

Combined drug design of potential *Mycobacterium tuberculosis* and HIV-1 inhibitors: 3',4'-di-substituted -4'*H*-spiro [isothiochromene-3,5'-isoxazol]-4(1*H*)-one

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Abstract

We report herein the design and synthesis of 17 new spiroheterocycles **10-26**, on the basis of two hypothetical pharmacophore structures designed to interact with both of *Mycobacterium tuberculosis* bacteria and HIV-1 virus. The *in vitro* biological evaluation of these compounds allowed us to point out seven new potential non-nucleoside hits, with MIC values in the range of 6.25 µg/mL and two new potential anti-HIV-1 inhibitors.

Keywords: Tuberculosis, HIV-1, spiro-isoxazolines, combined pharmacophore sites

Introduction

Isioxazoline derivatives have been shown to be efficient precursors for many synthetic intermediates including γ -amino alcohols and β -hydroxy ketones.¹⁻² Spiro-isoxazolines display interesting biological properties such as herbicidal, plant-growth regulatory and antitumour activities.³⁻⁴ Many 4-chromanone derivatives are versatile intermediates for the synthesis of natural products such as brazilline, hematoxylin, ripariochromene, clausenine, calanolide A and inophyllum.⁵⁻⁶ Chromanone heterocycles have also attracted much attention owing to their important pharmacological properties.⁵ Their high synthetic utility and pharmacological importance have prompted us to synthesize and to study the antitubercular activity of some new spiro-isoxazolidine compounds **10-26** (Scheme 1).

Previously antitubercular screening of 3-armed imidazo[1,2-*a*]pyrimidines showed that

compounds bearing a formyl, hydroxy or nitroso side chain in position 3 are highly active.⁷⁻⁸ From general structure/activity relationship observations, it appears that functionalized side chain(s) such as $[X-(C)_n-Y]$, where $X,Y = O, N$ and $n = 2$ or 3 , are crucial for bioactivity. These atoms or centres that have critical interactions with the bacterial cell receptor constitute the pharmacophore and are vital for antimicrobial activities. These interactions must have typically precise geometric requirements that are readily described in terms of the distances between the terminal atoms and their orientation in the pharmacophore sites ($O^1-C^1-C^2-N^1-H^{1c}$) for (S,S)-2,2'-(1,2-ethanediyl-diimino)dibutan-1-ol (**Ethambutol**)^{9a} and ($O^1-N^1-C^1O^5$) for 2-phenyl-3-nitrozo-imidazo[1,2-a]pyrimidine (**IMP**)^{9b}. The dihedral angle is around $1-3^\circ$. The (X--Y) distance is in the range of 2.5-2.8 Å and the charges are different as shown in Figures 1 and 2.

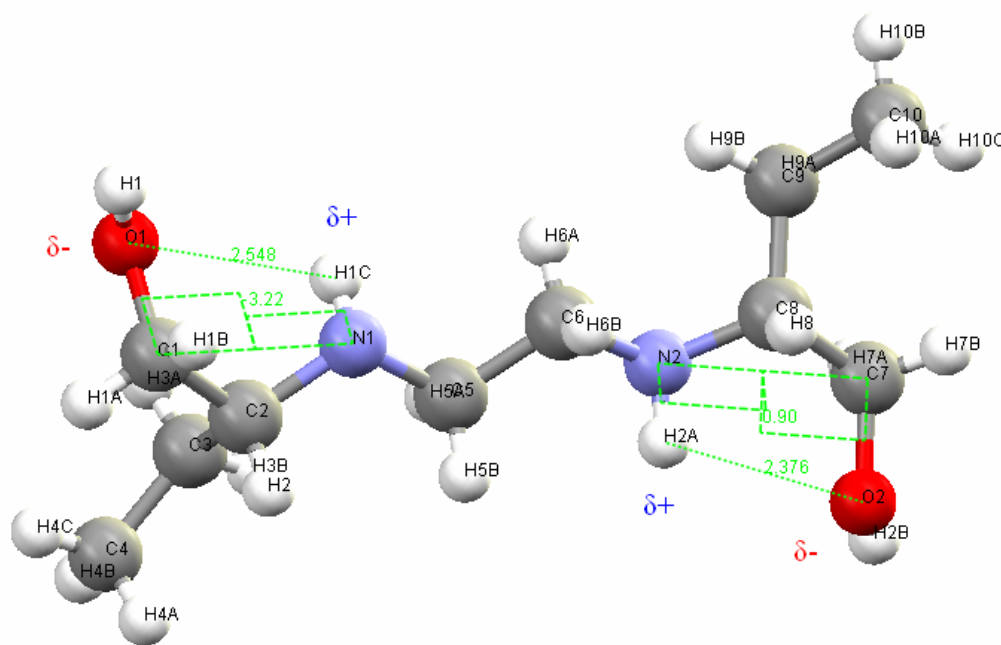


Figure 1. A view of clinical antitubercular agent (**Ethambutol**).^{9a} $O1\cdots H1 = 2.55$ Å; Torsion angle ($O1,C1,C2,N1$) = 3.22° .

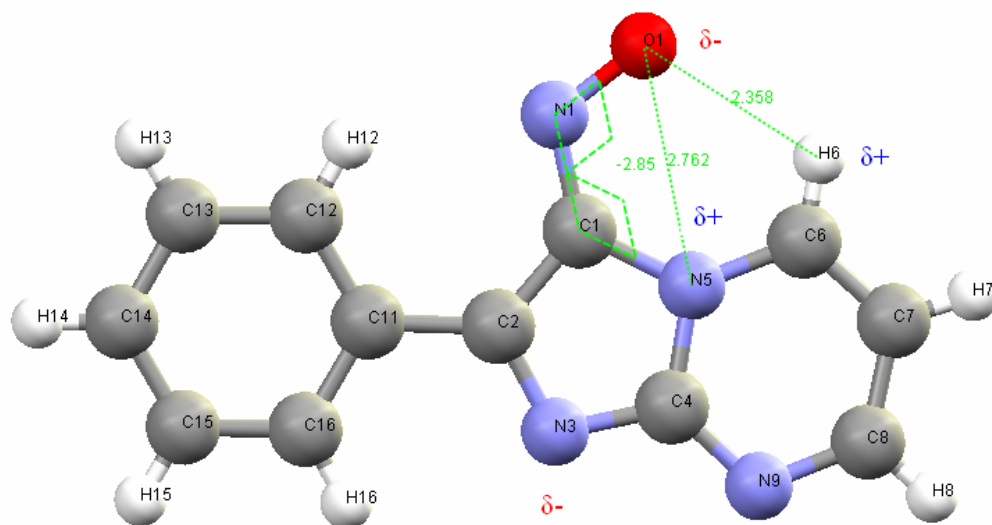


Figure 2. A view of antitubercular agent (IMP).^{9b} O1----N5 = 2.76 Å; Torsion angle (O1,N1,C1,N5) = 2.85°.

As an extensive continuation of our study on the structure-antibacterial activity relationships in 3-nitroso-imidazo[1,2-a]pyridine (-pyrimidine) derivatives,^{8-9b} we performed an investigation of spiroheterocycles **10-26** because they represent an attractive model for a theoretical and experimental study of the pharmacophore and their medical applications because of the large variability and combination in their substituents (R^1 and R^2). Moreover, given the current interest in selective drugs through the development of molecules that recognize simultaneously specific tuberculosis and HIV-1 sites, we became interested in the combined synthesis, antitubercular and anti-HIV screenings of spiroheterocycles having two rigid pharmacophore systems (O=C-C-O and O-C-O-N) as it was postulated in Figure 3.

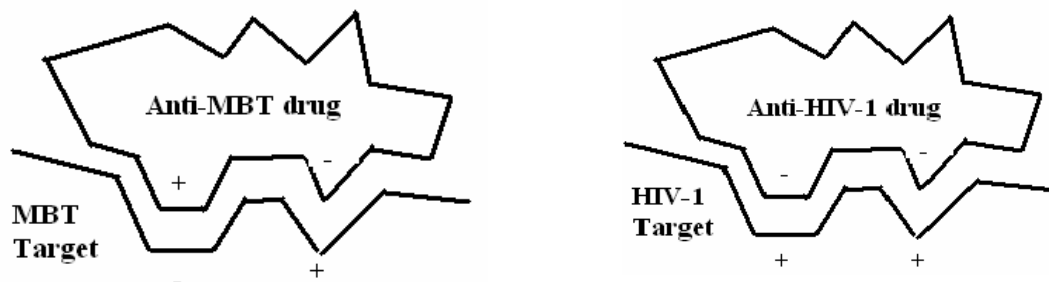


Figure 3. Possible interactions between antitubercular or anti-HIV-1 drugs and their specific biological targets.

The main interesting tasks of this work were: (i) Develop robust prediction models for the heterocycles/ *Mycobacterium tuberculosis* inhibitory properties (solubility, MT inhibition,

stability, selectivity, etc) of small molecules with the estimation of confidence of these models. (ii) Interpret the calculated/predicted results for the design of new compounds. (iii) Perform docking or pharmacophore modelling based on crystal structures to support the lead optimization process. (iiii) Combine the antitubercular pharmacophore site with a selective anti-HIV pharmacophore site without altering activity of the first one.

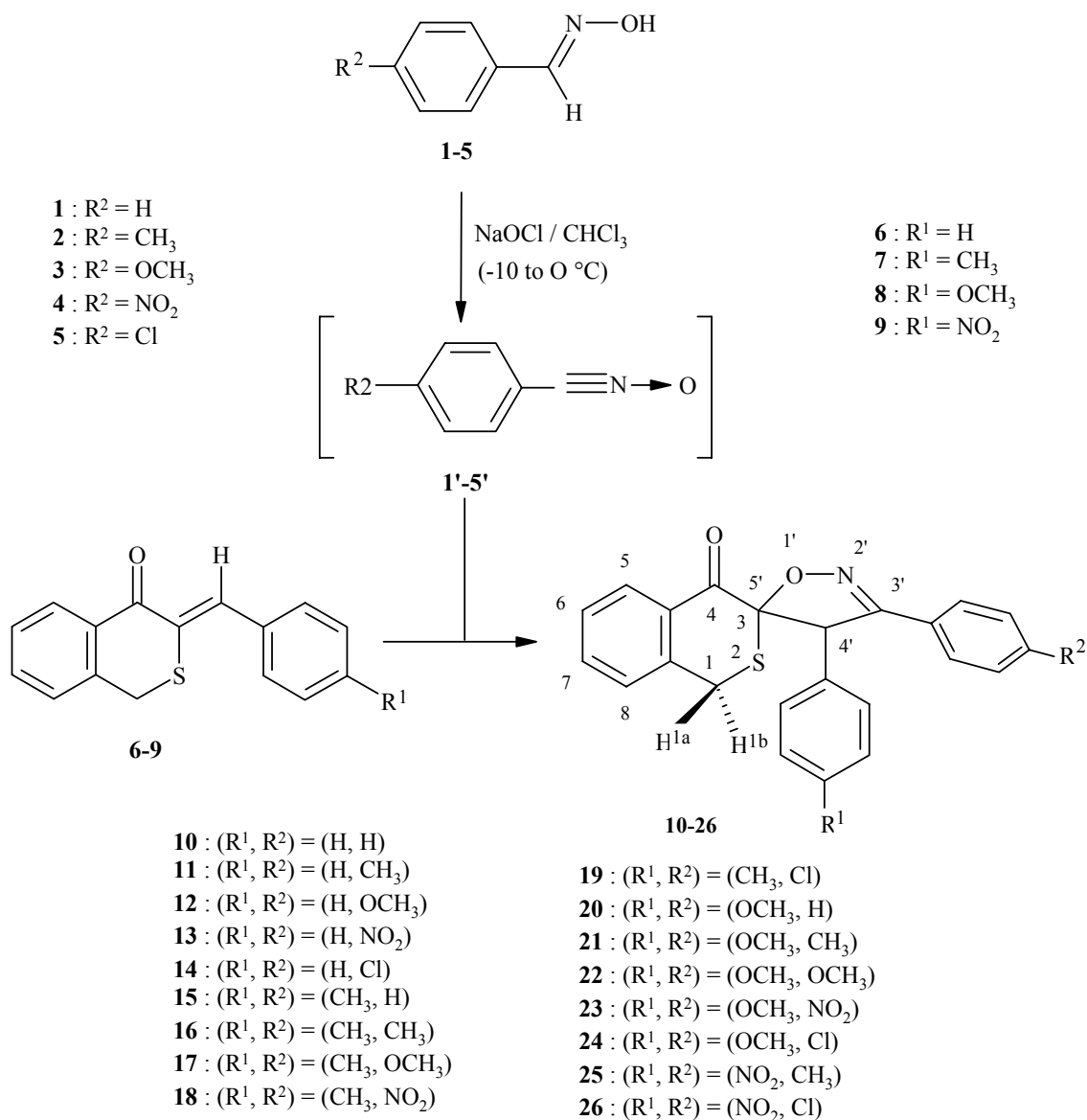
Results and Discussion

Chemistry

1,3-Dipolar cycloaddition reaction of *p*-substituted-benzaldoximes (**1-5**) (with (3*Z*)-3-(4-methylbenzylidene)-1*H*-isothiochromen-4(3*H*)-ones (**6-9**) in biphasic medium of sodium hypochloride and chloroform (NaOCl/ H₂O/ CHCl₃) has been demonstrated to be a powerful and optimal method for the cycloadduct heterocyclic family; 3',4'-diphenyl-4'*H*-spiro[isothiochromene-3,5'-isoxazol]-4(1*H*)-ones **10-26** (Scheme 1).

All compounds were characterized using ¹H and ¹³C NMR data's which are in agreement with previously reported similar compounds.^{11,12} Compounds (**10-26**) are stable at ambient temperature. Their structures have been determined by IR, MS and NMR (¹H and ¹³C) spectroscopy. The selective NMR data are regrouped in Tables 1 and 2.

The isothiochromanone moiety consists of a benzene ring fused with a six-membered heterocyclic ring which adopts a sofa conformation. The five-membered spiro-isoxazoline ring is in the same plan of the phenyl ring. But the *p*-R¹-phenyl and *p*-R²-phenyl rings bridged by the five-membered ring are nearly perpendicular to each other (Figure 3).



Scheme 1. Synthesis of spiroheterocycles **10-26**.

Table 1. Selected IR and ¹H NMR data of compounds **10-26**

Compd.	(R ¹ , R ²)	IR (ν CO)	δR ¹ δR ²	δH ^{1a}	δH ^{1b}	δH ^{4'}	δH (Arom)
10	(H,H)	1665	-	3.5 (d, 1H)	4.7 (d, 1H)		7.2-8.1
			-	² JH ^{1a} H ^{1b} = 16.8		6.2 (s, 1H)	(m, 14H)
11	(H,CH ₃)	1680	-	3.5 (d, 1H)	4.7 (d, 1H)		7.1-8.2
			2.3 (s, 3H)	² JH ^{1a} H ^{1b} = 16.8		6.2 (s, 1H)	(m, 13H)
12	(H,OCH ₃)	1661	-	3.4 (d, 1H)	4.5 (d, 1H)		6.4-8.3
			3.7 (s, 3H)	² JH ^{1a} H ^{1b} = 16.7		6.1 (s, 1H)	(m, 13H)
13	(H,NO ₂)	1670	-	3.5 (d, 1H)	4.5 (d, 1H)		7.2-8.1
			-	² JH ^{1a} H ^{1b} = 16.5		6.2 (s, 1H)	(m, 13H)
14	(H,Cl)	1665	-	3.5 (d, 1H)	4.7 (d, 1H)		7.2-8.3
			-	² JH ^{1a} H ^{1b} = 16.8		6.2 (s, 1H)	(m, 13H)
15	(CH ₃ ,H)	1660	2.3 (s, 3H)	3.5 (d, 1H)	4.7 (d, 1H)		7.1-8.2
			-	² JH ^{1a} H ^{1b} = 16.7		6.1 (s, 1H)	(m, 13H)
16	(CH ₃ ,CH ₃)	1675	2.5 (s, 3H)	3.5 (d, 1H)	4.7 (d, 1H)		7.1-8.1
			2.3 (s, 3H)	² JH ^{1a} H ^{1b} = 16.8		6.1 (s, 1H)	(m, 12H)
17	(CH ₃ ,OCH ₃)	1675	2.3 (s, 3H)	3.5 (d, 1H)	4.7 (d, 1H)		6.7-8.1
			3.7 (s, 3H)	² JH ^{1a} H ^{1b} = 16.8		6.1 (s, 1H)	(m, 12H)
18	(CH ₃ ,NO ₂)	1680	2.3 (s, 3H)	3.6 (d, 1H)	4.7 (d, 1H)		7.1-8.1
			-	² JH ^{1a} H ^{1b} = 16.8		6.1 (s, 1H)	(m, 12H)
19	(CH ₃ ,Cl)	1680	2.3 (s, 3H)	3.5 (d, 1H)	4.7 (d, 1H)		7.1-8.1
			-	² JH ^{1a} H ^{1b} = 16.8		6.1 (s, 1H)	(m, 12H)
20	(OCH ₃ ,H)	1680	3.7 (s, 3H)	3.5 (d, 1H)	4.5 (d, 1H)		6.6-8.0
			-	² JH ^{1a} H ^{1b} = 16.6		6.1 (s, 1H)	(m, 13H)
21	(OCH ₃ ,CH ₃)	1675	3.7 (s, 3H)	3.5 (d, 1H)	4.7 (d, 1H)		6.8-8.2
			2.3 (s, 3H)	² JH ^{1a} H ^{1b} = 16.8		6.1 (s, 1H)	(m, 12H)
22	(OCH ₃ ,OCH ₃)	1665	3.7 (s, 3H)	3.5 (d, 1H)	4.7 (d, 1H)		6.8-8.1
			3.7 (s, 3H)	² JH ^{1a} H ^{1b} = 16.8		6.1 (s, 1H)	(m, 12H)
23	(OCH ₃ ,NO ₂)	1670	3.7 (s, 3H)	3.5 (d, 1H)	4.6 (d, 1H)		6.7-8.1
			-	² JH ^{1a} H ^{1b} = 16.8		6.1 (s, 1H)	(m, 12H)
24	(OCH ₃ ,Cl)	1670	3.8 (s, 3H)	3.6 (d, 1H)	4.7 (d, 1H)		6.8-8.2
			-	² JH ^{1a} H ^{1b} = 16.8		6.1 (s, 1H)	(m, 12H)
25	(NO ₂ , CH ₃)	1680	-	3.6 (d, 1H)	4.7 (d, 1H)		7.1-8.2
			2.3 (s, 3H)	² JH ^{1a} H ^{1b} = 16.8		6.2 (s, 1H)	(m, 12H)
26	(NO ₂ ,Cl)	1665	-	3.5 (d, 1H)	4.7 (d, 1H)		7.2-8.1
			-	² JH ^{1a} H ^{1b} = 16.7		6.2 (s, 1H)	(m, 12H)

Table 2. Selected ^{13}C NMR data of compounds **10-26**

Comp.	(R ¹ , R ²)	δC (R ¹)	δC (R ²)	δC^1 (-SCH ₂ -)	$\delta\text{C}^{3,5'}$ (spiro-C)	δC^4 (-C=O)	$\delta\text{C}^{4'}$ (-CH-)
10	(H,H)	-	-	29.0	94.7	183.5	56.6
11	(H,CH ₃)	-	21.6	28.2	94.6	184.1	56.7
12	(H,OCH ₃)	-	55.4	28.2	95.0	183.7	56.5
13	(H,NO ₂)	-	-	28.0	95.6	183.5	56.1
14	(H,Cl)	-	-	28.1	94.9	183.8	55.4
15	(CH ₃ ,H)	21.3	-	28.9	94.7	183.5	56.6
16	(CH ₃ ,CH ₃)	28.5	21.5	28.2	94.6	184.1	56.4
17	(CH ₃ ,OCH ₃)	21.3	55.2	28.1	94.9	184.1	56.6
18	(CH ₃ ,NO ₂)	21.3	-	28.1	95.6	183.6	55.9
19	(CH ₃ ,Cl)	21.4	-	28.1	95.0	183.7	56.2
20	(OCH ₃ ,H)	55.4	-	28.1	95.6	183.6	58.8
21	(OCH ₃ ,CH ₃)	55.0	21.1	28.1	95.0	180.8	57.0
22	(OCH ₃ ,OCH ₃)	55.3	55.3	28.2	95.0	184.2	56.2
23	(OCH ₃ ,NO ₂)	55.3	-	28.1	95.8	183.6	55.5
24	(OCH ₃ ,Cl)	55.2	-	28.2	95.1	183.6	55.7
25	(NO ₂ , CH ₃)		21.5	28.0	94.1	183.6	56.2
26	(NO ₂ ,Cl)	-	-	28.0	94.4	183.4	56.0

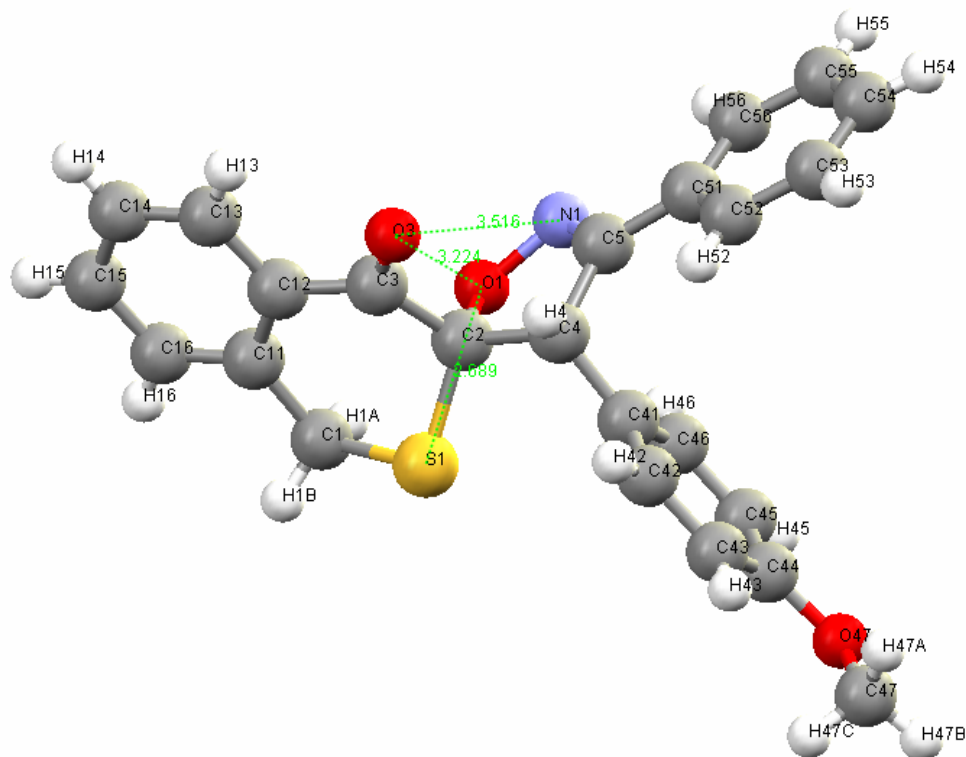


Figure 3. A view of compound **20** with the atom-numbering scheme.¹⁰

The (X---Y) distance length of most significant pharmacophore site to inhibit *Mycobacterium tuberculosis* is probably ($O^1-C^2-S^1$) which possesses the requested geometrical parameters and alternative charges (Table 3).

Table 3. Selected crystallographic data¹⁰ and Petra calculations¹⁹ of compound **20**

Pharmacophore site	(X, Y)	D (X---Y) (Å)	Dihedral angle (°)	Total charge (e-)		Partial π -charge (e-)	
				X	Y	X	Y
A	(O^1 , O^3)	3.22	107.9	-0.196	-0.369	+0.027	-0.083
B	(N^1 , O^3)	3.51	1.1	-0.207	-0.369	-0.083	-0.073
C	(O^1 , S^1)	2.680	0.0	-0.196	-0.110	+0.027	-0.0000
D	(N^1 , S^1)	3.85	145.2	-0.207	-0.110	-0.073	-0.000

Evaluation of anti-tuberculosis activity *in vitro*

Fifteen compounds **10-26** have been evaluated as anti-tuberculosis agents through the TAACF tuberculosis screening program, but only half of them have been shown to inhibit significantly the growth of *Mycobacterium tuberculosis* H₃₇Rv using the Alamar assay at the first level adopted for *in vitro* screening. Just one compound **10** displayed modest *in vitro* activity (less than 50%). Not surprisingly, some of these spiro-isoxazoline derivatives are currently being modified in the goal to be examined at the *in vivo* stage of the tuberculosis screening program. The anti-tuberculosis data are summarised in Table 4.

The most effective inhibitors are **24**, **19**, **21**, **22**, **15**, **17** and **16** which produce 90-93% inhibition of the *Mycobacterium tuberculosis*. Molecules **11**, **14**, **26**, **25** and **18** are less effective but produce appreciable growth inhibition (74-78 %inh.), at comparable concentrations. Compounds **12** and **13** only inhibit growth weakly and are considered essentially inactive (23-53 %inh.).

The molecules with the activity contain two substituted phenyl groups on each of the C^{3'} and C^{4'} atoms of the semi-conjugated isoxazoline ring. The replacement of the two phenyl groups by *para*-R¹-phenyl and *para*-R²-phenyl in compound **10** or *para*-R¹-phenyl in **15** results in a drastic change of antitubercular activity (from 23 to 91 %inh.).

The increase in activity could be due to the increase of the hydrophobic character that the alkyl and alkyloxy groups confer on the molecule. Hydrophobic molecules with rigid, planar structures such as aromatic rings, have been shown to have the ability to insert into membranes and induce localized permeability changes leading to leakage out of the membrane.¹⁵ The combination of methyl and nitro groups (**18** and **24**) while also hydrophobic and very easily inserted into the membrane, are much less likely to cause disruption of the lipid packing order. That can be explained by possible cell membrane disturbances as the nitro group is too small to have this type of effect. The enhanced antitubercular inhibition observed in the presence of (**15**, **16**, **17**, **19**, **21**, **22** and **24**) is then more likely due to its interaction with some intracellular target. The presence of a strong or poor electron-withdrawing group must alter the nature of the compound in such a way as to promote binding to the target(s).

Evaluation of anti-HIV-1 activity *in vitro*

As shown in Table 4, two of these spiranic compounds show anti-HIV-1 activity. In this series however, neither the degree of lipophilicity (cLogP) nor the molecular polar surface area (TPSA) correlates positively with the anti-HIV-1 activity. These parameters were computed using AM1 geometries optimised using the GAUSSIAN03 package,²² for which more information can be found elsewhere.²³

Interestingly, **25-26** that has NO₂ groups has a smaller Log P value and a very high hydration and TPSA value (of 95 compared to 47.7). These are also the most active species, and should be related to these properties. Although, no relationship was obtained we can suggest that high Log P and hydration energies will lead to active molecules.

Table 4. Antitubercular activity of spiro-isoxazoline compounds **10-26**^a

TAACF Code	Compd.	R ¹	R ²	Tuberculosis screening		
				Assay	MIC ($\mu\text{g/mL}$)	%Inhib. ¹⁶
157162	24	O-CH ₃	Cl	Alamar	< 6.25	93
157166	19	CH ₃	Cl	Alamar	< 6.25	92
157161	21	O-CH ₃	CH ₃	Alamar	< 6.25	91
157165	22	O-CH ₃	O-CH ₃	Alamar	< 6.25	91
157172	15	CH ₃	H	Alamar	< 6.25	91
157163	17	CH ₃	O-CH ₃	Alamar	< 6.25	90
157169	16	CH ₃	CH ₃	Alamar	< 6.25	90
157170	11	H	CH ₃	Alamar	> 6.25	87
157171	14	H	Cl	Alamar	> 6.25	87
157167	26	NO ₂	Cl	Alamar	> 6.25	78
157168	25	NO ₂	CH ₃	Alamar	> 6.25	77
157164	18	CH ₃	NO ₂	Alamar	> 6.25	74
157174	13	H	NO ₂	Alamar	> 6.25	53
157173	12	H	OCH ₃	Alamar	> 6.25	32
157160	10	H	H	Alamar	> 6.25	23

^a the antitubercular screening of compounds **20** and **23** has not been done.

A comparison of compounds with regard to their *in vitro* activity against HIV-1 reveals specificity notably when R¹ is nitro substituent. Compounds **25** and **26** have 11 micromolar activity against HIV-1 while others have no activity against HIV-1. It appears that in this limited series, the presence of a more polar substituent on the phenyl of oxazoline ring (compound **25** and **26**) enhances the activity (Table 5).

OSIRIS Calculations

The OSIRIS Property Explorer used in this paper is an integral part of Actelion's inhouse substance registration system. It lets you draw chemical structures and calculates on-the-fly various drug-relevant properties whenever a structure is valid. Prediction results of compounds **10-26** molecular properties (solubility, druglikeness and drug-score) are abstracted (Table 6).

Properties with high risks of undesired effects like mutagenicity or a poor intestinal absorption are shown in red. Whereas a green color indicates drug-conform behaviour.¹⁷

Table 5. Anti-HIV activity of selected compounds against HIV-1 cell line *in vitro*

Compd. (TAAC Code) (NCI Code)	(R1,R2)	Calculated properties ^b			Anti-HIV-1 screening ^c			
		W ^a	E ^{HYD}	LogP ¹³	IC50 (Mol)	EC50 (Mol)	TI50 (Mol)	Activity
11 (723011)	(H,CH ₃)	W=1813	-5.64	4.98005 (5.38)	3.38 10 ⁻⁵	ND	ND	-
14 (723012)	(H,Cl)	W= 1813	-6.47	5.33405 (5.00)	1.17 10 ⁻⁵	ND	ND	-
15 (723013)	(CH ₃ ,H)	W= 1803	-5.73	4.98005 (5.38)	>2.00 10 ⁻⁴	ND	ND	-
16 (723014)	(CH ₃ ,C H ₃)	W= 2008	-4.57	5.47905 (5.53)	>2.00 10 ⁻⁴	ND	ND	-
19 (723008)	(CH ₃ ,Cl)	W= 2008	-5.41	5.83305 (5.15)	>2.00 10 ⁻⁴	ND	ND	-
25 (723010)	(NO ₂ ,C H ₃)	W= 2458	-10.08	1.68805 (2.63)	1.18 10 ⁻⁵	2.14 10 ⁻⁶	5.50	+++
26 (723009)	(NO ₂ , Cl)	W= 2458	-10.88	2.04205 (2.25)	1.16 10 ⁻⁵	3.32 10 ⁻⁶	3.49	+++

^a (W): Wiener Index. ^b E^{HYD} is the hydration energy in kcal/mol, the TPSA²¹ is 47.7 for **11**, **14**, **15**, **16**, **19**, and 95.0 for **25-26**. The octanol/water partition coefficient is shown, where the values in parenthesis are regular LogP.

Molinspiration calculations

cLogP (octanol/water partition coefficient) is calculated by the methodology developed by Molinspiration as a sum of fragment-based contributions and correction factors. The method is very robust and is able to process practically all organic, and most organometallic molecules. Molecular Polar Surface Area TPSA is calculated based on the methodology published by Ertl et al.¹⁸ as a sum of fragment contributions. O- and N- centered polar fragments are considered. PSA has been shown to be a very good descriptor characterizing drug absorption, including intestinal absorption, bioavailability, Caco-2 permeability and blood-brain barrier penetration.¹⁸ Prediction results of compounds **10-26** molecular properties (TPSA, GPCR ligand and ICM) are valued (Table 7).

Table 6. OSIRIS calculations of spiro-isoxazoline compounds **10-26**^b

TAACF Code	Compd	R ¹	R ²	Osiris calculations ¹⁷				
				MW	cLogP	Solubil.	Druglikness	Drug-Score
157162	24	O-CH ₃	Cl	435	7.33	-8.40	3.60	0.24
157166	19	CH ₃	Cl	419	7.75	-8.52	2.09	0.22
157161	21	O-CH ₃	CH ₃	415	7.03	-7.81	1.01	0.22
157165	22	O-CH ₃	O-CH ₃	431	6.61	-7.48	2.27	0.25
157172	15	CH ₃	H	385	7.13	-7.79	1.30	0.24
157163	17	CH ₃	O-CH ₃	415	7.03	-7.81	1.01	0.22
157169	16	CH ₃	CH ₃	399	7.45	-8.13	0.73	0.21
157170	11	H	CH ₃	385	7.13	-7.79	1.40	0.24
157171	14	H	Cl	405	7.43	-8.18	4.00	0.24
157167	26	NO ₂	Cl	450	7.30	-8.64	-6.62	0.12
157168	25	NO ₂	CH ₃	430	7.00	-8.25	-9.24	0.12
157164	18	CH ₃	NO ₂	430	7.00	-8.25	-9.24	0.12
157174	13	H	NO ₂	416	6.69	-7.90	-7.31	0.13
157173	12	H	O-CH ₃	401	6.71	-7.46	2.93	0.27
157160	10	H	H	371	6.82	-7.44	-0.69	0.19

^bThe Osiris calculation of compounds **20** and **23** has not been done.

The 3',4'-di-unsubstituted-aryl spiro-isoxazoline compound **10** is among the least active substances to have been evaluated as antituberculosis agents in this series. Accordingly, an effort was initiated to establish a pharmacophore hypothesis to delineate the requirements of the active site via a comprehensive program of analogue synthesis and evaluation of the effects of structural modification(s) on anti-tuberculosis activity of **10**.

We then set out to determine the resultant *in vitro* and *in vivo* effects of chemical alterations in each region. The unsubstituted phenyl-rings of **10** endowed anti-tuberculosis activity. The modulating anti-tuberculosis effect(s) of substituents having different electronegative properties, located at the *para*-phenyls sites comprising the two phenyl-rings of region III, were ascertained next. A chloro substituent located at the 4-position on the phenyl ring of **24** generated higher anti-tuberculosis activity relative to **26** and **10** in the Alamar test. However, in general *p*-nitro-phenyl or *p*-methoxy-phenyl proved to be unhelpful as substituents R². We postulate that the strong tendency to form a (O=C-C-O) dipolar pharmacophore site in the predominant (keto-oxonium) form is likely to be responsible for the lack of biological activity observed with these semi π -conjugated isoxazoline derivatives. If this hypothesis is correct, by modifications of **10** we may be able to modulate the degree of interaction of the compound with *Mycobacterium tuberculosis* bacteria.

Table 7. Molinspiration calculations of spiro-isoxazoline compounds **10-26**

TAACF Code	Compd	R ¹	R ²	Molinspiration calculations ¹⁸				
				CLogP	TPSA	GPCR Ligand	ICM	Kinase Inhibitor
157162	24	O-CH ₃	Cl	6.097	47.903	-0.26	-0.45	-0.62
157166	19	CH ₃	Cl	6.477	38.669	-0.31	-0.47	-0.67
157161	21	O-CH ₃	CH ₃	5.795	47.903	-0.30	-0.51	-0.65
157165	22	O-CH ₃	O-CH ₃	5.415	57.137	-0.26	-0.44	-0.60
157172	15	CH ₃	H	5.849	38.669	-0.31	-0.48	-0.68
157163	17	CH ₃	O-CH ₃	5.795	47.903	-0.30	-0.51	-0.65
157169	16	CH ₃	CH ₃	6.175	38.669	-0.30	-0.47	-0.66
157170	11	H	CH ₃	5.849	38.669	-0.31	-0.48	-0.68
157171	14	H	Cl	6.152	38.669	-0.27	-0.42	-0.65
157167	26	NO ₂	Cl	6.098	84.493	-0.42	-0.48	-0.69
157168	25	NO ₂	CH ₃	5.796	84.493	-0.45	-0.53	-0.72
157164	18	CH ₃	NO ₂	5.796	84.493	-0.45	-0.53	-0.72
157174	13	H	NO ₂	5.471	84.493	-0.42	-0.49	-0.70
157173	12	H	O-CH ₃	5.470	47.903	-0.26	-0.46	-0.63
157160	10	H	H	5.524	38.669	-0.26	-0.43	-0.66

Petra calculations

PETRA is a program package comprising various empirical methods for the calculation of physicochemical properties in organic molecules. All methods are empirical in nature and have been developed over the last 20 years in the research group of Prof. J. Gasteiger.¹⁹ The following chemical effects can be quantified: heats of formation, bond dissociation energies, sigma charge distribution, π -charge distribution, inductive effect, resonance effect and delocalization energies and polarizability effect.¹⁹

So to check the apparent antitubercular activity of compounds (**24**, **19**, **21**, **22**, **15**, **17** and **16**) and to look for evidence of structure-activity via *Mycobacterium tuberculosis* bacteria we performed *Petra Calculations*¹⁹ with **10-26** (Table 8).

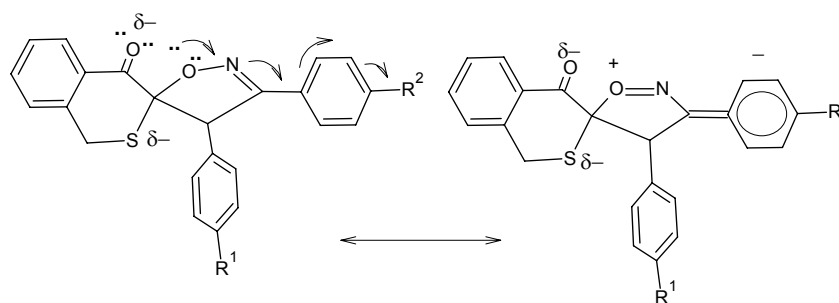


Table 8. Petra calculations of π -charges of heteroatoms of compounds **10-26**

TAACF Code	Compd.	R ¹	R ²	Petra calculations ¹⁹ (partial π -charge, e)			
				N ^{2'}	O ^{1'}	O ⁴	S ²
157162	24	O-CH ₃	Cl	-0.0947	+0.0309	-0.0827	0.00
157166	19	CH ₃	Cl	-0.0947	+0.0309	-0.0827	0.00
157161	21	O-CH ₃	CH ₃	-0.0735	+0.0272	-0.0827	0.00
157165	22	O-CH ₃	O-CH ₃	-0.0979	+0.0296	-0.0827	0.00
157172	15	CH ₃	H	-0.0732	+0.0275	-0.0827	0.00
157163	17	CH ₃	O-CH ₃	-0.0979	+0.0296	-0.0827	0.00
157169	16	CH ₃	CH ₃	-0.0735	+0.0272	-0.0827	0.00
157170	11	H	CH ₃	-0.0735	+0.0272	-0.0827	0.00
157171	14	H	Cl	-0.0947	+0.0309	-0.0827	0.00
157167	26	NO ₂	Cl	-0.0947	+0.0309	-0.0827	0.00
157168	25	NO ₂	CH ₃	-0.0735	+0.0272	-0.0827	0.00
157164	18	CH ₃	NO ₂	-0.0562	+0.0599	-0.0827	0.00
157174	13	H	NO ₂	-0.0562	+0.0590	-0.0827	0.00
157173	12	H	O-CH ₃	-0.0979	+0.0296	-0.0827	0.00
157160	10	H	H	-0.0732	+0.0275	-0.0827	0.00

The Petra software calculations confirmed that all compounds **10-26** have a clear preference for forming two antibacterial dipolar pharmacophore sites ($O^4=C^4-C^3-O^{1'}$) and ($S^2-C^3-O^{1'}$) though their estimated partial π charges respectively for O^4 , $O^{1'}$ are of different charges (-0.0827 e and $+0.0309$ e) for compound **21** for example. The Petra calculations with the other spiro-isoxazoline **10-26** are shown and summarised as follows in Table 7.

Compound **21** has the same partial π -charges [$+0.0275$ for O^4 and -0.0827 for $O^{1'}$] almost as tightly as **10** but its activity, as evidenced by a lack of observable antitubercular results, is considerably better (91% inhib. for **21** instead of 23% inhib. for **10**). Comparison of the couples of compounds (**11,15**), (**17,21**) and (**18,25**) with both the same substituents shows no detectable change in the antitubercular activity. In the light of these observations, the lack of antitubercular activity by the latter seven compounds (**15,16,17,19,21,22** and **24**) is explicable in terms of the existence of dipolar pharmacophore site ($O^4=C^4-C^3-O^{1'}$).

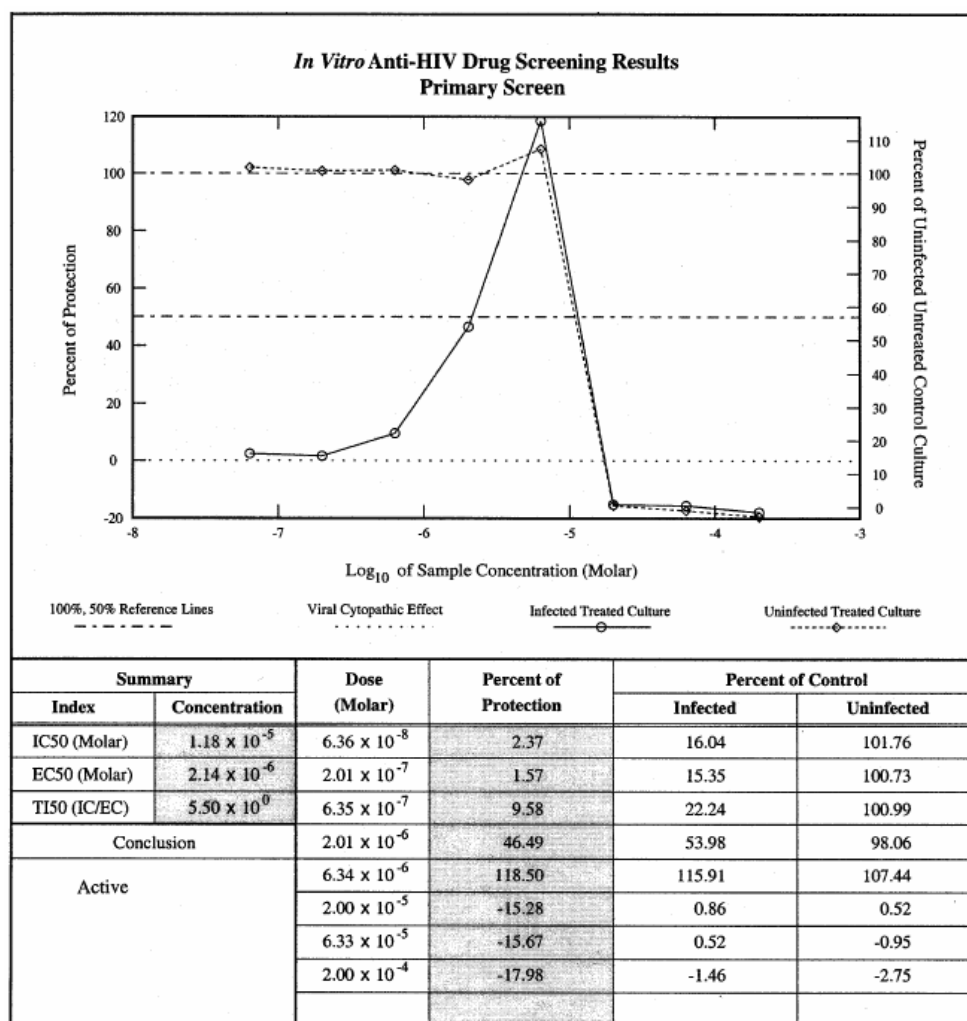


Figure 3. *In vitro* activity against HIV-1 results of active compound **25**.

Conclusions

The 3',4'-di-substituted spiroheterocycles **10-26** can easily be prepared by 1,3-dipolar cycloaddition reaction of *p*-substituted-benzaldoximes (**1-5**) with the appropriate (3*Z*)-3-(4-methylbenzylidene)-1*H*-isothiochromen-4(3*H*)-ones (**6-9**). These compounds typically form the highly interesting combined two pharmacophore sites in one molecule: S-C-O (antitubercular site; $d_{S...O} = 2.689$ Å) and O=C...N (anti-HIV site; $d_{O...N} = 3.516$ Å).

A number of important points emerge concerning their antitubercular properties. The positive results we have recorded, while encouraging for purposes of new drug design, confirm that very likely most of these compounds could be used without great risk of toxicity in diverse antibacterial activity. Based on their structural properties, these compounds may be useful as antitubercular agents with high activity or as potential antiviral agent.²⁰

These results prompt several pertinent observations: (i) This type of spiro-isoxazoline can furnish an interesting model for studying the interaction of spiro-isoxazoline antibiotics with DNA because the possible positive centre-bonding of a O¹ to the negatively charged centers of DNA is generally favoured; (ii) The rigid spiro geometric configuration enables us to prepare specific molecules for multi-therapeutic materials.

Acknowledgements

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Experimental Section

Antitubercular evaluation

Anti-tuberculosis activity assays were provided by the Tuberculosis Antimicrobial Acquisition and Coordinating Facility (TAACF) at the Southern Research Institute, Birmingham, AL, USA. Screening was conducted at 6.25 µg/mL against *Mycobacterium tuberculosis* H37Rv (ATCC 27294) using the Microplate Alamar Blue Assay.¹⁶ Physicochemical proprieties of compounds **10-26** such as (cLogP, solubility, Druglikeness, Drug-Score) and (cLogP, TPSA, GPCR Ligand, ICM, Kinase Inhibitor) and Atomic π -charges of heteroatoms are calculated by using respectively the Osiris, Molinspiration and Petra Calculations Programs.

The procedure used in anti-HIV screening

(a). Candidate agent is dissolved in dimethyl sulfoxide (unless otherwise instructed) then diluted 1:100 in cell culture medium before preparing serial half-Log₁₀ dilutions. T4 lymphocytes (CEM cell line) are added and after a brief interval HIV-1 is added, resulting in a 1:200 final dilution of the compound. Uninfected cells with the compound serve as a toxicity control, and infected and uninfected cells without the compound serve as basic controls. (b). Cultures are incubated at 37° in a 5% carbon dioxide atmosphere for 6 days. (c). The tetrazolium salt, XTT, is added to all wells, and cultures are incubated to allow formazan color development by viable cells. (d). Individual wells are analyzed spectrophotometrically to quantitate formazan production, and in addition are viewed microscopically for detection of viable cells and confirmation of protective activity. (e). Drug-treated virus-infected cells are compared with drug-treated noninfected cells and with other appropriate controls (untreated infected and untreated noninfected cells, drug-containing wells without cells, etc.) on the same plate. (f). Data are

reviewed in comparison with other tests done at the same time and a determination about activity is made.

General procedure

^1H and ^{13}C NMR spectra were recorded on a Bruker Spectrospin spectrometer operating at 80 MHz for ^1H (Université Paul Sabatier, Toulouse, France), an AC 200 spectrometer (operating at 200.12 MHz for ^1H , 50.32 MHz for ^{13}C), (Université de Franche-Comté, Besançon, France). Chemical shifts are listed in ppm and are reported relative to tetramethylsilane (^1H , ^{13}C), residual solvent peaks being used as internal standard. Complete assignments of the ^{13}C spectra required non-decoupled ^{13}C -NMR spectra with selective ^1H decoupling. Spectrometers, mass spectra on a Platform II Micro Mass spectrometer, Infrared spectra were obtained on a BECKMAN 310 spectrometer. Mass spectra on a HEWLETTPACKARD 5989A Mass spectrometer (70 eV) and elemental analysis (Université Paul Sabatier, Toulouse, France).

General synthesis

Synthesis of 3',4'-diaryl-4'*H*-spiro[isothiochromene-3,5'-isoxazol]-4(1*H*)-ones (10-26)

In an erlenmeyer equipped with a bulb for addition, a mixture of 10 mmol of the 3-arylideneisothiochroman-4-one **1-5** and 12 mmol of oxime **6-9** in 20 ml of chloroform was placed in a ice-salt bath, and under magnetic agitation 10 ml of a sodium hypochlorite solution (NaOCl , 18°) was added. Agitation was maintained for one hour after the addition. The organic phase was separated, washed several times with water, and dried on sodium sulphate. The residue obtained after evaporation of the solvent was recrystallized from ethanol.

3',4'-Di-phenyl-4'*H*-spiro[isothiochromene-3,5'-isoxazol]-4(1*H*)-one (10). M.p. = 158 °C. White powder. Yield 80%. IR (KBr; ν cm^{-1}): 1665 ($\text{C}=\text{O}$). ^1H NMR (CDCl_3 , δ ppm, J Hz): 8.1-7.2 (m, 14H, aromatic H); 6.2 (s, 1H, CH^4); 4.7 (d, 1H, H^{1a} , $^2\text{JH}^{1a}-\text{H}^{1b}$ = 16.8); 3.5 (d, 1H, H^{1b} , $^2\text{JH}^{1b}-\text{H}^{1a}$ = 16.8). ^{13}C NMR: 183.5 ($\text{C}^4=\text{O}$) ; 94.7 (spiro- $\text{C}^{3,5'}$); 56.6 (C^4) 28.9 ($-\text{SC}^1\text{H}_2-$); 127.85; 131.30; 140.85; 160.25 (quaternary aromatic C); 127.65; 127.80; 1280.10; 128.85; 128.70; 130.20; 130.45; 133.10 (tertiary aromatic C). MS, m/z (%): M^+ = 371 [$\text{C}_{23}\text{H}_{17}\text{NSO}_2$, (6%)], 118 (100%). Anal. Calcd. For $\text{C}_{23}\text{H}_{17}\text{NSO}_2$: C, 74.37; H, 4.61; N, 3.77. Found: C, 73.85; H, 4.81; N, 3.55.

3'-Phenyl-4'-(4-methyl-phenyl)-4'*H*-spiro[isothiochromene-3,5'-isoxazol]-4(1*H*)-one (11)

White powder. M.p. = 203 °C. Yield 90%. IR (KBr; ν cm^{-1}): 1680 ($\text{C}=\text{O}$). ^1H NMR (CDCl_3 , δ ppm, J Hz): 8.2-7.1 (m, 13H, aromatic H); 6.2 (s, 1H, CH^4); 4.7 (d, 1H, H^{1a} , $^2\text{JH}^{1a}-\text{H}^{1b}$ = 16.8); 3.5 (d, 1H, H^{1b} , $^2\text{JH}^{1b}-\text{H}^{1a}$ = 16.8); 2.3 (s, 3H, CH_3). ^{13}C NMR: 184.0 ($\text{C}^4=\text{O}$); 94.6 (spiro- $\text{C}^{3,5'}$); 56.7 (C^4); 28.2 ($-\text{SC}^1\text{H}_2-$); 21.6 (CH_3); 125.15; 131.30; 133.15; 140.65; 140.80; 160.10 (Quaternary aromatic C); 127.65; 127.70; 128.55; 128.80; 129.35; 129.50; 130.15; 130.45; 132.90 (tertiary aromatic C). MS, m/z (%): M^+ = 385 [$\text{C}_{24}\text{H}_{19}\text{NSO}_2$, (6%)], 118 (100%). Anal. Calcd. For $\text{C}_{24}\text{H}_{19}\text{NSO}_2$: C, 74.78; H, 4.97; N, 3.63. Found: C, 73.95; H, 5.12; N, 3.52.

3'-Phenyl-4'-(4-methoxy-phenyl)-4'*H*-spiro[isothiochromene-3,5'-isoxazol]-4(1*H*)-one (12).

White powder. M.p. = 178 °C. Yield 83%. IR (KBr; ν cm^{-1}): 1660 ($\text{C}=\text{O}$). ^1H NMR (CDCl_3 , δ

ppm, J Hz): 8.3-6.4 (m, 12H, aromatic H); 6.1 (s, 1H, CH^{4'}); 4.5 (d, 1H, H^{1a}, ²JH^{1a}-H^{1b} = 16.7); 3.7 (s, 3H, OCH₃); 3.4 (d, 1H, H^{1b}, ²JH^{1b}-H^{1a} = 16.7). ¹³C NMR: 183.7 (C⁴=O); 95.0 (spiro-C^{3,5'}); 56.5 (C^{4'}); 55.4 (OCH₃); 28.2 (-SC¹H₂-); 120.40; 130.45; 131.30; 133.25; 140.85; 159.75; 161.20 (Quaternary aromatic C); 114.10; 127.55; 127.80; 128.55; 128.60; 129.35; 130.20; 130.40; 132.95 (tertiary aromatic C). MS m/z: M⁺ = 401 [C₂₄H₁₉NSO₃, (6%)]; 118 (100%). Anal. Calcd. For C₂₄H₁₉NSO₃: C, 71.80; H, 4.77; N, 3.49. Found: C, 71.35; H, 4.67; N, 3.65.

3'-Phenyl-4'-(4-nitro-phenyl)-4'-H-spiro[isothiochromene-3,5'-isoxazol]-4(1H)-one (13).

White powder. M.p. = 174 °C. Yield = 60%. IR (KBr, ν cm⁻¹): 1670 (C=O). ¹H NMR (CDCl₃, δ ppm, J Hz): 8.4-7 (m, 13H, aromatic H); 6.20 (s, 1H, CH⁴); 4.5 (d, 1H, H^{1a}, ²JH^{1a}-H^{1b} = 16.5); 3.5 (d, 1H, H^{1b}, ²JH^{1b}-H^{1a} = 16.5). ¹³C NMR: 183.1 (C⁴=O); 94.6 (spiro-C^{3,5'}); 56.7 (C^{4'}); 28.0 (-SC¹H₂-) 132.10; 138.65; 140.65; 148.35; 158.60; 159.70 (quaternary aromatic C); 122.10; 126.40; 127.75; 127.90; 128.50; 128.90; 129.20; 130.10; 133.10 (tertiary aromatic C). MS m/z: M⁺ = 416 [C₂₃H₁₆SN₂O₄, (1%)], 118 (100%). Anal. Calcd. For C₂₃H₁₆N₂SO₄: C, 66.33; H, 3.87; N, 6.73. Found: C, 66.81; H, 3.56; N, 6.67.

3'-Phenyl-4'-(4-chloro-phenyl)-4'-H-spiro[isothiochromene-3,5'-isoxazol]-4(1H)-one (14).

White powder. M.p. = 198 °C. Yield = 70%. IR (KBr, ν cm⁻¹): 1665 (C=O). ¹H NMR (CDCl₃, δ ppm, J Hz): 8.3-7.2 (m, 13H, aromatic H); 6.2 (s, 1H, CH⁴); 4.7 (d, 1H, H^{1a}, ²JH^{1a}-H^{1b} = 16.8); 3.6 (d, 1H, H^{1b}, ²JH^{1b}-H^{1a} = 16.8). ¹³C NMR: 183.8 (C⁴=O); 94.9 (spiro-C^{3,5'}); 55.4 (C^{4'}); 28.1 (-SC¹H₂-). 126.55; 131.15; 132.65; 136.40; 140.75; 159.25 (Quaternary aromatic C); 127.60; 127.80; 128.70; 128.80; 129.00; 130.10; 130.45; 133.10 (tertiary aromatic C). MS m/z: M⁺ = 406 [C₂₃H₁₆SClNO₂, (3%)], 118 (100%). Anal. Calcd. For C₂₃H₁₆NSClO₂: C, 68.06; H, 3.97; N, 3.45. Found: C, 68.35; H, 3.52; N, 3.57.

3'-(4-Methoxy-phenyl)-4'-phenyl-4'-H-spiro[isothiochromene-3,5'-isoxazol]-4(1H)-one (15).

White powder. M.p. = 158 °C. Yield = 90%. IR (KBr, ν cm⁻¹): 1660 (C=O). ¹H NMR (CDCl₃, δ ppm, J Hz): 8.2-7.1 (m, 13H, aromatic H); 6.1 (s, 1H, CH^{4'}); 4.7 (d, 1H, H^{1a}, ²JH^{1a}-H^{1b} = 16.7); 3.5 (d, 1H, H^{1b}, ²JH^{1b}-H^{1a} = 16.7); 2.3 (s, 3H, CH₃). ¹³C NMR: 183.5 (C⁴=O); 94.7 (spiro-C^{3,5'}); 56.6 (C^{4'}); 28.9 (-SC¹H₂-); 21.3 (CH₃); 128.15; 129.90; 130.00; 131.30; 138.50; 140.85; 160.30 (quaternary aromatic C); 127.55; 127.80; 127.75; 130.00; 130.30; 130.45; 132.85 (tertiary aromatic C). MS, m/z (A.R%): M = 385 [C₂₄H₁₉NSO₂, (5%)], 118 (100%). Anal. Calcd. For C₂₄H₁₉NSO₂: C, 74.78; H, 4.97; N, 3.63. Found: C, 73.95; H, 4.81; N, 3.25.

3',4'-Di-(4-methyl-phenyl)-4'-H-spiro[isothiochromene-3,5'-isoxazol]-4(1H)-one (16).

White powder. M.p. = 179 °C. Yield = 85%. IR (KBr, ν cm⁻¹): 1675 (C=O). ¹H NMR (CDCl₃, δ ppm, J Hz): 8.1-7.1 (m, 12H, aromatic H); 6.1 (s, 1H, CH^{4'}); 4.7 (d, 1H, H^{1a}, ²JH^{1a}-H^{1b} = 16.8); 3.50 (d, 1H, H^{1b}, ²JH^{1b}-H^{1a} = 16.8); 2.5 (s, 3H, CH₃); 2.3 (s, 3H, CH₃). ¹³C NMR: 184.1 (C⁴=O); 94.6 (spiro-C^{3,5'}); 56.4 (C^{4'}); 28.8 (-SC¹H₂-); 21.5 (2CH₃); 125.30; 130.10; 131.40; 138.45; 140.60; 140.85; 160.25 (quaternary aromatic C); 127.50; 127.70; 127.75; 129.30; 129.35; 130.00; 130.45; 132.90 (tertiary aromatic C). MS, m/z: M⁺ = 399 [C₂₅H₂₁NSO₂, (7%)], 118 (100%). Anal. Calcd. For C₂₅H₂₁NSO₂: C, 75.16; H, 5.30; N, 3.51. Found: C, 74.95; H, 5.27; N, 3.54.

3'-(4-Methyl-phenyl)-4'-(4-methoxy-phenyl)-4'*H*-spiro[isothiochromene-3,5'-isoxazol]-4(1*H*)-one (17). White powder. M.p. = 153 °C. Yield = 90%. IR (KBr; ν cm⁻¹): 1675 (C=O). ¹H NMR (CDCl₃, δ ppm, J Hz): 8.1-6.7 (m, 12H, aromatic H); 6.1 (s, 1H, CH^{4'}); 4.7 (d, 1H, H^{1a}, ²JH^{1a}-H^{1b} = 16.8); 3.70 (s, 3H, OCH₃); 3.5 (d, 1H, H^{1b}, ²JH^{1b}-H^{1a} = 16.8); 2.3 (s, 3H, CH₃). ¹³C NMR: 184.1 (C⁴=O); 94.4 (spiro-C^{3,5'}); 56.4 (C^{4'}); 55.2 (OCH₃); 28.1 (-SC¹H₂-); 21.3 (CH₃); 120.50; 130.20; 131.35; 138.45; 140.90; 159.85; 161.20 (quaternary aromatic C); 114.10; 127.55; 127.80; 129.35; 130.05; 130.40; 132.95 (tertiary aromatic C). MS m/z (%): M⁺ = 415 [C₂₅H₂₁NSO₃, (9%)]; 132 (100%). Anal. Calcd. For C₂₅H₂₁NSO₂: C, 72.27; H, 5.09; N, 3.37. Found: C, 71.98; H, 4.97; N, 3.41.

3'-(4-Methyl-phenyl)-4'-(4-nitro-phenyl)-4'*H*-spiro[isothiochromene-3,5'-isoxazol]-4(1*H*)-one (18). White powder. M.p. = 187 °C. Yield = 65%. IR (KBr; ν cm⁻¹): 1680 (C=O). ¹H NMR (CDCl₃, δ ppm, J Hz): 8.1-7.1 (m, 12H, aromatic H); 6.1 (s, 1H, CH^{4'}); 4.7 (d, 1H, H^{1a}, ²JH^{1a}-H^{1b} = 16.8); 3.6 (d, 1H, H^{1b}, ²JH^{1b}-H^{1a} = 16.8); 2.3 (s, 3H). ¹³C NMR: 183.6 (C⁴=O); 95.6 (spiro-C^{3,5'}); 55.9 (C^{4'}); 28.1 (-SC¹H₂-); 21.3 (CH₃); 128.50; 130.00; 131.00; 139.15; 140.70; 148.60; 158.80 (quaternary aromatic C); 123.90; 127.75; 127.90; 128.10; 128.80; 129.05; 129.65; 130.90 (tertiary aromatic C). MS m/z: M⁺ = 430 [C₂₄H₁₈N₂SO₄, (3%)]; 132 (100%). Anal. Calcd. For C₂₄H₁₈N₂SO₄: C, 66.96; H, 4.21; N, 6.51. Found: C, 67.12; H, 4.18; N, 5.95.

3'-(4-Methyl-phenyl)-4'-(4-chloro-phenyl)-4'*H*-spiro[isothiochromene-3,5'-isoxazol]-4(1*H*)-one (19). White powder. M.p. = 148 °C. Yield = 67%. IR (KBr; ν cm⁻¹): 1680 (C=O). ¹H NMR (CDCl₃, δ ppm, J Hz): 8.1-7.1 (m, 12H, aromat. H); 6.1 (s, 1H, CH^{4'}); 4.7 (d, 1H, H^{1a}, ²JH^{1a}-H^{1b} = 16.8); 3.5 (d, 1H, H^{1b}, ²JH^{1b}-H^{1a} = 16.8); 2.3 (s, 3H, CH₃). ¹³C NMR: 183.7 (C⁴=O); 95.0 (spiro-C^{3,5'}); 56.2 (C^{4'}); 28.1 (-SC¹H₂-); 21.1 (CH₃); 126.70; 129.60; 131.20; 136.40; 138.75; 140.80; 159.35 (quaternary aromatic C); 127.60; 127.80; 129.00; 129.40; 130.00; 130.45; 133.05 (tertiary aromatic C). MS m/z: M⁺ = 420 [C₂₄H₁₈NSClO₂, (3%)]; 132 (100%). Anal. Calcd. For C₂₄H₁₈NSClO₂: C, 68.65; H, 4.32; N, 3.34. Found: C, 69.05; H, 4.17; N, 3.52.

3'-(4-Methoxy-phenyl)-4'-phenyl-4'*H*-spiro[isothiochromene-3,5'-isoxazol]-4(1*H*)-one (20). White powder. M.p. = 124 °C. Yield = 90%. IR (KBr; ν cm⁻¹): 1660 (C=O). ¹H NMR (CDCl₃, δ ppm, J en Hz): 8-6.6 (m, 13H, aromat. H); 6.1 (s, 1H, CH^{4'}); 4.7 (d, 1H, H^{1a}, ²JH^{1a}-H^{1b} = 16.8); 3.7 (s, 3H, OCH₃); 3.5 (d, 1H, H^{1b}, ²JH^{1b}-H^{1a} = 16.8);. ¹³C NMR: 183.6 (C⁴=O); 95.6 (spiro-C^{3,5'}); 55.8 (C^{4'}); 55.4 (OCH₃); 28.1 (-SC¹H₂-); 123.90; 131.00; 134.35; 140.70; 148.50; 158.70; 160.10 (quaternary aromatic C); Tertiary aromatic C : 114.25; 123.85; 127.70; 127.90; 128.50; 130.45; 131.25; 133.25 (tertiary aromatic C). MS m/z: M⁺ = 401 [C₂₄H₁₉NSO₃, (6%)]; 118 (100%). Anal. Calcd. For C₂₄H₁₉NSO₃: C, 71.80; H, 4.77; N, 3.49. Found: C, 72.08; H, 4.62; N, 3.47.

3'-(4-Methoxy-phenyl)-4'-(4-methyl-phenyl)-4'*H*-spiro[isothiochromene-3,5'-isoxazol]-4(1*H*)-one (21). White powder. M.p. = 150 °C. Yield = 90%. IR (KBr; ν cm⁻¹): 1675 (C=O). ¹H NMR (CDCl₃, δ ppm, J Hz): 8.2-6.8 (m, 12H, aromat. H); 6.1 (s, 1H, CH^{4'}); 4.7 (d, 1H, H^{1a}, ²JH^{1a}-H^{1b} = 16.8); 3.7 (s, 3H, OCH₃); 3.5 (d, 1H, H^{1b}, ²JH^{1b}-H^{1a} = 16.8); 2.3 (s, 3H, CH₃). ¹³C NMR: 180.8 (C⁴=O); 95.0 (spiro-C^{3,5'}); 57.0 (C^{4'}); 55.0 (OCH₃); 28.1 (-SC¹H₂-); 21.1 (CH₃); 120.50; 130.15; 131.35; 138.40; 140.90; 159.80; 161.20 (quaternary aromatic C); 114.10;

127.50; 127.80; 129.30; 129.35; 130.10; 131.40; 132.90 (tertiary aromatic C). MS m/z : M^+ = 415 [$C_{25}H_{21}NSO_3$, (25%)] ; 148 (100%); 132 (100%). Anal. Calcd. For $C_{25}H_{21}NSO_3$: C, 72.27; H, 5.09; N, 3.37. Found: C, 70.08; H, 4.97; N, 3.41.

3',4'-Di-(4-methoxy-phenyl)-4'H-spiro[isothiochromene-3,5'-isoxazol]-4(1H)-one (22).

White powder. M.p. = 154 °C. Yield = 85%. IR (KBr; ν cm^{-1}): 1665 (C=O). 1H NMR ($CDCl_3$, δ ppm, J Hz): 8.1-6 (m, 12H, aromat. H); 6.1 (s, 1H, CH^4); 4.7 (d, 1H, H^{1a} , $^2JH^{1a}-H^{1b}$ = 16.8); 3.7 (s, 6H, 2 OCH_3); 3.5 (d, 1H, H^{1b} , $^2JH^{1b}-H^{1a}$ = 16.8). ^{13}C NMR: 184.2 ($C^4=O$); 95.0 (spiro- $C^{3,5'}$); 56.2 (C^4); 55.3 (2x OCH_3); 28.2 ($-SC^1H_2-$); 120.50; 125.15; 130.40; 131.40; 140.90; 159.70; 159.80; 161.20 (quaternary aromatic C); Tertiary aromatic C : 113.90; 114.10; 127.50; 127.75; 129.35; 130.40; 131.30; 132.90 (tertiary aromatic C). MS m/z : M^+ = 431 [$C_{25}H_{21}NSO_4$, (5%)] ; 132 (100%). Anal. Calcd. For $C_{25}H_{21}NSO_4$: C, 69.59; H, 4.91; N, 3.25. Found: C, 69.83; H, 5.13; N, 3.15.

3'-(4-Methoxy-phenyl)-4'-(4-nitro-phenyl)-4'H-spiro[isothiochromene-3,5'-isoxazol]-4(1H)-one (23). White powder. M.p. = 150 °C. Yield = 60%. IR (KBr, ν cm^{-1}): 1670 (C=O). 1H NMR ($CDCl_3$, δ ppm, J Hz): 8.1-6.7 (m, 12H, aromat. H); 6.8 (s, 1H, CH^4); 4.6 (d, 1H, H^{1a} , $^2JH^{1a}-H^{1b}$ = 16.8); 3.7 (s, 3H, OCH_3); 3.5 (d, 1H, H^{1b} , $^2JH^{1b}-H^{1a}$ = 16.8). ^{13}C NMR: 183.6 ($C^4=O$); 95.8 (spiro- $C^{3,5'}$); 55.5 (C^4); 55.3 (OCH_3); 28.1 ($-SC^1H_2-$); 127.80; 130.95; 131.30; 140.75; 146.50; 158.60; 160.10 (quaternary aromatic C); 114.26; 123.89; 127.96; 128.54; 130.45; 130.95; 131.30; 133.30; 134.35 (tertiary aromatic C). MS m/z : M^+ = 446 [$C_{24}H_{18}N_2SO_5$, (5%)] ; 132 (100%). Anal. Calcd. For $C_{24}H_{18}N_2SO_5$: C, 64.56; H, 4.06; N, 6.27. Found: C, 63.95; H, 3.87; N, 5.95.

3'-(4-Methoxy-phenyl)-4'-(4-chloro-phenyl)-4'H-spiro[isothiochromene-3,5'-isoxazol]-4(1H)-one (24). White powder. M.p. = 132 °C. Yield = 80%. IR (KBr, ν cm^{-1}): 1675 (C=O). 1H NMR ($CDCl_3$, δ ppm, J Hz): 8.2-6.8 (m, 12H, aromat. H); 6.1 (s, 1H, CH^4); 4.7 (d, 1H, H^{1a} , $^2JH^{1a}-H^{1b}$ = 16.8); 3.8 (s, 3H, OCH_3); 3.6 (d, 1H, H^{1b} , $^2JH^{1b}-H^{1a}$ = 16.8). ^{13}C NMR: 183.6 ($C^4=O$); 95.1 (spiro- $C^{3,5'}$); 55.7 (C^4); 55.2 (OCH_3); 28.1 ($-SC^1H_2-$); 124.45; 126.60; 131.30; 136.30; 140.75; 159.30; 159.80 (quaternary aromatic C); 114.00; 127.55; 127.75; 128.90; 130.40; 131.20; 133.00 (tertiary aromatic C). MS m/z : M^+ = 436 [$C_{24}H_{18}NSClO_3$, (7%)]. Anal. Calcd. For $C_{24}H_{18}NSClO_3$: C, 66.13; H, 4.16; N, 3.21. Found: C, 65.98; H, 4.18; N, 3.15.

3'-(4-Nitro-phenyl)-4'-(4-methyl-phenyl)-4'H-spiro[isothiochromene-3,5'-isoxazol]-4(1H)-one (25). White powder. M.p. = 169 °C. Yield = 60%. IR (KBr; ν cm^{-1}): 1665 (C=O). 1H NMR ($CDCl_3$, δ ppm, J Hz): 8.2-7.2 (m, 12H, aromat. H); 6.2 (s, 1H, CH^4); 4.7 (d, 1H, H^{1a} , $^2JH^{1a}-H^{1b}$ = 16.8); 3.6 (d, 1H, H^{1b} , $^2JH^{1b}-H^{1a}$ = 16.8); 2.3 (s, 3H, CH_3). ^{13}C NMR: 183.4 ($C^4=O$); 94.4 (spiro- $C^{3,5'}$); 56.0 (C^4); 28.0 ($-SC^1H_2-$); 123.80; 140.35; 140.50; 141.30; 159.40 (quaternary aromatic C); 123.70; 127.55; 127.75; 127.80; 129.60; 130.50; 131.10; 133.25 (tertiary aromatic C). MS m/z : M^+ = 430 [$C_{24}H_{18}N_2SO_4$, (16%)] ; 89 (100%). Anal. Calcd. For $C_{24}H_{18}N_2SO_4$: C, 66.96; H, 4.21; N, 6.51. Found: C, 67.15; H, 4.18; N, 6.43.

3'-(4-Nitro-phenyl)-4'-(4-chloro-phenyl)-4'H-spiro[isothiochromene-3,5'-isoxazol]-4(1H)-one (26). White powder. M.p. = 149 °C. Yield = 60%. IR (KBr; ν cm^{-1}): 1665 (C=O). 1H NMR ($CDCl_3$, δ ppm, J Hz): 8.2-7.2 (m, 12H, aromat. H); 6.2 (s, 1H, CH^4); 4.7 (d, 1H, H^{1a} , $^2JH^{1a}-H^{1b}$

= 16.8); 3.6 (d, 1H, H^{1b} , $^2JH^{1b}-H^{1a}$ = 16.8); ^{13}C NMR: 183.4 ($C^4=O$); 94.4 ($C^{3,5'}$); 56.0 ($C^{4'}$); 28.0 ($-SC^1H_2-$); 125.90; 130.75; 137.60; 139.80; 140.40; 148.10; 158.55 (quaternary aromatic C); 123.80; 127.80; 127.90; 128.80; 129.25; 130.50; 131.10; 133.40 (tertiary aromatic C). MS m/z (%): M^+ = 451 [$C_{23}H_{15}N_2O_4ClS$, (16%)]; 89 (100%). Anal. Calcd. For $C_{23}H_{15}N_2O_4ClS$: C, 61.27; H, 3.35; N, 6.21. Found: C, 60.95; H, 3.26; N, 6.28.

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