Article

Synthesis and Cytotoxicity Evaluation of Spirocyclic Bromotyrosine Clavatadine C Analogs

Piyush A. Patel 1*, Tanja Bruun 1*, Polina Ilina 2, Heidi Mäkkylä 2, Antti Lempinen 1, Jari Yli-Kauhaluoma 1, Päivi Tammela 2* and Paula S. Kiuru 1,2*

1 Drug Research Program, Division of Pharmaceutical Chemistry and Technology, Faculty of Pharmacy, University of Helsinki, FI-00014 Helsinki, Finland; piyushkumar.patel@helsinki.fi (P.A.P.); tanja.bruun@helsinki.fi (T.B.); antti.lempinen@helsinki.fi (A.L.); jari.yli-kauhaluoma@helsinki.fi (J.Y.-K.)
2 Drug Research Program, Division of Pharmaceutical Biosciences, Faculty of Pharmacy, University of Helsinki, FI-00014 Helsinki, Finland; polina.ilina@helsinki.fi (P.I.); heidi.makkyila@helsinki.fi (H.M.); paivi.tammela@helsinki.fi (P.T.)
* Correspondence: paula.kiuru@helsinki.fi

Abstract: Marine-originated spirocyclic bromotyrosines are considered as promising scaffolds for new anticancer drugs. In a continuation of our research to develop potent and more selective anticancer compounds, we synthesized a library of 32 spirocyclic clavatadine analogs by replacing the agmatine, i.e., 4-(aminobutyl)guanidine, side chain with different substituents. These compounds were tested for cytotoxicity against skin cancer using the human melanoma cell line (A-375) and normal human fibroblast cell line (Hs27). The highest cytotoxicity against the A-375 cell line was observed for dichloro compound 18 (CC50 0.4 ± 0.3 µM, selectivity index (SI) 2). The variation of selectivity ranged from SI 0.4 to reach 2.4 for the pyridin-2-yl derivative 29 and hydrazide analog of 2-picoline 37. The structure–activity relationships of the compounds in respect to cytotoxicity and selectivity toward cancer cell lines are discussed.

Keywords: Clavatadine C; spirocyclic bromotyrosines; cytotoxicity; cancer selectivity; marine compounds; melanoma A-375 cell line

1. Introduction

Natural products have a long history of use in the treatment of various diseases, including cancer [1]. Marine organisms are a rich source of novel compounds with medicinally relevant properties. Many marine-derived bioactive terpenes, alkaloids, macrolides, and other compounds isolated from aquatic fungi, cyanobacteria, algae, sponges, and tunicates have been found to exhibit anticancer activities [2,3]. To date, 15 drugs with marine origins have been approved by the U.S. Food and Drug Administration and/or European Medicines Agency. Nine of these drugs are registered for the treatment of different cancer types [4,5]. At present, 33 marine-based compounds are in clinical trials, out of which 29 are being evaluated for cancer therapy. Bromotyrosine alkaloids have acquired special importance in medicinal chemistry since the vast majority of these secondary metabolites possess potential anticancer [6], antimicrobial, antiviral, and antifungal activities [7,8]. Quinn and co-workers identified two new spirocyclic bromotyrosine compounds, clavatadine C 1 and clavatadine D 2 (Figure 1). Both were isolated as trifluoroacetic acid (TFA) salts from the marine sponge Suberea clavata and their anticoagulative properties were described. [9]. Furthermore, moderate activity against MCF7, MDA-MB-231 (breast), and A549 (lung) cancer cell lines have been observed for clavatadine C 1-TFA [10]. Marine bromotyrosines with an isoxazoline moiety attached to the spiro center have exhibited anticancer properties [11,12]. The spirocyclic bromotyrosine is structurally more rigid and better occupies the chemical space than the open-chain bromotyrosine, making it an interesting scaffold for medicinal chemistry [13]. We have previously reported a set of simplified open-chain bromotyrosine...
analogs of purpurealidin I 3 (Figure 1) with potential antiproliferative activity [14]. As purpurealidin analogs are E-isomers having free rotation around the C-C σ bond, introduction of conformational restriction in the form of a spiro ring fusion offers a good strategy to improve selectivity toward the target cell of interest [15].

Figure 1. Clavatadine C, D, and purpurealidin I.

Cytotoxicity toward normal cells is a major challenge with anticancer compounds. Therefore, different approaches are required to develop a target-specific anticancer treatment. The structural simplification of natural products is one of the well-known strategies to improve pharmacokinetic profiles and to reduce side effects [16]. Initially, anticancer activity of the first simplified spiroisoxazolines 4 and 5 (Figure 2) was reported in Ehrlich ascites tumor cells in mice [17,18].

In order to understand the structure–activity relationships (SARs) of spirocyclic bromotyrosines as cytotoxic agents toward cancer cells, we synthesized a library of simplified spirocyclic clavatadine analogs 11–42 (Table 1). The agmatine, i.e., 4-(aminobutyl)guanidine, side chain was replaced with different amino and hydrazide substituents. These analogs were tested against a melanoma cell line (A-375) and normal human skin fibroblast cell line (Hs27) for cytotoxicity. The clavatadine scaffold was selected for the library synthesis to limit the free C-C rotation of purpurealidin analogs, to have a stereochemically simpler spiro core than the one in other spirocyclic bromotyrosines, and to build on the proven anticancer activity of simplified clavatadine analogs, such as compound 4.

The compounds were synthesized according to the route presented in Scheme 1. Synthetic procedures and analytical data of the compounds are given in the Supporting Information.
Table 1. Structures of the target spirocyclic bromotyrosine analogs 1-TFA and 11–42.

| No | X   | -R       | No | X   | -R       | No | X   | -R       | No | X   | -R       |
|----|-----|----------|----|-----|----------|----|-----|----------|----|-----|----------|
| 1-TFA | Br  | H        | 19 | Br  | H        | 28 | H   | H        | 37 | H   | H        |
| 11  | H   | H        | 20 | H   | H        | 29 | Br  | H        | 38 | Br  | H        |
| 12  | H   | N        | 21 | Br  | H        | 30 | Br  | N        | 39 | Br  | N        |
| 13  | Br  | H        | 22 | H   | H        | 31 | Br  | N        | 40 | H   | N        |
| 14  | Br  | N        | 23 | H   | H        | 32 | H   | N        | 41 | Br  | N        |
| 15  | H   | O        | 24 | Br  | H        | 33 | Br  | H        | 42 | Br  | O        |
| 16  | H   | OH       | 25 | H   | H        | 34 | Br  | H        |    |     |          |
| 17  | H   | N        | 26 | H   | N        | 35 | H   | N        |    |     |          |
| 18  | H   | Cl       | 27 | Br  | H        | 36 | Br  | H        |    |     |          |
reported open-chain bromotyrosine analogs [14], we found that most spirocyclic bromotyrosine analogs have lower CC50 to both cell lines tested. The lower SIs were observed for the aliphatic analogs compared to the hydrazide spacer. We also introduced ethylene and methylene groups as spacers in the pharmacologically important trifluoromethyl group [22] to pyridine in the synthesized libraries. The 3-chloro-4-methoxyphenyl analog without a spacer exhibited relatively similar cytotoxicity, the 3-chloro-4-methoxyphenyl analog without a spacer exhibited relatively similar cytotoxicity, the 3-chloro-4-methoxyphenyl analog without a spacer exhibited relatively similar cytotoxicity, the 3-chloro-4-methoxyphenyl analog without a spacer exhibited relatively similar cytotoxicity, the 3-chloro-4-methoxyphenyl analog without a spacer exhibited relatively similar cytotoxicity, the 3-chloro-4-methoxyphenyl analog without a spacer exhibited relatively similar cytotoxicity, the 3-chloro-4-methoxyphenyl analog without a spacer exhibited relatively similar cytotoxicity, the 3-chloro-4-methoxyphenyl analog without a spacer exhibited relatively similar cytotoxicity.

**Scheme 1.** General scheme for synthesis of spirocyclic bromotyrosines 1-TFA and 11–42. Reagents and conditions: a) HClO4, tert-butyl acetate, 20–25 °C, 18 h; b) Na2WO4·2H2O, H2O2, EtOH, 20–25 °C, 7 h; c) PIFA, pyridine, TFE, 0 °C, 1 h, or NBS, DMF, 0 °C, 20 min; d) TFA, DCM, 20–25 °C, 6 h; e) amine, EDC·HCl, HOBt, DCM, 20–25 °C, 5–15 h. The R substituents are given in Table 1.

### 2. Results

#### 2.1. Chemistry

The synthesis of the spirocyclic bromotyrosine scaffold started with esterification of L-tyrosine 6 using tert-butyl acetate in the presence of perchloric acid to give L-tyrosine tert-butyl ester 7. The ester 7 was oxidized with sodium tungstate and H2O2 to give the oxime 8 [10]. This resulting oxime was subjected to oxidative spirocyclization via treatment with [bis(trifluoro-acetoxy)iodo]benzene (PIFA) in 2,2,2-trifluoroethanol (TFE) in the case of non-halogenated compounds or N-bromosuccinimide (NBS) in N,N-dimethylformamide (DMF) in the case of brominated compounds to provide spirocyclic esters 9a/9b. The tert-butyl esters 9a/9b were deprotected with trifluoroacetic acid in dichloromethane (DCM) to give the spirocyclic carboxyl core 10a/10b. This spirocyclic core was coupled with various amines or hydrazides in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC·HCl) and 1-hydroxybenzotriazole hydrate (HOBt·H2O) to give the target spirocyclic bromotyrosine analogs 1-TFA and 11–42 (Table 1) with yields ranging 10–91%. We observed that the yields were typically higher in the case of dihydro carboxyl core 10a compared to the dibromo core 10b, and heterocyclic and aromatic amines compared to aliphatic amines and hydrazines.

#### 2.2. Biological Activity

The cytotoxicities of the synthetic clavatadine C 1-TFA, dihydroclavatadine C 11, and compounds 12–42 against cancer cells were primarily evaluated in the human malignant melanoma A-375 cell line at the single concentration of 50 µM (Table 2). The compounds demonstrating over 80% cytotoxicity were selected for confirmatory dose-response experiments in the same cell line and CC50 (cytotoxic concentration that caused death of 50% of cells) was calculated (Table 2). The observed cytotoxicity (CC50) against the A-375
melanoma cell line for the compounds 1-TFA and 11–42 was in the range of 0.4–12.3 µM (Table 2). Furthermore, we aimed to evaluate the potential of the compounds to selectively perturb the growth of skin cancer cells. Therefore, the compounds were tested for cytotoxicity in normal human fibroblast cell line Hs27 (Table 2).

Table 2. Cytotoxicity of compounds 1-TFA and 11–42 against human malignant melanoma cell line A-375 and normal human fibroblast Hs27 cells. Camptothecin, a compound with high selectivity to cancer cells, was used as a positive control. Purpurealidin I 3 was used as a reference compound [14]. CC50 = cytotoxic concentration that caused death of 50% of cells. ND = not determined. The primary screening was performed as a single experiment with three technical replicates per sample. The CC50 values are arithmetic means from 2–3 independent experiments performed in triplicate. The values in parentheses are standard deviations.

| Compound | Primary Test Results (Cytotoxicity % at 50 µM in A-375 Cells) | CC50 (µM) in A-375 Cells | CC50 (µM) in Hs27 Cells | Selectivity Index (SI) |
|----------|---------------------------------------------------------------|--------------------------|------------------------|-----------------------|
| 1-TFA    | 0.5                                                          | ND                       | ND                     | ND                    |
| 11       | −8.2                                                         | ND                       | ND                     | ND                    |
| 12       | 95.1                                                         | 2.1 (1.2)                | 3.8 (0.7)              | 1.8 (0.6)             |
| 13       | 99.8                                                         | 3.7 (1.9)                | 7.2 (6.2)              | 1.9 (1.0)             |
| 14       | 99.9                                                         | 7.2 (3.2)                | 7.8 (1.8)              | 1.1 (0.5)             |
| 15       | 96.1                                                         | 3.8 (0.7)                | 2.5 (0.3)              | 0.7 (0.2)             |
| 16       | 97.1                                                         | 4.9 (1.0)                | 7.4 (0.5)              | 1.5 (0.2)             |
| 17       | 20.8                                                         | ND                       | ND                     | ND                    |
| 18       | 100.0                                                        | 0.4 (0.3)                | 0.8 (0.2)              | 2.0 (0.8)             |
| 19       | 94.9                                                         | 9.2 (2.9)                | 6.8 (2.8)              | 0.7 (0.5)             |
| 20       | 100.0                                                        | 1.2 (0.1)                | 1.5 (0.7)              | 1.3 (0.5)             |
| 21       | 101.1                                                        | 2.3 (0.4)                | 5.0 (1.1)              | 2.2 (0.3)             |
| 22       | 99.8                                                         | 0.7 (0.1)                | 0.7 (0.2)              | 1.0 (0.3)             |
| 23       | 100.0                                                        | 1.2 (0.2)                | 1.4 (0.8)              | 1.2 (0.6)             |
| 24       | 97.8                                                         | 3.5 (1.1)                | 3.3 (1.1)              | 0.9 (0.3)             |
| 25       | 100.0                                                        | 1.2 (0.1)                | 2.0 (0.6)              | 1.7 (0.3)             |
| 26       | 99.2                                                         | 2.0 (1.2)                | 1.9 (0.9)              | 1.0 (0.8)             |
| 27       | 100.7                                                        | 3.0 (0.3)                | 6.6 (3.1)              | 2.2 (0.5)             |
| 28       | 99.6                                                         | 1.9 (0.2)                | 1.5 (0.6)              | 0.8 (0.4)             |
| 29       | 100.6                                                        | 2.4 (0.3)                | 5.8 (2.5)              | 2.4 (0.4)             |
| 30       | 98.1                                                         | 3.6 (0.1)                | 2.3 (0.4)              | 0.6 (0.2)             |
| 31       | 98.6                                                         | 5.3 (0.7)                | 2.1 (0.1)              | 0.4 (0.1)             |
| 32       | 97.5                                                         | 1.5 (1.0)                | 2.2 (1.3)              | 1.5 (0.9)             |
| 33       | 98.9                                                         | 3.6 (1.9)                | 6.6 (1.3)              | 1.9 (0.6)             |
| 34       | 97.0                                                         | 5.1 (2.8)                | 8.8 (1.8)              | 1.7 (0.6)             |
| 35       | 97.5                                                         | 1.1 (0.1)                | 2.5 (0.2)              | 2.3 (0.1)             |
| 36       | 100.7                                                        | 3.4 (0.2)                | 6.1 (0.9)              | 1.8 (0.2)             |
| 37       | 89.3                                                         | 2.5 (0.6)                | 6.0 (1.3)              | 2.4 (0.3)             |
| 38       | 93.5                                                         | 12.3 (1.3)               | 6.4 (3.0)              | 0.5 (0.5)             |
| 39       | 75.2                                                         | ND                       | ND                     | ND                    |
| 40       | 99.9                                                         | 0.5 (0.1)                | 0.5 (0.4)              | 1.0 (0.8)             |
| 41       | 97.6                                                         | 1.3 (0.4)                | 1.4 (1.0)              | 1.1 (0.8)             |
| 42       | 97.5                                                         | 3.5 (1.2)                | 4.4 (1.4)              | 1.3 (0.5)             |
| Purpurealidin I 3 | 98.9 | 3.7 (0.5) | 4.7 (0.2) | 1.3 (0.1) |
| Camptothecin | 94.7 | 0.05 (0.02) | 3.6 (1.0) | 70.9 (0.5) |

The degree of selectivity toward cancer cells can be expressed by the selectivity index (SI). The SI was calculated as a ratio of CC50 values between Hs27 fibroblasts and A-375 melanoma cells. High SI values show selectivity toward cancer cells, while values <2 show low selectivity [19] (Table 2). The highest but still moderate selectivity to cancer cells (SI 2.4, Table 2) was observed for the pyridin-2-yl compound 29 and hydrazide analog of 2-picoline 37. To further elucidate mechanisms of cytotoxicity mediated by these compounds, we tested their ability to induce apoptosis. Apoptosis, or programmed cell
death, is a mechanism utilized in the body for elimination of unwanted or damaged cells during development and aging. In cancer cells, apoptosis is typically inhibited and most selective anticancer agents act via induction of this pathway [20]. To elucidate potential effects of spirocyclic bromotyrosines on apoptosis, we tested their ability to induce the activity of caspases 3/7, a key protease involved in the apoptotic pathway. The results show that spirocyclic bromotyrosines induced the caspase pathway about twofold after 24 h, whereas no induction was observed after 48 h (Figure 3A). Treatment with a positive control camptothecin resulted in about a 20-fold caspase induction after 24 h of treatment and a nearly fivefold induction after 48 h of treatment. The data obtained correlated with microscopic observations. Cell rounding up and shrinkage, typical for apoptosis, was observed for both camptothecin-treated cells and the cells treated with spirocyclic bromotyrosines. However, for camptothecin the effect was more profound (Figure 3B–E).

**Figure 3.** Induction of apoptosis in A-375 melanoma cells by compounds 29 and 37, which demonstrated the highest selectivity, and positive control camptothecin. Cells were treated with compounds at 1 × CC_{50} concentration for 24 and 48 h. (A) Activity of caspase 3/7 apoptotic pathway shown as a fold change relative to DMSO-treated control, indicated with a horizontal line. Bars show mean values of two independent experiments (each performed in triplicate wells) ± SD. Representative microscopic images taken after a 24-h incubation with camptothecin (B), compound 29 (C), and 37 (D) show morphological changes characteristic for apoptosis; (E) shows cell morphology in DMSO-treated control wells. RLU = relative light units.
3. Discussion

To understand the preliminary cytotoxicity of clavatidine C analogs comprised of dibromo and dihydro spirocyclic cores, we synthesized clavatidine C 1-TFA (overall yield 38%) along with various amides 11–42 having aliphatic, aromatic, and heterocyclic substitution with or without a carbon spacer. We then evaluated their effect on cytotoxicity of the melanoma cell line A-375 and the normal skin fibroblast cell line Hs27. Though the clavatidine C 1-TFA and its dihydro analog 11 did not show any primary cytotoxicity, its analogs showed cytotoxicity, as shown in Table 2. Clavatidine C 1-TFA, dihydroclavatidine C 11 and aliphatic morpholinoacetyl carbohydrazide 17 showed less than 25% primary cytotoxicity (at 50 \( \mu \text{M} \) in A-375 cells) while the rest of the compounds had over 75% primary cytotoxicity. This result was somewhat unexpected when compared to the known activity of clavatidine C 1-TFA against breast and colon cancer cell lines [10]. Binneweg et al. recently found spiro-structured isofistularin-3 to display cell line dependent effects, and the three melanoma cell lines tested (SKMel-147, Mel-Juso, and Malme-3M) showed no significant decrease (and even increased in the case of SKMel-147) in cell viability, but on the contrary isofistularin-3 reduced cell viability of breast cancer cell line MCF-7 [21]. The highest cytotoxicity against the A-375 cell line was observed in dihydro 2,4-dichloro compound 18 (CC\( _{50} \) 0.4 ± 0.3 \( \mu \text{M} \), with moderate SI 2). The selectivity indices of the most simplified dimethyl amides 13 and 14 were 1.8 and 1.9, respectively, and amides 14–16 containing aliphatic substituents showed low SI. While the introduction of aromatic substituent in 18–30 with or without a spacer exhibited relatively similar cytotoxicity, the 3-chloro-4-methoxyphenyl analog 21 showed a slight improvement in the selectivity index. As seen in our open chain library [14], the introduction of pyridinyl substituent 26–29 at the amide showed improvement in SI value to 2.4 in the case of pyridin-2-yl analog 29, whereas the introduction of the pharmacologically important trifluoromethyl group [22] to pyridine in 30 and 31 lowered the selectivity. We also introduced ethylene and methylene groups as spacers in 32–36 along with hydrazide spacers in 37–39 to pyridine to evaluate their effect on selectivity and cytotoxicity. Cytotoxicity and selectivity were increased in analogs 32–36 having a spacer compared to the analogs 14–17 having aliphatic substituents. In the case of the dihydro pyridin-2-yl analogs 32 and 37, the change of spacers to hydrazide 37 led to a modest improvement in selectivity, but in case of the corresponding bromo analogs 33 and 38 the SI was lower in hydrazide 38. The introduction of other heterocycles 40–42 gave similar lower SIs as observed for the aliphatic analogs 14–16. In comparison with our earlier reported open-chain bromotyrosine analogs [14], we found that most spirocyclic bromotyrosine analogs have lower CC\( _{50} \) to both cell lines tested.

Overall, the SIs for this set of compounds stayed below 2.5, indicating relatively low selectivity toward A-375 cell line. The cytotoxic effect of the two most selective compounds was partially mediated by caspase-dependent apoptosis, although the low (twofold) level of apoptosis induction at CC\( _{50} \) concentration suggests predominantly an unspecific cytotoxicity mechanism. Taking into account considerable dissimilarities in the biology of different cancer types [23], future work may include testing of the compounds in a panel of cancer cell lines representing various malignancies. In summary, biological data of synthesized spiro-structured clavatidine C 1 analogs demonstrate low selectivity toward skin cancer. However, structure–activity relationships indicate further structural optimization by modification of the side chain for potential development of these analogs into anticancer agents should be explored.

4. Materials and Methods

4.1. Materials and Methods for Biological Testing

Analysis of selectivity to cancer cells. The cells were seeded to white frame and clear bottom 96-well plates (Perkin Elmer) at a density of 10,000 cells/well for the human malignant melanoma A-375 cell line (ATCC CRL-1619) and 7500 cells/well for the human skin fibroblast Hs27 cell line (ATCC CRL-1634). The cells were grown at 37 °C, 5% CO\(_2\) until they reached 70–80% confluence (approximately 24 h). Stock solutions of test compounds
and a positive control (camptothecin, Sigma-Aldrich, St. Louis, MO, USA) were prepared in DMSO and diluted into the assay medium (growth medium with 5% FBS) to the final concentration. Final DMSO concentration was 0.5% in all samples. The culture medium was removed from the plate and compounds added, 200 μL/well. After a 48-h incubation, the amount of ATP, which is directly proportional to the number of viable cells present in culture, was quantified using CellTiter-Glo® Luminescent Cell Viability kit (Promega, Madison, WI, USA), according to manufacturer’s instructions. Origin Graphing and Analysis, version 9.55 (OriginLab, Northampton, MA, USA), was used for determination of CC₅₀ values. The cancer cell selectivity index was calculated as a ratio of CC₅₀ values between Hs27 fibroblasts and A-375 melanoma cells. Standard deviation of selectivity indices was calculated using Equation (1)

\[
\sigma\left(\frac{x}{y}\right) = \sqrt{\left(\frac{\sigma(x)}{x}\right)^2 + \left(\frac{\sigma(y)}{y}\right)^2}
\]

(1)

In the Equation (1), \(x\) and \(y\) are average CC₅₀ values in Hs27 and A-375 cells, respectively, and \(\sigma(x)\) and \(\sigma(y)\) are their standard deviations.

Apoptosis induction assay and imaging. A-375 cells were seeded to white 96-well plates and treated with compounds at 1 × CC₅₀ concentration, 100 μL/well, following the procedure described above. After 24 and 48 h, caspase-3/7 activity was measured using the ApoTox-Glo kit (Promega) following the manufacturer’s instructions. The light microscopy images were taken using 4× phase contrast objective and Cytation5 automated imaging reader (Biotek).

4.2. Synthesis Experimental

4.2.1. General

All reactions were carried out using commercially available starting materials unless otherwise stated. The melting points were measured with a Stuart SMP40 automated melting point apparatus and were uncorrected. ¹H NMR and ¹³C NMR spectra in CDCl₃, d₆-DMSO, d₆-acetone, or CD₃OD at ambient temperature were recorded on a Bruker Ascend 400 spectrometer. Chemical shifts (\(\delta\)) are given in parts per million (ppm) relative to the NMR reference solvent signals (CDCl₃: 7.26 ppm, 77.16 ppm; CD₃OD: 3.31 ppm, 49.00 ppm; d₆-DMSO: 2.50 ppm, 39.52 ppm; d₆-acetone: 2.05 ppm, 29.84 ppm). Multiplicities are indicated by s (singlet), br s (broad singlet), d (doublet), dd (doublet of doublets), t (triplet), dt (doublet of triplets), q (quartet), p (pentet), and m (multiplet). The coupling constants \(J\) were quoted in hertz (Hz). LC-MS and HRMS-spectra were recorded using a Waters Acquity UPLC®-system (with Acquity UPLC® BEH C18 column, 1.7 μm, 50 mm × 2.1 mm, Waters, Milford, MA, USA) with Waters Synapt G2 HDMS with the ESI (+), high resolution mode, and PDA. The mobile phase consisted of H₂O (A) and acetonitrile (B), both containing 0.1% HCOOH. Microwave syntheses were performed in sealed tubes using a Biotage Initiator+ instrument equipped with an external IR sensor. The flash chromatography was performed with a Biotage Isolera One flash chromatography purification system with a 200–800 nm UV-VIS detector using SNAP KP-Sil 10 g, 25 g, or 50 g cartridges. The TLC plates were provided by Merck (Silica gel 60-F254) and visualization of the amine compounds was conducted using ninhydrin (a 0.2% w/v solution in a 3% solution of acetic acid in 1-butanol) staining.

4.2.2. Experimental Procedures and Characterization Data

\textbf{tert-Butyl L-tyrosinate (7)}

![Chemical Structure Image]
To a stirred suspension of L-tyrosine 6 (25.0 g, 0.138 mol) in tert-butyl acetate (100 mL) in an ice bath (0 °C), perchloric acid (15.7 mL, 0.276 mol, 2.0 equiv) was added dropwise. The reaction mixture was allowed to warm to room temperature and was stirred for 17 h. The mixture was washed with H₂O (300 mL) and a 1 M solution of HCl in H₂O (250 mL). The aqueous phase was diluted with H₂O (300 mL), followed by the addition of solid K₂CO₃ until the pH was 7. The resulting mixture was filtered, the filtrate was made alkaline (pH 9) by adding solid K₂CO₃, and then it was extracted with EtOAc (3 × 300 mL). The combined organic phases were washed with brine (300 mL), dried over anhydrous Na₂SO₄, filtered, and concentrated in vacuo to give an off-white solid; crude yield: 29 g (86%). The crude product was purified with automated flash chromatography (DCM/MeOH, gradient: 0→10%) to give the product 7 as a white solid (25 g, 76%). ¹H NMR (400 MHz, CDCl₃) δ 7.01 (d, J = 8.5 Hz, 2H), 6.65 (d, J = 8.5 Hz, 2H), 3.60 (dd, J = 7.7, 5.3 Hz, 1H), 3.00 (dd, J = 13.8, 5.3 Hz, 1H), 2.77 (dd, J = 13.8, 7.7 Hz, 1H), 1.45 (s, 9H). ¹³C NMR (101 MHz, CDCl₃) δ 174.2, 155.5, 130.5, 128.2, 115.8, 81.8, 56.2, 40.0, 28.2. ¹H NMR is in accordance with the literature [24].

tert-Butyl (E)-2-(hydroxyimino)-3-(4-hydroxyphenyl)propanoate (8)

![Image of tert-Butyl (E)-2-(hydroxyimino)-3-(4-hydroxyphenyl)propanoate (8)]

To a stirred solution of tert-butyl L-tyrosinate 7 (1.20 g, 5.06 mmol) in EtOH (20 mL) in an ice bath (0 °C), Na₂WO₄·2H₂O (1.83 g, 10.4 mmol, 1.1 equiv), a 30% solution of H₂O₂ in H₂O (9 mL), and H₂O (14 mL) were added. The resulting mixture was stirred for 8 h and slowly allowed to reach room temperature. The mixture was diluted with EtOAc (25 mL) and washed with a 10% solution of Na₂SO₃ in H₂O (2 × 15 mL), H₂O (14 mL), and NaOH (10 mL), and then it was extracted with EtOAc (3 × 25 mL), and the combined organic phases were washed with brine (10 mL), dried over anhydrous Na₂SO₄, filtered, and concentrated in vacuo. The crude product was purified with automated flash chromatography (n-heptane/EtOAc gradient: 5→50%) to give the compound 8 as a white solid (1.00 g, 79%). ¹H NMR (400 MHz, CD₃OD) δ 7.05 (d, J = 8.7 Hz, 2H), 6.67 (d, J = 8.6 Hz, 2H), 3.79 (s, 2H), 1.43 (s, 9H). ¹³C NMR (101 MHz, CD₃OD) δ 164.6, 156.9, 153.6, 131.0, 128.6, 116.1, 83.3, 30.3, 28.1. ¹H NMR is in accordance with the literature [10].

tert-Butyl 8-oxo-1-oxa-2-azaspiro[4.5]deca-2,6,9-triene-3-carboxylate (9a)

![Image of tert-Butyl 8-oxo-1-oxa-2-azaspiro[4.5]deca-2,6,9-triene-3-carboxylate (9a)]

tert-Butyl (E)-2-(hydroxyimino)-3-(4-hydroxyphenyl)propanoate 8 (0.40 g, 1.6 mmol) was dissolved in 2,2,2-trifluoroethanol (7.7 mL), followed by the addition of anhydrous pyridine (0.39 mL, 4.8 mmol, 2 equiv). The mixture was cooled in an ice bath (0 °C) for 5 min. Phenylidonium bis(trifluoroacetate) (0.76 g, 1.8 mmol, 1.1 equiv) was added to the cooled mixture, and stirring was continued for 1.5 h. The reaction was quenched with a 10% solution of Na₂S₂O₃ in H₂O (13 mL), and the resulting mixture extracted with EtOAc (3 × 15 mL). The combined organic phases were washed with brine, dried over anhydrous Na₂SO₄, filtered, and concentrated in vacuo. The crude product was purified with automated flash chromatography (n-heptane/EtOAc gradient: 12→100%)


To give the compound 9a as a yellow oil (0.33 g, 84%). \(^1\)H NMR (400 MHz, \(d_6\)-acetone) \(\delta\) 7.12–7.05 (m, 2H), 6.24–6.18 (m, 2H), 3.48 (s, 2H), 1.52 (s, 9H). \(^13\)C NMR (101 MHz, \(d_6\)-acetone) \(\delta\) 184.9, 159.5, 153.7, 145.7, 129.2, 83.6, 83.6, 44.3, 28.1. \(^1\)H NMR is in accordance with the literature [10].

8-Oxo-1-oxa-2-azaspiro[4.5]deca-2,6,9-triene-carboxylic acid (10a)

\[
\text{O}
\begin{array}{c}
\text{\(\text{N}\)}
\end{array}
\text{\(\text{O}\)}
\begin{array}{c}
\text{\(\text{O}\)}
\end{array}
\text{\(\text{OH}\)}
\]

To a solution of tert-butyl 8-oxo-1-oxa-2-azaspiro[4.5]deca-2,6,9-triene-carboxylate 9a (0.33 g, 1.3 mmol) in anhydrous DCM (10 mL), trifluoroacetic acid (5.0 mL) was added dropwise. The resulting mixture was stirred at room temperature for 3 h. The solvent was removed in vacuo to give the compound 10a as a light brown solid (0.24 g, 93%). \(^1\)H NMR (400 MHz, \(d_6\)-acetone) \(\delta\) 7.15–7.08 (m, 1H), 6.26–6.19 (m, 1H), 3.51 (s, 1H). \(^13\)C NMR (101 MHz, \(d_6\)-acetone) \(\delta\) 184.9, 161.0, 153.0, 145.7, 129.3, 116.5, 83.8.

tert-Butyl 7,9-dibromo-8-oxo-1-oxa-2-azaspiro[4.5]deca-2,6,9-triene-carboxylate (9b)

\[
\text{O}
\begin{array}{c}
\text{\(\text{Br}\)}
\end{array}
\text{\(\text{Br}\)}
\begin{array}{c}
\text{\(\text{O}\)}
\end{array}
\text{\(\text{N}\)}
\begin{array}{c}
\text{\(\text{O}\)}
\end{array}
\text{\(\text{O}\)}
\]

To a solution of tert-butyl (E)-2-(hydroxyimino)-3-(4-hydroxyphenyl)propanoate 8 (0.59 g, 2.348 mmol) in anhydrous DMF (10 mL) in an ice bath (0 °C), N-bromosuccinimide (1.35 g, 7.58 mmol, 3.25 equiv) in anhydrous DMF (5 mL) was added dropwise (15 min). The mixture was diluted with EtO (25 mL), and washed with H₂O (2 × 15 mL) and a 10% solution of Na₂S₂O₃ in H₂O (2 × 15 mL). The aqueous phase was back-extracted with EtO (2 × 30 mL). The combined organic phases were washed with brine (20 mL), dried over anhydrous Na₂SO₄, filtered, and concentrated in vacuo. The crude product was purified with automated flash chromatography (isocratic DCM) to give the compound 9b as a white solid (0.58 g, 62%). \(^1\)H NMR (400 MHz, CDCl₃) \(\delta\) 7.32 (s, 2H), 3.42 (s, 2H), 1.57 (s, 9H). \(^13\)C NMR (101 MHz, CDCl₃) \(\delta\) 171.5, 158.2, 152.3, 144.1, 123.4, 85.8, 84.9, 43.2, 27.8. \(^1\)H NMR is in accordance with the literature [10].

7,9-Dibromo-8-oxo-1-oxa-2-azaspiro[4.5]deca-2,6,9-triene-carboxylic acid (10b)

\[
\text{O}
\begin{array}{c}
\text{\(\text{Br}\)}
\end{array}
\text{\(\text{Br}\)}
\begin{array}{c}
\text{\(\text{O}\)}
\end{array}
\text{\(\text{O}\)}
\text{\(\text{N}\)}
\begin{array}{c}
\text{\(\text{O}\)}
\end{array}
\text{\(\text{OH}\)}
\]
To a solution of tert-butyl 7,9-dibromo-8-oxo-1-oxa-2-azaspiro[4.5]deca-2,6,9-triene-3-carboxylate 9b (0.58 g, 1.4 mmol) in anhydrous DCM (10 mL), trifluoroacetic acid (5.0 mL) was added dropwise. The resulting mixture was stirred at room temperature for 1 h, after which the solvent was removed in vacuo. The product was purified via trituration with Et₂O to give the compound 10b as a white solid (0.50 g, 98%). ¹H NMR (400 MHz, d₆-DMSO) δ 7.81 (s, 2H), 3.52 (s, 2H). ¹H NMR is in accordance with the literature [22]. ¹³C NMR (101 MHz, d₆-acetone) δ 172.3, 160.7, 153.6, 146.8, 123.2, 87.3, 43.7. HRMS (ESI-): calculated 303.8609 (C₁₇H₁₂Br₂N₂O), found 303.8609. LC-MS: [M-CO₂]⁻ m/z 304 (t₀ = 2.12 min), >99%. Mp: 174–175 °C (Lit. Mp: 167–168 °C) [10].

The spiro carbamate core was mainly constructed using the synthetic method given below, followed by N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide (EDC)-mediated coupling (General procedures for coupling A and B) to give the corresponding trifluoroacetate salts (General procedure C).

**General procedure for EDC-mediated coupling (A).** To a stirred solution of carboxylic acid 10a or 10b (0.36 mmol) in DCM (5 mL), 1-hydroxybenzotriazole (HOBt) hydrate (0.036 mmol, 0.1 equiv) and N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide (EDC) hydrochloride (0.39 mmol, 1.1 equiv) at 0–5 °C were added and stirred for 15 min. After this, the amine/hydrazide (0.36 mmol, 1.0 equiv) was added. The reaction mixture was allowed to reach room temperature and was stirred for a further 8–16 h. The mixture was diluted with DCM (10 mL) and washed with 1 M hydrochloric acid (5 mL), a saturated solution of NaHCO₃ in H₂O (5 mL), and brine (5 mL). The organic layer was dried over anhydrous Na₂SO₄, filtered, and concentrated in vacuo. The crude product was purified by automated flash chromatography (n-heptane/EtOAc gradient: 0→100%) to give the pure product.

**General procedure for EDC-mediated coupling (B).** Carboxylic acid 10a or 10b (0.3 mmol), amine (0.45 mmol, 1.5 equiv), HOBt hydrate (0.45 mmol, 1.5 equiv), and EDC·HCl (0.45 mmol, 1.5 equiv) were dissolved in anhydrous DCM (3 mL). The mixture was irradiated under microwave conditions at 60 °C for 2 h, after which it was diluted with DCM (10 mL). The solution was washed with a saturated solution of NH₄Cl in H₂O, water, and brine. The organic phase was dried over anhydrous Na₂SO₄, filtered, and concentrated in vacuo. The crude product was purified with automated flash column chromatography (n-heptane/EtOAc/EtOH 3:1 (12→100%)) to give the pure product.

**General procedure for deprotection of the Boc groups (C).** To a solution of clavatadine bis-Boc-derivative (0.28 mmol) in DCM (2 mL), TFA (1 mL) at 0–5 °C was added dropwise. The reaction mixture was allowed to reach room temperature. The resulting mixture was stirred for 3 h at room temperature. The solvent was removed in vacuo to give the crude product, which was triturated in Et₂O to give a solid trifluoroacetate salt.

**7,9-Dibromo-N-(4-guanidinobutyl)-8-oxo-1-oxa-2-azaspiro[4.5]deca-2,6,9-triene-3-carboxamide TFA salt (I-TFA)**

![Chemical structure of 7,9-Dibromo-N-(4-guanidinobutyl)-8-oxo-1-oxa-2-azaspiro[4.5]deca-2,6,9-triene-3-carboxamide TFA salt (I-TFA)]

General procedures A and C were followed to give the clavatadine C TFA salt 12 as an off-white solid (0.10 g, 77%). ¹H NMR (400 MHz, d₆-DMSO) δ 8.65 (t, J = 5.9 Hz, 1H), 7.80 (s, 2H), 7.59 (t, J = 5.7 Hz, 1H), 3.55 (s, 2H), 3.23–3.06 (m, 4H), 1.48 (m, 4H). ¹³C NMR (101 MHz, d₆-DMSO) δ 171.6, 158.2, 156.7, 155.0, 146.7, 121.6, 85.2, 43.2, 40.4, 38.4, 26.0,
25.9. Spectra are in accordance with the literature [10]. HRMS (ESI+): Calculated 463.9757 (C_{14}H_{18}Br_{2}N_{5}O_{3}), found 463.9755. LC-MS: [M + H]^+ m/z 464 (t_R = 2.25 min), >91%. Mp: 115–118 °C, decomp. (Lit. Mp: 130–140 °C, decomp.) [10].

**N-(4-Guanidinobutyl)-8-oxo-1-oxa-2-azaspiro[4.5]deca-2,6,9-triene-3-carboxamide TFA salt (11)**

![Diagram of N-(4-Guanidinobutyl)-8-oxo-1-oxa-2-azaspiro[4.5]deca-2,6,9-triene-3-carboxamide TFA salt (11)](image)

General procedures A and C were followed to give the compound 11 as an off-white solid (0.11 g, 34%). 1H NMR (400 MHz, CD3OD) δ 7.04–7.00 (m, 2H), 6.26–6.22 (m, 2H), 3.44 (s, 2H), 3.36–3.34 (m, 2H), 3.23–3.20 (m, 2H), 1.64 (m, 4H). 13C NMR (101 MHz, CD3OD) δ 186.4, 161.3, 158.6, 153.3, 146.7, 129.4, 83.6, 44.4, 42.0, 39.8, 27.5, 27.1. HRMS (ESI+): calculated 306.1566 (C_{14}H_{20}N_{5}O_{3}), found 306.1567. LC-MS: [M + H]^+ m/z 306 (t_R = 0.92 min), >98%. Mp: 170–173 °C.

**N,N-Dimethyl-8-oxo-1-oxa-2-azaspiro[4.5]deca-2,6,9-triene-3-carboxamide (12)**

![Diagram of N,N-Dimethyl-8-oxo-1-oxa-2-azaspiro[4.5]deca-2,6,9-triene-3-carboxamide (12)](image)

General procedure A was followed to give the compound 12 as an off-white amorphous solid (0.011 g, 28%). 1H NMR (400 MHz, CD3OD) δ 7.05 (d, J = 10.1 Hz, 2H), 6.25 (d, J = 10.1 Hz, 2H), 3.50 (s, 2H), 3.28 (s, 3H), 3.06 (s, 3H). 13C NMR (101 MHz, CD3OD) δ 185.1, 160.7, 153.4, 145.5, 128.7, 80.6, 45.2, 37.6, 34.7. HRMS (ESI+): calculated 221.0926 (C_{11}H_{13}N_{3}O_{3}), found 221.0927. LC-MS: [M + H]^+ m/z 221 (t_R = 1.85 min), >99%.

**7,9-Dibromo-N,N-dimethyl-8-oxo-1-oxa-2-azaspiro[4.5]deca-2,6,9-triene-3-carboxamide (13)**

![Diagram of 7,9-Dibromo-N,N-dimethyl-8-oxo-1-oxa-2-azaspiro[4.5]deca-2,6,9-triene-3-carboxamide (13)](image)

General procedure A was followed to give the compound 13 as a white amorphous solid (0.12 g, 37%). 1H NMR (400 MHz, CDCl3) δ 7.32 (s, 2H), 3.55 (s, 2H), 3.32 (s, 3H), 3.09 (s, 3H). 13C NMR (101 MHz, CDCl3) δ 171.6, 159.3, 153.7, 144.9, 123.8, 84.1, 45.8, 38.8, 36.5. HRMS (ESI+): calculated 378.9117 (C_{11}H_{11}Br_{2}N_{2}O_{3}), found 378.9119.
7,9-Dibromo-N-isopropyl-N-methyl-8-oxo-1-oxa-2-azaspiro[4.5]deca-2,6,9-triene-3-carboxamide (14)

![Chemical structure of 7,9-Dibromo-N-isopropyl-N-methyl-8-oxo-1-oxa-2-azaspiro[4.5]deca-2,6,9-triene-3-carboxamide (14)](image)

General procedure A was followed to give the compound 14 as an off-white amorphous solid (0.061 g, 35%). $^1$H NMR (400 MHz, CDCl$_3$) (1:1 mixture of rotamers) $\delta$ 7.33 (7.32) (s, 2H), 4.84 (4.66) (d, $J = 6.8$ Hz, 1H), 3.56 (3.55) (s, 2H), 3.10 (2.92) (s, 3H), 1.26 (1.19) (d, $J = 6.8$ Hz, 6H). $^{13}$C NMR (101 MHz, CDCl$_3$) $\delta$ 171.6, 159.0 (158.9), 154.2 (153.5), 144.98 (144.96), 123.71 (123.69), 84.0 (83.9), 46.03 (45.97), 30.1 (27.0), 20.9 (19.3). HRMS (ESI$^+$): calculated 406.9430 (C$_{13}$H$_{15}$Br$_2$N$_2$O$_3$), found 406.9426. LC-MS: [M + H]$^+$ m/z 407 ($t_R = 4.31$ min), >99%. Mp: 148–153 °C.

3-(Morpholine-4-carbonyl)-1-oxa-2-azaspiro[4.5]deca-2,6,9-trien-8-one (15)

![Chemical structure of 3-(Morpholine-4-carbonyl)-1-oxa-2-azaspiro[4.5]deca-2,6,9-trien-8-one (15)](image)

General procedure A was followed to give the compound 15 as an off-white amorphous solid (0.012 g, 12%). $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 3.50 (s, 2H), 3.28 (s, 3H), 3.06 (s, 3H). $^{13}$C NMR (101 MHz, CDCl$_3$) $\delta$ 186.4, 171.6, 160.2, 154.3, 146.6, 129.5, 83.5, 67.7, 61.5, 54.7, 44.4. HRMS (ESI$^+$): calculated 306.1566 (C$_{14}$H$_{20}$N$_5$O$_3$), found 306.1567. LC-MS: [M + H]$^+$ m/z 307 ($t_R = 5.39$ min), >99%.

3-(4-Hydroxypiperidine-1-carbonyl)-1-oxa-2-azaspiro[4.5]deca-2,6,9-trien-8-one (16)

![Chemical structure of 3-(4-Hydroxypiperidine-1-carbonyl)-1-oxa-2-azaspiro[4.5]deca-2,6,9-trien-8-one (16)](image)

General procedure A was followed to give the compound 16 as an off-white amorphous solid (0.024 g, 24%). $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 6.92–6.83 (m, 2H), 6.33–6.25 (m, 2H), 4.02–3.95 (m, 2H), 3.80–3.70 (m, 6H), 3.48 (s, 2H). $^{13}$C NMR (101 MHz, CDCl$_3$) $\delta$ 184.5, 158.9, 153.2, 144.1, 129.5, 80.9, 67.1, 66.8, 47.4, 46.2, 43.3. HRMS (ESI$^+$): calculated 263.1032 (C$_{13}$H$_{15}$N$_2$O$_4$), found 263.1036. LC-MS: [M + H]$^+$ m/z 264 ($t_R = 0.92$ min), >99%.

3-(4-Hydroxypiperidine-1-carbonyl)-1-oxa-2-azaspiro[4.5]deca-2,6,9-trien-8-one (16)
N’-(2-Morpholinoacetyl)-8-oxo-1-oxa-2-azaspiro[4.5]deca-2,6,9-triene-3-carboxydrazide (17)

General procedure A was followed to give the compound 17 as an amorphous off-white solid (0.030 g, 17%). 1H NMR (400 MHz, CD3OD) δ 7.09–7.00 (m, 2H), 6.29–6.20 (m, 2H), 3.77–3.61 (m, 4H), 3.47 (s, 2H), 3.17 (s, 2H), 2.68–2.55 (m, 4H). 13C NMR (101 MHz, CD3OD) δ 186.4, 171.6, 160.2, 154.3, 146.6, 129.5, 83.5, 67.7, 61.5, 54.7, 44.4. HRMS (ESI+): calculated 337.0147 (C15H11Cl2N2O3), found 337.0148. LC-MS: [M + H]+ m/z 335 (tR = 0.53 min), >96%.

N-(3,5-Dichlorophenyl)-8-oxo-1-oxa-2-azaspiro[4.5]deca-2,6,9-triene-3-carboxamide (18)

General procedure A was followed to give the compound 18 as an off-white amorphous solid (0.098 g, 51%). 1H NMR (400 MHz, CDCl3) δ 8.36 (br s, 1H), 7.56 (d, J = 1.8 Hz, 2H), 7.17 (t, J = 1.8 Hz, 1H), 6.89–6.84 (m, 2H), 6.35–6.29 (m, 2H), 3.45 (s, 2H). 13C NMR (101 MHz, CDCl3) δ 184.3, 156.4, 154.0, 152.7, 143.7, 138.2, 135.4, 129.4, 125.0, 117.9, 83.6, 42.7. HRMS (ESI+): calculated 337.0147 (C15H11Cl2N2O3), found 337.0148. LC-MS: [M + H]+ m/z 335 (tR = 0.53 min), >91%.

7,9-Dibromo-N-(3,5-dichlorophenyl)-8-oxo-1-oxa-2-azaspiro[4.5]deca-2,6,9-triene-3-carboxamide (19)

General procedure B was followed to give the compound 19 as a white amorphous solid (0.0045 g, 13%). 1H NMR (400 MHz, d6-acetone) δ 9.78 (br s, 1H), 7.93 (d, J = 1.9 Hz, 2H), 7.77 (s, 2H), 7.26 (t, J = 1.8 Hz, 1H), 3.76 (s, 2H). 13C NMR (101 MHz, d6-acetone) δ 172.2, 158.3, 155.8, 146.7, 141.3, 135.7, 124.7, 123.3, 119.1, 119.0, 87.4, 43.4. HRMS (ESI−): calculated 490.8200 (C15H10Br2Cl2N2O3), found 490.8198. LC-MS: [M-H]− m/z 491 (tR = 5.84 min), >99%.
N-(3-Chloro-4-methoxyphenyl)-8-oxo-1-oxa-2-azaspiro[4.5]deca-2,6,9-triene-3-carboxamide (20)

General procedure A was followed to give the compound 20 as an off-white amorphous solid (0.025 g, 13%). $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 8.23 (br s, 1H), 7.68 (d, $J$ = 2.6 Hz, 1H), 7.43 (dd, $J$ = 8.9, 2.6 Hz, 1H), 6.92 (d, $J$ = 8.9 Hz, 1H), 6.90–6.85 (m, 2H), 6.34–6.28 (m, 2H), 3.91 (s, 3H), 3.46 (s, 2H). $^{13}$C NMR (101 MHz, CDCl$_3$) $\delta$ 184.3, 156.4, 154.0, 152.7, 143.7, 130.2, 129.6, 123.0, 122.5, 119.6, 112.4, 83.5, 56.5, 43.3. HRMS (ESI$^+$): calculated 333.0642 (C$_{16}$H$_{13}$Cl$_2$N$_2$O$_3$), found 333.0639. LC-MS: [M + H]$^+$ m/z 333 ($t_R$ = 3.68 min), >99%.

7,9-Dibromo-N-(3-chloro-4-methoxyphenyl)-8-oxo-1-oxa-2-azaspiro[4.5]deca-2,6,9-triene-3-carboxamide (21)

General procedure B was followed to give the compound 21 as a white amorphous solid (0.037 g, 18%). $^1$H NMR (400 MHz, $d_6$-DMSO) $\delta$ 10.59 (br s, 1H), 7.88 (d, $J$ = 2.6 Hz, 1H), 7.85 (s, 2H), 7.68 (dd, $J$ = 9.0, 2.6 Hz, 1H), 7.15 (d, $J$ = 9.1 Hz, 1H), 3.83 (s, 3H), 3.65 (s, 2H). $^{13}$C NMR (101 MHz, $d_6$-DMSO) $\delta$ 171.6, 156.8, 155.4, 151.2, 146.5, 131.6, 121.8, 121.8, 120.5, 120.2, 112.8, 85.6, 56.2, 43.0. HRMS (ESI$^+$): calculated 486.8696 (C$_{16}$H$_{14}$Cl$_2$N$_2$O$_4$), found 486.8696. LC-MS: [M + H]$^+$ m/z 487 ($t_R$ = 4.95 min), >94%.

N-[2-(1H-Indol-3-yl)ethyl]-8-oxo-1-oxa-2-azaspiro[4.5]deca-2,6,9-triene-3-carboxamide (22)

General procedure A was followed to give the compound 22 as a white amorphous solid (0.058 g, 56%). $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 8.12 (br s, 1H), 7.65–7.59 (m, 1H), 7.39 (m, 1H), 7.23 (ddd, $J$ = 8.2, 7.2, 1.1 Hz, 1H), 7.15 (dd, $J$ = 8.0, 7.1, 1.0 Hz, 1H), 7.08 (d, $J$ = 2.3 Hz, 1H), 6.84–6.78 (m, 2H), 6.67 (brs, 1H), 6.30–6.23 (m, 2H), 3.71 (q, $J$ = 6.8 Hz, 2H), 3.36 (s, 2H), 3.06 (t, $J$ = 6.8 Hz, 2H). $^{13}$C NMR (101 MHz, CDCl$_3$) $\delta$ 184.4, 158.7, 153.8, 144.0, 136.5, 129.3, 127.2, 122.5, 119.7, 118.8, 112.5, 111.4, 82.7, 43.7, 39.8. HRMS (ESI$^+$): calculated 336.1348 (C$_{19}$H$_{18}$N$_3$O$_3$), found 336.1349. LC-MS: [M-H]$^-$ m/z 336 ($t_R$ = 4.95 min), >99%.
N-(4-Methoxybenzyl)-8-oxo-1-oxa-2-azaspiro[4.5]deca-2,6,9-triene-3-carboxamide (23)

General procedure A was followed to give the compound 23 as an off-white amorphous solid (0.13 g, 67%). $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 7.27–7.22 (m, 2H), 6.91–6.87 (m, 3H), 6.86–6.81 (m, 2H), 6.30–6.24 (m, 2H), 4.48 (d, $J = 5.9$ Hz, 2H), 3.81 (s, 3H), 3.41 (s, 2H). $^{13}$C NMR (101 MHz, CDCl$_3$) $\delta$ 184.4, 159.5, 158.5, 153.7, 143.9, 129.5, 129.4, 114.5, 114.4, 82.9, 55.4, 43.6, 41.7. HRMS (ESI$^+$): calculated 492.9197 (C$_{16}$H$_{11}$Br$_2$ClN$_2$O$_4$), found 492.9197.

7,9-Dibromo-N-(4-methoxybenzyl)-8-oxo-1-oxa-2-azaspiro[4.5]deca-2,6,9-triene-3-carboxamide (24)

General procedure A was followed to give the compound 24 as an off-white amorphous solid (0.047 g, 68%). $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 8.72 (d, $J = 2.5$ Hz, 1H), 8.44 (dd, 2H), 3.81 (s, 3H), 3.41 (s, 2H).

N-(4-Methoxyphenethyl)-8-oxo-1-oxa-2-azaspiro[4.5]deca-2,6,9-triene-3-carboxamide (25)

General procedure A was followed to give the compound 25 as an off-white amorphous solid (0.042 g, 41%). $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 7.15–7.11 (m, 2H), 6.89–6.85 (m, 2H), 6.85–6.81 (m, 2H), 6.63 (app. t, 1H), 6.31–6.24 (m, 2H), 3.80 (s, 3H), 3.60 (q, $J = 7.0$ Hz, 2H), 3.38 (s, 2H), 2.83 (t, $J = 7.0$ Hz, 2H). $^{13}$C NMR (101 MHz, CDCl$_3$) $\delta$ 184.4, 158.6, 158.6, 153.8, 143.9, 130.2, 129.7, 129.4, 114.3, 82.8, 55.4, 43.6, 41.0, 34.8. HRMS (ESI$^+$): calculated 327.1345 (C$_{18}$H$_{19}$N$_2$O$_4$), found 327.1348. LC-MS: [M + H]$^+$ m/z 327 ($t_R = 3.35$ min), >99%.
8-Oxo-N-(pyridin-3-yl)-1-oxa-2-azaspiro[4.5]deca-2,6,9-triene-3-carboxamide (26)

![Chemical structure of 8-Oxo-N-(pyridin-3-yl)-1-oxa-2-azaspiro[4.5]deca-2,6,9-triene-3-carboxamide (26)]

General procedure A was followed to give the compound 26 as an off-white amorphous solid (0.047 g, 68%). $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 8.72 (d, $J = 2.5$ Hz, 1H), 8.44 (dd, $J = 4.7$, 1.4 Hz, 1H), 8.40 (br s, 1H), 8.14 (m, 1H), 7.82 (s, 1H), 7.12 (ddd, $J = 8.4$, 7.4, 1.9 Hz, 1H), 7.10 (dd, $J = 7.4$, 5.0, 1.0 Hz, 1H), 6.92–6.86 (m, 2H), 6.30–6.24 (m, 2H), 4.48 (d, $J = 5.9$ Hz, 1H), 3.73 (s, 3H), 3.56 (s, 2H). $^{13}$C NMR (101 MHz, CDCl$_3$) $\delta$ 184.2, 157.0, 153.8, 146.4, 143.5, 133.7, 129.7, 127.2, 124.0, 83.8, 43.1. HRMS (ESI$^+$): calculated 270.0879 (C$_{14}$H$_{12}$N$_3$O$_3$), found 270.0881. LC-MS: [M + H]$^+$ $m/z$ 270 ($t_R = 2.49$ min), >99%.

7,9-Dibromo-8-oxo-N-(pyridin-3-yl)-1-oxa-2-azaspiro[4.5]deca-2,6,9-triene-3-carboxamide (27)

![Chemical structure of 7,9-Dibromo-8-oxo-N-(pyridin-3-yl)-1-oxa-2-azaspiro[4.5]deca-2,6,9-triene-3-carboxamide (27)]

General procedure B was followed to give the compound 27 as a white amorphous solid (0.042 g, 34%). $^1$H NMR (400 MHz, $d_6$-DMSO) $\delta$ 10.81 (br s, 1H), 8.93 (dd, $J = 2.6$, 0.8 Hz, 1H), 8.33 (dd, $J = 4.7$, 1.5 Hz, 1H), 8.14 (ddd, $J = 8.4$, 2.6, 1.5 Hz, 1H), 7.86 (s, 2H), 7.40 (ddd, $J = 8.3$, 4.7, 0.8 Hz, 1H), 3.66 (s, 2H). $^{13}$C NMR (101 MHz, $d_6$-DMSO) $\delta$ 171.6, 157.4, 155.2, 146.4, 145.1, 142.0, 134.9, 127.4, 123.6, 121.8, 85.8, 42.9. HRMS (ESI$^+$): calculated 425.9089 (C$_{14}$H$_{10}$Br$_2$N$_3$O$_3$), found 425.9090. LC-MS: [M + H]$^+$ $m/z$ 426 ($t_R = 2.49$ min), >99%.

8-Oxo-N-(pyridin-2-yl)-1-oxa-2-azaspiro[4.5]deca-2,6,9-triene-3-carboxamide (28)

![Chemical structure of 8-Oxo-N-(pyridin-2-yl)-1-oxa-2-azaspiro[4.5]deca-2,6,9-triene-3-carboxamide (28)]

General procedure A was followed to give the compound 28 as an off-white amorphous solid (0.040 g, 53%). $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 9.01 (br s, 1H), 8.35 (ddd, $J = 5.0$, 1.9, 1.0 Hz, 1H), 8.18–8.16 (m, 1H), 7.76 (ddd, $J = 8.4$, 7.4, 1.9 Hz, 1H), 7.12 (ddd, $J = 7.4$, 5.0, 1.0 Hz, 1H), 6.92–6.85 (m, 2H), 6.35–6.28 (m, 2H), 3.46 (s, 2H). $^{13}$C NMR (101 MHz, CDCl$_3$) $\delta$ 184.3, 156.9, 153.7, 150.2, 148.4, 143.7, 138.7, 129.5, 120.7, 114.2, 83.6, 43.1. HRMS (ESI$^+$): calculated 270.0879 (C$_{14}$H$_{12}$N$_3$O$_3$), found 270.0881. LC-MS: [M + H]$^+$ $m/z$ 270 ($t_R = 2.11$ min), >99%.
7,9-Dibromo-8-oxo-N-(pyridin-2-yl)-1-oxa-2-azaspiro[4.5]deca-2,6,9-triene-3-carboxamide (29)

![Diagram of 7,9-Dibromo-8-oxo-N-(pyridin-2-yl)-1-oxa-2-azaspiro[4.5]deca-2,6,9-triene-3-carboxamide](image)

General procedure B was followed to give the compound 29 as a white amorphous solid (0.016 g, 13%). $^1$H NMR (400 MHz, d$_6$-acetone) $\delta$ 9.14 (br s, 1H), 8.62 (d, $J$ = 2.3 Hz, 1H), 8.32 (d, $J$ = 8.7 Hz, 1H), 7.99 (dd, $J$ = 8.7, 2.4 Hz, 1H), 7.34 (s, 2H), 3.56 (s, 2H). $^{13}$C NMR (101 MHz, d$_6$-acetone) $\delta$ 171.3, 156.7, 153.6, 152.6, 146.0, 144.5 (q, $J_{C,F} = 40.4$ Hz), 143.9, 136.2 (q, $J_{C,F} = 33.3$ Hz), 124.3, 123.9, 113.6, 87.0, 42.4. HRMS (ESI$^+$): calculated 495.8643 (C$_{14}$H$_9$Br$_2$F$_3$N$_3$O$_3$), found 495.8941. LC-MS: [M + H]$^+$ m/z 496 and 498 ($t_R = 5.30$ and 5.15 min), >97%.

7,9-Dibromo-8-oxo-N-[5-(trifluoromethyl)pyridin-2-yl]-1-oxa-2-azaspiro[4.5]-deca-2,6,9-triene-3-carboxamide (30)

![Diagram of 7,9-Dibromo-8-oxo-N-[5-(trifluoromethyl)pyridin-2-yl]-1-oxa-2-azaspiro[4.5]-deca-2,6,9-triene-3-carboxamide](image)

General procedure A was followed to give the compound 30 as an off-white amorphous solid (0.012 g, 10%). $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 9.14 (br s, 1H), 8.62 (d, $J$ = 2.3 Hz, 1H), 8.32 (d, $J$ = 8.7 Hz, 1H), 7.99 (dd, $J$ = 8.7, 2.4 Hz, 1H), 7.34 (s, 2H), 3.56 (s, 2H). $^{13}$C NMR (101 MHz, CDCl$_3$) $\delta$ 172.3, 157.4, 155.9, 151.6, 150.7, 149.4, 146.7, 139.3, 123.3, 121.3, 114.5, 87.5, 43.3. HRMS (ESI$^+$): calculated 495.8643 (C$_{14}$H$_9$Br$_2$F$_3$N$_3$O$_3$), found 495.8941. LC-MS: [M + H]$^+$ m/z 496 and 498 ($t_R = 5.30$ and 5.15 min), >97%.

7,9-Dibromo-8-oxo-N-[6-(trifluoromethyl)pyridin-3-yl]-1-oxa-2-azaspiro[4.5] deca-2,6,9-triene-3-carboxamide (31)

![Diagram of 7,9-Dibromo-8-oxo-N-[6-(trifluoromethyl)pyridin-3-yl]-1-oxa-2-azaspiro[4.5] deca-2,6,9-triene-3-carboxamide](image)

General procedure A was followed to give the compound 31 as an off-white amorphous solid (0.015 g, 13%). $^1$H NMR (400 MHz, d$_6$-acetone) $\delta$ 10.09 (br s, 1H), 9.14 (d, $J$ = 2.4 Hz, 1H), 8.55 (dd, $J$ = 8.7, 2.5 Hz, 1H), 7.87 (d, $J$ = 8.6 Hz, 1H), 7.78 (s, 2H), 3.80 (s, 2H). $^{13}$C NMR (101 MHz, d$_6$-acetone) $\delta$ 172.3, 158.7 (m), 155.8 (m), 146.7, 143.4 (q, $J_{C,F} = 34.7$ Hz), 142.6 (m), 138.5 (m), 128.4 (m), 123.4, 122.8 (q, $J_{C,F} = 272.6$ Hz), 121.9 (q, $J_{C,F} = 2.9$ Hz), 87.5, 43.4. HRMS (ESI$^+$): calculated 495.8943 (C$_{15}$H$_9$Br$_2$F$_3$N$_3$O$_3$), found 495.8948. LC-MS: [M + H]$^+$ m/z 496 and 498 ($t_R = 4.80$ and 4.72 min), >91%.
8-Oxo-N-[2-(pyridin-2-yl)ethyl]-1-oxa-2-azaspiro[4.5]deca-2,6,9-triene-3-carboxamide (32)

General procedure A was followed to give the compound 32 as an off-white amorphous solid (0.076 g, 91%). \(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta\) 8.59–8.54 (m, 1H), 7.71 (app. t, 1H), 7.65 (td, \(J = 7.7, 1.8\) Hz, 1H), 7.21–7.19 (m, 1H), 7.19–7.16 (m, 1H), 6.87–6.81 (m, 2H), 6.29–6.23 (m, 2H), 3.81 (dt, \(J = 6.8, 5.9\) Hz, 2H), 3.39 (s, 2H), 3.07 (t, \(J = 6.3\) Hz, 2H). \(^{13}\)C NMR (101 MHz, CDCl\(_3\)) \(\delta\) 184.5, 159.0, 158.6, 153.9, 149.4, 144.1, 137.0, 129.3, 123.6, 121.9, 82.7, 43.8, 38.6, 36.5. HRMS (ESI\(^+\)): calculated 453.9402 \(\delta\) 136.9, 123.8, 123.5, 122.0, 85.8, 43.4, 38.7, 36.4. HRMS (ESI\(^+\)) \(\delta\): calculated 298.1192 (C\(_{15}\)H\(_9\)Br\(_2\)N\(_3\)O\(_3\)), found 298.1191. LC-MS: \([M + H]^+ m/z\) 298 (\(t_R = 0.74\), salt and 1.15 min), >99%.

7,9-Dibromo-8-oxo-N-[2-(pyridin-2-yl)ethyl]-1-oxa-2-azaspiro[4.5]deca-2,6,9-triene-3-carboxamide (33)

General procedure B was followed to give the compound 33 as a white amorphous solid (0.062 g, 24%). \(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta\) 8.59–8.54 (m, 1H), 7.79 (app. t, 1H), 7.64 (td, \(J = 7.7, 1.8\) Hz, 1H), 7.30 (s, 2H), 7.21–7.15 (m, 2H), 3.81 (q, \(J = 6.0\) Hz, 2H), 3.47 (s, 2H), 3.06 (t, \(J = 6.2\) Hz, 2H). \(^{13}\)C NMR (101 MHz, CDCl\(_3\)) \(\delta\) 171.5, 159.1, 158.0, 154.1, 149.5, 144.6, 136.9, 123.8, 122.0, 85.8, 43.4, 38.7, 36.4. HRMS (ESI\(^+\)) \(\delta\): calculated 453.9402 (C\(_{15}\)H\(_9\)Br\(_2\)N\(_3\)O\(_3\)), found 453.9403. LC-MS: \([M + H]^+ m/z\) 454 (\(t_R = 2.27\) min), >90%.

7,9-Dibromo-8-oxo-N-[2-(pyridin-3-yl)ethyl]-1-oxa-2-azaspiro[4.5]deca-2,6,9-triene-3-carboxamide (34)

General procedure A was followed to give the compound 34 as an off-white solid (0.075 g, 39%). \(^1\)H NMR (400 MHz, d\(_6\)-DMSO) \(\delta\) 8.72 (t, \(J = 5.8\) Hz, 1H), 8.44 (dq, \(J = 6.5, 2.6, 1.7\) Hz, 2H), 7.80 (s, 2H), 7.66 (dt, \(J = 7.8, 2.0\) Hz, 1H), 7.33 (ddd, \(J = 7.8, 4.8, 0.9\) Hz, 1H), 3.54 (s, 2H), 3.48–3.38 (m, 2H), 2.83 (t, \(J = 7.2\) Hz, 2H). \(^{13}\)C NMR (101 MHz, d\(_6\)-DMSO) \(\delta\) 171.6, 158.2, 154.9, 149.8, 147.5, 146.7, 146.7, 146.1, 134.6, 123.4, 121.6, 85.2, 43.1, 31.7. HRMS (ESI\(^+\)) \(\delta\): calculated 492.9199 (C\(_{16}\)H\(_{14}\)Br\(_2\)N\(_3\)O\(_3\)), found 492.9197. Mp: 202–205 °C.
8-Oxo-N-(pyridin-3-ylmethyl)-1-oxa-2-azaspiro[4.5]deca-2,6,9-triene-3-carboxamide (35)

General procedure B was followed to give the compound 35 as a white amorphous solid (0.13 g, 61%). $^1$H NMR (400 MHz, d$_6$-acetone) δ 8.59 (dd, $J = 2.3, 0.9$ Hz, 1H), 8.47 (dd, $J = 4.8, 1.7$ Hz, 1H), 7.86 (br s, 1H), 7.32–7.05 (m, 2H), 6.25–6.15 (m, 2H), 4.55 (d, $J = 6.3$ Hz, 2H), 4.39 (s, 2H), 3.57 (s, 2H). $^{13}$C NMR (101 MHz, d$_6$-acetone) δ 184.9, 159.9, 155.1, 150.3, 149.4, 145.9, 145.8, 148.9, 148.2, 146.7, 135.3, 134.3, 123.4, 121.6, 85.4, 43.0, 40.0. HRMS (ESI$^+$): calculated 439.9245 (C$_{15}$H$_{12}$Br$_2$N$_3$O$_3$), found 439.9244. LC-MS: [M + H]$^+$ m/z 440 ($t_R = 2.08$ min), >94%.

7,9-Dibromo-8-oxo-N-(pyridin-3-ylmethyl)-1-oxa-2-azaspiro[4.5]deca-2,6,9-triene-3-carboxamide (36)

General procedure B was followed to give 36 as a white amorphous solid (0.068 g, 55%). $^1$H NMR (400 MHz, d$_6$-DMSO) δ 8.53 (dd, $J = 2.3, 0.9$ Hz, 1H), 8.47 (dd, $J = 4.8, 1.7$ Hz, 1H), 7.86 (s, 2H), 7.34–7.08 (m, 2H), 6.31–6.22 (m, 2H), 3.54 (s, 2H). $^{13}$C NMR (101 MHz, d$_6$-DMSO) δ 171.6, 158.4, 158.4, 154.9, 154.8, 148.8, 146.7, 135.3, 134.3, 123.4, 121.6, 85.4, 43.0, 40.0. HRMS (ESI$^+$): calculated 439.9245 (C$_{15}$H$_{12}$Br$_2$N$_3$O$_3$), found 439.9244. LC-MS: [M + H]$^+$ m/z 440 ($t_R = 2.08$ min), >94%.

8-Oxo-N$^\circ$-picolinoyl-1-oxa-2-azaspiro[4.5]deca-2,6,9-triene-3-carboxyhydrazide (37)

General procedure A was followed to give the compound 37 as an off-white solid (0.025 g, 16%). $^1$H NMR (400 MHz, d$_6$-DMSO) δ 10.71 (br s, 1H), 10.65 (br s, 1H), 8.75–8.67 (m, 1H), 8.10–8.00 (m, 2H), 7.69–7.63 (m, 1H), 7.22–7.13 (m, 2H), 6.31–6.22 (m, 2H), 3.54 (s, 2H). $^{13}$C NMR (101 MHz, d$_6$-DMSO) δ 184.5, 163.0, 157.8, 153.2, 149.1, 148.7, 145.6, 137.9, 128.2, 127.1, 122.5, 81.6, 43.3. HRMS (ESI$^+$): calculated 313.0937 (C$_{15}$H$_{13}$N$_4$O$_4$), found 313.0937. LC-MS: [M + H]$^+$ m/z 313 ($t_R = 1.51$ min), >93%. Mp: 191–193 °C.
7,9-Dibromo-8-oxo-N’-picolinoyl-1-oxa-2-azaspiro[4.5]deca-2,6,9-triene-3-carbohydrazide (38)

General procedure A was followed to give the compound 38 as an off-white solid (0.1 g, 50%). 1H NMR (400 MHz, d6-DMSO) δ 10.74 (s, 1H), 10.65 (s, 1H), 8.70 (dt, J = 4.7, 1.4 Hz, 1H), 8.08–7.99 (m, 2H), 7.88 (s, 2H), 7.71–7.63 (m, 1H), 3.63 (s, 2H). 13C NMR (101 MHz, d6-DMSO) δ 171.7, 163.0, 157.5, 153.6, 149.0, 148.6, 146.7, 137.9, 127.1, 122.5, 121.7, 85.2, 43.5. HRMS (ESI+): calculated 470.9128 (C15H11Br2N4O4), found 470.9127. LC-MS: [M + H]+ m/z 471 and 473 (tR = 2.48 and 2.37 min), >99%. Mp: 199–202 °C.

7,9-Dibromo-8-oxo-N’-picolinoyl-1-oxa-2-azaspiro[4.5]deca-2,6,9-triene-3-carboxamide (39)

General procedure A was followed to give the compound 39 as an off-white solid (0.025 g, 14%). 1H NMR (400 MHz, d6-DMSO) δ 10.73 (br s, 2H), 9.06–9.01 (m, 1H), 8.78 (dd, J = 4.9, 1.7 Hz, 1H), 8.23 (ddd, J = 8.0, 2.3, 1.7 Hz, 1H), 7.88 (s, 2H), 7.62–7.53 (m, 2H), 3.64 (s, 2H). 13C NMR (101 MHz, d6-DMSO) δ 171.6, 164.1, 157.7, 153.5, 152.6, 148.4, 146.6, 135.2, 128.0, 123.7, 121.77, 85.2, 43.0. HRMS (ESI+): calculated 439.9128 (C15H11Br2N4O4), found 470.9129. LC-MS: [M + H]+ m/z 471 and 473 (tR = 3.14 and 3.11 min), >98%. Mp: 163–166 °C.

N-(3-(4-Chlorophenyl)-1-methyl-1H-pyrazol-5-yl)-8-oxo-1-oxa-2-azaspiro[4.5]deca-2,6,9-triene-3-carboxamide (40)

General procedure A was followed to give the compound 40 as an off-white solid (0.021 g, 18%). 1H NMR (400 MHz, CDCl3) δ 8.25 (br s, 1H), 7.74–7.68 (m, 2H), 7.40–7.33 (m, 2H), 6.91–6.87 (m, 2H), 6.67 (s, 1H), 6.36–6.31 (m, 2H), 3.86 (s, 3H), 3.48 (s, 2H). 13C NMR (101 MHz, CDCl3) δ 184.0, 156.2, 152.9, 149.2, 143.1, 134.9, 133.7, 131.5, 129.6, 128.8, 126.6, 96.9, 83.8, 42.8, 35.8. HRMS (ESI+): calculated 383.0911 (C19H16ClN4O3), found 383.0911. LC-MS: [M + H]+ m/z 383 and 385 (tR = 4.21 and 4.14min), >99%. 

Mar. Drugs 2021, 19, x FOR PEER REVIEW
7,9-Dibromo-N-[3-(4-chlorophenyl)-1-methyl-1H-pyrazol-5-yl]-8-oxo-1-oxa-2-azaspiro[4.5]deca-2,6,9-triene-3-carboxamide (41)

General procedure A was followed to give the compound 41 as an off-white solid (0.075 g, 35%). $^1$H NMR (400 MHz, $d_6$-DMSO) $\delta$ 10.74 (br s, 1H), 7.88 (s, 2H), 7.85–7.76 (m, 2H), 7.50–7.42 (m, 2H), 3.74 (s, 3H), 3.65 (s, 2H). $^{13}$C NMR (101 MHz, $d_6$-DMSO) $\delta$ 171.6, 157.6, 154.6, 147.1, 146.4, 136.4, 132.0, 132.0, 128.7, 126.5, 121.9, 98.3, 85.9, 42.8, 36.0. Mp: 251–254 °C. HRMS (ESI$^+$): calculated 538.9121 ($C_{19}H_{14}Br_2ClN_4O_3$), found 538.9120. LC-MS: [M + H]$^+$ m/z 541 ($t_R = 5.29$ min), >99%.

7,9-Dibromo-8-oxo-N-(4,5,6,7-tetrahydrobenzo[d]thiazol-2-yl)-1-oxa-2-azaspiro[4.5]deca-2,6,9-triene-3-carboxamide (42)

General procedure A was followed to give the compound 42 as a pale yellow solid (0.085 g, 40%). $^1$H NMR (400 MHz, $d_6$-acetone) $\delta$ 10.92 (br s, 1H), 7.78 (s, 2H), 3.79 (s, 2H), 2.72–2.69 (m, 2H), 2.62–2.59 (m, 2H), 1.84 (p, $J = 3.2$ Hz, 4H). $^{13}$C NMR (101 MHz, $d_6$-acetone) $\delta$ 172.29, 157.75, 155.14, 155.10, 146.75, 145.75, 145.21, 123.69, 123.34, 87.35, 43.40, 29.84, 26.92, 24.02, 23.67, 23.33. HRMS (ESI$^+$): calculated 487.9102 ($C_{16}H_{14}Br_2N_2O_3S$), found 487.9109. LC-MS: [M + H]$^+$ m/z 488 and 490 ($t_R = 4.92$ and 5.03 min), >99%. Mp: 185–190 °C.

5. Conclusions

In summary, the highest cytotoxicity against the A-375 cell line was observed in 2,4-dichloro compound 29 (CC$_{50}$ 0.4 ± 0.3 μM, SI 2) and the highest SI (2.4) was observed for the pyridin-2-yl derivative 29 and hydrazide analog of 2-picoline 37. The results of these spirocyclic clavatadine analogs provide a path for further mechanistic studies and optimization of simplified spirocyclic bromotyrosine derivatives to understand the elements of their SARs and improve the selectivity.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/md19070400/s1. Experimental data: $^1$H and $^{13}$C spectra of all compounds.

Author Contributions: The manuscript was written with contributions from all authors. Conceptualization: P.S.K., P.A.P., P.T., and T.B.; synthesis, P.A.P., T.B. and A.L.; biological screening, P.I., H.M. and T.B.; writing—original draft preparation, P.A.P., P.S.K., P.I. and T.B.; writing—review and editing, P.S.K., P.A.P., P.I., T.B., J.Y.-K. and P.T.; supervision, P.S.K. and P.T.; funding acquisition: P.S.K., P.T. and J.Y.-K. All authors have read and agreed to the published version of the manuscript.
Funding: This research was supported by the Academy of Finland (Grant Nos. 307464 and 315937). We confirm we support the Open access funding provided by University of Helsinki.

Institutional Review Board Statement: Not applicable.

Data Availability Statement: Data is contained within the article and Supplementary Material.

Acknowledgments: We thank Andrew Neal for revising the language, Antti Lehtinen for the synthesis assistance, Nina Sipari from the Viikki Metabolomics Unit (Helsinki Institute of Life Science, University of Helsinki; Biocenter Finland) for her expertise with the LC-MS analyses, and the DDCB core facility supported by the University of Helsinki (HiLIFE) and Biocenter Finland for access to bioactivity testing facilities. We confirm we support the Open access funding provided by University of Helsinki.

Conflicts of Interest: The authors declare no competing financial interest.

References
1. Newman, D.J.; Cragg, G.M. Natural Products as Sources of New Drugs over the Nearly Four Decades from 01/1981 to 09/2019. J. Nat. Prod. 2020, 83, 770–803. [CrossRef] [PubMed]
2. Kiuru, P; D’Auria, V.M.; Muller, C.D.; Tammela, P.; Vuorela, H.; Yli-Kauhaluoma, J. Exploring Marine Resources for Bioactive Compounds. Planta Med. 2014, 80, 1234–1246. [CrossRef] [PubMed]
3. Khalifa, S.M.; Elias, N.; Farag, M.A.; Chen, L.; Saeed, A.; Hegazy, M.F.; Moustafa, M.S.; Abd El-Wahed, A.; Al-Mousawi, S.M.; Musharraf, S.G.; et al. Marine Natural Products: A Source of Novel Anticancer Drugs. Mar. Drugs 2019, 17, 491. [CrossRef] [PubMed]
4. Jimenez, P.C.; Wilke, D.V.; Branco, P.C.; Bauermeister, A.; Rezende-Teixeira, P.; Gaudino, S.P.; Costa-Lotufo, L.V. Enriching Cancer Pharmacology with Drugs of Marine Origin. Brit. J. Pharmacol. 2020, 177, 3–27. [CrossRef] [PubMed]
5. Marine Pharmacology, Approved Marine Drugs. Available online: https://www.marinepharmacology.org/approved (accessed on 26 May 2021).
6. Calcabrini, C.; Catanzano, E.; Bishayee, A.; Turrini, E.; Fimognari, C. Marine Sponge Natural Products with Anticancer Potential: An Updated Review. Mar. Drugs 2017, 15, 310. [CrossRef] [PubMed]
7. Peng, J.; Li, J.; Hamann, M.T. The Marine Bromotyrosine Derivatives. In The Alkaloids: Chemistry and Biology; Cordell, G.A., Ed.; Elsevier: Amsterdam, The Netherlands, 2007; Volume 61, pp. 59–262.
8. Niemann, H.; Marmann, A.; Lin, W.; Proksch, P. Sponge Derived Bromotyrosines: Structural Diversity through Natural Combinatorial Chemistry. Nat. Prod. Commun. 2015, 10. [CrossRef]
9. Buchanan, M.; Carroll, A.; Wessling, D.; Hooper, J.; Quinn, R.J. Clavatadines C–E, Guanidine Alkaloids from the Australian Sponge Suberosa clavata. J. Nat. Prod. 2009, 72, 973–975. [CrossRef] [PubMed]
10. Badart, M.; Squires, C.; Baird, S.; Hawkins, B. The Synthesis of Clavatadine C. Tetrahedron Lett. 2016, 57, 5108–5111. [CrossRef]
11. Kaur, K.; Kumar, V.; Sharma, A.K.; Gupta, G.K. Isoxazoline Containing Natural Products as Anticancer Agents: A Review. Eur. J. Med. Chem. 2014, 77, 121–133. [CrossRef] [PubMed]
12. Drechsel, A.; Helm, J.; Ehrlich, H.; Pantovic, S.; Bornstein, S.R.; Bechmann, N. Anti-Tumor Activity vs. Normal Cell Toxicity: Therapeutic Potential of the Bromotyrosines Aerothionin and Homoaerothionin In Vitro. Mar. Drugs 2020, 18, 236. [CrossRef] [PubMed]
13. Zheng, Y.J.; Tice, C.M. The Utilization of Spirocyclic Scaffolds in Novel Drug Discovery. Expert Opin. Drug Dis. 2016, 11, 831–834. [CrossRef] [PubMed]
14. Bhat, C.; Ilina, P.; Tili, I.; Voráčová, M.; Bruun, T.; Barva, V.; Hribernik, N.; Lillsunde, K.-E.; Mäki-Lohiluoma, E.; Rüffer, T.; et al. Synthesis and Antiproliferative Activity of Marine Bromotyrosine Purpurealidin I and Its Derivatives. Mar. Drugs 2018, 16, 481. [CrossRef] [PubMed]
15. Yajun, Z.; Tice, C.; Singh, S. The Use of Spirocyclic Scaffolds in Drug Discovery. Bioorg. Med. Chem. Lett. 2014, 24, 3673–3682. [CrossRef]
16. Wang, S.; Dong, G.; Sheng, C. Structural Simplification: An Efficient Strategy in Lead Optimization. Acta Pharm. Sin. B 2019, 9, 880–901. [CrossRef]
17. Banyu Pharmaceutical Co., Ltd. Spiroisoxazoline Derivatives. Japanese Patent JP 59176268 A, 28 March 1983.
18. Banyu Pharmaceutical Co., Ltd. Spiroisoxazoline Derivatives. Japanese Patent JP 59190978 A, 13 April 1983.
19. Badisa, R.B.; Darling-Reed, S.F.; Joseph, P.; Cooperwood, J.S.; Latiniow, L.M.; Goodman, C.B. Selective Cytotoxic Activities of Two Novel Synthetic Drugs on Human Breast Carcinoma MCF-7 Cells. Anticancer Res. 2009, 29, 2993–2996. [PubMed]
20. Fulda, S.; Debatin, K.M. Extrinsic versus Intrinsic Apoptosis Pathways in Anticancer Chemotherapy. Oncogene 2006, 25, 4798–4811. [CrossRef] [PubMed]
21. Binneweg, B.; Schubert, M.; Voronkina, A.; Muzychka, L.; Wysokowski, M.; Petrenko, I.; Djurović, M.; Kovalchuk, V.; Tsurkan, M.; Martinovic, R.; et al. Biomaterials: Biomimetic and Pharmacological Potential of Cultivated Aplysina aerophoba Marine Demosponge. Mater. Sci. Eng. C 2020, 109, 110566. [CrossRef] [PubMed]
22. Bassetto, M.; Ferla, S.; Pertusati, F. Polyfluorinated Groups in Medicinal Chemistry. *Future Med. Chem.* **2015**, *7*, 527–546. [CrossRef] [PubMed]

23. López-Lázaro, M. A Simple and Reliable Approach for Assessing Anticancer Activity In Vitro. *Curr. Med. Chem.* **2015**, *22*, 1324–1334. [CrossRef] [PubMed]

24. Rerat, V.; Dive, G.; Cordi, A.; Tucker, G.; Bareille, R.; Amedee, J.; Bordenave, L.; Marchand-Brynaert, J. αvβ3 Integrin-targeting Arg-Gly-Asp (RGD) Peptidomimetics Containing Oligoethylene Glycol (OEG) Spacers. *J. Med. Chem.* **2009**, *52*, 7029–7043. [CrossRef] [PubMed]