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# Identification of a Potent and Selective Oxytocin Antagonist, from Screening a Fully Encoded Differential Release Combinatorial Chemical Library

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**Abstract**—A library of 1296 1,4-benzodiazepines was prepared on 160 μM Tentagel beads. Compounds are attached to the beads using orthogonally cleavable linkers. The library was first screened as pools of 30 beads where 50% of the material is released and screened. GW405212X, a selective oxytocin antagonist, was identified by picking single beads from active pools. 

## Introduction

The growth in the number of therapeutic targets emerging from the Human Genome Initiative and a parallel explosion in the number of chemical entities becoming available for high throughput screening means that drug discovery is entering a new arena. Combinatorial chemistry coupled with the judicious choice[1] of building blocks gives the medicinal chemist the ability to prepare many thousands of 'drug-like molecules'. Developments in automated screening and automated chemical synthesis means that there is a need to devise an efficient interface between combinatorial chemistry and biological assays.[2] Synthesising and screening a library as pooled samples offers major advantages. The number of chemical reactions to produce the library and the number of primary assay points to evaluate the compounds is drastically reduced. However, there is an accompanying problem. Each of the chemical entities in the pool has to be identified to elucidate which gave rise to the biological activity.

A combination of the traditional split-pool methodology[3] and advances in automated chemical delivery robotics[4] has enabled the efficient synthesis of large combinatorial chemical libraries. In a classical unencoded library[5] synthesised by the split-pool methodology, each pool can be efficiently screened as a mixture of $n$ components where the number of monomers used in the first synthetic step defines $n$. Powerful and efficient as this is, there are two major disadvantages with this approach.

Firstly, to identify the active components in a pool, each has to be synthesised in an iterative deconvolution[6] and re-screened. This is time consuming and uses large quantities of the common monomer.

Secondly, the measured biological activity is often not due to a potent individual component. Additive contributions from each of the components in the pool give rise to a cumulative biological activity. This is because the final monomer is common to each member of the pool.

Screening a library initially as pools, then as discrete compounds remains an attractive screening method. The approach where portions of the compound attached to the solid support are released under chemically controlled conditions is extremely attractive.[7,8] Peptides were synthesised on beads and released using controlled pH for the first and second tier of release. The identity of the peptide from a bead that gave rise to biological activity was elucidated by peptide sequencing of the peptide that remained on the bead attached to an orthogonal site. This method of identifying the biologically active entity would not be suitable for 'small molecule' libraries. An alternative approach has been suggested[9] whereby 50% of the compound is released

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from the bead by a controlled photocleavable tiered release. In our experience, different molecules were released at very different rates and a time point where 50% of each compound in the library could be reliably released was not achievable. Chemical encoding of each monomer by tagging an orthogonal position on a bead[10] and subsequently releasing the code is a simple and efficient procedure for identifying products. We have combined chemical encoding technology with two orthogonally cleavable linkers on each bead and termed these Fully Encoded Differential Release Libraries. Synthesis and screening of this library format offers distinct advantages over unencoded and encoded single linker libraries

## Results and Discussion

As an illustration of this process, we synthesised a benzodiazepine diamide library consisting of 1296 discrete compounds on Tentagel 160 μm HL beads (loading 0.46 mmol/g giving around 1 nmol/bead). The library was then screened in a scintillation proximity binding assay, using membranes prepared from Chinese hamster ovary cells expressing human oxytocin receptors.[11]

The initial stage of the synthesis is to differentiate beads so that about 10% of the sites are available for encoding and the remainder are used to link the compound. Beads are differentiated by coupling Boc glycine and Fmoc glycine in a 14:1 ratio. This gives a final ratio of 10:1 on the bead due to the different rates of reaction. The Fmoc group is removed and the first code is added to the beads. Each monomer in the library has its own unique code assigned to it. The Boc group is removed with TFA and the resin is neutralised using 10% diisopropylethylamine in dimethylformamide. The acid cleavable and photo cleavable linkers are mixed in a 1:1 ratio in dimethylformamide and coupled onto the resin using diisopropylcarbodiimide and hydroxybenzotriazole to afford a 1:1 ratio of linkers on each bead. Bradley[8] has reported that oxyacetate and oxybutyrate linkers couple at different rates. By using the oxybutyrate spacer on each linker, the linkers couple at approximately equal rates and the desired 1:1 ratio is readily achieved (Fig. 1).

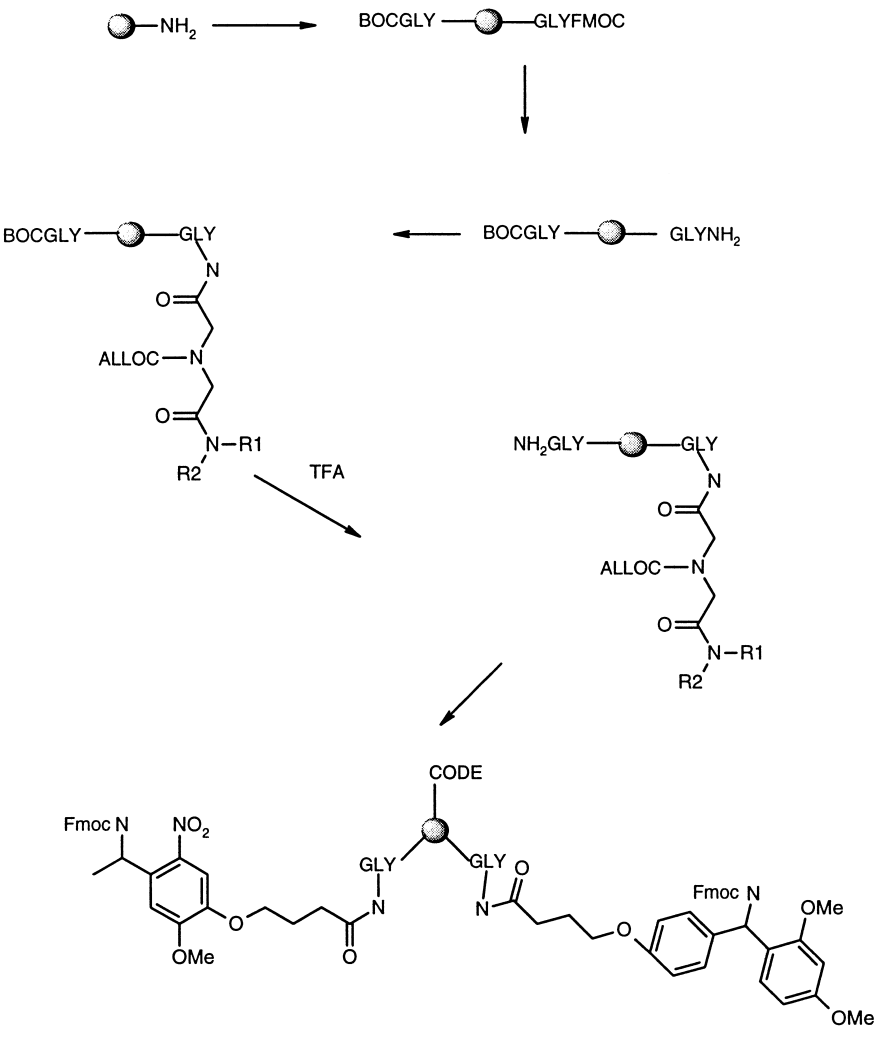



**Figure 1.** Construction of differentiated resin.

Library synthesis is accomplished by removing the Fmoc group with 20% piperidine in dimethylformamide. The first set of 36 amino acids, as their Fmoc derivatives, are coupled to the linkers using *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluoro phosphate (HATU) and diisopropylethylamine in dimethylformamide.[12] The beads are mixed and the Fmoc groups are removed. The common benzodiazepine core is coupled onto the amino acids using HATU. Equal portions of beads are distributed to 36 reaction vessels. The alloc group is removed[10] and the second set of codes coupled to the encoding strand. The Fmoc group is removed from the core and 36 carboxylic acids are coupled, again using HATU (Fig. 2). The amino acids and carboxylic acids used in the library were selected to display a range of functionality. Where appropriate, functional groups were protected as their *t*-butyl derivatives. Each monomer used in the library was rehearsed in the chemistry and selected on the basis of achieving a purity criterion (typically in the 80–100% range measured by LCMS). Final library quality control was tested by cleaving compound from a single bead and identifying a molecular ion. The code was read from the same bead and correlated with the molecular ion identified.

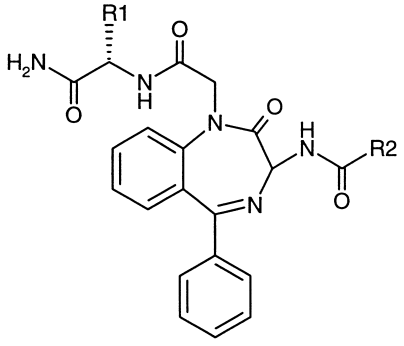
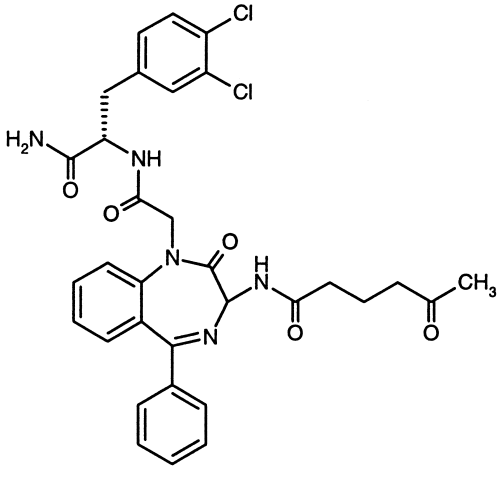


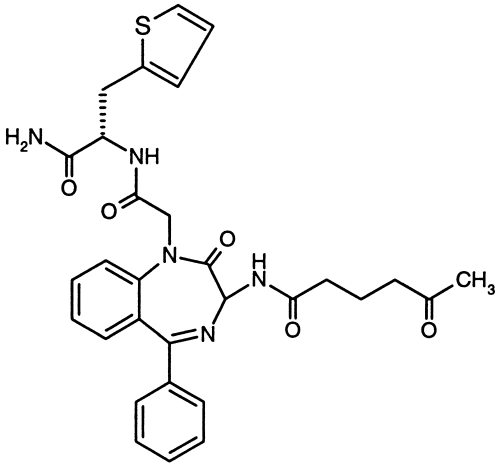

**Figure 2.** Library based on a 1,4-benzodiazepine core.

At the end of the synthesis all of the beads are combined as one large pool. Storing a library as a single pool of beads is efficient in terms of storage space and a single bar code can be used to identify each library. Redistribution of the beads as small pools of beads gives a random distribution. This will minimise the chance of additive effects from the pooled compounds. Although this particular library only uses two points of diversity the technique can be used for multiple positions of diversity, with appropriate encoding for each position.

This library was prepared on 3.6 g of resin that contains around 1.8 million beads. There are a large number of copies of each component of the library. The bead mass is over-sampled[13] for screening and 3888 beads are distributed at 30 beads per well. Beads are suspended in a neutrally buoyant medium and distributed in aliquots, containing the required number of beads, to bead cup plates.[14] The technique is flexible and adjusting the volume of the aliquot that is dispensed can vary the number of beads per well. Distribution is simple and easily automated. The technique is flexible so any number of beads per pool can be used during the first round of screening. The beads are treated with 90:10 TFA/$H_2O$ which removes the compounds from the acid cleavable linker and also cleaves the acid labile protecting groups on the monomers. The TFA is removed by centrifugation,[14] the dried material is dissolved in dimethylsulphoxide and the pooled samples assayed. Sample from the bead can be dissolved in a range of solvents dependent on the tolerance of the assay. For any active well, the beads that gave rise to the sample are distributed as single beads. This can either be carried out manually or using an automated bead picker capable of redistributing every bead from the pool. The remaining 50% of compound is now photolytically cleaved. Beads are photolysed at 365 nm at 9.5 mW $cm^2$ for 4 h in 0.2% hydrazine in dimethylsulphoxide. The cleaved single component is then assayed. The most potent compound identified from the library was the dichlorophenyl derivative, GW405212X, however we also identified




GW405212X $IC_{50}$ 5nM




GW428043X $IC_{50}$ 1μM

**Figure 3.** Affinities at the oxytocin receptor.

the much less potent thienyl derivative, GW428043X (Fig. 3)

## Conclusions

We have demonstrated differential release as a paradigm that provides an efficient interface between combinatorial chemistry and high throughput screening. The power of combinatorial chemistry can be used to prepare large libraries containing drug-like molecules. Aligned with the throughput of modern screening techniques a process can be developed to rapidly identify many novel lead series for the medicinal chemist. Identification of compounds from encoded libraries avoids costly and inefficient chemical deconvolutions. Mixing all the beads after completion of the synthesis reduces the possibility of additive effects in the screening process.

## References and Notes

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11. The assay is based on the binding of [$^3$H]-[leucyl]-oxytocin (specific activity 5.11 TBq/mmol), and wheat-germ agglutinin coated SPA beads (Amersham Pharmacia Biotech, Bucks, UK), to recombinant CHO-OT membrane preparations. All other reagents were of analytical grade (Sigma). The assay was performed in a total volume of 100 μL in 50 mM HEPES/KOH, 10 mM $MgCl_2$ at pH 7.4. Typically, the assay contained 10 μg CHO-OT membranes and 1 mg WGA-SPA beads that had been pre-incubated together for 1 h at 4 °C. Together with 0.3 KBq [$^3$H]-oxytocin this membrane–receptor complex was incubated in the presence of cleaved compounds at room temperature. After 4 h, DPM was counted for 1 min per well using a Wallac Trilux scintillation counter, with the appropriate colour quench correction. Nonspecific binding was determined in the presence of 10 μM oxytocin.
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14. Bead cup plates are modified micro-titre plates pierced to allow liquid to be spun through a hole and yet be small enough to retain the bead in the well. Neutrally buoyant fluid is removed by centrifugation. The beads are washed with water that is removed by centrifugation and are then dried in a vacuum centrifuge. Cleavage of compounds from the resin is carried out in the well. The hole is small enough that cleavage medium is retained in the well until the plates are placed in a centrifuge. After 2 h of cleavage time, the medium is spun through into a collection plate.