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Synthesis and biological evaluation of 3-hydroxymethyl-5-(1*H*-1,2,3-triazol) isoxazolidines

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ABSTRACT

A synthetic approach towards a series of 3-hydroxymethyl-5-(1*H*-1,2,3-triazol)isoxazolidines has been reported, according to a procedure based on the cycloaddition reaction, under microwave irradiation, of a nitrone with 1-vinyl triazoles, prepared by a click reaction of azides with alkynes. Biological tests show that the synthesized compounds are able to inhibit proliferation of follicular and anaplastic human thyroid cancer cell lines, with IC_{50} values ranging from 3.87 to 8.76 μM. The obtained compounds induce caspase-3 activation and DNA fragmentation prevalently in follicular human thyroid cancer cell lines.

1. Introduction

In the search for new antiviral/antitumor nucleoside analogues, structural modifications of the heterocyclic bases and/or the sugar moiety of natural nucleosides is a widely explored area in the medicinal chemistry.[1–6] In the latter, the main modifications involve changes in the (2-deoxy)-D-ribofuranose unit such as the substitution/functionalisation of hydroxyl groups, the cleavage of the sugar ring leading to acyclic nucleosides, the replacement by a methylene group of the endocyclic oxygen, the transposition of the latter and/or the insertion of additional heteroatoms in the sugar moiety.[7–10]

With regard to the modification of the natural purine/pyrimidine nucleobases, a variety of unnatural nucleobases have been evaluated and shown to lead to clinically useful nucleoside analogues. Bredinine[11] is an imidazole nucleoside antibiotic clinically used as an immunosuppressant; showdomycin,[12] ribavirin[13] and pyrazofurin[14] have been shown to possess a wide range of medicinal properties, including antibiotic, antiviral and antitumor activities (Fig. 1).

In particular, among antitumor nucleosides, those anchoring a five-membered heterocyclic ring are of great interest. Thus, tiazofurin[15] is a synthetic C-nucleoside recently approved for treatment of chronic myelogenous leukemia in accelerated phase or blast crisis; Eicar[16] is another five membered N-nucleoside with a potent antiviral and antitumor activity; compound **1**[17] exhibits a potent antiviral activity against vaccinia virus with high selectivity index (EC_{50} = 0.4 μM, SI >750); compound **2**[18] shows significant improved activity towards a broad range of tumor cell lines (Fig. 1).

Our interest in the chemistry of modified nucleosides[19–21] has led to the synthesis of a series of new nucleoside analogues where the furanose ring has been replaced by heterocyclic systems. ADFU,[22] an analogue of citofur, is a good inductor of apoptosis on lymphoid and monocytoid cells, with low cytotoxicity;[23] azanucleosides **3** showed anti-HCV activity,[24] phosphonated carbocyclic 2′-oxa-3′-azanucleosides **4** have shown to be potent inhibitors of rt of different retroviruses, following incubation with human PBMCs crude extract;[25] truncated phosphonated azanucleosides **5** are able to inhibit HIV and HTLV-1 viruses at concentration in the nanomolar range;[26] truncated phosphonated N,O-psiconucleosides **6** inhibit HIV in vitro infection with low or absent cytotoxicity[27] (Fig. 2).

Our encouraging results suggested us to investigate the synthesis of new N,O-nucleosides in the triazolyl series.[28] 1,2,3-Triazoles recently have gained significant interest in various fields of drug discovery and bioconjugation: introduction of a triazole ring into nucleosides to improve bioactivity in antitumor and/or antiviral agents has become widespread in drug design practices since the first synthetic nucleoside drug, ribavirin,[13] showed a broad spectrum of antiviral activity against many RNA and DNA viruses. The family of triazole nucleosides may provide a new structural

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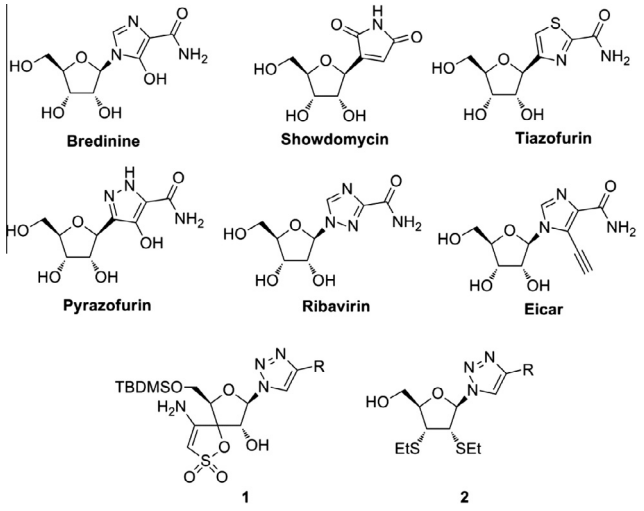


Figure 1. Structure of relevant nucleobase-modified nucleosides.

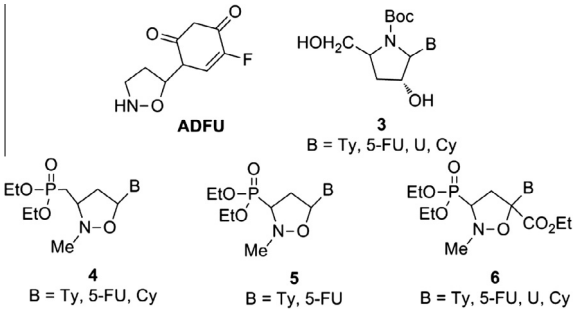


Figure 2. Sugar-modified nucleosides.

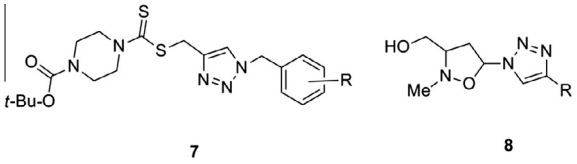


Figure 3. Triazole nucleosides.

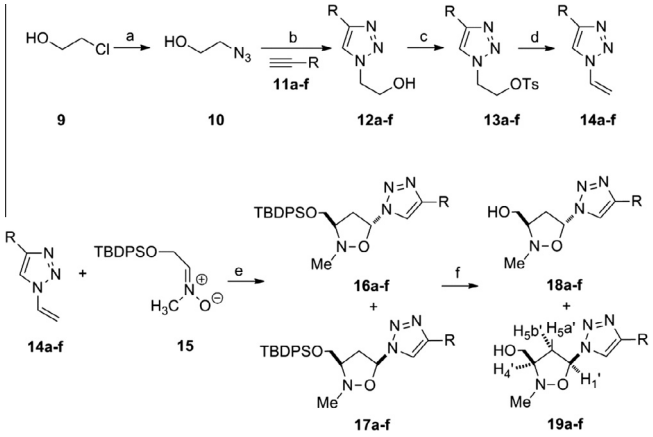
lead in the search for drug candidates: recently, novel 1,2,3-triazole-dithiocarbamate hybrids **7** have been shown to be endowed with excellent broad spectrum anticancer activity[29] (Fig. 3).

On these basis we have developed the synthesis of 3-hydroxymethyl-5-(1*H*-1,2,3-triazol-1-yl)isoxazolidines **8** (Fig. 3), as potential antitumor agents, according to a synthetic approach based on the combination of two methodologies: an azide-alkyne click-chemistry reaction to give the suitably 4-substituted 1-vinyl-1*H*-1,2,3-triazoles; a 1,3-dipolar cycloaddition of nitrones[30–35] with vinyl triazoles to afford the isoxazolidine ring nucleus.

The effect of the synthesized compounds on the follicular (FTC-133) and anaplastic (8305C) human thyroid cancer cell lines,[36,37] as well as on the activation of the apoptotic pathway has been evaluated. The obtained results indicate that the synthesized compounds are endowed with an interesting cytotoxic effect for these two types of thyroid cancer cell lines.

2. Results and discussion

N-Vinyl triazoles **14** have been prepared according to Scheme 1.[38] Thus, 2-chloroethanol **9** was reacted with sodium azide to give 2-azidoethanol **10**. The click reaction of **10** with a variety of alkynes **11a–f**, performed in H_2O/*tert*-BuOH (1:1) in the presence of sodium ascorbate, copper(II) sulfate and TEA at room temperature, afforded triazole derivatives **12a–f** in 90–95% yields. Compounds **12a–f** have been tosylated and then converted into vinyl triazoles **14a–f** by reaction with potassium *tert*-butoxide in *tert*-butanol (88–95% yields).



Scheme 1. Synthesis of isoxazolidinyl-triazoles **18** and **19**. Reagents and conditions: (a) NaN_3, NaOH; (b) $CuSO_4{\cdot}5H_2O$, sodium ascorbate, TEA, rt, 4 h; (c) tosyl chloride, TEA, CH_2Cl_2, rt, 12 h; (d) *t*-BuOK, *t*-BuOH, 40 °C, 12 h; (e) compound **15**, $CHCl_3$, 150W, 80 °C, 2 h, 85% yield; (f) TBAF, THF, rt, 4–5 h, overall yield of 93–96% in relative ratio 1:1.3 or 1:1.

The 1,3-dipolar cycloaddition of vinyl triazoles **14a–f** with *C*-[(*tert*-butyldiphenylsilyl)oxy]-*N*-methylnitrone **15**,[35] at 150 W, 80 °C for 2 h in $CHCl_3$, proceeded with a good yield and a complete regioselectivity to give a mixture of *trans*/*cis* isoxazolidines **16a–f** and **17a–f** in a 1:1.3 relative ratio.

The same mixture was reacted at room temperature: after 24 h only reagents were isolated, while, at reflux for 12 h, isoxazolidines **16a–f** and **17a–f** were obtained with very low yields.

Removal of the TBDPS protecting group was accomplished under standard conditions by treating the anomeric mixture with TBAF in THF, to afford the triazolyl nucleosides **18a–f** and **19a–f**, which were separated by silica gel chromatography (Scheme 1; Table 1).

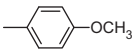
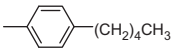
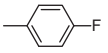
The diastereomeric ratio of the products was determined by ^{1}H NMR spectroscopy of the crude reaction mixture, whereas the relative configuration was assigned by NOEDS spectra (NOE Difference Spectroscopy). In particular, in **19a**, chosen as model compound, a positive NOE effect observed for H-4′ and H-5′b (the downfield resonance of protons at C-5′, 2.87 ppm) upon irradiation of H-1′ (δ = 6.17 ppm), is clearly indicative of their *cis* relationship. Analogously, irradiation of H-1′ in the same compound gives rise to an enhancement in the signals corresponding to H-4′ and H-5′b (δ = 3.54 and 2.87 ppm, respectively). On the contrary, no NOE effect was detected between H-4′ and H-1′ in compound **18a**.

The absence of *cis*/*trans* diastereoselectivity can be rationalized by assuming that the *E*-endo attack of the dipolarophile on the nitrone, which leads to *cis* adducts, competes efficiently with the *E*-exo attack, the preferred reaction pathway (steric control) leading to *trans* adducts, because of secondary orbital interactions exerted by the triazole ring. This behavior is also in agreement with literature data.[39]

3. Biological assay

To evaluate the biological properties of the synthesized compounds **18a–f** and **19a–f**, we have selected two cell lines, the follicular (FTC-133) and the anaplastic (8305C) human thyroid cancer

Table 1
Vinyl triazoles **14a–f** and isoxazolidinyl-triazoles **18a–f** and **19a–f** produced via Scheme 1

Alkyne	R	Product	Yield[b] (%)	Product	Ratio *trans*:*cis*	Yield[b] (%)
11a		**14a**	88	**18a, 19a**	1:1.3	79
11b	—TMS	**14b**[a]	80	**18b, 19b**	1:1.3	81
11c	$-CH_2CH_2CH_3$	**14c**	78	**18c, 19c**	1:1.3	79
11d		**14d**	79	**18d, 19d**	1:1	81
11e		**14e**	68	**18e, 19e**	1:1.3	82
11f		**14f**	77	**18f, 19f**	1:1.3	81

[a] The basic conditions lead to deprotection of the TMS in **14b** (R = H).
[b] Combined yield.

cell lines, as representatives of two aggressive types of thyroid cancer, poorly differentiated and dedifferentiated, respectively.[36,37]

3.1. Cytotoxic effect of the compounds

To monitor cell viability, both observations through fluorescent microscope analysis and MTT assays have been used. In preliminary experiments, FTC-133 and 8305C cell line cultures were exposed to different concentrations (0.5–20 μM) of the synthesized compounds for 12, 24 or 48 h, in order to establish the optimal concentrations and exposure times.[40–42] The observed effects were compared with that of gemcitabine, a well-known antitumor nucleoside. A significant reduction in cellular viability was observed in both FTC-133 and 8305C cell lines treated with all the compounds **18a–f** and **19a–f**, at 10 μM concentrations for 24 h, when compared with respective controls. The effect appeared more evident in FTC-133 cell lines.

The 50% cytotoxic inhibitory concentration (IC_{50}), causing 50% decreasing in cell proliferations, obtained graphically from dose–effect curves using Prism 5.0 (GraphPad Software Inc.), is reported in Table 2.

All compounds displayed cytotoxic effects on both cell lines, at concentrations ranging from 3.36 to 8.76 μM. FTC-133 cells were more susceptible to treatment with the synthesized derivatives than the 8305 cells. In particular, compounds **18c**, **19c**, **18e** and **19e** displayed the greatest activity by inhibiting the cancer growth rate in the range of 3.87–6.87 μM. Among these compounds, **18c** and **19c**, which possess a long alkyl group-substituted triazole ring, and **18e** and **19e**, which contain an alkyl-substituted phenyl ring linked to the triazole system, are the most active derivatives. The lack of the alkyl chain, as in compounds **18a** and **19a**, **18b** and **19b**, induces a decrease of the anticancer activity. Noteworthy, the relative *cis*, *trans* configuration of **18** and **19** does not seem to affect the biological activity.

Table 2
Concentrations (μM) of investigated compounds **18a–f** and **19a–f**, that induced 50% decrease (IC_{50}) in FTC-133 and 8305C cell proliferation[a]

Compd	FTC-133[b]	8305C[b]
Untreated (control)	>100	>100
Gemcitabine	3.36	4.53
18a	6.03	7.14
19a	6.33	8.76
18b	6.53	7.96
19b	6.30	8.13
18c	3.87	5.82
19c	4.71	6.87
18d	7.00	8.01
19d	6.48	7.18
18e	3.95	5.96
19e	4.49	6.24
18f	6.07	6.76
19f	5.85	6.85

[a] FTC-133 and 8305C cell lines were incubated with drug compounds in concentration ranging from 0.5 to 20 μm at 37 °C in a 5% CO_2 atmosphere for 24 h. Viability was determined by MTT assay.
[b] Each data represents mean value from four independent experiments, performed in triplicate. Gemcitabine was used as positive control.

3.2. Evaluation of the apoptotic pathway activation

We have also examined the effect of the synthesized compounds on the apoptotic pathway by evaluating caspase-3 cleavage by immunocytochemical analysis and DNA fragmentation by TUNEL test (terminal deoxynucleotidyl-transferase mediated dUTP nick-end-labeling test).

Treatment with gemcitabine and all the synthesized triazole derivatives **18** and **19**, after 24 h of cell incubation at 5 μM concentration, induced a significant enhancement of caspase-3 positive cells in FTC-133 and 8305C cell lines, when compared to the untreated controls (see Supplementary data).

These results were also confirmed by evaluating DNA fragmentation for compound **18c** chosen as model compound.

The effect appeared more evident in FTC-133 cell lines. Figure 4A and C show the fluorescent microscope analysis of caspase-3 cleavage in FCT-133 and 8305C human thyroid cancer cell lines, untreated (control) and treated with 5 μM Gemcitabine or **18c**, chosen as model compound.

The quantification and statistical analysis of caspase-3 immunolabeling is reported in Figure 4 B and D.

The treatment of both culture cell lines for 24 h with 5 μM of **18c**, induced a significant increase of DNA fragmentation, when compared with the respective controls, even if the more evident effect appeared in FTC-133 cell lines (Fig. 5).

The percentage of the apoptotic cells compared to the non apoptotic cells is reported in Table 3.

4. Conclusion

In summary, we report an efficient synthesis of 3-hydroxymethyl-5-(*1H*-1,2,3-triazol) isoxazolidines, according to a procedure based on the cycloaddition reaction of a nitrone with 1-vinyl triazoles, prepared by exploiting a click reaction of azides with a range of alkynes. Biological tests indicate that the obtained compounds are endowed with an interesting antitumor activity against two aggressive types of thyroid cancer. In particular, they are able to inhibit cell proliferation by 50% at concentrations ranging from 3.87 to 8.76 μM. The synthesized compounds activates the apoptotic pathway by caspase-3 cleavage, inducing also DNA fragmentation, prevalently in follicular human thyroid cancer cell lines.

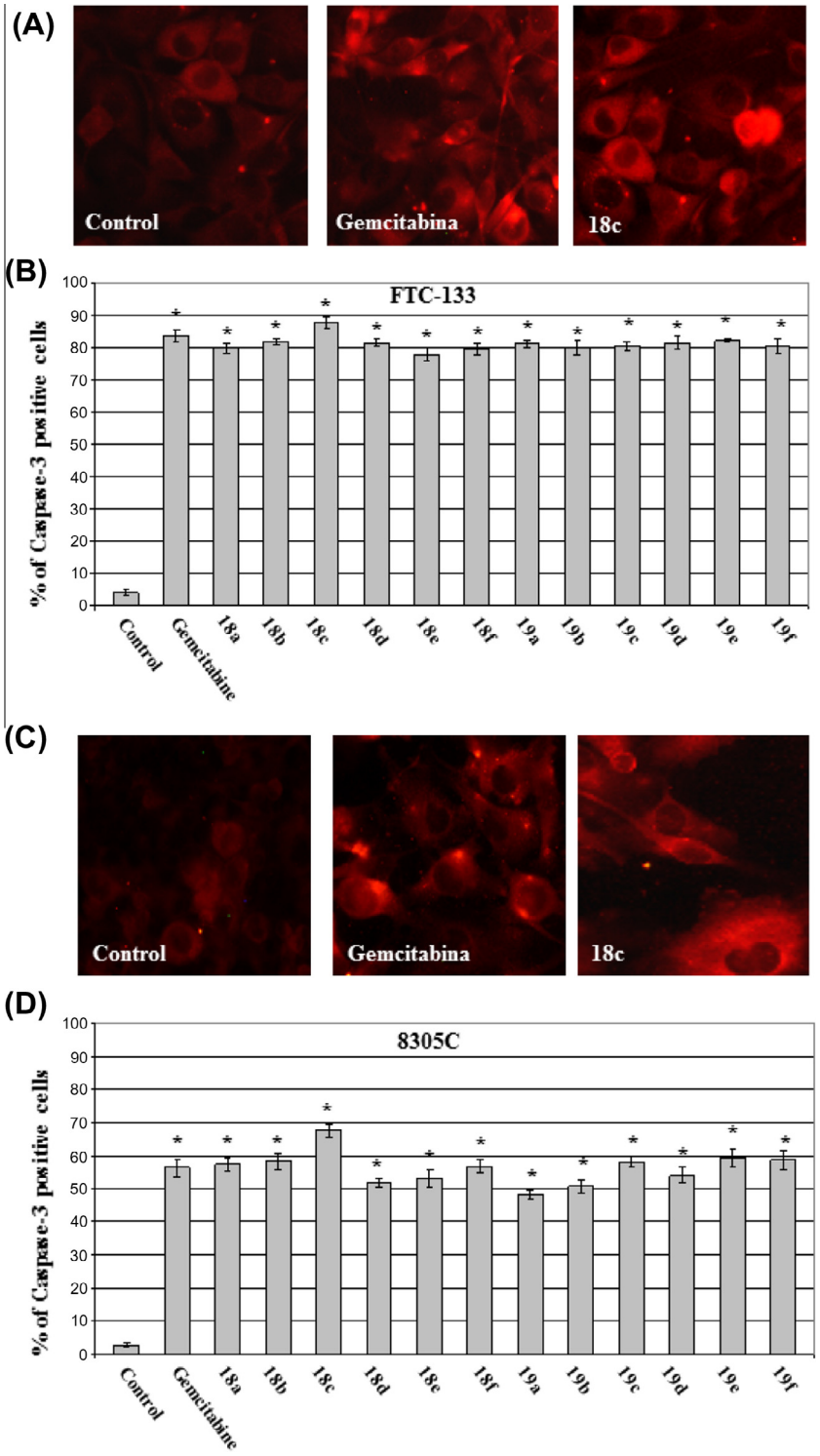


Figure 4. Fluorescent microscope analysis of caspase-3 cleavage in (A) FCT-133 and (C) 8305C human thyroid cancer cell lines, untreated (control) and treated with 5 μM Gembcitabine or **18c** for 24 h. % Caspase-3 positive cell in (B) FTC-133 and (D) 8305C, for all compounds. Scale bars = 50 μm. *p <0.001 Versus respective control by one-way ANOVA followed by post hoc Holm–Sidak test. Data were collected from four fields/coverslip in four separated experiments.

5. Experimental section

5.1. General

Solvents and reagents were used as received from commercial sources. Melting points were determined with a Kofler apparatus. Elemental analyses were performed with a Perkin–Elmer elemental analyzer. NMR spectra (^{1}H NMR recorded at 300 and 500 MHz, ^{13}C NMR recorded at 75 and 126 MHz) were obtained with Varian instruments and are reported in ppm relative to TMS. Thin-layer chromatographic separations were performed on Merck silica gel 60-F254 precoated aluminum plates. Flash chromatography was accomplished on Merck silica gel (200–400 mesh). Preparative separations were carried out by a

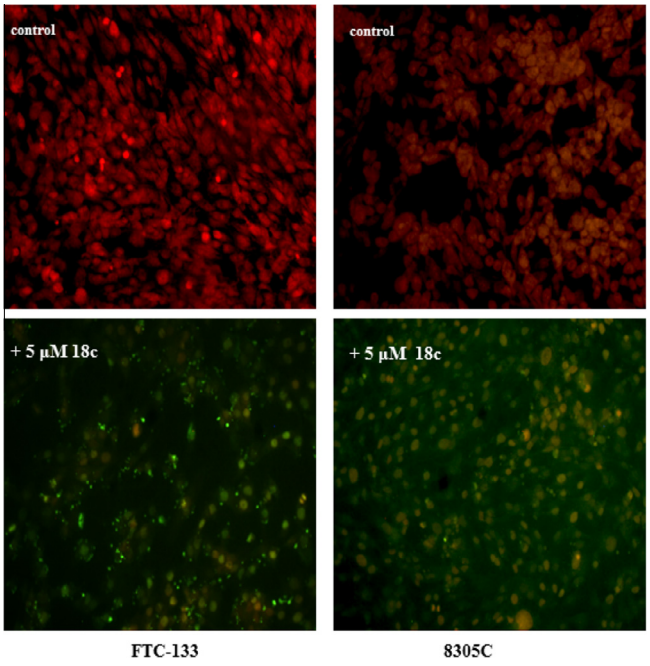


Figure 5. Representative pictures of TUNEL assay performed in follicular (FTC-133) and anaplastic (8305C) human thyroid cancer cell lines unexposed (control) and exposed to 5 μM **18c** for 24 h. Immunostaining of nonapoptotic (red) and apoptotic (green) cells is shown. Scale bars = 20 μm.

Table 3
Percentage of the apoptotic follicular (FTC-133) and anaplastic (8305C) human thyroid cancer cell lines treated with 5 μM **18c** for 24 h compared with the untreated controls by TUNEL test

Treatment	% of apoptotic cells FTC-133[a]	% of apoptotic cells 8305C[a]
Control	3 ± 1	2 ± 1
18c	95 ± 2	90 ± 3

[a] The ratio is expressed as a percentage, taking the total number of cells as 100 and comparing 10 random microscopic fields for each dish. The DNA fragmentation assay incorporates fluorescein-dUTP at the free 3′-hydroxyl ends of the fragmented DNA using TUNEL. Apoptotic cells appear green, while non apoptotic cells appear red: $^{*}p$ <0.05, significant differences versus the controls.

MPLC Büchi C-601 by using Merck silica gel 0.040–0.063 mm and the eluting solvents were delivered by a pump at the flow rate of 3.5–7.0 mL/min. UV spectra were recorded on a JASCO V 650 spectrophotometer. *C*-[(*tert*-Butyldiphenylsilyl)oxy]-*N*-methyl nitrone was prepared according to described procedures.[35]

5.2. General click procedure

5.2.1. 2-(4-Phenyl-1H-1,2,3-triazol-1-yl)ethanol (12a)

To a solution of **11a** (4.16 mL, 37.9 mmol), **10** (3.0 g, 34.45 mmol) and triethylamine (4.8 mL, 34.45 mmol) in *tert*-BuOH (40 mL) and H_20 (40 mL), $CuSO_4{\cdot}5H_2O$ (2.15 g, 8.8 mmol) and sodium ascorbate (3.41 g, 17.22 mmol) were added. The mixture was allowed to stir at rt for 4 h under N_2 and concentrated. Then the mixture was diluted with water and extracted with ethyl acetate (3 × 30 mL). The organic layer was dried over $MgSO_4$, filtered and concentrated at reduced pressure to yield 5.86 g (90%) of **12a** as green powder, mp = 89–92 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 4.09 (2H, t, J = 5.1 Hz), 4.44 (2H, t, J = 5.1 Hz), 4.82 (1H, br s), 7.15–7.43 (3H, m), 7.49–7.63 (2H, m), 7.76 (1H, s). ^{13}C NMR (126 MHz, $CDCl_3$) δ: 53.0, 61.0, 121.1, 125.4, 128.1, 128.7, 130.1, 147.2. Anal. Calcd for $C_{10}H_{11}N_3O$: C, 63.48; H, 5.86; N, 22.21. Found: C, 63.39; H, 5.81; N, 22.16.

5.2.2. 2-(4-(Trimethylsilyl)-1*H*-1,2,3-triazol-1-yl)ethanol (12b)

Compound **12b** was prepared by the general click procedure in 90% yield as colorless oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 0.21 (9H, s), 3.95 (2H, t, J = 5.1 Hz), 4.43 (2H, t, J = 5.1 Hz), 4.94 (1H, br s), 7.67 (1H, s). ^{13}C NMR (75 MHz, $CDCl_3$) δ: −1.1, 52.3, 61.1, 130.6, 134.0. Anal. Calcd for $C_7H_{15}N_3OSi$: C, 45.37; H, 8.16; N, 22.68. Found: C, 45.31; H, 8.15; N, 22.65.

5.2.3. 2-(4-Propyl-1*H*-1,2,3-triazol-1-yl)ethanol (12c)

Compound **12c** was prepared by the general click procedure in 92% yield as yellow oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 0.95 (3H, t, J = 7.4 Hz), 1.62–1.70 (2H, m), 2.64 (2H, t, J = 7.6), 3.14 (1H, br s), 4.05 (2H, t, J = 4.8 Hz), 4.42 (2H, t, J = 4.8 Hz), 7.38 (1H, s). ^{13}C NMR (126 MHz, $CDCl_3$) δ: 13.9, 22.7, 27.7, 52.6, 61.2, 122.1, 148.2. Anal. Calcd for $C_7H_{13}N_3O$: C, 54.17; H, 8.44; N, 27.08. Found: C, 53.98; H, 8.41; N, 27.11.

5.2.4. 2-(4-(4-Methoxyphenyl)-1*H*-1,2,3-triazol-1-yl)ethanol (12d)

Compound **12d** was prepared by the general click procedure in 92% yield as white powder, mp = 112–113 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 3.84 (3H, s), 4.13 (2H, t, J = 4.5 Hz), 4.51 (2H, t, J = 4.5 Hz), 4.90 (1H, br s), 6.93 (2H, d, J = 8.9 Hz), 7.43 (2H, d, J = 8.9 Hz), 7.75 (1H, s). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 52.8, 55.5, 61.4, 114.4, 120.2, 123.2, 127.1, 147.7, 159.8. Anal. Calcd for $C_{11}H_{13}N_3O_2$: C, 60.26; H, 5.98; N, 19.17. Found: C, 60.08; H, 5.93; N, 19.05.

5.2.5. 2-(4-(4-Pentylphenyl)-1*H*-1,2,3-triazol-1-yl)ethanol (12e)

Compound **12e** was prepared by the general click procedure in 95% yield as yellow powder, mp = 85–88 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 0.90 (3H, t, J = 6.6 Hz), 1.27–1.40 (4H, m), 1.52–1.71 (2H, m), 2.60 (2H, d, J = 7.4 Hz), 4.10 (2H, t, J = 4.6 Hz), 4.25 (1H, br s), 4.44 (2H, t, J = 4.6 Hz), 7.15 (2H, d, J = 7.8 Hz), 7.53 (2H, d, J = 7.8 Hz), 7.73 (1H, s). ^{13}C NMR (126 MHz, $CDCl_3$) δ: 14.2, 22.7, 31.2, 31.6, 35.8, 53.1, 61.2, 120.8, 125.6, 127.6, 128.9, 143.1, 147.5. Anal. Calcd for $C_{15}H_{21}N_3O$: C, 69.47; H, 8.16; N, 16.20. Found: C, 69.28; H, 8.15; N, 16.18.

5.2.6. 2-(4-(4-Fluorophenyl)-1*H*-1,2,3-triazol-1-yl)ethanol (12f)

Compound **12f** was prepared by the general click procedure in 95% yield as white powder, mp = 101–104 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 4.12 (2H, dd, J = 9.8, 5.2 Hz), 4.46–4.50 (2H, m), 7.05 (2H, t, J = 8.5 Hz), 7.63 (1H, dd, J = 8.5, 5.3 Hz), 7.76 (1H, s). ^{13}C NMR (126 MHz, $CDCl_3$) δ: 53.1, 61.3, 116.0 (d, J = 20.9 Hz), 120.9, 126.5 (d, J = 2.4 Hz), 127.3 (d, J = 8.0 Hz), 146.7, 162.7 (d, J = 247.5 Hz). Anal. Calcd for $C_{10}H_{10}FN_3O$: C, 57.97; H, 4.86; N, 20.28. Found: C, 58.01; H, 4.88; N, 20.26.

5.3. General tosylation procedure

5.3.1. 2-(4-Phenyl-1*H*-1,2,3-triazol-1-yl)ethyl-4-methyl benzene-sulfonate (13a)

To a solution of **12a** (2.0 g, 10.57 mmol) and TEA (3.24 mL, 23.25 mmol) in DCM (40 mL), tosyl chloride (2.42 g, 12.68 mmol) was added and the resulting mixture was stirred at rt for 12 h. Then the mixture was diluted with water and extracted with ethyl acetate (3 × 30 mL). The organic layer was dried over $MgSO_4$, filtered and concentrated at reduced pressure. The crude product was purified via flash column chromathography (7:3 cyclohexane/ethyl acetate) to yield 3.37 g (93%) of **13a** as white powder, mp = 127–129 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 2.32 (3H, s), 4.46 (2H, t, J = 5.1 Hz), 4.66 (2H, t, J = 5.1 Hz), 7.22 (2H, d, J = 8.4 Hz), 7.32–7.39 (1H, m), 7.40–7.47 (2H, m), 7.64 (2H, d, J = 8.4 Hz), 7.74 (1H, s), 7.76–7.80 (2H, m). ^{13}C NMR (126 MHz, $CDCl_3$) δ: 21.7, 49.3, 68.0, 120.7, 125.8, 127.8, 128.4, 129.1, 130.2, 130.4,

132.1, 145.7, 148.1. Anal. Calcd for $C_{17}H_{17}N_3O_3S$: C, 59.46; H, 4.99; N, 12.24. Found: C, 59.31; H, 4.94; N, 12.13.

5.3.2. 2-(4-(Trimethylsilyl)-1*H*-1,2,3-triazol-1-yl)ethyl-4-methylbenzenesulfonate (13b)

Compound **13b** was prepared by the general tosylation procedure in 91% yield as colorless oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 0.30 (9H, s), 2.42 (3H, s), 4.37 (2H, t, J = 5.1 Hz), 4.65 (2H, t, J = 5.1 Hz), 7.29 (2H, d, J = 8.3 Hz), 7.56 (1H, s), 7.64 (2H, d, J = 8.3 Hz). Anal. Calcd for $C_{14}H_{21}N_3O_3SSi$: C, 49.53; H, 6.23; N, 12.38. Found: C, 49.44; H, 6.17; N, 12.37.

5.3.3. 2-(4-Propyl-1*H*-1,2,3-triazol-1-yl)ethyl-4-methyl benzenesulfonate (13c)

Compound **13c** was prepared by the general tosylation procedure in 87% yield as orange oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 0.95 (3H, t, J = 7.4 Hz), 1.48–1.81 (2H, m), 2.43 (3H, s), 2.66 (2H, t, J = 7.5 Hz), 4.35 (2H, t, J = 4.9 Hz), 4.58 (2H, t, J = 4.9 Hz), 7.28–7.33 (3H, m), 7.66 (2H, d, J = 8.3 Hz). Anal. Calcd for $C_{14}H_{19}N_3O_3S$: C, 54.35; H, 6.19; N, 13.58. Found: C, 54.21; H, 6.14; N, 13.57.

5.3.4. 2-(4-(4-Methoxyphenyl)-1*H*-1,2,3-triazol-1-yl)ethyl-4-methylbenzenesulfonate (13d)

Compound **13d** was prepared by the general tosylation procedure in 85% yield as white powder, mp = 135–137 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 2.32 (3H, s), 3.85 (3H, s), 4.44 (2H, t, J = 4.8 Hz), 4.67 (2H, t, J = 4.8 Hz), 6.97 (2H, d, J = 8.8 Hz), 7.22 (2H, d, J = 8.3 Hz), 7.63 (2H, d, J = 8.3 Hz), 7.65 (1H, s), 7.70 (2H, d, J = 8.8 Hz). ^{13}C NMR (126 MHz, $CDCl_3$) δ: 21.7, 49.2, 55.5, 68.1, 114.4, 119.9, 123.1, 127.1, 127.8, 130.2, 132.0, 145.6, 147.9, 159.8. Anal. Calcd for $C_{18}H_{19}N_3O_4S$: C, 57.89; H, 5.13; N, 11.25. Found: C, 57.81; H, 5.10; N, 11.26.

5.3.5. 2-(4-(4-Pentylphenyl)-1*H*-1,2,3-triazol-1-yl)ethyl-4-methylbenzenesulfonate (13e)

Compound **13e** was prepared by the general tosylation procedure in 75% yield as white powder, mp = 108–110 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 0.90 (3H, t, J = 6.5 Hz), 1.29–1.37 (4H, m), 1.61–1.67 (2H, m), 2.29 (3H, s), 2.63 (2H, t, J = 7.4 Hz), 4.51 (2H, t, J = 4.9 Hz), 4.65 (2H, t, J = 4.9 Hz), 7.20 (2H, d, J = 8.0 Hz), 7.24 (2H, d, J = 8.0 Hz), 7.62 (2H, d, J = 8.0 Hz), 7.67 (2H, d, J = 8.0 Hz), 7.70 (1H, s). ^{13}C NMR (126 MHz, $CDCl_3$) δ: 14.1, 21.6, 22.7, 31.2, 31.6, 35.8, 49.2, 68.1, 120.4, 125.7, 127.6, 127.8, 129.0, 130.1, 132.0, 143.4, 145.6, 148.0. Anal. Calcd for $C_{22}H_{27}N_3O_3S$: C, 63.90; H, 6.58; N, 10.16. Found: C, 63.82; H, 6.55; N, 10.15.

5.3.6. 2-(4-(4-Fluorophenyl)-1*H*-1,2,3-triazol-1-yl)ethyl-4-methylbenzenesulfonate (13f)

Compound **13f** was prepared by the general tosylation procedure in 86% yield as white powder, mp = 140–141 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 2.34 (3H, s), 4.45 (2H, td, J = 5.3, 1.5 Hz), 4.64–4.70 (2H, m), 7.13 (2H, t, J = 8.7 Hz), 7.23 (2H, d, J = 7.9 Hz), 7.65 (2H, d, J = 7.9 Hz), 7.72 (1H, s), 7.75 (2H, dd, J = 8.9, 5.3 Hz). ^{13}C NMR (126 MHz, $CDCl_3$) δ: 21.7, 49.3, 68.1, 116.1 (d, J = 21.8 Hz), 120.5, 126.6 (d, J = 2.7 Hz), 127.6 (d, J = 8.0 Hz), 1127.8, 130.2, 132.1, 145.7, 147.2, 162.87 (d, J = 247.4 Hz). Anal. Calcd for $C_{17}H_{16}FN_3O_3S$: C, 56.50; H, 4.46; N, 11.63. Found: C, 56.42; H, 4.39; N, 11.60.

5.4. General elimination procedure

5.4.1. 4-Phenyl-1-vinyl-1*H*-1,2,3-triazole (14a)

To a solution of **13a** (1.0 g, 2.91 mmol) in *tert*-BuOH (20 mL), *tert*-BuOK (0.49 g, 4.36 mmol) was added and the resulting mixture was stirred at 40 °C for 12 h. The mixture was diluted with water and extracted with DCM (3 × 10 mL). The organic layer was dried over $MgSO_4$, filtered and concentrated at reduced pressure to yield 475 mg (95%) of **14a** as white solid, mp = 94–97 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 5.17 (1H, dd, J = 8.9, 1.9 Hz), 5.71 (1H, dd, J = 15.9, 1.9 Hz), 7.33–7.45 (4H, m), 7.85 (2H, dd, J = 8.3, 1.2 Hz), 8.01 (1H, s). ^{13}C NMR (126 MHz, CDCl3) δ: 104.8, 116.3, 126.0, 128.6, 129.0, 130.1, 130.4, 148.0. Anal. Calcd for $C_{10}H_9N_3$: C, 70.16; H, 5.30; N, 24.54. Found: C, 70.02; H, 5.25; N, 24.53.

5.4.2. 1-Vinyl-1*H*-1,2,3-triazole (14b)

Compound **14b** was prepared by the general elimination procedure in 88% yield as white oil. The basic conditions lead to deprotection by TMS. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 5.03 (1H, d, J = 9.0 Hz), 5.60 (1H, d, J = 15.9 Hz), 7.24 (1H, dd, J = 15.9 e 8.9), 7.58 (1H, s), 7.80 (1H, s). ^{13}C NMR (126 MHz, $CDCl_3$) δ: 104.9, 120.5, 130.1, 133.9. Anal. Calcd for $C_4H_5N_3$: C, 50.52; H, 5.30; N, 44.18. Found: C, 50.48; H, 5.29; N, 44.13.

5.4.3. 4-Propyl-1-vinyl-1*H*-1,2,3-triazole (14c)

Compound **14c** was prepared by the general elimination procedure in 90% yield as colorless oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 0.84 (3H, t, J = 7.4 Hz), 1.52–1.64 (2H, m), 2.58 (2H, t, J = 7.6 Hz), 4.97 (1H, dd, J = 9.0, 1.8 Hz), 5.50 (1H, dd, J = 16.0, 1.8 Hz), 7.20 (1H, dd, J = 16.0, 9.0 Hz), 7.52 (1H, s). ^{13}C NMR (126 MHz, $CDCl_3$) δ: 13.5, 22.4, 27.4, 103.8, 117.6, 130.2, 148.3. Anal. Calcd for $C_7H_{11}N_3$: C, 61.29; H, 8.08; N, 30.63. Found: C, 61.24; H, 8.01; N, 30.57.

5.4.4. 4-(4-Methoxyphenyl)-1-vinyl-1*H*-1,2,3-triazole (14d)

Compound **14d** was prepared by the general elimination procedure in 91% yield as white solid, mp = 100–103 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 3.84 (3H, s), 5.16 (1H, dd, J = 8.9, 2.0 Hz), 5.67 (1H, dd, J = 16.0, 2.0 Hz), 6.96 (2H, d, J = 8.9 Hz), 7.38 (1H, dd, J = 16.0, 9.0 Hz), 7.78 (2H, d, J = 8.9 Hz), 7.92 (1H, s). ^{13}C NMR (126 MHz, $CDCl_3$) δ: 55.5, 104.5, 114.4, 115.3, 122.8, 127.3, 130.5, 148.0, 160.0. Anal. Calcd for $C_{11}H_{11}N_3O$: C, 65.66; H, 5.51; N, 20.88. Found: C, 65.53; H, 5.48; N, 20.85.

5.4.5. 4-(4-Pentylphenyl)-1-vinyl-1*H*-1,2,3-triazole (14e)

Compound **14e** was prepared by the general elimination procedure in 93% yield as white solid, mp = 78–81 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 0.90 (3H, t, J = 7.0 Hz), 1.24–1.41 (4H, m), 1.54–1.69 (2H, m), 2.63 (2H, t, J = 7.4 Hz), 5.17 (1H, dd, J = 8.9, 1.9 Hz), 5.69 (1H, dd, J = 16.0, 1.9 Hz), 7.25 (2H, d, J = 8.1 Hz), 7.38 (1H, dd, J = 16.0, 9.0 Hz), 7.76 (2H, d, J = 8.1 Hz), 7.97 (1H, s). ^{13}C NMR (126 MHz, $CDCl_3$) δ: 14.2, 22.7, 31.2, 31.6, 35.9, 104.6, 115.8, 126.0, 127.5, 129.1, 130.5, 143.7, 148.3. Anal. Calcd for $C_{15}H_{19}N_3$: C, 74.65; H, 7.94; N, 17.41. Found: C, 74.57; H, 7.89; N, 17.40.

5.4.6. 4-(4-Fluorophenyl)-1-vinyl-1*H*-1,2,3-triazole (14f)

Compound **14f** was prepared by the general elimination procedure in 90% yield as white solid, mp = 123–126 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 5.21 (1H, d, J = 8.9 Hz), 5.71 (1H, dd, J = 15.9, 1.9 Hz), 7.14 (2H, t, J = 8.7 Hz), 7.39 (1H, dd, J = 15.9, 8.9 Hz), 7.82–7.85 (2H, m), 7.97 (1H, s). ^{13}C NMR (126 MHz, $CDCl_3$) δ: 104.9, 116.0 (d, J = 8.9 Hz), 116.1, 126.4 (d, J = 2.9 Hz), 127.8 (d, J = 8.3 Hz), 130.4, 147.2, 163.0 (d, J = 248.1 Hz). Anal. Calcd for $C_{10}H_8FN_3$: C, 63.49; H, 4.26; N, 22.21. Found: C, 65.53; H, 5.48; N, 20.85.

5.5. General 1,3-dipolar cycloaddition procedure

A solution of **14a** (475 mg, 2.77 mmol) and nitrone **15** (1.04 g, 3.3 mmol) in $CHCl_3$ (5 mL) was put in a sealed tube and irradiated under microwave conditions at 150 W, 80 °C, for 2 h. The removal of the solvent in vacuo afforded a crude material which, after flash chromatography purification by using as eluent a mixture

of cyclohexane/ethyl acetate 7:3, gave the unseparable mixture (*trans*/*cis*) of compound **16a** and **17a**, yield 1.17 g (85%), as yellow oil, that was used for the next reaction. The ^{1}H NMR spectrum of the crude reaction mixture shows the presence of *trans* and *cis* isomer respectively in 1:1.3 ratio. Compounds **16b**–**f** and **17b**–**f** were prepared by the 1,3-dipolar cycloaddition procedure in 85% yield as yellow oil and then used for the next reaction.

5.6. General desilylation of the hydroxymethyl group procedure: synthesis of 18 and 19

A solution of compounds **16a** and **17a** (1.17 g, 2.35 mmol) and TBAF (0.853 mL, 3.52 mmol) in freshly distilled THF (30 mL) was stirred until desilylation was completed (TLC, 4–5 h). Volatiles were flash evaporated, and the residue was purified by MPLC (CH_2Cl_2/MeOH, 98:2) to afford **18a** (*trans* isomer) and **19a** (*cis* isomer) in 93% yield.

5.6.1. ((3*RS*,5*RR*)-2-Methyl-5-(4-phenyl-1*H*-1,2,3-triazol-1-yl)isoxazolidin-3-yl)methanol (18a)

Colorless oil, 40.4% yield. UV (hexane) λ_{max} ($\log\varepsilon$): 243 (4.51). ^{1}H NMR (500 MHz, $CDCl_3$) δ: 2.83 (3H, s), 2.90–2.98 (3H, m), 3.02–3.11 (1H, m), 3.78 (1H, ddd, J = 16.0, 11.8, 3.6 Hz), 6.32–6.35 (1H, m), 7.28–7.43 (2H, m), 7.77–7.84 (3H, m), 8.21 (1H, s). ^{13}C NMR (126 MHz, $CDCl_3$) δ: 39.0, 44.4, 61.1, 68.9, 86.1, 118.6, 125.9, 128.3, 128.9, 130.6, 148.1. Anal. Calcd for $C_{13}H_{16}N_4O_2$: C, 59.99; H, 6.20; N, 21.52. Found: C, 59.96; H, 6.17; N, 21.51.

5.6.2. ((3*RS*,5*SR*)-2-Methyl-5-(4-phenyl-1*H*-1,2,3-triazol-1-yl)isoxazolidin-3-yl)methanol (19a)

White solid, 52.6% yield, mp = 98–100 °C. UV (hexane) λ_{max} ($\log\varepsilon$): 245 (4.53) ^{1}H NMR (500 MHz, $CDCl_3$) δ: 2.78 (3H, s), 2.81–2.94 (1H, m), 3.50–3.59 (2H, m), 3.70 (2H, m, ddd, J = 16.2, 11.5, 4.0 Hz), 6.16–6.20 (1H, m), 7.31–7.46 (3H, m), 7.85 (2H, d, J = 7.3 Hz). ^{13}C NMR (126 MHz, $CDCl_3$) δ: 36.7, 46.6, 62.0, 67.9, 88.1, 119.5, 126.0, 128.6, 129.0, 130.3 148.6. Anal. Calcd for $C_{13}H_{16}N_4O_2$: C, 59.99; H, 6.20; N, 21.52. Found: C, 59.89; H, 6.18; N, 21.49.

5.6.3. ((3*RS*,5*RR*)-2-Methyl-5-(1*H*-1,2,3-triazol-1-yl)isoxazolidin-3-yl) methanol (18b)

Compound **18b** was prepared by the general desilylation procedure in 41.3% yield as yellow oil. UV (EtOH) λ_{max} ($\log\varepsilon$): 219 (4.07). ^{1}H NMR (500 MHz, $CDCl_3$) δ: 2.76 (3H, s), 2.77–2.82 (1H, m), 2.86–2.91 (1H, m), 3.02 (1H, dt, J = 13.4, 8.3 Hz), 3.71 (2H, ddd, J = 16.5, 11.8, 4.1 Hz), 3.86 (1H, br s), 6.30 (1H, dd, J = 8.1, 2.9 Hz), 7.61 (1H, s), 8.03 (1H, s). ^{13}C NMR (126 MHz, $CDCl_3$) δ: 39.1, 42.3, 60.7, 70.0, 85.8, 122.6, 133.8. Anal. Calcd for $C_7H_{12}N_4O_2$: C, 45.64; H, 6.57; N, 30.42. Found: C, 45.59; H, 6.52; N, 30.39.

5.6.4. ((3*RS*,5*SR*)-2-Methyl-5-(1*H*-1,2,3-triazol-1-yl)isoxazolidin-3-yl)methanol (19b)

Compound **19b** was prepared by the general desilylation procedure in 53.7% yield as yellow oil. UV (EtOH) λ_{max} ($\log\varepsilon$): 221 (4.32). ^{1}H NMR (500 MHz, $CDCl_3$) δ: 2.69 (3H, s), 2.74–2.84 (1H, m), 3.28–3.49 (2H, m), 3.65 (2H, ddd, J = 16.9, 11.5, 4.8 Hz), 6.14 (1H, dd, J = 7.2, 2.1 Hz), 7.67 (1H, d, J = 1.1 Hz), 7.78 (1H, d, J = 1.1 Hz). ^{13}C NMR (126 MHz, $CDCl_3$) δ: 37.3, 46.5, 61.9, 67.9, 87.6, 123.5, 134.2. Anal. Calcd for $C_7H_{12}N_4O_2$: C, 45.64; H, 6.57; N, 30.42. Found: C, 45.62; H, 6.55; N, 30.41.

5.6.5. ((3*RS*,5*RR*)-2-Methyl-5-(4-propyl-1*H*-1,2,3-triazol-1-yl)isoxazolidin-3-yl)methanol (18c)

Compound **18c** was prepared by the general desilylation procedure in 40.4% yield as yellow oil. UV (EtOH) λ_{max} ($\log\varepsilon$): 220 (4.06). ^{1}H NMR (500 MHz, $CDCl_3$) δ: 0.94 (3H, t, J = 7.1 Hz), 1.58–1.73 (2H, m), 2.65 (2H, t, J = 7.5 Hz), 2.80 (3H, s), 2.81–2.96 (2H, m), 2.97–3.06 (1H, m), 3.75 (2H, ddd, J = 16.3, 11.7, 3.6 Hz), 6.26 (1H, dd, J = 7.8, 2.2 Hz), 7.71 (1H, s). ^{13}C NMR (126 MHz, $CDCl_3$) δ: 13.9, 22.7, 27.7, 39.0, 44.4, 61.1, 69.0, 85.8, 119.6, 148.5. Anal. Calcd for $C_{10}H_{18}N_4O_2$: C, 53.08; H, 8.02; N, 24.76. Found: C, 52.89; H, 7.96; N, 24.68.

5.6.6. ((3*RS*,5*SR*)-2-Methyl-5-(4-propyl-1*H*-1,2,3-triazol-1-yl)isoxazolidin-3-yl)methanol (19c)

Compound **19c** was prepared by the general desilylation procedure in 52.6% yield as yellow oil. UV (EtOH) λ_{max} ($\log\varepsilon$): 222 (4.40). ^{1}H NMR (500 MHz, $CDCl_3$) δ: 0.94 (3H, t, J = 7.4 Hz), 1.61–1.71 (2H, m), 2.67 (2H, td, J = 7.7, 3.1 Hz), 2.72 (3H, s), 2.73–2.83 (2H, m), 3.36–3.51 (2H, m), 3.60–3.74 (2H, m), 6.07–6.09 (1H, m), 7.48 (1H, s). ^{13}C NMR (126 MHz, $CDCl_3$) δ: 13.8, 22.6, 27.6, 29.7, 37.0, 62.1, 68.0, 87.7, 120.7, 148.9. Anal. Calcd for $C_{10}H_{18}N_4O_2$: C, 53.08; H, 8.02; N, 24.76. Found: C, 52.96; H, 8.00; N, 24.75.

5.6.7. ((3*RS*,5*RR*)-5-(4-(4-Methoxyphenyl)-1*H*-1,2,3-triazol-1-yl)-2-methylisoxazolidin-3-yl methanol (18d)

Compound **18d** was prepared by the general desilylation procedure in 47.5% yield as white solid, mp = 131–133 °C. UV (EtOH) λ_{max} ($\log\varepsilon$): 247 (4.61). ^{1}H NMR (500 MHz, $CDCl_3$) δ: 2.81 (3H, s), 2.88–3.06 (3H, m), 3.64–3.80 (2H, m), 3.81 (3H, s), 6.32 (1H, m), 6.93 (2H, d, J = 8.5 Hz), 7.74 (2H, d, J = 8.5 Hz), 8.11 (1H, s). ^{13}C NMR (126 MHz, $CDCl_3$) δ: 29.8, 39.0, 44.4, 55.4, 61.1, 69.0, 86.1, 114.3, 117.8, 123.3, 127.2, 148.0, 159.7. Anal. Calcd for $C_{14}H_{18}N_4O_3$: C, 57.92; H, 6.25; N, 19.30. Found: C, 57.91; H, 6.22; N, 19.29.

5.6.8. ((3*RS*,5*SR*)-5-(4-(4-Methoxyphenyl)-1*H*-1,2,3-triazol-1-yl)-2-methylisoxazolidin-3-yl)methanol (19d)

Compound **19d** was prepared by the general desilylation procedure in 47.5% yield as white solid, mp = 110–112 °C. UV (EtOH) λ_{max} ($\log\varepsilon$): 250 (4.68). ^{1}H NMR (500 MHz, $CDCl_3$) δ: 2.76 (3H, s), 2.80–2.93 (1H, m), 3.46–3.64 (2H, m), 3.69 (2H, ddd, J = 16.5, 11.5, 4.3 Hz), 3.85 (3H, s), 6.14 (1H, d, J = 6.5 Hz), 6.97 (2H, d, J = 8.9 Hz), 7.77 (2H, d, J = 8.9 Hz), 7.90 (1H, s). ^{13}C NMR (126 MHz, $CDCl_3$) δ: 29.7, 37.0, 55.4, 62.1, 68.0, 87.9, 114.4, 118.8, 122.9, 127.1, 148.2, 159.8. Anal. Calcd for $C_{14}H_{18}N_4O_3$: C, 57.92; H, 6.25; N, 19.30. Found: C, 57.88; H, 6.23; N, 19.31.

5.6.9. ((3*RS*,5*RR*)-2-Methyl-5-(4-(4-pentylphenyl)-1*H*-1,2,3-triazol-1-yl)isoxazolidin-3-yl)methanol (18e)

Compound **18e** was prepared by the general desilylation procedure in 41.7% yield as white solid, mp = 69–71 °C. UV (EtOH) λ_{max} ($\log\varepsilon$): 242 (4.72). ^{1}H NMR (500 MHz, $CDCl_3$) δ: 0.87 (3H, t, J = 6.9 Hz), 1.25–1.37 (4H, m), 1.55–1.64 (2H, m), 2.53–2.61 (2H, m), 2.77 (3H, s), 2.79–2.92 (2H, m), 2.98 (1H, dt, J = 12.7, 8.1 Hz), 3.74 (2H, ddd, J = 16.2, 11.8, 3.9 Hz), 6.27 (1H, dd, J = 8.0, 2.6 Hz), 7.17 (2H, d, J = 8.0 Hz), 7.69 (2H, d, J = 8.0 Hz), 8.22 (1H, s). ^{13}C NMR (126 MHz, $CDCl_3$) δ: 14.1, 22.5, 31.1, 31.5, 35.7, 39.1, 44.3, 60.9, 67.0, 86.0, 118.4, 125.7, 127.7, 128.9, 143.2, 148.0. Anal. Calcd for $C_{18}H_{26}N_4O_2$: C, 65.43; H, 7.93; N, 16.96. Found: C, 65.36; H, 7.89; N, 16.91.

5.6.10. ((3*RS*,5*SR*)-2-Methyl-5-(4-(4-pentylphenyl)-1*H*-1,2,3-triazol-1-yl)isoxazolidin-3-yl)methanol (19e)

Compound **19e** was prepared by the general desilylation procedure in 54.3% yield as white solid, mp = 99–100 °C. UV (EtOH) λ_{max} ($\log\varepsilon$): 244 (4.67). ^{1}H NMR (500 MHz, $CDCl_3$) δ: 0.89 (3H, t, J = 6.9 Hz), 1.26–1.40 (4H, m), 1.60–1.66 (2H, m), 2.53 (2H, t, J = 7.4 Hz), 2.76 (3H, s), 2.80–2.93 (2H, m), 3.48–3.58 (1H, m), 3.70 (2H, ddd, J = 16.5, 11.5, 4.2 Hz), 6.12–6.21 (1H, m), 7.24 (2H, d, J = 8.0 Hz), 7.74 (2H, d, J = 8.0 Hz), 7.94 (1H, s). ^{13}C NMR (126 MHz, $CDCl_3$) δ: 14.1, 22.7, 31.1, 31.6, 35.8, 36.8, 46.7, 62.1,

68.0, 88.1, 119.2, 125.8, 127.7, 129.1, 143.5, 148.6. Anal. Calcd for $C_{18}H_{26}N_4O_2$: C, 65.43; H, 7.93; N, 16.96. Found: C, 65.41; H, 7.90; N, 16.89.

5.6.11. ((3*RS*,5*RR*)-5-(4-(4-Fluorophenyl)-1*H*-1,2,3-triazol-1-yl)-2-methylisoxazolidin-3-yl)methanol (18f)

Compound **18f** was prepared by the general desilylation procedure in 41.3% yield as white oil. UV (EtOH) λ_{max} (log ε): 240 (4.40). ^{1}H NMR (500 MHz, $CDCl_3$) δ: 2.77 (3H, s), 2.80–2.93 (2H, m), 2.95–3.02 (1H, m), 3.74 (2H, ddd, J = 16.0, 11.8, 3.6 Hz), 6.29 (1H, dd, J = 8, 2.6 Hz), 7.03 (2H, t, J = 8.7), 7.73 (2H, dd, J = 8.7, 5.4 Hz), 8.23 (1H, s). ^{13}C NMR (126 MHz, $CDCl_3$) δ: 29.7, 39.0, 44.3, 60.7, 69.0, 86.5, 115.8 (d, J = 21.7 Hz), 118.6, 126.7 (d, J = 3.0 Hz), 127.6 (d, J = 8.1 Hz), 134.9, 147.1, 162.7 (d, J = 247.4 Hz). Anal. Calcd for $C_{13}H_{15}FN_4O_2$: C, 56.11; H, 5.43; N, 20.13. Found: C, 56.06; H, 5.41; N, 20.11.

5.6.12. ((3*RS*,5*SR*)-5-(4-(4-Fluorophenyl)-1*H*-1,2,3-triazol-1-yl)-2-methylisoxazolidin-3-yl)methanol (19f)

Compound **19f** was prepared by the general desilylation procedure in 53.7% yield as white solid, mp = 112–114 °C. UV (EtOH) λ_{max} (log ε): 239 (4.61). ^{1}H NMR (500 MHz, $CDCl_3$) δ: 2.77 (3H, s), 2.82–2.89 (2H, m), 3.51–3.59 (2H, m), 3.71 (2H, ddd, J = 16.1, 11.5, 3.9 Hz), 6.17 (1H, d, J = 6.0 Hz), 7.12 (2H, t, J = 8.7 Hz), 7.81 (2H, dd, J = 8.7, 5.3 Hz), 7.96 (1H, s). ^{13}C NMR (126 MHz, $CDCl_3$) δ: 29.8, 37.0, 46.6, 62.0, 68.0, 88.0, 116.0 (d, J = 21.8 Hz), 119.3, 126.5 (d, J = 2.8 Hz), 127.6 (d, J = 8.1 Hz), 147.6, 162.9 (d, J = 247.6 Hz). Anal. Calcd for $C_{13}H_{15}FN_4O_2$: C, 56.11; H, 5.43; N, 20.13. Found: C, 56.04; H, 5.42; N, 20.09.

5.7. General protocol for inhibition assays

Dulbecco's modified Eagle medium (DMEM) and Minimum essential Medium (MEM) containing 2 mM GlutaMAX (GIBCO), Ham's F12 (GIBCO), non-essential amino acids, heat inactivated-Foetal Bovine Serum (FBS, GIBCO), Normal Goat Serum (NGS, GIBCO), Streptomycin and penicillin antibiotics, Trypsin–EDTA 0.05% solution, and Mouse anti-Human Transferrin Receptor (TfR-1) monoclonal antibody were obtained from Invitrogen (Milano, Italia). Lab-Tek™ Chamber Slides II, 3(4,5-dimethyl-thiazol-2-yl)2,5-diphenyl-tetrazolium bromide salts (MTT), and other chemicals of analytical grade were obtained from Sigma–Aldrich (Milano, Italy). Mouse monoclonal antibody against caspase-3 was from Becton–Dickinson (Milan, Italy). Tetrarhodamine isothiocyanate (TRITC)-conjugated anti-mouse IgG polyclonal antibody, were from Chemicon (Prodotti Gianni, Milan, Italy). ApoAlert DNA fragmentation assay kit was from Clontech (Milan, Italy).

5.7.1. Cell cultures

FTC-133 and 8305C cell lines were suspended in appropriate medium and plated in flasks at a final density of 2×10^6 cells or in Lab-Tek™ Chamber Slides II at a final density 0.5×10^5 cells/well. Specifically the medium for FTC-133 cell lines was: DMEM containing 2 mM Gluta-MAX, 10% FBS, streptomycin (50 μg/mL), penicillin (50 U/mL); whereas the medium for 8305C cell lines was: MEM containing 2 mM Gluta-MAX, 10% FBS, streptomycin (50 μg/mL), penicillin (50 U/mL), and 1% non-essential amino acids. Cell lines were then incubated at 37 °C in humidified atmosphere containing 5% CO_2 and the medium was replaced every 2 or 3 days. When the cultures were about 85–90% confluent, cells were trypsinized by 0.05% trypsin and 0.53 mM EDTA at 37 °C in humidified atmosphere containing 5% CO_2 for 5 min. Trypsinization was stopped by adding 20% FBS, resuspended and plated in flasks fed with fresh basic complete media. Cells were seeded again at 1:4 density ratio and incubated at 37 °C in humidified atmosphere containing 5% CO_2.

5.7.2. Treatment of the cells

FTC-133 and 8305C were replated on to Lab-Tek™ Chamber Slides II at a final density of 1×10^4 cells/well, and fed in fresh complete medium. In preliminary experiments, we exposed the both cultures both in absence or in presence of different concentrations of **18a–f** or **19a–f** (0.5, 1, 5, 10, 25, 50, 100 μM) for 12, 24, 48 h, in order to establish the optimal concentrations and their exposure times to all synthesized compounds. For this purpose, MTT test and morphological characterization were utilized.[43]

MTT bioassay. Cell survival analysis was performed by MTT reduction assay, evaluating mitochondrial dehydrogenase activity.[42–44] Cells were set up 6×10^5 cells per well of a 96-multiwell, flat-bottomed, 200-μL microplate, and maintained at 37 °C in a humidified 5% CO_2/95% air mixture At the end of treatment time, 20 μL of 0.5% MTT in (pH 7.4) PBS were added to each microwell. After 1 h of incubation with the reagent, the supernatant was removed and replaced with 200 μL of dimethyl sulfoxide (DMSO). The optical density of each well was measured with a microplate spectrophotometer reader (Titertek Multiskan; Flow Laboratories, Helsinki, Finland) at 570 nm.

5.7.3. Immunocytochemistry

Expression of caspase-3 in FTC-133 and 8305C was identified by immunocytochemical procedures.[42–44] Untreated or **18c** treated FTC-133 and 8305C were fixed by exposing to 4% paraformaldehyde in 0.1 M PBS for 20 min. Then, cells were washed three times with PBS and incubated for 1 h at 37 °C in humidified air and 5%CO_2 with 1% NGS in PBS to block unspecific sites. The cells were successively incubated overnight at 37 °C in humidified air and 5%CO_2 with mouse monoclonal antibody against caspase-3 (1:200). Finally, the slides were washed three times with PBS, mounted in PBS/glycerol (50:50), and analyzed on a Leica fluorescent microscopy (Germany). No non-specific staining of hMSCs was observed in control incubations in which the primary antibody was omitted.

5.7.4. TUNEL test

The ApoAlert DNA fragmentation assay kit detecting nuclear DNA fragmentation, a hallmark of apoptosis, was used. The ApoAlert DNA fragmentation assay incorporates fluorescein-dUTP at the free 3′-hydroxyl ends of the fragmented DNA using TUNEL and was performed according to the user's manual. FTC-133 and 8305C cell cultures, untreated and treated for 24 h with 5 μM of **18c** were made up according to the user's manual. Cells were mounted and visualized directly by fluorescence microscopy (Leika, Germany) with either a propidium iodide (PI) filter alone or a FITC filter alone. According to the user's manual, apoptotic cells appear green with the FITC filter alone while nonapoptotic cells appear red under the dual-pass FITC/PI filter set. We focused on 10 random microscopic fields for each dish. In each microscopic field we counted the number of apoptotic cells and we compared this number with all the non-apoptotic cells visualized in the same microscopic field; the ratio is expressed as a percentage.

5.7.5. Statistical analysis

Data were statistically analysed using one-way analysis of variance (one-way ANOVA) followed by post hoc Holm–Sidak test to estimate significant differences among groups. Data were reported as mean of four experiments in duplicate, and differences between groups were considered to be significant at $^*p < 0.05$.

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Supplementary data

Supplementary data (^{1}H and ^{13}C NMR data, for new compounds. Caspase-3 test) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2013.10.001.

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