First Non-ATP Competitive Glycogen Synthase Kinase 3 β (GSK-3β) Inhibitors: Thiadiazolidinones (TDZD) as Potential Drugs for the Treatment of Alzheimer's Disease

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Glycogen synthase kinase 3 β (GSK-3β) has a central role in Alzheimer's disease (AD). Selective inhibitors which avoid τ hyperphosphorylation may represent an effective therapeutical approach to the AD pharmacotherapy and other neurodegenerative disorders. Here, we describe the synthesis, biological evaluation, and SAR of the small heterocyclic thiadiazolidinones (TDZD) as the first non-ATP competitive inhibitor of GSK-3β. Their synthesis is based on the reactivity of sulfenyl chlorides. In GSK-3β assays, TDZD derivatives showed IC_{50} values in the micromolar range, whereas in other protein kinases assays they were devoid of any inhibitory activity. SAR studies allowed the identification of the key structural features. Finally, a possible enzymatic binding mode is proposed.

Introduction

Neurofibrillary tangles (NFTs) are one of the characteristic neuropathological lesions of Alzheimer's disease (AD) and other neurodegenerative processes such as frontotemporal dementia, Pick's disease, progressive supranuclear palsy, and corticobasal degeneration.[1] They are composed of a highly phosphorylated form of the microtubule-associated protein τ.[2] τ, in the adult human brain, is a set of six protein isoforms, whose binding affinity to microtubules can be modulated by phosphorylation. A total of 25 sites of aberrant phosphorylation has so far been identified in τ protein. Many of these sites are serine or threonine residues that are immediately followed in the sequence by proline residues and, hence, are candidate phosphorylation sites for proline-directed kinases.[3]

There is some evidence to suggest that τ hyperphosphorylation is an early event in the development of neurofibrillary pathology. Thus, the blockade of this hyperphosphorylation step may be a prime target at which to interrupt the pathogenic cascade.[4, 5]

Although many protein kinases are known to phosphorylate τ in vitro,[6] the in vivo players contributing to the hyperphosphorylation of τ remain elusive. Recent studies have demonstrated that glycogen synthase kinase 3 β (GSK-3β) and cyclin dependent kinase 5 (cdk5) most readily contribute to the ATP-induced "AD-like" phosphorylation of τ,[7] while studies on transfected cells and on cultured neurones showed that GSK-3β activity controls τ phosphorylation and τ functional interaction with microtubules.[8] GSK-3β binds to presenilin 1 and plays a role in Wnt and insulin signaling cascades, both of which have been proposed to be linked to AD.[9] Moreover GSK-3β activity may be altered in AD brain, and it has been postulated that GSK-3β mediates β-amyloid-induced neurotoxicity and presenilin-1 mutation pathogenic effects.[10,11] These observations suggest a central role for GSK-3β in AD.

Human GSK-3 is a ubiquitously expressed serine/threonine protein kinase implicated in the regulation of many physiological responses in mammalian cells by phosphorylating a variety of cytoplasmic and nuclear proteins.[12] It also phosphorylates components of the neuronal cytoskeleton including the microtubule-associated protein τ and neurofilament heavy chain. Two nearly identical forms of GSK-3 exist: GSK-3α and GSK-3β. Both are constitutively active in resting cells, but their differential expression and the lack of relation between transcription and translation in some tissues indicate that GSK-3α and GSK-3β have different means of regulation.[13]

Advances in genetics and transgenic approaches have a continuous impact on our understanding of AD and related disorders, especially since aspects of the histopathology and neurodegeneration can be reproduced in animal models. The recent transgenic mice overexpressing GSK-3β in the brain during adulthood are a valuable tool for the study of the pathogenesis and potential drug therapy of AD.[14]

Recently, the 3D crystalline structure of GSK-3β has been described[15,16] and has now begun to illuminate a sophisticated regulatory mechanism which is capable of processing different signals within the same cell.[17] These results may also lead to the design of function-specific drugs that disrupt some actions of GSK-3β but leave others intact.[18]

There is no doubt that potential selective inhibitors of GK3β which avoid τ hyperphosphorylation may represent an effective new therapeutical approach to the treatment of AD. Up to now, few compounds are known with this inhibitory enzymatic property. Lithium behaves as a specific inhibitor of the GSK-3 family of protein kinases in vitro and in intact cells,[19] and recent studies suggested a possible role of lithium in the

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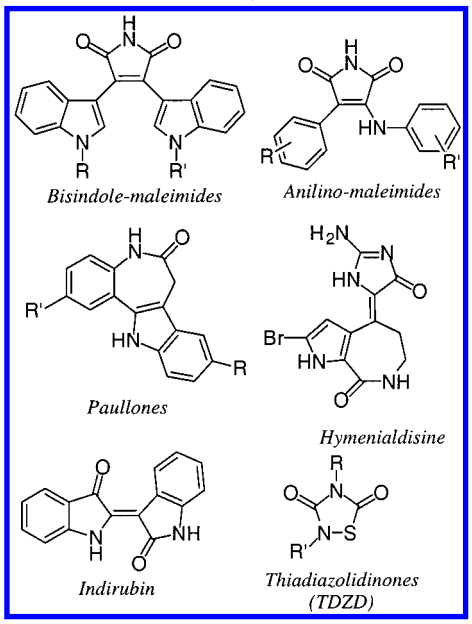
Chart 1. Inhibitors of GSK-3β



inhibition of GSK-3-induced τ phosphorylation.[20] However, its high milimolar inhibitory concentration prevents lithium from its potential therapeutical use.

Some compounds as bisindole[21] or anilino[22] maleimides, kenpaullone,[23] indirubin,[24] or the marine natural product hymenialdisine[25] (Chart 1) have recently been reported as GSK-3β inhibitors. All of them compete reversibly with ATP in their GSK-3β inhibition, showing a wide protein kinase inhibition profile. Here, we describe the small heterocyclic thiadiazolidinones (TDZD) as the first non-ATP competitive GSK-3β inhibitors as promising candidates for further development of AD pharmacotherapy.[26] Their synthesis, structure−activity relationships, and a hypothetical binding mode will be described.

Chemistry

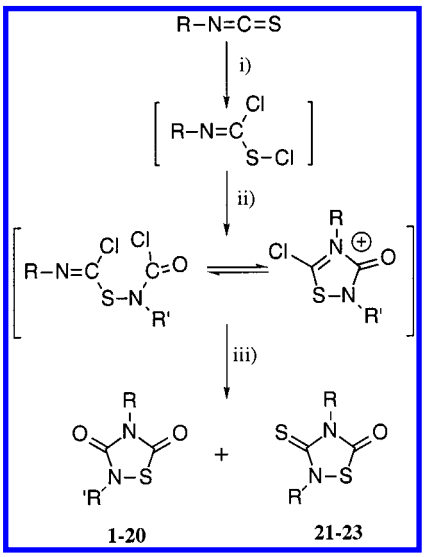
Thiadiazolidinones **1**−**20** were synthesized following a pathway which is based on the reactivity of *N*-alkyl-*S*-[*N*′-(chlorocarbonyl)amino]isothiocarbamoyl chlorides with isocyanates.[27] This synthesis was previously used in our laboratory for the efficient preparation of potassium channel openers[28] and cholinergic drugs.[29,30] Thus, chlorination of *N*-alkyl or *N*-arylisothiocyanates in an inert atmosphere and subsequent reaction with *N*-alkyl or *N*-arylisocyanates produces sparingly soluble 3-oxothiadiazolium salts via the intermediate iminochloromethylsulfenyl chloride. These heterocyclic salts are exceptionally reactive, and in the presence of moist air, it was possible to obtain through hydrolysis the 1,2,4-thiadiazolidine-3,5-diones **1**−**20** as white crystalline solids after evolution of hydrogen chloride (Scheme 1). In all the reactions assayed, a minoritary product could be detected by TLC which could be isolated by silica gel column chromatography only in some cases. The structural elucidation by analytical and spectroscopic (^{1}H and ^{13}C NMR) data revealed a 5-oxo-1,2,4-thiadiazolidin-3-thione chemical structure for the minoritary derivatives **21**−**23**.

The formation of these thione compounds could initially be explained by addition of the starting isothiocyanate to the corresponding iminosulfenyl chloride. To check this hypothesis, chlorination of benzylisothiocyanate and subsequent reactions with different alkylisothiocyanates were assayed. In all cases, a complex reaction mixture was obtained, and only the thione benzylated derivative **23** was isolated after several chromatographic purifications. Only when ethylisothiocyanate was used as the second reagent was the thione derivative **24** obtained as the minoritary product in low yield (Scheme 2). These results led us to discard the first proposed reaction mechanism. The reactivity of iminosulfenyl chlorides with isothiocyanates is lower than with isocyanates, and probably the thione derivatives **21**−**23** result from autocondensation of the corresponding iminosulfenyl chlorides.

Introduction of an imino moiety at the 5-position of the thiadiazolidine framework was achieved by the basic hydrolysis of the urea derivative **26** (Scheme 3). This last compound was easily obtained following a described procedure of 5-aminophenyl-1,2,3,4-thiatriazole rearrangement with isocyanates and isothiocyanates[31] (Scheme 3).

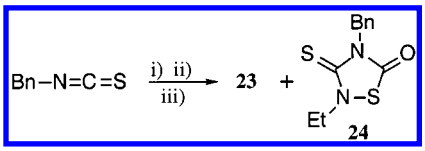
The structure of all new compounds was elucidated from their analytical and spectroscopic data (^{1}H and ^{13}C NMR) which are collected in the Experimental Section. Unequivocal assignment of all chemical shifts (^{1}H and ^{13}C NMR) was done using bidimensional experiments such as HMQC for one bond correlation and HMBC for long distance proton/carbon correlation. Thus, *N*2-CH$_x$ correlated exclusively with the quaternary carbon C-3, while *N*4-CH$_x$ correlated with C-3 and C-5 heterocyclic carbons, providing an unequivocal structure assignation.

Scheme 1[a]



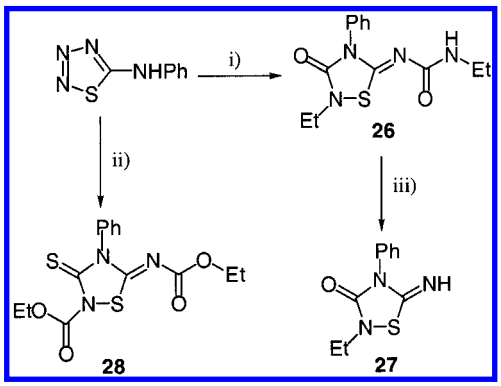
[a] Reagents: (i) Cl_2/hexane/N_2/-15 °C; (ii) R′−N=C=O/hexane/N_2/rt; (iii) air/rt.

Scheme 2[a]



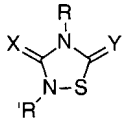
[a] Reagents: (i) Cl_2/hexane/N_2/-15 °C; (ii) Et−N=C=S/hexane/N_2/rt; (iii) air/rt.

Scheme 3[a]



[a] Reagents: (i) Et−N=C=O/Et_3N/dry THF/70 h/rt; (ii) $EtCO_2$−N−C=S//Et_3N/dry THF/70 h/rt; (iii) NaOH/MeOH/10 h.

Table 1. Glycogen Synthase Kinase-3β Inhibition of Thiadiazolidinones **1**−**28**



compd	R	R′	X	Y	IC_{50} (μM)[a]
1	Et	Me	O	O	5
2	Et	Et	O	O	25
3	Et	Pr	O	O	10
4	Et	^{i}Pr	O	O	35
5	Et	cyclohex	O	O	10
6	^{n}Bu	Et	O	O	70
7	cyclohex	Me	O	O	>100
8	Bn	Me	O	O	2
9	Bn	Et	O	O	7
10	Bn	Bn	O	O	10
11	Ph	Me	O	O	2
12	4-BrPh	Me	O	O	3
13	3-BrPh	Me	O	O	4
14	2-BrPh	Me	O	O	6
15	4-ClPh	Me	O	O	4
16	4-FPh	Me	O	O	4
17	4-CF_3Ph	Me	O	O	6
18	4-MePh	Me	O	O	5
19	4-OMePh	Me	O	O	2
20	1-naphthyl	Me	O	O	3
21	Et	Et	S	O	20
22	Ph	Ph	S	O	8
23	Bn	Bn	S	O	10
24	Bn	Et	S	O	6
25[b]	Et	Me	S	N-3Pyr	10
26[c]	Ph	Et	O	NCONHEt	75
27	Ph	Et	O	NH	65
28[c]	Ph	CO_2Et	S	NCO_2Et	100

[a] IC_{50}: 50% inhibitory concentration of glycogen synthase kinase-3β activity (μM). Assays were performed in triplicate. [b] Reference 28. [c] Reference 31.

Biological Results and SAR

The GSK-3β activity of the new TDZD derivatives here synthesized (**1**−**27**) was determined following a method described in the experimental part. Briefly, GSK-3β enzyme (Sigma) was incubated with ATP and GS-1 as substrate[32] in the presence and in the absence of the corresponding test compound. Finally, the GSK-3β activity was expressed in picomoles of phosphate incorporated per 20 min of incubation or in the percentage of maximal activity.

Results are collected in Table 1 where IC_{50} represents the compound concentration that inhibits the 50% of the enzyme activity. As it is shown, a wide range of TDZD inhibits GSK-3β with IC_{50} values in the micromolar range.

Table 2. IC_{50} Values for Kinase Inhibition of TDZD **8**

protein kinases	IC_{50} (μM)	specific activities
Cdk-1/cyclin B	>100	0.4 nmol Pi/min/mg protein
CK-II	>100	0.4 μmol Pi/min/mg protein
PKA	>100	4 nmol Pi/min/mg protein
PKC	>100	0.5 nmol Pi/min/mg protein

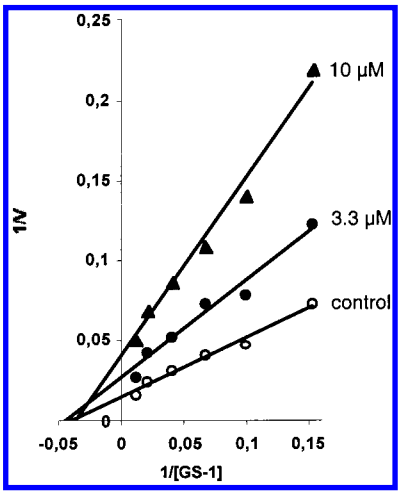


Figure 1. Double-reciprocal plot of kinetic data from assays of GSK-3β protein kinase activity at different concentrations of TDZD **8**. GS-1 concentrations in the reaction mixture varied from 6.5 to 100 μM, TDZD **8** concentrations are 3.3 and 10 μM, and the concentration of ATP was kept constant at 15 μM. *V* is picomoles of phosphate/20 min.

Selectivity of protein kinase inhibition[33] is critical for pathway analysis in cellular systems and to avoid widespread effects in a potentially therapeutical treatment. For these reasons, TDZD **8** was assayed against other kinases as protein kinases A and C (PKA and PKC), casein kinase II (CK-II), and cyclin dependent kinase 1 (Cdk-1/cyclin B). Although a number of recent articles[23−25] described potent inhibitors of Cdk-2 which also inhibit GSK-3β, all of the TDZD derivatives here evaluated showed IC_{50} values of >100 μM (see Table 2). These results point to a specific GSK-3β inhibition probably out of the ATP binding region of the enzyme.

To investigate the mechanism of TDZD's action on GSK-3β, several kinetic experiments were performed. First, we varied both GS-1 (6.5, 10, 15, 25, 50, and 100 μM) and TDZD **8** concentrations (3.3 and 10 μM) with a constant concentration of ATP (15 μM). Double-reciprocal plotting of the data (Figure 1), in which each point is mean of two different experiments, suggests that TDZD **8** acts as a noncompetitive inhibitor of GS-1 binding.

Moreover, kinetic experiments varying both ATP levels (6.5, 10, 15, 25, 50, and 100 μM) and inhibitor concentrations were performed. In these cases, two different GSK-3β inhibitors were used: our TDZD **8** and the known ATP competitive inhibitor Ro 31-8220.[21] We have measured the kinase activity increasing ATP up to 100 μM where enzyme saturation could be ensured. Double-reciprocal plotting of the data (Figure 2), in which each point is mean of two different experiments, suggests that TDZD **8** act as a noncompetitive inhibitor

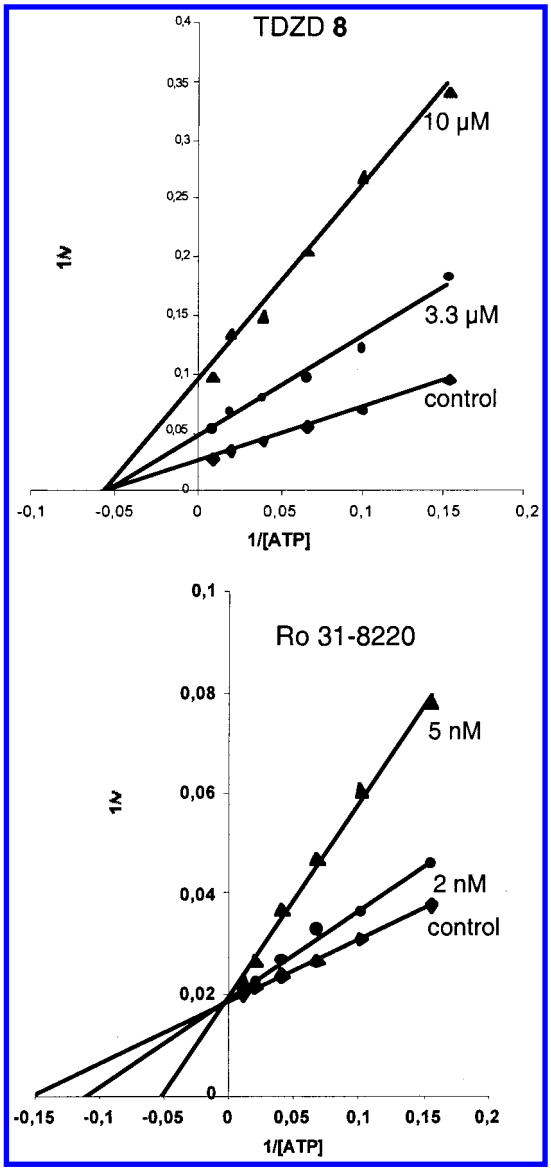


Figure 2. Double-reciprocal plot of kinetic data from assays of GSK-3β protein kinase activity at different concentrations of inhibitors. ATP concentrations in the reaction mixture varied from 6.5 to 100 μM, and inhibitor concentrations are 3.3 and 10 μM for TDZD **8** and 2 and 5 μM for Ro 31-8220. The concentration of GS-1 was kept constant in both experiments at 15 μM. *V* is picomoles of phosphate/20 min.

of ATP binding while the ATP competitive binding mode of Ro 31-8220 is shown.

These results provide the first non-ATP competitive inhibitors of GSK-3β. To date, all the GSK-3β inhibitors described compete with the ATP in their binding mode to the enzyme. The property here described for TDZD inhibitors could represent an important hit in their potential use in pharmacotherapy.

Taking into account the interesting enzymatic profile found in these TDZD compounds, preliminary structure−activity relationships can be done.

The size of the substituent attached to the *N*2 of the thiadiazolidinone ring seems to be crucial for GSK-3β inhibition, the methyl moiety being the best substitution obtained in this test set (see compound **1** versus **2**−**5**, or TDZD **8** versus **9** and **10**). This fact could point to a steric hindrance in the enzyme.

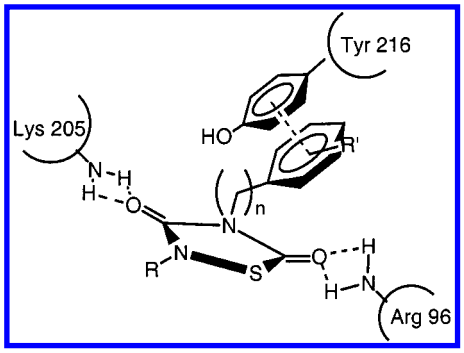


Figure 3. Proposed binding mode of TDZD to GSK-3β.

On the other hand, the nature of the substituent of *N*4 is also important for inhibition. When comparisons allow, potency is always enhanced with the introduction of an aromatic moiety, such as phenyl, methylphenyl, or naphthyl groups (compounds **8** and **11**−**20**), suggesting favorable hydrophobic interactions with the protein. Regarding the alkyl substituents in this *N*4 position, only the smallest ones, such as ethyl moiety, result in a GSK-3β inhibitor (compounds **1** versus **6** or **7**).

Substitution of the carbonyl group attached to the *C*3 position of the heterocycle by a thiocarbonyl fragment led to derivatives that mainly retain the inhibitory potency (compounds **21**, **23**, and **24** versus **2**, **10**, and **9**, respectively) but decreased compound solubility and stability. However, when the *C*5 carbonyl group is replaced by the bioisosteric imino moiety, a dramatic decrease in the inhibition is observed (compounds **25**−**28**). This fact might suggest that the *C*5-carbonyl group is critical for binding.

During the preparation of this manuscript, the 3D crystalline structure of GSK-3β has been reported by two different groups.[15,16] In both cases, an oxyanion binding site was determined, and a small molecule (HEPES or phosphate) is necessary for the formation of crystals and the stabilization of the active conformation. According to these authors, the convergence of three basic residues (Arg 96, Arg 180, and Lys 205) is required for the active conformation, and it can only be achieved by counterbalancing negative charge provided by the oxyanion. Regarding the chemical structure and the SAR observed in the TDZD-specific GSK-3β inhibitors here described, we propose a hypothetical binding mode that must be further theoretically and experimentally confirmed. In this model, the negative charge on the TDZD heterocycle may be the driving force to recognize the oxyanion binding site of the enzyme. Moreover, interactions between the side chain of residues Arg 96 and Lys 205 and the crucial carbonyl groups of TDZD might be possible (Figure 3). An additional disposition of the inhibitor with the *N*4 pointing out to the inside of the activation loop toward Tyr 216 might be explain the potency of the *N*4-aryl TDZD by potential π−π nonbonded interactions.

Conclusions

An efficient synthetic approach based on the reactivity of sulfenyl chlorides to derivatives of a novel kinase inhibitor template, the thiadiazolidin-3,5-dione (TDZD) derivatives, is described. These compounds represent the first non-ATP competitive inhibitors of GSK-3β reported to date. Preliminary structure−activity rela-

tionships show the key features for inhibition and have been used to propose a hypothetically enzymatic binding mode. The potency and the selectivity of TDZD should allow their use as tool compounds to aid resolution of the complex signaling pathway where GSK-3β is implicated and as new drugs for the effective treatment of Alzheimer's disease and other neurodegenerative process in which the τ protein is involved.

Experimental Section

Chemical Procedures. Melting points were determined with a Reichert-Jung Thermovar apparatus and are uncorrected. Flash column chromatography was carried out at medium pressure using silica gel (E. Merck, Grade 60, particle size 0.040−0.063 mm, 230−240 mesh ASTM) and preparative centrifugal circular thin-layer chromatography (CCTLC) on a circular plate coated with a 1 mm layer of Kieselgel 60 PF254, Merk, by using a Chromatotron with the indicated solvent as eluent. Compounds were detected with UV light (254 nm). ^{1}H NMR spectra were obtained on Varian XL-300 and Gemini-200 spectrometers working at 300 and 200 MHz, respectively. Typical spectral parameters were as follows: spectral width 10 ppm, pulse width 9 μs (57°), data size 32 K. ^{13}C NMR experiments were carried out on the Varian Gemini-200 spectrometer operating at 50 MHz. The acquisition parameters were as follows: spectral width 16 kHz, acquisition time 0.99 s, pulse width 9 μs (57°), data size 32 K. Chemical shifts are reported in δ values (ppm) relative to internal Me_4Si, and J values are reported in hertz. Elemental analyses were performed by the analytical department at C.N.Q.O. (CSIC), and the results obtained were within ±0.4% of the theoretical values.

General Procedure for the Synthesis of 1,2,4-Thiadiazolidine-3,5-dione. Chlorine was bubbled slowly through a solution of aryl or alkyl isothiocyanate in dry hexane (25 mL), under nitrogen atmosphere, at −15 °C to −10 °C. Chlorine was generated by the addition of 35% HCl to $KMnO_4$. The temperature of the reaction mixture was carefully controlled during the addition step. At this point, the *N*-aryl or *N*-alkyl-*S*-chloroisothiocarbamoyl chloride was formed. Afterward, alkyl or aryl isocyanate was added. The mixture was stirred at room temperature between 8 and 10 h. After this time, the resulting product was purified by suction filtration and recrystallization or silica gel column chromatography using the appropriate eluent. In some cases, it was possible to isolate 5-oxo-1,2,4-thiadiazolidine-3-thione as secondary product as is indicated below.

4-Ethyl-2-methyl-1,2,4-thiadiazolidine-3,5-dione (1). Reagents: Ethyl isothiocyanate (0.56 mL, 6.5 mmol), 35% HCl (3.1 mL), $KMnO_4$ (0.5 g), methyl isocyanate (0.38 mL, 6.5 mmol). Conditions: rt, 8 h. Purification: CCTLC using AcOEt/hexane (1:1). Yield: 0.30 g (29%) as orange oil. ^{1}H NMR ($CDCl_3$): 1.22 (t, 3H, CH_2CH_3, J = 6.9 Hz); 3.12 (s, 3H, CH_3); 3.71 (c, 2H, CH_2CH_3, J = 6.9 Hz). ^{13}C NMR ($CDCl_3$): 13.1 (CH_2CH_3); 31.3 (CH_3); 46.5 (CH_2CH_3); 153.2 (3-C=O); 165.5 (5-C=O). Anal. ($C_5H_8N_2SO_2$) C, H, N, S.

2,4-Diethyl-1,2,4-thiadiazolidine-3,5-dione (2). Reagents: Ethyl isothiocyanate (0.56 mL, 6.5 mmol), 35% HCl (3.1 mL), $KMnO_4$ (0.5 g), ethyl isocyanate (0.51 mL, 6.5 mmol). Conditions: rt, 8 h. Purification: silica gel column chromatography using AcOEt/hexane (1:1). Yield: 0.43 g (38%) as yellow oil. ^{1}H NMR ($CDCl_3$): 1.24 (t, 3H, CH_2CH_3, J = 7.1 Hz); 1.24 (t, 3H, CH_2CH_3, J = 7.1 Hz); 3.71 (c, 2H, CH_2CH_3, J = 7.1 Hz); 3.66 (c, 2H, $'CH_2CH_3$, J = 7.1 Hz). ^{13}C NMR ($CDCl_3$): 13.1 (CH_2CH_3); 13.8 ($CH_2'CH_3$); 37.7 ($'CH_2CH_3$); 39.8 (CH_2CH_3); 152.7 (3-C=O); 165.9 (5-C=O). Anal. ($C_6H_{10}N_2SO_2$) C, H, N, S.

4-Ethyl-2-propyl-1,2,4-thiadiazolidine-3,5-dione (3). Reagents: Ethyl isothiocyanate (0.56 mL, 6.5 mmol), 35% HCl (3.1 mL), $KMnO_4$ (0.5 g), propyl isocyanate (0.62 mL, 6.5 mmol). Conditions: rt, 9 h. Purification: silica gel column chromatography using AcOEt/hexane (1:3). Yield: 0.44 g (36%) as orange oil. ^{1}H NMR ($CDCl_3$): 0.9 (t, 3H, $CH_2CH_2'CH_3$, J = 7.3 Hz); 1.24 (t, 3H, CH_2CH_3, J = 6.9 Hz); 1.61 (m, 2H, $CH_2'CH_2CH_3$); 3.71 (c, 2H, CH_2CH_3, J = 6.9 Hz); 3.9 (t, 2H, $'CH_2CH_2CH_3$, J = 7.3 Hz). ^{13}C NMR ($CDCl_3$): 2.2 ($CH_2CH_2'CH_3$); 13.1 (CH_2CH_3); 22.1 ($CH_2'CH_2CH_3$); 39.2 ($'CH_2CH_2CH_3$); 46.5 (CH_2CH_3); 152.2 (3-C=O); 166.1 (5-C=O). Anal. ($C_7H_{12}N_2SO_2$) C, H, N, S.

4-Ethyl-2-isopropyl-1,2,4-thiadiazolidine-3,5-dione (4). Reagents: Ethyl isothiocyanate (0.56 mL, 6.5 mmol), 35% HCl (3.1 mL), $KMnO_4$ (0.5 g), isopropyl isocyanate (0.63 mL, 6.5 mmol). Conditions: rt, 9 h. Purification: silica gel column chromatography using AcOEt/hexane (1:3). Yield: 0.37 g (30%) as yellow oil. ^{1}H NMR ($CDCl_3$): 1.20 (d, 6H, $CH(CH_3)_2$, J = 6.6 Hz); 1.31 (t, 3H, CH_2CH_3, J = 6.2 Hz); 3.73 (c, 2H, CH_2CH_3, J = 6.2 Hz); 4.62 (m, $CH(CH_3)_2$). ^{13}C NMR ($CDCl_3$): 13.1 (CH_2CH_3); 21.2 ($CH(CH_3)_2$); 37.4 ($CH(CH_3)_2$); 46.9 (CH_2CH_3); 152.2 (3-C=O); 166.1 (5-C=O). Anal. ($C_7H_{12}N_2SO_2$) C, H, N, S.

2-Cyclohexyl-4-ethyl-1,2,4-thiadiazolidine-3,5-dione (5) and 2,4-Diethyl-5-oxo-1,2,4-thiadiazolidine-3,5-dione (21). Reagents: Ethyl isothiocyanate (0.56 mL, 6.5 mmol), 35% HCl (3.1 mL), $KMnO_4$ (0.5 g), cyclohexyl isocyanate (0.825 mL, 6.5 mmol). Conditions: rt, 10 h. Purification: silica gel column chromatography using AcOEt/hexane (1:10). Yield: The first fraction yielded 0.12 g of **5** (2%) as yellow oil. ^{1}H NMR ($CDCl_3$): 1.20 (t, 3H, CH_2CH_3, J = 7.1 Hz); 1.31 (t, 3H, $CH_2'CH_3$, J = 7.2 Hz); 3.33 (c, 2H, $'CH_2CH_3$, J = 7.2 Hz); 3.89 (c, 2H, CH_2CH_3, J = 7.1 Hz). ^{13}C NMR ($CDCl_3$): 12.2 (CH_2CH_3); 15.7 ($CH_2'CH_3$); 42.4 (CH_2CH_3); 45.8 ($'CH_2CH_3$); 146.3 (3-C=S); 168.2 (5-C=O). Anal. ($C_6H_{10}N_2OS_2$) C, H, N, S.

The second fraction yielded 0.73 mg of **21** as white solid (49%); mp = 45−48 °C. ^{1}H NMR ($CDCl_3$): 1.20 (t, 3H, CH_2CH_3, J = 7.1 Hz); 1.31−1.92 (m, 5H, chex); 3.72 (c, 2H, CH_2CH_3, J = 7.1 Hz). ^{13}C NMR ($CDCl_3$): 13.0 (CH_2CH_3); 39.8 (CH_2CH_3); 24.7; 25.1; 31.73; 53.71 (C chex); 152.2 (3-C=O); 166.2 (5-C=O). Anal. ($C_{10}H_{16}N_2O_2S$) C, H, N, S.

4-*n*-Butyl-2-ethyl-1,2,4-thiadiazolidine-3,5-dione (6). Reagents: *n*-Butyl isothiocyanate (0.78 mL, 6.5 mmol), 35% HCl (3.1 mL), $KMnO_4$ (0.5 g), ethyl isocyanate (0.51 mL, 6.5 mmol). Conditions: rt, 8 h. Purification: silica gel column chromatography using CH_2Cl_2/hexane (1:1). Yield: 0.51 g (44%) as yellow oil. ^{1}H NMR ($CDCl_3$): 0.92 (t, 3H, $CH_2CH_2CH_2CH_3$, J = 7.14 Hz); 1.25 (t, 3H, CH_2CH_3, J = 7.14 Hz); 1.32 (m, 2H, $CH_2CH_2CH_2CH_3$); 1.55−1.72 (m, 2H, $CH_2CH_2CH_2CH_3$); 3.65 (t, 2H, $CH_2CH_2CH_2CH_3$, J = 7.14 Hz); 3.66 (c, 2H, CH_2CH_3, J = 7.14 Hz). ^{13}C NMR ($CDCl_3$): 13.3 ($CH_2CH_2CH_2CH_3$); 13.5 (CH_2CH_3); 19.5 ($CH_2CH_2CH_2CH_3$); 29.5 ($CH_2CH_2CH_2CH_3$); 39.7 (CH_2CH_3); 42.1 ($CH_2CH_2CH_2CH_3$); 152.7 (3-C=O); 165.7 (5-C=O). Anal. ($C_8H_{14}N_2SO_2$) C, H, N, S.

4-Cyclohexyl-2-methyl-1,2,4-thiadiazolidine-3,5-dione (7). Reagents: Cyclohexyl isothiocyanate (0.78 mL, 6.5 mmol), 35% HCl (3.1 mL), $KMnO_4$ (0.5 g), methyl isocyanate (0.38 mL, 6.5 mmol). Conditions: rt, 10 h. Purification: silica gel column chromatography using AcOEt/hexane (1:10). Yield: 0.045 g (3%) as yellow oil. ^{1}H NMR ($CDCl_3$): 1.25−2.20 (m, 5H, chex); 3.1 (s, 3H, CH_3).^{13}C NMR ($CDCl_3$): 24.9; 25.8; 28.8; 55.0 (C chex); 31.3 (CH_3); 153.4 (3-C=O); 165.7 (5-C=O). Anal. ($C_9H_{14}N_2SO_2$) C, H, N, S.

4-Benzyl-2-methyl-1,2,4-thiadiazolidine-3,5-dione (8) and 2,4-Dibenzyl-5-oxothiadiazolidine-3-thione (23). Reagents: Benzyl isothiocyanate (0.86 mL, 6.5 mmol), 35% HCl (3.1 mL), $KMnO_4$ (0.5 g), methyl isocyanate (0.38 mL, 6.5 mmol). Conditions: rt, 8 h. Isolation **(8)**: filtration of reaction mixture. Purification: recrystallization from hexane. Yield: 0.75 g (35%) as white solid; mp 60−61 °C. ^{1}H NMR ($CDCl_3$): 3.2 (s, 3H, CH_3); 4.8 (s, 2H, CH_2−Bn); 7.31−7.45 (m, 5H, arom.).^{13}C NMR ($CDCl_3$): 31.4 (CH_3); 46.0 (CH_2−Bn) 128.2; 128.6; 128.8; 135.1 (C arom.); 155.2 (3-C=O); 165.6 (5-C=O). Anal. ($C_{10}H_{10}N_2SO_2$) C, H, N, S.

Isolation of (**23**): The filtrate was evaporated. Purification: silica gel column chromatography using CH_2Cl_2/hexane (1:1). Yield: 0.08 g (8%) as yellow solid; mp 91−95 °C. ^{1}H NMR ($CDCl_3$): 4.52 (s, 2H, $'CH_2$−Bn); 5.10 (s, 2H, CH_2−Bn); 7.31−7.52 (m, 10H, arom.). ^{13}C NMR ($CDCl_3$): 50.1 (CH_2−Bn); 54.3 ($'CH_2$−Bn); 128.1; 128.4; 128.9; 135.4 (C arom.); 127.1; 127.4;

128.4; 138.6 (′C arom.); 148.1 (3-C═S); 169.0 (5-C═O). Anal. ($C_{16}H_{14}N_2S_2O$) C, H, N, S.

4-Benzyl-2-ethyl-1,2,4-thiadiazolidine-3,5-dione (9). Reagents: Benzyl isothiocyanate (0.86 mL, 6.5 mmol), 35% HCl (3.1 mL), $KMnO_4$ (0.5 g), ethyl isocyanate (0.51 mL, 6.5 mmol). Conditions: rt, 10 h. Purification: silica gel column chromatography using CH_2Cl_2/hexane (1:1) and CCTLC using CH_2Cl_2. Yield: 0.39 g (25%) as yellow oil; ^{1}H NMR ($CDCl_3$): 1.22 (t, 3H, $CH_2'CH_3$, J = 7.2 Hz); 3.7 (c, 2H, $'CH_2CH_3$, J = 7.2 Hz); 4.8 (s, 2H, CH_2−Bn); 7.32−7.44 (m, 5H, arom.). ^{13}C NMR ($CDCl_3$): 13.7 ($CH_2'CH_3$); 39.9 ($'CH_2CH_3$); 45.8 (CH_2−Bn); 128.1; 128.6; 128.8; 135.1 (C arom.); 152.6 (3-C═O); 165.9 (5-C═O). Anal. ($C_{11}H_{12}N_2SO_2$) C, H, N, S.

2,4-Dibenzyl-1,2,4-thiadiazolidine-3,5-dione (10). Reagents: Benzyl isothiocyanate (0.86 mL, 6.5 mmol), 35% HCl (3.1 mL), $KMnO_4$ (0.5 g), benzyl isocyanate (0.80 mL, 6.5 mmol). Conditions: rt, 9 h. Isolation: filtration of reaction mixture. Purification: recrystallization from ethanol. Yield: 0.57 g (30%) as white solid; mp 78−80 °C. ^{1}H NMR ($CDCl_3$): 4.7 (s, 2H, ′CH_2−Bn); 4.8 (s, 2H, CH_2−Bn); 7.32−7.55 (m, 10H, arom.). ^{13}C NMR ($CDCl_3$): 46.0 (CH_2−Bn); 48.8 (′CH_2−Bn); 128.3; 128.5; 128.7; 134.4 (′C arom.); 128.3; 128.8; 128.9; 135.2 (C arom.); 153.1 (3-C═O); 165.9 (5-C═O). Anal. ($C_{16}H_{14}N_2SO_2$) C, H, N, S.

4-Phenyl-2-methyl-1,2,4-thiadiazolidine-3,5-dione (11) and 2,4-Diphenyl-5-oxothiadiazolidine-3-thione (22). Reagents: Phenyl isothiocyanate (0.78 mL, 6.5 mmol), 35% HCl (3.1 mL), $KMnO_4$ (0.5 g), methyl isocyanate (0.38 mL, 6.5 mmol). Conditions: rt, 8 h. Isolation **(11)**: filtration of reaction mixture. Purification: recrystallization from methanol. Yield: 0.25 g (30%) as white solid; mp 174−179 °C. ^{1}H NMR ($CDCl_3$): 3.21 (s, 3H, CH_3); 7.31−7.50 (m, 5H, arom.). ^{13}C NMR ($CDCl_3$): 31.7 (CH_3); 127.2; 129.2; 129.4; 132.7 (C arom.); 152.7 (3-C═O); 165.3 (5-C═O). Anal. ($C_8H_8N_2SO_2$) C, H, N, S.

Isolation of (**22**): The filtrate was evaporated. Purification: silica gel column chromatography using CH_2Cl_2. Yield: 0.14 g (15%) as yellow solid; mp 105−110 °C. ^{1}H NMR ($CDCl_3$): 6.70−7.01 (m, 5H, arom); 7.12−7.33 (m, 5H, ′arom.). ^{13}C NMR ($CDCl_3$): 127.2; 128.6; 129.4; 132.7 (C arom.); 128.7; 129.2; 129.7; 146.7 (′C arom.); 152.4 (3-C═S); 169.3 (5-C═O). Anal. ($C_{14}H_{10}N_2S_2O$) C, H, N, S.

4-(4-Bromophenyl)-2-methyl-1,2,4-thiadiazolidine-3,5-dione (12). Reagents: 4-Bromophenyl isothiocyanate (1.4 g, 6.5 mmol), 35% HCl (3.1 mL), $KMnO_4$ (0.5 g), methyl isocyanate (0.38 mL, 6.5 mmol). Conditions: rt, 9 h. Isolation: filtration of reaction mixture. Purification: recrystallization from hexano/CH_2Cl_2. Yield: 0.32 g (20%) as white solid; mp 182−184 °C. ^{1}H NMR ($CDCl_3$): 3.25 (s, 3H, CH_3); 7.25−7.61 (2 d, 4H, arom., J = 8.6 Hz). ^{13}C NMR ($CDCl_3$): 31.6 (CH_3); 123.0; 128.6; 131.6; 132.5 (C arom.); 153.4 (3-C═O); 165.7 (5-C═O). Anal. ($C_9H_7BrN_2SO_2$) C, H, N, S.

4-(3-Bromophenyl)-2-methyl-1,2,4-thiadiazolidine-3,5-dione (13). Reagents: 3-Bromophenyl isothiocyanate (1.4 g, 6.5 mmol), 35% HCl (3.1 mL), $KMnO_4$ (0.5 g), methyl isocyanate (0.38 mL, 6.5 mmol). Conditions: rt, 8 h. Isolation: filtration of reaction mixture. Purification: recrystallization from *n*-propanol. Yield: 0.40 g (21%) as white solid; mp 113−116 °C. ^{1}H NMR ($CDCl_3$): 3.15 (s, 3H, CH_3); 7.22−7.51 (m, 4H, arom.). ^{13}C NMR ($CDCl_3$): 31.6 (CH_3); 122.5; 130.3; 130.5; 132.5; 133.7 (C arom.); 152.1 (3-C═O); 164.9 (5-C═O). Anal. ($C_9H_7BrN_2SO_2$) C, H, N, S.

4-(2-Bromophenyl)-2-methyl-1,2,4-thiadiazolidine-3,5-dione (14). Reagents: 2-Bromophenyl isothiocyanate (0.87 mL, 6.5 mmol), 35% HCl (3.1 mL), $KMnO_4$ (0.5 g), methyl isocyanate (0.38 mL, 6.5 mmol). Conditions: rt, 9 h. Isolation: filtration of reaction mixture. Purification: recrystallization from *n*-butanol. Yield: 0.41 g (22%) as white solid; mp 136−140 °C. ^{1}H NMR ($CDCl_3$): 3.15 (s, 3H, CH_3); 7.22−7.63 (m, 4H, arom.). ^{13}C NMR ($CDCl_3$): 31.8 (CH_3); 123.0; 128.7; 130.6; 131.5; 132.2 (C arom.); 151.7 (3-C═O); 164.4 (5-C═O). Anal. ($C_9H_7BrN_2SO_2$) C, H, N, S.

4-(4-Chlorophenyl)-2-methyl-1,2,4-thiadiazolidine-3,5-dione (15). Reagents: 4-Chlorophenyl isothiocyanate (1.1 g, 6.5 mmol), 35% HCl (3.1 mL), $KMnO_4$ (0.5 g), methyl isocyanate (0.38 mL, 6.5 mmol). Conditions: rt, 6 h. Isolation: filtration of reaction mixture. Purification: recrystallization from ethanol. Yield: 0.47 g (30%) as white solid; mp 175−178 °C. ^{1}H NMR ($CDCl_3$): 3.25 (s, 3H, CH_3); 7.32−7.44 (2 d, 4H, arom., J = 8.9 Hz). ^{13}C NMR ($CDCl_3$): 31.7 (CH_3); 128.4; 129.6; 131.2; 135.1 (C arom.); 152.3 (3-C═O); 165.0 (5-C═O). Anal. ($C_9H_7ClN_2SO_2$) C, H, N, S.

4-(4-Fluorophenyl)-2-methyl-1,2,4-thiadiazolidine-3,5-dione (16). Reagents: 4-Fluorophenyl isothiocyanate (1.1 g, 6.5 mmol), 35% HCl (3.1 mL), $KMnO_4$ (0.5 g), methyl isocyanate (0.38 mL, 6.5 mmol). Conditions: rt, 8 h. Isolation: filtration of reaction mixture. Purification: recrystallization from ethanol. Yield: 0.37 g (25%) as white solid; mp 178−180 °C. ^{1}H NMR ($CDCl_3$): 3.25 (s, 3H, CH_3); 7.13−7.36 (m, 4H, arom.). ^{13}C NMR ($CDCl_3$): 31.7 (CH_3); 116.3; 129.1; 160.9; 164.2 (C arom.); 152.5 (3-C═O); 165.2 (5-C═O). Anal. ($C_9H_7FN_2SO_2$) C, H, N, S.

2-Methyl-4-(4-trifluoromethylphenyl)-1,2,4-thiadiazolidine-3,5-dione (17). Reagents: 4-Trifluoromethylphenyl isothiocyanate (0.66 g, 3.25 mmol), 35% HCl (1.5 mL), $KMnO_4$ (0.25 g), methyl isocyanate (0.14 mL, 3.25 mmol). Conditions: rt, 10 h. Isolation: solvent evaporation. Purification: silica gel column chromatography using CH_2Cl_2. Yield: 0.05 g (5%) as white solid; mp 164−166 °C. ^{1}H NMR ($CDCl_3$): 3.32 (s, 3H, CH_3); 7.51−7.73 (2 d, 4H, arom., J = 8.6 Hz). ^{13}C NMR ($CDCl_3$): 31.7 (CH_3); 123.8 (CF_3); 126.5; 127.4; 130.9; 147.6 (C arom.); 153.1 (3-C═O); 164.9 (5-C═O). Anal. ($C_{10}H_7F_3N_2SO_2$) C, H, N, S.

4-(4-Methylphenyl)-2-methyl-1,2,4-thiadiazolidine-3,5-dione (18). Reagents: 4-Methylphenyl isothiocyanate (0.88 mL, 6.5 mmol), 35% HCl (3.1 mL), $KMnO_4$ (0.5 g), methyl isocyanate (0.38 mL, 6.5 mmol). Conditions: rt, 6 h. Isolation: filtration of reaction mixture. Purification: recrystallization from CH_2Cl_2/hexane. Yield: 0.29 g (21%) as white solid; mp 182−184 °C. ^{1}H NMR ($CDCl_3$): 2.4 (s, 3 H, p-CH_3−Ph); 3.25 (s, 3H, CH_3); 7.20−7.34 (m, 4H, arom.). ^{13}C NMR ($CDCl_3$): 21.1 (p-CH_3−Ph); 31.7 (CH_3); 126.7; 130.0; 130.3; 139.3 (C arom.); 152.9 (3-C═O); 165.3 (5-C═O). Anal. ($C_{10}H_{10}N_2SO_2$) C, H, N, S.

4-(4-Methoxylphenyl)-2-methyl-1,2,4-thiadiazolidine-3,5-dione (19). Reagents: 4-Methoxylphenyl isothiocyanate (0.89 mL, 6.5 mmol), 35% HCl (3.1 mL), $KMnO_4$ (0.5 g), methyl isocyanate (0.38 mL, 6.5 mmol). Conditions: rt, 8 h. Isolation: filtration of reaction mixture. Purification: recrystallization from CH_2Cl_2/hexane. Yield: 0.44 g (30%) as white solid; mp 140−144 °C. ^{1}H NMR ($CDCl_3$): 3.31 (s, 3H, CH_3); 3.80 (s, 3 H, p-CH_3O−Ph); 7.02−7.32 (m, 4H, arom.). ^{13}C NMR ($CDCl_3$): 31.7 (CH_3); 55.5 (p-CH_3O−Ph); 114.7; 125.3; 128.5; 159.9 (C arom.); 152.9 (3-C═O); 165.5 (5-C═O). Anal. ($C_{10}H_{10}N_2SO_3$) C, H, N, S.

2-Methyl-4-naphthyl-1,2,4-thiadiazolidine-3,5-dione (20). Reagents: Naphthyl isothiocyanate (1.2 g, 6.5 mmol), 35% HCl (3.1 mL), $KMnO_4$ (0.5 g), methyl isocyanate (0.38 mL, 6.5 mmol). Conditions: rt, 8 h. Isolation: filtration of reaction mixture. Purification: recrystallization from methanol. Yield: 0.05 g (8%) as brown crystal; mp 191−193 °C. ^{1}H NMR ($CDCl_3$): 3.31 (s, 3H, CH_3); 7.49−8.02 (m, 7H, arom.). ^{13}C NMR ($CDCl_3$): 31.8 (CH_3); 125.4; 126.7; 127.1; 127.5; 128.7; 129.5; 129.6; 130.6; 131.6 (C arom.); 152.9 (3-C═O); 165.1 (5-C═O). Anal. ($C_{13}H_{10}N_2SO_2$) C, H, N, S.

4-Benzyl-2-ethyl-5-oxo-1,2,4-thiadiazolidine-3-thione (24). Reagents: Benzyl isothiocyanate (0.86 mL, 6.5 mmol), 35% HCl (3.1 mL), $KMnO_4$ (0.5 g), ethyl isothiocyanate (0.57 mL, 6.5 mmol). Conditions: rt, 12 h. Isolation: solvent evaporation. Purification: silica gel column chromatography using CH_2Cl_2/hexane (1:2) first and preparative thin-layer chromatography using CH_2Cl_2/hexane (1:10) after. Yield: 0.04 g (3%) as yellow oil. ^{1}H NMR ($CDCl_3$): 1.2 (t, 3H, CH_2CH_3, J = 7.0 Hz); 4.25 (c, 2H, CH_2CH_3, J = 7.0 Hz); 4.5 (s, 2H, CH_2−Bn); 7.11−7.31 (m, 5H, arom.). ^{13}C NMR ($CDCl_3$): 11.2 (CH_2CH_3); 46.1 (CH_2−Bn); 56.2 (CH_2CH_3); 127.2; 127.3; 128.6; 138.3 (C arom.); 154.3 (3-C═S); 168.7 (5-C═O). Anal. ($C_{11}H_{12}N_2S_2O$) C, H, N, S.

2-Ethyl-3-oxo-4-phenyl-1,2,4-thiadiazolidin-5-imine (27). A solution of ethyl-(2-ethyl-3-oxo-4-phenyl-1,2,4-thiadiazolidin-5-ylidene)urea (**26**)[31] (0.06 g, 0.2 mmol) and NaOH (0.2 mL, 0.2 mmol) in 20 mL of MeOH was stirred at room temperature for 18 h. Purification: CCTLC using AcOEt /hexane (1:1). Yield: 0.022 g (50%) as white solid; mp: 200−202 °C. ^{1}H NMR ($CDCl_3$): 1.25 (t, 3H, CH_2CH_3, J = 7.14 Hz); 3.71 (c, 2H, J = 7.14); 7.25−7.42 (m, 5H, arom.). ^{13}C NMR ($CDCl_3$): 14.7 (CH_2-CH_3); 39.1 (CH_2CH_3); 121.0; 126.1; 129.9 138.0 (C arom.); 164.5 (3-C═O); 170.7 (5-C═NH). Anal. ($C_{10}H_{11}N_3OS$) C, H, N, S.

Biological Evaluation. Materials: The recombinant rabbit glycogen synthase kinase 3β (catalog number G 1663) and the catalytic subunit of cAMP dependent protein kinase (P 2645), phenylmethylsulfonyl fluoride, benzamidine, leupeptin, aprotinin, pepstatin, sodium fluoride, 2-mercaptoethanol, EDTA, EGTA, and MES were obtained from Sigma (St. Louis, MO). Cyclin dependent kinase 1-cyclin B, (catalog number 14-103) was purchased from Upstate Biotechnology, Inc. (New York). Histone H1 and Ro 31-8220 were supplied by Calbiochem. DEAE-cellulose and phosphocellulose were from Whatman Ltd. (London). ATP was purchased from Boehringer-Mannheim. (γ-^{32}P)ATP was purchased from Amersham. Alkaline phosphatase was obtained from Roche (catalog number 713023, Germany). Stathmin was purified from bovine brain essentially as described by Belmont and Mitchinson[34] with some modifications. As an additional step in the purification, proteins were chromatographed on DEAE-cellulose, before the HPLC step. The pellet was dissolved in 50 mL of 10 mM Tris-HCl, 1 mM EDTA, 15 mM 2-mercaptoethanol, pH 7.5 (buffer A), and applied to DEAE-cellulose, equilibrated in the same buffer. Stathmin was eluted with a gradient from 0 to 1 M NaCl.

GSK-3β Inhibition: GSK-3β enzyme (Sigma) was incubated with 15 μM of ATP, 0.2 μC$_i$ of [γ-^{32}P]ATP, GS-1 as substrate,[32] and different concentrations of the test compound.

GSK-3 activity was assayed in 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM EGTA, and 1 mM EDTA buffer, at 37 °C, in the presence of 15 μM GS-1 (substrate), 15 μM [γ-^{32}P]ATP in a final volume of 12 μL. After 20 min incubation at 37 °C, 4 μL aliquots of the supernatant were spotted onto 2 × 2 cm pieces of Whatman P81 phosphocellulose paper, and 20 s later, the filters were washed four times (for at least 10 min each time) in 1% phosphoric acid. The dried filters were transferred into scintillation vials, and the radioactivity was measured in a liquid scintillation counter. Blank values were subtracted, and the GSK-3β activity was expressed in picomoles of phosphate incorporated in GS-1 per 20 min or in percentage of maximal activity. The IC_{50} (concentration at which a 50% of enzyme inhibition is shown) values are gathered in Table 1.

Protein Kinase A (PKA) Inhibition: The potential inhibition of this enzyme is evaluated by determining the stathmin phosphorylation by the protein kinase A (PKA). The stathmin was purified following the procedure described by Belmont and Mitchinson.[34]

Purified PKA (Sigma, catalytic subunit from bovine heart (p 2645)) and 10−15 μg of substrate (stathmin) were used in a 12 μL total volume of buffer solution containing 15 μM ATP and 0.2 μCi of (γ-^{32}P)ATP. The cAMP kinase protein (100 ng/reaction) was performed in 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM EGTA, and 1 mM EDTA buffer, at 37 °C. After 20 min incubation at 37 °C, 4 μL aliquots of the supernatant were spotted onto 2 × 2 cm pieces of Whatman P81 phosphocellulose paper. After 20 s later, the filters were washed four times (for at least 10 min each time) in 1% phosphoric acid. The dried filters were transferred into scintillation vials, and the radioactivity was measured in a liquid scintillation counter

In these conditions none of the compounds essayed gathered in Table 1 showed any inhibition of PKA.

Protein Kinase C (PKC) Inhibition: The potential inhibition of this enzyme is evaluated by determining the phosphorylation of the peptide PANKTPPKSPGEPAK by the protein kinase C (PKC) using phosphatidyl serine as stimulating agent. The method followed is the same described above for GSK-3β.

PKC purified from rat brains following the method described by Walsh[35] and 1 mM of substrate were used in a total volume of 12 μL in 25 mM Tris-HCl, pH 7.5, 20 mM $MgCl_2$, 0.5 mM EGTA, 0.5 mM EDTA, 7 mM $CaCl_2$, and 100 mg/mL PS buffer solution containing 10 μM (γ-^{32}P)ATP.

In these conditions, none of the compounds essayed gathered in Table 1 showed any inhibition of PKC.

Casein Kinase II (CK-II) Inhibition: The phosphorylating activity of this enzyme against stathmin has been measured using CK-II purified from bovine brains, following the method described by Alcazar,[36] with 10−15 μg of substrate (stathmin) in a total volume of 12 μL of 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM EGTA, and 1 mM EDTA buffer solution, at 37 °C, containing 15 μM (γ-^{32}P)ATP and 100 ng of purified CK-2. After the reaction took place, it followed the same method described for PKA.

In these conditions, none of the compounds essayed gathered in Table 1 showed any inhibition of CK-II.

Cyclin Dependent Kinase 1-Cyclin B Inhibition: The potential inhibition of this enzyme was evaluated by determining the histone H1 phosphorylation by cyclin dependent kinase 1-cyclin B.

UBI (an enzyme composed of an equal amount of the Cdk-1 and the 45 kDa cyclin B subunit, catalog number 14-103) and 10−15 μg of substrate (histone H1) were used in a 12 μL total volume of buffer solution containing 15 μM ATP and 0.5 μCi of (γ-^{32}P)ATP. The kinase activity (50 ng/reaction) was performed in 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 0.01% Brij 35, and 1 mM EGTA buffer, at 37 °C. After 20 min incubation at 37 °C, 4 μL aliquots of the supernatant were spotted onto 2 × 2 cm pieces of Whatman P81 phosphocellulose paper, and 20 s later, the filters were washed four times (for at least 10 min each time) in 1% phosphoric acid. The dried filters were transferred into scintillation vials, and the radioactivity was measured in a liquid scintillation counter

In these conditions, none of the compounds essayed gathered in Table 1 showed any inhibition of Cdk-1/cyclin B.

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