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Graphical Abstract

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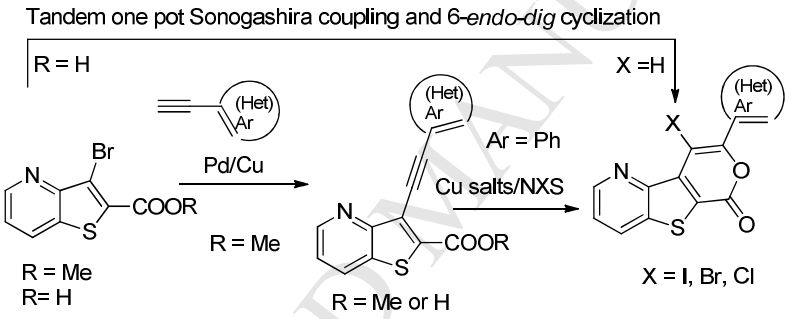

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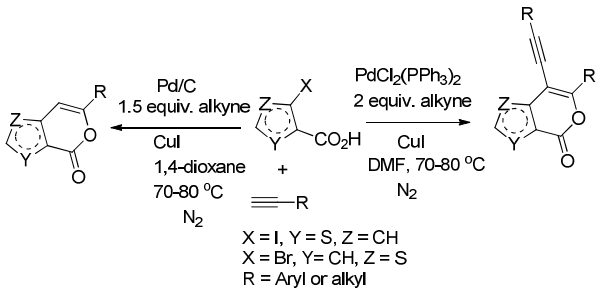
ABSTRACT

Novel 8-(het)aryl-6*H*-pyrano[4',3':4,5]thieno[3,2-*b*]pyridines were prepared in good to high yields by a tandem one-pot procedure of Sonogashira coupling and 6-*endo-dig* lactonization from 3-bromothieno[3,2-*b*]pyridine-2-carboxylic acid and (het)arylalkynes. Sonogashira coupling products were also prepared from the corresponding methyl ester giving in the same reaction the corresponding 6-*endo-dig* compounds as minor products. The Sonogashira phenyl ester product gave cyclization with electrophiles only in low to moderate yields. Nevertheless, halolactonizations using Cu(I) or (II) salts/*N*-halosuccinimides (NXS) from either the phenyl ester or the carboxylic acid derivatives occurred in good to high yields. The growth inhibition potential of the compounds was evaluated using human tumor cell lines, HCT-15 (colorectal adenocarcinoma) and NCI-H460 (non-small cell lung cancer) and studies of apoptosis induction were performed for the three most promising compounds in HCT-15 cells. Two of them caused almost 40% of cell death by apoptosis when tested at their 1.5×GI_{50} concentrations. The tricyclic lactone with a F atom in the *meta* position showed to be the most promising one.

1. Introduction

2*H*-Pyran-2-ones (2-pyranones) represent an important class of naturally occurring δ-lactones together with their benzo derivatives isocoumarins and have shown a wide range of pharmacological activities.[1,2]

The thiophene ring is a bioisostere of benzene and is present in many bioactive agents and drugs. Nevertheless, thienopyranones are rather unusual. In 2006 Pal *et al.* reported a one-pot regioselective synthesis of the 6-*endo-dig* products, alkynylated or not in the pyranone ring, using the Sonogashira coupling[3a,b] of 3-iodothiophene-2-carboxylic acid or 2-bromothiophene-3-carboxylic acid with several terminal alkynes in the presence of different palladium catalysts and solvents (Scheme 1).[4] These authors studied some of the compounds for their *in vitro* antitumor activity and concluded that further exploration of the scaffolds was necessary for the synthesis of more promising antitumor compounds.



Scheme 1. Pal's *et al.* work.[4]

Our research group in 2009 reported the synthesis of several 3-arylbenzothieno[2,3-*c*]pyran-1-ones iodinated or not in the pyranone ring from Sonogashira coupling products of methyl 3-bromobenzo[*b*]thiophene-2-carboxylates with several arylalkynes, followed by electrophilic 6-*endo-dig* cyclizations[5]

using I_2 or TFA. The Sonogashira coupling of the 3-bromobenzo[*b*]thiophene-2-carboxylic acid with arylalkynes gave in a one-pot procedure the corresponding tricyclic 6-*endo-dig* lactones. The synthesized compounds showed to have a promising antitumor activity with some derivatives (either Sonogashira products or lactones) presenting GI_{50} values approaching 10 μM in the cell lines tested.[6]

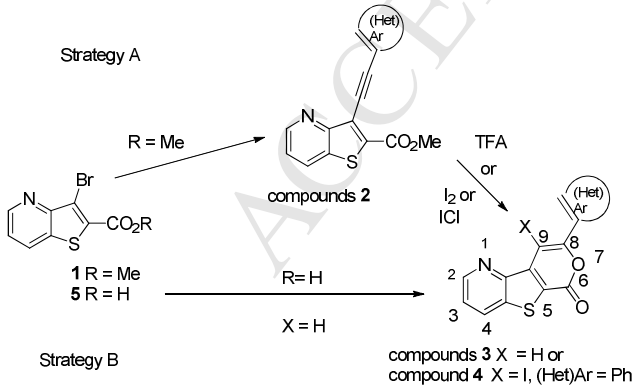
For some years now we have been interested in the synthesis and antitumor evaluation of new functionalized thieno[3,2-*b*]pyridines either on the pyridine ring by C-C Suzuki-Miyaura[7] and Sonogashira,[8] C-N Buchwald-Hartwig,[9,10a] and C-O Ullmann couplings[10a,b] or on the thiophene ring by C-N coupling.[11] Some of the compounds synthesized by the different metal catalyzed couplings showed GI_{50} values below 10 μM in different human tumor cell lines and for those some insights on the mechanism of action were achieved.

Herein the synthesis of novel tricyclic lactones 8-(het)aryl-6*H*-pyrano[4',3':4,5]thieno[3,2-*b*]pyridines in a tandem one-pot procedure of Sonogashira coupling of 3-bromothieno[3,2-*b*]pyridine-2-carboxylic acid with several (het)arylalkynes followed by a 6-*endo-dig* lactonization, is reported. The cyclization of the methyl 3-(phenylethynyl)thieno[3,2-*b*]pyridine-2-carboxylate with electrophiles occurred only in low yields. Nevertheless, the halolactonizations to the tricyclic lactones were achieved in good to high yields from the latter or the corresponding carboxylic acid using Cu(I) or Cu(II) salts and the corresponding *N*-halosuccimide (NXS).[12]

The Sonogashira compounds and the tricyclic lactones were evaluated for their in vitro cell growth inhibition ability, using the human tumor cell lines HCT-15 (colorectal adenocarcinoma) and NCI-H460 (non-small cell lung cancer). For the most promising compounds, studies of induction of apoptosis were also performed.

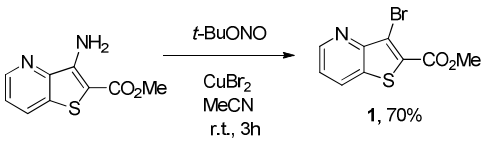
2. Resuts and Discussion

Initially we envisaged two possible strategies (Scheme 2, A and B) to obtain 8-(het)aryl-6*H*-pyrano[4',3':4,5]thieno[3,2-*b*]pyridines iodinated or not at position 9.



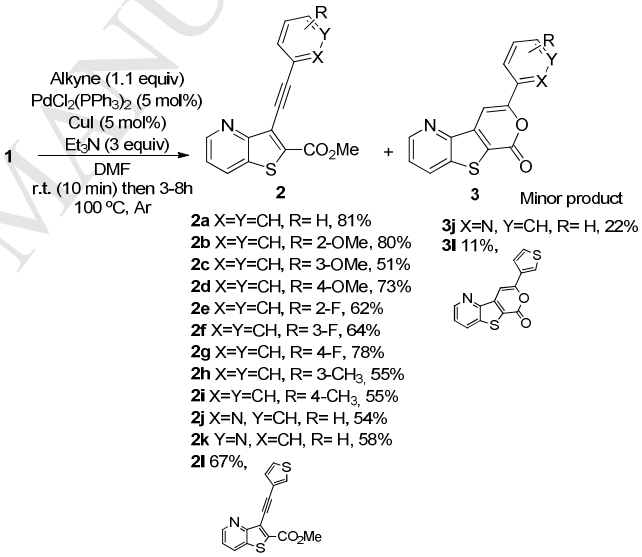
Scheme 2. Envisaged strategies to obtain 8-(het)aryl-6*H*-pyrano[4',3':4,5]thieno[3,2-*b*]pyridines iodinated or not at position 9.

For strategy A, the methyl 3-bromothieno[3,2-*b*]pyridine-2-carboxylate **1** was prepared from the methyl 3-aminothieno[3,2-*b*]pyridine-2-carboxylate[13] using *t*-BuONO and $CuBr_2$ (Scheme 3), following a procedure found for the synthesis of the corresponding ethyl ester.[14]



Scheme 3. Synthesis of 3-bromo compound **1** from 3-aminothieno[3,2-*b*]pyridine-2-carboxylate.

The reaction of compound **1** with several (het)arylalkynes by Sonogashira coupling, using $PdCl_2(PPh_3)_2$, CuI, Et_3N as a base and DMF as solvent, gave the new Sonogashira products 2 in good to high yields and also the corresponding 6-endo-dig lactones **3** as minor products. The conditions used avoided the synthesis in high extent of the (het)arylalkyne dimer which is always formed. Among the cyclized products only compounds **3j** and **3l** were obtained in quantifiable yields (Scheme 4).



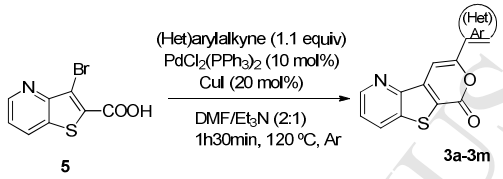
Scheme 4. Synthesis of methyl 3-[(het)arylethynyl]thieno[3,2-*b*]pyridine-2-carboxylates **2** as major products and 8-(het)aryl-6*H*-pyrano[4',3':4,5]thieno[3,2-*b*]pyridines **3** as minor products, **3j** and **3l** with quantifiable yields.

Several attempts were performed to obtain only compounds **3** from compound **1**. Different Cu catalysts and/or TFA were added after the evidence of the formation of compounds **2** by TLC. However, most of the attempts gave only the cyclized compounds in low to moderate yields or were unsuccessful.

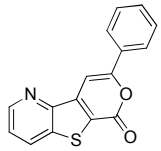
The next efforts consisted in the use of compound **2a** as starting material, either to obtain compound **3a** with TFA at rt (in 30% yield) or the corresponding 9-iodo tricyclic lactone **4** by halocyclization in CH_2Cl_2 at r.t. with I_2 or ICl (Scheme 2). However, contrary to the expected, the yields of the products were low (24 and 19%, respectively). Thus, for the thieno[3,2-*b*]pyridine system strategy A of (halo)lactonatization was not very successful.

For strategy B, the carboxylic acid **5** was prepared from compound **1** using LiOH in THF/MeOH/H_2O 6:1:1 at r.t. for 3h,[15] and was reacted with several (het)arylalkynes to give the tricyclic lactones **3a-m** in a tandem one-pot procedure of Sonogashira coupling followed by a 6-*endo-dig* lactonization. Compounds **3** were obtained in good to high yields, except the 2-ethynylpyridine derivative **3j** that was isolated only in 20% yield (Table 1). The compounds obtained in the highest yields were those resulting from the reaction with phenylacetylene (**3a**) or with arylacetylenes bearing a *meta*-substituent relative to the triple bond (**3c**, **3f** and **3h**). The *para*-substituents used appear to diminish the yields due to the increase of electron density on the triple bond either by the electron donating resonance effects of the OMe, NH_2 and the lone-pairs of electrons of the F atom or by the positive inductive effect and hyperconjugation of the methyl group, which do not benefit the cyclization reaction. These effects can also decrease the deprotonation of the *p*-substituted arylalkynes in the Sonogashira coupling, but this was not observed in the synthesis of compounds **2** from compound **1** (Scheme 4). The lactones **3k** and **3l** derivatives of 3-ethynylpyridine and 3-ethynylthiophene were obtained in similar good yields despite the different electronic character of the rings.

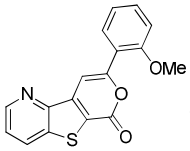
Table 1. Synthesis of lactones **3a**-**3m** from compound **5** and several (het)arylalkynes in a tandem one pot procedure of Sonogashira coupling and 6-*endo-dig* cyclization.



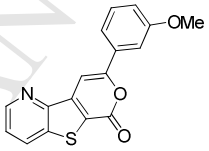
5 **3a-3m**



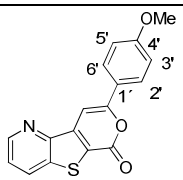
3a, 80%



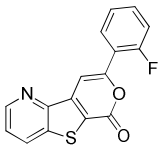
3b, 59%



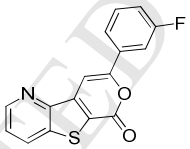
3c, 78%



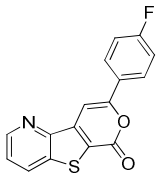
3d, 58%



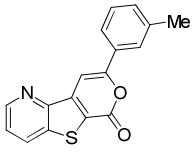
3e, 55%



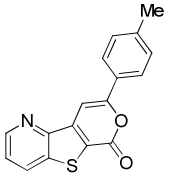
3f, 62%



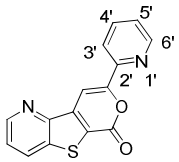
3g, 55%



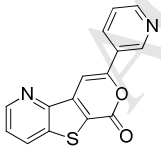
3h, 76%



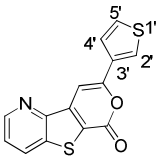
3i, 53%



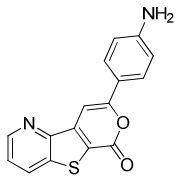
3j, 20%



3k, 54%



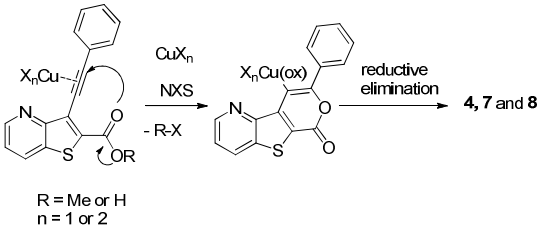
3l, 50%



3m, 50%

After testing strategies A and B (Scheme 2) only strategy B showed to succeful. Thus, other reactions were performed from **2a** or the corresponding carboxylic acid **6** to obtain the 9-halogenated tricyclic lactones **4**, **7** and **8** using Cu salts/NXS (Table 2). The conditions used were based on the work of Miyata *et al.* that regioselectively synthesized the 4-chloro-3-phenylisocoumarin by chlorocyclization of the methyl 2-(phenylethynyl)benzoate with $CuCl_2$/*N*-chlorosuccinimide (NCS). Although these authors referred that the mechanism was not fully elucidated, they suggested that the activation of the alkyne moiety by $CuCl_2$ was followed by the nucleophilic addition of the carbonyl oxygen to form a Cu intermediate *via* a 6-*endo-dig* cyclization in the case of the methyl 2-(phenylethyny) benzoate. The Cu species formed are then oxidized by another equiv. of $CuCl_2$ to generate Cu(III) species. After chlorination at the copper atom by NCS, reductive elimination affords the

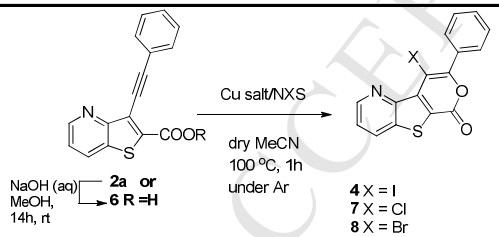
product.[12] An adjustment of this proposal to our starting materials using Cu(I) or (II) salts/NXS is depicted in Scheme 5.



Scheme 5. Proposed mechanism of halocyclization of thieno[3,2-*b*]pyridine alkynyl ester or acid derivatives with Cu salts/NXS.

In order to compare results Cu(I) and (II) chloride and bromide salts were used with the corresponding NXS. In the case of copper iodide salts only CuI is stable. From analysis of table 2 it is only possible to conclude which are the best conditions to obtain the chloro or bromo tricyclic lactones **7** or **8** from each substrate and not find a general conclusion about the best copper species to be used. The best yields for the 9-chloro tricyclic lactone **7** were achieved from the ester **2a** using $CuCl_2$/NCS (entry 1, 80%) and from the acid **6** using CuCl/NCS (entry 4, 63%). On the contrary, the bromolactone **8** was obtained in an excellent yield from the acid **6** using $CuBr_2$/*N*-bromosuccinimide (NBS) (entry 9, 86%) but it was not detected (ND) from **2a** using the same conditions (entry 5). Nevertheless, lactone **8** was obtained in 35% yield using CuBr/NBS (entry 6). An acidic medium appears to benefit the system $CuBr_2$/NBS and CuCl/NCS in the synthesis of **8** and **7**, respectively from compound 6 (entries 4 and 7). The same was not observed for the synthesis of **8** from **6** using CuBr/NBS (entry 8, 42%). The iodo tricyclic lactone **4** was obtained in good yields from both starting materials **2a** and **6** using CuI/*N*-iodosuccinimide (NIS) (entries 9 and 10) proving that the nature of the substrate has no effect on these reagents. These halocyclizations will allow further functionalizations of the tricyclic lactones.

Table 2. Conditions and yields for the synthesis 9-halogenated lactones **4a-c**.



Entry	Starting material	Compound obtained	Reagents	Yield (%)
1	**2a**	**7**	$CuCl_2$/NCS	80
2	**2a**	**7**	CuCl/NCS	20
3	**6**	**7**	$CuCl_2$/NCS	35
4	**6**	**7**	CuCl/NCS	63
5	**2a**	**8**	$CuBr_2$/NBS	ND
6	**2a**	**8**	CuBr/NBS	35
7	**6**	**8**	$CuBr_2$/NBS	86
8	**6**	**8**	CuBr/NBS	42
9	**2a**	**4**	CuI/NIS	46
10	**6**	**4**	CuI/NIS	52

The proportions used were Cu salt (2.5 equiv.)/ NXS (2 equiv.)[12]
The product yields were determined after column chromatography.

The antitumor potential of compounds **2** and **3** was evaluated by their cell growth inhibitory effect in two different important human tumor cell lines, HCT-15 (colorectal adenocarcinoma) and NCI-H460 (non-small cell lung cancer), performing a screening colorimetric assay with sulforhodamine B.[16,17] The results, including the GI_{50} concentrations, which inhibited 50% of the cell growth, are presented in Table 3. The GI_{50} concentrations could not be determined for some compounds, since they formed crystals at lower concentrations. In these cases, the GI_{50} was considered higher than the highest concentration tested before crystals were formed. Compounds **3d**, **3j** and **3k** were not soluble in DMSO and could not be included in this study.

Table 3. Results of the cell growth inhibitory effect of compounds **2** and **3** on HCT-15 and NCI-H460 cell lines.

	GI_{50} (μM)	
	HCT-15	**NCI-H460**
2a	28 ± 0.8	49 ± 14.3
2b	17.7 ± 1.9	54.7 ± 9.0
2c	18.5 (n=2)	> 20
2d	17.7 ± 0.5	27.7 ± 2.2
2e	> 18.75	> 18.75
2f	> 5	> 5
2g	> 18.75	> 18.75
2h	> 10	> 10
2i	5.6 ± 0.6	> 75
2j	10.8 ± 1.1	17.0 ± 1.2
2k	28.3 ± 1.8	54.0 ± 2.9
2l	41.7 ± 0.7	45 ± 1.4
3a	> 10	> 10
3b	> 5	> 5
3c	> 0.625	> 0.625
3e	> 5	> 5
3f	7.4 ± 1.1	7.3 ± 0.1
3g	> 2.5	> 2.5
3h	> 10	> 10
3i	> 2.34	> 2.34
3l	29.3 ± 2.6	26.3 ± 2.8
3m	12.6 ± 2.9	11.4 ± 0.9

[a] GI_{50} concentrations correspond to the mean ± S.E.M. of at least three independent experiments (except when crystals were formed bellow the GI_{50} concentration, in which case only one experiment was performed).

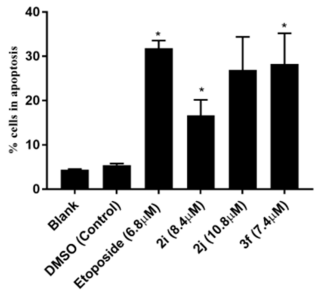
Doxorubicin was used as a positive control (353.3 ± 24.2 nM in HCT-15 cells and 25 ± 0.8nM in NCI-H460 cells).

From the results obtained (Table 3) it is possible to establish some structure-activity relationships for the compounds with determined GI_{50}. Among the aryl derivatives Sonogashira coupling products, the functionalizations decrease in general the GI_{50} values and turn the compounds more selective for the HCT-15 cell line, with compound **2i** (with a Me group in the *para* position) being completely selective. Among the Sonogashira hetaryl derivatives, only compound **2j** (obtained from 2-ethynylpyridine) presented a low GI_{50} value in HCT-15 cells. It was very difficult to determine the GI_{50} values of the tricyclic lactones, but for the ones whose GI_{50} concentrations were determined it is possible to conclude that they are not selective between the two cell lines and only compound **3f** (with a F atom in the *meta* position) presented a GI_{50} below 10 µM.

In order to understand if the most potent compounds **2i**, **2j** and **3f** had an effect on apoptosis, the Annexin-V/PI assay was performed in the most sensitive cell line HCT-15. This was treated with the compounds at two different concentrations (one equal or close to the GI_{50}, and another 1.5 × higher than the previous concentration) to see if there was a dose-dependent effect, or with appropriate controls. Representative histograms of the flow cytometry analysis of apoptotic cell death following AnnexinV-FITC/PI staining in HCT-15 cells are shown in the Supp. Material.

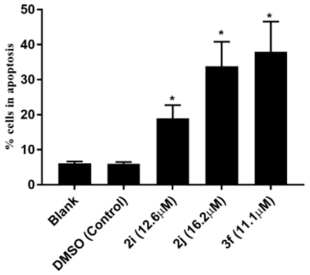
The results presented as graphs with the calculated % of cell death by apoptosis, clearly demonstrated that the three compounds tested caused a statistically significant increase in the % of cell death by apoptosis in the HCT-15 cells, when compared to the Blank cells. However, the results visibly show a dose-dependent apoptotic effect for the three compounds, since the % of apoptotic cells increased with increasing concentrations (Figure 1).

A.



	% cells in apoptosis
Blank	4.4 ± 0.1
DMSO (control)	5.4 ± 0.4
Etoposide	31.8 ± 1.7
2i (8.4µM)	16.7 ± 3.5
2j (10.8µM)	26.9 ± 7.5
3f (7.4µM)	28.3 ± 6.9

B.



	% cells in apoptosis
Blank	6.1 ± 0.6
DMSO (control)	6.1 ± 0.5
2i (12.6µM)	19.0 ± 3.8
2j (16.2µM)	33.9 ±6.9
3f (11.1µM)	38.0 ± 8.6

Figure 1. Effect of the compounds **2i**, **2j** and **3f** on the levels of HCT-15 cell death by apoptosis. Cells were treated for 48h with medium (Blank), the compounds vehicle (DMSO), etoposide at its GI_{50} (6.8 µM in HCT-15)[18] was used as positive control or with the compounds. **(A)** The graph shows the effect of **2i** at 8.4 µM (corresponding to 1.5 × GI_{50}), **2j** at its GI_{50} concentration (10.8 µM), **3f** at its GI_{50} concentration (7.4 µM) and respective controls; the table presents the % of cells undergoing apoptosis for each treatment. Results are the mean ± S.E.M. of three independent experiments. *p ≤ 0.05 treatment vs the respective control. (B) The graph shows the effect of **2i**, **2j**, **3f** at 1.5 × the concentrations tested in (A) and respective controls; The table presents the % of cells undergoing apoptosis for each treatment. Results are the mean ± S.E.M of three independent experiments. *p ≤ 0.05 treatment vs the respective control.

Compound **3f** is a strong inducer of apoptosis causing similar levels of apoptosis (28.3%) as the positive control etoposide (31.8%), when tested at their respective GI_{50} concentrations. Compound **2j** and compound **3f** caused apoptosis in more than 30% and almost 40% of the treated cells, respectively, at their 1.5× GI_{50} values.

3. Conclusions

In conclusion we were able to synthesize novel 8-(het)aryl-6*H*-pyrano[4',3':4,5]thieno[3,2-*b*]pyridines by a tandem one-pot procedure of Sonogashira coupling and 6-*endo-dig* cyclization from 3-bromothieno[3,2-*b*]pyridine-2-carboxylic acid and several (het)arylalkynes, in good to high yields. Sonogashira coupling products were also prepared from the methyl 3-bromothieno[3,2-*b*]pyridine-2-carboxylate with several (het)arylalkynes, giving in the same reaction the 6-*endo-dig* lactones as minor products. The cyclization of the Sonogashira phenyl ester product with electrophiles (I_2, ICl, TFA) only occurred in low to moderate yields. The halolactonizations were achieved with Cu(I) and/or (II) salts and the corresponding NXS from the methyl 3-(phenylethynyl)thieno[3,2-*b*]pyridine-2-carboxylate or the corresponding carboxylic acid. It was possible to conclude which were the best starting materials and Cu salts to obtain the chloro and bromolactones in the highest yields, but it was not possible to reach an overall conclusion about the use of Cu(I) or (II) salts. The iodocyclization occurred using CuI/NIS in good yields, independently of the substrate. This methodology opens avenues for further functionalizations of these tricyclic lactones.

The antitumor potential of the Sonogashira compounds **2** and lactones **3** was evaluated on two human tumor cell lines and the three most potent compounds **2i**, **2j** and **3f** in HCT-15 cells (GI_{50}<10 µM) showed to induce apoptosis in a dose dependent manner. The Sonogashira product **2j** and the lactone **3f** caused apoptosis in nearly 40% of cells, when tested at their 1.5×GI_{50} concentrations. The latter, with a F atom in the meta position, was shown to be the most promising compound.

4. Experimental section.

Melting points (°C) were determined in open capillary tubes and are uncorrected. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance III 400 or on a Bruker Avance III HD nanobay 400 (^{1}H at 400, ^{13}C at 100.6 and ^{19}F at 376.48 MHz), and the chemical shifts were quoted in parts per million (ppm) referenced to the appropriate non-deuterated solvent peak relative to 0.0 ppm for tetramethylsilane. Two dimensional correlations ^{1}H-^{1}H (COSY) and ^{1}H–^{13}C (HSQC and HMBC) were performed to attribute some signals. Low resolution MS/ESI of compounds **3k**, **3i**, **4**, **7**, **8** and **6** were performed by direct injection on a ThermoFinigan spectrophotometer LC–MS and high-resolution mass spectra (HRMS) were performed on a Maxis UHR-q-TOF mass spectrometer Bruker 4G (Bruker, Wissembourg, France), with an electrospray ionisation (ESI) mode. Elemental Analysis

was performed on a LECO CHNS 932 Elemmental Analyser. The reactions were monitored by thin layer chromatography

(TLC) using Macherey-Nagel pre-coated aluminium silica gel 60 sheets (0.20 mm) with UV254 indicator. Column chromatography was performed on Panreac, Silica Gel 60, 230–400 mesh. Ether refers to diethylether. Petroleum ether refers to the boiling range 40–60 °C.

4.1. Synthesis of precursors 1, 5 and 6

*4.1.1. Methyl 3-bromothieno[3,2-b]pyridine-2-carboxylate (**1**)*

To a round bottom flask with dry MeCN (5 mL), $CuBr_2$ (237 mg; 1.15 mmol; 1.2 equiv) and *tert*-butyl nitrite (0.197 mL; 1.44 mmol; 1.5 equiv)[14] and finally the amine **2**[13] (200 mg; 0.960 mmol; 1.0 equiv) were added, the latter in small portions. The mixture was stirred at r.t. for 1 hour. Saturated $NH_4Cl_{(aq)}$ solution (10 mL) was added and mixture was extracted with dichoromethane (4 × 10mL). The organic fractions were collected, washed with water (4 × 10 mL) and brine (10 mL), dried ($MgSO_4$), filtered and evaporated to give a dark brown oil. This was submitted to column chromatography using a solvent gradient from petroleum ether to 4:6 ether:petroleum ether, to give compound **1** as a white solid (183 mg, 70%) m.p. 141-143 ºC. ^{1}H NMR (400 MHz, DMSO-d_6): δ = 3.93 (s, 3H, OMe), 7.64 (dd, *J* = 8.0 and 4.4 Hz, 1H, 6-H), 8.63 (dd, *J* = 8.0 and 1.6 Hz, 1H, 7-H), 8.87 (dd, 1H, *J* = 4.4 and 1.6 Hz, 5-H) ppm. ^{13}C NMR (100.6 MHz, DMSO-d_6): δ = 53.1 (OMe), 116.7 (C), 122.8 (6-CH), 130.6 (C), 132.3 (7-CH), 133.3 (C), 149.6 (5-CH), 151.3 (C), 160.9 (C=O) ppm. MS (ESI): m/z (%) 274 [M ^{81}Br+H]$^+$ (100), 272 [M ^{79}Br +H]$^+$ (96). HRMS (ESI)**:** [M ^{81}Br+H]$^+$ calcd for $C_9H_7{}^{81}BrNO_2S$: 273.9355; found 273.9354. [M ^{79}Br +H]$^+$ Calcd. for $C_9H_7{}^{79}BrNO_2S$: 271.9376, found: 271.9375.

*4.1.2. 3-bromothieno[3,2-b]pyridine-2-carboxylic acid (**5**)*

In a round bottom flask compound **1** (500 mg, 1.840 mmol) and LiOH (165 mg, 6.900 mmol, 3.75 equiv) were added to a mixture of THF/methanol/water (12: 2: 2 mL).[15] The reaction was stirred at r.t. for 3h. After that the solution was concentrated under vacuum and HCl 1M was added until pH=5. A precipitate came out and it was filtered and dried in an oven overnight at 50 ºC to give compound **5** as a yellowish solid (460 mg; 97%), m.p. 221-222 ºC. ^{1}H NMR (400 MHz, DMSO-d_6): δ = 7.61 (dd, *J* = 8.4 and 4.4 Hz, 1H, 6-H), 8.60 (dd, *J* = 8.4 and 1.6 Hz, 1H, 7-H), 8.85 (dd, *J* = 4.4 and 1.6 Hz, 1H, 5-H) ppm. ^{13}C NMR (100.6 MHz, DMSO-d_6): δ = 116.0 (C), 122.5 (6-CH), 132.2 (7-CH), 133.0 (C), 133.3 (C), 149.3 (5-CH), 152.0 (C), 162.0 (C=O) ppm. MS-ESI: m/z (%): 260 [M ^{81}Br+H]$^+$ (100), 258 [M ^{79}Br+H]$^+$ (99).

*4.1.3. 3-(phenylethynyl)thieno[3,2-b]pyridine-2-carboxylic acid (**6**)*

To a round bottom flask with methanol, compound **2a** (100 mg, 0.341 mmol) and $NaOH_{(aq)}$ 1M (2.7 equiv, 0.9 mL) were added. The reaction was stirred at rt for 14h and then water (10 mL) and ethyl acetate (10 mL) were added and the phases were separated. The aqueous phase was acidified with HCl until pH = 5 and it was extracted again with ethyl acetate (2 × 5mL). The combined organic phases were dried ($MgSO_4$) and evaporated under reduced pressure to give compound **6** as a bright yellow solid (quantitative yield), m.p. 155-157 ºC. ^{1}H NMR (400 MHz, DMSO-d_6): δ = 7.43-7.53 (m, 3H, 3 × ArH), 7.56-7.64 (m, 3H, 2 × ArH and 6-H), 8.60 (br d, 1H, 7-H), 8.85 (br d, 1H, 5-H) ppm. ^{13}C NMR (100.6 MHz, DMSO-d_6): δ = 83.0 (C), 98.5 (C), 122.2 (6-CH), 122.3 (C), 122.5 (C), 128.9 (2×CH), 129.4 (CH), 131.5 (2×CH), 132.2 (7-CH), 133.8 (C), 139.2 (C), 149.0 (5-CH), 153.9 (C), 162.5 (C=O) ppm. MS-ESI: m/z (%): 280 [M+H]$^+$ (100).

4.2. General procedure for the synthesis of methyl 3-[(het)arylethynyl]thieno[3,2-b]pyridine-2-carboxylates (2a-l)

A dried *Schlenk* tube was filled with dry DMF (2 mL), compound **1** (1 equiv.), $PdCl_2(PPh_3)_2$ (5 mol%), and then with a mixture of CuI (5 mol%), the (het)arylalkyne (1.1 equiv.) and Et_3N (3 equiv.) in DMF (1 mL), under Argon. The reaction was stirred for 10 min at r.t. and then it was heated at 100 °C for 3-8h. The reactions were monitored by TLC. After cooling, water was added (10 mL) and the mixture was extracted with ethyl acetate (3 × 10 mL). The organic phase was washed with water (4 × 10 mL) and brine (3 × 10 mL), dried ($MgSO_4$) and then evaporated. A purification by column chromatography using a gradient of solvents from petroleum ether to 50:50 ether:petroleum ether increasing 10% of ether each time, was carried out to isolate the products. A dimer of the corresponding (het)arylalkynes was always isolated in a little extent as the less polar product. The corresponding lactones were also formed as shown by ^{1}H-NMR, but only with measured yields for **3j** and **3l**, that allowed their fully characterization.

*4.2.1. Methyl 3-(phenylethynyl)thieno[3,2-b]pyridine-2-carboxylate (**2a**)*

Compound **1** (120 mg, 0.441 mmol), $PdCl_2(PPh_3)_2$, (16.0 mg, 0.0220 mmol), phenylacetylene (54.0 µL, 0.485 mmol), CuI (4.00 mg, 0.0220 mmol), Et_3N (180.0 µL, 1.32 mmol) in DMF (3 mL) were heated for 5h. Compound **2a** was obtained as a beige solid (105 mg, 81%), m.p. 123-124 ºC. ^{1}H NMR (400 MHz, DMSO-d_6): δ = 3.97 (s, 3H, OMe), 7.46-7.52 (m, 3H, 3×ArH), 7.61-7.65 (m, 3H, 2×ArH and 6-H), 8.63 (dd, *J* = 8.4 and 1.6 Hz, 1H, 7-H), 8.88 (dd, *J* = 4.4 and 1.6 Hz, 1H, 5-H) ppm. ^{13}C NMR (100.6 MHz, DMSO-d_6): δ = 53.0 (OMe), 82.7 (C), 98.9 (C), 122.0 (C), 122.4 (6-CH), 123.2 (C), 128.9 (2×CH), 129.5 (CH), 131.6 (2×CH), 132.2 (7-CH), 133.8 (C), 136.7 (C), 149.3 (5-CH), 153.6 (C), 161.4 (C=O) ppm. HRMS (ESI): [M+H]$^+$ calcd for $C_{17}H_{12}NO_2S$: 294.0583; found: 294.0581.

*4.2.2. Methyl 3-[(2-methoxyphenyl)ethynyl]thieno[3,2-b]pyridine-2-carboxylate (**2b**)*

Compound **1** (120 mg, 0.440 mmol), $PdCl_2(PPh_3)_2$ (16.0 mg, 0.0220 mmol), 1-ethynyl-2-methoxybenzene (50.0 mg, 0.490 mmol), CuI (4.00 mg, 0.0220 mmol), Et_3N (180.0 µL, 1.32 mmol) in DMF (3 mL) were heated for 5h. Compound **2b** was obtained as a white solid (114 mg, 80%), m.p. 136-138 ºC. ^{1}H NMR (400 MHz, DMSO-d_6): δ = 3.89 (s, 3H, 2'-OMe), 3.95 (s, 3H, OMe), 7.02 (apparent td, *J* = 7.6 and 1.2 Hz, 1H, 5'-H), 7.13 (br d, *J* = 8.0 Hz, 1H, 3'-H), 7.43-7.47 (m, 1H, 4'-H), 7.56 (dd, *J* = 7.6 and 1.6 Hz, 1H, 6'-H), 7.61 (dd, *J* = 8.4 and 4.4 Hz, 1H, 6-H), 8.61 (dd, *J* = 8.4 and 1.6 Hz, 1H, 7-H), 8.86 (dd, *J* = 4.4 and 1.6 Hz, 1H, 5-H) ppm. ^{13}C NMR (100.6 MHz, DMSO-d_6): δ = 53.0 (OMe), 56.0 (2'-OMe), 86.1 (C), 96.1 (C), 111.2 (C), 111.6 (3'-CH), 120.6 (5'-CH), 122.3 (6-CH), 123.6 (C), 131.1 (4'-CH), 132.1 (7-CH), 133.6 (6'-CH), 133.8 (C), 136.3 (C), 149.2 (5-CH), 153.7 (C), 159.8 (C), 161.6 (C=O) ppm. HRMS (ESI): [M+H]$^+$ calcd for $C_{18}H_{14}NO_3S$: 324.0689; found: 324.0689.

*4.2.3. Methyl 3-[(3-methoxyphenyl)ethynyl]thieno[3,2-b]pyridine-2-carboxylate (**2c**)*

Compound **1** (93.0 mg, 0.340 mmol), $PdCl_2(PPh_3)_2$ (12.0 mg, 0.0170 mmol), 1-ethynyl-3-methoxybenzene (50.0 µL, 0.377 mmol), CuI (3.00 mg, 0.0170 mmol), Et_3N (143.0 µL, 1.03

mmol) in DMF (3 mL) were heated for 8h. Compound **2c** was obtained as a white solid (56.0 mg, 51%), m.p. 120-122 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ = 3.82 (s, 3H, 3'-OMe), 3.97 (s, 3H, OMe), 7.04-7.10 (m, 1H, ArH), 7.12-7.15 (1H, m, ArH), 7.21-7.24 (m, 1H, ArH), 7.40 (apparent t, *J* = 8Hz, 1H, 5'-H), 7.62 (dd, *J* = 8.4 and 4.4 Hz, 1H, 6-H), 8.62 (dd, *J* = 8.4 and 1.6 Hz, 1H, 7-H), 8.87 (dd, *J* = 4.4 and 1.6 Hz, 1H, 5-H) ppm. ^{13}C NMR (100.6 MHz, DMSO-d_6): δ = 53.0 (OMe), 55.3 (3'-OMe), 82.5 (C), 98.8 (C), 116.0 (CH), 116.1 (CH), 122.4 (6-CH), 123.12 (C), 123.13 (C), 124.2 (CH), 130.1 (5'-CH), 132.2 (7-CH), 133.9 (C), 136.9 (C), 149.2 (5-CH), 153.6 (C), 159.2 (C), 161.4 (C=O) ppm. HRMS (ESI): $[M+H]^+$ calcd for $C_{18}H_{14}NO_3S$: 324.0689; found: 324.0689.

*4.2.4. Methyl 3-[(4-methoxyphenyl)ethynyl]thieno[3,2-b]pyridine-2-carboxylate (**2d**)*

Compound **1** (120 mg, 0.440 mmol), $PdCl_2(PPh_3)_2$ (16.0 mg, 0.0220 mmol), 1-ethynyl-4-methoxybenzene (63 µL, 0.485 mmol), CuI (4.00 mg, 0.0220 mmol), Et_3N (180 µL, 1.32 mmol) in DMF (3 mL) were heated for 8h. Compound **2d** was obtained as a white solid (104 mg, 73%), m.p. 123-125 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ = 3.82 (s, 3H, 4'-OMe), 3.96 (s, 3H, OMe), 7.05 (d, *J* = 8.4 Hz, 2H, 3' and 5'-H), 7.58 (d, *J* = 8.4 Hz, 2H, 2' and 6'-H), 7.62 (dd, *J* = 8.0 and 4.4 Hz, 1H, 6-H), 8.61 (dd, *J* = 8.0 and 1.6 Hz, 1H, 7-H), 8.87 (dd, *J* = 4.4 and 1.6 Hz, 1H, 5-H) ppm. ^{13}C NMR (100.6 MHz, DMSO-d_6): δ = 53.0 (OMe), 55.4 (4'-OMe), 81.8 (C), 99.5 (C), 114.0 (C), 114.6 (3' and 5'-CH), 122.3 (6-CH), 123.7 (C), 132.1 (7-CH), 133.4 (2' and 6'-CH), 133.8 (C), 135.7 (C), 149.1 (5-CH), 153.6 (C), 160.1 (C), 161.5 (C=O) ppm. HRMS (ESI): $[M+H]^+$ calcd for $C_{18}H_{14}NO_3S$: 324.0689; found: 324.0688.

*4.2.5. Methyl 3-[(2-fluorophenyl)ethynyl]thieno[3,2-b]pyridine-2-carboxylate (**2e**)*

Compound **1** (134 mg, 0.492 mmol), $PdCl_2(PPh_3)_2$ (17.0 mg, 0.0246 mmol),1-ethynyl-2-fluorobenzene (61.0 µL, 0.540 mmol), CuI (5.00 mg, 0.0246 mmol), Et_3N (206 µL, 1.48 mmol) in DMF (3 mL) were heated for 5h. Compound **2e** was obtained as a white solid (95.0 mg, 62%), m.p. 148-150 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ = 3.96 (s, 3H, OMe), 7.33 (apparent td, *J* = 7.6 and 1.2 Hz, 1H, 5'-H), 7.36-7.42 (m, 1H, 3'-H), 7.52-7.58 (m, 1H, ArH), 7.63 (dd, *J* = 8.0 and 4.4 Hz, 1H, 6-H), 7.70 (apparent td, *J* = 7.6 and 2 Hz, 1H, ArH), 8.64 (dd, *J* = 8.0 and 1.6 Hz, 1H, 7-H), 8.88 (dd, *J* = 4.4 and 1.6 Hz, 1H, 5-H) ppm. ^{13}C NMR (100.6 MHz, DMSO-d_6): δ = 53.0 (OMe), 87.2 (d, *J* = 3 Hz, C), 92.0 (C), 110.5 (d, *J* = 15 Hz, 1'-C), 115.9 (d, *J* = 20 Hz, 3'-CH), 122.4 (6-CH), 122.6 (C), 124.9 (d, *J* = 4 Hz, 5'-CH), 131.7 (d, *J* = 8 Hz, CH), 132.2 (7-CH), 133.7 (CH), 133.8 (C), 137.5 (C), 149.3 (5-CH), 153.5 (C), 161.4 (C=O), 161.9 (d, *J* = 250 Hz, C-F) ppm. ^{19}F NMR (376.48 MHz, DMSO-d_6): δ = -109.3 (s) ppm. HRMS (ESI): $[M+H]^+$ calcd for $C_{17}H_{11}FNO_2S$: 312.0489; found: 312.0486.

*4.2.6. Methyl 3-[(3-fluorophenyl)ethynyl]thieno[3,2-b]pyridine-2-carboxylate (**2f**)*

Compound **1** (134 mg, 0.492 mmol), $PdCl_2(PPh_3)_2$ (17.0 mg, 0.0246 mmol), 1-ethynyl-3-fluorobenzene (63.0 µL, 0.540 mmol), CuI (5.00 mg, 0.0246 mmol), Et_3N (206 µL, 1.48 mmol), DMF (3 mL) were heated for 5h. Compound **2f** was obtained as a white solid (98.0 mg, 64%), m.p. 139-141 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ = 3.97 (s, 3H, OMe), 7.33-7.39 (m, 1H, ArH), 7.42-7.46 (m, 1H, ArH), 7.48-7.58 (m, 2H, 5'-H and 6'-H), 7.63 (dd, *J* = 8.4 and 4.4 Hz, 1H, 6-H), 8.64 (dd, *J* = 8.4 and 1.2 Hz, 1H, 7-H), 8.88 (dd, *J* = 4.4 and 1.2 Hz, 1H, 5-H) ppm. ^{13}C NMR (100.6 MHz, DMSO-d_6): δ = 53.0 (OMe), 83.5 (C), 97.3 (d, *J* = 4 Hz, C), 116.9 (d, *J* = 21 Hz, CH), 117.9 (d, *J* = 23 Hz, CH), 122.4 (6-CH), 122.6 (C), 123.9 (d, *J* = 10 Hz, 1'-C), 128.1 (d, *J* = 3 Hz, 6'-CH), 131.1 (d, *J* = 9 Hz, 5'-CH), 132.2 (7-CH), 133.8 (C), 137.4 (C), 149.3 (5-CH), 153.5 (C), 161.3 (C=O), 161.9 (d, *J* = 246 Hz, C-F) ppm. ^{19}F NMR (376.48 MHz, DMSO-d_6): δ = -112.1 (s) ppm. HRMS (ESI): $[M + H]^+$ calcd for $C_{17}H_{11}FNO_2S$: 312.0489; found: 312.0487.

*4.2.7. Methyl 3-[(4-fluorophenyl)ethynyl]thieno[3,2-b]pyridine-2-carboxylate (**2g**)*

Compound **1** (134 mg, 0.492 mmol), $PdCl_2(PPh_3)_2$ (17.0 mg, 0.0246 mmol), 1-ethynyl-4-fluorobenzene (62.0 µL, 0.540 mmol), CuI (5 mg, 0.0246 mmol), Et_3N (206 µL, 1.48 mmol) in DMF (3 mL) were heated for 5h. Compound **2g** was obtained as a white solid (120 mg, 78%), m.p. 138-140 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ = 3.97 (s, 1H, OMe), 7.33 (m, 2H, 3' and 5'-H), 7.63 (dd, *J* = 8.4 and 4.4 Hz, 1H, 6-H), 7.70 (m, 2H, 2' and 6'-H), 8.63 (dd, *J* = 8.4 and 1.6 Hz, 1H, 7-H), 8.86 (dd, *J* = 4.4 and 1.6 Hz, 1H, 5-H) ppm. ^{13}C NMR (100.6 MHz, DMSO-d_6): δ = 53.0 (OMe), 82.5 (C), 97.9 (C), 116.2 (d, *J* = 22 Hz, 3' and 5'-CH), 118.5 (d, *J* = 3Hz, 1'-C), 122.4 (6-CH), 123.1 (C), 132.2 (7-CH), 133.8 (C), 134.0 (d, *J* = 9Hz, 2' and 6'-CH), 136.8 (C), 149.2 (5-CH), 153.3 (C), 161.4 (C=O), 162.4 (d, *J* = 249 Hz, C-F) ppm. ^{19}F NMR (376.48 MHz, DMSO-d_6): δ = -109.3 (s) ppm. HRMS (ESI): $[M+H]^+$ calcd for $C_{17}H_{11}FNO_2S$: 312.0489; found: 312.0486.

*4.2.8. Methyl 3-(m-tolylethynyl)thieno[3,2-b]pyridine-2-carboxylate (**2h**)*

Compound **1** (120 mg, 0.440 mmol), $PdCl_2(PPh_3)_2$ (16.0 mg, 0.0220 mmol), 3-ethynyltoluene (63.0 µL, 0.480 mmol), CuI (4.00 mg, 0.0220 mmol), Et_3N (180 µL, 1.32 mmol) in DMF (3 mL) were heated for 3h. Compound **2h** was obtained as a white solid (75.0 mg, 55%), m.p. 133-134 °C. ^{1}H NMR (400 MHz,$CDCl_3$): δ = 2.38 (s, 3H, Me), 4.04 (s, 3H, OMe), 7.20 (br d, 1H, ArH), 7.28 (m, 1H, ArH), 7.46 (dd, *J* = 8.2 and 4.4 Hz, 1H, 6-H),7.56-7.59 (m, 2H, 2×ArH), , 8.25 (dd, *J* = 8.2 Hz and 1.2 Hz, 1H, 7-H), 8.92 (dd, *J* = 4.4 and 1.2 Hz, 1H, 5-H) ppm. ^{13}C NMR (100.6 MHz, $CDCl_3$): δ = 21.2 (Me), 53.0 (OMe), 82.0 (C), 100.1 (C), 121.6 (6-CH), 122.4 (C), 124.3 (C), 128.2 (CH), 129. 4 (CH), 130.0 (CH), 132.0 (7-CH), 133.0 (CH), 134.5 (C), 137.0 (C), 138.0 (C), 148.5 (5-CH), 154.0 (C), 162.0 (C=O) ppm. HRMS (ESI): $[M+H]^+$ calcd for $C_{18}H_{14}NO_2S$: 308.0740; found: 308.0739.

*4.2.9. Methyl 3-(p-tolylethynyl)thieno[3,2-b]pyridine-2-carboxylate (**2i**)*

Compound **1** (120 mg, 0.440 mmol), $PdCl_2(PPh_3)_2$ (16.0 mg, 0.0220 mmol), 4-ethynyltoluene (62.0 µL, 0.480 mmol), CuI (4.00 mg, 0.0220 mmol), Et_3N (180 µL, 1.32 mmol) in DMF (3 mL) were heated for 3h. Compound **2i** was obtained as a white solid (75.0 mg, 55%), m.p. 139-140 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ = 2.37 (s, 3H, Me), 3.96 (s, 3H, OMe), 7.30 (d, *J* = 8.4 Hz, 2H, 3' and 5'-H), 7.53 (d, *J* = 8.4 Hz, 2H, 2' and 6'-H), 7.62 (dd, *J* = 8.4 and 4.4 Hz, 1H, 6-H), 8.62 (dd, *J* = 8.4 Hz and 1.6 Hz, 1H, 7-H), 8.87 (dd, *J* = 4.4 and 1.6 Hz, 1H, 5-H) ppm. ^{13}C NMR (100.6 MHz, DMSO-d_6): δ = 21.1 (Me), 52.9 (OMe), 82.3 (C), 99.3 (C), 119.1 (C), 122.4 (6-H), 123.5 (C), 129.6 (3' and 5'-CH), 131.6 (2' and 6'-CH), 132.2 (7-CH), 133.8 (C), 136.3 (C), 139.4 (C), 149.2 (5-CH), 153.6 (C), 161.5 (C=O) ppm. HRMS (ESI): $[M+H]^+$ calcd for $C_{18}H_{14}NO_2S$: 308.0740; found: 308.0738.

*4.2.10. Methyl 3-(pyridin-2-ylethynyl)thieno[3,2-b]pyridine-2-carboxylate (**2j**) and 8-(pyridine-2-yl)-6H-pyrano[4',3':4,5]thieno[3,2-b]pyridine-6-one (**3j**)*

Compound **1** (120 mg, 0.440 mmol), $PdCl_2(PPh_3)_2$ (16.0 mg, 0.0220 mmol), 2-ethynylpyridine (49.0 µL, 0.480 mmol), CuI (4.00 mg, 0.0220 mmol), Et_3N (180 µL, 1.32 mmol) in DMF (3 mL), were heated for 8h. Compound **2j** was obtained as a beige solid (70.0 mg, 54%), mp 90-92 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ = 3.96 (s, 3H, OMe), 7.47 (ddd, *J* = 7.6, 4.8 and 1.6 Hz, 1H, 5'-H), 7.63 (dd, *J* = 8.4 and 4.4 Hz, 1H, 6-H), 7.70-7.73 (m, 1H, 3'-H), 7.91 (apparent td, *J* = 7.6 and 1.6 Hz, 1H, 4'-H), 8.64 (dd, *J* = 8.4 and 1.6 Hz, 1H, 7-H), 8.66-8.68 (m, 1H, 6'-H), 8.87 (dd, *J* = 4.4 and 1.6 Hz, 1H, 5-H) ppm. ^{13}C NMR (100.6 MHz, DMSO-d_6): δ = 53.1 (OMe), 81.5 (C), 98.0 (C), 122.4 (C), 122.5 (6-CH), 124.1 (5'-CH), 128.0 (3'-CH), 132.4 (7-CH), 133.9 (C), 137.0 (4'-CH), 138.1 (C), 142.1 (C), 149.4 (5-CH), 150.4 (6'-CH), 153.7 (C), 161.3 (C=O) ppm. HRMS (ESI): $[M+H]^+$ calcd for $C_{16}H_{11}N_2O_2S$: 295.0536; found: 295.0535.

Compound **3j** was also isolated as a slightly less polar product, as a white solid (27.0 mg, 22%), m.p. 286-287 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ = 7.54 (dd, *J* = 8.8 and 4.4 Hz, 1H, 5'-H), 7.74 (dd, *J* = 8.2 and 4.4 Hz, 1H, 3-H), 8.02-8.04 (m, 2H, 3' and 4'-H), 8.20 (s, 1H, 9-H), 8.76-8.79 (m, 2H, 4-H and 6'-H), 8.96 (dd, *J* = 4.4 and 1.6 Hz, 1H, 2-H) ppm. ^{13}C NMR (100.6 MHz, DMSO-d_6): δ = 98.1 (9-CH), 120.0 (3' or 4'-CH), 123.7 (3-CH), 125.2 (5'-CH), 125.4 (C), 132.8 (4-CH), 137.7 (C), 137.9 (3' or 4'-CH), 142.9 (C), 148.4 (C), 149.3 (2-CH), 149.4 (C), 150.2 (6'-CH), 156.0 (C), 157.7 (C) ppm. HRMS (ESI): $[M+H]^+$ calcd for $C_{15}H_9N_2O_2S$: 281.0379; found: 281.0379.

*4.2.11. Methyl 3-(pyridin-3-ylethynyl)thieno[3,2-b]pyridine-2-carboxylate (**2k**)*

Compound **1** (120 mg, 0.440 mmol), $PdCl_2(PPh_3)_2$ (16.0 mg, 0.0220 mmol), 3-ethynylpyridine (46.0 mg, 0.480 mmol), CuI (4.00 mg, 0.0220 mmol), Et_3N (180 µL, 1.32 mmol) in DMF (3 mL) were heated for 5h. Compound **2k** was obtained as a white solid (75.0 mg, 58%), m.p. 167-168 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ = 3.98 (s, 3H, OMe), 7.53 (ddd, *J* = 8.0, 4.8 and 0.8 Hz, 1H, 5'-H), 7.64 (dd, *J* = 8.4 and 4.4 Hz, 1H, 6-H), 8.05 (apparent dt, *J* = 8.0 and 2.0Hz, 1H, 4'-H), 8.63-8.66 (m, 2H, 7-H and 6'-H), 8.82 (dd, *J* = 2.0 and 0.8Hz, 1H, 2'-H), 8.88 (dd, *J* = 4.4 and 1.6 Hz, 1H, 5-H) ppm. ^{13}C NMR (100.6 MHz, DMSO-d_6): δ = 53.1 (OMe), 85.5 (C), 95.4 (C), 119.2 (C), 122.45 (6-CH), 122.5 (C), 123.9 (5'-CH), 132.3 (7-CH), 133.9 (C), 137.6 (C), 138.8 (4'-CH), 149.4 (5-CH), 149.6 (6'-CH), 151.7 (2'-CH), 153.5 (C), 161.3 (C=O) ppm. HRMS (ESI): $[M+H]^+$ calcd for $C_{16}H_{11}N_2O_2S$: 295.0536; found: 295.0536.

*4.2.12. Methyl 3-(thien-3-ylethynyl)thieno[3,2-b]pyridine-2-carboxylate (**2l**) and 8-(thien-3-yl)-6H-pyrano[4',3':4,5]thieno[3,2-b]pyridine-6-one (**3l**)*

Compound **1** (120 mg, 0.440 mmol), $PdCl_2(PPh_3)_2$ (16.0 mg, 0.0220 mmol), 3-ethynylthiophene (48.0 µL, 0.480 mmol), CuI (4.00 mg, 0.0220 mmol), Et_3N (180 µL, 1.32 mmol) in DMF (3 mL) were heated for 4h 30 min. Compound **2l** was obtained as a white solid (88.0 mg, 67%), m.p. 137-139 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ = 3.96 (s, 3H, OMe), 7.32 (dd, *J* = 5.2 and 1.2 Hz, 1H, 5'-H), 7.62 (dd, *J* = 8.4 and 4.4 Hz, 1H, 6-H), 7.71 (dd, *J* = 5.2 and 2.8 Hz, 1H, 4'-H), 8.02 (dd, *J* = 2.8 and 1.2 Hz, 1H, 2'-H), 8.62 (dd, *J* = 8.4 and 1.6 Hz, 1H, 7-H), 8.86 (dd, *J* = 4.4 and 1.6 Hz, 1H, 5-H) ppm. ^{13}C NMR (100.6 MHz, DMSO-d_6): δ = 52.9 (OMe), 82.1 (C), 94.6 (C), 121.0 (C), 122.4 (6-CH), 123.3 (C), 127.3 (4'-CH), 129.6 (5'-CH), 131.2 (2'-CH), 132.2 (7-CH), 133.8 (C), 136.4 (C), 149.2 (5-CH), 153.6 (C), 161.5 (C=O) ppm. HRMS (ESI): $[M+H]^+$ calcd for $C_{15}H_{10}NO_2S_2$: 300.0147; found: 300.0147.

Compound **3l** was also isolated as a slightly more polar product, as a white solid (14.0 mg, 11%), m.p. 137-138 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ = 7.73-7.75 (m, 2H, 3-H and 5'-H), 7.81-7.84 (m, 2H, 9-H and 4'-H), 8.26 (dd, *J* = 3 and 1.6 Hz, 1H, 2'-H), 8.74 (dd, *J* = 8.4 and 1.6 Hz, 1H, 4-H), 8.92 (dd, *J* = 4.4 and 1.6 Hz, 1H, 2-H) ppm. ^{13}C NMR (100.6 MHz, DMSO-d_6): δ = 96.7 (9-CH), 123.0 (C), 123.6 (CH), 125.1 (4'-CH), 125.6 (2'-CH), 128.3 (CH), 132.8 (4-CH), 133.6 (C), 137.7 (C), 143.6 (C), 148.8 (2-CH), 149.4 (C), 154.0 (C), 157.8 (C) ppm. HRMS (ESI): $[M+H]^+$ calcd for $C_{14}H_8NO_2S_2$: 285.9991; found: 285.9990.

4.3. General procedure for the one-pot synthesis of 8-(het)aryl-6H-pyrano[4',3':4,5]thieno[3,2-b]pyridin-6-one (3a-3m) from compound 5

To a *Schlenk* tube filled with DMF/Et_3N (2:1, 3 mL), compound **5** (1 equiv.), $PdCl_2(PPh_3)_2$ (10 mol%), CuI (20 mol%) and the (het)arylalkyne (1.1 equiv.) were added under argon. The mixture was stirred for 1h 30min at 120 °C. The reactions were monitored by TLC. After cooling, the solvents were evaporated and the mixture was purified by column chromatography using different gradients of ethyl acetate/petroleum ether to isolate the products.

*4.3.1. 8-phenyl-6H-pyrano[4',3':4,5]thieno[3,2-b]pyridin-6-one (**3a**)*

A mixture of compound **5** (100 mg, 0.390 mmol), $PdCl_2(PPh_3)_2$ (27.0 mg, 0.0390 mmol), CuI (15.0 mg, 0.0775 mmol) and phenylacetylene (47.0 µL, 0.430 mmol) in DMF/Et_3N, was heated. After purification using solvent gradient from ethyl acetate/petroleum ether 10/90 to 25/75 compound **3a** was obtained as a brown solid (87.0 mg, 80%), mp 195-196 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.49-7.55 (m, 4H,3×ArH and 3-H), 7.76 (s, 1H, 9-H), 8.00 (br d, 2H, 2×ArH), 8.33 (br d, *J* = 8.0 Hz, 1H, 4-H), 8.88 (br d, *J* = 4.0 Hz, 1H, 2-H) ppm. ^{13}C NMR (100.6 MHz, $CDCl_3$): δ = 97.0 (9-CH), 122.9 (3-CH), 124.3 (C), 125.6 (2×CH), 129.0 (2×CH), 130.5 (CH), 131.6 (C), 131.7 (4-CH), 138.4 (C), 143.8 (C), 148.5 (2-CH), 150.1 (C), 158.0 (C), 158.7 (C) ppm. HRMS (ESI): $[M+H]^+$ calcd for $C_{16}H_{10}NO_2S$: 280.0427; found: 280.0426.

*4.3.2. 8-(2-methoxyphenyl)-6H-pyrano[4',3':4,5]thieno[3,2-b]pyridin-6-one (**3b**)*

A mixture of compound **5** (100 mg, 0.390 mmol), $PdCl_2(PPh_3)_2$ (27.0 mg, 0.0390 mmol), CuI (15.0 mg, 0.0775 mmol) and 2-ethynylanisole (55.0 µL, 0.430 mmol) in DMF/Et_3N, was heated. After purification using solvent gradient from ethyl acetate/petroleum ether 10/90 to 25/75, compound **3b** was obtained as a yellow solid (71.0 mg, 59%), mp 220-222 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 4.02 (s, 3H, OMe), 7.03-7.11(m, 2H, 3' and 5'-H), 7.42 (apparent td, *J* = 8.0 and 1.6 Hz, 1H, 4'-H), 7.50 (dd, *J* = 8.0 and 4.4 Hz, 1H, 3-H), 8.03 (dd, *J* = 8.0 and 1.6 Hz, 1H, 6'-H), 8.12 (s, 1H, 9-H), 8.30 (dd, *J* = 8.0 and 1.2 Hz, 1H, 4-H), 8.87 (dd, *J* = 4.4 and 1.2 Hz, 1H, 2-H) ppm. ^{13}C NMR (100.6 MHz, $CDCl_3$): δ = 55.7 (OMe), 102.1 (9-CH), 111.4 (CH), 120.4 (C), 120.8 (CH), 122.7 (3-CH), 124.1 (C), 129.1 (6'-CH), 131.3 (4'-CH), 131.5 (4-CH), 138.3 (C), 144.3 (C), 148.4 (2-CH), 150.3 (C), 154.8 (C), 157.4 (C), 159.0 (C) ppm. HRMS (ESI): $[M+H]^+$ calcd for $C_{17}H_{12}NO_3S$: 310.0532; found: 310.0532.

*4.3.3. 8-(3-methoxyphenyl)-6H-pyrano[4',3':4,5]thieno[3,2-b]pyridin-6-one (**3c**)*

A mixture of compound **5** (100 mg, 0.390 mmol), $PdCl_2(PPh_3)_2$ (27.0 mg, 0.0390 mmol), CuI (15.0 mg, 0.0775 mmol), 3-ethynylanisole (54.0 µL, 0.430 mmol) in DMF/Et_3N, was heated. After purification using solvent gradient from ethyl acetate/petroleum ether 10/90 to 30/70, compound **3c** was obtained as a beige solid (93.0 mg, 78%), mp 199-201 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 3.91 (s, 3H, OMe), 7.03 (dd, *J* = 8.4 and 2.0 Hz, 1H, 4'-H), 7.41 (apparent t, *J* = 8.0 Hz, 1H, 5'-H), 7.51-7.56 (m, 2H, 3-H and 2'-H), 7.59 (br d, *J* = 8.0 Hz, 1H, 6'-H), 7.75 (s, 1H, 9-H), 8.34 (dd, *J* = 8.4 and 1.2 Hz, 1H, 4-H), 8.90 (br d, *J* = 4.4 Hz, 1H, 2-H) ppm. ^{13}C NMR (100.6 MHz, $CDCl_3$): δ = 55.5 (OMe), 97.2 (9-CH), 110.4 (2'-CH), 116.9 (4'-CH), 118.1 (6'-CH), 122.9 (3-CH), 124.5 (C), 130.0 (5'-CH), 131.7 (4-CH), 132.9 (C), 138.4 (C), 143.8 (C), 148.5 (2-CH), 150.1 (C), 157.8 (C), 158.7 (C), 160.1 (C). HRMS (ESI): $[M+H]^+$ calcd for $C_{17}H_{12}NO_3S$: 310.0532; found: 310.0534.

*4.3.4. 8-(4-methoxyphenyl)-6H-pyrano[4',3':4,5]thieno[3,2-b]pyridin-6-one (**3d**)*

A mixture of compound **5** (100 mg, 0.390 mmol), $PdCl_2(PPh_3)_2$ (27.0 mg, 0.0390 mmol), CuI (15.0 mg, 0.0775 mmol), 4-ethynylanisole (55.0 µL, 0.430 mmol) in DMF/Et_3N, was heated. After purification using solvent gradient from ethyl acetate/petroleum ether 10/90 to 35/65, compound **3d** was obtained as a light yellow solid (70.0 mg, 58%), mp 212-214 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 3.90 (s, 3H, OMe), 7.01 (d, *J* = 8.8 Hz, 2H, 3' and 5'-H), 7.53 (dd, *J* = 8.2 and 4.4 Hz, 1H, 3-H), 7.64 (s, 1H, 9-H), 7.95 (d, *J* = 8.8 Hz, 2H, 2' and 6'-H), 8.32 (dd, *J* = 8.2 and 1.6 Hz, 1H, 4-H), 8.88 (dd, *J* = 4.4 and 1.6 Hz, 1H, 2-H) ppm. ^{13}C NMR (100.6 MHz, $CDCl_3$): δ = 55.5 (OMe), 95.5 (9-CH), 114.4 (3' and 5'-CH), 122.8 (3-CH), 123.2 (C), 124.2 (C), 127.2 (2' and 6'-CH), 131.7 (4-CH), 138.4 (C), 144.2 (C), 148.4 (2-CH), 150.1 (C), 158.2 (C), 158.9 (C), 161.5 (C) ppm. HRMS (ESI): $[M+H]^+$ calcd for $C_{17}H_{12}NO_3S$: 310.0532; found: 310.0533.

*4.3.5. 8-(2-fluorophenyl)-6H-pyrano[4',3':4,5]thieno[3,2-b]pyridin-6-one (**3e**)*

A mixture of compound **5** (100 mg, 0.390 mmol), $PdCl_2(PPh_3)_2$ (27,0 mg, 0.0390 mmol), CuI (15.0 mg, 0.0775 mmol), 1-ethynyl-2-fluorobenzene (48.0 µL, 0.430 mmol) in DMF/Et_3N, was heated. After purification using solvent gradient from ethyl acetate/petroleum ether 10/90 to 30/70, compound **3e** was obtained as a beige solid (64.0 mg, 55%), mp 190-191 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.21-7.33 (m, 2H, 2×ArH), 7.42-7.48 (m, 1H, ArH), 7.54 (dd, *J* = 8.4 and 4.4 Hz, 1H, 3-H), 7.97 (s, 1H, 9-H), 8.08 (td, *J* = 8.0 and 1.6 Hz, 1H, 5'-H), 8.34 (dd, *J* = 8.4 and 1.2 Hz, 1H, 4-H), 8.90 (dd, *J* = 4.4 and 1.2 Hz, 1H, 2-H) ppm. ^{13}C NMR (100.6 MHz, $CDCl_3$): δ = 102.1 (d, *J* = 16 Hz, 9-CH), 116.7 (d, *J* = 22 Hz, 3'-CH), 119.9 (d, *J* = 10 Hz, 1'-C), 122.9 (3-CH), 124.7 (d, *J* = 4Hz, CH), 125.0 (C), 128.7 (d, *J* = 1 Hz, 5'-CH), 131.6 (4-CH), 131.7 (d, *J* = 9 Hz, CH), 138.3 (C), 143.8 (C), 148.7 (2-CH), 150.1 (C), 152.4 (d, *J* = 4 Hz, 8-C), 158.5 (C), 160.1 (d, *J* = 254 Hz, C-F) ppm. ^{19}F NMR (376.48 MHz, $CDCl_3$): δ = - 110.9 (s) ppm. HRMS (ESI): $[M+H]^+$ calcd for $C_{16}H_9FNO_2S$: 298.0332; found: 298.0333.

*4.3.6. 8-(3-fluorophenyl)-6H-pyrano[4',3':4,5]thieno[3,2-b]pyridin-6-one (**3f**)*

A mixture of compound **5** (100 mg, 0.390 mmol), $PdCl_2(PPh_3)_2$ (27.0 mg, 0.0390 mmol), CuI (15 mg, 0.0775 mmol), 1-ethynyl-3-fluorobenzene (49.0 µL, 0.430 mmol) in DMF/Et_3N, was heated. After purification using solvent gradient from ethyl acetate/petroleum ether 20/80 to 40/60, compound **3f** was obtained as a brown solid (71.0 mg, 62%), mp 209-210 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ = 7.14-7.19 (m, 1H, ArH), 7.44-7.50 (m, 1H, 5'-H), 7.54 (dd, *J* = 8.4 and 4.4 Hz, 1H, 3-H), 7.67-7.70 (m, 1H, ArH), 7.74 (s, 1H, 9-CH), 7.77 (br d, *J* = 8 Hz, 1H, ArH), 8.33 (dd, *J* = 8.4 and 1.2 Hz, 1H, 4-H), 8.88 (dd, *J* = 4.4 and 1.2 Hz, 1H, 2-H) ppm. ^{13}C NMR (100.6 MHz, $CDCl_3$) δ: 97.7 (9-CH), 112.6 (d, *J* = 24 Hz, 2' or 4'-CH), 117.4 (d, *J* = 21 Hz, 2' or 4'-CH), 121.1 (d, *J* = 3 Hz, 6'-CH), 123.0 (3-CH), 124.9 (C), 130.6 (d, *J* = 8 Hz, 5'-CH), 131.7 (4-CH), 133.7 (d, *J* = 8Hz, 1'-C), 138.4 (C), 143.5 (C), 148.6 (2-CH), 149.9 (C), 156.4 (d, *J* = 3 Hz, 8-C), 158.3 (C), 163.2 (d, *J* = 247 Hz, C-F) ppm. ^{19}F NMR (376.48 MHz, $CDCl_3$): δ = - 111.6 (s) ppm. HRMS (ESI): $[M+H]^+$ calcd for $C_{16}H_9FNO_2S$: 298.0332; found: 298.0332.

*4.3.7. 8-(4-fluorophenyl)-6H-pyrano[4',3':4,5]thieno[3,2-b]pyridin-6-one (**3g**)*

A mixture of compound **5** (100 mg, 0.390 mmol), $PdCl_2(PPh_3)_2$ (27.0 mg, 0.0390 mmol), CuI (15.0 mg, 0.0775 mmol), 1-ethynyl-4-fluorobenzene (49.0 µL, 0.430 mmol) in DMF/Et_3N, was heated. After purification using solvent gradient from ethyl acetate/petroleum ether 10/90 to 30/70, compound **3g** was obtained as a yellow solid (64.0 mg, 55%), mp 248-249 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.16-7.22 (m, 2H, 3' and 5'-H), 7.54 (dd, *J* = 8.4 and 4.4 Hz, 1H, 3-H), 7.68 (s, 1H, 9-H), 7.97-8.00 (m, 2H, 2' and 6'-H), 8.33 (dd, *J* = 8.4 and 1.6 Hz, 1H, 4-H), 8.88 (dd, *J* = 4.4 and 1.6 Hz, 1H, 2-H). ^{13}C NMR (100.6 MHz, $CDCl_3$): δ = 96.8 (9-CH), 116.2 (d, *J* = 22 Hz, 3' and 5'-CH), 123.0 (3-CH), 124.2 (C), 127.7 (d, *J* = 9 Hz, 2' and 6'-CH), 127.9 (d, *J* = 3 Hz, 1'-C), 131.7 (4-CH), 138.4 (C), 143.8 (C), 148.6 (2-CH), 150.0 (C), 157.0 (C), 158.5 (C), 164.1 (d, *J* = 252 Hz, C-F). ^{19}F NMR (376.48 MHz, $CDCl_3$): δ = - 109.2 (s) ppm. HRMS (ESI): $[M+H]^+$ calcd for $C_{16}H_9FNO_2S$: 298.0332; found: 298.0333.

*4.3.8. 8-(m-tolyl)-6H-pyrano[4',3':4,5]thieno[3,2-b]pyridin-6-one (**3h**)*

A mixture of compound **5** (100 mg, 0.390 mmol), $PdCl_2(PPh_3)_2$ (27.0 mg, 0.0390 mmol), CuI (15.0 mg, 0.0775 mmol), 3-ethynyltoluene (55.0 µL, 0.430 mmol) in DMF/Et_3N, was heated. After purification using solvent gradient from ethyl acetate/petroleum ether 10/90 to 35/65, compound **3h** was obtained as a beige solid (86.0 mg, 76%), mp 141-142 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 2.44 (s, 3H, Me), 7.29 (br d, 1H, 4'-H), 7.38 (apparent t, *J* = 8.0 Hz, 1H, 5'-H), 7.52 (dd, *J* = 8.2 and 4.4 Hz, 1H, 3-H), 7.70 (s, 1H, 9-H), 7.77 (br d, *J* = 8.0 Hz, 1H, 6'-H), 7.81 (br s, 1H, 2'-H), 8.31 (dd, *J* = 8.2 and 1.6 Hz, 1H, 4-H), 8.86 (dd, *J* = 4.4 and 1.6 Hz, 1H, 2-H). ^{13}C NMR (100.6 MHz, $CDCl_3$): δ = 21.4 (Me), 96.8 (9-CH), 122.6 (6'-CH), 122.8 (3-CH), 124.1 (C), 126.1 (2'-CH), 128.8 (5'-CH), 131.3 (4'-CH), 131.4 (C), 131.6 (4-CH), 138.3 (C), 138.7 (C), 143.8 (C), 148.4 (2-CH), 149.9 (C), 158.1 (C), 158.7 (C). HRMS (ESI): $[M+H]^+$ calcd for $C_{17}H_{12}NO_2S$: 294.0583; found: 294.0584.

*4.3.9. 8-(p-tolyl)-6H-pyrano[4',3':4,5]thieno[3,2-b]pyridin-6-one (**3i**)*

A mixture of compound **5** (100 mg, 0.390 mmol), $PdCl_2(PPh_3)_2$ (27.0 mg, 0.0390 mmol), CuI (15.0 mg, 0.0775 mmol), 4-ethynyltoluene (54.0 µL, 0.430 mmol) in DMF/Et_3N, was heated. After purification using solvent gradient from ethyl acetate/petroleum ether 20/80 to 50/50, compound **3i** was obtained as a yellow solid (60.0 mg, 53%), mp 210-212 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 2.43 (s, 3H, Me), 7.31 (d, *J* = 8.0 Hz, 2H, 3' and 5'-H), 7.53 (dd, *J* = 8.4 and 4.4 Hz, 1H, 3-H), 7.72 (s, 1H, 9-H), 7.89 (d, *J* = 8.0 Hz, 2H, 2' and 6'-H), 8.33 (dd, *J* = 8.4 and 1.2 Hz, 1H, 4-H), 8.88 (dd, *J* = 4.4 and 1.2 Hz, 1H, 2-H). ^{13}C NMR (100.6 MHz, $CDCl_3$): δ = 20.4 (Me), 96.3 (9-CH), 122.9 (3-CH), 123.9 (C), 125.5 (2' and 6'-CH), 128.8 (C), 129.7 (3' and 5'-CH), 131.7 (4-CH), 138.4 (C), 141.0 (C), 144.0 (C),148.5 (2-CH), 150.0 (C), 158.3 (C), 158.8 (C). MS-ESI m/z

(%): 294 $[M+H]^+$ (100). Anal. calcd for $C_{17}H_{11}NO_2S$: C, 69.61%; H, 3.78%; N, 4.77%; S, 10.93%. Found C, 69.81%; H, 3.95%; N, 4.65%; S 10.46%.

*4.3.10. 8-(pyridine-2-yl)-6H-pyrano[4',3':4,5]thieno[3,2-b]pyridine-6-one (**3j**)*

A mixture of compound **5** (100 mg, 0.390 mmol), $PdCl_2(PPh_3)_2$ (27.0 mg, 0.0390 mmol), CuI (15.0 mg, 0.0775 mmol), 2-ethynylpyridine (43.0 µL, 0.430 mmol) in DMF/Et_3N, was heated. After purification using solvent gradient from ethyl acetate/petroleum ether 10/90 to 50/50, compound **3j** was obtained as a white solid (22.0 mg, 20%). The ^{1}H NMR spectrum is identical to the one described in section *4.2.10.*where compound **3j** was fully characterized.

*4.3.11. 8-(pyridin-3-yl)-6H-pyrano[4',3':4,5]thieno[3,2-b]pyridin-6-one (**3k**)*

A mixture of compound **5** (100 mg, 0.390 mmol), $PdCl_2(PPh_3)_2$ (27.0 mg, 0.0390 mmol), CuI (15.0 mg, 0.0775 mmol), 3-ethynylpyridine (44.0 mg, 0.430 mmol) in DMF/Et_3N, was heated. After purification using solvent gradient from ethyl acetate/petroleum ether 10/90 to 50/50, compound **3k** was obtained as a beige solid (59.0 mg, 54%), mp 270-271 °C. ^{1}H NMR: (400 MHz, DMSO-d_6, 70 °C): δ = 7.69-7.74 (m, 2H, 2×Ar-H), 8.08 (s, 1H, 9-H), 8.56 (br d, *J* = 8.0 Hz, 1H, ArH), 8.72-8.80 (m, 2H, ArH), 8.94 (dd, *J* = 4.0 and 1.2 Hz, 1H, 2-H), 9.31 (broad s, 1H, ArH) ppm.^{13}C NMR (100.6 MHz, DMSO-d_6, 70 °C): δ = 98.5 (9-CH), 123.2 (CH), 124.5 (CH), 124.6 (C), 128.1 (C), 132.4 (CH), 134.4 (CH), 137.4 (C), 142.6 (C), 144.6 (CH), 148.8 (2-CH), 149.0 (CH), 153.8 (C), 155.7 (C), 157.2 (C) ppm. MS-ESI m/z (%): 281 $[M+H]^+$ (100). Anal. calcd for $C_{15}H_8N_2O_2S$: C, 64.27%; H, 2.88%; N, 9,99%; S, 11.44%. Found C, 64.01%; H, 2.52%; N, 10.30%; S 11.90%.

*4.3.12. 8-(thien-3-yl)-6H-pyrano[4',3':4,5]thieno[3,2-b]pyridine-6-one (**3l**)*

A mixture of compound **5** (100 mg, 0.390 mmol), $PdCl_2(PPh_3)_2$ (27.0 mg, 0.0390 mmol), CuI (15.0 mg, 0.0775 mmol), 3-ethynylthiophene (42.0 µL, 0.430 mmol) in DMF/Et_3N, was heated. The purification using solvent gradient from ethyl acetate/petroleum ether 10/90 to 30/70, compound **3l** was obtained as a brown solid (55.0 mg, 50%). The ^{1}H NMR spectrum is identical to the one described in section *4.2.12* where compound **3l** was fully characterized.

*4.3.13. 8-(4-aminophenyl)-6H-pyrano[4',3':4,5]thieno[3,2-b]pyridin-6-one (**3m**)*

A mixture of compound **5** (100 mg, 0.390 mmol), $PdCl_2(PPh_3)_2$ (27.0 mg, 0.0390 mmol), CuI (15.0 mg, 0.0775 mmol), 4-ethynylaniline (50.0 mg, 0.430 mmol), in DMF/Et_3N, was heated. After purification using solvent gradient from ethyl acetate/petroleum ether 20/80 to 60/40, compound **3m** was obtained as an orange solid (57.0 mg, 50%), mp 257-259 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ = 5.83 (br s, 2H, NH_2), 6.67 (d, *J* = 8.8 Hz, 2H, 3' and 5'-H), 7.57 (s, 1H, 9-CH), 7.68-7.73 (m, 3H, 2', 6' and 3-H), 8.70 (dd, *J* = 8.4 and 1.2 Hz, 1H, 4-H), 8.90 (dd, *J* = 4.4 and 1.2 Hz, 1H, 2-CH) ppm. ^{13}C NMR (100.6 MHz, DMSO-d_6): δ = 92.9 (9-CH), 113.7 (3' and 5'-CH), 117.8 (C), 120.3 (C), 123.5 (3-CH), 126.9 (2' and 6'-CH), 132.7 (4-CH), 137.6 (C), 144.4 (C), 148.6 (2-CH), 149.4 (C), 151.5 (C), 158.2 (C), 159.0 (C) ppm. HRMS (ESI): m/z $[M+H]^+$ calcd for $C_{16}H_{12}N_2O_2S$: 295.0536; found: 295.0536.

4.4. General procedure for the synthesis of 9-halo-8-phenyl-6H-pyrano[4',3':4,5]thieno[3,2-b]pyridin-6-ones 4, 7 and 8

To a *Schlenk* tube filled with dry MeCN (3 mL), compound **2a** or **6** (1 equiv.), Cu(I) or (II) salt (2.5 equiv) and the corresponding NXS (2 equiv.) were added under argon. The mixture was stirred for 1h at 100 °C. The reactions were monitored by TLC. After cooling, the solvents were evaporated and the mixture was purified by column chromatography using different gradients of ethyl acetate/petroleum ether to isolate the products.

Only the experiments that gave the best yields are described. The complete results are presented in Table 2.

*4.4.1. 9-iodo-8-phenyl-6H-pyrano[4',3':4,5]thieno[3,2-b]pyridin-6-one (**4**)*

A mixture of compound **6** (50.0 mg, 0.180 mmol), CuI (85.0 mg, 0.450 mmol) and *N*-iodosuccinimide (81.0 mg, 0.360 mmol) in dry MeCN (3 mL), was heated. After purification using solvent gradient from ethyl acetate/petroleum ether 10/90 to 40/60, compound **4** was obtained as a white solid (38.0 mg, 52%), mp 217-219 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.50-7.53 (m, 3H, 3×ArH), 7.57 (dd, *J* = 8.4 and 4.4 Hz, 1H, 3-H), 7.73-7.75 (m, 2H, 2×ArH), 8.35 (dd, *J* = 8.4 and 1.2 Hz, 1H, 4-H), 9.02 (dd, *J* = 8.4 and 1.2 Hz, 1H, 2-H) ppm. ^{13}C NMR (100.6 MHz,$CDCl_3$): δ = 122.6 (3-CH), 127.0 (C), 128.17 (2×CH), 130.3 (2×CH), 130.4 (CH), 131.5 (4-CH), 134.6 (C), 139.1 (C), 140.3 (C), 147.1 (2-CH), 148.1 (C), 150.3 (C), 158.1 (C), 158.4 (C) ppm. MS-ESI m/z (%): 406 $[M+H]^+$ (100). Anal. cald for $C_{16}H_8INO_2S$: C, 47.43%; H, 1.99%; N, 3.46%; S, 7.91%. Found C, 47.13%; H, 2.40%; N, 3.71%; S 7.82%.

*4.4.2. 9-chloro-8-phenyl-6H-pyrano[4',3':4,5]thieno[3,2-b]pyridin-6-one (**7**)*

A mixture of compound **2a** (50.0 mg, 0.170 mmol), $CuCl_2$ (57.0 mg, 0.426 mmol) and *N*-chlorosuccinimide (46.0 mg, 0.340 mmol) in dry MeCN (3 mL), was heated. After purification by using solvent gradient from ethyl acetate/petroleum ether 10/90 to 40/60, compound **7** was obtained as a yellow solid (42.0 mg, 80%), mp 189-191 ºC. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.52-7.58 (m, 4H, 3×ArH and 3-H), 7.91-7.93 (m, 2H, 2×ArH), 8.37 (dd, *J* = 8.4 and 1.6 Hz, 1H, 4-H), 9.01 (dd, *J* = 4.4 and 1.6 Hz, 1H, 2-H) ppm. ^{13}C NMR (100.6 MHz, $CDCl_3$): δ = 109.8 (C), 122.5 (3-CH), 126.4 (C), 128.3 (2×CH), 129.6 (2×CH), 130.5 (CH), 130.8 (C), 131.5 (4-CH), 138.5 (C), 139.4 (C), 148.5 (2-CH), 150.3 (C), 153.8 (C), 157.7 (C) ppm. MS-ESI m/z (%):316 $[M^{37}Cl+H]^+$ (37), 314 $[M^{35}Cl+H]^+$ (100). Anal. calcd for $C_{16}H_8ClNO_2S$: C, 61.25%; H, 2.57%; N, 4.46%; S, 10.22%. Found C, 60.91%; H, 2.92%; N, 4.80%; S 9.72%.

*4.4.3. 9-bromo-8-phenyl-6H-pyrano[4',3':4,5]thieno[3,2-b]pyridin-6-one (**8**)*

A mixture of compound **6** (50.0 mg, 0.180 mmol), $CuBr_2$ (101 mg, 0.450 mmol) and *N*-bromosuccinimide (64.0 mg, 0.360 mmol) in dry MeCN (3 mL), was heated. After purification using solvent gradient from ethyl acetate/petroleum ether 10/90 to 40/60, compound **8** was obtained as a light yellow solid (55.0 mg, 86%), mp 241-243 ºC. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.51-7.54 (m, 3H, 3×ArH), 7.57 (dd, *J* = 8.4 and 4.4 Hz, 1H, 3-H), 7.83-7.86 (m, 2H, 2×ArH), 8.37 (dd, *J* = 8.4 and 1.2 Hz, 1H, 4-H), 9.01 (dd, *J* = 4.4 and 1.2 Hz, 1H, 2-H) ppm. ^{13}C NMR (100.6 MHz, $CDCl_3$): δ = 95.8 (C), 122.5 (3-CH), 126.8 (C), 128.2 (2×CH), 129.6 (2×CH), 130.5 (CH), 131.5 (4-CH), 132.2 (C), 138.7 (C), 139.7 (C), 148.1 (2-CH), 150.5 (C), 155.1 (C), 158.0 (C), 1ppm. MS-ESI m/z (%): 360 $[M^{81}Br+H]^+$ (99.5), 358 $[M^{79}Br+H]^+$ (100). Anal calcd. for $C_{16}H_8BrNO_2S$: C, 53.65%; H, 2.25%; N, 3.91%; S, 8.95%. Found C, 53.27%; H, 2.45%; N, 4.25%; S 9.36%.

4.5. Biological assays

4.5.1. Cell culture

The antitumor potential of the compounds previously synthetized was tested in two different human tumor cell lines: NCI-H460 (non-small cell lung cancer) and HCT-15 (human colorectal adenocarcinoma). Both cell lines were maintained in RPMI-1640 medium supplemented with Ultraglutamine I and 25mM HEPES (Lonza) and Fetal Bovine Serum (FBS, Biowest). The concentration of FBS used was 5% (v/v) for the Sulforhodamine B (SRB) assays and 10% (v/v) for the apoptosis assays. Cells were routinely monitored and kept at 37 °C in a humidified incubator with 5% CO_2. Cell viability was determined with the Trypan Blue exclusion assay and experiments were only performed when exponentially growing cells presented more than 90% viability. The cell lines were genotyped and frequently monitored for mycoplasma contamination by PCR.

4.5.2. Cell growth inhibition assay

The potential cytotoxicity of the compounds was tested with the Sulforhodamine B colorimetric assay, according to a previously described protocol.[16,17] Briefly, cells were seeded at an appropriate concentration previously optimized (5×10^4 cells/mL for NCI-H460 cells and 1×10^5 cells/mL for HCT-15 cells) and allowed to adhere for 24 hours.[19] All experiments were performed in two 96 well-plates, a T0 plate to be analysed immediately after the compounds addition and another one (T48 plate) to be analysed 48h after the compounds addition. Cells were treated with five serial dilutions of each compound (with the final concentrations depending on their initial concentration). The solvent of the compounds (DMSO) and the cell culture medium (RPMI with 5% FBS) were used as negative controls and doxorubicin in five serial dilutions was used as positive control for both cell lines. Following 48h incubation with the compounds for the T48 plate (or at time zero for the T0 plate) cells were fixed by adding ice-cold 10% trichloroacetic acid (TCA, w/v, Panreac, Barcelona, Spain) and afterwards were stained with 0.4% sulforhodamine B reagent (SRB, Sigma Aldrich, St Louis, MO, USA) in 1% (v/v) acetic acid. The bound dye was then solubilized by adding 10mM of Tris-base solution (pH 10.5, Sigma Aldrich, St Louis, MO, USA) and the absorbance was measured at 510 nm in a microplate reader (BioTek® Synergy HT, Winooski, VT, USA). Finally, the GI_{50} (concentration that inhibits 50% of cell growth) was determined for each compound in both cell lines, by assessing the dose-response curves obtained.

4.5.3. Analysis of apoptosis with the Annexin V-FITC/PI assay

The HCT-15 cells were plated in 6 well-plates at a concentration of 2.0×10^5 cells/well. Cells were allowed to adhere for 24h and then treated with three of the compounds that showed the lowest GI_{50} in the screening assay (**2i, 2j** and **3f**) at two different concentrations: a lower concentration equal or close to the GI_{50} concentration, and another concentration 1.5 × higher than the previous one. As controls, cells were treated with RPMI with 10% (v/v) FBS (Blank) and with the highest concentration used of the solvent of the compounds (DMSO control). Etoposide was used as a positive control (at its GI_{50} concentration, previously determined by some of us to be 6.8 μM in the HCT-15 cells.[18] Following 48h treatment, cells were trypsinized and centrifuged at 290×g for 5 minutes. The cell pellets were re-suspended in a binding buffer solution from the Annexin V-FITC Apoptosis Detection Kit (eBioscience, Bender MedSystems), as indicated by the manufacturer. Then, cells were incubated with 5μL of human Annexin V-FICT for 10 minutes in the dark and further incubated with 10 μL of propidium iodide for 5 minutes.[8] Samples were then analyzed by flow cytometry using the BD Accuri™ C6 flow cytometry, plotting at least 20 000 events per sample. Results were analyzed using the FlowJo software (version 7.6.5, Tree Star, Inc., Ashland, USA).

4.5.4. Statistical Analysis

Results from the SRB assay are represented as mean ± S.E.M. from at least three independent experiments (except when crystals were formed bellow the GI_{50} concentration, in which case only one experiment was performed). The results from the apoptosis analysis were statistically analysed using a two-tailed paired Student's t-test, comparing treated versus Blank cells.

5. References

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Supplementary Material

^{1}H, ^{13}C and ^{19}F NMR spectra of all new compounds and key intermediates. Representative histograms of flow cytometry analysis of apoptotic cell death following annexinV/FITC/PI staining in HCT-15 cells treated with compounds **2i**, **2j** and **3f** and respective controls.