91          |
| 5     | L-l | L-tryptophan | 7 (5b)          | -              | +44.9  | 67:33                | 94          |
| 6     | D-l | L-tryptophan | 10 (5b)         | -              | +89.7  | 50:50                | 94          |
| 7     | L-l | D-tryptophan | 8 (5c)          | -              | −61.7  | 31:69                | 90          |
| 8     | D-l | D-tryptophan | 10 (5c)         | -              | −142.9 | 46:54                | 86          |
| 9     | L-v | L-tryptophan | -               | 28 (4a)        | +300.5 | 100:0                | 94          |
| 10    | D-v | L-tryptophan | -               | 21(4d)         | −210.5 | 7:93                 | 89          |
| 11    | L-l | L-tryptophan | -               | 28 (5a)        | +81.8  | 90:10                | 89          |
| 12    | D-l | D-tryptophan | -               | 40 (5d)        | −186.0 | 17:83                | 85          |

a  R residual of amino acid at C-1 position; b Optical rotation, concentration (g/100 mL); c Calculated from the peak area from chiral HPLC experiments (by using equation X × 100/Xn in which X is the peak area of each peak and Xn is the total peak area); d Calculated from the peak area from reversed-phase HPLC experiments; * Entry of Mazurkiewicz–Ganesan approach.

The overall yield of this one-step method ranged from 7 to 14% with different enantiomeric ratios (Table 1). The low yields of this one-pot reaction were attributed to a high temperature applied in microwave irradiation to convert the intermediate Boc-protected-benzoxazin-4-one to the final products. Moreover, the steric hindrance at C-1 could also be a reason, as previously noted by Liu et al. [26] and Wang et al. [24] in the synthesis of similar compounds. Although a partial epimerization was observed under these conditions, this one-pot procedure was proved successful in providing the pyrazinoquinazolinone scaffold and a series of compounds for further biological investigations. On the other hand, the moderate yields of compounds obtained by Mazurkiewicz–Ganesan method were related to the mild conditions and a multistep approach (Table 1 entry 9–12). In this study, the coupling agent 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide
(ECD) was replaced by TBTU, which gave a similar yield for the dipeptide iv (81–94%) when compared to the previous reports. The tripeptides vi were also obtained in a very high yield (84–94%). The bottleneck of this multistep approach was the conversion of the intermediates vii to the final products since their decomposition to form the precursors vi, similar to what has been previously report by Ganesan et al. [25], was observed. Compounds 4a/d and 5a/d were purified by preparative TLC (EtOAc:CH₂Cl₂:MeOH: 50:47.5:2.5) after refluxing in CH₃CN in the presence of 4-(dimethylamino)pyridine. The degrees of epimerization of 4a/d and 5a/d by Mazurkiewicz–Ganesan method were found to be lower than those obtained by the microwave method (Table 1). These results could be also associated with the mild conditions used in the multistep approach.

Scheme 1. Synthesis of the pyrazinoquinazolinone alkaloids 4 and 5. (Method A) One-pot microwave-assisted approach. Reagents and conditions: (a) dried-pyridine, (PhO)₃P, 55 °C, 16–24 h; (b) dried-pyridine, (PhO)₃P, 220 °C, 1.5 min; (Method B) Mazurkiewicz–Ganesan approach. Reagents and conditions (a) CH₃CN, TBTU, Et₃N, rt, 5 h; (b) CH₂Cl₂/aq.Na₂CO₃, rt, 3 h; (c) dried CH₂Cl₂, Ph₃P, I₂, EtN(i-Pr)₂, rt, overnight; (d) piperidine in CH₂CH₂, rt, 12 min, then CH₃CN, DMAP, reflux 19 h. i-Pr = isopropyl; i-Bu = isobutyl, Boc = tert-butyloxycarbonyl; Fmoc = fluorenylmethyloxycarbonyl; DMAP = 4-(dimethylamino) pyridine, TBTU = 1,1,3,3-tetramethylaminium tetrafluoroborate.

2.2. Structure Elucidation

The structures of the new compounds, 4a, 4b, and 4d, and of the synthetic fiscalin B (4c) and their homologues 5a, 5b, 5d, and 5c, were established by extensive analyses of 1D and 2D NMR spectra and high-resolution mass spectrometry. The ¹H (Table 2, Supplementary Materials, Figures S17–S30) and ¹³C NMR data (Table 3, Supplementary Materials, Figures S31–S44) of 4c were in agreement with those reported in the literature for fiscalin B, which was obtained from the marine sources [24,25]. The chemical shift values of some key protons allowed the determination of the relative configurations of both stereogenic centres as well as the conformation of the piperazine ring in 4 and 5, as shown in 4a, 4b and 4c, 4d (Figure 2). The substituent on C-4 is always in a pseudoaxial position while H-4 is shifted to δH 5.52–5.68 in all compounds, showing the characteristic anisotropic effect of the coplanar carbonyl group at C-14 on the quasi-equatorial proton, as explained by Hernández et al. [28].
The difference between anti and syn enantiomers were observed on H-1. In the antienantiomers, the chemical shift value of the axial H-1 of 4\(_b/c\) and 5\(_b/c\) at \(\delta\) 2.69–2.73 indicates the folding of the C-4-indolyl substituent over the piperazine ring and the isopropyl group. This phenomenon was also reported by Hernández and coworkers for H-1 signals of quinazolinones whose chemical shift values were ca. 3 ppm for the boat conformation and the antienantiomers, due to the absence of the shielding effect by the aromatic ring [28]. Meanwhile, for the syn enantiomers, 4\(_a/d\) and 5\(_a/d\), H-1 chemical shift values were at ca. \(\delta\) 3.95–4.32, indicating the shielding effect from the aromatic ring over H-1. The chemical shifts of H-1\('\) of the isopropyl group were also different between the anti- and the syn enantiomers, being ca. \(\delta\) 0.94–1.06 for 4\(_a/d\) and \(\delta\) 2.61–2.66 for 4\(_c/d\), indicating the different shielding effect of the aromatic ring on the group on C-1.

Table 2. \(^1\)H NMR data (300 MHz, CDCl\(_3\)) for 4 and 5.

| Position | 4a | 4b | 4c | 4d |
|----------|----|----|----|----|
| H-1      | 3.95, dd (8.4, 3.5) | 2.69, d (2.4) | 2.69, d (2.4) | 3.95, dd (8.5, 3.6) |
| H-2      | 5.60, d (3.0) | 5.85, br | 5.80, br | 6.39, d (3.2) |
| H-4      | 5.52, dd (6.3, 3.8) | 5.68, dd (5.0, 2.8) | 5.67, dd (5.0, 2.8) | 5.52, dd (6.3, 3.8) |
| H-8      | 8.08, br | 8.09, br | 8.07, br | 8.03, br |
| H-9      | 7.54, d (3.0) | 7.54, t (5.2) | 7.54, d (8.1, 7.3, 1.1) | 7.54, d (8.1, 7.3, 1.1) |
| H-10     | 7.79, d (8.7, 7.7, 1.9) | 7.77, d (7.9, 7.7, 1.9) | 7.77, d (8.6, 2.1, 1.9) | 7.79, d (8.5, 7.1, 1.2) |
| H-11     | 7.62, d (7.7) | 7.57, d (3.4) | 7.57, d (3.5) | 7.62, d (7.7) |
| H-1’     | 1.06–0.94, m | 2.66–2.61, m | 2.66–2.61, m | 1.06–0.94, m |
| H-2’     | 0.48, d (6.6) | 0.63, d (2.3) | 0.63, d (4.0) | 0.48, d (6.6) |
| H-3’     | 0.75, d (6.8) | 0.66, d (4.3) | 0.75, d (6.8) | 0.66, d (4.3) |
| H-4’a    | 3.72, dd (15.0, 2.9) | 3.77, dd (14.8, 3.7) | 3.72, dd (14.8, 3.7) | 3.72, dd (14.8, 3.7) |
| H-4’b    | 3.80, dd (14.9, 6.3) | 3.77, dd (14.8, 3.4) | 3.80, dd (14.8, 3.4) | 3.80, dd (14.8, 3.4) |
| H-6’     | 6.89, d (2.4) | 6.60, d (2.3) | 6.60, d (2.4) | 6.90, d (2.3) |
| H-7’     | 8.06, br | 8.03, br | 8.03, br | 8.03, br |
| H-9’     | 7.28, d (8.4) | 7.28, d (8.2) | 7.28, d (8.2) | 7.28, d (8.2) |
| H-10’    | 7.10, d (8.6, 7.6, 1.0) | 7.13, t (8.0) | 7.13, t (8.1) | 7.10, d (8.5, 7.5, 1.9) |
| H-11’    | 6.93, d (8.0) | 7.55, d (8.3) | 7.55, d (8.3) | 6.94, d (8.2, 7.2, 1.0) |
| H-12’    | 7.49, d (8.0) | 7.44, d (8.0) | 7.44, d (8.1) | 7.49, d (7.9) |

| Position | 5a | 5b | 5c | 5d |
|----------|----|----|----|----|
| H-1      | 4.32, dt (10.7, 3.2) | 2.73, dd (9.7, 3.4) | 2.72, d (9.7) | 4.31, dt (11.0, 3.2) |
| H-2      | 5.45, d (2.5) | 5.79, br | 5.75, br | 6.21, d (2.5) |
| H-4      | 5.54, dd (5.2, 3.3) | 5.68, dd (5.2, 3.0) | 5.68, dd (5.0, 2.9) | 5.55, dd (5.2, 3.3) |
| H-8      | 8.29, d (8.0) | 8.37, d (8.0) | 8.37, d (8.0) | 8.39, dd (8.0, 1.1) |
| H-9      | 7.41, d (8.0) | 7.55, d (8.1) | 7.55, d (8.0) | 7.42, d (8.0) |
| H-10     | 7.78, d (8.5, 7.2, 1.5) | 7.78, d (8.5, 7.1, 1.5) | 7.78, d (8.5, 7.1, 1.5) | 7.79, d (8.5, 7.1, 1.6) |
| H-11     | 5.60, d (7.8) | 7.60, d (7.8) | 7.60, d (7.9) | 7.59, d (7.8) |
| H-1’     | 2.84–2.44, m | 1.43–1.33, m | 1.44–1.36, m | 2.89–2.56, m |
| H-2’     | 2.08–2.00, m | 2.01, d (14.8, 12.5, 4.2) | 2.01, d (14.0, 9.4) | 2.07–2.00, m |
| H-3’     | 0.75, d (6.0) | 0.77, d (6.4) | 0.77, d (6.3) | 0.74, d (6.0) |
| H-4’a    | 0.14, d (6.1) | 0.28, d (6.5) | 0.28, d (6.4) | 0.49, d (6.0) |
| H-4’b    | 3.75, dd (15.0, 5.3) | 3.65, dd (14.9, 5.2) | 3.65, dd (14.9, 5.2) | 3.75, dd (15.0, 5.3) |
| H-6’     | 6.68, d (2.3) | 6.64, d (2.3) | 6.64, d (2.0) | 6.68, d (2.4) |
| H-7’     | 8.08, br | 8.09, br | 8.07, br | 8.03, br |
| H-9’     | 7.28, d (8.1) | 7.29, d (8.2) | 7.29, d (8.2) | 7.29, d (8.3) |
| H-10’    | 7.12, d (8.5, 7.1, 1.1) | 7.13, t (8.0) | 7.13, t (8.0) | 7.12, d (8.1, 7.0, 1.1) |
| H-11’    | 6.98, d (8.5, 7.0, 1.1) | 6.98, t (7.5) | 6.98, t (7.5) | 6.93, d (8.0, 7.1, 1.0) |
| H-12’    | 7.53, d (8.1, 7.1, 1.0) | 7.50, d (8.0) | 7.50, d (8.0) | 7.53, d (8.2, 7.2, 1.1) |
HMBC correlations were also used to distinguish the anti-isomers from the syn counterparts. For the anti isomer 4b (whose indole moiety derived from L-Trp), H-4 exhibited correlations to C-14, C-5', C-4', and C-3 whereas H-1 showed correlations to C-14, C-2', C-3', and C-1'. On the contrary, in 4c (whose indole moiety is derived from D-Trp), the HMBC correlations from H-4 to C-14, C-5', C-4' and from H-1 to C-14, C-3', and C-2', were observed. For the syn isomer 4a (whose indole moiety derived from L-Trp), the HMBC correlations from H-1 to C-14, and from H-4 to C-14, C-4', and C-5' were observed while the HMBC correlations from H-1 to C-3 and C-14 and from H-4 to only C-4' were observed in the syn isomer 4d. Moreover, the NOESY spectrum revealed the cross peak between the C-1' methyl groups and H-4 for the anti isomer 4c, while for the syn isomer 4a that correlation was absent (Supplementary Materials, Figures S45–S46). These observations support the identity/identification of the syn and anti conformational isomers.

Figure 2. Most relevant chemical shifts and key HMBC correlations of the protons to the stereogenic centers on the piperazine ring of 4a–d.
Table 3. $^{13}$C NMR data (75 MHz, CDCl$_3$) for 4 and 5.

| Position | $^{13}$C, Type | $\delta$C (ppm) |
|----------|----------------|-----------------|
| C-1      | 61.9, CH       | 58.1            |
| C-3      | 167.8, CO      | 169.5           |
| C-4      | 57.5, CH       | 56.8            |
| C-6      | 161.4, CO      | 160.9           |
| C-7      | 120.2, C       | 120.2           |
| C-8      | 126.8, CH      | 126.9           |
| C-9      | 127.1, CH      | 127.2           |
| C-10     | 134.7, CH      | 134.7           |
| C-11     | 127.1, CH      | 127.0           |
| C-12     | 146.8, C       | 147.1           |
| C-14     | 149.3, C       | 150.3           |
| C-1'     | 34.5, CH       | 29.4            |
| C-2'     | 18.0, CH$_3$   | 14.8            |
| C-3'     | 19.6, CH$_3$   | 18.8            |
| C-4'     | 27.4, CH$_2$   | 27.4            |
| C-5'     | 110.2, C       | 109.3           |
| C-6'     | 123.5, CH      | 123.6           |
| C-8'     | 136.0, C       | 136.0           |
| C-9'     | 110.9, CH      | 111.1           |
| C-10'    | 122.3, CH      | 122.6           |
| C-11'    | 119.9, CH      | 120.0           |
| C-12'    | 118.7, CH      | 118.7           |
| C-13'    | 127.8, C       | 127.2           |
whose C-1 bears a hydrogen atom, showed no antitumor activity (GI$_{50} > 100$ µM) while the analogue, with the phenyl group on C-1, was more active (GI$_{50} = 15$ µM) [25]. Moreover, fumiquinazolines F (3) and G (4), whose C-1 bears a methyl group, showed moderate activity against P-388 cells (GI$_{50} = 13.5$ µM) [31]. Likewise, differences in the inhibitory effects against the two cell lines were observed between enantiomeric pairs; i.e., $4a$ (1S,4S)/$4d$ (1R,4R) and $4b$ (1S,4R)/$4c$ (1R,4S). Significant differences were detected for GI$_{50}$ concentration values in the pair $4a$/$4d$ in NCI-H460 cells ($p = 0.026$).

Among the fiscalin series such as epi-fiscalin A (16, 1S,4S), epi-fiscalin C (17, 1S,4S), fiscalin F (1S,4S), and fiscalin C (15, 1R,4S), the configurations of the stereogenic carbons of the isopropyl pyrazinone and imidazolone moieties have already been found to influence the antitumor activity [21,32]. Although this study brought insights into the antitumor activity of fiscalin B (4c) and the synthetic analogues, none of the compounds showed any effect on the intracellular accumulation of Rh123, when tested at 10 µM concentrations using verapamil as a positive control for P-gp inhibition (Figure 3).

Table 4. The GI$_{50}$ of $4a$–$d$ and $5a$–$d$ in the NCI-H460 and HCT-15 human tumor cell lines.

| Compound | NCI-H460 (µM) | HCT-15 (µM) |
|----------|---------------|-------------|
| 4a       | 81.33 ± 1.55  | 40.33 ± 3.12|
| 4b       | 70.20 ± 3.15  | 38.15 ± 0.29|
| 4c       | 57.62 ± 2.08  | 31.78 ± 1.21|
| 4d       | 60.10 ± 2.61  | 33.30 ± 1.37|
| 5a       | 32.52 ± 4.24  | 48.18 ± 2.51|
| 5b       | 41.52 ± 2.52  | 51.94 ± 4.26|
| 5c       | 31.19 ± 3.01  | 43.63 ± 0.25|
| 5d       | 36.47 ± 3.98  | 47.00 ± 1.47|

Values were determined with the SRB assay and are the mean ± SEM of three independent experiments. Doxorubicin was used as a positive control, with the following GI$_{50}$ concentrations: 23.02 ± 0.54 nM in NCI-H460 cells, 331.49 ± 49 nM in HCT-15 cells.

Figure 3. Accumulation ratio of Rh123 in K562 and K562Dox cell lines. Cells were incubated for 1 h with $4a$–$d$, $5a$, $5b$, and $5d$ at a final concentration of 10 µM. The activity of $5c$ was not analyzed due to its quantity we have obtained. Verapamil (10 µM) was used as a positive control (known P-gp inhibitor), and K562 cells were used as a negative control. The accumulation ratio in the untreated K562Dox cells was defined as zero; any value higher than that represents a potential inhibition of P-gp. Results are the mean of two independent experiments.
3. Materials and Methods

3.1. General Procedure

All reagents were from analytical grade. Dried pyridine and triphenylphosphite were purchased from Sigma (Sigma-Aldrich Co. Ltd., Gillingham, UK). Protected amino acids (ii) and anthranilic acid (i) were purchased from TCI (Tokyo Chemical Industry Co. Ltd., Chuo-ku, Tokyo, Japan). Column chromatography purifications were performed using flash silica Merck 60, 230–400 mesh (EMD Millipore corporation, Billerica, MA, USA) and preparative TLC was carried out on precoated plates Merck Kieselgel 60 F254 (EMD Millipore corporation, Billerica, MA, USA), spots were visualized with UV light (Vilber Lourmat, Marne-la-Vallée, France). Melting points were measured in a Kölfer microscope and are uncorrected. Infrared spectra were recorded in a KBr microplate in a FTIR spectrometer Nicolet iS10 from Thermo Scientific (Waltham, MA, USA) with Smart OMNI-Transmission accessory (Software 188 OMNIC 8.3). 1H and 13C NMR spectra were recorded in CDCl3 (Deutero GmbH, Kastellaun, Germany) at room temperature unless otherwise mentioned on Bruker AMC instrument (Bruker Biosciences Corporation, Billerica, MA, USA), operating at 300 MHz for 1H and 75 MHz for 13C. Carbons were assigned according to HSQC and or HMBC experiments. Optical rotation was measured at 25 °C using the ADP 410 polarimeter (Bellingham + Stanley Ltd., Tunbridge Wells, Kent, UK), using the emission wavelength of sodium lamp, concentrations are given in g/100 mL. Qualitative GC-MS analyses were performed on a Trace GC 2000 Series ThermoQuest gas chromatography (Thermo Fisher Scientific Inc., Austin, TX, USA) equipped with ion-trap GCQ Plus ThermoQuest Finnigan mass detector (Thermo Fisher Scientific Inc., Austin, TX, USA). Chromatographic separation was achieved using a capillary column (30 m × 0.25 mm × 0.25 µm, cross-linked 5% diphenyl and 95% dimethyl polysiloxane) from Thermo Scientific™ (Thermo Fisher Scientific Inc., Austin, TX, USA) and high-purity helium C-60 as carrier gas. High resolution mass spectra (HRMS) were measured on a Bruker FTMS APEX III mass spectrometer (Bruker Corporation, Billerica, MA, USA) recorded as ESI (Electrospray) made in Centro de Apoio Científico e Tecnolóxico á Investigación (CACTI, University of Vigo, Pontevedra, Spain). The purity of synthesized compounds was determined by reversed-phase HPLC with diode array detector (DAD) using C18 column (Kimetex®, 2.6 EVO C18 100 Å, 150 × 4.6 mm), and the mobile phase was methanol: water (60:40) or acetonitrile:water (50:50). Enantiomeric ratio was determined by chiral HPLC (LCMS-2010EV, Shimadzu, Lisbon, Portugal), employing a system equipped with a chiral column (Lux® 5 µm Amylose-1, 250 × 4.6 mm) and UV-detection at 254 nm, mobile phase was hexane:ethanol (80:20) and the flow rate was 0.5 mL/min.

3.2. General Conditions for the Synthesis of 4-(1H-Indol-3-ylmethyl)-1-isopropyl-2H-pyrazino[2,1-b]quinazoline-3,6-(1H,4H)-diones (4b/4c)

In a closed vial, anthranilic acid (i) (28 mg, 200 µmol), N-Boc-L-valine (iia) for 4c or N-Boc-D-valine (iib) for 4b (44 mg, 200 µmol), and triphenylphosphite (63 µL, 220 µmol) were added along with 1 mL of dried pyridine. The vial was heated in heating block with stirring at 55 °C for 16–24 h. After cooling the mixture to room temperature, L-tryptophan methyl ester hydrochloride (iiia) for 4b or D-tryptophan methyl ester hydrochloride (iiib) for 4c (51 mg, 200 µmol) was added, and the mixture was irradiated in the microwave at a constant temperature at 220 °C for 1.5 min. Four reaction mixtures were prepared in the same conditions and treated in parallel. After removing the solvent with toluene, the crude product was purified by flash column chromatography using hexane: EtOAc (60:40) as a mobile phase. The preparative TLC was performed using CH2Cl2:Me2CO (95:5) as mobile phase. The major compound appeared as a black spot with no fluorescence under the UV light. The desired compounds 4b/4c were collected as yellow solids. Before analysis, compounds were recrystallized from methanol.
3.2.1. (1R,4S)-4-(1H-Indol-3-ylmethyl)-1-isopropyl-2H-pyrazino[2,1-b]quinazoline-3,6-(1H,4H)-dione (4b)

Yield: 14%; m.p.: 168–169 °C; [α]D = +65.21 (c 0.46; CHCl3); IR νmax (KBr) 3411, 3066, 1684, 1596, 1471, 1389, 1293 cm−1; 1H NMR see Table 2; 13C NMR see Table 3; m/z (rel. intensity, %): 385.9 (M+, 2), 257.1 (29), 214.1 (3), 202.0 (7), 171.1 (10), 143.1 (4), 130.1 (100), 103.0 (18), 77.0 (16); (+)-HRESIMS m/z 387.1810 (M + H)+ (calculated for C23H22N4O2, 387.1776).

3.2.2. (1S,4R)-4-(1H-Indol-3-ylmethyl)-1-isopropyl-2H-pyrazino[2,1-b]quinazoline-3,6-(1H,4H)-dione (4c)

Yield: 8%; m.p.: 168–169 °C; [α]D = −248.1 (c 0.43; CHCl3); IR νmax (KBr) 3346, 3066, 1683, 1596, 1471, 1389, 1293 cm−1; 1H NMR see Table 2; 13C NMR see Table 3; m/z (rel. intensity, %): 385.9 (M+, 5), 257.1 (32), 298.9 (37), 284.2 (6), 254.0 (15), 238.8 (12), 201.8 (18), 189.0 (35), 171.1 (25), 149.1 (32), 130.1 (100), 103.0 (9), 77.0 (13); (+)-HRESIMS m/z 387.1809 (M + H)+ (calculated for C23H22N4O2, 387.1776).

3.3. General Condition for the Synthesis of 4-(1H-Indol-3-ylmethyl)-1-isobutyl-2H-pyrazino[2,1-b]quinazoline-3,6-(1H,4H)-diones (5b/5c)

In a closed vial, anthranilic acid (i) (28 mg, 200 µmol), N-Fmoc-L-leucine (ii) for 5c or N-Fmoc-L-4-tryptophan methyl ester (iii) for 5b (70.68 mg, 200 µmol), and triphenylphosphite (63 µL, 220 µmol) were added along with 1 mL of dried pyridine. The vial was heated in heating block at 55 °C for 16–24 h. After cooling the mixture to room temperature, we added 1% MeOH in CH2Cl2. The mixture was irradiated in the microwave at a constant temperature at 220 °C for 1.5 min. Four reaction mixtures were prepared in the same conditions and treated in parallel. After removing the solvent with toluene, the mixture was purified by flash column chromatography using hexane: EtOAc (60:40) as a mobile phase. The preparative TLC was performed using CH2Cl2 as a mobile phase. The major compound appeared as a black spot with no fluorescence under UV light. The desired compounds, 5b/5c, were collected as yellow solids. Before analysis, compounds were recrystallized from methanol.

3.3.1. (1R,4S)-4-(1H-Indol-3-ylmethyl)-1-isobutyl-2H-pyrazino[2,1-b]quinazoline-3,6-(1H,4H)-dione (5b)

Yield: 10%; m.p.: 220 °C; [α]D = +89.74 (c 0.52; CHCl3); IR νmax (KBr) 3334, 3060, 1686, 1457, 1386, 1291 cm−1; 1H NMR see Table 2; 13C NMR see Table 3; (+)-HRESIMS m/z 401.1967 (M + H)+ (calculated for C29H24N4O2, 401.1933).

3.3.2. (1S,4R)-4-(1H-Indol-3-ylmethyl)-1-isobutyl-2H-pyrazino[2,1-b]quinazoline-3,6-(1H,4H)-dione (5c)

Yield: 8%; m.p.: 219 °C; [α]D = −61.72 (c 0.54; CHCl3); IR νmax (KBr) 3334, 3060, 1682, 1587, 1396, 1298 cm−1; 1H NMR see Table 2; 13C NMR see Table 3; (+)-HRESIMS m/z 401.1966 (M + H)+ (calculated for C29H24N4O2, 401.1933).

3.4. General Condition for the Synthesis of Compounds 4a and 5a

3.4.1. Synthesis of N-(2-Aminobenzoyl)-L-tryptophan methyl ester (iv-a)

To a mixture of anthranilic acid (287 mg, 2.39 mmol) and TBTU (920 mg, 2.86 mmol, 1.2 equiv) in acetonitrile (20 mL) was added Et3N (833 µL, 4.78 mmol, 2 equiv) and L-tryptophan methyl ester (521 mg, 2.39 mmol) at room temperature. After stirring for 5 h, the reaction mixture was concentrated under reduced pressure. The residue was dissolved in CH2Cl2 and washed with 1 M HCl, extracted with CH2Cl2 (3 × 100 mL), dried with Na2SO4, filtered, and concentrated. The residue was purified by flash chromatography (eluent 1% MeOH in CH2Cl2) to yield iv-a as a white solid (675.5 mg, 96%), m.p. 134–135 °C, IR νmax (KBr): 3424, 1746, 1727, 1645, 1611, 1581; 1H NMR (300 MHz, CDCl3): 8.15 (s, 1H), 7.56 (d, 1H, J = 7.9 Hz), 7.35 (d, 1H, J = 8.1 Hz), 7.19–7.15 (m, 3H), 7.09 (t, 1H, J = 7.4 Hz), 7.01 (d, 1H,
J = 2.3 Hz), 6.65 (dd, 1H, J = 8.7 and 1.1 Hz), 6.56 (ddd, 1H, J = 7.5 and 0.8 Hz), 5.08 (dt, 1H, J = 7.5 and 5.3 Hz), 3.71 (s, 3H), 3.43 (m, 2H); 13C NMR (75, MHz, CDCl3) 172.6 (CO), 168.8 (CO), 148.8 (C), 136.1 (C), 132.6 (CH), 127.6 (CH), 127.6 (C), 122.8 (CH), 122.3 (CH), 119.8 (CH), 118.7 (CH), 117.3 (CH), 116.7 (CH), 115.3 (C), 111.3 (C), 110.1 (C), 53.1 (CH), 52.7 (CH3), 27.7 (CH2).

3.4.2. Synthesis of N-[9H-Fluoren-9-ylmethoxy]carbonyl]-L-valinyl-2-aminobenzoyl-L-tryptophan methyl ester (vi-a)

To a solution of iv-a (160 mg, 0.474 mmol) in dried CH2Cl2 (10 mL) was added N-Fmoc-L-valine-Cl [27] (v-a, 182 mg, 0.5 mmol). The mixture was stirred for 30 min, followed by addition of aqueous Na2CO3 (1 M, 8 mL, 8 mmol). After continuous stirring for 3 h, the mixture was extracted with CH2Cl2 (4 × 100 mL), dried with Na2SO4, filtered, and concentrated. The residue was purified by flash chromatography (eluent: 5% MeOH in CH2Cl2 with aqueous NaOH (10 mL) in the presence of DMAP (64 mg, 0.53 mmol) and refluxed for 19 h. The reaction mixture was stirred at room temperature for 5 h, quenched with aqueous NaCl, treated with CH2Cl2 (2 × 100 mL), dried with Na2SO4, filtered and concentrated. The residue was triturated with hexane (1 × 200 mL), and hexane was added to remove an excess of PhCl. The reaction mixture was stirred at room temperature for 20 min, followed by solvent evaporation to provide the solid which was triturated with hexane (1 × 200 mL), CH2Cl2/PhMe (1 × 200 mL), and hexane (1 × 200 mL). The vacuum-dried crude residue was dissolved in CH3CN (10 mL) in the presence of DMAP (64 mg, 0.53 mmol) and refluxed for 19 h. The reaction mixture was purified by preparative TLC (EtOAc: MeOH: CH2Cl2, 50:2.5:47.5) to afford 4a (45.5 g, 28%); m.p.: 112.8–114.1 °C, [α]D30 = +300.55 (c 0.061, CHCl3), IR vmax (KBr) 3417, 3068, 1683, 1594, 1471, 1387, 1333 cm−1; 1H NMR see Table 2; 13C NMR see Table 3; m/z (rel. intensity, %): 386.3 (M+, 8), 341.0 (5), 315 (10), 282.0 (8), 257.1 (55), 241.9 (7), 217.1 (17), 186.1 (10), 171.0 (18), 130.1 (100), 103.0 (14), 77.0 (16); (+)-HRESIMS m/z 387.1810 (M + H)+ (calculated for C23H22N4O2, 387.1776).

3.4.3. Synthesis of (15,4S)-4-(1H-Indol-3-ylmethyl)-1-isopropyl-2H-pyrazino[2,1-b]quinazolin-3,6-(1H, 4H)-dione (4a)

To a solution of vi-a (290 mg, 0.440 mmol) in dried CH2Cl2 (20 mL) was added Ph3P (576 mg, 2.2 mmol, 5 equiv), I2 (448 mg, 2.16 mmol. 4.9 equiv), and N,N-diisopropylethylamine (774 µL, 4.44 mmol, 10 equiv). The reaction mixture was stirred at room temperature for 5 h, quenched with aqueous Na2CO3, and extracted with CH2Cl2 (3 × 100 mL), dried with Na2SO4, filtered and concentrated. Hexane was added to remove an excess of Ph3P; the precipitate was filtered and was treated with CH2Cl2 (10 mL) and piperidine (2.5 mL, 20%), at room temperature for 20 min, followed by solvent evaporation to provide the solid which was triturated with hexane (1 × 200 mL), CH2Cl2/PhMe (1 × 200 mL), and hexane (1 × 200 mL). Hexane was added to remove an excess of Ph3P. The precipitate was filtered and was treated with CH2Cl2 (10 mL) and piperidine (2.5 mL, 20%) at room temperature for 20 min, followed by solvent evaporation to provide the solid which was triturated with hexane (1 × 200 mL), CH2Cl2/PhMe (1 × 200 mL), and hexane (1 × 200 mL). The vacuum-dried crude residue was dissolved in CH3CN (10 mL) in the presence of DMAP (64 mg, 0.53 mmol) and refluxed for 19 h. The reaction mixture was purified by preparative TLC (EtOAc: MeOH: CH2Cl2, 50:2.5:47.5) to afford 4a (45.5 g, 28%); m.p.: 112.8–114.1 °C, [α]D30 = +300.55 (c 0.061, CHCl3), IR vmax (KBr) 3417, 3068, 1683, 1594, 1471, 1387, 1333 cm−1; 1H NMR see Table 2; 13C NMR see Table 3; m/z (rel. intensity, %): 386.3 (M+, 8), 341.0 (5), 315 (10), 282.0 (8), 257.1 (55), 241.9 (7), 217.1 (17), 186.1 (10), 171.0 (18), 130.1 (100), 103.0 (14), 77.0 (16); (+)-HRESIMS m/z 387.1810 (M + H)+ (calculated for C23H22N4O2, 387.1776).

3.4.4. Synthesis of N-[9H-Fluoren-9-ylmethoxy]carbonyl]-L-methylpentanyl-2H-aminobenzoyl-L-tryptophan methyl ester (vi-b)

To a solution of compound iv-a (129 mg, 0.382 mmol) in dried CH2Cl2 (10 mL) was added N-Fmoc-L-leucine-Cl [27] (v-b, 171 mg, 0.458 mmol). The mixture was stirred for 30 min, followed by addition of aqueous Na2CO3 (1 M, 7.6 mL, 7.6 mmol). After being stirred for a total 3 h, the mixture was extracted with CH2Cl2 (4 × 100 mL), dried with Na2SO4, filtered, and concentrated. The residue was purified by flash chromatography (eluent: 5% MeOH in CH2Cl2) to give vi-b as a white solid (231.7 mg, 92%); m.p.: 193.7–194.9 °C, [α]D30 = +18.42 (c 0.398, CHCl3), IR vmax (KBr) 3405, 1744, 1695, 1586 cm−1; 1H NMR (300, MHz, CDCl3): 11.4 (s, 1H), 8.59 (d, 1H, J 8.3 Hz), 8.19 (s, 1H), 7.76 (d, 2H,
1H, 7.3 Hz), 7.65 (d, 1H, J = 7.6 Hz), 7.59 (d, 1H, J = 7.4 Hz), 7.52-7.28 (m, 8H), 7.17 (t, 1H, J = 7.3 Hz), 7.06 (d, 1H, J = 7.4 Hz), 7.00 (d, 1H, J = 7.7 Hz) 6.96 (s, 1H), 6.73 (d, 1H, J = 7.6 Hz), 5.55 (d, 1H, J = 8.5 Hz), 5.05 (dd, 1H, J = 12.6 and 5.2 Hz), 4.39 (d, 2H, J = 6.6 Hz), 4.28 (m, 2H), 3.72 (s, 3H), 3.38 (m, 2H), 2.33 (dt, 1H, J = 13.0 and 6.4 Hz), 1.06 (d, 3H, J = 6.8 Hz), 0.98 (d, 3H, J = 6.8 Hz); 13C NMR (75 MHz, CDCl3): 172.1 (CO), 170.2 (CO), 168.3 (CO), 156.5 (CO), 143.8 (2C), 141.3 (2C), 139.0 (C), 136.1 (C), 132.9 (CH), 127.7 (2CH), 127.5 (C), 127.1 (2CH), 126.9 (CH), 125.3 (CH), 125.2 (CH), 123.2 (CH), 122.8 (CH), 122.4 (CH), 121.4 (C), 120.2 (2CH), 119.8 (CH), 118.5 (C), 111.4 (CH), 109.7 (CH), 67.2 (CH2), 61.3 (CH), 53.3 (CH), 52.6 (CH3), 38.6 (CH), 47.3 (CH2), 31.4 (CH), 27.3 (CH2), 19.4 (CH3), 17.5 (CH3).

3.4.5. Synthesis of (15,4S)-4-(1H-Indol-3-ylmethyl)-1-isobutyl-2H-pyrazino[2,1-b]quinazolin-3,6-(1H,4H)-dione (5a)

To a solution of vi-b (232 mg, 0.344 mmol) in dried CH2Cl2 (20 mL) was added Ph3P (451 mg, 1.72 mmol, 5 equiv), I2 (428 mg, 1.68 mmol, 4.9 equiv), and N,N-diisopropylethylamine (605 µL, 3.47 mmol, 10 equiv). The reaction mixture was stirred at room temperature for 5 h, quenched with aqueous Na2CO3, and extracted with CH2Cl2 (3 × 100 mL), dried with Na2SO4, filtered and concentrated. Hexane was added to remove an excess of Ph3P; the precipitate was filtered and was treated with CH2Cl2 (10 mL) and piperidine (2.5 mL, 20%) at room temperature for 20 min, followed by solvent evaporation to provide the solid which was triturated with hexane (1 × 200 mL), CH2Cl2/PhMe (1 × 200 mL), and hexane (1 × 200 mL). The vacuum-dried crude residue was dissolved in CH3CN (10 mL) in the presence of DMAP (80 mg, 0.66 mmol) and refluxed for 19 h. The reaction mixture was purified by preparative TLC (EtOAc:MeOH:CH2Cl2 = 7:3:2) to afford 5a (39 mg, 28%); m.p.: 131.9–134.3 °C; [α]D20 = +81.76 (c 0.106, CHCl3), IR νmax (KBr) 3435, 3060, 1686, 1602, 1387, 1292 cm–1; 1H NMR see Table 2; 13C NMR see Table 3; (+)-HRESIMS m/z 401.1933 (M + H)+ (calculated for C24H24N4O2, 401.1933).

3.5. General Condition for the Synthesis Compound 4d and 5d

3.5.1. Synthesis of N-(2-Aminobenzoyl)-D-tryptophan methyl ester (iv-b)

To a mixture of anthranilic acid (287 mg, 2.39 mmol) and TBTU (920 mg, 2.86 mmol, 1.2 equiv) in CH2CN (20 mL) was added Et3N (833 µL, 4.78 mmol, 2 equiv) and D-tryptophan methyl ester (521 mg, 2.39 mmol) at room temperature with stirring. After being stirred for 5 h, the reaction mixture was concentrated under reduced pressure. The residue was dissolved in CH2Cl2 and washed with 1M HCl, extracted with CH2Cl2 (3 × 100 mL), dried with Na2SO4, filtered and concentrated. The residue was purified by flash chromatography (eluent: 1% MeOH in CH2Cl2) to yield iv-b as a white solid (569.7 mg, 81%), m.p.: 131.9–134.3 °C, IR νmax (KBr) 3423, 1746, 1644 cm–1, 1H NMR (300 MHz, CDCl3): 8.14 (s, 1H), 7.56 (d, 1H, J = 7.9 Hz), 7.35 (d, 1H, J = 8.1 Hz), 7.19–7.15 (m, 3H), 7.10 (ddd, 1H, J = 8.0, 7.1, and 1.1 Hz), 7.01 (d, 1H, J = 2.4 Hz), 6.65 (dd, 1H, J = 8.7 and 1.1), 6.60 (s, 1H), 6.59–6.52 (ddd, 2H, J = 7.5 and 0.8 Hz), 5.08 (dt, 1H, J = 7.5 and 5.3 Hz), 3.72 (s, 3H), 3.43 (m, 2H); 13C NMR (75 MHz, CDCl3): 172.6 (CO), 168.8 (CO), 148.8 (C), 136.1 (C), 132.6 (CH), 127.6 (CH), 127.5 (C), 122.8 (CH), 122.3 (CH), 119.8 (CH), 118.7 (CH), 117.3 (CH), 116.7 (CH), 115.3 (C), 111.3 (CH), 110.1 (C), 53.1 (CH), 52.5 (CH3), 27.7 (CH2).

3.5.2. Synthesis of N-[9H-Fluoren-9-ylmethoxy]carbonyl]-D-valinyl-2-aminobenzoyl-D-tryptophan methyl ester (vi-c)

To a solution of iv-b (140 mg, 0.416 mmol) in dried CH2Cl2 (10 mL) was added N-Fmoc-D-valine-Cl [27] (v-c, 182 mg, 0.5 mmol). The mixture was stirred for 30 min, followed by addition of aqueous Na2CO3 (1 M, 8 mL, 8 mmol). After continuous stirring for 3 h, the mixture was extracted with CH2Cl2 (4 × 100 mL), dried with Na2SO4, filtered, and concentrated. The residue was purified by flash chromatography (eluent: 5% MeOH in CH2Cl2 to give vi-c as a white solid (220.4 mg, 84%), m.p.: 197.8–200.2 °C, [α]D20 = −22.72 (c 0.088, CHCl3), IR νmax (KBr) 3423, 1724, 1670, 1589 cm–1;
1H NMR (300, MHz, CDCl3): 11.42 (s, 1H), 8.59 (d, 1H, J = 8.3 Hz), 8.18 (s, 1H), 7.76 (d, 2H, J = 7.4 Hz), 7.66 (d, 1H, J = 7.3 Hz), 7.60 (d, 1H, J = 7.3 Hz); 7.53–7.28 (m, 9H), 7.17 (t, 1H, J = 7.3 Hz), 7.08 (d, 1H, J = 7.4 Hz), 7.02 (m, 1H), 6.96 (s, 1H), 6.72 (d, 1H, J = 7.6 Hz), 5.55 (d, 1H, J = 8.5 Hz), 5.06 (dd, 1H, J = 12.5 and 5.2 Hz), 4.40 (d, 1H, J = 6.6 Hz), 4.32–4.24 (m, 1H), 3.73 (s, 3H), 3.45–3.31 (m, 2H), 1.65 (s, 3H) 2.40–2.31 (m, 1H), 1.06 (d, 3H, J = 6.8 Hz), 0.98 (d, 3H, J = 6.9 Hz). \[^{13}\text{C}\] NMR (75, MHz, CDCl3) 172.0 (CO), 170.2 (CO), 168.3 (CO), 156.5 (C), 144.1 (2C), 141.3 (2C), 139.0 (C), 136.1 (C), 132.9 (CH), 136.1 (C), 132.9 (CH), 136.1 (C), 132.9 (CH), 136.1 (C), 132.9 (CH), 136.1 (C), 132.9 (CH), 129.7 (2CH), 127.5 (C), 127.1 (2CH), 126.9 (CH), 125.3 (CH), 125.2 (CH), 123.2 (CH), 122.8 (CH), 122.4 (CH), 121.4 (C), 120.2 (2CH), 119.8 (CH), 118.5 (C), 114.1 (CH), 109.7 (C), 67.2 (CH2), 61.3 (CH), 53.3 (CH), 52.6 (CH3), 47.3 (CH), 31.3 (CH), 27.3 (CH2), 19.4 (CH2), 17.6 (CH3).

3.5.3. Synthesis of (1R,4R)-4-[(1H-Indol-3-ylmethyl)-1-isopropyl-2H-pyrazino[2,1-b]quinoxalin-3,6-(1H,4H)-dione (4d)

To a solution of vi-c (183 mg, 0.278 mmol) in dried CH2Cl2 (20 mL) was added Ph3P (365 mg, 1.4 mmol, 5 equiv), I2 (345 mg, 1.36 mmol, 4.9 equiv), and N,N-diisopropylethylamine (489 µL, 2.81 mmol, 10 equiv). The reaction mixture was stirred at room temperature for 5 h, quenched with aqueous Na2CO3, and extracted with CH2Cl2 (3 × 100 mL), dried with Na2SO4, filtered, and concentrated. Hexane was added to remove an excess of Ph3P, the precipitate was filtered and treated with CH2Cl2 (10 mL) and piperidine (2.5 mL, 20%) at room temperature for 20 min, followed by solvent evaporation to provide the solid which was triturated with hexane (1 × 200mL), CH2Cl2/PhMe (1 × 200 mL), and hexane (1 × 200 mL). The vacuum-dried crude residue was dissolved in CH3CN (10 mL in the presence of DMAP (64 mg, 0.53 mmol) and refluxed for 19 h. The reaction mixture was purified by preparative TLC (EtOAc:MeOH:CH3N= 7.4 Hz), 7.00 (t, 1H, J = 7.4 Hz), 7.66 (d, 1H, J = 7.3 Hz), 7.53–7.28 (m, 9H), 7.17 (t, 1H, J = 7.3 Hz), 7.08 (d, 1H, J = 7.4 Hz), 7.02 (m, 1H), 6.96 (s, 1H), 6.72 (d, 1H, J = 7.6 Hz), 5.55 (d, 1H, J = 8.5 Hz), 5.06 (dd, 1H, J = 12.5 and 5.2 Hz), 4.40 (d, 1H, J = 6.6 Hz), 4.32–4.24 (m, 1H), 3.73 (s, 3H), 3.45–3.31 (m, 2H), 1.65 (s, 3H) 2.40–2.31 (m, 1H), 1.06 (d, 3H, J = 6.8 Hz), 0.98 (d, 3H, J = 6.9 Hz).

3.5.4. Synthesis of N-[9H-Fluoren-9-yldimethoxy)carbonyl]-D-methylpentyl-2-aminobenzoyl-D-tryptophan methyl ester (vi-d)

To a solution of iv-b (130 mg, 0.386 mmol) in dried CH2Cl2 (10 mL) was added N-Fmoc-D-leucine-Cl [27] (v-d, 172.5 mg, 0.464 mmol). The mixture was stirred for 30 min, followed by addition of aqueous Na2CO3 (1 M, 7.7 mL, 7.7 mmol). After continuous stirring for 3 h, the mixture was extracted with CH2Cl2 (4 ×), dried with Na2SO4, filtered, and concentrated. The residue was purified by flash chromatography (eluent: 5% MeOH in CH2Cl2) to give vi-d as a white solid (251 mg, 98%), m.p.: 194.9–196.3 °C, [α]D30 = −31.75 (c 0.105, CHCl3), IR max (KBr) 3321, 3068, 1683, 1593, 1471, 1387, 1291 cm−1; 1H NMR see Table 2; 13C NMR see Table 3; m/z (rel. intensity, %): 385.9 (M+), 127.1, 257.1 (29), 214.1 (3), 202.0 (7), 171.1 (10), 143.1 (4), 130.1 (100), 103.0 (18), 77 (16); (+)-HRESIMS m/z 389.1776 (M + H)+ (calculated for C23H22N4O2, 387.1809).

3.5.5. Synthesis of (1R,4R)-4-[(1H-Indol-3-ylmethyl)-1-isobutyl-2H-pyrazino[2,1-b]quinoxalin-3,6-(1H,4H)-dione (5d)

To a solution of vi-d (251 mg, 0.373 mmol) in dried CH2Cl2 (20 mL) was added Ph3P (489 mg, 1.9 mmol, 5 equiv), I2 (464 mg, 1.83 mmol, 4.9 equiv), and N,N-diisopropylethylamine (656 µL,
3.77 mmol, 10 equiv). The reaction mixture was stirred at room temperature for 5 h, quenched with aqueous Na₂CO₃, and extracted with CH₂Cl₂ (3 × 100 mL), dried with Na₂SO₄, filtered, and concentrated. Hexane was added to remove an excess of Ph₃P, the precipitate was filtered and was treated with CH₂Cl₂ (10 mL) and piperidine (2.5 mL, 20%) at room temperature for 20 min, followed by solvent evaporation to provide the solid which was triturated with hexane (1 × 200 mL), CH₂Cl₂/PhMe (1 × 200 mL), and hexane (1 × 200 mL). The vacuum-dried crude residue was dissolved in CH₃CN (10 mL in the presence of DMAP (84 mg, 0.82 mmol) and refluxed for 19 h. The reaction mixture was purified by preparative TLC (EtOAc:MeOH:CH₂Cl₂, 50:2.5:47.5) to afford 5d (61.6 mg, 40%), m.p.: 103.2–105.6 ℃, [α]₃₀°D = −186.04 (c 0.086, CHCl₃), IR νmax (KBr) 3333, 3061, 1687, 1603, 1296 cm⁻¹; ¹H NMR see Table 2; ¹³C NMR see Table 3; (+)-HRESIMS m/z 401.1966 (M + H)⁺ (calculated for C₂₄H₂₄N₄O₂, 401.1933).

3.6. Screening Test for Antitumor and Anti-P-Glycoprotein Activity

Compounds 4a–d and 5a–d were reconstituted in sterile DMSO to the final concentration of 60 mM, and several aliquots were made and stored at −20 ℃ to avoid repeated freeze-thaw cycles. For experiments, the compounds were freshly diluted in medium to the desired concentration. Screening for tumor cell growth inhibition was carried out in two human tumor cell lines (NCI-H460 and HCT-15), with the sulforhodamine B (SRB) assay, as previously described [30]. Briefly, tumor cells were plated in 96-well plates, incubated at 37 ℃ for 24 h, and then treated for 48 h with 5 serial dilutions (1:2) of each compound (ranging from 150 µM to 9.375 µM). The effect of the vehicle solvent (DMSO) was also analyzed as a control. Cells were fixed with 10% ice-cold trichloroacetic acid, washed with water and stained with SRB. Finally, the plates were washed with 1% acetic acid and the bound SRB was solubilized with 10 mM Tris Base. Absorbance was measured in a microplate reader (Synergy Mx, Biotek Instruments Inc., Winooski, VT, USA) at 510 nm. For each compound, the corresponding GI₅₀ (concentration which inhibited 50% of net cell growth) was determined, as previously described [33]. For the screening of compounds for drug-efflux inhibitory activity, the flow cytometry determination of rhodamine-123 cellular accumulation was carried out as previously described [34]. Briefly, K562 and K562Dox cells were incubated for 1 h at 37 ℃ with 20 µM of the compounds, and 1 µM of rhodamine-123 (Rh123, from Sigma, USA). Verapamil was used as a positive control. Cells were then washed, resuspended in ice cold PBS, and analyzed in a BD Accuri™ C6 Flow Cytometer (BD Biosciences, San Jose, CA, USA). Data were analyzed using the FlowJo software (version 7.6.1, Tree Star, Inc.). The ratio of Rh123 accumulation in the cells was then calculated as MFIK562Dox+Compound -MFIK562Dox)/MFIK562Dox [30].

4. Conclusions

Inspired by the marine-derived fiscalin B (4c), quinazolinone alkaloid derivatives were synthesized using two different methodologies: a highly efficient and straightforward three-component one-pot microwave-assisted approach and also a multistep Mazurkiewicz–Ganesan approach. While the former proved to be efficient and practical for broad screening libraries of the compounds, the latter, although with a more intricate methodology, proved to be a good approach for the synthesis of the syn enantiomers. Moreover, we have found that partial epimerization under the reaction conditions could occur. In vitro growth inhibitory activity of two tumor cell lines revealed that among this series of synthesized compounds, six new analogues were found to exhibit tumor cell growth inhibitory activity. Consequently, this marine-inspired synthesis can bring new insights into discovery of new lead compounds in the oncology area.

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Author Contributions: E.S. and A.K. conceived the study design. S.L. synthesized the compounds and elucidated their structure and, A.M.S.S., E.S., and M.M.M.P. analysed the data. D.I.S.P. performed the HPLC analysis. A.P. and T.F.-M. performed the cytotoxicity and anti-Pgp assays, and T.F.-M. and M.H.V. analyzed the data and
discussed results. S.L. and E.S. wrote the manuscript, while all authors gave significant contributions in discussion and revision. All authors agreed to the final version of the manuscript.

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