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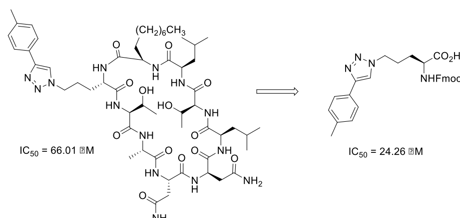

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Identification of a new p53/MDM2 inhibitor motif inspired by studies of chlorofusin.

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ABSTRACT

Previous studies on the natural product chlorofusin have shown that the full peptide and azaphilone structure are required for inhibition of the interaction between MDM2 and p53. In the current work, we utilized the cyclic peptide as a template and introduced an azidonorvaline amino acid in place of the ornithine/azaphilone of the natural product and carried out click chemistry with the resulting peptide. From this small library the first ever non-azaphilone containing chlorofusin analogue with MDM2/p53 activity was identified. Further studies then suggested that the simple structure of the Fmoc-norvaline amino acid that had undergone a click reaction was also able to inhibit MDM2/p53 interaction. This is an example where studies of a natural product have led to the serendipitous identification of a new small molecule inhibitor of a protein-protein interaction.

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Chlorofusin is a natural product that was first identified in a screen for inhibitors of the MDM2/p53 interaction.[1, 2] It is a cyclic peptide in which one of the amino acids, an ornithine, is condensed with a densely functionalized azaphilone. Interest in both its biological activity, as an inhibitor of a protein-protein interaction, and its structure initiated a series of studies of the natural compound. The cyclic peptide was synthesized by both solid[3] and solution phase[4-6] methods, its stereochemistry and its lack of inherent biological activity were established. The total synthesis initially proved elusive as the first approach gave an erroneous structure to the azaphilone portion,[7] although this was closely followed by the first total synthesis from the Boger laboratory.[8,9] Interestingly, this approach allowed access to all the diastereomers of the azaphilone structure and showed that the *presence* of the azaphilone was required for MDM2/p53 inhibition but the overall stereochemistry of the molecule had little effect.[10] This has recently been confirmed through a further total synthesis.[11] We have also shown that the introduction of substituents on the ornithine in order to generate simpler analogues does not lead to active MDM2/p53 inhibitors.[12] Yao and co-workers have recently described a synthesis of chlorofusin analogues that incorporate a triazole moiety in the linker between the peptide and the azaphilone and these are the first chlorofusin analogues to show inhibitory activity in the MDM2/p53 assay.[13] This has prompted us to disclose our own studies of a click chemistry-based approach to chlorofusin analogues that gave us the first simple non-azaphilone peptide analogues of chlorofusin with anti-MDM2 activity. However, on further probing, we found that the simple "clicked" amino acid that we incorporated into the chlorofusin peptide also had activity and was a relatively potent inhibitor of MDM2/p53 interactions. In order to facilitate these studies, we have also developed a fluorescence polarization based assay for screening purposes that is simpler than the previously described[12] ELISA based assay.

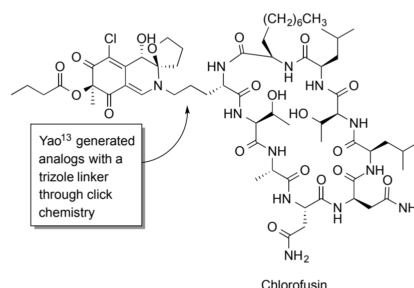
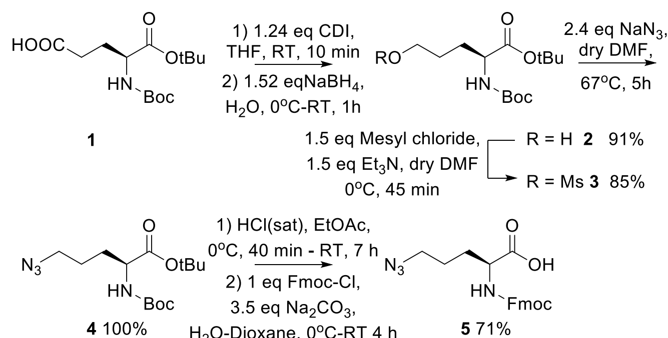


Figure 1. Chlorofusin.

Incorporation of an azide moiety into our solid phase protocol would give us a compound that could undergo click chemistry[14] either while still on the resin or following cleavage. For this, we required an Fmoc protected azide-containing amino acid. We utilized a route from Boc-glutamic acid α-t-butyl ester **1** to generate an azide analogue of ornithine in a modest yield, but through a scalable process (Scheme 1). Boc-glutamic acid α-t-

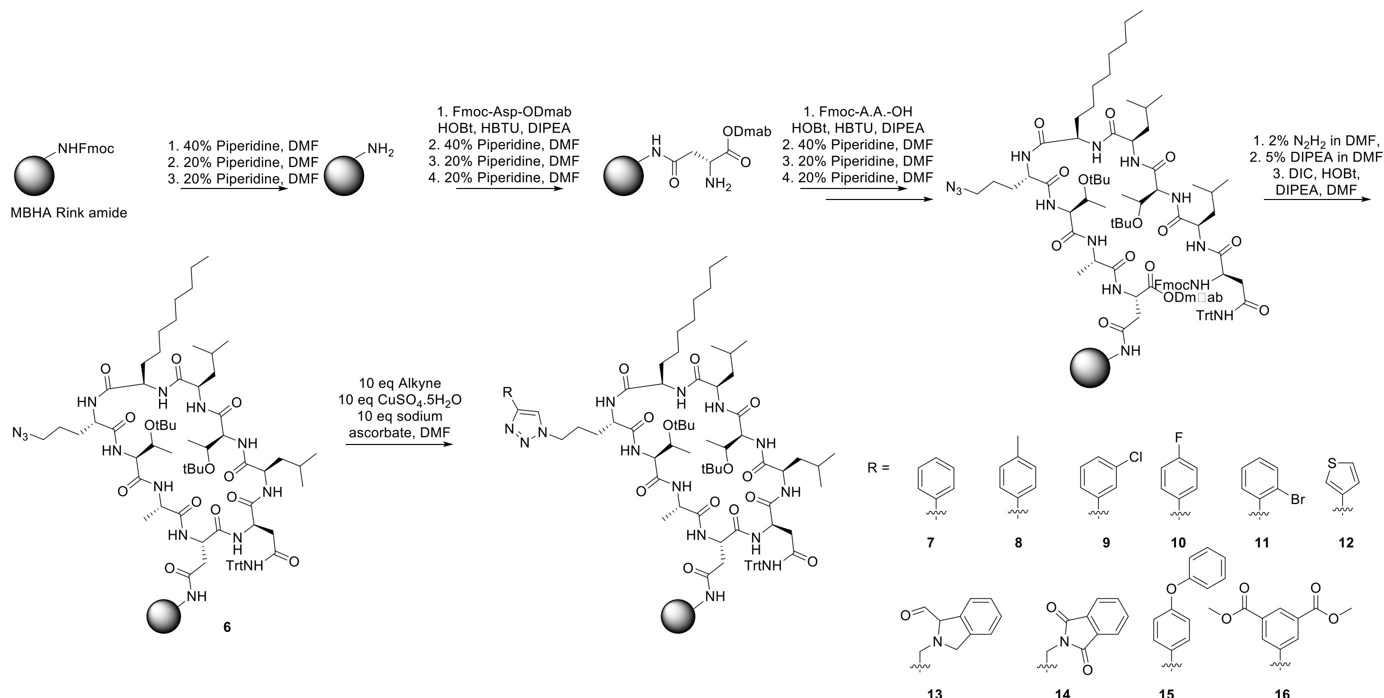
butyl ester **1** was treated with carbonyldiimidazole and then reduced to alcohol **2** with sodium borohydride in a one pot, two step procedure, producing the expected compound in excellent yield. Mesylation and subsequent treatment of the crude with sodium azide[15] generated the required side chain on the amino acid (**4**). The product does not require purification and was treated with a saturated solution of hydrochloric acid in ethyl acetate at room temperature to remove both the Boc and tBu protecting groups. Evaporation of the solvent and reaction of the residue with Fmoc-Cl in the presence of sodium carbonate gave the target amino acid **5**.



Scheme 1. Synthesis of the azide analogue of ornithine.

Incorporation of **5** in to the solid phase synthesis[3] proceeded smoothly (Scheme 2) and the azide was formed on resin, as demonstrated by cleavage of a small sample and analysis by mass spectrometry. A convenient route to derivatising this compound would be to carry it out on resin, provided the triazole-forming reaction proceeded under these conditions. We had previously found that acylation of the ornithine on resin was a convenient approach to generate analogues.[12] To our satisfaction, treatment of the resin bound peptide (**6**) with a selection of alkynes with different functional groups led to the successful generation of click analogues. These could be removed from the resin using a standard cleavage cocktail (95% TFA, 2.5 % H_2O, 2.5% TIPS) and purified by preparative HPLC.

The compounds were screened by a convenient fluorescence polarization (FP) assay, adapted from several that have been previously published.[16] The high affinity peptide LTFEHYWAQLTS was synthesised on Rink amide resin to generate the amide at the C-terminus and was tagged with FAM at the N-terminus. Human MDM2 protein (17-125) was used in the polarization assay and the wildtype p53 peptide (residues 15-27) was used as a positive control and had an IC_{50} of 14.45 μM and K_i of 9.19 μM. Screening the click chlorofusin analogues **7-16** at 100 μM concentration revealed that only the 4-methylphenyl (**8**) inhibited the MDM2/peptide interaction. A full inhibition curve for compound **8** revealed the methylphenyl compound had an IC_{50} of 66.01 μM and K_i of 8.309 μM. This is the first time that simple analogues of chlorofusin have been shown to have activity against MDM2 and compound **8** has a similar affinity to the wildtype p53 peptide.

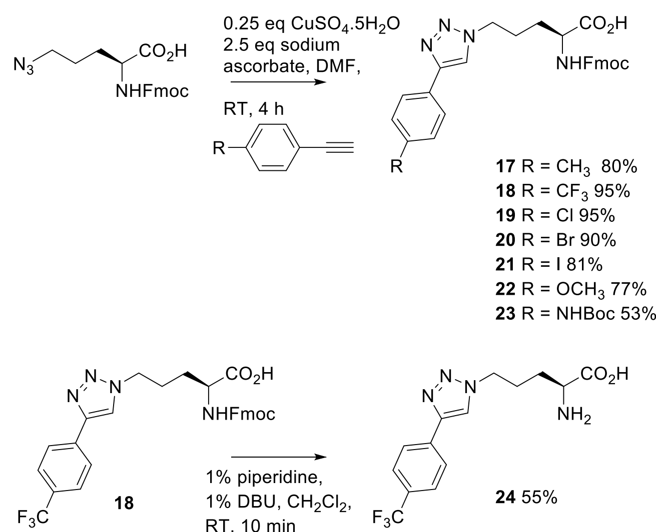


Scheme 2. Solid phase synthesis of analogues of chlorofusin peptide.

It seemed reasonable to explore the binding of the ornithine amino acid analogue to confirm that the peptide structure of chlorofusin was contributing to the binding of the click chemistry product. The 4-(4-methylphenyl)triazole structure is significantly different from the azaphilone of the natural product and it was a possibility that the side chain itself was responsible for the biological activity. With this in mind, the ornithine azide with Fmoc-protection **5** was treated with 4-ethynyltoluene and, as a further novel analogue, 4-trifluoromethylphenylethylene under the conditions of the click chemistry reaction (Scheme 3). The yields were excellent and the products could be isolated without chromatographic purification. Nonetheless, to ensure ideal purity, they were additionally subjected to preparative HPLC. When these compounds were tested in the FP assay, we were surprised to discover that they also inhibited the MDM2/p53 interaction, with the 4-methylphenyl compound having improved activity in comparison with the full peptide structure (Table 1).

The presence of halides is often seen as preferential for MDM2 binding ligands and so we also generated a number of analogues containing the Cl, Br and I phenyl systems. In order to probe the potential size of the binding site, we made a methoxy analogue as the methylphenyl- compound was more active than the trifluoromethylphenyl. When screened in the FP assay, all of

these compounds except for the chlorophenyl- compound were inhibitors of the p53 peptide/MDM2 interaction. The most potent compound with the highest binding affinity was the iodo-derivative although the binding affinity of the bromo compound was very similar. Binding of the 4-methoxy compound was also enhanced compared with the methyl, suggesting that the larger group is filling a binding pocket on the MDM2 protein more efficiently than the smaller CH_3. All of these compounds compare well with the p53 peptide, with higher affinity. When the well-known anti-MDM2 compound nutlin 3a was compared in the same assay, its IC50 value was about 5 fold lower than that of the iodo compound, suggesting that it is maintaining a slightly higher potency than these new compounds in this assay.



Scheme 3. Synthesis of compounds **19-26**

Compounds	IC_{50} (μM)[a]	K_i (μM)[b]
17	24.26	3.05
18	49.80	6.96
20	5.51	0.69
21	2.90	0.36
22	5.66	0.71
23	57.36	7.22
Nutlin 3A	0.61	

[a]Concentration of substrate required to decrease polarization fluorescence by 50%.[b]Apparent inhibition constant.

Removal of the Fmoc group from the compound **18** to generate **24** leads to a complete loss of activity, suggesting that a hydrophobic group is required in this position of the molecule. Screening of the t-Bu ester version of the iodo compound, which we suspected would lead to cellular uptake of these compounds, also led to loss of activity for binding.

A preliminary screen in a proliferation assay was also undertaken. Compounds **19-23** showed no activity against either SJSA-1 (osteosarcoma, amplification of MDM2) or A375 cell lines (melanoma, wild type p53, normal MDM2). However, both compounds **17** and **18** showed some activity, with **17** being active in the SJSA-1 cell line (33.1 μM) and inactive in A375 whereas **18** was active in both cell lines (31.2 μM, 49.3 μM). Cell-based assays such as these are not direct indicators of whether the compounds are acting through the inhibition of MDM2/p53 and the fact that **18** is active in both cell lines is indicative of a different mechanism or mechanisms of action. Cell based assays that offer a more definitive demonstration of intracellular activity have recently been described[17] and we will explore the utility of these approaches.

In conclusion, we have developed new analogues of chlorofusin via click chemistry that have the ability to prevent the binding of the p53 peptide to the MDM2 protein. Chlorofusin could be considered to be the product of a natural "fragment-based" approach to small molecule development – with the condensation of an azaphilone and a cyclic peptide leading to an active molecule. In a similar vein, we have used the cyclic peptide as a template to generate new molecules to bind to MDM2. Further investigation of those molecules has led to the identification of a new small molecule motif, based around the click product of an Fmoc-protected ornithine, which can also inhibit the p53/MDM2 interaction. It would be interesting to see if the azaphilone click analogues recently described[12] also have activity as the Fmoc-amino acid structure. Studies are continuing to identify the binding site and the structure-activity relationship for this new class of compound.

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Supplementary Material

Supplementary material describing the experimental details is available.