European Journal of Medicinal Chemistry 83 (2014) 508–515

Contents lists available at ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Original article

Microwave-assisted synthesis of 3,5-disubstituted isoxazoles and evaluation of their anti-ageing activity

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ARTICLE INFO

Article history:
Received 28 February 2014
Received in revised form
17 June 2014
Accepted 23 June 2014
Available online 24 June 2014

Keywords:
Isoxazole
Microwave
Antioxidant
Anti-ageing
Caenorhabditis elegans

ABSTRACT

One-pot uncatalysed microwave-assisted 1,3-dipolar cycloaddition reactions between *in situ* generated nitrile oxides and alkynes bearing protected antioxidant substituents, were regioselectively afforded 3,5-disubstituted isoxazoles. The yields were moderate, based on the starting aldehydes, while the reaction times were in general shorter than those reported in the literature.

The cytoprotective and anti-ageing effect of the final deprotected compounds was evaluated *in vitro*, on cellular survival following oxidative challenge and *in vivo*, on organismal longevity using the nematode *Caenorhabditis elegans*. The activity of the isoxazole analogues depends on the nature and the number of the antioxidant substituents. Analogue **17** bearing a phenolic group and a 6-OH-chroman group is a promising anti-ageing agent, since it increased survival of human primary fibroblasts following treatment with H_2O_2 and extended *C. elegans* lifespan.

1. Introduction

Isoxazoles, have attracted an increasing research interest, as non classical amide or ester bioisosteres and potential pharmacophores endowed with anticancer [1], neuroprotective [2], anti-obesity [3], antidepressant [4], insecticidal [5], antidiabetic [6] and anti-inflammatory [7] activities. The major synthetic strategies to construct this heterocycle are: i) condensation of a 1,3-dicarbonyl compound with hydroxylamine and ii) 1,3-dipolar cycloaddition of an alkyne with a nitrile oxide, which is regioselective in the presence of copper(I), giving 3,5-disubstituted isoxazoles [8].

Nitrile oxides can react with simple terminal alkynes without the need of a catalyst, because of their increased reactivity, compared to azides. Very recently, several groups [1a,c,d,3,6,9–12] accessed 3,5-disubstituted isoxazoles through a metal-free cycloaddition of alkynes with nitrile oxides, usually in modest yields or long reaction times.

In general the 3,5-regioisomer was favoured under uncatalyzed conditions. Use of organocatalysts [7] or hypervalent iodine [13] reagents improved the yield/regioselectivity of the reaction. Moreover, the regiospecific synthesis of novel isoxazolines and isoxazoles of N-substituted saccharin derivatives, using a microwave oven, was also described [14].

Our group has been involved in the synthesis of neuroprotective antioxidants and we reported that the presence of isoxazole scaffold results in higher *in vitro* neuroprotective activity, compared to other nitrogen heteroaromatics. Isoxazole analogues were prepared by conventional Cu catalyzed cycloadditions [2] or by using dual-frequency ultrasound irradiation [15].

Although the isoxazole pharmacophore has been incorporated into a wide range of bioactive agents, the effect of isoxazole analogues on the cellular or organismal lifespan has not yet been reported.

Ageing is an inevitable natural biological process that is linked to the gradual deterioration of organismal homeostasis and the increasing accumulation of damaged macromolecules [16]. The progression of ageing has been highly correlated with increased levels of reactive oxygen species (ROS) and the extent of ROS formation and oxidative damage has been inversely correlated with longevity in different species [17]. Increased oxidative stress promotes the deterioration of all biomolecules including DNA, lipids and proteins thus leading to a global failure of cellular and organismal homeostasis [18]. There are various models used to study ageing *in vitro* and *in vivo*. Human primary fibroblasts that age *in vitro*, the so called replicative senescence model constitute the best accepted model to study human ageing *in vitro* [19]. Moreover, human primary fibroblasts can easily be used in different assays to

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http://dx.doi.org/10.1016/j.ejmech.2014.06.046
0223-5234/

reveal antioxidant properties on top of anti-ageing properties of different compounds. The nematode *Caenorhabditis elegans* is also a prominent model to study organismal ageing due to its short lifespan, the fast generation time and the multiple experimental applications [20,21].

The discovery of agents which could slow down the deleterious effects of ageing *in vitro* and/or *in vivo* has attracted an increasing research interest. Since ageing is associated with increased incidence of diseases related to elevated levels of reactive oxygen species (ROS), dietary phenolic antioxidants have emerged as promising candidates [22–27] while there is one recent report on the anti-ageing properties of synthetic compounds applying the aforementioned *in vivo* model [28]. Therefore bioactive isoxazoles bearing antioxidant groups would represent an interesting approach towards the development of anti-ageing compounds.

Collectively, the aim of the present study was i) the green regiospecific microwave-assisted one-pot synthesis of isoxazoles from *in situ* generated nitrile oxides and alkynes, in the presence or absence of Cu(I) as catalyst and ii) the investigation of the effects of the derived compounds on the cellular survival following oxidative challenge and on organismal longevity.

2. Results

We first investigated the cycloaddition reaction between the *in situ* generated 4-methoxy-phenyl nitrile oxide and phenyl acetylene under conventional heating and microwave irradiation in the presence or absence of Cu catalyst. The reaction was performed in a mixture of *tert*-butanol/water (Scheme 1). Specifically, 4-methoxybenzaldehyde was first converted to the corresponding aldoxime via reaction with hydroxylamine. Without isolation, the aldoxime was converted to the corresponding nitrile oxide using chloramine-T trihydrate which acts as both a halogenating agent and a base. The results are shown in Table 1.

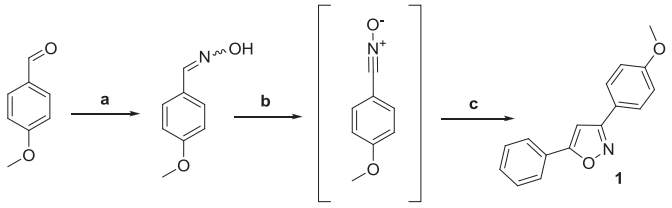
The Cu catalyzed reaction at ambient temperature gave 45% of the desired isoxazole after 24 h (entry 1). The yield was slightly improved when the temperature was increased to 90 °C (entry 2) whereas the use of microwave irradiation significantly improved the yield and shortened the reaction time (entry 7).

Concerning the amount of the catalyst, the use of 0.3 equivalents of $CuSO_4$ and 0.6 equivalents of sodium ascorbate (entry 6) gave higher yields than lower catalyst loading (entry 5).

On the contrary, the use of larger excess of chloramine-T (1.5 equivalents, entry 8) did not affect the yield of the reaction.

The best results of the Cu catalyzed reaction were obtained at 90 °C and 80 W. Lower (60 °C, entry 6) or higher (100 and 110 °C) temperatures (entries 13 and 14) gave lower yields. The yield was further decreased when 120 °C and 100 W were applied (entry 15). Solid additives [29] such as silica gel, Al_2O_3 or NaCl had a detrimental effect on the reaction yield.

Although washing with NH_4OH, ensures quantitative removal of the copper salt during the reaction work-up, Cu-free cycloaddition strategy, not requiring metals and additives, is a promising approach. Thus, we set out to examine the feasibility and the regioselectivity of the microwave-assisted 1,3-dipolar cycloaddition reactions, *tert*-butanol/water, under metal free conditions.



Scheme 1. One-pot isoxazole synthesis. Reagents and conditions: **a**: $NH_2OH{\cdot}HCl$, *t*-BuOH:H_2O (1:1), NaOH 1 N, rt, **b**: $TsN(Cl)Na{\cdot}3H_2O$, *t*-BuOH:H_2O (1:1), rt, **c**: $CuSO_4{\cdot}5H_2O$/Sodium ascorbate, phenyl acetylene, MW irradiation.

Table 1
Formation of isoxazole **1** under conventional heating or microwave irradiation in the presence or absence of Cu catalyst.

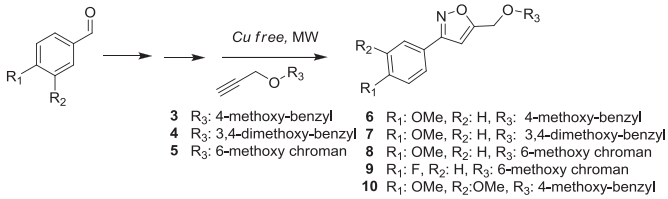
Entry	Chloramine-T	Catalyst	Method	Time	Yield %[b]
1	1.05 eq	*a*[#]	Room temperature	24 h	45
2	1.05 eq	*a*[#]	90 °C	24 h	55
3	1.05 eq	*a*[#]	90 °C	30 min	35
4	1.05 eq	*Cu free*	90 °C	30 min	47
5	1.05 eq	*a*	MW (60 °C, 80 W)	30 min	30
6	1.05 eq	*a*[#]	MW (60 °C, 80 W)	30 min	68
7	**1.05 eq**	***a*[#]**	**MW (90 °C, 80 W)**	**30 min**	**72**
8	1.5 eq	*a*[#]	MW (90 °C, 80 W)	30 min	72
9	1.05 eq	*a*[#]	+Silica gel MW (90 °C, 80 W)	30 min	Traces
10	1.05 eq	*a*[#]	+Al_2O_3 MW (90 °C, 80 W)	30 min	45
11	1.05 eq + Al_2O_3	*a*[#]	MW (90 °C, 80 W)	30 min	45
12	1.05 eq	*a*[#] + NaCl	MW (90 °C, 80 W)	30 min	50
13	1.05 eq	*a*[#]	MW (100 °C, 80 W)	30 min	58
14	1.05 eq	*a*[#]	MW (110 °C, 80 W)	30 min	62
15	1.05 eq	*a*[#]	MW (120 °C, 100 W)	30 min	53
16	1.05 eq	*Cu free*	MW (90 °C, 80 W)	30 min	57
17	**1.05 eq**	***Cu free***	**MW (90 °C, 80 W)**	**45 min**	**68**
18	1.05 eq	*Cu free*	MW (90 °C, 80 W)	60 min	62
19	1.05 eq	*Cu free* + NaCl	MW (90 °C, 80 W)	30 min	26
20	**1.05 eq**	***Cu free***	**MW (90 °C, 100 W)**	**22 min**	**65**
21	1.05 eq	*Cu free*	MW (90 °C, 100 W)	30 min	45

Solvent: *t*-BuOH:H_2O (1:1), (*a*) $CuSO_4{\cdot}5H_2O$/Sodium ascorbate (0.05 eq/0.1 eq), [#](0.3 eq/0.6 eq). [b]isolated yields based on 4-methoxy-benzaldehyde, after column chromatography. Optimal conditions for catalyzed and Cu free reaction are shown in bold.

Microwave irradiation increased the yield of the uncatalysed reaction (entries 4 and 16). The optimum reaction time at 90 °C and 80 W was 45 min (entry 17) giving 68% of isoxazole. Similar yields were obtained using 90 °C, 100 W for 22 min (entry 20).

Using the optimal conditions for the metal free reaction, we synthesized the compounds depicted in Scheme 2 and Table 2. Although reaction time of 22 min and 100 W, did not significantly affect the yield of the reaction in the case of phenyl acetylene (Table 1 entry 20), when aliphatic alkyne was used (Table 2, entries 1, 4) the yield was decreased. The low yield of the entry 6 of Table 2 (compound **10**), is due to the removal of the 4-methoxybenzyl group, under these reaction conditions. After purification by column chromatography, compound **10** and 3-(3,4-dimethoxyphenyl)-5-isoxazolyl-methanol were isolated.

It should be noted that all the yields are based on the aldehydes and not on oximes or imidoyl chlorides. Thus, the low to moderate yields of the Cu-free reactions are overall yields of a three step reaction. As we have previously reported [15], in our experiments the *in situ* generation of hydroximoyl chlorides and their conversion to nitrile oxides was fast, followed by addition of terminal alkynes which are trapping agents to avoid the dimerization of nitrile oxides to furoxans.



Scheme 2. Uncatalysed synthesis of isoxazoles.