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## Graphical Abstract

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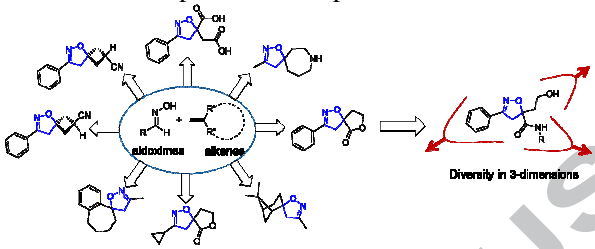

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# Synthesis of functionalized 2-isoxazolines as three-dimensional fragments for fragment-based drug discovery

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ABSTRACT

The design of new sp$^3$ and spiro-enriched fragments has been achieved from 1,3-dipolar cycloaddition between alkenes and chloro-oximes. The selection of reagents was performed to afford a panel of 2-isoxazoline-containing fragments that show desirable three dimensional (3D) characteristics to allow the probing of biologically-relevant chemical space. Principal moments of inertia (PMI) were calculated to evaluate the 3D diversity. The resulting 3D fragments with suitable physicochemical properties, especially a good solubility, will be used to improve the hit rate of our fragment-based screening.



The approval of anti-melanoma drug vemurafenib in 2011 and the development of several drug candidates that originated from fragment-based lead discovery are testaments of the efficacy of the use of fragments in the drug-discovery process. Fragment-based approach relies on the screening of small chemical entities that can easily probe specific biological targets. Identified hits are then rapidly optimized using rational design to drug-like compounds showing a better pharmacological profile.[1]

The design and availability of fragment libraries is therefore considered as a pivotal element in the success of fragment-based screenings. However, current fragment libraries have been limited to planar *sp*$^2$-rich scaffolds which may not always allow generating hits for several challenging targets.[2] Less planar fragments are also better starting point to explore the spatial features of target proteins during hit-to-lead process. Therefore, strategies to increase the proportion of *sp*$^3$-rich fragments are now considered key to improve hit-rate in fragment-based screening.[3]

Many papers have accounted in the last decade for the synthesis and occurrence of 2-isoxazoline in Nature and medicinal chemistry (Figure 1).[4] The most notable compounds are acivicin and (+)-calafianin. Acivicin[5] is a fermentation product of *Streptomyces sviceus* which has shown promising anti-neoplastic activity and is under clinical trials. (+)-calafianin[6], a bromotyrosine-derived spiro-isoxazoline has been isolated from marine sponge *Aplysina gerardogreeni*. Recently, Kaur *et al.* have pointed out many 2-isoxazoline-containing natural products which exhibit potential anticancer activity.[7] Beside its natural occurence, 2-isoxazoline is also an important skeleton found in many synthetic bioactive compounds. Many isoxazoline derivatives have been reported in the literature to exhibit a broad range of biological activities, e.g. antimicrobial[8], anticancer[9], antiplatelet[10], anti-inflammatory[11] activities, and regular effects on central nervous system[12] or sympathetic nervous system.[13]

Prompted by these observations, we focused our work on the design of new three-dimensional fragments based on 2-isoxazoline motif and we report herein their synthesis. The key element of our approach was the optimization of the one-pot 1,3-dipolar cycloaddition starting from aldoximes and chloro-oximes to yield a diverse set of 3D-fragments.

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Several synthetic methods used for the formation of 2-isoxazoline have been described in the literature.[4,14] Among them, 1,3-dipolar cycloaddition has proved to be an excellent and general synthetic route.[15] The retrosynthetic analysis of 2-isoxazoline is depicted in Scheme 1. Our objective was to generate 2-isoxazoline *via* 1,3-dipolar cycloaddition between nitrile oxide (dipole) and 1,1-disubstitued alkene (dipolarophile). Many studies on the regioselectivity of isoxazoline synthesis by intermolecular 1,3-dipolar cycloaddition emphasized that 1,1-disubstituted alkenes react with nitrile oxides to predominantly afford 5-subtituted isoxazolines.[16] Nitrile oxides are reactive intermediates that are usually generated *in situ* from stable precursors. They can be generated from primary nitroalkane *via* dehydration or by a direct oxidation of aldoxime or halogenation of aldoxime to *N*-hydroxyiminoyl halides followed by dehydro-halogenation.[4] The latter is the most usual method in nitrile oxide generation.[17]

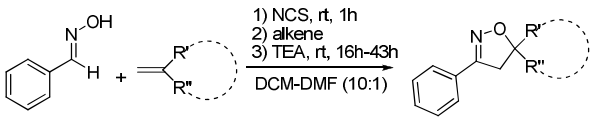
Benzaldoxime **2a** and *N*-Boc-4-methylenepiperidine **3** served as model substrates in the initial experiments (Scheme 2). 2-Isoxazoline was firstly prepared using an aqueous solution sodium hypochlorite (1.6N).[19] Unfortunately, the reaction mixture contained by-products and the targeted isoxazoline **4a-Boc** was obtained in poor yield (32%). To improve yield, we considered other described conditions.[20] Benzaldoxime was firstly chlorinated by *N*-chlorosuccinimide (NCS) in *N*,*N*-dimethylformamide (DMF). The *N*-hydroxyiminoyl chloride **2a'** was isolated with quantitative yield. This intermediate was then converted to nitrile oxide followed by a 1,3-dipolar cycloaddition with *N*-Boc-4-methylenepiperidine **3** in tetrahydrofurane (THF) to yield 47% of the desired isoxazoline **4a**-**Boc**.

However, the main inconvenience of this synthetic pathway is that this process comprises two steps where the intermediate and the final product must be separately purified. This drawback becomes more challenging when a large number of isoxazoline fragments are synthesized. Furthermore, several studies in the literature have reported that some hydroxyiminoyl chloride (chloro-oxime) are unstable and must be used shortly after preparation.[21]

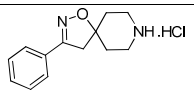
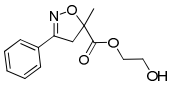
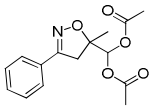
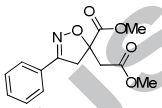
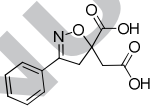
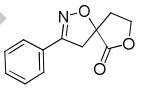
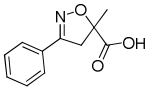
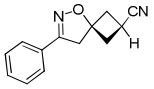
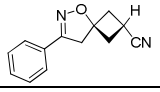
Finally, an improvement of this synthetic method was achieved. The reaction was conducted using a one-pot procedure with a mixture of dichloromethane (DCM) and DMF (10:1) thanks to the adaptation of a published protocol.[22] Benzaldoxime was converted after 1h into hydroxy-iminoyl chloride *in situ* using NCS. Addition of Boc-4-methylidenepiperidine and triethylamine allowed the formation of the targeted spiro-isoxazoline **4a-Boc** in an overall yield of 60%. Interestingly, the tert-butoxycarbonyl (Boc) protecting group was not cleaved under these mild conditions. **4a-Boc** was then deprotected using acidic conditions to yield **4a**.

With the optimized reagents and synthetic conditions in hands (method 3, Scheme 2), the scope of the reaction was explored with benzaldoxime and a large set of gem-disubstituted alkenes in order to have at least one tetra substituted carbon atom, or one spiranic carbon in the structure (Table 1). Itaconic acid dimethyl ester was successfully synthesized according to a published protocol.[23] Non-commercially available alkenes were obtained from the corresponding ketones using Wittig conditions.[18] Original 2-isoxazoline containing fragments **4a-4h'** were obtained in good to high yields.

**Table 1**. 3D-fragments synthesized from benzaldoxime




**2a** **4a - 4h**

| Name | Structure | Yield[a] (%) | MW (Da) | Fsp$^3$ | Sol. (mM)[d] |
|---|---|---|---|---|---|
| **4a** |  | 44[b] | 216.3[c] | 0.46 | >2 |
| **4b** |  | 93 | 249.3 | 0.38 | 1.0 |
| **4c** |  | 35 | 291.3 | 0.40 | 1.0 |
| **4d** |  | 88 | 277.3 | 0.36 | 1.5 |
| **4e** |  | 60 | 249.2 | 0.25 | >2 |
| **4f** |  | 94 | 217.2 | 0.33 | - |
| **4g** |  | 56 | 205.2 | 0.27 | 1.8 |
| **4h** |  | 16 | 212.3 | 0.38 | 0.4 |
| **4h'** |  | 44 | 212.3 | 0.38 | 0.3 |

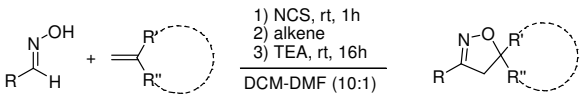
[a] Isolated yield after purification
[b] Yield obtained after 3 steps including Boc deprotection.
[c] Molecular weight calculated as free base
[d] Measured in PBS at pH 7.4

As expected, 1,3-dipolar cycloaddition is controlled by the reactivity of alkenes. When alkenes are substituted with electron withdrawing groups (**4b**, **4d**, **4e**, **4f** and **4g**), the reaction was completed after 16 hours and isoxazolines were obtained in moderate to good yield (56 to 94%). Interestingly, the presence of carboxylic function (compounds **4e or 4g**) did not affect the reactivity. On the contrary, a low reactivity was observed with alkene **4c**. In the case of 3-methylenecyclobutane carbonitrile used as starting alkene, reaction yielded two diastereomers **4h** and **4h'**. After separation by flash-chromatography, the allocation of their structures was achieved using 1D-NOESY.

F$sp^3$ value is defined as the ratio of $sp^3$-hybridized carbon atoms on the total carbon count. This value was calculated for fragments **4a-4h'**.[24] The result showed that F$sp^3$ range from 0.25 to 0.46 (Table 1). In an effort to generate more $sp^3$-rich fragments, benzaldoxime was then replaced by commercially available acetaldoxime or synthesized cyclopropylcarbaldoxime.[25] This led to the synthesis of nine spiroisoxazoline-containing fragments (Table 2) with higher F$sp^3$, ranging from 0.42 to 0.92. Fragments **5e** and **5g** were obtained in good yields thanks to the high reactivity of the

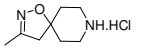
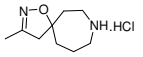
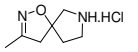
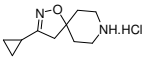
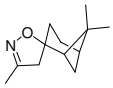
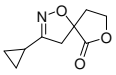
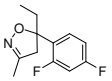
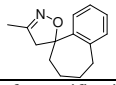
starting alkene, where electron density is lowered by the lactone. The seven remaining fragments were synthesized with low to moderate yields. Boc derivatives (**5a-Boc to 5d-Boc**) were then deprotected using acidic conditions to afford the corresponding hydrochloride salts.[26]

**Table 2**. 3D-fragments synthesized from acetaldoxime or cyclopropylcarbaldoxime




**2b**: R = Me
**2c**: R = cyclopropyl

**5a - 5d (Boc)**
**5e - 5i**

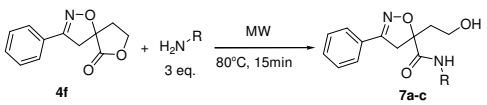
| Name | Structure | Yield[a] (%) | MW (Da) | Fsp$^3$ | Sol. (mM)[d] |
|---|---|---|---|---|---|
| **5a** |  | 30[b] | 154.2[c] | 0.88 | >2 |
| **5b** |  | 31[b] | 168.2[c] | 0.89 | >2 |
| **5c** |  | 50[b] | 140.2[c] | 0.86 | >2 |
| **5d** |  | 47[b] | 180.3[c] | 0.90 | >2 |
| **5e** |  | 72 | 155.1 | 0.71 | - |
| **5f** |  | 10 | 193.3 | 0.92 | 1.9 |
| **5g** |  | 87 | 181.2 | 0.78 | - |
| **5h** |  | 57 | 225.2 | 0.42 | 0.8 |
| **5i** |  | 32 | 215.3 | 0.50 | 1.6 |

[a]Isolated yield after purification
[b]Yield obtained after 3 steps including Boc deprotection.
[c]Molecular weight calculated as free base
[d]Measured in PBS at pH 7.4

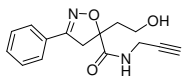
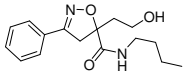
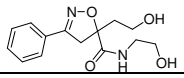
In some of the 1,3-dipolar cycloadditions, we observed the formation of a by-product. This side-product was supposed to arise from the dimerization of the corresponding nitrile oxide in the absence of a reactive dipolarophile as trapping agent to yield 1,2,5-oxadiazole-2-oxide derivative.[27] To confirm this hypothesis, we conducted reactions with benzaldoxime and acetaldoxime in the absence of alkene. We only observed the formation of furoxan **6a** which is consistent with the literature (Scheme 3).[17]

In an effort to exemplify how diverse structures can be rapidly obtained from **4f**, we performed the opening of the lactone with amines to yield isoxazolines **7a-7c** (Table 3). This reaction was carried out using 3 equivalents of the corresponding amine. The mixtures were heated at 80°C under microwave irradiations to afford the targeted compounds in good yields. This method could also be applied to intermediates **5e** and **5g** to extend the scope of the reaction and increase diversity.

**Table 3**. Synthesis of isoxazolines **7a-7c** starting from lactone **4f**




**4f** **7a-c**

| Name | Structure | Yield[a] (%) | MW (Da) | Fsp$^3$ | Sol. (mM)[b] |
|---|---|---|---|---|---|
| **7a** |  | 82 | 272.3 | 0.33 | >2 |
| **7b** |  | 78 | 276.3 | 0.47 | >2 |
| **7c** |  | 65 | 278.3 | 0.43 | 1.9 |

[a]Isolated yield after purification
[b]Measured in PBS at pH 7.4

Finally, we analyzed the overall shape coverage of our entire set of fragments by monitoring normalized PMI ratios (npr1/npr2), according to Sauer's method.[28] The PMI plot is shown in Figure 2. The data obtained for our 3D-fragments were compared to a set of 471 members of our in house library of commercially available fragments, whose molecular weight was equivalent to the synthesized compounds.

The graph shows that our set of substituted isoxazoline fragments (in pink) cover a large space of shapes (Figure 2) with a greater proportion of rod-like or sphere-like shapes than commercially available fragments (in cyan). The calculation of the mean of NPR1+NPR2 with both sets: 1.19 ± 0.08 for 2-isoxazoline containing fragments and 1.09 ± 0.07 for the 471 commercially available fragments confirms this tendency. Interestingly, two fragments **5h** and **5i** which were designed from aromatic alkenes (with medium Fsp$^3$) showed also sphere-like and rod-like properties. These results are conformed to a recent study in which the authors showed that a positive-correlation between Fsp$^3$ and 3-dimentionality of molecule are not always observed.[29]

Finally, a critical property of fragments for screening is their solubility in aqueous media. To validate the impact of the three-dimensionality on solubility, we measured this parameter in an aqueous phosphate buffered saline solution (PBS) at pH =7.4 for a set of 18 fragments. Interestingly, 15 fragments (83%) show solubility higher than 1 mM, including aliphatic or aromatic fragments **5f** and **5i**.In summary, we optimized a reliable protocol based on 1,3-dipolar cycloaddition for the synthesis of a diverse set of fragments containing 2-isoxazoline motif. Among the 21 compounds described, 16 (**4b**, **4c**, **4h**, **4h'**, **5a-i**, **7a-c**) are original. Lactone containing fragment **4f** was shown to be further functionalized with amines under microwave irradiation. Using chemoinformatic analysis, we quantified the shapes of the novel synthesized fragments. Finally, the majority of these fragments were proved to be soluble in PBS at a concentration higher than 1 mM. The resulting compounds will be screened for their affinity to therapeutically relevant proteins using Thermal Shift Assay and the results will be reported in due course.

## Acknowledgments

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## Supplementary data

Supplementary data (experimental procedures, analytical and spectral data of all synthesized compounds) associated with this article can be found in the online version.

## References and notes


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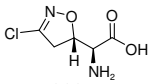
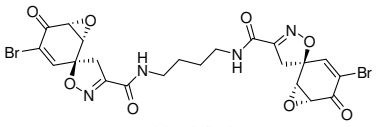
**Captions**

Figure 1. Structure of natural products containing 2-isoxazoline motif.
Figure 2. Molecular shape analysis of set isoxazoline containing 3D-fragments
Scheme 1. Retrosynthetic analysis of 2-isoxazoline
Scheme 2. Synthetic conditions used for the optimization of the synthesis of compound **4a-Boc**
Scheme 3. Formation of the by-product furoxan **6a**.

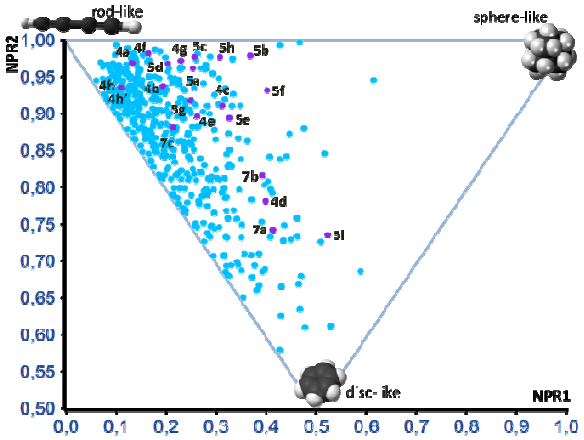
2-isoxazoline

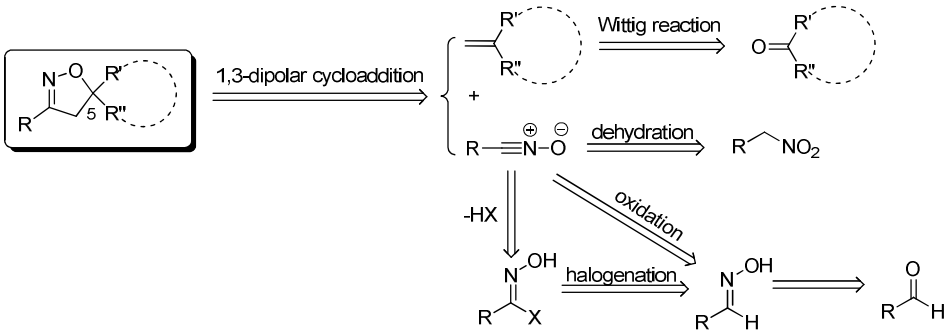


acivicin

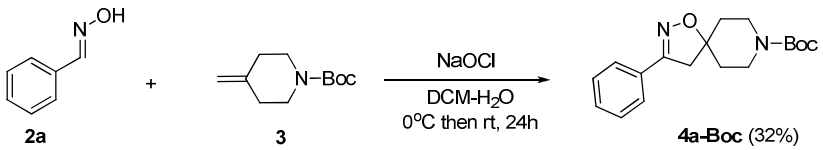


(+)-calafianin



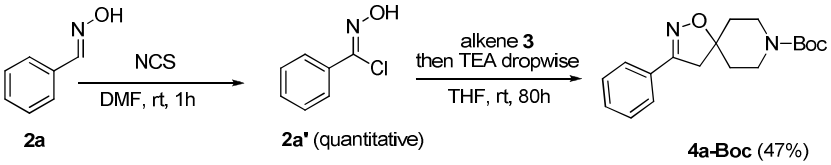


**Method 1:**



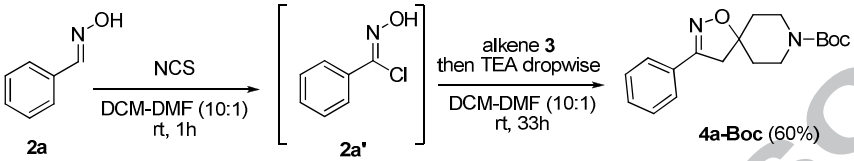

**2a** **3** **4a-Boc** (32%)

**Method 2:**

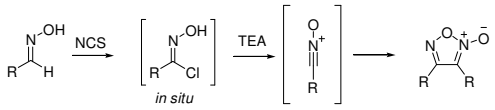



**2a** **2a'** (quantitative) **4a-Boc** (47%)

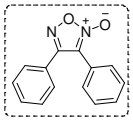
**Method 3:**




**2a** **2a'** (formed *in situ*) **4a-Boc** (60%)




furoxan **6a**



**6b** (not formed)