International Journal of
Molecular Sciences

MDPI

Article

Tricyclic Pyrrole-Based Compounds as Zika Virus Inhibitors

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Abstract

A small library of 23 pyrrole-based tricyclic derivatives bearing bulky amine moieties was synthesized, and all were evaluated for their antiviral activities against ZIKV and SARS-CoV. Three compounds, derivatives **2g**, **2h** and **2j**, elicited interesting activity against ZIKV: compound **2g**, containing a bornylamine residue, showed the best activity against Huh-7 cells with EC_{50} and CC_{50} values of 0.4 μM and 230.5 μM, respectively, and a Selectivity Index (SI) of 501. All three compounds reduce ZIKV yield primarily by impairing viral protein.

Keywords: synthesis of tricyclic pyrroles; antiviral activity; Western blot analysis

1. Introduction

Viral infections [1] include common viral diseases, such as respiratory infections (flu, colds), but also dangerous viral infections that emerge each year, such as SARS, the Middle East respiratory syndrome (MERS), or Ebola, which can spread epidemically worldwide. The first two are caused by coronaviruses and the third by Ebolavirus. Their treatment primarily involves the use of antiviral agents reducing the incidences of some of them, vaccination playing a pivotal preventive measure. Unfortunately, some viruses can reduce the effectiveness of vaccines through mutations, which currently represent one of the most significant problems of antiviral therapy [2] and, therefore, there is a continuous search for new antiviral drugs [3,4].

Nitrogen heterocycles are scaffolds widely used in medicinal chemistry to identify drug candidates due to their chemical–physical properties that characterize the pharmacokinetics and pharmacodynamics of the drug [5]. Among them, the therapeutic potential [6] of pyrrole-containing compounds is widely reported in the literature as well as their use to treat infectious diseases [7]. These nitrogen-containing heterocyclic templates are expressed both in natural products [8], like heme and cobalamin (vitamin B12), and in clinically approved drugs [9], like atorvastatin, a potent competitive inhibitor of the enzyme 3-hydroxy-3-methylglutaryl-CoenzimeA (HMG-CoA) reductase, and finafloxacin, a fluoroquinolone antibiotic indicated in the treatment of acute otitis externa.

Recent research has demonstrated that several novel pyrrole-based compounds, as pyrrolo[2,3-*d*]pyrimidines, exhibit significant inhibitory activity against several viruses [10], and several studies have demonstrated that pyrrole-containing compounds show a broad spectrum of antiviral activities, and resulting amide derivatives are particularly interesting (Figure 1) [11].

Academic Editor: Dongwei Kang

Received: 26 January 2026
Revised: 21 February 2026
Accepted: 27 February 2026
Published: 28 February 2026

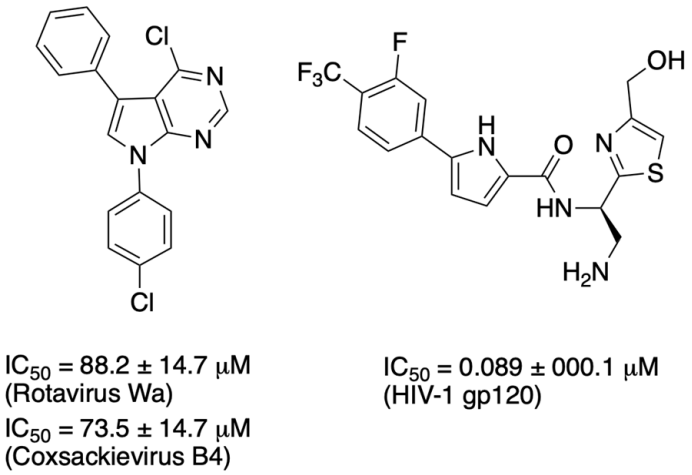


Figure 1. Pyrrole-based compounds endowed with antiviral activity.

As part of a research project aimed at investigating the biological activity of compounds with a 1,4-dihydroindeno[1,2-*b*]pyrrol-3-yl-substituted core, recently we have identified a series of derivatives endowed with interesting tubulin polymerization inhibition (Figure 2) [12]. Therefore, to further investigate the potential of 1,4-dihydroindenopyrrole, we modulate the tricyclic system by *N*-benzylation of pyrrole and by introduction of bulky substituents on 3 position, as several examples of antiviral compounds bearing bicyclic monoterpenes are reported in the literature [13,14]. These structural modifications led us to obtain 1-(4-methylbenzyl)-1,4-dihydroindeno[1,2-*b*]pyrrole-3-carboxamides and 3-carbohydrazides (Figure 2).

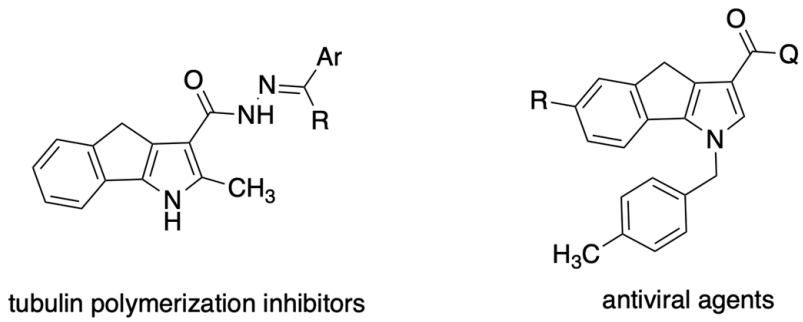


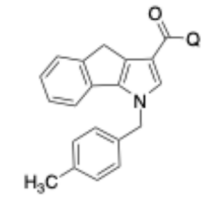
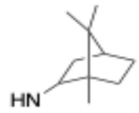
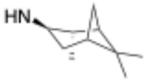
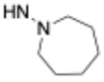
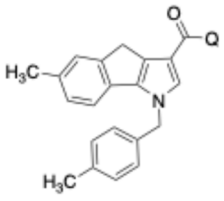
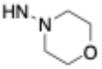
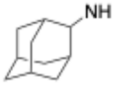
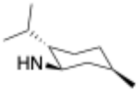
Figure 2. 1,4-Dihydroindeno[1,2-*b*]pyrrol-3-yl derivatives.

To evaluate the potential antiviral activity of these novel compounds, we characterized them against two distinct viruses that have caused global concern, like Zika virus (ZIKV), a flavivirus transmitted by *Aedes* mosquitoes and causes fever/rash with risks during pregnancy that may cause microcephaly, and SARS-CoV-2, that spreads through the respiratory tract causing COVID-19, most commonly manifesting acute respiratory syndrome.

2. Results

We describe the synthesis and anti ZIKV activity of two novel series of tricyclic pyrrole-based carbohydrazides and carboxamides **1a**–**k** and **2a**–**l**, (Table 1) which differ by the presence of a methyl group on 6-position of the tricyclic pyrrole.

Table 1. 1-(4-Methylbenzyl)-1,4-dihydroindeno[1,2-*b*]pyrrole-3-carbohydrazides **1a**–**d**, carboxamides **1e**–**k** and their 6-methyl-analogues **2a**–**l**.

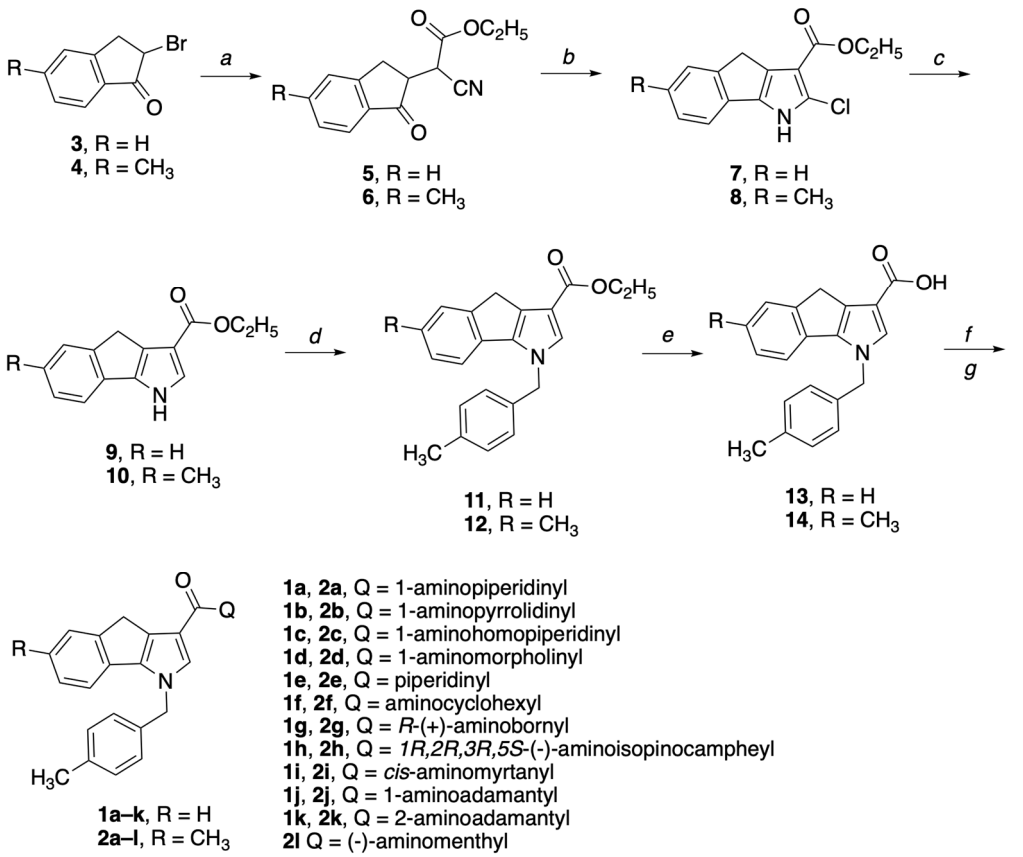
Structure	Compound	Q	Compound	Q
 **1a–k**	**1a,2a**		**1g,2g**	
	1b,2b		**1h,2h**	
	1c,2c		**1i,2i**	
 **2a–l**	**1d,2d**		**1j,2j**	
	1e,2e		**1k,2k**	
	1f,2f		**2l**	

2.1. Chemistry

Carbohydrazides **1**,**2a**–**d** and carboxamides **1e**–**k** and **2e**–**l** were synthesized by seven synthetic steps starting from bromo-ketone **3** or **4** (Scheme 1). Its alkylation with ethyl cyanoacetate and K_2CO_3 in acetone gave the corresponding cyano-ketoester (**5** or **6**), which served as precursor for the synthesis of the key intermediate tricyclic pyrrole 3-carboxilate (**9** or **10**) in the designed synthetic route.

The cyclization of cyano-ketoesters **5** or **6** furnished derivatives **7** and **8** which were dehalogenated in the presence of ammonium formate and 10% Pd/C in EtOH to yield the key intermediates **9** and **10** (Scheme 1). Their *N*-benzylation with 1-(chloromethyl)-4-methylbenzene in the presence of 60% NaH in mineral oil gave the corresponding *p*-methylbenzyl derivates (**11** or **12**), which were hydrolysed to acid **13** or **14**. Then, acid **13** was reacted with $SOCl_2$ to give the corresponding acyl chlorides (not isolated), whereas **14** was activated with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and 1-hydroxybenzotriazole (HOBt), followed by reaction with the appropriate amine, furnished derivatives **1a**–**k** and **2a**–**l**, respectively.

While the literature reporting the synthesis of pyrrole–polycyclic systems bearing substituents in both positions α and β of the pyrrole ring is extensive, analogues with the β-carboxy group as single substituent are less described. The 5-*exo-trig* cyclization process [15] necessary for the preparation of the 1,4-dihydroindeno[1,2-*b*]pyrrole-3-carboxylic system (**7** and **8**) precursor of **9** or **10**, proposes a single-stage mechanism, starting from cyano-ketoester (**5** or **6**), whose ethereal solution was insufflated with HCl. This process allows the isolation of ethyl 2-chloro-6-methyl-1,4-dihydroindeno[1,2-*b*]pyrrole-3-carboxylate (**6** or **7**) via imidoyl chloride (**5′** or **6′**), which, by iminium ion cyclization (**7′** or **8′**) followed by dehydration and deprotonation, gave the tricyclic ester (Figure 3).



Scheme 1. Synthesis of 1-(4-Methylbenzyl)-1,4-dihydroindeno[1,2-*b*]pyrrole-3-carbohydrazides **1a–d**, carboxamides **1e–k** and their 6-methyl-analogues **2a–l**. **Reagents and conditions:** (a) K_2CO_3, $NCCH_2COOEt$, $(CH_3)_2CO$, 45 °C, 1.5 h; (b) Et_2O, HCl_g, r.t., 24 h; (c) MeOH, $HCOONH_4$, Pd/C 10%, r.t., 4 h; (d) NaH, DMF, $ClCH_2C_6H_4CH_3$, r.t., 4h; (e) KOH, EtOH, H_2O, rfx, 12 h; (f) Toluene, $SOCl_2$, 3 h, rfx (for **1a–k**) or EDC, HOBt, CH_2Cl_2, r.t., 1.5 h (for **2a–l**); (g) CH_2Cl_2, TEA, H_2N-Q, 3 h, r.t.

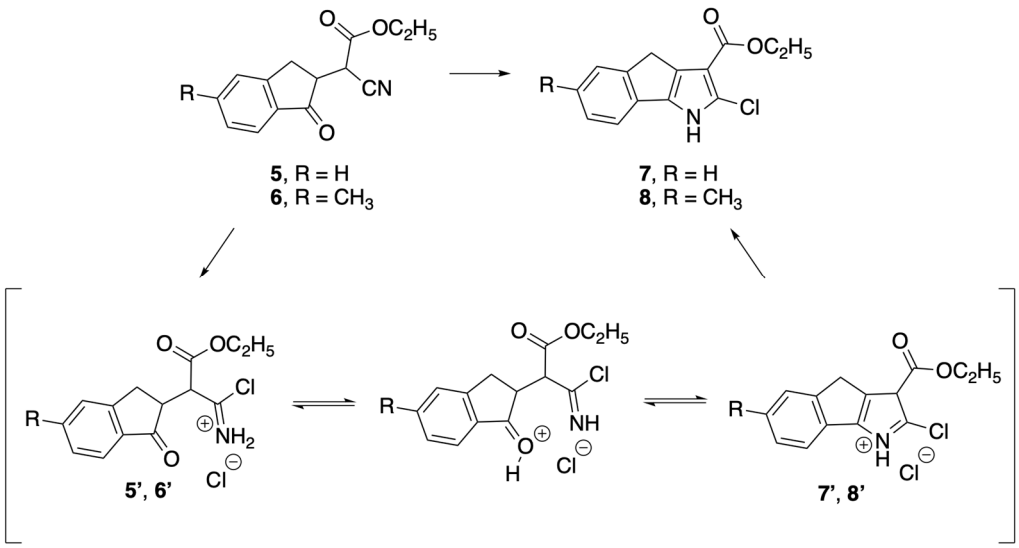


Figure 3. *5-Exo-trig* cyclization mechanism.

2.2. Biology

2.2.1. Antiviral Activity Against ZIKV and SARS-CoV-2

New compounds were screened for their potential antiviral activity against $ZIKV^{Br}$ on Huh-7 cells (preliminary results are reported in Table S1 in Supplementary Materials). Each

compound was tested in duplicate at concentrations from 180 µM to 0.003 µM. Treatment was applied immediately after infection, and at 24–48 h post-infection, supernatants were collected to assess viral yield for ZIKV determined as PFU/mL. The same treatments were applied to uninfected cells to evaluate the cytotoxicity of each compound, comparing them with reference sofosbuvir (SOF).

Compounds **2g**, **2h** and **2j** showed promising antiviral effects and were further characterized to determine their IC_{50}, CC_{50}, and selectivity index (SI) values.

Concerning the antiviral activity against ZIKV, compound **2g** showed the best values with a SI of 501 (EC_{50} = 0.4 µM; CC_{50} = 230.5 µM) (Figure 4 and Table 2). Derivative **2h** had a SI of 232.5 (EC_{50} = 0.9 µM; CC_{50} = 211.6 µM), and compound **2j** showed a good effect against ZIKV on Huh-7 cells, with a SI of 246.4 (EC_{50} = 0.6 µM; CC_{50} = 160.2 µM). In comparison, SOF exhibited an SI of 187.8 (EC_{50} = 1.5 µM; CC_{50} = 281.7 µM).

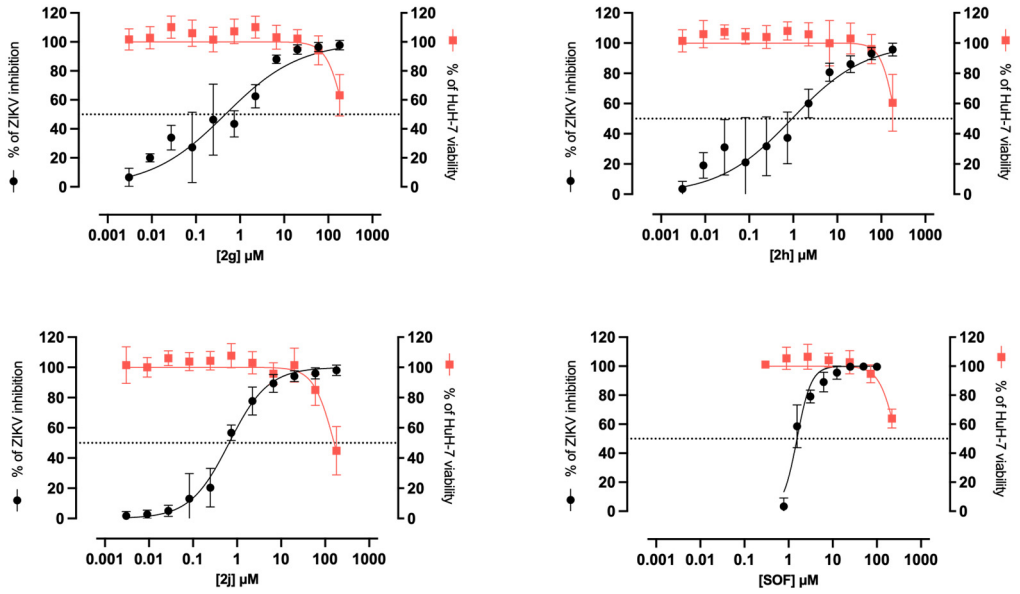


Figure 4. Dose–response curves of the % ZIKV replication inhibition (black line) and % cell viability (red line) for compound **2g**, **2h**, **2j** and for control drug SOF.

Table 2. ZIKV/Huh-7 antiviral screening results for compounds **2g**, **2h** and **2j**.

Compound	ZIKV/Huh-7		
	CC_{50} (µM)	EC_{50} (µM)	SI
2g	230.5 ± 13.3	0.4 ± 0.6	501
2h	211.6 ± 22.7	0.9 ± 0.8	232.5
2j	160.2 ± 31.1	0.6 ± 0.2	246.4
Sofosbuvir	281.7 ± 38	1.5 ± 0.2	187.8

Compounds **2g** and **2j** were selected to test their activity against SARS-CoV-2^{Mi} on Vero-TMPRSS, applying the same treatments described above for ZIKV, and using as reference compound EIDD-1931. Neither of them showed activity against this virus. Therefore, we determined the CC_{50} values for **2g** and **2j**, 220.7 and 432.4 µM, respectively, whereas the control EIDD-1931 had a CC_{50} of 28.1 µM.

2.2.2. Determination of Viral Protein Synthesis During Treatment with **2g**, **2h**, **2j**

To determine whether the reduction in ZIKV yield by **2g**, **2h** and **2j** was due to inhibition of viral protein synthesis or viral release, Huh-7 were infected with $ZIKV^{Br}$ and treated with **2g**, **2h**, **2j** or SOF at 1×, 5×, and 10× their EC_{50}. Viral protein levels

in cell lysates were analyzed by immunoblotting (Figure 5). Compounds **2g**, **2h** and **2j** downregulated viral protein expression in Huh-7 cells but did not fully suppress it; similarly, SOF strongly reduced protein synthesis at 7.5 µM (5 × EC_{50}) (Figure 5). These findings indicate that **2g**, **2h** and **2j** reduced viral protein synthesis.

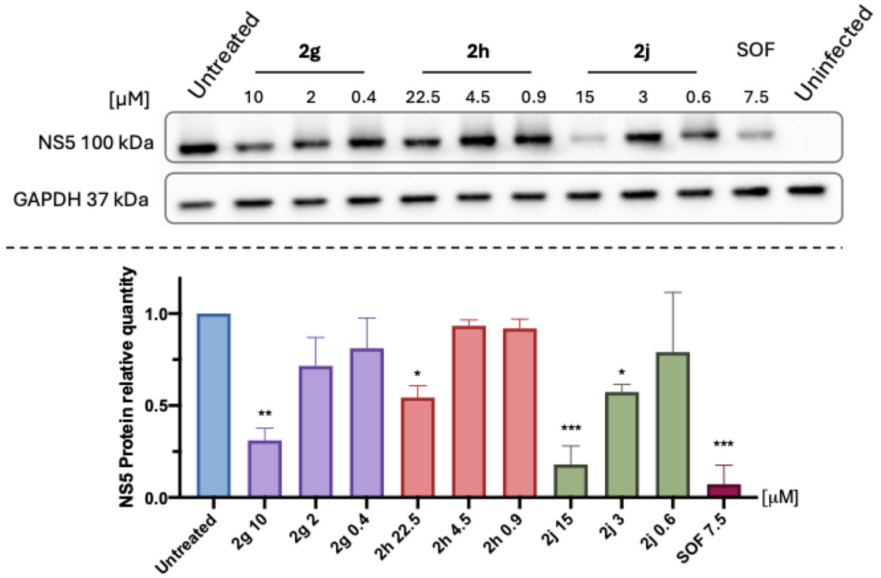


Figure 5. (**Up**) Western blot of Huh-7 lysed 48 h after treatment with **2g**, **2h**, **2j** or SOF developed with antibodies against ZIKV NS5 protein or GAPDH as a control. (**Down**) Quantitative analysis of the results in (**Up**): * p-value ≤ 0.05; ** p-value ≤ 0.01; *** p-value ≤ 0.001.

After testing compounds **2h**, **2g** and **2j** on SARS-CoV-2 and observing that it was not possible to calculate an IC_{50} based on viral yield because the compounds were not active against this virus, we decided to test them again, evaluating their effect on viral protein production. A Western blot analysis was performed to detect the viral nucleocapsid protein. In agreement with previous results, the levels of viral protein were comparable to those observed in the infected, untreated control, confirming that the compounds had no antiviral activity. In contrast, treatment with the reference compound EIDD-1931 resulted in an almost complete inhibition of viral protein synthesis, which was statistically significant (Figure 6).

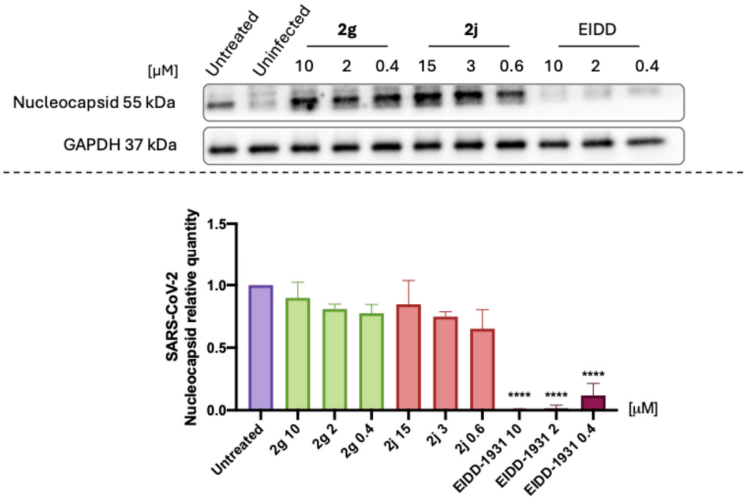


Figure 6. (**Up**) Western blot of Vero-TMPRSS cells lysed 48 h after treatment with **2g**, **2j** or EIDD-1931 developed with antibodies against SARS-CoV-2 Nucleocapsid protein or GAPDH as a control. (**Down**) Quantitative analysis of the results in (**Up**): **** p-value ≤ 0.0001.

3. Conclusions

We synthesized 23 novel 1,4-dihydroindeno[1,2-*b*]pyrrole-based derivatives (**1a**–**k**, **2a**–**l**) and evaluated their antiviral and cytotoxic activity against ZIKV and SARS-CoV-2.

In the series **1a**–**k** the introduction of different amines on the carboxylic function do not confer antiviral activity against the selected viruses. On the contrary, the introduction of a methyl group on C-6 position of the tricyclic system, as in series **2a**–**l**, is decisive for their activity against ZIKV. Three compounds with the best SI and the lowest IC_{50} values, **2g**, **2h** and **2j**, were selected to further evaluate their activity against ZIKV. Two, **2g** and **2j**, were used to assess the anti-SARS-CoV-2 activity. From these preliminary assays, we can assume that only compounds of series **2a**–**l**, bearing a methyl group on the C-6 position of the tricyclic system, showed antiviral activity against ZIKV, whereas SARS-CoV-2 was not affected. These preliminary data suggest that the methyl group on the phenyl ring of the tricyclic system, missing in series **1a**–**k**, plays a role in downregulation of viral protein expression in Huh-7 cells. Moreover, it was confirmed that bulky amines as bornyl (**2g**), isopinocampheyl (**2h**) and 1-adamantylbornyl (**2j**) in the 3-carboxy-position of the pyrrole-based structure are preferred for increased activity of such compounds. Particularly, derivative **2g** was identified as the most active of all the pyrrole-based compounds and the immunoblotting analyses showed a not fully suppression of viral protein expression in Huh-7 cells, suggesting that the reduction in ZIKV yield is due primarily to an impairing of viral protein synthesis.

Further studies are in progress to assess the impact of other structural modifications, with the aim of obtaining new information on this template and deepening the structure-activity relationships, aiming at the development of potential novel antiviral agents.

4. Experimental Section

4.1. Chemistry

4.1.1. General Methods

The melting point range of compounds was measured with a Köpfler hot stage microscopy and is uncorrected.

Thin Layer Cromatography (TLC) was performed on Poligram® SIL N-HR/HV_{254} silica plates (0.2 mm). Compounds were purified using flash chromatography (FC), either automatically on a Biotage® Flash-master system using pre-packed Biotage® SNAP silica gel cartridges or manually with Merck® Kieselgel 60 (0.040–0.063 mm) silica gel.

^{1}H and ^{13}C NMR spectra were acquired at room temperature using a Bruker AVANCE III Nanobay 400 MHz or a Varian Unity-200 MHz spectrophotometer. Tetramethylsilane (TMS) was used as internal standard. Spectra were acquired using as deuterated solvents dimethyl sulfoxide (DMSO-d_6) or chloroform ($CDCl_3$). Chemical shifts (δ) and coupling constants (J, expressed in Hz) were reported and multiplicities are indicated as s (singlet), br s (broad singlet), d (doublet), dd (double doublet), t (triplet) and m (multiplet). (Figures S1–S23 in Supplementary Materials).

Structures were confirmed by spectroscopic data.

IR spectra of compounds were recorded as KBr tablet using a Jasco FT/IR460 plus spectrophotometer and absorbance was indicated as wave number (ν, cm^{-1}).

Reactions with air- or moisture-sensitive compounds were conducted under a nitrogen or argon atmosphere.

Agilent 1100 LC/MSD system, consisting of a single quadrupole detector (SQD) mass spectrometer (MS) equipped with an electrospray ionization (ESI) interface and a photodiode array (PDA) detector, range 120–550 nm, was used to perform LC/MS analyses applying ESI in positive mode. Mobile phases: (A) MeOH in H_2O (8:2). Analyses were performed at a flow rate of 0.9 mL/min, temperature 350 °C. The purity of final compounds

was verified through elemental analysis (C, H, and N) using a PerkinElmer 240 B elemental analyser. All synthesized final compounds were found to have a purity exceeding 95%.

Reagents and solvents were purchased from Merck®, Alfa Aesar®, and Acros Organics®, and used without further purification.

Ethyl 1,4-dihydroindeno[1,2-*b*]pyrrole-3-carboxylate (**9**) and ethyl 6-methyl-1, 4-dihydroindeno[1,2-*b*]pyrrole-3-carboxylate (**10**) were prepared as reported in the literature [16] or by analogy, respectively.

4.1.2. General Synthesis of Esters **11** and **12**

To a solution of ester **9** or **10** (3.47 mmol) in DMF (11,56 mL) NaH 60% in mineral oil (4,16 mmol) was added under Argon and stirred for 30 min. Then, a solution of 4-methylbenzyl chloride (3.47 mmol) in THF (3.10 mL) was added to the suspension and the whole stirred at room temperature for 5h. The mixture was taken up with H_2O and extracted with $CHCl_3$, dried (Na_2SO_4) and concentrated, to give a crude solid which was purified by FC furnishing the desired ester.

4.1.3. Ethyl-1-(4-methylbenzyl)-1,4-dihydroindeno[1,2-*b*]pirrole-3-carboxilate (**11**)

A general procedure was used to prepare the title compound from ester **9**. Subsequent FC purification (petroleum benzine/EtOAc: 8/2) furnished **11** as a white solid (750 mg, 64.99%), mp = 106–108 °C. IR (cm^{-1}): 1702 (C=O). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.37 (t, 3H, J = 7.0 Hz), 2.31 (s, 3H), 3.69 (s, 2H), 4.31 (q, 2H, J 6.8 Hz), 5.31 (s, 2H), 7.02–7.38 (m, 7H), 7.35 (s, 1H), 7.46 (d, 1H, J = 6.8 Hz). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 14.15 (CH_3), 21.33 (CH_3), 22.54 (CH_2), 51.57 (CH_2), 56.12 (CH_2), 114.28 (C), 118.63 (C), 122.70 (CH), 124.62 (CH), 126.21 (CH), 127.25 (CH × 2), 128.63 (CH), 128.94 (CH × 2), 130.01 (CH), 134.36 (C), 135.42 (C), 137.60 (C), 139.41 (C), 140.85 (C), 165.90 (C=O). Anal. calcd for $C_{22}H_{21}NO$: C, 83.78; H, 6.71; N, 4.44. Found: C, 82.37; H, 6.60; N, 4.36.

4.1.4. Ethyl-6-methyl-1-(4-methylbenzyl)-1,4-dihydroindeno[1,2-*b*]pirrole-3-carboxilate (**12**)

General procedure was used to prepare title compound from ester **10**. Subsequent FC purification (petroleum benzine/EtOAc: 9/1) furnished **12** as a yellow solid (680 mg, 57.05%), mp = 127–129 °C. IR (cm^{-1}): 1706 (C=O). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.37 (t, 3H, J = 6.8 Hz), 2.31 (s, 3H), 2.36 (s, 3H), 3.66 (s, 2H), 4.30 (q, 2H, J = 6.6 Hz), 5.31 (s, 2H), 6.96–7.10 (m, 5H), 7.26 (d, 1H, J_m = 1.4 Hz), 7.31 (d, 2H, J = 6.6 Hz). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 14.21 (CH_3), 21.33 (CH_3), 21.65 (CH_3), 22.30 (CH_2), 51.46 (CH_2), 56.24 (CH_2), 114.10 (C), 118.62 (C), 122.75 (CH), 124.54 (CH), 126.53 (CH), 127.41 (CH × 2), 128.75 (CH × 2), 131.40 (CH), 134.44 (C), 134.62 (C), 135.35 (C), 137.31 (C), 138.34 (C), 141.02 (C), 165.71 (C=O). Anal. calcd for $C_{23}H_{23}NO$: C, 83.85; H, 7.04; N, 4.25. Found: C, 82.39; H, 7.07; N, 4.21.

4.1.5. General Synthesis of Carboxylic Acids **13** and **14**

To a suspension of ester **11** or **12** (1.25 mmol) in EtOH (5.30 mL), a solution of KOH (2.61 mmol) in EtOH (4.23 mL) and H_2O (4 drops) was added: the mixture was refluxed overnight. The solution was poured onto ice and acidified with 1N HCl and the resulting precipitate was filtered under vacuum and dissolved in $KHCO_3$ aq. The basic solution was acidified with 1N HCl to precipitate the desired compound which was washed (H_2O) and air dried to give the corresponding carboxylic acid **13** or **14**.

4.1.6. 1-(4-Methylbenzyl)-1,4-dihydroindeno[1,2-*b*]pirrole-3-carboxilic acid (**13**)

Title compound was prepared from ester **11** following the general procedure, to yield **13** as a white solid (380 mg, 99.73%), mp = 232–234 °C. IR (cm^{-1}): 1670 (C=O). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 2.32 (s, 3H), 3.72 (s, 2H), 5.35 (s, 2H), 7.05–7.30 (m, 6H), 7.42 (s, 1H),

7.62 (d, 2H, *J* = 8.0 Hz). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 21.32 (CH_3), 22.51(CH_2), 51.40 (CH_2), 56.22 (CH_2), 114.21 (C), 118.67 (C), 122.82 (CH), 124.53 (CH), 126.15 (CH), 127.21 (CH × 2), 128.50 (CH), 128.92 (CH × 2), 130.10 (CH), 134.33 (C), 135.42 (C), 137.53 (C), 139.34 (C), 140.81 (C), 166.25 (C=O). Anal. calcd for $C_{20}H_{17}NO_2$: C, 79.19; H, 5.65; N, 4.62. Found: C, 78.12; H, 5.57; N, 4.56.

4.1.7. 6-Methyl-1-(4-methylbenzyl)-1,4-dihydroindeno[1,2-*b*]pirrole-3-carboxilic acid (**14**)

Title compound was prepared from ester **12** following the general procedure, to yield **14** as a white solid (300 mg, 75.75%), mp = 230–232 °C. IR (cm^{-1}): 1670 (C=O). ^{1}H NMR (400 MHz, $CDCl_3$) *δ*: 2.31 (s, 3H), 2.36 (s, 3H), 3.69 (s, 2H), 5.32 (s, 2H), 6.97–7.12 (m, 5H), 7.28 (d, 2H, *J* = 8.2 Hz), 7.39 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 21.33 (CH_3), 21.72 (CH_3), 22.35 (CH_2), 51.40 (CH_2), 56.22 (CH_2), 114.10 (C), 118.67 (C), 122.72 (CH), 124.55 (CH), 126.53 (CH), 127.42 (CH × 2), 128.74 (CH × 2), 131.41 (CH), 134.42 (C), 134.74 (C), 135.32 (C), 137.21 (C), 138.23 (C), 141.02 (C), 166.31 (C=O). Anal. calcd for $C_{21}H_{19}NO_2$: C, 79.47; H, 6.03; N, 4.41. Found: C, 78.42; H, 5.95; N, 4.35.

4.1.8. General Synthesis of Carbohydrazides **1a**–**d**, **2a**–**d** and Carboxamides **1e**–**k**, **2e**–**l**

Method A. A solution of acid **13** (0.49 mmol) and $SOCl_2$ (1.47 mmol) in toluene (4 mL) was refluxed for 3 h. After evaporation of $SOCl_2$ in excess, the residue was dissolved in CH_2Cl_2 (5.5 mL) and the solution was dropwise added of the appropriate hydrazine or amine (0.637 mmol) and TEA (0.64 mmol for **1a**,**c**–**g**,**i**–**k**, or 1.27 mmol for **1b**,**h**), cooling with an ice bath. The resulting solution was stirred at room temperature for 3 h, and then washed (saturated $NaCl_{aq}$), dried (Na_2SO_4) and concentrated, to give a crude product which was purified by FC to yield compounds **1a**–**k**.

Method B. To a suspension of acid **14** (0.315 mmol) in CH_2Cl_2 (2.12 mL) HOBt (0.378 mmol) and EDC (0.378 mmol) were added and the whole stirred at room temperature for 1.5 h. The appropriate hydrazine or amine (0.63 mmol) and TEA (0.756 mmol only for **2a**,**k**) were added and the resulting solution was stirred at room temperature for another 2 h. Then it was washed (saturated $NaCl_{aq}$), dried (Na_2SO_4) and concentrated, to give a crude product which was purified by FC to give derivatives **2a**–**l**.

4.1.9. 1-(4-Methylbenzyl)-*N*-(piperidin-1-yl)-1,4-dihydroindeno[1,2-*b*]pirrole-3-carbohydrazide (**1a**)

Compound **1a** was prepared following general procedure I, Method A, by a reaction of acid **13** and 1-aminopiperidine. After FC purification (petroleum ether/EtOAc 3/7), carbohydrazide **1a** was isolated as a beige solid (28 mg, 14.81%). M.p.: 152.5–154.6 °C; IR (nujol) ν: 1629 (C=O), 3154 (NH); ^{1}H NMR (400 MHz, DMSO) δ: 1.32–1.44 (m, 2H), 1.47–1.71 (m, 4H), 2.25 (s, 3H), 2.79–3.03 (m, 4H), 3.63 (s, 2H), 5.42 (s, 2H), 7.05 (t, 2H, *J* = 7.6 Hz), 7.13–7.19 (m, 4H, NH exch. with D_2O), 7.32–7.34 (m, 1H), 7.40–7.46 (m, 2H), 7.53–7.56 (m, 1H); ^{13}C NMR (100 MHz, DMSO) δ: 21.07 (CH_3), 23.50 (CH_2), 25.74 (CH_2 × 2), 31.34 (CH_2), 51.51 (CH_2 × 2), 55.99 (CH_2), 114.80 (C), 117.13 (CH), 119.54 (CH), 123.73 (CH), 124.93 (CH), 125.75 (CH), 126.76 (CH), 127.16 (CH), 127.73 (CH), 128.28 (C), 134.87 (C), 135.26 (C), 137.21 (C), 137.94 (C), 146.94 (C), 162.18 (C=O). MS (ESI): $C_{25}H_{27}N_3O$ requires *m*/*z* 385.22, found 386.22 $[M + H]^+$. Anal. calcd for $C_{25}H_{27}N_3O$: C, 77.89; H, 7.06; N, 10.90. Found: C, 76.58; H, 7.03; N, 10.71.

4.1.10. 1-(4-Methylbenzyl)-*N*-(pyrrolidin-1-yl)-1,4-dihydroindeno[1,2-*b*]pyrrole-3-carbohydrazide (**1b**)

Compound **1b** was prepared following general procedure I, Method A, by a reaction of acid **13** and 1-aminopyrrolidine. After FC purification (petroleum ether/EtOAc 3/7), carbohydrazide **1b** was isolated as a beige solid (39 mg, 21.97%). M.p.: 120–124 °C; IR

(nujol) ν: 1629 (C=O), 3226 (NH); ^{1}H NMR (400 MHz, DMSO) δ: 1.86–1.99 (m, 4H), 2.24 (s, 3H), 3.05–3.11 (m, 4H), 3.61 (s, 2H), 5.46 (s, 2H), 7.09 (t, 1H, *J* = 7.6 Hz), 7.13–7.20 (m, 6H, NH exch. with D_2O), 7.36 (d, 1H, *J* = 7.2 Hz), 7.47 (d, 1H, *J* = 7.2 Hz), 7.74 (s, 1H); ^{13}C NMR (100 MHz, DMSO) δ: 20.61 (CH_3), 22.17 (CH_2 × 2), 30.89 (CH_2), 51.03 (CH_2), 51.17 (CH_2), 56.12 (CH_2), 111.78 (C), 116.96 (CH), 123.65 (CH), 125.39 (CH), 126.14 (CH), 126.42 (CH), 126.73 (CH × 2), 128.86 (C), 129.27 (CH × 2), 134.04 (C), 134.50 (C), 136.84 (C), 138.10 (C), 146.36 (C), 162.69 (C=O). MS (ESI): $C_{24}H_{25}N_3O$ requires *m*/*z* 371.20, found 372.20 $[M + H]^+$. Anal. calcd for $C_{24}H_{25}N_3O$: C, 77.60; H, 6.78; N, 11.31. Found: C, 77.37; H, 6.76; N, 11.28.

4.1.11. 1-(4-Methylbenzyl)-*N*-(homopiperidin-1-yl)-1,4-dihydroindeno[1,2-*b*]pyrrole-3-carbohydrazide (**1c**)

Compound **1c** was prepared following general procedure I, Method A, by a reaction of acid **13** and 1-aminohomopiperidine. After FC purification (petroleum ether/EtOAc 3/7), carbohydrazide **1c** was isolated as a beige solid (73 mg, 3.73%). M.p.: 169–173 °C; IR (nujol) ν: 1635 (C=O), 3226 (NH); ^{1}H NMR (400 MHz, DMSO) δ: 1.58–1.70 (m, 8H), 2.25 (s, 3H), 2.97–3.06 (m, 4H), 3.61 (s, 2H), 5.40 (s, 2H), 7.05 (t, 1H, *J* = 7.2 Hz), 7.12–7.16 (m, 5H), 7.32 (d, 1H, *J* = 7.2 Hz), 7.44 (d, 1H, *J* = 7.2 Hz), 7.49 (s, 1H), 8.90 (br s, 1H, NH exch. with D_2O); ^{13}C NMR (100 MHz, DMSO) δ: 21.09 (CH_3), 26.98 (CH_2), 27.10 (CH_2), 31.32 (CH_2), 51.49 (CH_2), 57.89 (CH_2), 59.52 (CH_2), 60.70 (CH_2), 72.72 (CH_2), 114.98 (C), 117.11 (CH), 123.69 (CH), 125.76 (CH), 126.76 (CH), 127.16 (CH), 127.50 (CH × 2), 129.04 (C), 129.70 (CH × 2), 134.89 (C), 135.31 (C), 137.18 (C), 137.84 (C), 146.95 (C), 162.45 (C=O). MS (ESI): $C_{26}H_{29}N_3O$ requires *m*/*z* 399.23, found 400.23 $[M + H]^+$. Anal. calcd for $C_{26}H_{29}N_3O$: C, 78.16; H, 7.32; N, 10.52. Found: C, 78.39; H, 7.34; N, 10.55.

4.1.12. 1-(4-Methylbenzyl)-*N*-(morpholin-1-yl)-1,4-dihydroindeno[1,2-*b*]pyrrole-3-carbohydrazide (**1d**)

Compound **1d** was prepared following general procedure I, Method A, by a reaction of acid **13** and 1-aminomorpholine. After FC purification (chloroform/acetone 8/2), carbohydrazide **1d** was isolated as a beige solid (44 mg, 23.28%). M.p.: 198–205 °C; IR (nujol) ν: 1629 (C=O), 3193 (NH); ^{1}H NMR (400 MHz, DMSO) δ: 2.25 (s, 3H), 2.90–3.00 (m, 4H), 3.55–3.70 (m, 6H), 5.43 (s, 2H), 7.05 (t, 1H, *J* = 8.0 Hz), 7.13–7.21 (m, 5H,), 7.33 (d, 1H, *J* = 8.0 Hz), 7.41–7.53 (m, 2H), 9.00 (br s, 1H, NH exch. with D_2O); ^{13}C NMR (100 MHz, DMSO) δ: 21.09 (CH_3), 51.51 (CH_2), 54.57 (CH_2), 54.73 (CH_2), 55.16 (CH_2), 66.19 (CH_2), 66.33 (CH_2), 112.13 (C), 117.22 (CH), 125.79 (CH × 2), 126.70 (CH), 126.80 (CH), 127.53 (CH), 128.00 (C), 129.72 (CH × 2), 129.76 (CH), 134.79 (C), 135.20 (C), 136.62 (C), 137.23 (C), 146.91 (C), 162.18 (C=O). MS (ESI): $C_{24}H_{25}N_3O$ requires *m*/*z* 387.19, found 388.19 $[M + H]^+$. Anal. calcd for $C_{24}H_{25}N_3O_2$: C, 74.39; H, 6.50; N, 10.84. Found: C, 74.20; H, 6.48; N, 10.80.

4.1.13. 1-(4-Methylbenzyl)-*N*-(piperidin-1-yl)-1,4-dihydroindeno[1,2-*b*]pyrrole-3-carboxamide (**1e**)

Compound **1e** was prepared following general procedure I, Method A, by a reaction of acid **13** and piperidine. After FC purification (petroleum ether/EtOAc 7/3), carbohydrazide **1e** was isolated as a beige solid (83 mg, 46.41%). M.p.: 102–104 °C; IR (nujol) ν: 1606 (C=O); ^{1}H NMR (400 MHz, DMSO) δ: 1.53–1.54 (m, 4H), 1.62–1.63 (m, 2H), 2.24 (s, 3H), 3.51 (s, 2H), 3.59 (t, 4H, *J* = 5.2 Hz), 5.42 (s, 2H), 7.04 (t, 1H, *J* = 7.6 Hz), 7.10–7.18 (m, 5H), 7.31 (d, 1H, *J* = 7.6 Hz), 7.33 (s, 1H), 7.42 (d, 1H, *J* = 7.6 Hz); ^{13}C NMR (100 MHz, DMSO) δ: 21.08 (CH_3), 24.71 (CH_2 × 2), 26.41 (CH_2), 31.56 (CH_2 × 2), 51.24 (CH_2 × 2), 115.08 (C), 117.16 (CH), 123.71 (CH), 125.72 (CH), 126.83 (CH), 127.09 (CH × 2), 128.67 (C), 128.82 (CH), 129.68 (CH × 2), 134.83 (C), 135.43 (C), 137.11 (C), 137.19 (C), 146.59 (C), 165.21 (C=O). MS (ESI): $C_{25}H_{26}N_2O$ requires *m*/*z* 370.20, found 371.20 $[M + H]^+$. Anal. calcd for $C_{25}H_{26}N_2O$: C, 81.05; H, 7.07; N, 7.56. Found: C, 79.81; H, 7.05; N, 7.54.

4.1.14. *N*-Cyclohexyl-1-(4-methylbenzyl)-1,4-dihydroindeno[1,2-*b*]pyrrole-3-carboxamide (**1f**)

Compound **1f** was prepared following general procedure I, Method A, by a reaction of acid **13** and cyclohexylamine. After FC purification (petroleum ether/EtOAc 8/2), carboxamide **1f** was isolated as a beige solid (69 mg, 36.17%). M.p.: 189–190 °C; IR (nujol) ν: 1617 (C=O), 3272 (NH); ^{1}H NMR (400 MHz, DMSO) δ: 1.11–1.38 (m, 5H), 1.58–1.86 (m, 5H), 2.25 (s, 3H), 3.67 (s, 2H), 3.70–3.73 (m, 1H), 5.41 (s, 2H), 6.67 (d, 1H, *J* = 7.6 Hz, NH exch. with D_2O), 7.04 (t, 1H, *J* = 7.6 Hz), 7.10–7.19 (m, 5H), 7.30–7.33 (m, 1H), 7.43 (d, 1H, *J* = 7.6 Hz), 7.54 (s, 1H); ^{13}C NMR (100 MHz, DMSO) δ: 21.09 (CH_3), 25.41 (CH_2 × 2), 25.79 (CH_2), 31.29 (CH_2), 33.17 (CH_2 × 2), 47.92 (CH), 51.49 (CH_2), 116.32 (C), 117.09 (CH), 123.65 (CH), 125.77 (CH), 126.76 (CH), 127.14 (CH × 2), 128.93 (CH), 129.13 (C), 129.70 (CH × 2), 134.95 (C), 135.39 (C), 137.16 (C), 137.82 (C), 146.97 (C), 163.22 (C=O). MS (ESI): $C_{26}H_{28}N_2O$ requires *m*/*z* 384.22, found 385.22 $[M + H]^+$. Anal. calcd for $C_{26}H_{28}N_2O$: C, 81.21; H, 7.34; N, 7.29. Found: C, 81.40; H, 7.32; N, 7.27.

4.1.15. *N*-Bornyl-1-(4-methylbenzyl)-1,4-dihydroindeno[1,2-*b*]pyrrole-3-carboxamide (**1g**)

Compound **1g** was prepared following general procedure I, Method A, by a reaction of acid **13** and bornylamine. After FC purification (petroleum ether/EtOAc 8/2), carboxamide **1g** was isolated as a beige solid (72 mg, 32.56%). M.p.: 151.7–153 °C; IR (nujol) ν: 1617 (C=O), 3222 (NH); ^{1}H NMR (400 MHz, DMSO) δ: 0.76 (s, 3H), 0.86 (s, 3H), 0.95 (s, 3H), 1.08 (dd, 1H, *J* = 12 Hz, *J* = 4 Hz), 1.27 (t, 1H, *J* = 8.00 Hz), 1.38 (t, 1H, *J* = 8.00 Hz), 1.63–1.70 (m, 3H), 1.79 (t, 1H, *J* = 4 Hz), 2.17 (t, 1H, *J* = 12 Hz), 2.24 (s, 3H), 3.71 (d, 1H, *J* = 21.6 Hz), 3.55 (d, 1 H, *J* = 21.6 Hz), 4.34 (br s, 1H, NH exch. with D_2O) 5.42 (s, 2H), 7.05 (t, 1H, *J* = 7.2 Hz), 7.11–7.19 (m, 5H), 7.31 (d, 1H, *J* = 7.6 Hz), 7.46 (d, 1H, *J* = 7.2 Hz), 7.65 (s, 1H); ^{13}C NMR (100 MHz, DMSO) δ: 14.41 (CH_3), 18.95 (CH_3), 20.28 (CH_3), 21.08 (CH_3), 28.15 (CH_2), 28.27 (CH_2), 31.27 (CH_2), 35.79 (CH_2), 44. 93 (CH), 48.23 (C), 49.86 (C), 51.50 (CH_2), 53.04 (CH), 116.33 (C), 117.09 (CH), 123.68 (CH), 125.79 (CH), 126.78 (CH), 127.04 (CH × 2), 127.95 (CH), 129.15 (C), 129.69 (CH × 2), 134.96 (C), 135.46 (C), 137.14 (C), 137.82 (C), 146.92 (C), 164.34 (C=O). MS (ESI): $C_{30}H_{34}N_2O$ requires m/z 438.27, found 439.27 $[M + H]^+$. Anal. calcd for $C_{30}H_{34}N_2O$: C, 82.15; H, 7.81; N, 6.39. Found: C, 81.99; H, 7.79; N, 6.38.

4.1.16. *N*-(*1S*,*2S*,*3S*,*5R*)-(+)-Isopinocampheyl-1-(4-methylbenzyl)-1,4-dihydroindeno[1,2-*b*]pyrrole-3-carboxamide (**1h**)

Compound **1h** was prepared following general procedure I, Method A, by a reaction of acid **13** and (1S,2S,3S,5R)-(+)-isopinocampheyamine. After FC purification (petroleum ether/EtOAc 8/2), carboxamide **1h** was isolated as a beige solid (52 mg, 23.72%). M.p.: 192–195 °C; IR (nujol) ν: 1619 (C=O), 3289 (NH); ^{1}H NMR (400 MHz, DMSO) δ: 1.04 (s, 3H), 1.06 (s, 3H), 1.23 (s, 3H), 1.64–1.68 (m, 1H), 1.88–1.92 (m, 1H), 1.90–1.95 (m, 2H), 1.96–2.06 (m, 1H), 2.25 (s, 3H), 2.30–2.46 (m, 2H), 3.62 (d, 2H, *J* = 5.2 Hz), 4.26–4.38 (m, 1H), 5.42 (s, 2H), 7.06 (d, 1H, *J* = 6.4 Hz), 7.03–7.19 (m, 5H), 7.32 (d, 1H, *J* = 7.6 Hz), 7.45 (d, 1H, *J* = 7.6 Hz), 7.49 (d, 1H, *J* = 8.0 Hz, 1H, NH exch. with D_2O), 7.57 (s, 1H); ^{13}C NMR (100 MHz, DMSO) δ: 20.61 (CH_3), 20.64 (CH_3), 23.17 (CH_3), 27.96 (CH_3), 30.84 (CH_2), 33.90 (CH_2), 36.41 (CH_2), 38.31 (C), 41.21 (CH), 43.98 (CH), 46.56 (CH), 47.34 (CH), 51.04 (CH_2), 115.83 (C), 116.61 (CH), 123.19 (CH), 125.31 (CH), 126.29 (CH), 126.64 (CH × 2), 128.44 (CH) 128.87 (C), 129.24 (CH × 2), 134.48 (C), 134.95 (C), 136.69 (C), 137.38 (C), 146.54 (C), 163.15 (C=O). MS (ESI): $C_{30}H_{34}N_2O$ requires *m*/*z* 438.27, found 439.27 $[M + H]^+$. Anal. Calcd for $C_{30}H_{34}N_2O$: C, 82.15; H, 7.81; N, 6.39. Found: C, 82.82; H, 7.78; N, 6.37.

4.1.17. 1-(4-Methylbenzyl)-*N*-myrtanyl-1,4-dihydroindeno[1,2-*b*]pyrrole-3-carboxamide (**1i**)

Compound **1i** was prepared following general procedure I, Method A, by a reaction of acid **13** and *cis*-myrtanylamine. After FC purification (petroleum ether/EtOAc 83/17), carboxamide **1i** was isolated as a beige solid (49 mg, 22.32%). M.p.: 88–91 °C; IR (nujol) ν:

1623 (C=O), 3284 (NH); ^{1}H NMR (200 MHz, $CDCl_3$) δ: 0.80–0.97 (m, 2H), 1.09 (s, 3H), 1.22 (s, 3H), 1.82–2.15 (m, 5H), 2.31 (s, 3H), 2.33–2.40 (m, 2H), 3.40–3.50 (m, 2H), 3.64 (s, 2H), 5.32 (s, 2H), 5.68 (br s, 1H, NH exch. with D_2O), 7.02–7.30 (m, 7H), 7.46 (d, 2H, *J* = 7.8 Hz). ^{13}C NMR (50 MHz, $CDCl_3$) δ: 19.93 (CH_3), 21.08 (CH_3), 23.26 (CH_3), 26.05 (CH_2), 28.02 (CH_2), 30.88 (CH_2), 33.30 (CH_2), 38.75 (C), 41.38 (CH), 41.64 (CH), 43.97 (CH), 45.00 (CH_2), 52.15 (CH_2), 95.87 (C), 115.79 (C), 116.77 (CH), 123.62 (CH), 125.40 (CH), 126.33 (C), 126.66 (CH), 126.73 (CH × 2), 127.96 (CH), 129.60 (CH × 2), 133.55 (C), 134.86 (C), 137.69 (C), 147.19 (C), 164.63 (C=O). MS (ESI): $C_{30}H_{34}N_2O$ requires *m*/*z* 438.27, found 439.27 $[M + H]^+$. Anal. calcd for $C_{30}H_{34}N_2O$: C, 82.15; H, 7.81; N, 6.39. Found: C, 82.08; H, 7.78; N, 6.37.

4.1.18. 1-Adamantyl-1-(4-methylbenzyl)-1,4-dihydroindeno[1,2-*b*]pyrrole-3-carboxamide (**1j**)

Compound **1j** was prepared following general procedure I, Method A, by a reaction of acid **13** and 1-adamantanamine. After FC purification (petroleum ether/EtOAc 85/15), carboxamide **1j** was isolated as a beige solid (83 mg, 39.43%). M.p.: 124–126 °C; IR (nujol) ν: 1631 (C=O), 3421 (NH); ^{1}H NMR (400 MHz, DMSO) δ: 1.66 (s, 6H), 2.06 (s, 8H), 2.24 (s, 4H), 3.59 (s, 2H), 5.39 (s, 2H), 6.67 (br s, 1H, NH exch with D_2O), 7.02 (t, 1H, *J* = 6.8 Hz), 7.09–7.18 (m, 5H), 7.31 (d, 1H, *J* = 7.6 Hz), 7.43 (d, 1H, *J* = 7.2 Hz), 7.55 (s, 1H); ^{13}C NMR (100 MHz, DMSO) δ: 21.08 (CH_3), 29.21 (CH × 3), 31.21 (CH_2), 36.60 (CH_2 × 3), 41.77 (CH_2 × 3), 51.35 (CH_2), 51.47 (C), 117.08 (C), 117.13 (CH), 123.63 (CH), 125.75 (CH), 126.77 (CH), 127.09 (CH × 2), 128.05 (CH), 128.91 (C), 129.67 (CH × 2), 134.96 (C), 135.43 (C), 137.13 (C), 137.73 (C), 146.93 (C), 163.69 (C=O). MS (ESI): $C_{30}H_{32}N_2O$ requires *m*/*z* 436.25, found 437.25 $[M + H]^+$. Anal. calcd for $C_{30}H_{32}N_2O$: C, 82.53; H, 7.39; N, 6.42. Found: C, 82.37; H,7.37; N, 6.41.

4.1.19. 2-Adamantyl-1-(4-methylbenzyl)-1,4-dihydroindeno[1,2-*b*]pyrrole-3-carboxamide (**1k**)

Compound **1k** was prepared following general procedure I, Method A, by a reaction of acid **13** and 2-adamantanamine. After FC purification (petroleum ether/EtOAc 75/25), carboxamide **1k** was isolated as a beige solid (43 mg, 20.66%). M.p.: 81.9–82.3 °C; IR (nujol) ν: 1639 (C=O), 3276 (NH); ^{1}H NMR (400 MHz, DMSO) δ: 1.55–2.16 (m, 14H), 2.24 (s, 3H), 3.66 (s, 2H), 4.04 (s, 1H,), 5.61 (s, 2H), 6.98 (d, 1H, *J* = 6.8 Hz, NH exch with D_2O), 7.06 (t, 1H, *J* = 7.6 Hz), 7.04–7.18 (m, 5H), 7.32 (d, 1H, *J* = 7.2 Hz), 7.47 (d, 1H, *J* = 7.6 Hz), 7.68 (s, 1H); ^{3}C NMR (100 MHz, DMSO) δ: 20.60 (CH_3), 26.80 (CH), 30.67 (CH_2), 31.28 (CH_2 × 2), 31.44 (CH × 3), 36.87 (CH_2 × 2), 37.19 (CH_2), 51.01 (CH_2), 52.93 (CH), 115.72 (C), 116.66 (CH), 123.23 (CH), 125.34 (CH), 126.33 (CH), 126.60 (CH × 2), 128.06 (CH), 128.39 (C), 129.20 (CH × 2), 134.47 (C), 134.94 (C), 136.66 (C), 137.36 (C), 146.34 (C), 163.17 (C=O). MS (ESI): $C_{30}H_{32}N_2O$ requires *m*/*z* 436.25, found 437.25 $[M + H]^+$. Anal. calcd for $C_{30}H_{32}N_2O$: C, 82.53; H, 7.39; N, 6.42. Found: C, 82.28; H, 7.37; N, 6.40.

4.1.20. 6-Methyl-1-(4-methylbenzyl)-*N*-(piperidin-1-yl)-1,4-dihydroindeno[1,2-*b*]pyrrole-3-carbohydrazide (**2a**)

Compound **2a** was prepared following general procedure I, Method B, by a reaction of acid **14** and 1-aminopiperidine. After FC purification (petroleum ether/EtOAc 3/7), carbohydrazide **2a** was isolated as a beige solid (20 mg, 16.80%). M.p.: 165–168 °C; IR (nujol) ν: 1614 (C=O); ^{1}H NMR (400 MHz, DMSO) δ: 0.84–0.89 (m, 2H), 1.22–1.30 (m, 2H), 1.40–1.60 (m, 2H), 1.89–1.74 (m, 4H), 2.25 (s, 3H), 2.30 (s, 3H), 3.63 (s, 2H), 5.45 (s, 2H), 7.02 (t, 1H, *J* = 8.0 Hz), 7.06 (s, 1H), 7.11–7.16 (m, 4H,), 7.25 (d, 1H, *J* = 7.6 Hz), 7.35 (d, 1H, *J* = 6.8 Hz), 7.66–7.68 (m, 1H, NH exch. with D_2O); ^{13}C NMR (100 MHz, DMSO) δ: 20.61 (CH_3), 20.94 (CH_3), 22.36 (CH_2), 22.90 (CH_2), 28.32 (CH_2), 29.76 (CH_2), 30.75 (CH_2), 51.19 (CH_2), 56.18 (CH_2), 114.80 (C), 116.74 (CH), 126.31 (CH), 126.77 (CH), 126.93 (CH), 128.21 (C), 128.81 (CH), 129.26 (CH × 2), 129.30 (CH), 131.42 (C), 134.45 (C × 2), 136.87 (C × 2),

146.67 (C), 162.11 (C=O). MS (ESI): $C_{26}H_{29}N_3O$ requires *m*/*z* 399.23, found 400.23 $[M + H]^+$. Anal. calcd for $C_{26}H_{29}N_3O$: C, 78.16; H, 7.32; N, 10.52. Found: C, 77.85; H, 7.29; N, 10.48.

4.1.21. 6-Methyl-1-(4-methylbenzyl)-*N*-(pyrrolidin-1-yl)-1,4-dihydroindeno[1,2-*b*]pyrrole-3-carbohydrazide (**2b**)

Compound **2b** was prepared following general procedure I, Method B, by a reaction of acid **14** and 1-aminopyrrolidine. After FC purification (petroleum ether/EtOAc 3/7), carbohydrazide **2b** was isolated as a white solid (30 mg, 25.34%). M.p.: 102–105 °C; IR (nujol) ν: 1460 (C=O); ^{1}H NMR (200 MHz, $CDCl_3$) δ: 1.80–2.10 (m, 4H), 2.30 (s, 3H), 2.36 (s, 3H), 2.98–3.20 (m, 4H), 3.64 (s, 2H), 5.30 (s, 2H), 6.86–7.38 (m, 8H), 7.62 (br s, 1H, NH exch. with D_2O). MS (ESI): $C_{26}H_{29}N_3O$ requires *m*/*z* 385.22, found 386.22 $[M + H]^+$. Anal. calcd for $C_{25}H_{27}N_3O$: C, 77.89; H, 7.06; N, 10.90. Found: C, 77.58; H, 7.03; N, 10.86.

4.1.22. 6-Methyl-1-(4-Methylbenzyl)-*N*-(homopiperidin-1-yl)-1,4-dihydroindeno[1,2-*b*]pirrole-3-carbohydrazide (**2c**)

Compound **2c** was prepared following general procedure I, Method B, by a reaction of acid **14** and 1-aminohomopiperidine. After FC purification (petroleum ether/EtOAc 1/1), carbohydrazide **1c** was isolated as a white solid (66 mg, 16.15%). M.p.: 118–120 °C; IR (nujol) ν: 1637 (C=O), 2360 (NH); ^{1}H NMR (200 MHz, $CDCl_3$) δ: 1.50–1,90 (m, 8H), 2.31 (s, 3H), 2.36 (s, 3H), 3.12–3.39 (m, 4H), 3.62 (s, 2H), 5.30 (s, 2H), 6.92–7.10 (m, 8H), 7.62 (br s, 1H, NH exch. with D_2O). MS (ESI): $C_{27}H_{31}N_3O$ requires *m*/*z* 431.25, found 432.25 $[M + H]^+$. Anal. calcd for $C_{27}H_{31}N_3O$: C, 78.42; H, 7.56; N, 10.16. Found: C, 78.11; H, 7.53; N, 10.12.

4.1.23. 1-(4-Methylbenzyl)-*N*-(morpholin-1-yl)-1,4-dihydroindeno[1,2-*b*]pyrrole-3-carbohydrazide (**2d**)

Compound **2d** was prepared following general procedure I, Method B, by a reaction of acid **14** and 1-aminomorpholine. After FC purification (chloroform/acetone 9/1), carbohydrazide **2d** was isolated as a white solid (26 mg, 20.39%). M.p.: 226–228 °C; IR (nujol) ν: 1631 (C=O), 3197 (NH); ^{1}H NMR (200 MHz, $CDCl_3$) δ: 2.31 (s, 3H), 2.36 (s, 3H), 2.83–3.17 (m, 4H), 3.65 (s, 2H), 3.70–4.05 (m, 4H), 5.31 (s, 2H), 6.22–6.57 (m, 1H, NH exch. with D_2O), 6.20–7.20 (m, 8H); ^{13}C NMR (50 MHz, $CDCl_3$) δ: 21.09 (CH_3), 21.38 (CH_3), 31.35 (CH_2), 52.18 (CH_2), 56.54 (CH_2 × 2), 66.50 (CH_2 × 2), 116.41 (CH), 118.08 (C), 125.76 (CH), 126.42 (CH × 2), 126.89 (CH), 127.00 (CH), 127.20 (C), 129.62 (CH × 2), 132.09 (C), 133.11 (C), 133.43 (C), 133.62 (C), 137.80 (C), 146.95 (C). MS (ESI): $C_{25}H_{27}N_3O_2$ requires *m*/*z* 401.21, found 402.21 $[M + H]^+$. Anal. calcd for $C_{25}H_{27}N_3O_2$: C, 74.79; H, 6.78; N, 10.47. Found: C, 74.49; H, 6.75; N, 10.43.

4.1.24. 6-Methyl-1-(4-Methylbenzyl)-*N*-(piperidin-1-yl)-1,4-dihydroindeno[1,2-*b*]pyrrole-3-carboxamide (**2e**)

Compound **2e** was prepared following general procedure I, Method B, by a reaction of acid **14** and piperidine. After FC purification (petroleum ether/EtOAc 1/1), carbohydrazide **2e** was isolated as a white solid (93 mg, 24.72%). M.p.: 125–130 °C; IR (nujol) ν: 1614 (C=O), 3430 (NH); ^{1}H NMR (200 MHz, $CDCl_3$) δ: 1.43–1.83 (m, 6H), 2.30 (s, 3H), 2.34 (s, 3H), 3.55 (s, 2H), 3.62–3.71 (m, 4H), 5.29 (s, 2H), 6.90–7.18 (m, 8H); ^{13}C NMR (50 MHz, $CDCl_3$) δ: 21.07 (CH_3), 21.35 (CH_3), 24.78 (CH_2 × 2), 26.29 (CH_2), 31.51 (CH_2 × 2), 51.91 (CH_2 × 2), 115.36 (C), 116.25 (CH), 126.30 (CH), 126.71 (CH × 2), 126.90 (CH), 127.18 (CH),127.56 (C), 129.52 (CH × 2), 132.25 (C), 133.11 (C), 133.89 (C), 137.50 (C), 137.78 (C), 147.01 (C), 166.37 (C=O). MS (ESI): $C_{25}H_{26}N_2O$ requires *m*/*z* 384.22, found 385.22 $[M + H]^+$. Anal. calcd for $C_{26}H_{28}N_2O$: C, 81.21; H, 7.34; N, 7.29. Found: C, 80.88; H, 7.31; N, 7.26.

4.1.25. 6-Methyl-*N*-Cyclohexyl-1-(4-methylbenzyl)-1,4-dihydroindeno[1,2-*b*]pyrrole-3-carboxamide (**2f**)

Compound **2f** was prepared following general procedure I, Method B, by a reaction of acid **14** and cyclohexylamine. After FC purification (petroleum ether/EtOAc 6/4), carboxamide **1f** was isolated as a white solid (104 mg, 86.66%). M.p.: 200–202 °C; IR (nujol) ν: 1627 (C=O), 3274 (NH); ^{1}H NMR (400 MHz, DMSO) δ: 1.14–1.28 (m, 6H), 1.58–1.82 (m, 4H), 2.24 (s, 3H), 2.28 (s, 3H), 3.56 (s, 2H), 3.70–3.72 (m, 1H), 5.37 (s, 2H), 6.97 (d, 1H, *J* = 8.0 Hz), 7.11 (q, 4H, *J* = 7.6 Hz), 7.20 (d, 1H, *J* = 7.6 Hz), 7.26 (s, 1H), 7.29 (d, 1H, *J* = 8.0 Hz, NH exch. with D_2O), 7.49 (s, 1H,); ^{13}C NMR (100 MHz, DMSO) δ: 20.60 (CH_3), 20.92 (CH_3), 24.94 (CH_2 × 2), 25.32 (CH_2), 30.70 (CH_2), 32.69 (CH_2 × 2), 47.44 (CH), 51.00 (CH_2), 115.86 (C), 116.28 (CH), 126.16 (CH), 126.68 (CH × 2), 126.71 (CH), 126.73 (CH), 127.89 (C), 129.18 (CH × 2), 131.96 (C), 132.28 (C), 134.96 (C), 136.65 (C), 137.44 (C), 146.86 (C), 162.80 (C=O). MS (ESI): $C_{27}H_{30}N_2O$ requires *m*/*z* 398.24, found 399.24 $[M + H]^+$. Anal. calcd for $C_{27}H_{30}N_2O$: C, 81.37; H, 7.59; N, 7.03. Found: C, 81.21; H, 7.57; N, 7.02.

4.1.26. 6-Methyl-*N*-Bornyl-1-(4-methylbenzyl)-1,4-dihydroindeno[1,2-*b*]pyrrole-3-carboxamide (**2g**)

Compound **2g** was prepared following general procedure I, Method B, by a reaction of acid **14** and bornylamine. After FC purification (petroleum ether/EtOAc 7/3), carboxamide **2g** was isolated as a white solid (117 mg, 83.80%). M.p.: 165–167 °C; IR (nujol) ν: 1619 (C=O), 3313 (NH); ^{1}H NMR (400 MHz, DMSO) δ: 0.76 (s, 3H), 0.86 (s, 3H), 0.94 (s, 3H), 1.07 (dd, 1H, *J* = 12.4 Hz, *J* = 4.8 Hz), 1.27 (t, 1H, *J* = 12 Hz), 1.40 (t, 1H, *J* = 8.8 H), 1.63–1.70 (m, 2H), 1.78 (t, 1H, *J* = 8.4 Hz), 2.16 (t, 1H, *J* = 12 Hz), 2.24 (s, 3H), 2.29 (s, 3H), 3.50 (d, 1 H, *J* = 21.6 Hz), 3.66 (d, 1 H, *J* = 21.6 Hz), 4.29–4.39 (m, 1H), 5.39 (s, 2H), 6.97 (d, 1H, *J* = 7.6 Hz), 7.09–7.20 (m, 6H), 7.27 (s, 1H, NH exch. with D_2O), 7.60 (s, 1H,); ^{13}C NMR (100 MHz, DMSO) δ: 14.41 (CH_3), 18.95 (CH_3), 20.28 (CH_3), 21.08 (CH_3), 21.40 (CH_3), 28.15 (CH_2), 28.26 (CH_2), 31.16 (CH_2), 35.78 (CH_2), 44.93 (CH), 48.22 (C), 49.86 (C), 51.49 (CH_2), 53.03 (CH), 116.35 (C), 116.77 (CH), 126.77 (CH), 127.06 (CH × 2), 127.23 (CH), 127.33 (CH), 128.45 (C), 129.66 (CH × 2), 132.44 (C), 132.78 (C), 135.51 (C), 137.10 (C), 137.92 (C), 147.29 (C), 164.38 (C=O). MS (ESI): $C_{31}H_{36}N_2O$ requires *m*/*z* 452.28, found 453.28 $[M + H]^+$. Anal. calcd for $C_{31}H_{36}N_2O$: C, 82.26; H, 8.02; N, 6.19. Found: C, 82.10; H, 8.00; N, 6.18.

4.1.27. 6-Methyl-N-(*1S*,*2S*,*3S*,*5R*)-(+)-Isopinocampheyl-1-(4-methylbenzyl)-1,4-dihydroindeno[1,2-*b*]pyrrole-3-carboxamide (**2h**)

Compound **2h** was prepared following general procedure I, Method B, by a reaction of acid **14** and (1S,2S,3S,5R)-(+)-isopinocampheyamine. After FC purification (petroleum ether/EtOAc 6/4), carboxamide **2h** was isolated as a white solid (92 mg, 65.50%). M.p.: 225–228 °C; IR (nujol) ν: 1617 (C=O), 3278 (NH); ^{1}H NMR (400 MHz, DMSO) δ: 1.03 (s, 3H), 1,06 (s, 3H), 1.22 (s, 3H), 1.62–1.67 (m, 1H), 1.79 (t, 1H, *J* = 4.8 Hz), 1.90–1.98 (m, 2H), 2.00–2.02 (m, 1H), 2.24 (s, 3H), 2.29 (s, 3H), 2.45–2.31 (m, 2H), 3.58 (d, 2 H, *J* = 4.8 Hz), 4.20–4.34 (m, 1H,) 5.38 (s, 2H), 6.97 (d, 1H, *J* = 7.6 Hz), 7.12 (q, 4H, *J* = 8.0 Hz,), 7.20 (d, 1H, *J* = 7.6 Hz), 7.27 (s, 1H), 7.49 (d, 1H, *J* = 8.4 Hz, NH exch. with D_2O), 7.52 (s, 1H); ^{13}C NMR (100 MHz, DMSO) δ: 20.61 (CH_3), 20.63 (CH_3), 20.93 (CH_3), 23.17 (CH_3), 27.96 (CH_3), 30.73 (CH_2), 33.89 (CH_2), 36.39 (CH_2), 38.31 (C), 41.20 (CH), 43.97 (CH), 46.54 (CH), 47.34 (CH), 51.02 (CH_2), 115.84 (C), 116.28 (CH), 126.19 (CH), 126.50 (CH), 126.66 (CH × 2), 126.73 (CH), 128.14 (C), 129.20 (CH × 2), 131.95 (C), 132.30 (C), 134.99 (C), 136.66 (C), 137.49 (C), 146.91 (C), 163.20 (C=O). MS (ESI): $C_{31}H_{36}N_2O$ requires *m*/*z* 452.28, found 453.28 $[M + H]^+$. Anal. calcd for $C_{31}H_{36}N_2O$: C, 82.26; H, 7.02; N, 6.19. Found: C, 82.01; H, 8.00; N, 6.17.

4.1.28. 6-Methyl-1-(4-Methylbenzyl)-*N*-myrtanyl-1,4-dihydroindeno[1,2-*b*]pyrrole-3-carboxamide (**2i**)

Compound **2i** was prepared following general procedure I, Method B, by a reaction of acid **14** and *cis*-myrtanylamine. After FC purification (petroleum ether/EtOAc 7/3), carboxamide **2i** was isolated as a white solid (119 mg, 85.38%). M.p.: 88–91 °C; IR (nujol) ν: 1623 (C=O), 3268 (NH); ^{1}H NMR (400 MHz, DMSO) δ: 0.81–0.87 (m, 3H), 1.06 (s, 3H), 1.18 (s, 3H), 1.49–1.59 (m, 1H), 1.79–1.97 (m, 4H), 2.24 (s, 3H), 2.28 (s, 3H), 2.30–2.36 (m, 1H), 3.23 (t, 2H, *J* = 6.0 Hz), 3.57 (s, 2H), 5.37 (s, 2H), 6.97 (d, 1H, *J* = 7.6 Hz), 7.11 (q, 4H, *J* = 8.4 Hz), 7.20 (d, 1H, *J* = 7.6 Hz), 7.26 (s, 1H), 7.44 (s, 1H), 7.52 (t, 1H, *J* = 6 Hz, NH exch. with D_2O). ^{13}C NMR (100 MHz, DMSO) δ: 19.64 (CH_2), 21.08 (CH_3), 21.39 (CH_3), 23.38 (CH_3), 26.18 (CH_2), 28.31 (CH_3), 31.17 (CH_2), 33.30 (CH_2), 38.75 (C), 41.32 (CH), 41.41 (CH), 43.63 (CH), 44.51 (CH_2), 51.45 (CH_2), 116.30 (C), 116.78 (CH), 126.64 (CH), 127.17 (CH × 2), 127.20 (CH), 127.23 (CH), 128.03 (C), 129.67 (CH × 2), 132.41 (C), 132.80 (C), 135.40 (C), 137.13 (C), 137.98 (C), 147.28 (C), 164.12 (C=O). MS (ESI): $C_{31}H_{36}N_2O$ requires *m*/*z* 452.28, found 453.28 $[M + H]^+$. Anal. calcd for $C_{31}H_{36}N_2O$: C, 82.26; H, 8.02; N, 6.19. Found: C, 82.10; H, 8.00; N, 6.18.

4.1.29. 6-Methyl-1-Adamantyl-1-(4-methylbenzyl)-1,4-dihydroindeno[1,2-*b*]pyrrole-3-carboxamide (**2j**)

Compound **2j** was prepared following general procedure I, Method B, by a reaction of acid **14** and 1-adamantanamine. After FC purification (petroleum ether/EtOAc 7/3), carboxamide **2j** was isolated as a white solid (114 mg, 81.76%). M.p.: 158–162 °C; IR (nujol) ν: 1770 (C=O), 3336 (NH); ^{1}H NMR (400 MHz, DMSO) δ: 1.65 (s, 6H), 2.06 (s, 9H), 2.27 (s, 3H), 2.32 (s, 3H), 3.55 (s, 2H), 5.36 (s, 2H), 6.65 (s, 1H, NH exch with D_2O), 6.97 (d, 1H, *J* = 7.6 Hz), 7.05–7.10 (m, 4H), 7.19 (d, 1H, *J* = 7.6 Hz), 7.25 (s, 1H), 7.50 (s, 1H); ^{13}C NMR (100 MHz, DMSO) δ: 20.60 (CH_3), 20.91 (CH_3), 28.91 (CH × 3), 30.62 (CH_2), 36.12 (CH_2 × 2), 41.29 (CH_2 × 3), 50.84 (CH_2), 50.97 (C), 116.28 (CH), 116.67 (C), 126.16 (CH), 126.63 (CH × 2), 126.74 (CH), 126.96 (CH), 127.69 (C), 129.16 (CH × 2), 131.96 (C), 132.27 (C), 135.00 (C), 136.62 (C), 137.36 (C), 146.81 (C), 163.28 (C=O). MS (ESI): $C_{31}H_{34}N_2O$ requires *m*/*z* 450.27, found 451.27 $[M + H]^+$. Anal. calcd for $C_{31}H_{34}N_2O$ C, 82.63; H, 7.61; N, 6.22. Found: C, 82.30; H, 7.58; N, 6.20.

4.1.30. 6-Methyl-2-Adamantyl-1-(4-methylbenzyl)-1,4-dihydroindeno[1,2-*b*]pyrrole-3-carboxamide (**2k**)

Compound **2k** was prepared following general procedure I, Method B, by a reaction of acid **14** and 2-adamantanamine. After FC purification (petroleum ether/EtOAc 6/4), carboxamide **2k** was isolated as a white solid (79 mg, 56.52%). M.p.: 178–180 °C; IR (nujol) ν: 1644 (C=O), 3440 (NH); ^{1}H NMR (400 MHz, DMSO) δ: 1.55 (d, 2H, *J* = 12.8 Hz), 1.73 (s, 1H), 1.78–1.84 (m, 5H), 1.94 (s, 1H), 2.05 (d, 2H, *J* = 12.4 Hz), 2.24 (s, 3H), 2.29 (s, 3H), 2.50–2.40 (m, 2H), 3.60 (s, 2H), 4.01–4.03 (m, 1H), 5.38 (s, 2H), 6.97 (t, 2H, *J* = 7.2 Hz), 7.12 (q, 4H, *J* = 8.4 Hz), 7.19 (d, 1H, *J* = 7.6 Hz), 7.28 (s, 1H), 7.44 (s, 1H, NH exch with D_2O), 7.63 (s, 1H); ^{3}C NMR (100 MHz, DMSO) δ: 21.08 (CH_3), 21.41 (CH_3), 27.25 (CH), 27.28 (CH), 31.04 (CH_2), 31.75 (CH_2 × 2), 31.93 (CH × 2), 37.35 (CH_2 × 2), 37.66 (CH_2), 51.47 (CH_2), 53.39 (CH), 116.21 (C), 116.81 (CH), 126.70 (CH), 127.10 (CH × 2), 127.26 (CH), 126.94 (CH), 127.96 (C), 129.64 (CH × 2), 132.43 (C), 132.82 (C), 135.46 (C), 137.11 (C), 137.94 (C), 147.18 (C), 163.69 (C=O). MS (ESI): $C_{31}H_{34}N_2O$ requires *m*/*z* 450.27, found 451.27 $[M + H]^+$. Anal. calcd for $C_{31}H_{34}N_2O$: C, 82.63; H, 7.61; N, 6.22. Found: C, 82.47; H, 7.60; N, 6.21.

4.1.31. 6-Methyl-*N*-((1*S*,2*R*,5*R*)-menthyl)-1-(4-methylbenzyl)-1,4-dihydroindeno[1,2-*b*]pyrrole-3-carboxamide (**2l**)

Compound **2l** was prepared following general procedure I, Method B, by a reaction of acid **14** and (1*S*,2*R*,5*R*)-menthyl-amine. After FC purification (petroleum ether/EtOAc 8/2), carboxamide **2l** was isolated as a white solid (99 mg, 70.43%). M.p.: 179–181 °C; IR (nujol) ν: 1640 (C=O), 3430 (NH); ^{1}H NMR (400 MHz, DMSO) δ: 0.74 (d, 3H, *J* = 6.8 Hz), 0.86–0.89 (m, 6H), 0.90–1.10 (m, 1H), 1.23–1.43 (m, 4H), 1.61–1.71 (m, 2H), 1.77–1.80 (m, 1H), 2.24 (s, 3H), 2.29 (s, 3H), 3.56 (q, 2H, *J* = 21.4 Hz), 3.70–3.78 (m, 1H), 5.37 (s, 2H), 6.97 (d, 2H, *J* = 7.6 Hz), 7.12 (q, 4H, J = 8.4 Hz), 7.19–7.23 (m, 2H), 7.26 (s, 1H, NH exch with D_2O), 7.47 (s, 1H); ^{13}C NMR (100 MHz, DMSO) δ: 16.04 (CH_3), 20.61 (CH_3), 20.92 (CH_3), 21.08 (CH_3), 22.24 (CH_3), 23.52 (CH_2), 26.12 (CH), 30.74 (CH_2), 31.70 (CH), 34.33 (CH_2), 42.66 (CH_2), 46.42 (CH), 48.75 (CH), 50.99 (CH_2), 115.97 (C), 116.28 (CH), 126.17 (CH), 126.47 (CH), 126.51 (CH), 126.72 (CH × 2), 127.84 (C), 129.20 (CH × 2), 131.95 (C), 132.29 (C), 134.95 (C), 136.66 (C), 137.47 (C), 146.88 (C), 162.92 (C=O). MS (ESI): $C_{31}H_{38}N_2O$ requires *m*/*z* 454.30, found 455.30 $[M + H]^+$. Anal. calcd for $C_{31}H_{38}N_2O$: C, 81.89; H, 8.42; N, 6.16. Found: C, 81.73; H, 8.40; N, 6.15.

4.2. Biology

4.2.1. Cell Culture

Huh-7 [17], and Vero E6 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Waltham, MA, USA), 1 mM glutamine, 1 mM sodium pyruvate, 7% fetal bovine serum (FBS, Gibco). Vero E6 TMPRSS2 (Vero TMPRSS) [18] were cultured like VeroE6 cells, with 1mg/mL of G418 (Merck) added once a month. All cells were cultured without antibiotics and checked for *Mycoplasma* as described [19].

4.2.2. Compounds Preparation and Storage

All compounds were stored at 10mM in DMSO at room temperature for 1 month. For longer storage, aliquots were kept at −20 °C.

4.2.3. Viruses

ZIKV, strain Brazil/2016/INMI1 ($ZIKV^{Br}$) ("Lazzaro Spallanzani"—National Institute for Infectious Diseases, Rome, Italy) was propagated on Huh-7 cells. SARS-CoV-2, strain VR PV10734 (SARS-CoV2-Mi, GISAID EPI_ISL_2544194) (Università San Raffaele, Milan, Italy) was propagated on Vero-TMPRSS cells. SARS-CoV-2 and all related experiments were conducted in a biosafety level 3 (BSL-3) laboratory available at AOUP, University Hospital of Pisa.

4.2.4. Determination of Compound Cytotoxicity and Cytotoxic Concentration 50 (CC_{50})

Cytotoxic effects on uninfected cells were evaluated using the Orangu WST-8 assay (Cell Guidance Systems, Cambridge, UK) and the crystal violet assay [20]. Cells were seeded in 96-well plates and treated with three-fold serial dilutions of the compounds (from 0.25 to 180 μM). Huh-7 and Vero TMPRSS cells were seeded in preparation for antiviral assays. The former were seeded at 1 × 10^4 cells/well and incubated for 48 h, whereas Vero TMPRSS cells were seeded at 1.2 × 10^4 cells/well and incubated for 24 h to match the specific conditions, in terms of timing and culture medium, required for their antiviral assays. After the incubation, the medium was replaced with a 10% WST-8 solution in DMEM and incubated for 1 h at 37 °C. Then, optical density was measured at 450 nm with a Varioskan® LUX multimode microplate reader (ThermoFisher Scientific®, Milan, Italy). Subsequently, cells were fixed with 4% buffered formalin solution (Merck®, Darmstadt, Germany), stained with 1% crystal violet (Merck®, Darmstadt, Germany), and the dye was solubilized in 30% acetic acid for 30 min under shaking. Optical density was then

measured at 595 nm to confirm experimental outcomes. The percentage of viability at each concentration was calculated using the formula:

$$\% \text{ viability} = 100 \times (OD_{compound}/OD_{untreated\ control})$$

where $OD_{compound}$ and $OD_{untreated}$ are the OD of the cells treated with compound or DMSO, respectively.

The 50% cytotoxic concentration (CC_{50}) value was calculated using nonlinear regression analysis on GraphPad Prism 7 (San Diego, CA, USA).

4.2.5. Determination of Viral Yield Reduction and Effective Concentration 50 (EC_{50})

Viral yield reduction assays for ZIKV and SARS-CoV-2 were performed as previously described [21]. Briefly, 1×10^4 Huh-7 cells/well and 1.2×10^4 Vero TMPRSS cells/well were infected with ZIKV (MOI 1) or SARS-CoV-2 (MOI 0.05) in a primary 96-well plate for 2 h at 37 °C, 5% CO_2. Following inoculum removal, cells were treated with serial dilutions of the compounds (from 0.5 to 50 µM for initial screening; from 0.12 to 180 µM for EC_{50} determination of the most promising compounds) in DMEM with 2% FBS. Supernatants were collected after 48 h (Huh-7) or 24 h (VERO TMPRSS), diluted three-fold, and added onto VERO E6 or VERO TMPRSS monolayers, respectively. After 2 h at 37 °C, the inoculum was replaced with 0.75% carboxymethyl cellulose (Merck®, Darmstadt, Germany) in DMEM 2% FBS. After 48–72 h, cells were then fixed with 4% buffered formalin solution (Merck®, Darmstadt, Germany) and stained with 1% crystal violet (Merck®, Darmstadt, Germany). Titers and percentage of inhibition were calculated as follows:

$$PFU/mL = (\text{number of plaques}*3\hat{}n)/\text{vol infection}$$

where n is the dilution where plaques were counted:

$$\% \text{ inhibition} = 100 \times (1 - \text{Titer-compound}/\text{Titer-untreated control})$$

where Titer-compound and Titer-untreated control are the titers (PFU/mL) obtained on cells treated with compound or DMSO, respectively.

The EC_{50} value was determined by nonlinear regression analysis on GraphPad Prism (San Diego, CA, USA). Selectivity index (SI) was obtained by dividing CC_{50} by EC_{50} for each drug/virus/cell line. Compounds with SI values ≥ 10 were active in vitro.

4.2.6. Western Blot Analysis

Immunoblotting was performed as described [22]. Briefly, cells were washed in phosphate-buffered saline (PBS) and lysed using RIPA lysis buffer added with protease and phosphatase inhibitors (ThermoFisher Scientific, Milano, Italy). Lysates were obtained in loading buffer, subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 12% PAGE gels, and transferred onto nitrocellulose (Millipore). The antibodies used (Table 3) were diluted in PBS, 5% Skim milk, 0.1% Tween 20.

Table 3. Antibodies used for Western blot.

Antigen	Host *Species*	Manufacturer	Use	Cat. Number	Dilution
ZIKV NS5	rabbit	GeneTex	WB	GTX 133328	1:1000
Nucleocapsid SARS-CoV-2	rabbit	Sino Biological	WB	40588-RC02	1:1000
GAPDH	mouse	Invitrogen	WB	MA5-15738	1:1000
rabbit IgG	Goat, HRPO	Merck	WB	A0545	1:20.000
mouse IgG	Rabbit, HRPO	Merck	WB	A9044	1:20.000

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijms27052306/s1.

Author Contributions: Conceptualization, G.M.; Data curation, P.C., B.A., G.L. and A.C.; Formal analysis, E.P. and C.D.M.; Methodology, E.P. and C.D.M.; Investigation, P.C., B.A., I.L. and S.P. Project administration, G.M.; Writing—original draft preparation, G.M. and E.P.; Writing—review and editing, G.M. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The original contributions presented in this study are included in the article/Supplementary Materials. Further inquiries can be directed to the corresponding author.

Conflicts of Interest: The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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