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Decorated macrocycles via ring closing double reductive amination. Identification of an apoptosis inducer of leukemic cells, which at least partially antagonises a 5-HT2 receptor.

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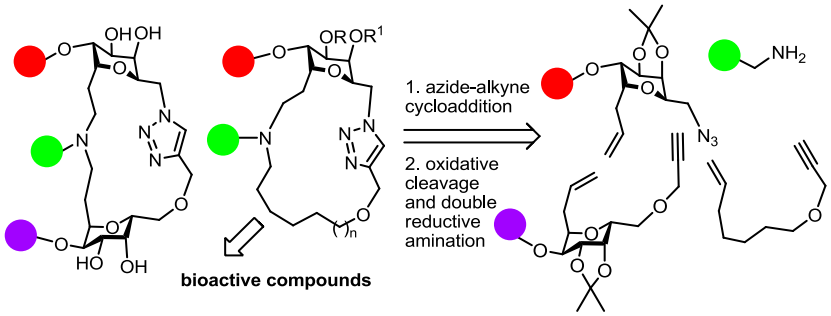
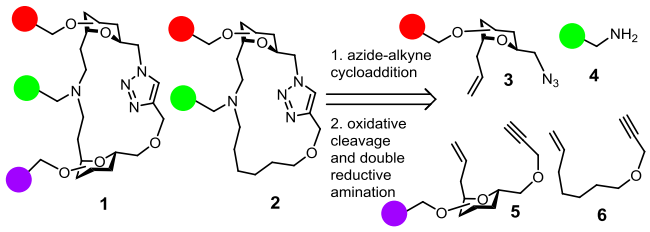
Supporting Information Placeholder



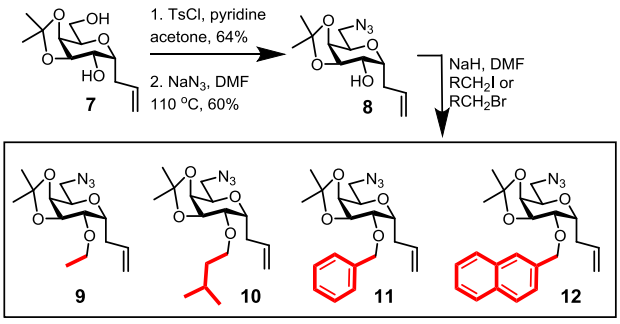
ABSTRACT: A build-couple-pair strategy, including double reductive amination macrocyclisation, has been used to generate decorated macrocycles (Eannaphanes) with an embedded triazole and monosaccharide. Biological screening led to the identification of an inducer of apoptosis in leukemic cells, which acts at least partially as a 5-HT2 antagonist.

Challenges encountered in the discovery of small molecule drugs[1] may be related to a relatively low number of scaffolds or frameworks investigated in medicinal chemistry programmes.[2] In this regard, the development of new macrocyclic scaffolds has been of interest[3,4] due to a view that there is a lack of macrocyclic scaffolds in screening collections.[5,6] Motivated to address this, we approached the convergent assembly of a macrocycle, which could be concisely assembled, and facilitate decoration of the scaffold with pharmacophoric groups. The Eannaphane scaffold reported herein contains an amine as well as triazole and saccharide groups, embedded into the macrocycle (**1-2**; Scheme 1).[7] The synthetic approach was based on assembling building blocks **3-6**. It was envisaged that those with the azide and alkyne groups would be first combined using the copper catalysed azide-alkyne cycloaddition to generate a 1,4-triazole and that this would be followed by oxidative cleavage of the alkene groups and subsequent ring closing double reductive amination reactions to give rise to the macrocycle (Scheme 1). Various pharmacophores could be incorporated by placing such groups strategically on **3-6**.

The synthesis of the bifunctional saccharide derived building blocks were first worked out. The allyl galactose derivative **7** was selective tosylated at the 6-OH group and subsequent reaction with sodium azide in DMF led to the azide **8.** Alkylation of the galactose 2-OH group with a variety of alkyl halides gave **9-12** (Scheme 2). In order to obtain **16** (Scheme 3) that has both alkyne and alkene groups, a TBS group was introduced at the primary alcohol group of **7** to give **14**. Next, alkylation, to introduce a pharmacophoric group at the 2-oxygen atom, followed by desilylation to give **15** and subsequent propargylation gave **16**. The simpler substrates **17/18**, were prepared from the alcohol precursor.

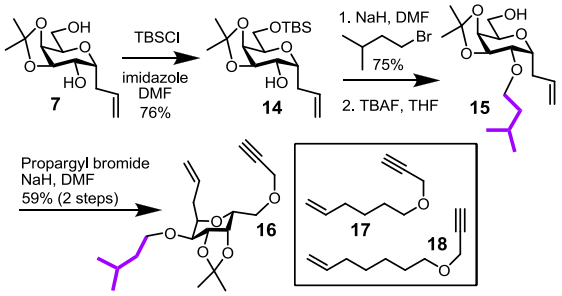


Scheme 1. Retrosynthetic analysis

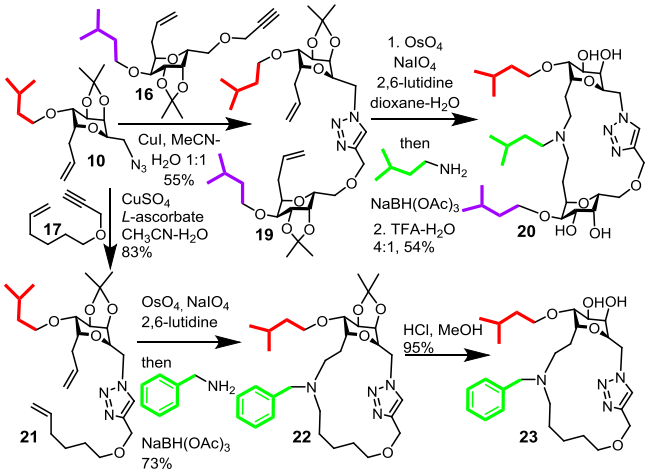


Scheme 2. Synthesis of **9-12**

Preparation of the macrocycles is exemplified in Scheme 4 with the synthesis of **20** and **23** shown. Hence the Cu(I) catalysed azide-alkyne cycloaddition[8] of **10** with **16** gave the 1,4-triazole **19**. Oxidative cleavage of the two alkenes of **19** gave a dialdehyde, which provided the macrocycle after double reductive amination cyclisation. This macrocycle with the acetonide protecting groups present can be isolated or the acetonides can be removed to give **20**, with three isopentyl groups. The reaction of **10** with **17** gave **21** and oxidative cleavage – double reductive amination ring closing reaction, this time using benzyl amine, gave **22**. Acetonide removal from **22** gave **23**.



Scheme 3. Synthesis of **16** and structures of **17-18**

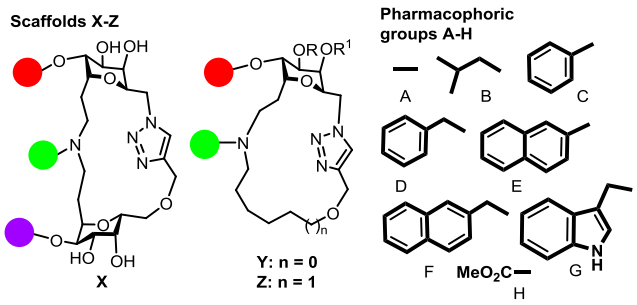


Scheme 4. Synthesis of macrocycles

By varying the structures of the building blocks it was possible to generate analogues or a series of structures for screening. Compounds **24-40** (Table 1) are among those to have been prepared by the strategy shown in Scheme 4. The macrocycle is decorated with pharmacophores, such as isopentyl, benzyl, indolylethyl, carboxyl, ethyl and napthylethyl groups. The strategy is relevant to peptidomimetic synthesis given that a number of these pharmacophores correspond to side chains of natural amino acids.

Once in hand, a preliminary assessment of selected biological properties of these compounds was carried out. Normal cells (primary human fibroblasts) and cancerous cells (leukemic cells) were treated with the compounds and while normal fibroblasts (non-cancerous cells) were not affected, the viability of Oci-AML2 leukemic cells (cancerous cells) sharply reduced upon exposure to 10 μM or higher concentrations of some compounds. The macrocycles with two saccharides (**20**, **24-33** and their protected derivatives) were generally found to be insoluble in assay conditions above 1 μM. However macrocycles with one embedded saccharide such as **22**, **23** and **34-38** and their acetonide derivatives had improved solubility and the biological assays were focused on these agents. The more potent were macrocycles decorated with both the isopentyl and naphthylethyl groups such as **38** and **40** and to a lesser extent **35**. The effects of **40** on both fibroblasts and Oci-AML2 cells are shown in Figure 1(A). In order to confirm the potential cytotoxic effect of **40** on leukemic cells, a panel of leukemic cell types were treated with a dosage of **40** and it reduced the viability of all four leukemic cell types by 80-90% compared to the untreated control sample (Figure 1B).

Table 1. Examples of Macrocycles Synthesized



compound	scaffold	●	●	●
24	X	A	B	B
25	X	A	C	B
26	X	B	C	B
27	X	B	F	B
28	X	C	B	B
29	X	C	D	B
30	X	C	F	B
31	X	C	G	B
32	X	E	B	B
33	X	E	C	B
34 (R, R^1 = H, H)	Y	B	B	-
35 (R, R^1 = H, H)	Y	B	F	-
36 (R, R^1 = H, H)	Z	B	B	-
37 (R, R^1 = H, H)	Z	B	G	-
38 (R, R^1 = H, H)	Z	B	F	-
39 (R, R^1 = H, H)	Z	E	H	-
40 (R, R = CMe_2)	Z	B	F	-

This observed reduction in viability could have been due to induction of necrotic cell death or apoptotic cell death or inhibition of cell growth. To identify the mechanism by which **40** reduced viability, Oci-AML2 cells were treated with the compound and cell morphology as well as exposure of the membrane lipid phosphatidyl serine to the outer leaflet of the plasma membrane was monitored using the Annexin V binding assay (Figure 2). Compound **40** induced phosphatidyl serine exposure, cell shrinkage and nuclear condensation, all typical events in cells dying through the natural (apoptotic)

cell death programme showing thar the cause of reduced viability was induction of apoptotic cell death (Figure 2).[9]

Compound **40** was next investigated for its inhibition of ligand binding to 55 different receptors[10] and the ability of **40** to displace an agonistic or antagonistic radiolabelled-ligand from each receptor was measured. Several of the hits for **40** in this screening were serotonin receptors (5-HT1A, 5-HT2A, 5-HT2B) and the serotonin transporter and the Ki values are shown in Table 2.[11] Serotonin has previously been associated with induction of apoptosis in leukemic cells.[12,13] Primary leukemic cells may express serotonin receptors and the serotonin transporter and serotonin is a survival signal for them.

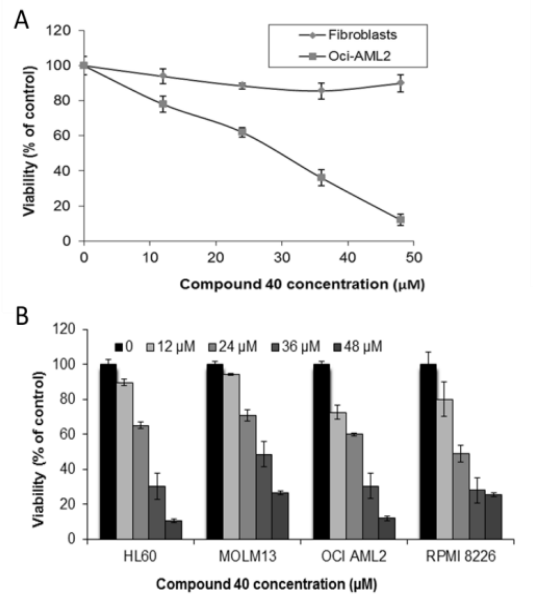
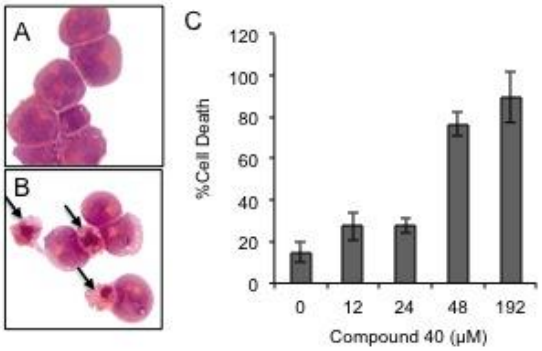


Figure 1. (A): Structure of **40** and its effect on normal, non-transformed human fibroblasts and Oci-AML2 leukemic cells. Cells were treated with the indicated doses of **40** for 24 h after which the viability of the cells was determined with MTT. **(B):** Compound **40** kills leukemic cells. Four leukemic cell lines (HL-60, Molm13, Oci-AML2, RPMI8226) were treated with 12, 24, 36 and 48 μM of **40** for 24 h after which cell viability was measured with MTT. All graphs show average percentage viability compared to the untreated control sample +/- Stdev from 3 independent repeat

Table 2. Binding of 40 to HT Receptors

HT receptor	K_i of **40**	Reference compd, K_i
5-HT1A (h)	0.31 μM	8-OH-DPAT, 0.44 nM
5-HT2A (h)	0.97 μM	Ketanserin, 0.44 nM
5-HT2B (h)	0.87 μM	Mesulergine, 2.9 nM
5-HT transporter (h)	3.4 μM	Imipramine, 0.99 nM

The possibility that the interaction of **40** with HT receptors is responsible for induction of apoptosis in leukemic cells was further explored. Oci-AML2 cells were treated with a series of known serotonin receptor agonists and antagonists for 24 h after which cell death was quantified by measuring the percentage of cells with depolarised mitochondria using TMRE staining and flow cytometry (Figure 3). Inhibition of 5-HT2 receptors with antagonists (ritanserine, imipramine) or a dual 5-HT1/5-HT2 antagonist (cyproheptadine) led to induction of cell death in Oci-AML2 leukemic cells. On the contrary, activation of neither 5-HT1 with 8-hydroxy-2-(di-N-propylamino)tetralin (8-OH-DPAT) nor 5-HT2 with 2,5-dimethoxy-4-iodoamphetamine (DOI) killed Oci-AML2 cells (Figure 3). To investigate whether **40** kills leukemic cells by inhibiting 5-HT2, we tested whether the cytotoxic action of **40** could be reversed by blocking its action on 5-HT2 using the 5-HT2 agonist, DOI. Oci-AML2 cells were pre-treated with DOI



for 2 h to allow occupation of the receptors then **40** was added for 22 h and induction of cell death measured (Figure 4). DOI reversed the cytotoxic effect of **40**, showing that **40** induced cell death at least partly by inhibiting either the 5-HT2A and/or 5-HT2B receptor.

Figure 2. Compound **40** induced apoptotic cell death in OCI AML2 leukemic cells. **(A)** and **(B):** OCI-AML2 cells were treated with 24 μM of **40** for 15 h after which the cells were collected on a microscope slide and with haematoxylin (nucleus) and eosin (cytosol). (A) control, untreated cells with homogeneously stained nuclei and a ring of pink-stained cytosol typical of live leukemic cells and (B) cells treated with **40** where some cells show shrunken and condensed (darkly stained) nuclei typical of apoptotic cells. **(C):** OCI AML2 cells were treated with the indicated doses of **40** for 24 h. Induction of apoptotic cell death was measured by detecting exposure of phosphatidyl serine in the outer layer of the plasma membrane using Annexin V staining.

In order to investigate the binding of **40**, molecular docking was performed, using MOE.[14] The coordinates for 5-HT2B were available in the RSCB-PDB (4NC3, resolution 2.80Å) and includes a co-crystal with ergotamine (4IB4).[15] Docking of ergotamine was first investigated and the binding pose obtained had almost same binding conformation and interaction as in the co-crystal structure, with a slight deviation of 0.477 Å (RMSD). Clustering of binding poses of the reference compound mesulergine (RMSD 0.736 Å) and **40** (RMSD 0.952 Å), showed that **40** may be able to display similar binding as the 5-HT2 antagonist (Fig. 3). For example, there was a π-π stacking interaction of the naphthalene group of **40** (3.80 Å & 3.95 Å) and the benzopyrrole residue of mesulergine (3.43 Å & 3.48 Å) with Phe340. Overall, the comparison of **40** with mesulergine indicates that **40** would utilize a wider volume of the cavity that potentially could be used to facilitate selective inhibitor design.

To conclude, we describe an efficient synthesis of a new chiral macrocyclic scaffold, adding to number of available macrocyclic frameworks. The synthesis route enabled the generation of analogues and decoration of the macrocycle in a concise manner. The ring closing double reductive amination macrocyclisation has been rarely used in macrocycle formation[16] and the results shown herein indicate it is worthy of

consideration more generally.[17] The utilization of the amine in the final ring-closing step provides an efficient way to expand the number of macrocyclic compounds for screening, given the wide number of available amines. The strategy used herein could potentially contribute to preparation of more macrocyclic scaffolds[18] and follows the build-couple-pair strategy[19] used in diversity-oriented synthesis. Evaluation of a preliminary set of molecules based on the macrocycle has led to identification of a compound selectively toxic to tumour cells and evidence is provided that the apoptosis induced is at least partially due to binding to either the 5-HT2A and/or 5-HT2B receptor. The pharmacology of 5-HT1/2 receptors is complex. The receptors may have ligand-independent activity and the functional efficacy of ligands depends not only on their binding affinity, but the nature of their interaction. The high concentration of 5-HT2-antagonists required to induce cell death indicate that basal, ligand-independent receptor activity is important for cell survival and blockage of this activity may reflect inverse agonistic action of the tested compounds, and not pure antagonism.[20] Full pharmacological investigation is necessary to confirm this. The macrocyclic scaffold and synthetic approach is relevant for medicinal chemistry or chemical biology, leading to bioactive compounds. Investigations are underway to generate more macrocyclic scaffolds by this and related approaches.

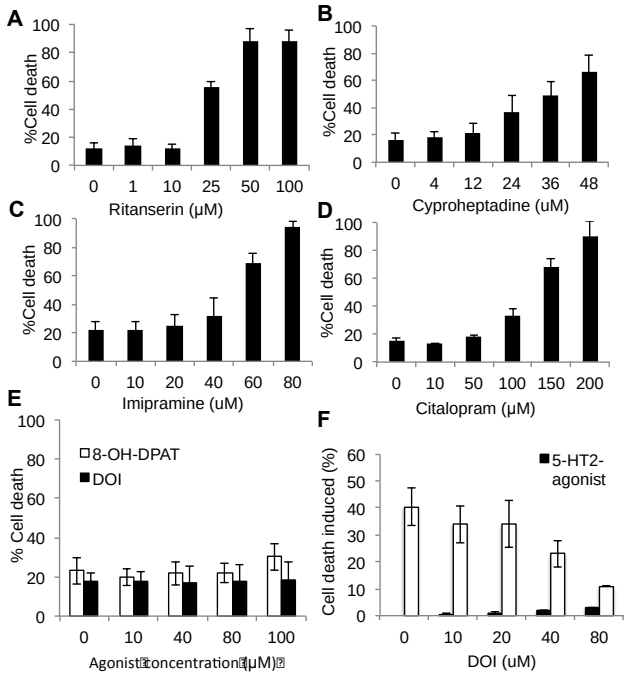


Figure 3. Effect of HT receptor agonists and antagonists on leukemic cell survival. (**A-D**): Ritanserin, cyproheptadine and imipramine are 5-HT2 receptor antagonists, while citalopram is a serotonin reuptake receptor inhibitor. Cells were treated with a dosage of each compound for 24 h after which the percentage of dead cells was determined with TMRE staining. **(E)** Activation of 5-HT receptors do not lead to cell death. Oci-AML2 cells were treated with the 5-HT1 and 5-HT2 agonists, 8-OH-DPAT and DOI, for 24 h and the cell death induced was determined (MTT assay). **(F)** Oci-AML2 cells were pre-treated with DOI for 2 h followed by 24 μM of **40** for a further 22 h after which cell death induced by the compounds was quantified using TMRE dye. All graphs show percentage cell death of 3 independent repeats +/- Stdev.

Supporting Information

NMR spectra, experimental procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

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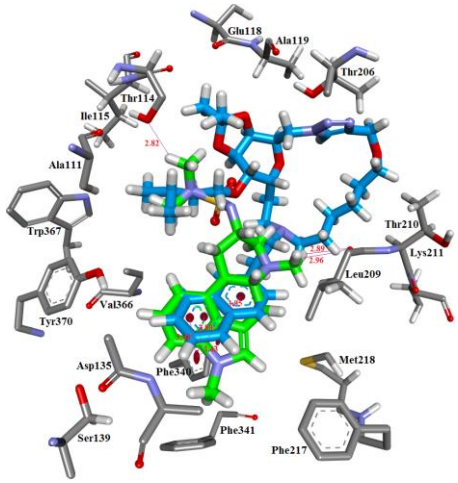

Figure 4. Docked mesulergine (green) and **40** (blue) with 5-HT2B.

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