Rapid Synthesis of Triazole-Modified Resveratrol Analogues via Click Chemistry

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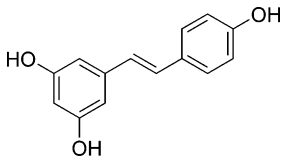
Received November 7, 2005

Abstract: Resveratrol is a phytoalexin able to display an array of biological activities. We decided to replace the double bond with a triazole ring using the archetypical click reaction: the Huisgen [3 + 2] cycloaddition. Seventy-two triazole derivatives were synthesized via a parallel combinatorial approach. Preliminary data suggest that this procedure can lead to the synthesis of compounds that display some, but not all, of resveratrol's actions with increased potency.

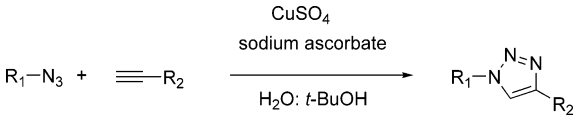
The increasing knowledge of molecular cell biology and the advances in biomedical research have increased exponentially the known targets for therapeutic intervention. Such an increase has been paralleled by an increased interest in natural compounds as lead compounds for novel pharmacological screenings. In this respect, there has been great interest in resveratrol (**1**). The relatively high concentrations in wine and the original



resveratrol **(1)**

observations of its cardioprotective role (which formed the basis of the French paradox)[1] brought this compound to notoriety. Most of initial work performed on this molecule was indeed on the cardiovascular system, including effects on lipid metabolism and platelet function,[2] but reports on cancer protection opened new avenues.[3−5] The main problem with resveratrol research is that this molecule exerts most of its effects at high micromolar concentrations,[4] and it is therefore difficult to ascertain which are the targets responsible for the single effects. Among the potential beneficial uses of resveratrol, it has been proposed that it might act as a cardioprotective and neuroprotective agent, as an antiinflammatory drug and in cancer chemoprotection.[4] The potential candidate targets are even greater: alongside the inhibition of lipid peroxidation, inhibition of nitric oxide synthase, inhibition of cyclooxygenases, and accumulation of ceramide,[4,6,7] there is growing evidence that a number of proteins involved in signal transduction, in the cell cycle, in regulating transcription, and in ionic conductance are modulated by resveratrol.[4,8−11] In this context, it has also been shown that resveratrol can mimic estrogens in activating ERα and β receptors.[4,12] In summary, therefore, resveratrol possesses the potential to intervene in a number of therapeutically interesting pathways, but its low potency and the plethora of actions limit its applications.

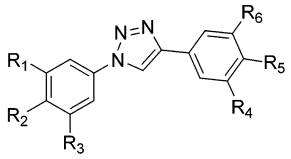
Scheme 1. Huisgen [3 + 2] Cycloaddition



The concept of click chemistry is experiencing a growing popularity.[13] This term, coined by Barry K. Sharpless,[14] now refers to reactions yielding the product in high yield without the need for further purification, generating inoffensive byproducts, and operating in a benign solvent, usually water. In this way, it is possible to generate a plethora of new compounds reliably and thereby accelerate the process of drug discovery.

In the present paper, we have synthesized a series of *trans*-resveratrol analogues replacing the double bond with a triazole ring. The substitution with a triazole ring would have the great advantage of making these analogues suitable for combinatorial chemistry. Indeed, we employed the archetypical "click" reaction: the Huisgen [3 + 2] cycloaddition between alkynes and azides catalyzed by copper(I) salts[14] (Scheme 1). The presence of the copper(I) salts at room temperature generates 1,4 regioselective products,[15] which contrasts with the thermal reaction, which demands high temperatures and gives equimolecular quantities of the two regioisomers, which are often difficult to separate. Indeed, out of 88 parallel reactions attempted, 74 yielded precipitates, and 72 of these were confirmed as the expected products. As a proof of principle, we also have evaluated the cytotoxicity of these compounds, and the preliminary biological analysis suggests that this procedure is capable of generating active triazole-substituted resveratrol analogues.

To establish whether the double bond of resveratrol is amenable to bioisosteric substitution with the triazole moiety, compounds **2**−**6** were synthesized (Figure 1) using the Huisgen [3 + 2] cycloaddition via the Sharpless protocol.[14,15] As a preliminary screening, these compounds were tested in a cytotoxicity/proliferation assay in neuroblastoma (SH-SY5Y), breast cancer (MDA-MB-231), basophilic leukemia (RBL 2H3), and human pancreatic carcinoma (FG2) cell lines. We employed a colorimetric method (MTT; based on mitochondrial activity), which does not distinguish between decreased proliferation and increased cell death.[16] In all the cell lines, resveratrol was capable of inducing a reduction of cell growth after 48 h incubation. Triazole-derivatives were not uniform in their behavior with some compounds not eliciting any effect and



2 R_1= OH; R_2= H; R_3= OH; R_4= H; R_5= OH; R_6= H

3 R_1= H; R_2= OH; R_3= H; R_4= OH; R_5= H; R_6= OH

4 R_1= OMe; R_2= H; R_3= OMe; R_4= H; R_5= OMe; R_6= H

5 R_1= H; R_2= OMe; R_3= H; R_4= OMe; R_5= H; R_6= OMe

6 R_1= OH; R_2= H; R_3= OH; R_4= H; R_5= OMe; R_6= H

Figure 1. Structure of triazole-modified resveratrol analogues.

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10.1021/jm051118z CCC: $33.50
Published on Web 12/21/2005

Table 1. Effect of Resveratrol and Triazole-Substituted Analogues on Viability in a Panel of Cell Lines[a]

	1	2	3	4	5	6
SH-SY5Y	33.5 ± 3.7	93.4 ± 4.7	86.6 ± 8.5	85.9 ± 1.0	78.6 ± 4.3	62.2 ± 2.1
MDA-MB-231	67.9 ± 0.9	97.0 ± 2.4	95.5 ± 0.8	99.3 ± 5.8	93.2 ± 2.1	72.2 ± 2.0
RBL 2H3	52.1 ± 2.3	110 ± 2.7	114 ± 4.6	113 ± 3.1	116 ± 1.4	99.6 ± 5.0
FG2	42.2 ± 1.6	93.7 ± 2.7	91.9 ± 2.8	100.8 ± 4.6	88.2 ± 2.3	83.7 ± 2.6

[a] Values represent the % viability, as measured by the MTT assay, of control or treated cells at 48 h and are mean ± SEM of four determinations. All compounds were used at a concentration of 100 μM.

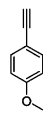
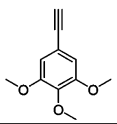
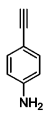
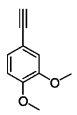
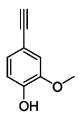
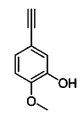
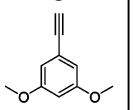
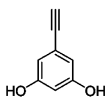
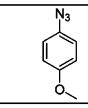
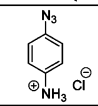
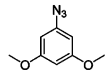
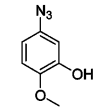
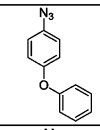
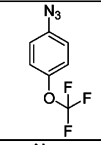
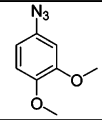
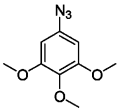
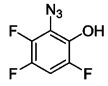
others appearing efficacious at 100 μM (Table 1). Yet, differences in the extent of resveratrol action were observable among the cell lines, as were differences in compounds **2**−**6**. For example, **6** did not have any effect on RBL 2H3 cells, it paralleled the effect of **1** in MDA-MB-231 cells, and it had a lower effect compared to **1** in the other two cell lines. To further understand these differences, a time-course (measuring cell number) up to 96 h was performed with compounds **1**, **4**, and **6** in MDA-MB-231 and SH-SY5Y cells. Indeed, major differences were observed in proliferation rates between control and treated cells with the three compounds, and these differences were more evident at 96 h treatment. Under these conditions, 100 μM resveratrol was capable of inhibiting completely cell proliferation, as was compound **6.** The per-methylated **4** was unable to affect MDA-MB-231 proliferation, while it reduced SH-SY5Y proliferation by 50% (data not shown). It has already been shown that methylated or per-methylated analogues of *trans*-resveratrol display biological activities, at times increased over the parent compound,[17] and therefore it was not surprising that **6** and **4** unmasked an activity that was not significant in the demethylated compounds (**2**,**3**). These preliminary data suggested that a combinatorial approach could be beneficial toward finding resveratrol analogues that maintained some, but not all, properties of the parent compound.

As a proof of principle that click chemistry could be feasible for rapid parallel combinatorial synthesis of resveratrol analogues, we used 8 alkynes and 11 azides. The feasibility of this approach is given by the precipitation of the resultant product. The product can then be purified by filtration and ether washes. This procedure resulted in 74 of the expected 88 compounds being formed, as detected by precipitation. All precipitated compounds were submitted to MS analysis to verify their authenticity. Indeed, 67/74 (91%) were confirmed as the expected product. Two of the compounds yielded no peak at the predicted mass. Compounds **Ia**, **Ic**, **Id**, **If**, and **Ig** displayed a mass fragmentation compatible with a triazole product yet failed the original mass verification, as we observed a base peak at m/z $[M - H]^+$ in positive mode and a $[M - 3H]^-$ in negative mode. Originally, we intended to use 2,3,5,6-tetrafluorophenyl azide synthesized from 2,3,5,6-tetrafluoroaniline. Yet, the mass suggested the presence of a hydroxyl group instead of a fluorine. Indeed, ^{13}C NMR, ^1H NMR, and MS analysis (see Supporting Information) of the azide **I** revealed that this compound displayed a hydroxyl group in the ortho position instead of the expected fluorine. This suggests that the original amine underwent an aromatic nucleophilic substitution of one of the four fluorine groups with water during the azidation protocol.[18] In conclusion, therefore, the procedure resulted in 72 triazole products.

As mentioned above, resveratrol possesses numerous potential molecular targets resulting in multiple potential cellular outcomes. It is therefore possible that all the compounds synthesized share with the original molecule at least one of its actions. To evaluate a potential biological effect of these triazole-substituted compounds, we decided to investigate cytotoxic effects in MDA-MB-231 cells. Although the antiproliferative/cytotoxic effect of **1** was modest in these cells (Table 2), this compound can also induce a morphological change in these cells that can be observed in phase contrast microscopy[19] (see Supporting Information). All compounds were tested at decreasing concentrations in 96-well plates to establish an automated procedure. Cells treated with **1** (100 μM) displayed 80.2% ± 2.7% viability of control ($n = 47$), while at 10 μM, they displayed 106% ± 7.2% viability of control ($n = 33$). Out of the 72 compounds tested, 54 were unable to reduce viability (a cutoff of 70% of control was arbitrarily chosen) at a concentration of 10 μM. It is interesting that some of these compounds (**Ic**, **Hc**, and **Mc**) induced a significant proliferation of cells (154% ± 12.8%, 123% ± 4.7%, and 174% ± 11.1% compared to control, respectively; $n = 5$). Since **1** has been shown to possess estrogenic activity, it would be appealing to speculate that these compounds retain this activity, while they do not retain the antiproliferative activity.[12,20] Of the remaining compounds, nine possessed an approximate IC_{50} between 1 and 10 μM, eight between 100 nM and 1 μM, and one between 10 nM and 100 nM (**Fc**). As a confirmation of the protocol, the lead compounds arising from this screening (**Aa**, **Ca**, **Da**, **La**, **Ac**, **Fc**, **Ad**, and **Ed**) were submitted to HPLC analysis to confirm purity. Compounds with a purity lower than 95% (**Aa**, **Ca**, **Da**, and **La**; all over 85%) were resynthesized, reverified, and reevaluated (**Aa *bis*, Ca *bis*, Da *bis*, La *bis***). The new compounds possessed identical biological activity (data not shown).

It has been previously shown that MDA-MB-231 cells change morphological characteristics upon resveratrol addition.[19] This change, which might or might not be correlated to the decreased proliferation/increased cell death, is represented by an increased filopodia extension.[19] When cells were incubated for 48 h with resveratrol this phenomenon could be reproduced at concentrations over 50 μM (see Supporting Information). We then attempted to establish whether the lead compounds were able to reproduce this effect at low concentrations. We therefore used, for each of the lead compounds, concentrations in the range of the approximate IC_{50} established in the cytotoxicity assay. Indeed, compounds **Ac** and **Ad** revealed similar processes (although not to the same extent compared to high concentrations of **1**), while no morphological changes appeared evident with **Aa**, **Ca**, **Da**, **Ed**, **Fc**, and **La** (see Supporting Information). Among other reports on resveratrol is its ability to induce a modest S-phase arrest of the cell cycle.[21] Indeed, in MDA-MB-231 cells, 31.5% ± 2.1% of control cells were in S-phase, while this number increased to 49.5% ± 5.6% in resveratrol-treated cells (10 μM). Of the lead compounds tested, only **Aa** (10 nM) and **Ad** (10 nM) induced an increase in the S-phase percentage (39% ± 4.2% and 41.5% ± 0.7%). Cells treated with the other lead compounds did not show any significant change in the cell cycle characteristics (S-phase **Ca** 100 nM 29% ± 1.4%; **Da** 100 nM 32.5% ± 6.4%; **La** 100 nM 32% ± 4.2%; **Ac** 10 nM 32% ± 8.5%; **Fc** 100 nM 35.5% ± 5.0%). It is interesting to note that compound **Ad** shared with **1** both characteristics investigated (morphology and cell cycle), while **Ac** and **Aa** shared just one. This would suggest that our aim of generating

Table 2. Cytotoxic/Antiproliferative Effect of Click Chemistry Generated Resveratrol Analogues[a]

	a 	b 	c 	d 	e 	f 	g 	h 
A 	**>100**	>10000	**>100**	**>100**	>10000	>10000	>10000	>10000
B 	**>100**	n.p.	n.p.	n.p.	m.f.	> 10000	m.f.	n.p.
C 	**>100**	>1000	>10000	>1000	≈10000	> 10000	> 10000	>10000
D 	**>100**	≈10000	> 10000	>1000	> 10000	> 10000	> 10000	> 10000
E 	**>100**	≈10000	> 10000	≈10000	n.p.	> 10000	> 10000	> 10000
F 	≈1000	> 10000	**≥10**	>1000	≈10000	> 10000	>1000	> 10000
G 	> 10000	n.p.	> 10000	n.p.	n.p.	**>100**	≈10000	> 10000
H 	> 10000	>10000	> 10000	n.p.	> 10000	> 10000	> 10000	> 10000
I 	>1000	n.p.	> 10000	n.p.	≈10000	> 10000	> 10000	n.p.
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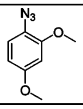
M 	> 10000	> 10000	> 10000	n.p.	n.p.	> 10000	> 10000	≈10000

[a] Experiments were performed at log intervals, and values represent the approximate IC_{50} values expressed as nM ($n = 4-6$ for log unit performed); np = not precipitated; mf = mass spectrometry failed to reveal the expected product.

potent analogues that did not display all the mechanisms of resveratrol was accomplished.

It would be appealing to draw a structure−activity relationship with the compounds synthesized and screened. Indeed, a few indications can be extrapolated or postulated. For example, compounds with a *para*-methoxy group on either ring appear to be cytotoxic. Yet, the cytotoxicity of these compounds is unlikely to resemble that of combretastatin A4,[22] because compounds **Hf** and **Db**, the closest analogues of this molecule, were devoid of cytotoxic activity. Furthermore, compounds with a Grimm's isosteric substitution (i.e., OH−NH_2 in the para position, **Ic**, **Hc**, and **Mc**) displayed a proliferative effect, and these might therefore be good lead compounds for resveratrol analogues with estrogenic activity. On the other hand, this preliminary screening should be extended to more sophisticated and aimed biological assays to unravel structure−activity relationships of other actions of resveratrol.

In conclusion, we have employed click chemistry to generate triazole-substituted resveratrol analogues. At high concentrations, resveratrol possesses numerous actions with a therapeutic potential, and therefore the rapid triazole synthesis of resveratrol analogues described here could be used to generate an enormous chemical database. In the present paper, as a proof of principle, we have generated a preliminary screening for analogues with an antitumoral potential. We have found that some of the compounds screened were more potent than resveratrol as

cytotoxic/antiproliferative agents. Because of the numerous actions of resveratrol, it is difficult to ascertain whether the effects observed are indeed correlated with a resveratrol-like action. Yet, it is suggestive that some of the lead compounds did display similarities compared to resveratrol. This protocol will allow us to generate agents that possess less promiscuity compared to resveratrol and therefore might prove therapeutically useful.

Acknowledgment. Financial support from Università del Piemonte Orientale and Regione Piemonte is gratefully acknowledged.

Supporting Information Available: Synthesis of alkyne and azide building blocks, characterization (MS and ^{1}H and ^{13}C NMR data), and HPLC purities of the selected compounds, raw data relative to Table 2, and morphological changes induced by the lead compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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JM051118Z