

Article

Pyrrolizine- and Indolizine-Derived Spirooxindoles: Synthesis, Antibacterial Activity and Inverse Docking Analysis

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Abstract: Spirooxindoles are a family of heterocyclic compounds which bear the oxindole nucleus in their structures, which have a considerable pharmaceutical potential and which have been linked to various drugs for the treatment of diverse diseases. In this work, a wide variety of spirooxindoles bearing a pyrrolizinic nucleus were obtained by a 1,3-dipolar cycloaddition reaction between substituted isatins, *trans*-3-benzoyl acrylic acid and L-proline. In this approach, the target products **9a–m** were obtained in 40–86% yields under heating to reflux in methanol over 2 h. Similarly, spirooxindoles containing an indolizinic nucleus **11a–j** were obtained in 45–69% yields by switching L-proline for pipercolic acid under heating to reflux in acetonitrile for 8 h. The antibacterial activity of the obtained products was evaluated against *P. aeruginosa*, *K. pneumoniae*, *E. coli*, *S. aureus*, and *N. gonorrhoeae*, also including an inverse docking analysis. Results show that **9f** and **11i**, were the most active compounds against *S. aureus*, while compounds **9d** and **9m** displayed the higher activity against *N. gonorrhoeae*. Inverse docking analysis showed that compounds **9b**, **11a** **11e**, and **11i** displayed high affinity to the target protein 6TYM and 7Q6S, which are involved in biological pathways of diverse cancer and Parkinson diseases.

Keywords: spirooxindoles; 1,3-dipolar cycloaddition; isatin; antibacterial activity; inverse docking analysis



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1. Introduction

Spirooxindoles are a family of compounds that have two important characteristics: they have a spiro carbon that gives them structural rigidity and an oxindole nucleus associated with their pharmacophoric properties. In this sense, spirooxindoles are widely distributed in nature (Figure 1a) [1], like the alkaloids elacomine [2,3] and horsfiline [4]; spirotryprostatin A and B, with inhibitory activity of the cellular cycle [5]; and rhynchophylline, with antihypertensive, hypotensive and neuroprotective properties [6–9].

Considering the pharmaceutical potential, and their ability to act as protein inhibitors that have been linked to various diseases, representing a growing area of research [10–13], these compounds could pave the way for novel therapeutic approaches and drug developments. Some outstanding examples are shown in Figure 1b: **MI-888** is a potent antitumor agent [14], the compound **1** is a HIV-1 reverse transcriptase inhibitor [15], and compound **2**

is active against the bacterium *Mycobacterium tuberculosis* (MTB) with an MIC of 2.8 μM [16]. On the other hand, compound **3** is an acetylcholinesterase inhibitor (AChE) with an IC_{50} of 0.11 μM [17] and the spirooxindole **4** [18] exhibited anticancer activity against cell lines of prostate cancer DU145 (IC_{50} : 8.02 μM), esophageal cancer line EC109 (IC_{50} : 6.62 μM), human gastric cancer cell line MGC803 (IC_{50} : 9.49 μM), and breast adenocarcinoma line MCF-7 (IC_{50} : 17.65 μM). Additionally, compound **5** displayed antibacterial activity against *Bacillus subtilis* and *Staphylococcus aureus* with an MIC of 0.291 μM (for both strains), and against *Pseudomonas aeruginosa*, *Salmonella typhimurium* and *Proteus vulgaris* with an MIC of 0.146 μM (for the three strains) [19].

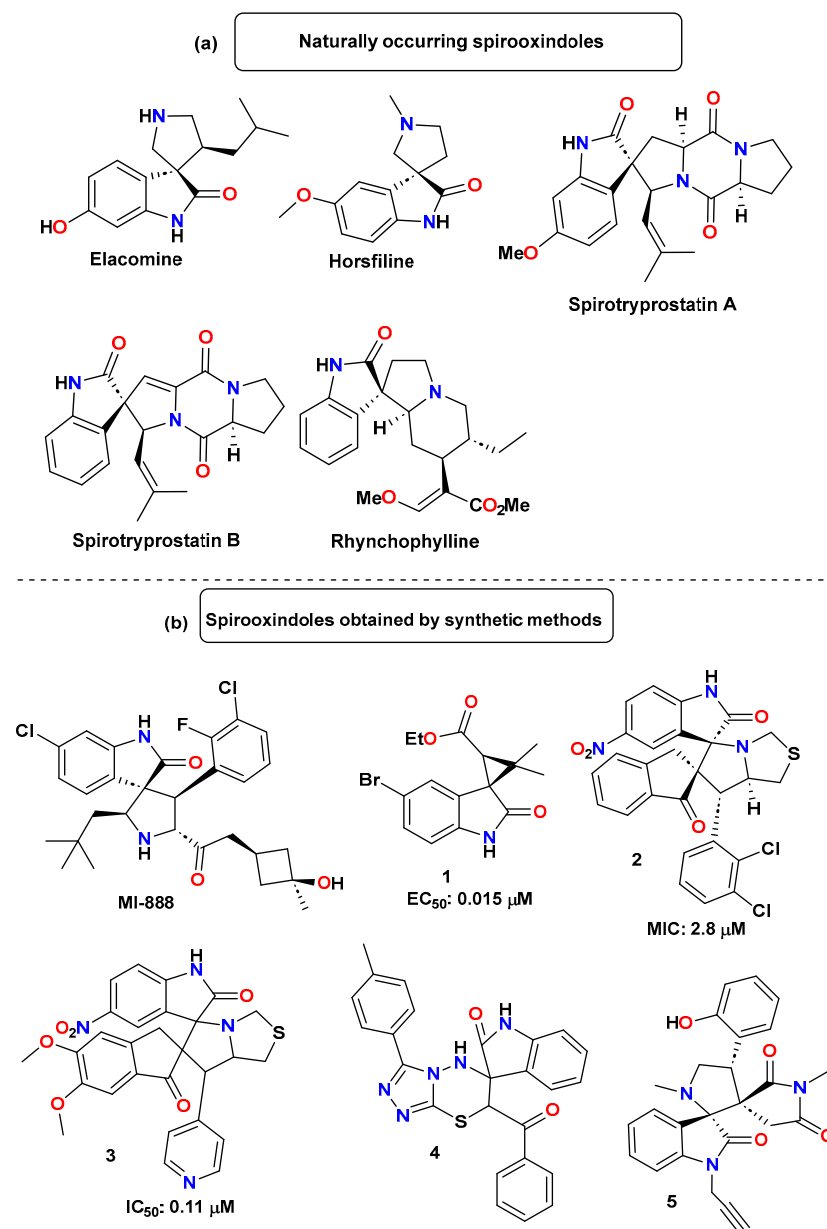


Figure 1. (a) Spirooxindole derivatives found in nature. (b) Pharmacologically active spirooxindole derivatives obtained by synthetic methods.

On the other hand, isatin is used as a building block for spirooxindoles, usually through multicomponent reactions such as cyclocondensation [20–25], and 1,3-dipolar cycloaddition [26–35], with the latter allowing one to obtain stereospecific products with high structural complexity. A fundamental feature of this process is the generation of azomethine ylides, as discovered by Grigg and co., using secondary amino acids and

carbonylic compounds. In this approach, the azomethine ylide is formed *in situ* after NH to C=O addition, condensation/dehydration and a decarboxylation process of the CO₂H functionality coming from the amino acid reagent. Finally, the azomethine ylide-formed (1,3-dipole species) reacts with different dipolarophiles to achieve highly substituted cycloaddition products [36,37].

For instance, in this research work we aimed to use this tricomponent methodology as a key strategy for creating new spirooxindoles with potential biological activity using substituted isatins, *trans*-3-benzoyl acrylic acid and two amino acids (i.e., L-proline and pipecolic acid) as starting materials, followed by the evaluation, both *in vivo* and *in silico*, of the antibacterial and toxicological properties of the obtained compounds, as well as their inverse docking analysis.

2. Materials and Methods

2.1. Chemistry

The reagents and solvents in this work were obtained from commercial sources such as Aldrich (St. Louis, MO, USA), Alfa Aesar (Haverhill, MA, USA), Merck (St. Louis, MO, USA), etc., the reactions were followed by thin layer chromatography (TLC) on silica gel plates 60GF254 (Merck, St. Louis, MO, USA). The melting points were measured using a Stuart SMP3 melting point apparatus (Cole-Parmer Instrument Company LLC, Burlington, MA, USA) and are uncorrected. FT-IR spectra were taken on a SHIMADZU IRAffinity-1 spectrophotometer (Shimadzu, Kyoto, Japan) with an ATR probe. ¹H and ¹³C NMR spectra were measured with a BRUKER DPX 400 spectrophotometer (Bruker, Bruker BioSpin GmbH, Rheinstetten, Germany) operating at 400 and 100 MHz, respectively, using dimethylsulfoxide-*d*₆ and chloroform-*d* as solvents. Mass spectra were obtained on a SHIMADZU-GCMS-QP2010 spectrometer (Shimadzu, Kyoto, Japan) operating between 20 and 70 eV. Microanalysis was performed on a Thermo Electron Flash EA 1112 analyzer (Thermo Fisher Scientific, Waltham, MA, USA).

2.1.1. General Procedure for the Synthesis of Spirooxindoles 9a–m

Equimolar amounts of the corresponding substituted isatins **6**, L-proline **7a** and *trans*-3-benzoyl acrylic acid **8** were dissolved in 10 mL of MeOH in a flat bottom flask and subjected to reflux for 2 h (TLC control). The solid formed was isolated by filtration on vacuum and washed with cold MeOH. The products did not require further purification.

(1'*RS*,2'*RS*,3*SR*,7*a'**SR*)-2'-Benzoyl-1-benzyl-2-oxo-1',2',5',6',7',7*a'*-hexahydrospiro[indoline-3,3'-pyrrolizine]-1'-carboxylic acid **9a**: White solid. Yield: 61%. M.p.: 228–229 °C (Decomp). FT-IR (ATR) ν (cm⁻¹): 2957, 2864, 1726, 1678, 1697. ¹H NMR (400 MHz, DMSO-*d*₆) δ (ppm): 1.67–1.79 (m, 1 H, H-6'), 1.80–1.94 (m, 2 H, H-6', H-7'), 2.01–2.13 (m, 1 H, H-7'), 2.24–2.34 (m, 1 H, H-5'), 2.34–2.44 (m, 1 H, H-5'), 3.62 (t, *J* = 10.4 Hz, 1 H, H-1'), 3.92–3.97 (m, 1 H, H-7*a'*), 4.22 (d, *J* = 15.8 Hz, 1 H, N-CH), 4.73 (d, *J* = 15.8 Hz, 1 H, N-CH), 4.87 (d, *J* = 11.3 Hz, 1 H, H-2'), 6.50 (d, *J* = 7.8 Hz, 1 H), 6.91–6.98 (m, 1 H), 7.00–7.11 (m, 3 H), 7.15 (d, *J* = 7.4 Hz, 1 H), 7.18–7.26 (m, 3 H), 7.30 (d, *J* = 3.9 Hz, 4 H), 7.52 (br. s., 1 H), 12.71 (br.s., COOH). ¹³C NMR (100 MHz, DMSO-*d*₆) δ (ppm): 27.4 (C-6'), 31.3 (C-7'), 43.2 (N-CH₂), 47.5 (C-5'), 51.4 (C-1'), 59.9 (C-2'), 67.4 (C-7*a'*), 71.7 (C-spiro), 109.6 (CH), 122.5 (CH), 124.4 (C), 127.3 (CH), 127.4 (CH), 127.8 (CH), 127.9 (CH), 128.8 (CH), 129.1 (CH), 129.9 (CH), 133.7 (CH), 136.3 (C), 136.8 (C), 142.7 (C), 173.4 (C), 178.0 (C), 197.2 (C). MS (EI, 70 eV) *m/z*: 466 (M⁺•, 2), 430 (5), 404 (66), 375 (11), 339 (28), 290 (78), 223 (25), 91 (100). Anal. Calcd. for C₂₉H₂₆N₂O₄: C: 74.66; H: 5.62; N: 6.00. Found: C: 74.54; H: 5.61; N: 6.02.

(1'*RS*,2'*RS*,3*SR*,7*a'**SR*)-2'-Benzoyl-1-methyl-2-oxo-1',2',5',6',7',7*a'*-hexahydrospiro[indoline-3,3'-pyrrolizine]-1'-carboxylic acid **9b**: White solid. Yield: 44%. M.p.: 214–216 °C (Decomp). FT-IR (ATR) ν (cm⁻¹): 3071, 2978, 2936, 2864, 1730, 1672. ¹H NMR (400 MHz, DMSO-*d*₆)

δ (ppm): 1.65–1.78 (m, 1 H, H-6'), 1.79–1.93 (m, 2 H, H-6', H-7'), 2.00–2.10 (m, 1 H, H-7'), 2.23–2.30 (m, 1 H, H-5'), 2.35–2.44 (m, 1 H, H-5'), 2.64 (s, 3 H, N-CH₃), 3.51 (t, J = 10.5 Hz, 1 H, H-1'), 3.88 (dt, J = 9.6, 6.1 Hz, 1 H, H-7a'), 4.73 (d, J = 11.3 Hz, 1 H, H-2'), 6.56 (d, J = 7.8 Hz, 1 H), 6.94–7.02 (m, 1 H), 7.07–7.20 (m, 4 H), 7.28 (t, J = 7.6 Hz, 2 H), 7.49 (t, J = 7.3 Hz, 1 H), 12.65 (br. s., 1 H, COOH). ¹³C NMR (100 MHz, DMSO-*d*₆) δ (ppm): 26.3 (CH₃), 27.5 (C-6'), 31.4 (C-7'), 47.7 (C-5'), 50.9 (C-1'), 61.2 (C-2'), 67.6 (C-7a'), 71.8 (C-spiro), 108.9 (CH), 122.4 (CH), 124.3 (C), 126.9 (CH), 127.6 (CH), 128.6 (CH), 130.0 (CH), 133.3 (CH), 136.9 (C), 143.5 (C), 173.4 (C), 177.8 (C), 197.5 (C). MS (EI, 70 eV) m/z : 390 (M⁺•, 2), 354 (7), 299 (46), 263 (31), 241 (44), 214 (100). Anal. Calcd. for C₂₃H₂₂N₂O₄: C: 70.75; H: 5.68; N: 7.18. Found: C: 70.82; H: 5.71; N: 7.14.

(1'*RS*,2'*RS*,3*SR*,7a'*SR*)-2'-Benzoyl-2-oxo-1',2',5',6',7',7a'-hexahydrospiro[indoline-3,3'-pyrrolizine]-1'-carboxylic acid **9c**: White solid. Yield: 72%. M.p.: 211–212 °C (Decomp). FT-IR (ATR) ν (cm⁻¹): 3310, 2874, 1699, 1678. ¹H NMR (400 MHz, DMSO-*d*₆) δ (ppm): 1.64–1.76 (m, 1 H, H-6'), 1.78–1.91 (m, 2 H, H-7', H-6'), 1.99–2.09 (m, 1 H, H-7'), 2.24–2.31 (m, 1 H, H-5'), 2.42 (q, J = 8.3, 7.4 Hz, 1 H, H-5'), 3.56 (t, J = 10.5 Hz, 1 H, H-1'), 3.88 (dt, J = 9.7, 6.2 Hz, 1 H, H-7a'), 4.77 (d, J = 11.3 Hz, 1 H, H-2'), 6.46 (d, J = 8.0 Hz, 1 H), 6.87 (t, J = 7.5 Hz, 1 H), 7.01–7.10 (m, 2 H), 7.26–7.39 (m, 4 H), 7.49 (t, J = 6.8 Hz, 1 H), 10.17 (s, 1 H, NH), 12.63 (s, 1 H, COOH). ¹³C NMR (100 MHz, DMSO-*d*₆) δ (ppm): 27.2 (C-6'), 31.2 (C-7'), 47.6 (C-5'), 51.4 (C-1'), 59.7 (C-2'), 67.3 (C-7a'), 72.0 (C-spiro), 110.1 (CH), 121.6 (CH), 124.9 (C), 127.6 (CH), 127.9 (CH), 128.8 (CH), 129.8 (CH), 133.6 (CH), 136.9 (C), 142.3 (C), 173.5 (C), 179.5 (C), 197.4 (C). MS (EI, 70 eV) m/z : 376 (M⁺•, 1), 314 (100), 285 (97), 257 (20), 200 (30), 105 (74), 77 (49). Anal. Calcd. for C₂₂H₂₀N₂O₄: C: 70.20; H: 5.36; N: 7.44. Found: C: 69.99; H: 5.32; N: 7.41.

(1'*RS*,2'*RS*,3*SR*,7a'*SR*)-2'-Benzoyl-1-hexyl-2-oxo-1',2',5',6',7',7a'-hexahydrospiro[indoline-3,3'-pyrrolizine]-1'-carboxylic acid **9d** [38]: White solid. Yield: 60%. M.p.: 196–197 °C. FT-IR (ATR) ν (cm⁻¹): 2957, 2932, 2866, 1738, 1676, 1604. ¹H NMR (400 MHz, DMSO-*d*₆) δ (ppm): 0.81 (t, J = 6.7 Hz, 3 H, CH₃), 1.03–1.24 (m, 9 H), 1.65–1.78 (m, 1 H), 1.79–1.92 (m, 2 H), 1.99–2.10 (m, 1 H), 2.24 (ddd, J = 10.0, 6.8, 4.1 Hz, 1 H), 2.36 (td, J = 8.7, 6.3 Hz, 1 H), 3.07 (dt, J = 13.6, 6.1 Hz, 1 H), 3.57 (dd, J = 11.1, 10.0 Hz, 1 H, H-1'), 3.89 (dt, J = 9.7, 6.2 Hz, 1 H, H-7a'), 4.79 (d, J = 11.3 Hz, 1 H, H-2'), 6.65 (d, J = 7.8 Hz, 1 H), 6.96 (t, J = 7.5 Hz, 1 H), 7.10–7.19 (m, 2 H), 7.22 (d, J = 7.4 Hz, 2 H), 7.29 (t, J = 7.7 Hz, 2 H), 7.47 (t, J = 7.4 Hz, 1 H), 12.66 (br. s., 1 H, COOH). ¹³C NMR (100 MHz, DMSO-*d*₆) δ (ppm): 14.3 (CH₃), 22.4 (CH₂), 26.2 (CH₂), 26.8 (CH₂), 27.5 (C-6'), 31.3 (CH₂), 31.4 (C-7'), 39.7 (CH₂), 47.4 (C-5'), 51.2 (C-1'), 60.1 (C-2'), 67.5 (C-7a'), 71.6 (C-spiro), 109.1 (CH), 122.1 (CH), 124.4 (C), 127.3 (CH), 127.8 (CH), 128.7 (CH), 130.0 (CH), 133.5 (CH), 137.0 (C), 143.0 (C), 173.4 (C), 177.5 (C), 197.3 (C). MS (EI, 70 eV) m/z : 460 (M⁺•, 3), 398 (100), 369 (12), 333 (18), 284 (44), 105 (40), 77 (27). Anal. Calcd. for C₂₈H₃₂N₂O₄: C: 73.02; H: 7.00; N: 6.10. Found: C: 73.10; H: 7.10; N: 6.20.

(1'*RS*,2'*RS*,3*SR*,7a'*SR*)-2'-Benzoyl-5-chloro-1-methyl-2-oxo-1',2',5',6',7',7a'-hexahydrospiro[indoline-3,3'-pyrrolizine]-1'-carboxylic acid **9e**: White solid. Yield: 58%. M.p.: 218–219 °C (Decomp.). FT-IR (ATR) ν (cm⁻¹): 3404, 3053, 1698, 1553. ¹H NMR (400 MHz, DMSO-*d*₆) δ (ppm): 1.67–1.79 (m, 1 H, H-6'), 1.82–1.97 (m, 2 H, H-6', H-7'), 1.98–2.08 (m, 1 H, H-7'), 2.23–2.32 (m, 1 H, H-5'), 2.36–2.44 (m, 1 H, H-5'), 2.64 (s, 3 H, N-CH₃), 3.56 (t, J = 10.6 Hz, 1 H, H-1'), 3.88 (dt, J = 9.7, 6.3 Hz, 1 H, H-7a'), 4.74 (d, J = 11.5 Hz, 1 H, H-2'), 6.60 (d, J = 8.4 Hz, 1 H), 7.14–7.19 (m, 3 H), 7.24 (dd, J = 8.4, 2.0 Hz, 1 H), 7.31 (t, J = 7.7 Hz, 2 H), 7.46–7.52 (m, 1 H). ¹³C NMR (100 MHz, DMSO-*d*₆) δ (ppm): 26.5 (N-CH₃), 27.7 (C-6'), 31.1 (C-7'), 47.6 (C-5'), 50.7 (C-1'), 61.3 (C-2'), 67.7 (C-7a'), 71.8 (C-spiro), 110.4 (CH), 126.3 (C), 126.8 (CH), 126.8 (C), 127.6 (CH), 128.8 (CH), 129.9 (CH), 133.6 (CH), 136.8 (C), 142.4 (C), 173.3 (C), 177.5 (C), 197.4 (C). MS (EI, 70 eV) m/z : 424 (M⁺•, 3), 362 (100), 313 (4), 297 (11), 248 (49), 105 (92), 77 (89). Anal. Calcd. for C₂₃H₂₁ClN₂O₄: C: 65.02; H: 4.98; N: 6.59. Found: C: 65.08; H: 4.96; N: 6.57.

(1'*RS*,2'*RS*,3*SR*,7*a'**SR*)-2'-Benzoyl-1-benzyl-5-chloro-2-oxo-1',2',5',6',7',7*a'*-hexahydrospiro[indoline-3,3'-pyrrolizine]-1'-carboxylic acid **9f**: White solid. Yield: 41%. M.p.: 222–224 °C. FT-IR (ATR) ν (cm⁻¹): 3169, 2860, 1662, 1585. ¹H NMR (400 MHz, DMSO-*d*₆) δ (ppm): 1.68–1.83 (m, 1 H), 1.83–1.98 (m, 2 H), 2.00–2.11 (m, 1 H), 2.26–2.35 (m, 1 H), 2.35–2.44 (m, 1 H), 3.67 (t, *J* = 10.4 Hz, 1 H, H-1'), 3.94 (dt, *J* = 9.6, 6.3 Hz, 1 H, H-7*a'*), 4.27 (d, *J* = 15.8 Hz, 1 H, N-CH), 4.72 (d, *J* = 15.8 Hz, 1 H, N-CH), 4.89 (d, *J* = 11.3 Hz, 1 H, H-2'), 6.54 (d, *J* = 8.4 Hz, 1 H), 6.97–7.04 (m, 2 H), 7.17 (d, *J* = 8.4 Hz, 1 H), 7.19–7.25 (m, 4 H), 7.30–7.38 (m, 4 H), 7.51–7.59 (m, 1 H). ¹³C NMR (100 MHz, DMSO-*d*₆) δ (ppm): 27.7 (C-6'), 31.0 (C-7'), 43.3 (N-CH₂), 47.4 (C-5'), 51.2 (C-1'), 60.0 (C-2'), 67.5 (C-7*a'*), 71.6 (C-spiro), 111.1 (CH), 126.5 (C), 126.9 (C), 127.3 (CHx2), 127.9 (CH), 129.0 (CH), 129.1 (CH), 129.9 (CH), 134.0 (CH), 136.0 (C), 136.6 (C), 141.6 (C), 173.3 (C), 177.7 (C), 197.1 (C). MS (EI, 70 eV) *m/z*: 500 (M⁺•, 1), 438 (30), 387 (9), 347 (15), 324 (48), 257 (16), 105 (86), 91 (100). Anal. Calcd. for C₂₉H₂₅ClN₂O₄: C: 69.53; H: 5.03; N: 5.59. Found: C: 69.48; H: 5.04; N: 5.62.

(1'*RS*,2'*RS*,3*SR*,7*a'**SR*)-2'-Benzoyl-5,7-dichloro-2-oxo-1',2',5',6',7',7*a'*-hexahydrospiro[indoline-3,3'-pyrrolizine]-1'-carboxylic acid **9g**: White solid. Yield: 58%. M.p.: 228–229 °C. FT-IR (ATR) ν (cm⁻¹): 2956, 2949, 2855, 1682, 1584, 1510. ¹H NMR (400 MHz, DMSO-*d*₆) δ (ppm): 1.68–1.81 (m, 1 H, H-6'), 1.81–1.96 (m, 2 H, H-6', H-7'), 1.96–2.07 (m, 1 H, H-7'), 2.28–2.36 (m, 1 H, H-5'), 2.36–2.45 (m, 1 H, H-5'), 3.60 (t, *J* = 10.5 Hz, 1 H, H-1'), 3.87 (dt, *J* = 9.6, 6.3 Hz, 1 H, H-7*a'*), 4.79 (d, *J* = 11.5 Hz, 1 H, H-2'), 7.15 (s, 1 H), 7.30–7.39 (m, 5 H), 7.54 (t, *J* = 6.9 Hz, 1 H), 10.76 (s, 1 H, NH). ¹³C NMR (100 MHz, DMSO-*d*₆) δ (ppm): 27.7 (C-6'), 30.9 (C-7'), 47.4 (C-5'), 50.9 (C-1'), 60.7 (C-2'), 67.6 (C-7*a'*), 72.6 (C-spiro), 115.3 (C), 126.1 (CH), 126.5 (C), 127.7 (CH), 128.3 (C), 129.0 (CH), 129.3 (CH), 133.8 (CH), 136.8 (C), 139.2 (C), 173.2 (C), 179.3 (C), 197.4 (C). MS (EI, 70 eV) *m/z*: 444 (M⁺•, 2), 382 (100), 353 (87), 325 (12), 268 (16), 105 (53), 77 (53). Anal. Calcd. for C₂₂H₁₈Cl₂N₂O₄: C: 59.34; H: 4.07; N: 6.29. Found: C: 59.39; H: 4.08; N: 6.31.

(1'*RS*,2'*RS*,3*SR*,7*a'**SR*)-2'-Benzoyl-5-methyl-2-oxo-1',2',5',6',7',7*a'*-hexahydrospiro[indoline-3,3'-pyrrolizine]-1'-carboxylic acid **9h**: White solid. Yield: 49%. M.p.: 222–224 °C. FT-IR (ATR) ν (cm⁻¹): 2956, 2939, 2854, 1683, 1583, 1510. ¹H NMR (400 MHz, DMSO-*d*₆) δ (ppm): 1.64–1.77 (m, 1 H), 1.78–1.93 (m, 2 H), 1.98–2.09 (m, 1 H), 2.21 (s, 3 H, 5-CH₃), 2.23–2.31 (m, 1 H), 2.38–2.48 (m, 1 H), 3.56 (t, *J* = 10.5 Hz, 1 H, H-1'), 3.87 (dt, *J* = 9.9, 6.0 Hz, 1 H, H-7*a'*), 4.75 (d, *J* = 11.3 Hz, 1 H, H-2'), 6.35 (d, *J* = 7.7 Hz, 1 H), 6.86 (d, *J* = 9.4 Hz, 2 H), 7.27–7.39 (m, 4 H), 7.49 (t, *J* = 7.1 Hz, 1 H), 10.06 (s, 1 H, NH), 12.62 (s, 1 H, COOH). ¹³C NMR (100 MHz, DMSO-*d*₆) δ (ppm): 21.2 (5-CH₃), 27.3 (C-7'), 31.1 (C-6'), 47.6 (C-5'), 51.4 (C-1'), 59.7 (C-2'), 67.3 (C-7*a'*), 72.1 (C-spiro), 109.8 (CH), 125.0 (C), 127.9 (CH), 128.0 (CH), 128.8 (CH), 130.0 (CH), 130.3 (C), 133.5 (CH), 137.0 (C), 139.9 (C), 173.6 (C), 179.4 (C), 197.5 (C). MS (EI, 70 eV) *m/z*: 368 (4), 328 (100), 299 (92), 277 (17), 223 (14), 105 (43), 77 (30). Anal. Calcd. for C₂₃H₂₂N₂O₄: C: 70.75; H: 5.68; N: 7.18. Found: C: 70.87; H: 5.65; N: 7.14.

(1'*RS*,2'*RS*,3*SR*,7*a'**SR*)-2'-Benzoyl-5-nitro-2-oxo-1',2',5',6',7',7*a'*-hexahydrospiro[indoline-3,3'-pyrrolizine]-1'-carboxylic acid **9i**: White solid. Yield: 40%. M.p.: 161–162 °C. FT-IR (ATR) ν (cm⁻¹): 3595, 3234, 1734, 1678, 1520. ¹H NMR (400 MHz, DMSO-*d*₆) δ (ppm): 1.68–1.82 (m, 1 H, H-6'), 1.82–1.96 (m, 2 H, H-6', H-7'), 2.01–2.13 (m, 1 H, H-7'), 2.29–2.37 (m, 1 H, H-5'), 2.41–2.48 (m, 1 H, H-5'), 3.63 (t, *J* = 10.5 Hz, 1 H, H-1'), 3.94 (dt, *J* = 9.8, 6.2 Hz, 1 H, H-7*a'*), 4.85 (d, *J* = 11.1 Hz, 1 H, H-2'), 6.69 (d, *J* = 8.6 Hz, 1 H), 7.30–7.40 (m, 4 H), 7.49–7.56 (m, 1 H), 7.83 (d, *J* = 2.2 Hz, 1 H), 8.06 (dd, *J* = 8.8, 2.2 Hz, 1 H), 10.98 (br. s., 1 H, NH). ¹³C NMR (100 MHz, DMSO-*d*₆, DEPT-135 experiment) δ (ppm): 27.4 (C-6'), 30.9 (C-7'), 47.7 (C-5'), 51.4 (C-1'), 59.9 (C-2'), 67.4 (C-7*a'*), 110.5 (CH), 122.7 (CH), 127.2 (CH), 127.9 (CH), 129.1 (CH), 134.1 (CH). MS (EI, 70 eV) *m/z*: 359 (100), 330 (70), 284 (22), 254 (10), 105 (24), 77 (18). Anal. Calcd. for C₂₂H₁₉N₃O₆: C: 62.70; H: 4.54; N: 9.97. Found: C: 62.77; H: 4.51; N: 9.92. The ¹³C spectrum could not be recorded due to solubility problems.

(1'*RS*,2'*RS*,3*SR*,7*a'**SR*)-2'-Benzoyl-1-benzyl-5,7-dichloro-2-oxo-1',2',5',6',7',7*a'*-hexahydrospiro[indoline-3,3'-pyrrolizine]-1'-carboxylic acid **9j**: White solid. Yield: 86%. M.p.: 226–227 °C. FT-IR (ATR) ν (cm⁻¹): 2968, 2872, 1728, 1705, 1676. ¹H NMR (400 MHz, DMSO-*d*₆) δ (ppm): 1.74–1.87 (m, 1 H, H-6'), 1.88–2.00 (m, 2 H, H-6', H-7'), 2.00–2.10 (m, 1 H, H-7'), 2.32–2.40 (m, 1 H, H-5'), 2.41–2.48 (m, 1 H, H-5'), 3.68 (t, *J* = 10.5 Hz, 1 H, H-1'), 3.93 (dt, *J* = 9.5, 6.5 Hz, 1 H, H-7*a'*), 4.60 (d, *J* = 16.8 Hz, 1 H, N-CH), 4.90 (d, *J* = 11.3 Hz, 1 H, H-2'), 4.99 (d, *J* = 16.6 Hz, 1 H, N-CH), 6.91 (d, *J* = 7.2 Hz, 2 H), 7.16–7.26 (m, 3 H), 7.27–7.33 (m, 4 H), 7.41 (t, *J* = 7.6 Hz, 2 H), 7.61 (t, *J* = 7.3 Hz, 1 H), 12.75 (br. s., 1 H, COOH). ¹³C NMR (100 MHz, DMSO-*d*₆) δ (ppm): 28.1 (C-6'), 30.9 (C-7'), 44.7 (N-CH₂), 47.1 (C-5'), 50.6 (C-1'), 61.4 (C-2'), 67.8 (C-7*a'*), 71.0 (C-spiro), 115.6 (C), 126.0 (CH), 126.4 (CH), 127.5 (CH), 127.7 (C), 127.7 (CH), 129.0 (CH), 129.1 (CH), 129.3 (C), 131.5 (CH), 134.0 (CH), 136.9 (C), 137.4 (C), 137.7 (C), 173.1 (C), 178.8 (C), 197.3 (C). MS (EI, 70 eV) *m/z*: 368 (2), 313 (3), 236 (6), 83 (52), 69 (100). Anal. Calcd. for C₂₉H₂₄Cl₂N₂O₄: C: 65.06; H: 4.52; N: 5.23. Found: C: 65.22; H: 4.54; N: 5.21.

(1'*RS*,2'*RS*,3*SR*,7*a'**SR*)-2'-Benzoyl-1-benzyl-5-fluoro-2-oxo-1',2',5',6',7',7*a'*-hexahydrospiro[indoline-3,3'-pyrrolizine]-1'-carboxylic acid **9k**: White solid. Yield: 80%. M.p.: 228–229 °C. FT-IR (ATR) ν (cm⁻¹): 2963, 2864, 1724, 1703, 1678. ¹H NMR (400 MHz, DMSO-*d*₆) δ (ppm): 1.68–1.83 (m, 1 H, H-6'), 1.83–1.99 (m, 2 H, H-6', H-7'), 1.99–2.11 (m, 1 H, H-7'), 2.27–2.35 (m, 1 H, H-5'), 2.35–2.45 (m, 1 H, H-5'), 3.69 (t, *J* = 10.5 Hz, 1 H, H-1'), 3.94 (dt, *J* = 9.6, 6.4 Hz, 1 H, H-7*a'*), 4.23 (d, *J* = 16.0 Hz, 1 H), 4.73 (d, *J* = 16.0 Hz, 1 H), 4.90 (d, *J* = 11.5 Hz, 1 H, H-2'), 6.51 (dd, *J* = 8.7, 4.2 Hz, 1 H), 6.95 (td, *J* = 9.0, 2.2 Hz, 1 H), 6.99–7.05 (m, 2 H), 7.13 (dd, *J* = 8.3, 2.2 Hz, 1 H), 7.18–7.25 (m, 3 H), 7.30–7.39 (m, 4 H), 7.55 (t, *J* = 6.6 Hz, 1 H), 12.70 (br. s., 1 H, COOH). ¹³C NMR (100 MHz, DMSO-*d*₆) δ (ppm): 27.7 (C-6'), 31.1 (C-7'), 43.3 (N-CH₂), 47.4 (C-5'), 51.1 (C-1'), 60.0 (C-2'), 67.5 (C-7*a'*), 71.9 (C-spiro), 110.5 (d, *J*_{C-F} = 8.0 Hz, CH), 115.3 (d, *J*_{C-F} = 24.8 Hz, CH), 116.4 (d, *J*_{C-F} = 23.3 Hz, CH), 126.3 (d, *J*_{C-F} = 7.3 Hz, C) (C), 127.3 (CH), 127.8 (CH), 127.9 (CH), 129.0 (CH), 129.1 (CH), 133.9 (CH), 136.1 (C), 136.7 (C), 138.9 (C), 157.3 (C), 159.7 (C), 173.4 (C), 177.9 (C), 197.1 (C). MS (EI, 70 eV) *m/z*: 422 (38), 371 (10), 331 (14), 308 (21), 105 (32), 91 (100). Anal. Calcd. for C₂₉H₂₅FN₂O₄: C: 71.89; H: 5.20; N: 5.78. Found: C: 71.78; H: 5.22; N: 5.76.

(1'*RS*,2'*RS*,3*SR*,7*a'**SR*)-2'-Benzoyl-5-fluoro-2-oxo-1',2',5',6',7',7*a'*-hexahydrospiro[indoline-3,3'-pyrrolizine]-1'-carboxylic acid **9l**: White solid. Yield: 71%. M.p.: 227–228 °C. FT-IR (ATR) ν (cm⁻¹): 3321, 2862, 1711, 1692, 1676. ¹H NMR (400 MHz, DMSO-*d*₆) δ (ppm): 1.64–1.80 (m, 1 H, H-6'), 1.80–1.96 (m, 2 H, H-6', H-7'), 1.96–2.08 (m, 1 H, H-7'), 2.24–2.36 (m, 1 H, H-5'), 2.36–2.46 (m, 1 H, H-5'), 3.61 (t, *J* = 10.5 Hz, 1 H, H-1'), 3.87 (dt, *J* = 9.6, 6.2 Hz, 1 H, H-7*a'*), 4.78 (d, *J* = 11.3 Hz, 1 H, H-2'), 6.45 (dd, *J* = 8.4, 4.3 Hz, 1 H), 6.91 (td, *J* = 9.0, 2.2 Hz, 1 H), 7.00 (dd, *J* = 8.4, 2.0 Hz, 1 H), 7.34 (t, *J* = 7.6 Hz, 2 H), 7.40 (d, *J* = 7.4 Hz, 2 H), 7.52 (t, *J* = 7.1 Hz, 1 H), 10.21 (s, 1 H, NH), 12.63 (br. s., 1 H, COOH). ¹³C NMR (100 MHz, DMSO-*d*₆) δ (ppm): 27.5 (C-6'), 31.0 (C-7'), 47.5 (C-5'), 51.2 (C-1'), 59.8 (C-2'), 67.4 (C-7*a'*), 72.3 (C-spiro), 110.8 (d, *J*_{C-F} = 8.0 Hz, CH), 115.2 (d, *J*_{C-F} = 24.8 Hz, CH), 116.3 (d, *J*_{C-F} = 22.6 Hz, CH), 126.7 (d, *J*_{C-F} = 7.3 Hz, C), 127.9 (CH), 128.9 (CH), 133.8 (CH), 136.8 (C), 138.5 (C), 156.7 (C), 159.1 (C), 173.4 (C), 179.5 (C), 197.2 (C). MS (EI, 70 eV) *m/z*: 394 (M⁺•, 1), 368 (2), 332 (83), 303 (75), 275 (10), 218 (29), 105 (46), 77 (33), 73 (55), 44 (100). Anal. Calcd. for C₂₂H₁₉FN₂O₄: C: 67.00; H: 4.86; N: 7.10. Found: C: 66.96; H: 4.83; N: 7.05.

(1'*RS*,2'*RS*,3*SR*,7*a'**SR*)-2'-Benzoyl-5-chloro-1-hexyl-2-oxo-1',2',5',6',7',7*a'*-hexahydrospiro[indoline-3,3'-pyrrolizine]-1'-carboxylic acid **9m**: White solid. Yield: 78%. M.p.: 167–170 °C. FT-IR (ATR) ν (cm⁻¹): 2930, 2855, 1724, 1680. ¹H NMR (400 MHz, DMSO-*d*₆) δ (ppm): 0.81 (t, *J* = 6.9 Hz, 3 H, CH₃), 0.99–1.20 (m, 8 H, CH₂ × 4), 1.27 (d, *J* = 15.8 Hz, 1 H), 1.68–1.80 (m, 1 H), 1.82–1.95 (m, 2 H), 1.97–2.10 (m, 1 H), 2.22–2.30 (m, 1 H), 2.32–2.41 (m, 1 H), 3.05–3.17 (m, 1 H), 3.62 (dd, *J* = 11.2, 9.9 Hz, 1 H, H-1'), 3.89 (dt, *J* = 9.7, 6.4 Hz, 1 H, H-7*a'*), 4.81 (d, *J* = 11.5 Hz, 1 H, H-2'), 6.71 (d, *J* = 8.4 Hz, 1 H), 7.19 (d,

$J = 1.95$ Hz, 1 H), 7.22–7.29 (m, 3 H), 7.30–7.35 (m, 2 H), 7.46–7.53 (m, 1 H), 12.65 (br.s., 1 H, COOH). ^{13}C NMR (100 MHz, DMSO- d_6) δ (ppm): 14.3 (CH₃), 22.4 (CH₂), 26.1 (CH₂), 26.7 (CH₂), 27.8 (CH₂), 31.1 (CH₂), 31.2 (CH₂), 39.9 (CH₂), 47.3 (CH₂), 51.0 (C-1'), 60.2 (C-2'), 67.5 (C-7a'), 71.6 (C-spiro), 110.6 (CH), 126.5 (C), 126.5 (C), 127.2 (CH), 127.8 (CH), 128.9 (CH), 129.9 (CH), 133.7 (CH), 136.8 (C), 142.0 (C), 173.3 (C), 177.2 (C), 197.2 (C). MS (EI, 40 eV) m/z : 494 ($\text{M}^{+\bullet}$, 1), 458 (2), 432 (30), 367 (25), 318 (100), 105 (67), 77 (39), 43 (62). Anal. Calcd. for C₂₈H₃₁ClN₂O₄: C: 67.94; H: 6.31; N: 5.66. Found: C: 67.82; H: 6.35; N: 5.61.

2.1.2. General Procedure for the Synthesis of Spirooxindoles 11a–j

Equimolar amounts of the corresponding substituted isatin **6**, pipecolic acid **7b** and *trans*-3-benzoyl acrylic acid **8** were dissolved in 10 mL of acetonitrile in a flat bottom flask and subjected to reflux for 8 h, (TLC control). The solid formed was isolated by filtration on vacuum and washed with cold acetonitrile. The products did not require further purification.

(1'*RS*,2'*RS*,3*SR*,8a'*SR*)-2'-Benzoyl-5-methyl-2-oxo-1',5',6',7',8',8a'-hexahydro-2'H-spiro[indoline-3,3'-indolizine]-1'-carboxylic acid **11a** [39]: White solid. Yield: 48%. M.p.: 256–257 °C. FT-IR (ATR) ν (cm⁻¹): 3364, 3225, 2957, 1741, 1704, 1668. ^1H NMR (400 MHz, DMSO- d_6) δ (ppm): 1.10–1.20 (m, 2 H, H-8', H-7'), 1.21–1.32 (m, 1 H, H-6'), 1.43–1.50 (m, 1 H, H-7'), 1.67–1.74 (m, 1 H, H-8'), 2.05–2.18 (m, 5H, 5-CH₃, H-5', H-6'), 2.23 (td, $J = 10.9$, 2.8 Hz, 1 H, H-5'), 3.22–3.29 (m, 1 H, H-8a'), 3.44 (t, $J = 9.8$ Hz, 1 H, H-1'), 4.47 (d, $J = 9.6$ Hz, 1 H, H-2'), 6.30 (d, $J = 7.8$ Hz, 1 H), 6.57 (s, 1 H), 6.71 (d, $J = 7.8$ Hz, 1 H), 7.28 (t, $J = 7.7$ Hz, 2 H), 7.38 (d, $J = 7.3$ Hz, 2 H), 7.43 (t, $J = 7.3$ Hz, 1 H), 10.35 (s, 1 H, NH), 12.62 (s, 1 H, COOH). ^{13}C NMR (100 MHz, DMSO- d_6) δ (ppm): 21.0 (CH₃), 23.8 (C-8'), 25.5 (C-7'), 31.8 (C-6'), 45.6 (C-5'), 50.5 (C-1'), 54.9 (C-2'), 61.4 (C-8a'), 71.4 (C-spiro), 109.1 (CH), 126.7 (CH), 127.5 (C), 127.6 (CH), 128.8 (CH), 129.7 (CH), 130.9 (C), 133.4 (CH), 137.2 (C), 140.3 (C), 173.6 (C), 179.3 (C), 197.6 (C). MS (EI, 70 eV) m/z : 404 ($\text{M}^{+\bullet}$, 9), 368 (5), 332 (39), 315 (15), 271 (30), 255 (17), 227 (69), 141 (33), 105 (100), 77 (69). Anal. Calcd. for C₂₄H₂₄N₂O₄: C: 71.27; H: 5.98; N: 6.93. Found: C: 71.18; H: 5.96; N: 6.89.

(1'*RS*,2'*RS*,3*SR*,8a'*SR*)-2'-Benzoyl-5-fluoro-2-oxo-1',5',6',7',8',8a'-hexahydro-2'H-spiro[indoline-3,3'-indolizine]-1'-carboxylic acid **11b** [39]: White solid. Yield: 68%. M.p.: 235–236 °C. FT-IR (ATR) ν (cm⁻¹): 3478, 3096, 2937, 1715, 1676. ^1H NMR (400 MHz, DMSO- d_6) δ (ppm): 1.11–1.23 (m, 2 H, H-8', H-7'), 1.23–1.33 (m, 1 H, H-6'), 1.42–1.54 (m, 1 H, H-7'), 1.66–1.79 (m, 1 H, H-8'), 2.05–2.19 (m, 2 H, H-5', H-6'), 2.20–2.32 (m, 1 H, H-5'), 3.25 (t, $J = 10.0$ Hz, 1 H, H-8a'), 3.45 (t, $J = 9.7$ Hz, 1 H, H-1'), 4.50 (d, $J = 9.7$ Hz, 1 H, H-2'), 6.41 (dd, $J = 8.6$, 4.3 Hz, 1 H), 6.54 (dd, $J = 8.2$, 2.7 Hz, 1 H), 6.78 (td, $J = 9.0$, 2.7 Hz, 1 H), 7.32 (t, $J = 7.6$ Hz, 2 H), 7.40–7.51 (m, 3 H), 10.52 (s, 1 H, NH), 12.69 (s, 1 H, COOH). ^{13}C NMR (100 MHz, DMSO- d_6) δ (ppm): 23.7 (C-8'), 25.5 (C-7'), 31.8 (C-6'), 45.6 (C-5'), 50.5 (C-1'), 55.1 (C-2'), 61.5 (C-8a'), 71.6 (C-spiro), 110.4 (d, $J_{\text{C-F}} = 6.9$ Hz, CH), 113.5 (d, $J_{\text{C-F}} = 24.7$ Hz, CH), 116.0 (d, $J_{\text{C-F}} = 23.6$ Hz, CH), 127.6 (CH), 129.0 (CH), 129.4 (d, $J_{\text{C-F}} = 7.4$ Hz, C), 133.8 (CH), 136.9 (C), 139.0 (C), 159.5 (C), 173.4 (C), 179.3 (C), 197.4 (C). MS (EI, 70 eV) m/z : 408 ($\text{M}^{+\bullet}$, 10), 368 (17), 336 (37), 313 (12), 275 (36), 259 (16), 231 (39), 141 (22), 105 (49), 77 (34). Anal. Calcd. for C₂₃H₂₁FN₂O₄: C: 67.64; H: 5.18; N: 6.86. Found: C: 67.54; H: 5.19; N: 6.84.

(1'*RS*,2'*RS*,3*SR*,8a'*SR*)-2'-Benzoyl-1-methyl-2-oxo-1',5',6',7',8',8a'-hexahydro-2'H-spiro[indoline-3,3'-indolizine]-1'-carboxylic acid **11c** [39]: White solid. Yield: 49%. M.p.: 219–220 °C. FT-IR (ATR) ν (cm⁻¹): 2941, 2356, 1709, 1677. ^1H NMR (400 MHz, DMSO- d_6) δ (ppm): 1.09–1.21 (m, 2 H, H-8', H-7'), 1.21–1.36 (m, 1 H, H-6'), 1.38–1.52 (m, 1 H, H-7'), 1.66–1.81 (m, 1 H, H-8'), 2.01–2.18 (m, 2 H, H-5', H-6'), 2.23 (t, $J = 10.2$ Hz, 1 H, H-5'), 2.98 (s, 3 H, N-CH₃), 3.22–3.29 (m, 1 H, H-8a'), 3.42 (t, $J = 10.0$ Hz, 1 H, H-1'), 4.47 (d, $J = 9.8$ Hz, 1 H, H-2'), 6.52 (d, $J = 7.8$ Hz, 1 H), 6.79–6.90 (m, 2 H), 7.01 (t, $J = 7.5$ Hz, 1 H), 7.20–7.31 (m, 4 H), 7.37–7.47 (m, 1 H), 12.66 (s, 1 H, COOH). ^{13}C NMR (100 MHz, DMSO- d_6) δ (ppm):

23.7 (C-8'), 25.4 (C-7'), 26.2 (CH₃), 31.8 (C-6'), 45.6 (C-5'), 50.4 (C-1'), 55.9 (C-2'), 61.8 (C-8a'), 71.1 (C-spiro), 108.2 (CH), 122.9 (CH), 125.6 (CH), 127.0 (C), 127.4 (CH), 128.7 (CH), 129.6 (CH), 133.5 (CH), 137.0 (C), 144.0 (C), 173.4 (C), 177.4 (C), 197.3 (C). MS (EI, 70 eV) *m/z*: 404 (M⁺, 1), 393 (12), 368 (22), 339 (26), 313 (70), 264 (34), 236 (16), 57 (100). Anal. Calcd. for C₂₄H₂₄N₂O₄: C: 71.27; H: 5.98; N: 6.93. Found: C: 71.09; H: 5.97; N: 6.91.

(1'*RS*,2'*RS*,3*SR*,8*a'SR*)-2'-Benzoyl-5-chloro-2-oxo-1',5',6',7',8',8*a'*-hexahydro-2'H-spiro[indoline-3,3'-indolizine]-1'-carboxylic acid **11d**: White solid. Yield: 68%. M.p.: 225 °C (Decomp). FT-IR (ATR) ν (cm⁻¹): 3370, 3218, 2955, 2360, 1742, 1707. ¹H NMR (400 MHz, DMSO-*d*₆) δ (ppm): 1.12–1.22 (m, 2 H, H-8', H-7'), 1.22–1.33 (m, 1 H, H-6'), 1.40–1.53 (m, 1 H, H-7'), 1.64–1.76 (m, 1 H, H-8'), 2.09–2.19 (m, 2 H, H-5', H-6'), 2.21–2.31 (m, 1 H, H-5'), 3.24 (t, *J* = 9.3 Hz, 1 H, H-8a'), 3.44 (t, *J* = 9.8 Hz, 1 H, H-1'), 4.50 (d, *J* = 9.6 Hz, 1 H, H-2'), 6.43 (d, *J* = 8.3 Hz, 1 H), 6.71 (s, 1 H), 6.99 (dd, *J* = 8.3, 2.3 Hz, 1 H), 7.32 (t, *J* = 7.6 Hz, 2 H), 7.41 (d, *J* = 7.3 Hz, 2 H), 7.47 (t, *J* = 7.3 Hz, 1 H), 10.66 (s, 1 H, NH). ¹³C NMR (100 MHz, DMSO-*d*₆) δ (ppm): 23.7 (C-8'), 25.5 (C-7'), 31.8 (C-6'), 45.7 (C-5'), 50.5 (C-1'), 55.2 (C-2'), 61.6 (C-8a'), 71.4 (C-spiro), 111.0 (CH), 126.0 (CH), 126.2 (C), 127.6 (CH), 129.1 (CH), 129.4 (CH), 129.7 (C), 133.8 (CH), 137.0 (C), 141.7 (C), 173.4 (C), 179.0 (C), 197.5 (C). MS (EI, 70 eV) *m/z*: 424 (M⁺, 19), 368 (14), 352 (73), 335 (30), 313 (38), 291 (65), 247 (71), 105 (100), 77 (76). Anal. Calcd. for C₂₃H₂₁ClN₂O₄: C: 65.02; H: 4.98; N: 6.59. Found: C: 65.20; H: 4.96; N: 6.57.

(1'*RS*,2'*RS*,3*SR*,8*a'SR*)-2'-Benzoyl-1-benzyl-2-oxo-1',5',6',7',8',8*a'*-hexahydro-2'H-spiro[indoline-3,3'-indolizine]-1'-carboxylic acid **11e**: White solid. Yield: 45%. M.p.: 203–205 °C. FT-IR (ATR) ν (cm⁻¹): 3225, 2962, 1738, 1681. ¹H NMR (400 MHz, DMSO-*d*₆) δ (ppm): 1.13–1.32 (m, 3 H, H-8', H-7', H-6'), 1.43–1.51 (m, 1 H, H-7'), 1.67–1.78 (m, 1 H, H-8'), 2.04–2.13 (m, 1 H, H-5'), 2.15–2.22 (m, 1 H, H-6'), 2.21–2.30 (m, 1 H, H-5'), 3.33 (t, *J* = 10.0 Hz, 1 H, H-8a'), 3.49 (t, *J* = 9.8 Hz, 1 H, H-1'), 4.53 (d, *J* = 9.5 Hz, 1 H, H-2'), 4.63 (d, *J* = 15.6 Hz, 1 H, CH), 4.89 (d, *J* = 15.6 Hz, 1 H, CH), 6.54 (d, *J* = 7.8 Hz, 1 H), 6.83 (d, *J* = 4.4 Hz, 2 H), 6.95 (dq, *J* = 8.1, 4.1, 3.6 Hz, 1 H), 7.13 (t, *J* = 7.7 Hz, 2 H), 7.23–7.37 (m, 7H), 7.39 (t, *J* = 7.4 Hz, 1 H). ¹³C NMR (100 MHz, DMSO-*d*₆) δ (ppm): 23.7 (C-8'), 25.5 (C-7'), 31.9 (C-6'), 43.3 (CH₂), 45.6 (C-5'), 50.6 (C-1'), 55.2 (C-2'), 61.6 (C-8a'), 71.1 (C-spiro), 109.0 (CH), 123.0 (CH), 126.1 (CH), 126.7 (C), 127.6 (CH), 128.0 (CH), 128.8 (CH), 129.2 (CH), 129.6 (CH), 133.5 (CH), 136.7 (C), 136.9 (C), 143.3 (C), 173.5 (C), 177.6 (C), 197.3 (C). MS (EI, 70 eV) *m/z*: 480 (M⁺, 9), 444 (12), 418 (95), 389 (10), 339 (80), 331 (100), 141 (28), 105 (39), 91 (35), 77 (18). Anal. Calcd. for C₃₀H₂₈N₂O₄: C: 74.98; H: 5.87; N: 5.83. Found: C: 75.07; H: 5.89; N: 5.80.

(1'*RS*,2'*RS*,3*SR*,8*a'SR*)-2'-Benzoyl-1-hexyl-2-oxo-1',5',6',7',8',8*a'*-hexahydro-2'H-spiro[indoline-3,3'-indolizine]-1'-carboxylic acid **11f** [39]: White solid. Yield: 48%. M.p.: 176–177 °C. FT-IR (ATR) ν (cm⁻¹): 2931, 2858, 1723, 1684, 1662. ¹H NMR (400 MHz, DMSO-*d*₆) δ (ppm): 0.79–0.91 (m, 3 H, CH₃), 1.08–1.22 (m, 2 H), 1.20–1.33 (m, 7H), 1.35–1.57 (m, 3 H), 1.66–1.78 (m, 1 H), 2.00–2.10 (m, 1 H), 2.11–2.27 (m, 2 H), 3.23–3.29 (m, 1 H, H-8a'), 3.37–3.51 (m, 2 H, NCH, H-1'), 3.60 (dt, *J* = 14.5, 7.4 Hz, 1 H, NCH), 4.48 (d, *J* = 9.7 Hz, 1 H, H-2'), 6.60 (d, *J* = 7.8 Hz, 1 H), 6.74–6.91 (m, 2 H), 7.01 (t, *J* = 7.5 Hz, 1 H), 7.19–7.32 (m, 4 H), 7.42 (t, *J* = 7.2 Hz, 1 H), 12.68 (s, 1 H, COOH). ¹³C NMR (100 MHz, DMSO-*d*₆) δ (ppm): 14.3 (CH₃), 22.5 (CH₂), 23.7 (C-8'), 25.5 (C-7'), 26.4 (CH₂), 27.4 (CH₂), 31.3 (CH₂), 31.8 (C-6'), 39.7 (CH₂), 45.5 (C-5'), 50.5 (C-1'), 55.4 (C-2'), 61.6 (C-8a'), 70.9 (C-spiro), 108.4 (CH), 122.7 (CH), 126.0 (CH), 126.9 (C), 127.5 (CH), 128.8 (CH), 129.6 (CH), 133.5 (CH), 143.5 (C), 173.5 (C), 177.2 (C), 197.4 (C). MS (EI, 70 eV) *m/z*: 475 (M⁺ + H, 3), 368 (22), 339 (35), 313 (75), 264 (39). Anal. Calcd. for C₂₉H₃₄N₂O₄: C: 73.39; H: 7.22; N: 5.90. Found: C: 73.55; H: 7.25; N: 5.93.

(1'*RS*,2'*RS*,3*SR*,8*a'SR*)-2'-Benzoyl-5-chloro-1-methyl-2-oxo-1',5',6',7',8',8*a'*-hexahydro-2'H-spiro[indoline-3,3'-indolizine]-1'-carboxylic acid **11g** [39]: White solid. Yield: 69%. M.p.: 224–226 °C. FT-IR (ATR) ν (cm⁻¹): 3378, 3227, 2957, 1744, 1708, 1668. ¹H NMR (400 MHz, DMSO-*d*₆) δ (ppm): 1.11–1.22 (m, 2 H), 1.22–1.36 (m, 1 H), 1.40–1.50 (m, 1 H), 1.66–1.79 (m, 1 H), 2.05–2.19 (m, 2 H), 2.19–2.29 (m, 1 H), 2.97 (s, 3 H, CH₃), 3.24 (td, *J* = 10.2,

2.5 Hz, 1 H, H-8a'), 3.43 (t, $J = 10.0$ Hz, 1 H, H-1'), 4.48 (d, $J = 9.9$ Hz, 1 H, H-2'), 6.56 (d, $J = 8.3$ Hz, 1 H), 6.79 (d, $J = 2.1$ Hz, 1 H), 7.09 (dd, $J = 8.3, 2.2$ Hz, 1 H), 7.23–7.35 (m, 4 H), 7.45 (td, $J = 7.0, 1.6$ Hz, 1 H). ^{13}C NMR (100 MHz, DMSO- d_6) δ (ppm): 23.6 (C-8'), 25.4 (C-7'), 26.4 (CH₃), 31.7 (C-6'), 45.7 (C-5'), 50.2 (C-1'), 56.1 (C-2'), 62.0 (C-8a'), 71.1 (C-spiro), 109.9 (CH), 125.5 (CH), 127.0 (C), 127.4 (CH), 128.9 (CH), 129.2 (C), 129.5 (CH), 133.8 (CH), 136.8 (C), 142.9 (C), 173.3 (C), 177.0 (C), 197.3 (C). MS (EI, 70 eV) m/z : 438 (M^+ , 1), 336 (17), 313 (18), 275 (17), 231 (30), 141 (41), 105 (91), 77 (55), 57 (87), 43 (100). Anal. Calcd. for C₂₄H₂₃ClN₂O₄: C: 65.68; H: 5.28; N: 6.38. Found: C: 65.56; H: 5.29; N: 6.35.

(1'*RS*,2'*RS*,3*RS*,8a'*SR*)-2'-Benzoyl-2-oxo-1',5',6',7',8',8a'-hexahydro-2'H-spiro[indoline-3,3'-indolizine]-1'-carboxylic acid **11h**: White solid. Yield: 53%. M.p.: 225–226 °C. FT-IR (ATR) ν (cm⁻¹): 3479, 3380, 2957, 1741, 1706. ^1H NMR (400 MHz, DMSO- d_6) δ (ppm): 1.11–1.20 (m, 2 H, H-8', H-7'), 1.21–1.32 (m, 1 H, H-6'), 1.43–1.52 (m, 1 H, H-7'), 1.68–1.76 (m, 1 H, H-8'), 2.08–2.19 (m, 2 H, H-5', H-6'), 2.20–2.29 (m, 1 H, H-5'), 3.22–3.30 (m, 1 H, H-8a'), 3.45 (t, $J = 9.9$ Hz, 1 H, H-1'), 4.48 (d, $J = 9.6$ Hz, 1 H, H-2'), 6.41 (d, $J = 7.6$ Hz, 1 H), 6.76 (d, $J = 4.3$ Hz, 2 H), 6.92 (dt, $J = 8.2, 4.4$ Hz, 1 H), 7.28 (t, $J = 7.5$ Hz, 2 H), 7.39 (d, $J = 7.8$ Hz, 2 H), 7.44 (t, $J = 7.3$ Hz, 1 H), 10.47 (s, 1 H, NH), 12.63 (s, 1 H, COOH). ^{13}C NMR (100 MHz, DMSO- d_6) δ (ppm): 23.7 (C-8'), 25.5 (C-7'), 31.8 (C-6'), 45.5 (C-5'), 50.6 (C-1'), 55.0 (C-2'), 61.4 (C-8a'), 71.3 (C-spiro), 109.4 (CH), 122.2 (CH), 126.2 (CH), 127.5 (C), 127.6 (CH), 128.9 (CH), 129.5 (CH), 133.5 (CH), 137.0 (C), 142.7 (C), 173.6 (C), 179.3 (C), 197.5 (C). MS (EI, 70 eV) m/z : 390 (M^+ , 1), 318 (7), 257 (13), 213 (23), 141 (44), 105 (100), 77 (68). Anal. Calcd. for C₂₃H₂₂N₂O₄: C: 70.75; H: 5.68; N: 7.18. Found: C: 70.54; H: 5.68; N: 7.16.

(1'*RS*,2'*RS*,3*SR*,8a'*SR*)-2'-Benzoyl-1-benzyl-5-chloro-2-oxo-1',5',6',7',8',8a'-hexahydro-2'H-spiro[indoline-3,3'-indolizine]-1'-carboxylic acid **11i**: White solid. Yield: 67%. M.p.: 218–219 °C. FT-IR (ATR) ν (cm⁻¹): 3225, 2944, 1735, 1677. ^1H NMR (400 MHz, DMSO- d_6) δ (ppm): 1.10–1.24 (m, 2 H, H-8', H-7'), 1.24–1.35 (m, 1 H, H-6'), 1.44–1.52 (m, 1 H, H-7'), 1.69–1.79 (m, 1 H, H-8'), 2.07–2.13 (m, 1 H, H-5'), 2.14–2.22 (m, 1 H, H-6'), 2.22–2.30 (m, 1 H, H-5'), 3.32 (t, $J = 9.9$ Hz, 1 H, H-8a'), 3.49 (t, $J = 9.7$ Hz, 1 H, H-1'), 4.55 (d, $J = 9.5$ Hz, 1 H, H-2'), 4.64 (d, $J = 15.5$ Hz, 1 H, CH), 4.88 (d, $J = 15.6$ Hz, 1 H, CH), 6.57 (d, $J = 9.5$ Hz, 1 H), 6.78 (s, 1 H), 7.04 (d, $J = 8.3$ Hz, 1 H), 7.17 (t, $J = 7.6$ Hz, 2 H), 7.25–7.37 (m, 7H), 7.42 (t, $J = 7.4$ Hz, 1 H). ^{13}C NMR (100 MHz, DMSO- d_6) δ (ppm): 23.6 (C-8'), 25.5 (C-7'), 31.8 (C-6'), 43.4 (CH₂), 45.8 (C-5'), 50.5 (C-1'), 55.3 (C-2'), 61.7 (C-8a'), 71.1 (C-spiro), 100.0 (C), 110.6 (CH), 126.0 (CH), 127.2 (C), 127.6 (CH), 128.0 (CH), 128.1 (CH), 128.9 (C), 129.0 (CH), 129.2 (CH), 129.5 (CH), 133.4 (CH), 136.3 (C), 136.8 (C), 142.2 (C), 173.4 (C), 177.1 (C), 197.3 (C). MS (EI, 70 eV) m/z : 514 (M^+ , 19), 478 (9), 452 (55), 373 (81), 365 (86), 141 (27), 105 (55), 91 (100), 77 (64). Anal. Calcd. for C₃₀H₂₇ClN₂O₄: C: 69.97; H: 5.28; N: 5.44. Found: C: 70.07; H: 5.33; N: 5.40.

(1'*RS*,2'*RS*,3*SR*,8a'*SR*)-2'-Benzoyl-5-chloro-1-hexyl-2-oxo-1',5',6',7',8',8a'-hexahydro-2'H-spiro[indoline-3,3'-indolizine]-1'-carboxylic acid **11j**: White solid. Yield: 65%. M.p.: 226–228 °C. FT-IR (ATR) ν (cm⁻¹): 2932, 2857, 1711, 1701, 1678. ^1H NMR (400 MHz, DMSO- d_6) δ (ppm): 0.80–0.88 (m, 3 H, CH₃), 1.14–1.31 (m, 9H), 1.33–1.52 (m, 3 H), 1.66–1.77 (m, 1 H), 2.02–2.11 (m, 1 H), 2.11–2.26 (m, 2 H), 3.21–3.29 (m, 1 H, H-8a'), 3.36–3.49 (m, 2 H, NCH, H-1'), 3.59 (dt, $J = 14.6, 7.5$ Hz, 1 H, NCH), 4.49 (d, $J = 9.8$ Hz, 1 H, H-2'), 6.65 (d, $J = 8.4$ Hz, 1 H), 6.78 (s, 1 H), 7.09 (dd, $J = 8.3, 2.3$ Hz, 1 H), 7.24–7.36 (m, 4 H), 7.44 (d, $J = 7.1$ Hz, 1 H), 12.71 (s, 1 H, COOH). ^{13}C NMR (100 MHz, DMSO- d_6) δ (ppm): 14.3 (CH₃), 22.5 (CH₂), 23.6 (C-8'), 25.4 (C-7'), 26.4 (CH₂), 27.3 (CH₂), 31.3 (CH₂), 31.7 (C-6'), 45.6 (C-5'), 50.3 (C-1'), 55.5 (C-2'), 61.8 (C-8a'), 70.9 (C-spiro), 110.1 (CH), 125.9 (CH), 126.9 (C), 127.5 (CH), 129.0 (CH), 129.1 (C), 129.5 (CH), 133.7 (CH), 142.4 (C), 173.3 (C), 176.8 (C), 197.4 (C). MS (EI, 70 eV) m/z : 508 (M^+ , 13), 472 (6), 446 (41), 367 (65), 359 (69), 332 (16), 141 (100), 105 (74), 77 (34). Anal. Calcd. for C₂₉H₃₃ClN₂O₄: C: 68.43; H: 6.53; N: 5.50. Found: C: 68.47; H: 6.57; N: 5.53.

2.2. Biology

2.2.1. Antibacterial Activity

The novel compounds were screened for bacterial activity using the agar diffusion method as described before [40]. Briefly, 10 μ L of each compound stock solution (100 mg/mL) were added into 6 mm wells in Mueller Hinton agar (MHA) inoculated with the selected bacterial strain and incubated 24 h at 35–37 °C. The solvent (dimethyl sulfoxide (DMSO)) was included as negative control and known antibiotics such as gentamicin and trimethoprim sulfamethoxazole were positive controls for inhibition. To test *N. gonorrhoeae*, this method was performed by inoculating 200 μ L of bacterial suspensions (1.5×10^8 CFU/mL) onto enriched gonococcal agar (GC) before adding the compounds into each well, then the plates were incubated up to 48 h at 35–37 °C in 5% CO₂ atmosphere. Tetracycline and penicillin were included as growth inhibition controls. Controls were interpreted using the quality ranges established by the standards [38], assays were considered valid as all the controls achieved the standard values.

Compounds inducing growth inhibition in more than one experiment and an inhibition diameter >13 mm were subjected to microdilution test to determine the minimum inhibitory concentration (MIC) according with the standard [38]. The final readings were performed using resazurin (125 μ g/mL). Known inhibitors were included as controls (gentamicin and tetracycline), assays were done at least twice. To define the MIC for *N. gonorrhoeae* the method was performed following the standard with modifications [40]. A 1% oxidase solution was used to determine endpoints of bacterial growth. Experiments were conducted in two different days.

2.2.2. Toxicity Studies Using the In Vivo Model

The level of toxicity was determined for the potentially active compounds. Six- instar larvae of *Galleria mellonella* were inoculated with 10 μ L of the selected compound up to 2000 mg/kg [41,42]. The larvae viability was checked every 24 h over 5 days and the half lethal dose (LD₅₀) was determined.

2.2.3. Hemolytic Activity

The ability to induce hemolysis in human red blood cells (huRBCs) of compounds with antibacterial effect was assessed using the spectrophotometry method [43]. Briefly, 240 μ L of 5% huRBC in phosphate buffer saline (PBS) were placed into 96-well plates and subsequently exposed to 200 μ g/mL of the selected compounds (i.e., 10 μ L of 5 mg/mL working solution of each compound in Mueller Hinton broth (MHB) with 5% DMSO 0.1% Tween-80). As positive control 10 μ L SDS 1% was added. For a negative control, only medium with no chemicals was added to huRBC. Measurements were performed at 540 nm in a microplate spectrophotometer. Experiments were performed at least twice.

2.3. Molecular Modeling

In Silico Analysis of Toxicity and Inverse Docking Analysis

Chemical structures from the most active compounds were examined using Protox II [44]. Protox II uses several models by comparison of similar structures to predict hepatotoxicity, immunotoxicity, carcinogenicity, mutagenicity, cytotoxicity and metabolic interactions [44].

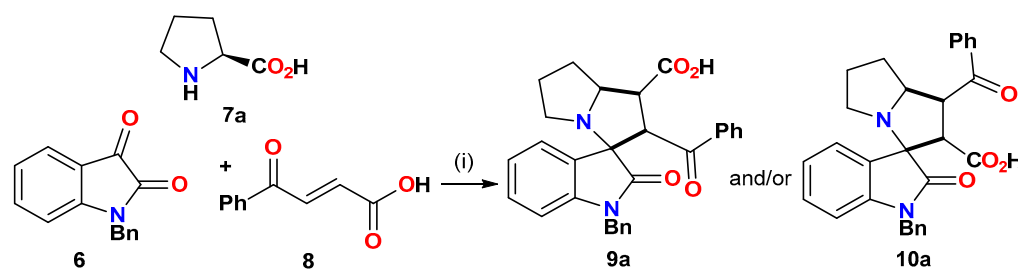
Inverse docking analysis was performed in triplicate using AutoDock Vina.(autodock vina 1.1.2) Crystallographic protein structures were downloaded from the Protein Data Bank. Target optimization was executed using a python script (Python 3.10.9) which removed water molecules, heteroatoms and non-polar hydrogens. A Chimera X command line was launched to minimize the energy and add the corresponding charges (AMBER).

Compounds' 3D structures were converted into the "sdf" format and using Gaussian 09, they were optimized with the B3LYP multiparameter functional and a 6–31 G Gaussian basis set. An Open Babel 2.4.1 command line was executed to add polar hydrogens, Gasteiger charges, and free bond rotation modes into the corresponding "pdbqt" format files. A bash script was designed in order to perform each protein docking. The docking site was settled up into a box located in the coordinates of the co-crystallized ligand found on the PDB protein file. Each run was executed with a total of 10 number modes, an exhaustiveness of 100, and using a total of 8 cpus for each target. A python script was designed in order to analyze, compare and sort the docking results [45]. Physicochemical descriptors were calculated using SwissADME online server (<http://www.swissadme.ch>, accessed on 24 June 2024) [46].

3. Results and Discussion

3.1. Synthetic Approaches

The 1,3-CD tricomponent reaction between *N*-benzylisatin **6** ($R^1 = \text{Bn}$, $R^2 = \text{H}$), L-proline **7a** and *trans*-3-benzoylacrylic acid **8** was performed as a model reaction (Scheme 1) under different reaction conditions (Table 1).



Scheme 1. Model reaction for the synthesis of spirooxindoles containing the pyrrolizine moiety.

Table 1. Setting up the reaction conditions for the synthesis of the spirooxindole **9a/10a**.

Entry	Condition (i)	Time (h)	Yield of 9a/10a (%)
1	EtOH, reflux	2	26
2	ACN, reflux	2	56
3	H ₂ O, reflux	3	59
4	MeOH, reflux	2	61
5	THF, reflux	4	45

All reactions were followed by TLC. Initially, the reaction was evaluated in ethanol as solvent under reflux for 2 h with the formation of a small amount of a new product along with unreacted precursors (Entry 1, Table 1). The isolation and preliminary characterization of this product by mass spectrometry (showing a molecular ion peak with $m/z = 466$), indicated that it could correspond to either structure **9a** or its regioisomeric analogue **10a** (in any case with a yield of 26%). In an attempt to improve the yield of the new product **9a/10a**, the reaction was carried out in acetonitrile, affording an improvement of the reaction yield (56%) (Entry 2, Table 1). When water and methanol were used as solvents we observed a significant increase in the reaction yields (i.e., 59% and 61%, respectively) (Entries 3 and 4, Table 1). Finally, when the reaction was performed in refluxing THF the isolated product **9a/10a** was obtained in 45% yield (Entry 5, Table 1). According to the above results, the refluxing methanol afforded the better yield for which this reaction condition was adopted as the optimal to continue with the experiments.

Regarding the structure of the isolated solid **9a/10a**, its elucidation was achieved by (1D, 2D)-NMR, FT-IR, and mass spectrometry. The mass spectrum of this compound showed a molecular ion peak with $m/z = 466$ which could be assigned to either spirooxindole **9a** or **10a** but served as initial evidence for us of the formation of the product of interest. Preliminary analysis of the NMR spectrum allowed us to rule out the formation of a mixture of both regioisomers (**9a** and **10a**), as no signal duplication was observed. In the ^1H NMR spectrum of product **9a/10a** (see Supplementary Data), the signals corresponding to the formation of the bicyclic pyrrolizinic system (i.e., H-2', H-1' and H-7a' protons) are highlighted (the numbering of the pyrrolizinic moiety is shown in Figure 2).

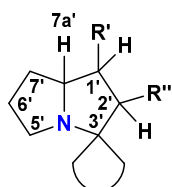
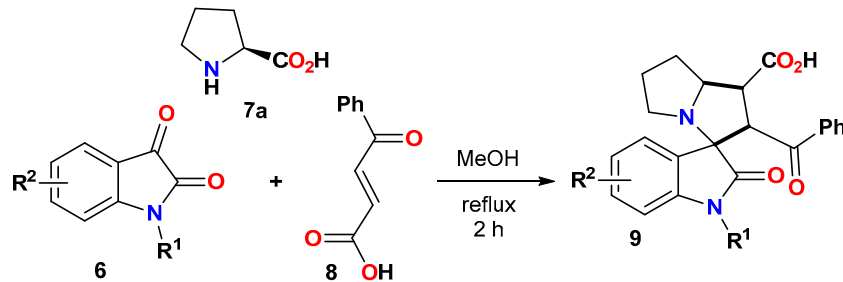


Figure 2. Numbering of positions in the pyrrolizine moiety.

Independent of the **9a/10a** regioisomer formed, the H-2' proton is observed as a doublet (by its neighboring with H-1' proton) at 4.87 ppm with $J = 11.3$ Hz, the H-1' proton appears as a false triplet at 3.62 ppm with $J = 10.4$ Hz, while the H-7a' proton appears as a multiplet in the region of 3.92–3.97 ppm. The signals of the six protons belonging to the three methylene groups were observed at high field in the region of 1.60–2.45 ppm. The signals of the diastereotopic protons of $\text{CH}_2\text{-Ph}$ (Bn) substituent were observed at 4.22 ppm and 4.73 ppm as doublets with $J = 15.8$ Hz for each one, while the proton signal of the CO_2H group is observed as a singlet at 12.71 ppm. The signals for the fourteen aromatic protons were observed in the region between 6.50–7.60 ppm. In the ^{13}C NMR spectrum and DEPT-135 of the product **9a** or **10a** (see Supplementary Data) the characteristic signals that gave us the confirmation of the formation of the pyrrolizinic ring were observed. In the ^{13}C NMR spectrum and DEPT-135 of the product **9a** or **10a** (see Supplementary Data) the characteristic signals that allowed us to confirm the formation of the bicyclic pyrrolizinic moiety are highlighted. Signals for the three methylene carbon atoms C-7', C-6' and C-5' are observed at high field at 31.3, 27.4, and 47.5 ppm, respectively. The spiro-carbon atom signal C-3' was observed at 71.7 ppm. The C-1', C-2' and C-7a' carbon atom signals were observed at 51.4, 59.9 and 67.4 ppm, respectively. The ten signals belonging to the CH aromatic carbon atoms were also observed at 109.6, 122.5, 127.3, 127.4, 127.8, 127.9, 128.8, 129.1, 129.9 and 133.7 ppm, and the three C=O carbon atoms at 173.4, 178.0 and 197.2 ppm. In the FT-IR spectrum is highlighted some relevant signals like the C-H stretching bands at 2957 and 2864 cm^{-1} , and the C=O stretching bands at 1726, 1697 and 1678 cm^{-1} . Resuming, the above spectroscopic data and the long range 2'-H-C correlations in the HMBC experiment suggests that the correct structure for the isolated solid is more likely associated to regioisomer **9a** than **10a**.

Once an acceptable structure of the isolated solid **9a** was established, the generality and scope of this reaction was extended to various *N*-alkyl- and aromatic ring-substituted isatins **6**, affording product **9** in 40–86% yields (Table 2).

All products were fully characterized by NMR, FT-IR, and MS (see Supplementary Data). Additionally, the growth of single crystals of product **9d** suitable for X-ray diffraction unequivocally confirmed the regioselective formation of the spiro-derivative **9** instead of **10**, as shown in Figure 3 [38].

Table 2. Synthesis of the series of spirooxindoles containing the pyrrolizine moiety **9a–m**.


Compound	R ¹	R ²	Yield of 9 (%)
9a	Bn	H	61
9b	CH ₃	H	44
9c	H	H	72
9d *	<i>n</i> -Hexyl	H	60
9e	CH ₃	5-Cl	58
9f	Bn	5-Cl	41
9g	H	5,7- <i>di</i> -Cl	58
9h	H	5-CH ₃	49
9i	H	5-NO ₂	40
9j	Bn	5,7- <i>di</i> -Cl	86
9k	Bn	5-F	80
9l	H	5-F	71
9m	<i>n</i> -Hexyl	5-Cl	78

* Structure of product **9d** re-confirmed by single-crystal X-ray diffraction analysis [45].

Subsequently, the synthetic utility of the established reaction conditions was extended to the synthesis of a new series of spirooxindoles, **11/12**, switching the L-proline **7a** by pipercolic acid **7b** (Table 3). Initially, a model reaction starting with pipercolic acid **7b**, 5-methylisatin **6** (R¹ = H, R² = 5-Me) and *trans*-3-benzoylacrylic acid **8** was subjected to the previously established reaction conditions (i.e., methanol under reflux for 6 h), but unfortunately, without any evident change in precursors. This drawback suggested that a detailed revision of the reaction conditions for this new approach must also be undertaken. Repeating the same reaction in refluxing ethanol for 6 h showed a similar behavior and no change in precursors was observed. When refluxing toluene and xylenes were used as solvents (8 h of heating), complex mixtures of reagents and products were observed. When the reaction was performed in an ethanol/acetic acid mixture (heating for 8 h), the formation of a small amount of a new product was observed, along with unreacted starting materials. After its isolation the corresponding structure could be assigned to either regioisomer **11a** or **12a** (25% yield in any case), as confirmed by mass spectrometry showing a molecular ion with *m/z* = 404.

While looking for an improvement of the reaction yield, additional solvents such as acetonitrile (8 h, 48%) and glacial acetic acid (8 h, 36%) under reflux were evaluated, indicating that acetonitrile afforded the best result. In addition to the mass spectrum, the NMR spectrum of the obtained solid showed the formation of a single regioisomer, **11a** or **12a**, as no duplicate signals were observed. Moreover, signals for the protons assigned to H-2' (doublet at 4.47 ppm with *J* = 9.6 Hz), H-1' (false triplet at 3.44 ppm with *J* = 9.8 Hz) and H-8a' (multiplet at 3.22–3.29 ppm), corroborated the formation of the

bicyclic spiro-indolizinic moiety, as shown in Figure 4. The previous structural findings for the spiro-derivative **9a** (Figure 3), and the long range 2'-H-C correlations in the HMBC experiment suggests that the correct structure for the isolated solid is more likely associated with regioisomer **11a**.

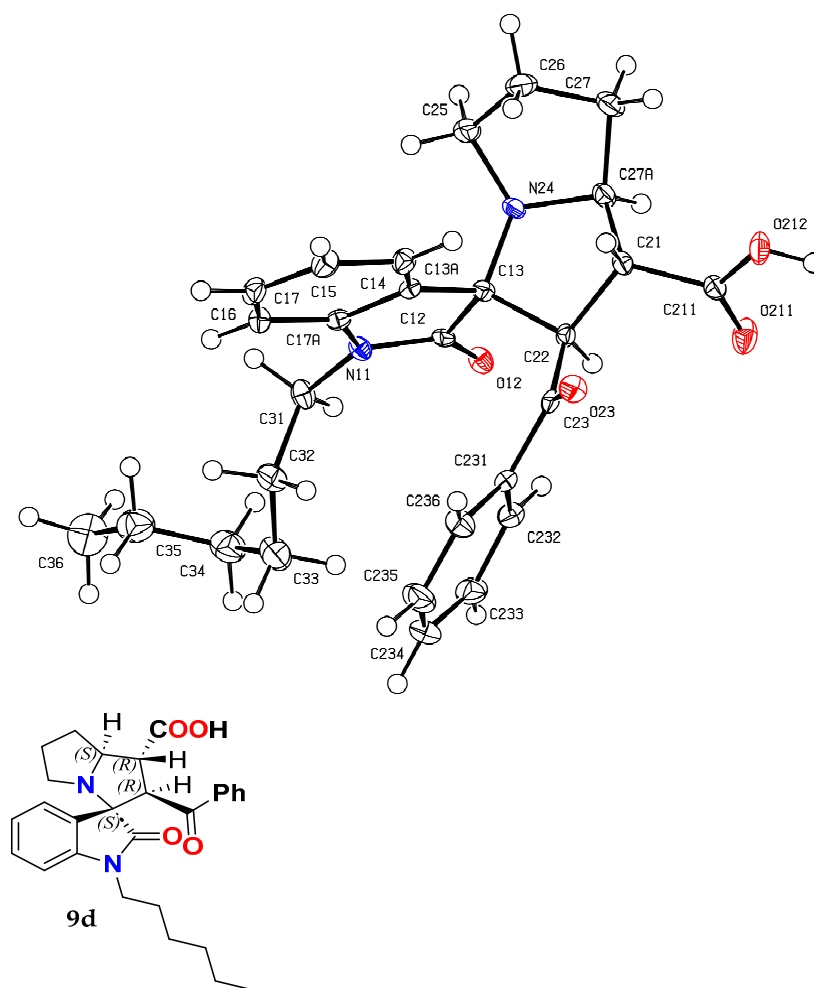
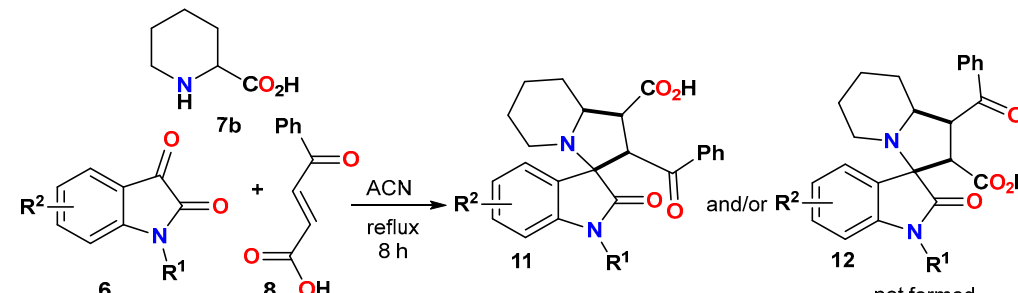


Figure 3. ORTEP diagram for the obtained product **9d**.

Table 3. Synthesis of the series of spirooxindoles containing the indolizine moiety **11a–j**.

Compound	R ¹	R ²	Yield of 11 (%)
11a *	H	5-CH ₃	48
11b *	H	5-F	68
11c *	CH ₃	H	49

Table 3. Cont.



Compound	R ¹	R ²	Yield of 11 (%)
11d	H	5-Cl	68
11e	Bn	H	45
11f *	<i>n</i> -Hexyl	H	48
11g *	CH ₃	5-Cl	69
11h	H	H	53
11i	Bn	5-Cl	67
11j	<i>n</i> -Hexyl	5-Cl	65

* Structure of products of 11 re-confirmed by single-crystal X-ray diffraction analysis [39].

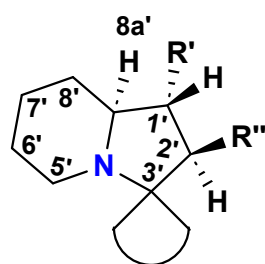


Figure 4. Numbering of positions in the indolizine moiety.

Nevertheless, it was not possible to discriminate conclusively by NMR techniques the correspondence of the structure of the isolated solid to either the **11a** or **12a** regioisomer. Fortunately, the growth of single-crystals of this compound suitable for X-ray diffraction unequivocally confirmed the regioselective formation of the spiro-derivative **11a** instead of **12a**, as shown in Figure 5 [39].

The scope of this reaction under the new established reaction conditions (i.e., reflux in acetonitrile), was extended to the diversely substituted isatin **6**, affording a new series of spirooxindolindolizine **11** in 45–69% yields (Table 3). Additionally, the growth of single crystals for some other derivatives of **11** and their X-ray diffraction analysis re-confirmed the regioselectivity of this reaction.

The possible mechanistic route for the formation of products **9/11** begins with the addition of the amino acid (i.e., L-proline **7a** or pipecolic acid **7b**) over the substituted isatin **6** at C-3, thus forming the additional products **I**, which, after cyclization and elimination of water, form the lactonic intermediates **II**. Subsequently, **II** may undergo decarboxylation process to generate the key azomethine ylides **AY-1** or **AY-2** (see Scheme 2).

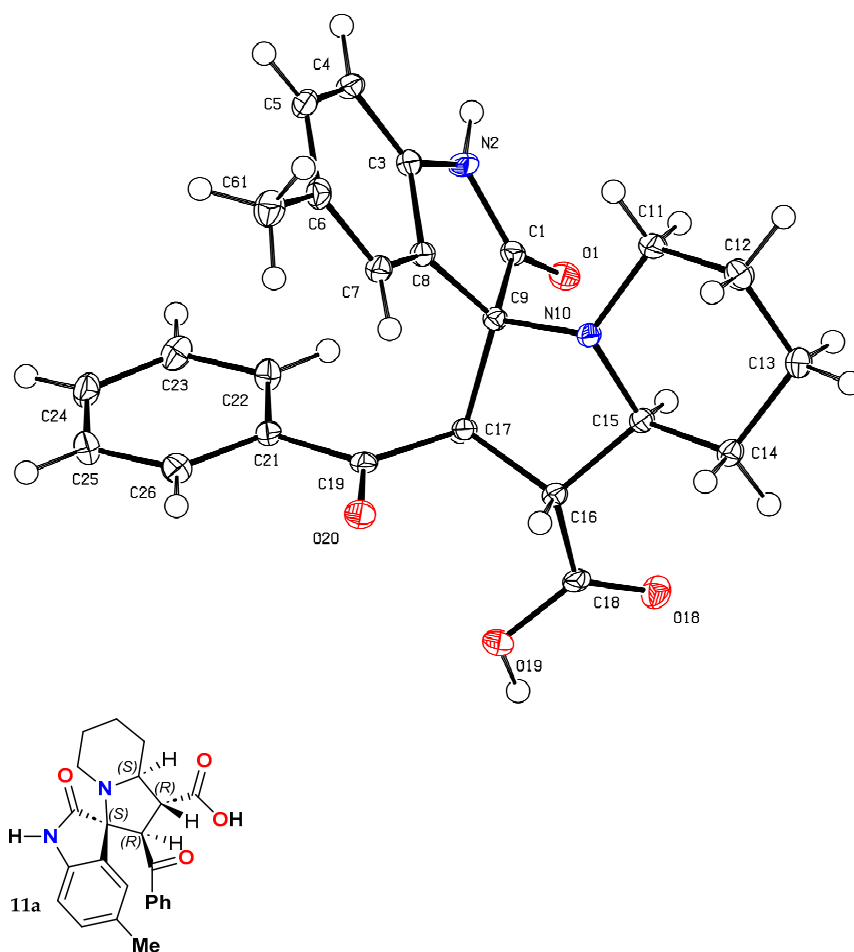
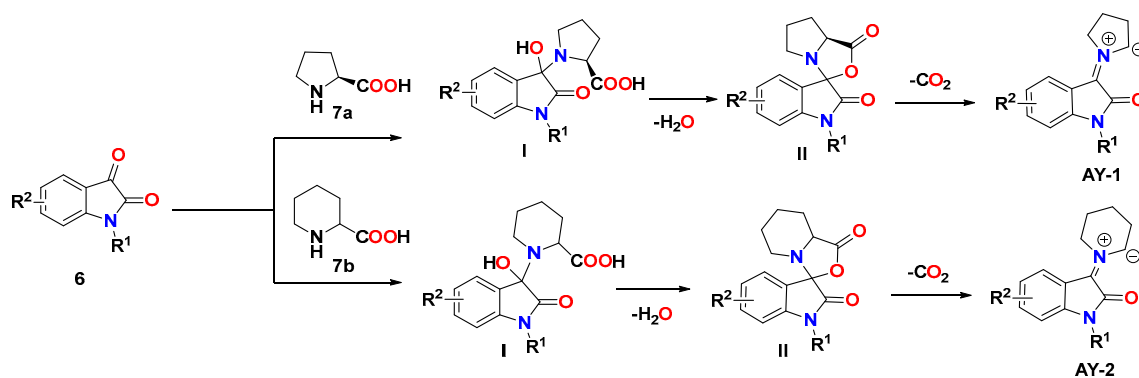


Figure 5. ORTEP diagram for the obtained product 11a.



Scheme 2. Plausible mechanistic route for the formation of azomethine ylides AY-1/AY-2.

In a second step, the azomethine ylides AY-1 or AY-2 react with the dipolarophile **8** via two possible transition states TS-1 or TS-2 (see Figure 6). The results obtained by X-ray diffraction allow us to confirm that the reaction passes through the TS-2 for the two products obtained, **9** and **11** [38,39]. Structures **9***/**11*** are stereoisomeric products of **9**/**11** but there is no evidence of their formation. Products **9** and **11** were obtained as enantiomeric pairs ($1'R,2'R,3S,7a'S$)/($1'S,2'S,3R,7a'R$) and ($1'R,2'R,3S,8a'S$)/($1'S,2'S,3R,8a'R$), respectively.

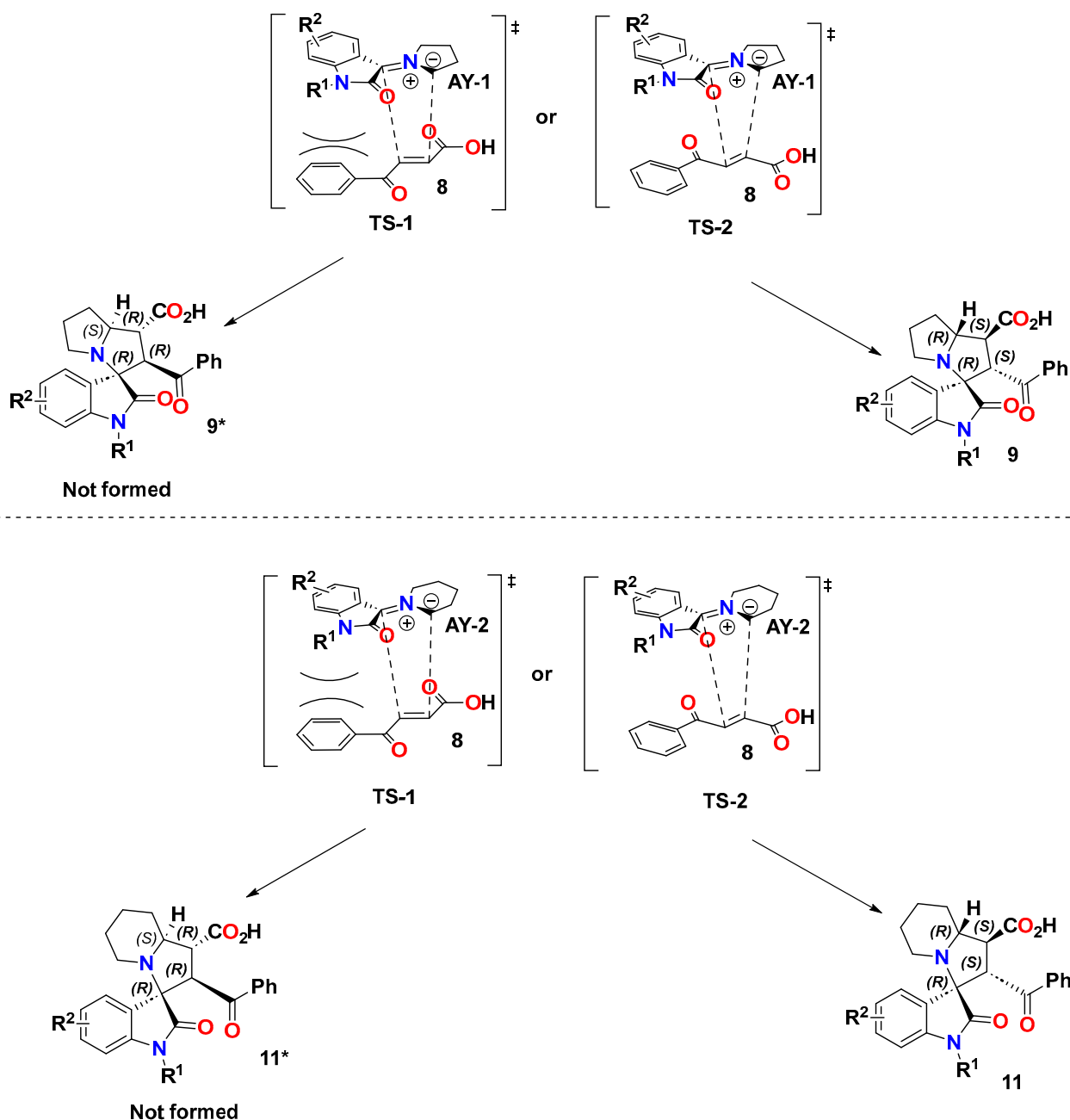


Figure 6. Plausible transition states for the 1,3-dipolar cycloaddition between the in situ generated azomethine ylides AY-1 and AY-2 and the dipolarophile 8.

3.2. Biological Evaluation

3.2.1. Antibacterial Activity

The antibacterial activities of the obtained compounds against *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Escherichia coli*, *Staphylococcus aureus*, and *Neisseria gonorrhoeae* (see Tables 4 and 5) were evaluated. The compounds showing inhibitory activity against *S. Aureus* were 9f, 9j, 11i, and 11j. Among these, the most active were 11i (*S. aureus* ATCC 25923 MIC: 4 µg/mL, *S. aureus* ATCC 43300 MIC: 16.12 µg/mL) and 9f (*S. aureus* ATCC 25923 MIC: 8 µg/mL, *S. aureus* ATCC 43300 MIC: 8 µg/mL) (see Figure 7). Interestingly, the most active ones contain in their structures the *N*-Bn group and 5-Cl substituent on the benzene ring. Whereas the compounds active against *N. gonorrhoeae* were the products 9d, 9f, 9j, 9m, 11f, 11i, 11j, with the most active compounds being 9d (MIC: 62.5 µg/mL) and 9m (MIC: 62.5 µg/mL). These compounds stand out for presenting an *N*-hexyl group, and,

in the case of **9m**, a 5-Cl substituent on the benzene ring, and were more active than their **9f** homologues (*N*-Bn and 5-Cl), and **9j** (*N*-Bn and 5,7-di-Cl) homologues.

Table 4. Antibacterial activity of spirooxindoles **9** containing the pyrrolizine moiety.

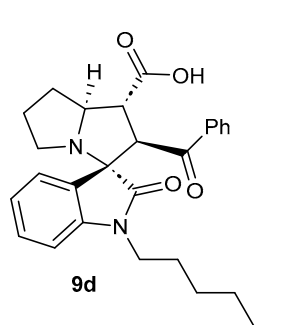
Compound	R ¹	R ²	Result
9a	Bn	H	Not measured
9b	CH ₃	H	Not active against <i>P. aeruginosa</i> , <i>K. pneumoniae</i> , <i>E. coli</i> , <i>S. aureus</i> , <i>N. gonorrhoeae</i>
9c	H	H	Not active against <i>P. aeruginosa</i> , <i>K. pneumoniae</i> , <i>E. coli</i> , <i>S. aureus</i> , <i>N. gonorrhoeae</i>
9d	Hexyl	H	Not active against <i>P. aeruginosa</i> , <i>K. pneumoniae</i> , <i>E. coli</i> , <i>S. aureus</i> , Active against <i>N. gonorrhoeae</i> MIC. 62.5 µg/mL
9e	CH ₃	5-Cl	Not active against <i>P. aeruginosa</i> , <i>K. pneumoniae</i> , <i>E. coli</i> , <i>S. aureus</i> , <i>N. gonorrhoeae</i>
9f	Bn	5-Cl	Not active against <i>P. aeruginosa</i> , <i>K. pneumoniae</i> , <i>E. coli</i> Active against <i>S. aureus</i> ATCC 25923 MIC: 8 µg/mL <i>S. aureus</i> ATCC 43300 MIC: 8 µg/mL <i>N. gonorrhoeae</i> MIC: 250 µg/mL
9g 9h	H H	5,7-di-Cl 5-CH ₃	Not active against <i>P. aeruginosa</i> , <i>K. pneumoniae</i> , <i>E. coli</i> , <i>S. aureus</i> , <i>N. gonorrhoeae</i>
9i	H	5-NO ₂	Not measured
9j	Bn	5,7-di-Cl	Not active against <i>P. aeruginosa</i> , <i>K. pneumoniae</i> , <i>E. coli</i> Active against <i>S. aureus</i> ATCC 25923 MIC: >500 µg/mL <i>S. aureus</i> ATCC 43300 MIC > 500 µg/mL <i>N. gonorrhoeae</i> MIC. 125 µg/mL
9k 9l	Bn H	5-F 5-F	Not active against <i>P. aeruginosa</i> , <i>K. pneumoniae</i> , <i>E. coli</i> , <i>S. aureus</i> , <i>N. gonorrhoeae</i>
9m	Hexyl	5-Cl	Not active against <i>P. aeruginosa</i> , <i>K. pneumoniae</i> , <i>E. coli</i> , <i>S. aureus</i> , Active against <i>N. gonorrhoeae</i> MIC. 62.5 µg/mL

Table 5. Antibacterial activity of spirooxindoles **11** containing the indolizine moiety.

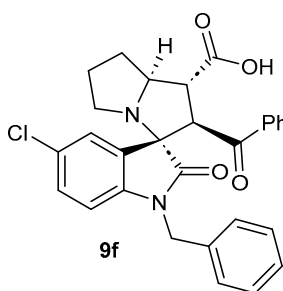
Product	R ¹	R ²	Result
11a 11b 11c 11d 11e	H H CH ₃ H Bn	5-CH ₃ 5-F H 5-Cl H	Not active against <i>P. aeruginosa</i> , <i>K. pneumoniae</i> , <i>E. coli</i> , <i>S. aureus</i> , <i>N. gonorrhoeae</i>
11f	Hexyl	H	Not active against <i>P. aeruginosa</i> , <i>K. pneumoniae</i> , <i>E. coli</i> , <i>S. aureus</i> Active against <i>N. gonorrhoeae</i> MIC: 1mg/mL
11g 11h	CH ₃ H	5-Cl H	Not active against <i>P. aeruginosa</i> , <i>K. pneumoniae</i> , <i>E. coli</i> , <i>S. aureus</i> , <i>N. gonorrhoeae</i>
11i	Bn	5-Cl	Not active against <i>P. aeruginosa</i> , <i>K. pneumoniae</i> , <i>E. coli</i> . Active against <i>S. aureus</i> ATCC 25923 MIC: 4 µg/mL <i>S. aureus</i> ATCC 43300 MIC: 16.12 µg/mL <i>N. gonorrhoeae</i> MIC: 500 µg/mL

Table 5. Cont.

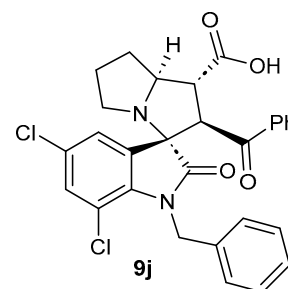
Product	R ¹	R ²	Result
11j	Hexyl	5-Cl	Not active against <i>P. aeruginosa</i> , <i>K. pneumoniae</i> , <i>E. coli</i> . Active against <i>S. aureus</i> ATCC 25923 MIC: >500 µg/mL <i>S. aureus</i> ATCC 43300 MIC > 500 µg/mL <i>N. gonorrhoeae</i> MIC 250 µg/mL



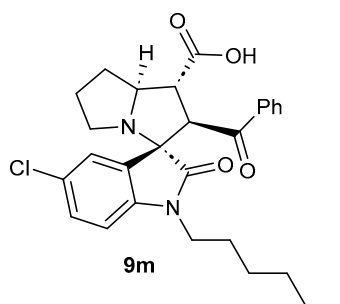
N. gonorrhoeae
MIC: 62.5 µg/mL



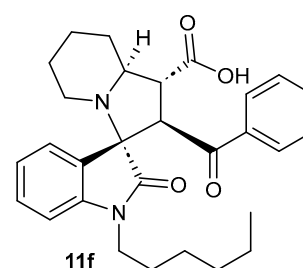
S. aureus ATCC 25923
MIC : 8 µg/mL
S. aureus ATCC 43300
MIC : 8 µg/mL
N. gonorrhoeae
MIC: 250 µg/mL



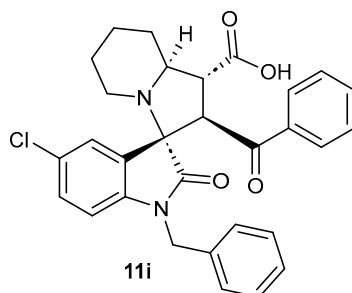
S. aureus ATCC 25923
MIC: >500 µg/mL
S. aureus ATCC 43300
MIC>500 µg/mL
N. gonorrhoeae
MIC: 125 µg/mL



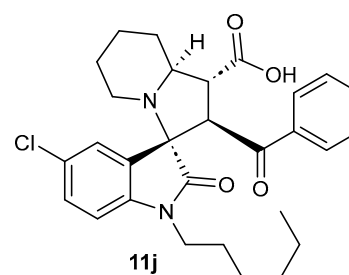
N. gonorrhoeae MIC: 62.5 µg/mL



N. gonorrhoeae MIC: 1 mg/mL



S. aureus ATCC 25923 MIC: 4 µg/mL
S. aureus ATCC 43300 MIC: 16.12 µg/mL
N. gonorrhoeae MIC: 500 µg/mL



S. aureus ATCC 25923 MIC: >500 µg/mL
S. aureus ATCC 43300 MIC >500 µg/mL
N. gonorrhoeae MIC: 250 µg/mL

Figure 7. Structure–activity relationship of spirooxindoles **9d**, **9f**, **9j**, **9m**, **11f**, **11i**, and **11j** against *S. aureus* and *N. gonorrhoeae*.

3.2.2. Toxicological Activity

Toxicity parameters, median lethal dose (LD₅₀) and toxicity classification were predicted for the active compounds using Protox II [44]. All compounds (6/6) showed predicted neurotoxicity, respiratory and clinical toxicity and, though less likely, nephrotoxicity and immunotoxicity. Some compounds showed alerts for blood–brain barrier toxicity (**9f**,

9j, 11i, 11j) and GABA receptor events (**9d, 9m, 11j**), though with a predicted low likelihood (CS < 0.7), as well as metabolic interference by cytochrome inhibition, as shown in Table 6. Most compounds (5/6) were classified as toxicity class 4.

Table 6. Toxicological data predicted with Protox II for active compounds: **9d, 9f, 9j, 9m, 11i, and 11j**.

Compound	9d	9f	9j	9m	11i	11j
Hepatotoxicity Consensus scoring (CS)	Inactive 0.77	Inactive 0.70	Inactive 0.70	Inactive 0.72	Inactive 0.70	Inactive 0.71
Neurotoxicity Consensus scoring (CS)	Active 0.62	Active 0.73	Active 0.73	Active 0.75	Active 0.77	Active 0.77
Cardiotoxicity Consensus scoring (CS)	Inactive 0.80	Inactive 0.79	Inactive 0.79	Inactive 0.80	Inactive 0.80	Inactive 0.81
Nephrotoxicity Consensus scoring (CS)	Active 0.60	Active 0.63	Active 0.63	Active 0.60	Active 0.63	Active 0.59
Respiratory toxicity Consensus scoring (CS)	Active 0.75	Active 0.72	Active 0.72	Active 0.77	Active 0.78	Active 0.79
Immunotoxicity Consensus scoring (CS)	Active 0.71	Inactive 0.76	Inactive 0.65	Active 0.88	Inactive 0.85	Active 0.81
Carcinogenicity Consensus scoring (CS)	Inactive 0.56	Inactive 0.61	Inactive 0.61	Inactive 0.59	Inactive 0.62	Inactive 0.60
Mutagenicity Consensus scoring (CS)	Inactive 0.67	Inactive 0.68	Inactive 0.68	Inactive 0.63	Inactive 0.68	Inactive 0.63
Cytotoxicity Consensus scoring (CS)	Inactive 0.62	Inactive 0.58	Inactive 0.58	Inactive 0.58	Inactive 0.61	Inactive 0.59
Clinical toxicity Consensus scoring (CS)	Active 0.69	Active 0.74	Active 0.74	Active 0.67	Active 0.78	Active 0.70
Blood brain barrier toxicity Consensus scoring (CS)	Inactive 0.57	Active 0.54	Active 0.54	Inactive 0.56	Active 0.53	Active 0.57
GABA receptor event Consensus scoring (CS)	Active 0.57	Inactive 0.61	Inactive 0.61	Active 0.53	Inactive 0.59	Active 0.52
Cytochrome inhibition (P450 2C9) Consensus scoring (CS)	Inactive 0.50	Active 0.57	Active 0.57	Active 0.60	Active 0.55	Active 0.57
LD ₅₀ (mg/Kg)	208	769	769	500	729	325
Toxicity class	3	4	4	4	4	4

LD₅₀: 50% lethal dose. GABA: gamma aminobutyric acid.

Consensus score is indicated as follows: <0.3 not good prediction, 0.3–0.7 marginal prediction. > 0.7 strong predictions. Toxicity alerts with consensus scoring (CS) > 0.7 are highlighted in bold. Toxicity classification: Class I: fatal if swallowed (LD₅₀ ≤ 5 mg/kg), II: fatal if swallowed (5 < LD₅₀ ≤ 50), III: toxic if swallowed (50 < LD₅₀ ≤ 300), IV: harmful if swallowed (300 < LD₅₀ ≤ 2000), V: may be harmful if swallowed (2000 < LD₅₀ ≤ 5000), VI: non-toxic (LD₅₀ > 5000). Toxicity *in vivo* of the compounds was also tested in the *Galleria mellonella* model. Five out of six compounds were at least at toxicity class 4 as no larval killing was observed when larvae were inoculated with 500 mg/kg. The toxicity class was the same *in vivo* and *in silico* for five out of six compounds. Although larval killing was observed at 500 mg/Kg for **11i** and **9d**, they showed lower LD₅₀ *in vivo* than toxicity predicted *in silico*.

The ability to induce membrane damage was tested *in vitro* by assessing the hemolytic activity of the compounds against human red blood cells (RBCs). The hemolytic activity

of the active compounds ranged from 2.4% to 100%. The spirooxindoles containing the pyrrolizine moiety showed higher hemolytic activity, with the compounds **9j** and **9f** having 2.4% and 16.6% hemolytic activity, respectively. By contrast, the spirooxindol-indolizines had lower hemolytic effects, and compounds **11f** and **11i** induced 2.3% and 8% hemolysis, respectively, suggesting that they have lower membrane interactions and toxicity.

3.3. Inverse Docking Analysis and Computing the Physicochemical Descriptors

The new spirooxindoles **9a–m** and spirooxindoles **11a–j** were docked against a total of 129 biological pathways and 26 human diseases reported in the Kyoto Encyclopedia of Genes and Genomes (KEGG) data base (see the list on the Supporting Information). The compounds were docked into the protein crystallographic structures available in the PDB database, at the exact position where the native ligands were coupled. The best docking results are shown in Table 7 (see the Supporting Information for more details). Comparison with the native ligands is shown in bold. While it is generally expected that native ligands exhibit high affinity for their respective protein targets, this assumption was only validated for the target protein 7Q6S.

Table 7. Highest docking affinities, spirooxindoles **9a–m** and spirooxindoles **11a–j**.

Protein Target	Compound	Docking Score (Kcal/mol) ^a
6TYM	11e	−10.4
6TYM	11i	−10.6
6TYM	Native ligand ^b (08A)	−10.7
4JYG	9b	−10.5
4JYG	Native ligand (1NY)	−9.7
5KIR	9c	−10.6
5KIR	9l	−10.2
5KIR	Native ligand (RCX)	−6.4
5UZK	9g	−10
5UZK	Native ligand (504)	−9.8
7Q6S	9b	−10
7Q6S	11e	−10.1
7Q6S	11a	−10.1
7Q6S	9h	−10
7Q6S	Native ligand (91M)	−12.6

^a Docking score (kcal/mol) calculated using AutoDock Vina. ^b Reported native ligand co-crystallized, PDB database.

Additionally, physicochemical descriptors, pharmacokinetics and druglikeness were assessed using the SwissADME database tool [46]. A summary is shown in Table 8 (see Supplementary Data). From these results, the best molecules were **9b**, **9c**, **9d**, **9g**, **9l**, and **11j**, as these molecules were predicted as noninhibitors of CYP3A4 and CYP2D6, which are the two most abundant cytochrome P450 enzymes that are involved in drug metabolism.

Table 8. Physicochemical descriptors of spirooxindoles **9a–m** and spirooxindoles **11a–j**.

Compound	GI Absorption	MW	#H-Bond Acceptors	#H-Bond Donors	Log P	CYP2D6 Inhibitor	CYP3A4 Inhibitor
9a	High	466.53	5	1	2.74	Yes	Yes
9b	High	390.43	5	1	1.47	No	No
9c	High	376.41	5	2	1.37	No	No

Table 8. Cont.

Compound	GI Absorption	MW	#H-Bond Acceptors	#H-Bond Donors	Log P	CYP2D6 Inhibitor	CYP3A4 Inhibitor
9d	High	460.56	5	1	3.28	No	No
9e	High	424.88	5	1	2.10	Yes	Yes
9f	High	500.97	5	1	3.24	Yes	Yes
9g	High	445.3	5	2	2.48	No	No
9h	High	390.43	5	2	1.69	Yes	Yes
9i	High	421.4	7	2	0.63	Yes	Yes
9j	High	535.42	5	1	3.72	No	No
9k	High	484.52	6	1	3.01	Yes	Yes
9l	High	394.4	6	2	1.68	No	No
9m	High	495.01	5	1	3.76	Yes	No
11a	High	404.46	5	2	1.98	Yes	Yes
11b	High	408.42	6	2	1.98	Yes	Yes
11c	High	404.46	5	1	1.82	Yes	Yes
11d	High	424.88	5	2	2.16	Yes	Yes
11e	High	480.55	5	1	2.91	Yes	Yes
11f	High	474.59	5	1	3.55	Yes	No
11g	High	438.9	5	1	2.41	Yes	Yes
11h	High	390.43	5	2	1.62	Yes	Yes
11i	High	515	5	1	3.51	Yes	Yes
11j	High	509.04	5	1	4.02	No	No

4. Conclusions

New and diversely substituted spirooxindoles **9** and **11** were synthesized by a 1,3-dipolar cycloaddition reaction between substituted isatins with two amino acids (i.e., L-proline, and pipecolic acid) and *trans*-3-benzoyl acrylic acid as dipolarophile. Spirooxindoles **9a–m** were obtained in 40–86% yields by reflux in methanol, while spirooxindoles **11a–j** were obtained in 45–69% yields by reflux in acetonitrile.

The most active products against *S. Aureus* were **11i** (*S. aureus* ATCC 25923 MIC: 4 µg/mL, *S. aureus* ATCC 43300 MIC: 16.12 µg/mL) and **9f** (*S. aureus* ATCC 25923 MIC: 8 µg/mL, *S. aureus* ATCC 43300 MIC: 8 µg/mL) and the most active compounds against *N. gonorrhoeae* were **9d** (MIC: 62.5 µg/mL) and **9m** (MIC: 62.5 µg/mL). Toxicological analysis in vivo and in silico of active compounds **9d**, **9f**, **9j**, **9m**, **11i**, and **11j** showed that 5/6 compounds were at toxicity class 4, and compounds **11i** and **9d** showed lower LD₅₀ in vivo than in silico. The hemolytic activity of the active compounds ranged from 2.4% to 100%. Overall, the most active compounds against *S. aureus* **9f** and **11i** and *N. gonorrhoeae* **9m** and **9d** may be starting points for further improvement of the activity and lowering of potential toxicity.

Inverse docking analysis showed that compounds **11e**, **11i**, **11a**, and **9b** displayed high affinity toward the protein targets 6TYM and 7Q6S (both are a KEAP1, INrf2, KLHL19), these proteins are involved in biological pathways such as those associated with Parkinson's disease, different pathways in cancer, chemical carcinogenesis with reactive oxygen species, hepatocellular carcinoma, and fluid shear stress and atherosclerosis. On the

other hand, compounds **9c** and **9l** reported a high affinity to the protein 5KIR (a human cyclooxygenase-2) displaying higher affinity than the Rofecoxib, a COX-2-selective nonsteroidal anti-inflammatory drug (NSAID).

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/chemistry7010018/s1>, Chemistry: ^1H NMR, ^{13}C NMR, DEPT-135 (1D NMR) spectra for compounds **9a–m** and **11a–j** (Figures S1–S76), 2D NMR spectra for compounds **9a** and **11a** (Figures S4–S7, Figures S46–S49); FT-IR spectra of compounds **9a–m** and **11a–j** (Figures S77–S99). Antibacterial activity: Antimicrobial screening of the novel heterocycles **9a–m** and **11a–j** (Table S1). Inverse docking analysis: Tested biological pathways and diseases, KEGG database (Table S2). Predicted physicochemical descriptors, pharmacokinetic properties, druglike nature and medicinal chemistry friendliness of spirooxindoles **9a–m** and spirooxindoles **11a–j** (Table S3). Docking scores of the evaluated spirooxindoles **9a–m** and spirooxindoles **11a–j** (Table S4).

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