Marked Small Molecule Libraries: A New Approach to Molecular Probe Design

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DECLARATION

This thesis is submitted in part fulfilment of the requirements for the degree of Doctor of Philosophy at the University of Edinburgh. Unless otherwise stated the work described in this thesis is original and has not been submitted previously in whole or in part for any degree or other qualification at this, or any other university. In accordance with the regulations this thesis does not exceed 70,000 words in length.

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ABSTRACT

This thesis documents a new approach for the identification of a small biologically active molecule's site of interaction, through the rapid synthesis of molecular probes. A marked library approach has been developed whereby a biocompatible marker is attached onto the small molecule's molecular scaffold. This marker plays no role in the screening process itself, but facilitates the formation of a range of molecular probes from active marked library members. As an example of molecular probe formation, site selective biotinylation will be discussed in the introduction.
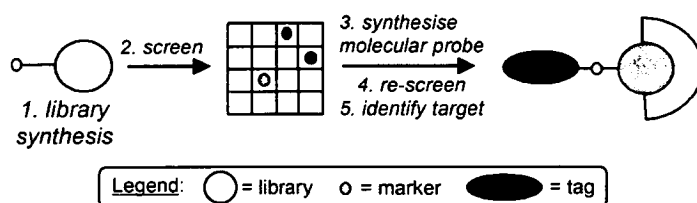
This marked library concept has been applied to the natural product anisomycin **A**. Investigations focused on development of a detailed structure activity relationship for anisomycin's activation of the stress activated protein kinase (SAPK) pathway, along with the synthesis of a number of marked library analogues. The active marked library members were then converted to a range of functional molecular probes utilising the copper(I) catalysed Huisgen cycloaddition as the key coupling step. These molecular probes are being used in the elucidation of anisomycin's biological target for activation of the SAPK pathway.
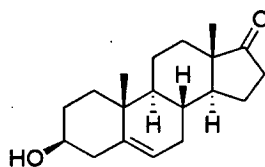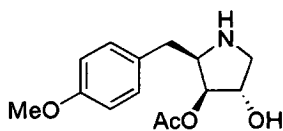
In a further demonstration of this strategy, a focused library of marked steroids has been synthesised based on the functionalisation of dehydroepiandrosterone **B**. Directed by the results of preliminary biological screening, a number of marked library members have been converted into fluorescent molecular probes. These probes will be used in future applications to probe the biological action of the dehydroepiandrosterone.

CONTENTS

1   Introduction

1.1   Biotin

Biotin **1**, also known as vitamin H, is a water-soluble co-factor that is extensively utilised by Nature.[1] It is involved in a number of biologically important processes including gluconeogenesis, lipogenesis, amino acid metabolism and energy transduction. The structure comprises two fused heterocycles, a tetrahydrothiophene and an imidazolidinone. Biotin possesses three contiguous stereocentres and a pendant five carbon atom chain that ends in a carboxylate group (**Figure 1.1**). The chain acts as a swinging arm and the carboxyl functionality allows the coupling of biotin to proteins. This structural arrangement allows for eight possible stereoisomers of biotin to exist however only D-biotin, the naturally occurring isomer is biologically active.
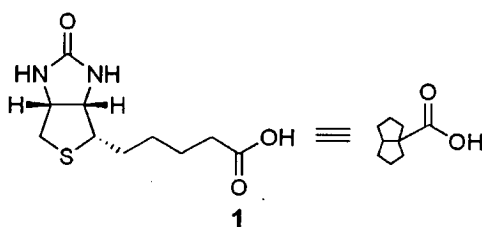
Figure 1.1: Structure of D-biotin **1** and the abbreviation that will be used in this review.

Biotin is synthesised by most plants and bacteria and is one of only a handful of co-factors required by Nature.[*] It is covalently linked to proteins *via* its carboxylate through the action of a biotin protein ligase (BPL). This coupling reaction is a remarkably specific process with most organisms having fewer than five biotinylated proteins present.[2] Once coupled, biotin is involved as a carbon dioxide carrier in a number of biologically important processes.

BPL catalyses the formation of a new amide bond between the carboxylate of biotin and the ε-amino group of a specific lysine residue of the biotin carrier protein (BCP). The coupling occurs in a two step ATP dependent process, in which biotin **1** is firstly

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[*] Co-factors are non-protein components of enzymes that are required to catalyse certain biochemical reactions.

activated as the mixed anhydride by reaction with ATP to form biotinoyl-AMP **2**. The activated intermediate **2** then reacts rapidly with the ε-amine of the lysine residue to form the biocytin linkage **3** (**Scheme 1.1**).[2-7]
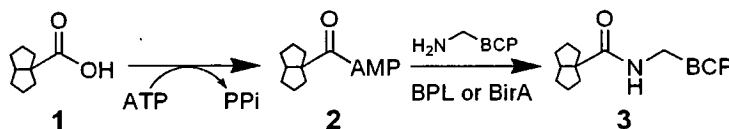
Scheme 1.1: Coupling of biotin **1** to the biotin carrier protein, *via* activation of biotin with ATP.

Once coupled to the BCP the biotin moiety plays a key role in the carboxylation of acetyl-SCoA in the fatty acid biosynthetic pathway.[8, 9] This involves the reaction of hydrogencarbonate **4** with ATP to form the short-lived mixed anhydride **5** (**Scheme 1.2**). This anhydride **5** subsequently reacts selectively with the N_1 of **3** in a process catalysed by a carboxylase enzyme, forming carboxy-biotin **6**. The 16 Å 'swing' arm provided by the biocytin moiety allows the flexibility to move between different active sites; thus allowing carboxy-biotin **6** to move from the carboxylase site to the transcarboxylase site.[10] A formal Claisen condensation then takes place between the enolate of acetyl-SCoA and **6** in a sequence catalysed by the transcarboxylase enzyme. This leads to the formation of malonyl co-enzyme A **7** and the regeneration of the BCP-biotin unit **3**.
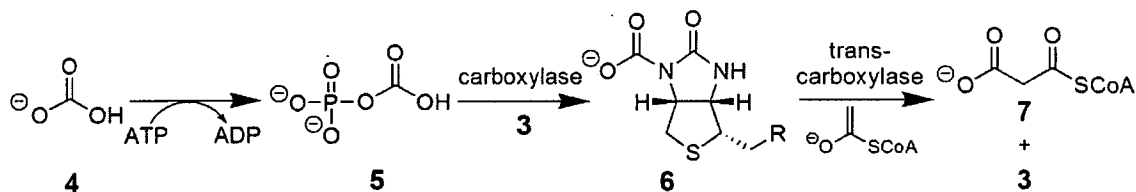
Scheme 1.2: Illustration of biotin's role within the fatty acid synthesis pathway. Activation of hydrogencarbonate **4** forms the mixed anhydride **5**, which reacts with biocytin to form carboxy-biotin **6**. A Claisen condensation then extends the acetyl-SCoA carbon backbone by coupling a one carbon carboxyl unit from carboxy-biotin **6** with the enolate of acetyl-SCoA to form malonyl SCoA **7**.

Recently biotin has been applied in a range of scientific settings including chemical biology, drug delivery and materials chemistry.[11-13] This primarily exploits the interaction between biotin and the protein avidin which is one of the strongest non-

covalent interactions known at $K_a = 2.5 \times 10^{15}$ M^{-1}.[14] Avidin is a tetrameric protein which can bind a single biotin molecule per subunit. The exceptional binding affinity and slow dissociation rates of the ligand result from a network of hydrogen bonds between the protein and the heterocyclic core of biotin. The pair of ureido nitrogens form hydrogen bonds with Thr35 and Asn118, the ureido oxygen forms contacts with Ser16 and Tyr33, while the hydrophobic tetrahydrothiophene interacts with Phe79, Trp97, and Trp110. In addition, hydrogen bonding occurs between the biotin carboxylic acid and avidin residues Ala39, Thr40, and Ser75.[15]

The related tetrameric bacterial protein streptavidin shares 33% of the conserved amino acids with that of avidin and a strong binding affinity for biotin ($K_a = 1.0 \times 10^{12}$ M^{-1}) (**Figure 1.2, part a**).[16] Specific interactions include hydrogen bonding between the nitrogens of the urea with Ser45 and Asp128 and oxygen contacts with Asn23, Ser27, and Tyr43 (**Figure 1.2, part b**). This network of hydrogen bonding in conjunction with hydrophobic interactions with four Trp residues (Trp 79, 92, 108, and 120) and the tetrahydrothiophene are responsible for the high binding affinity.[17, 18]
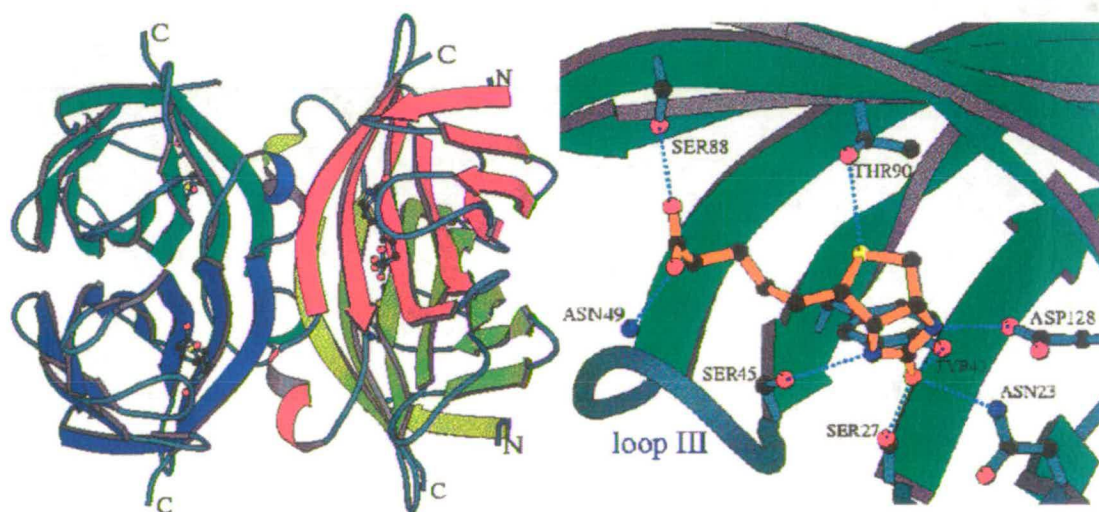
Figure 1.2: Illustration of (a) the streptavidin tetramer with a molecule of biotin bound per monomer; and (b) showing the distinct hydrogen bonding interactions between biotin and streptavidin.[19, 20]

Therefore, biotin-(strept)avidin systems have been used in a plethora of biotechnological applications such as affinity isolation and purification, immunoassay, diagnostics, localisation and biosensors. However, the incorporation of a biotin unit still poses a number of challenges. Herein, we review the strategies

utilised for the site specific (and not so specific) incorporation of biotin into a range of molecules over the last few years.

1.2 Biotin Linkers

In order to couple biotin to the molecule of interest, whether it is a protein or a small molecule, a linker component is typically inserted to separate biotin from the other functionalities of the target molecule. The linker has a number of further roles to play including controlling the flexibility, influencing the cell permeability and regulating the binding of biomolecules to the molecular probe.

The two main categories of linker typically used in the formation of biotinylated molecular probes are based on carbon and polyethylene glycol (PEG) backbones (**Figure 1.3; 8 and 9**).[21] In addition to the constituents of the linker, the length of the backbone plays an important role in regulating the properties of the biotinylated species. Biotin itself has a 5-carbon side arm that provides a natural 13.5 Å spacer, and this can then be extended by coupling to an appropriate linker. Typically the linker varies from 4-atoms which extends the arm to ~17.5 Å, up to 14-atoms which has a length of ~30.5 Å.[22] Control of the linker's length allows for the regulation of target binding, with a longer linker allowing the capture of molecules with greater steric hindrance. However, the downside in using a long linker is that it often leads to the binding of proteins and small peptides to the linker itself. Therefore, the choice of linker length has to be selected on a case by case basis, based on the desired application of the biotinylated molecule.
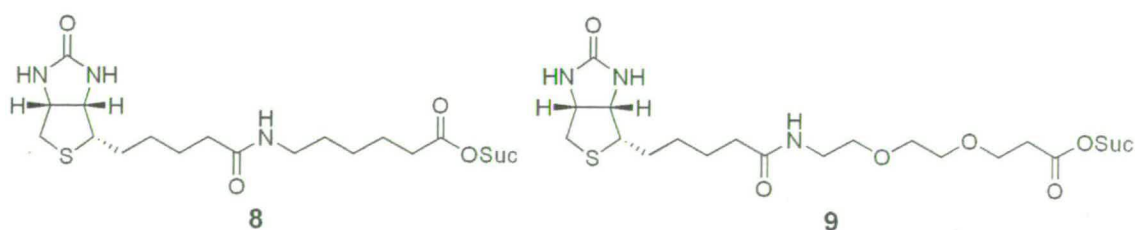
Figure 1.3: Structure of two biotin-NHS reagents that vary in the type of linker unit. (a) **8**, has a carbon linker and (b) **9** has a polyethyleneglycol (PEG) linker.

A novel class of linkers has also been developed which contain a cleavable unit within the linker. This allows the biotinylated molecule to be attached to a strept(avidin) column, but under specific conditions the linker can be cleaved leading to elution. This strategy allows the molecule to be purified and identified without the need for the harsh elution conditions (8 M guanidine•HCl, pH 1.5; excess biotin or boiling in SDS) usually required to release biotin from an strept(avidin) column.[23] This cleavable linker strategy takes a number of forms. However, the use of the disulfide functionality is by far the most popular (**Figure 1.4; 10**). This S-S bond is stable over a range of physiological conditions but can be selectively cleaved by reduction.[24] Other cleavable linkers have been developed which utilise photoreactive, oxidative, alkali sensitive and fluoride sensitive moieties.[25-28]†

10
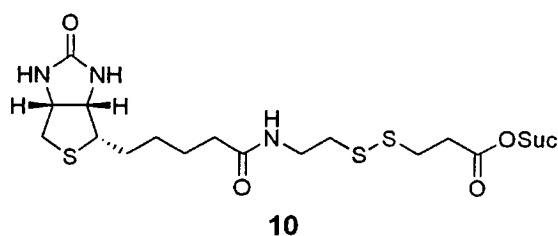
Figure 1.4: Structure of a biotinylation reagent **10** containing a disulfide linker that can be selectively cleaved. This allows release of the bound protein of interest from the strept(avidin) binding protein, thus removing the need for harsh eluting conditions that are often required.

1.3   Biotinylation Through a Range of Functionalities

The biotinylation of biological molecules has been used as a tagging technique for over 30 years.[29-31] It has several advantages over techniques such as radiolabeling, as it removes the hazard and disposal issues associated with radioisotopes.[32, 33] Therefore, a number of biotinylation reagents have been developed, thus allowing a range of synthetic approaches to be used to incorporate biotin into a molecular scaffold. This section will focus on the range of reactive functionalities that have been successfully employed over the last ten years, as well as the alternatively useful biotinylation techniques that have emerged during the last decade.

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† For the purpose of this review the linkers are abbreviated to L; however, the specific structures of the linkers are shown in the appropriate schemes.

1.3.1.1 Coupling with Biotin

With the development of an array of coupling reagents and additives that can be used to form amide/ester bonds,[34, 35] one of the most common methods of biotinylation is to couple a molecule of interest directly to biotin **1**. This direct coupling approach has been applied to a range of molecules including polysaccharides and cyclodextrins with high yields of the resultant products (**Scheme 1.3**; **11→12, 13→14**).[36, 37]
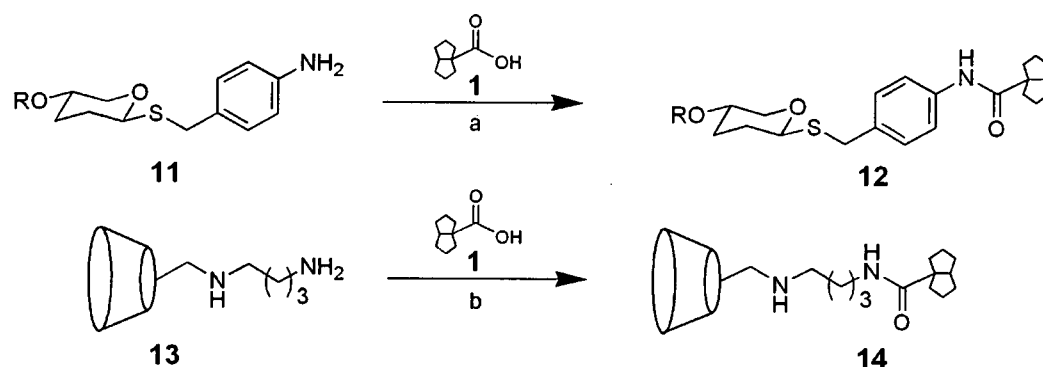
Scheme 1.3: (a) **1**, TBTU, DMF, Et₃N (85%);[36] (b) **1**, DCC, HOBt, DMF (85%).[37]

The same amide coupling strategy with extended linkers (L) has been utilised by Rando to form molecular probes for identification of the retinol binding protein,[38] and by Waldmann in the formation of biotinylated dipeptides (**Scheme 1.4**; **15→17, 18→20**).[39]
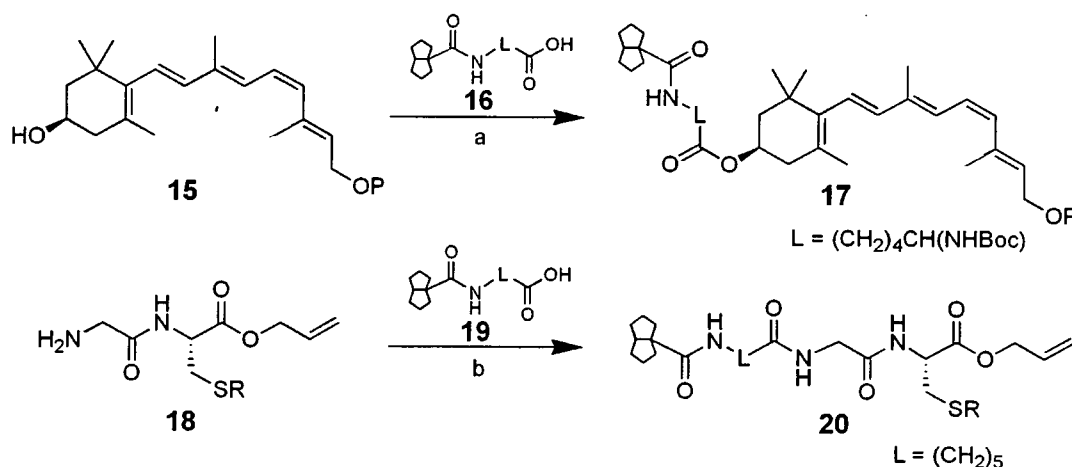
Scheme 1.4: (a) **16**, EDC, DMAP, DMSO/DCM (78%);[38] (b) **19**, DIC, HOBt, Et₃N, DMF (42%).[39]

However, transfer of the use of coupling reagents to complex multi-functional biological substrates is not directly applicable due to the presence of numerous acid, amine and alcohol functionalities. This leads to a lack of selectivity in the coupling reaction and biotinylation at a number of sites within the biomolecule.

1.3.1.2 Biochemical Coupling using BPL and BirA

The incorporation of biotin into a protein's structure in a site specific manner is successfully achieved in Nature in a range of biological systems using BPL. The use of BPL as a catalyst for introducing biotin into protein scaffolds has been investigated. Characterisation of the amino acid sequence required by naturally biotinylated proteins has led to the development of peptide tags that direct biotinylation. Biotinylation can be carried out by the cell's endogenous biosynthetic machinery or through co-expression of an exogenous biotin ligase, in most cases to date this has been the 35 kDa *E. coli* biotin ligase enzyme (BirA) (**Scheme 1.5**).[4]
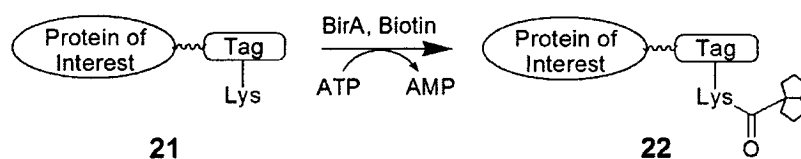
Scheme 1.5: Conversion of a tagged protein of interest **21** into the analogous biotinylated protein **22**. Coupling takes place between biotin and the ε-amine of the lysine residue within the tag in a process catalysed by BirA.

The discovery of a highly conserved Ala-Met-Lys-Met recognition sequence within the C-terminus of the (de)carboxylases has allowed the development of a number of fusion tags.[2, 40-44] These tags can be used at the C-terminus, N-terminus and within a protein's structure allowing *in vivo* or *in vitro* site specific biotinylation of recombinant proteins. Moreover, enzymatic biotinylation of these tags has been shown to have kinetics comparable to that of the natural biotin acceptor sequence.

The commercially available BioEase™ tag is based on a 72 amino acid domain that is derived from the α subunit of *K. pneumoniae* oxalacetate decarboxylase, with the whole 72 amino acid sequence being required for recognition (**Figure 1.5**).[45] This approach provides a significant reduction in bulk over other widely utilised fusion

sequences such as GFP (238 amino acids, 27 kDa). However, a 72 amino acid sequence is still a substantial size to append to a biomolecule for the purpose of introducing the 244 Da biotin motif.

BioEase™
Gly-Ala-Gly------------Ala-Met-**Lys**-Met------------Thr-Leu-Ala

AviTag™
Gly-Leu-Asn-Asp-Ile-Phe-Glu-Ala-Gln-**Lys**-Ile-Glu-Trp-His-Glu

Figure 1.5: The abbreviated 72 amino acid BioEase™ tag and the full 15 amino acid AviTag™ sequence with the key lysine residue highlighted.

There are a number of advantages to using a smaller tag; it is much less likely to affect the structure and thus properties of the tagged protein, it is less likely to be recognised by endogenous biotin ligases; and their small size means there is no need for the extra complication of protecting groups associated with some larger tags. Consequently, a series of smaller tags have been developed over a number years, through numerous screening rounds of combinatorial peptide libraries for sequences biotinylated by BirA.[46-49] The resultant peptides have been used to carry out site specific biotinylation of fusion proteins in *E. coli* cells. A 15 amino acid sequence derived from the *E. coli* BCP subunit of acetyl CoA carboxylase has been commercialised under the name AviTag™ [also known as acceptor protein (AP)] (**Figure 1.5**).[50, 51] Within the 15 amino acid sequence, the two Met residues flanking the Lys in the natural sequence have been altered to Gln and Ilu. This, provides a robust, highly efficient tag that allows the site specific biotinylation of proteins *in vivo* in both bacterial and mammalian cells.[50-52]

The BirA technology has recently been expanded by Ting *et al.* to the coupling of ketone biotin **24**.[53] As ketone functionalities are not normally present in cell surfaces, this probe makes an excellent bioorthogonal substrate which can be site specifically conjugated to hydroxylamine or hydrazide functionalised molecules. Therefore, AP-fused recombinant proteins **23** can be coupled to ketone biotin **24** in the presence of BirA in a highly specific manner. The coupled ketone biotin unit **25** can then be modified using Schiff base reactions with hydroxylamine or hydrazide probes **26** under physiological conditions (**Scheme 1.6**).[53] However, this methodology is

limited by the lack of affinity ketone biotin has for streptavidin and the extended timescale associated with a two step coupling strategy, which prevents the investigation of rapid biological processes. In addition it is also restricted to investigating molecules on cell surfaces due to the number of ketone and aldehyde containing small molecules within the cell that would remove the specificity of the Schiff base condensation. However, it seems likely that the development of this new coupling approach utilising ketone biotin will provide a useful extension to the BirA methodology and a new method for investigating cell surfaces.
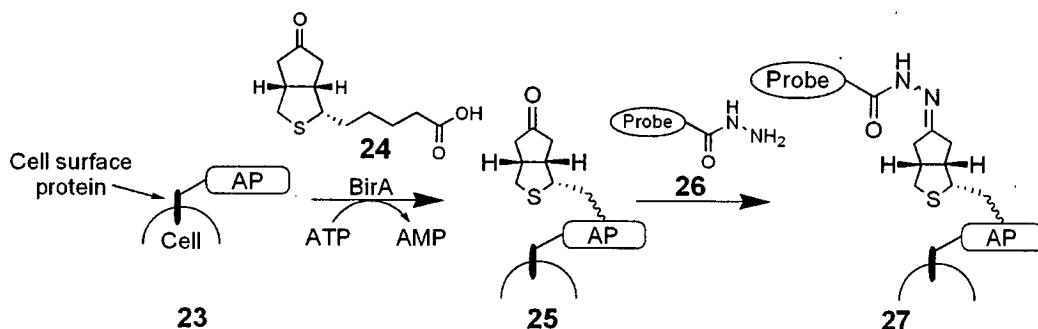
Scheme 1.6: BirA catalysed coupling of ketone biotin **24** to the AP **23**. Followed by a Schiff base condensation between ketone **25** and hydrazide **26** (or hydroxylamine) to form **27**.[53]

1.3.1.3 Coupling with Activated Esters of Biotin to Form Amides

The most widely utilised reactive functionality for biotinylation is an activated acid in the form of a hydroxyl succinimidyl ester. This functionality reacts readily with primary and secondary amines to form amides, and has been used in conjunction with a variety of linker types and lengths.

The coupling of biotin-*N*-hydroxysuccinimide (biotin-NHS) has been extensively used to tag proteins, peptides and antibodies and is used as a standard technique in the purification of biomolecules.[29] Biotinylation proceeds readily at pH 6-8 over a short period of time. More recently, this has been expanded to the solid phase synthesis of biotinylated antibodies, whereby the biotin-NHS derivative **29** has been coupled to a primary amino functionality **28** to form **30**, prior to release from the solid support (**Scheme 1.7**).[54]

$L= CH_2-[CH_2OCH_2]_4-CH_2$
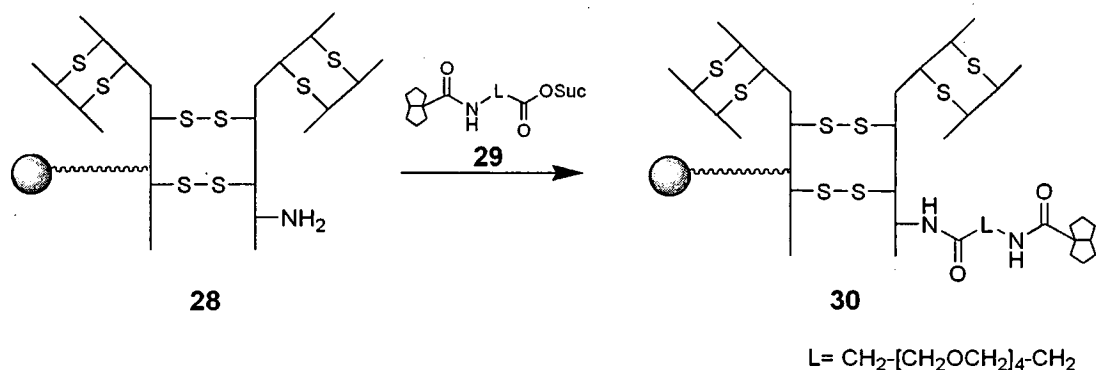
Scheme 1.7: Illustration of amine **28** directed biotinylation of antibodies on solid support.[54]

Modification of the succinimide structure by incorporation of a sulfo group onto the ring alters the properties of the biotinylation reagent dramatically; stopping the reagent from crossing the cell membrane.[55] This modification allows the specific labelling of extra-cellular biomolecules. For example, Scheurer *et al.* recently labelled membrane proteins in metastatic and nonmetastatic teratocarcinoma cells, thus facilitating their capture on streptavidin sepharose, before digestion and subsequent analysis of the isolated proteins by MS-MS.[56]

In contrast, more synthetic applications have used biotin-NHS derivatives to biotinylate small molecule scaffolds in high yields, often as the final step in a synthetic sequence. Peterson introduced the biotin moiety in the final step in his synthesis of tagged estradiol derivatives (**Scheme 1.8**; **31→33**), which were then used in yeast three-hybrid screens.[57] This NHS coupling strategy has been widely applied to a range of biologically interesting molecules including carbohydrates **34**, steroids **35** and a number of drugs including ezetimibe **36**, a cholesterol inhibitor and paclitaxel **37**, an anti-cancer compound (**Figure 1.6**).[58-61]
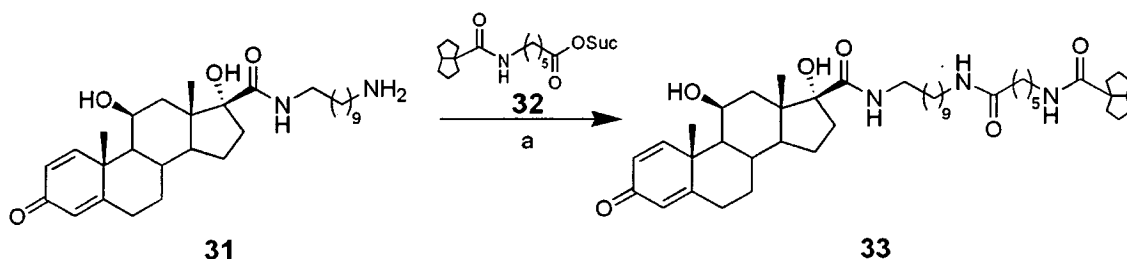
Scheme 1.8: (a) **32**, DIEA, DCM/MeOH, (93%).[57]
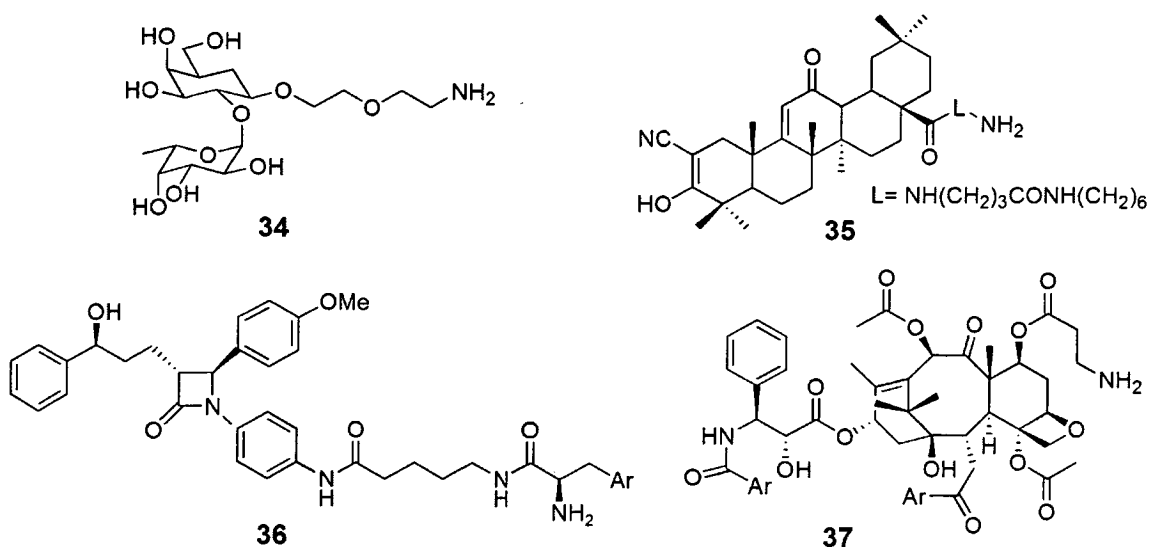
Figure 1.6: Structures of modified (a) Fucose α(1→2) galactose **34**, (b) 2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oic acid **35**, (c) 2-azetidinone **36** and (d) paclitaxel **37** which have all been coupled with biotin-NHS.[58-61]

1.3.2.1 Coupling with Biotinylated Amines to form Amides

In an alternative approach, a number of biotinylated amines have been developed to allow coupling of small molecules, peptides and the C-terminal of proteins to biotin. This procedure, orthogonal to the NHS approach, was utilised by the researchers at Merck who were investigating the biological target of L-685,485, a potent γ-secretase inhibitor.[62] A pair of biologically active biotinylated molecular probes were synthesised, with the coupling of biotinylated amine **39** with a functionalised acid **38** shown in **Scheme 1.9**. Subsequent affinity chromatography utilising both biotinylated molecular probes led to the isolation of the same labelled protein, presenilin 1 (PS1); allowing the conclusion to be drawn that PS1 contains the active site of the γ-secretase.[63]
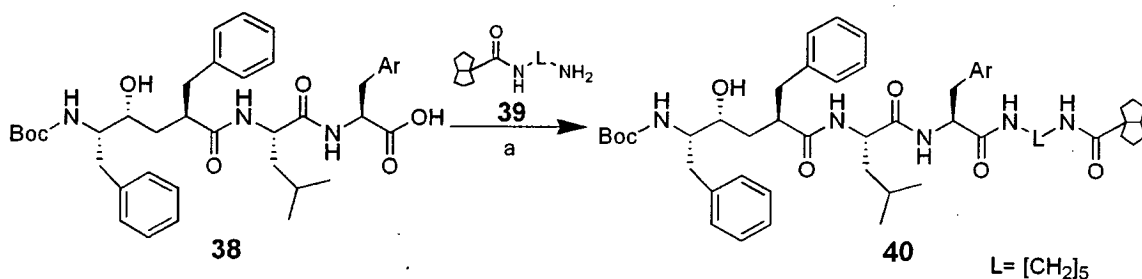
Scheme 1.9: (a) **39**, EDC, HOBT, Et$_3$N, DMF (63%).[63]

1.3.2.2 Coupling with Biotinylated Amines to form Amines

Welzel *et al.* utilised a biotinylated amine **42** in the formation of a multifunctional target identification reagent **43** which includes a biotin moiety, a photoreactive functionality and an attachment point for the ligand under investigation.[64] The amine **42** was coupled to **41** by reductive amination in the presence of sodium cyanoborohydride in good yield (**Scheme 1.10**).

$$L = CH_2\text{-}[CH_2OCH_2]_2\text{-}CH_2$$
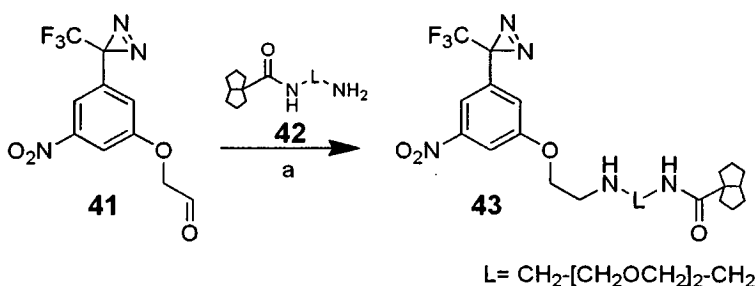
Scheme 1.10: (a) **42**, NaCNBH$_3$, MeOH, TFA (60%).[64]

1.3.3.1 Coupling with Biotinylated Hydroxylamines and Hydrazines to form Oximes and Hydrazones

The use of biotinylated hydroxylamine and hydrazine reagents has also been exploited as a method to introduce biotin into molecular architectures. This approach relies on the high selectivity of the reagent to undergo Schiff base condensations with a carbonyl functionality within the parent structure. This condensation reaction has recently been used in the development of a new strategy for the detection of *O*-GlcNAc proteins, whereby a modified keto-sugar has been incorporated onto the protein's structure using the natural biosynthetic machinery.[65] The glycosylated protein **44** can then be tagged selectively using biotinylated hydroxylamine **45**, which reacts with the ketone marker within the sugar moiety to form **47** (**Scheme 1.11**). Addition of the biotin component allowed for the subsequent detection of the proteins using streptavidin-conjugated HRP as an antibody.
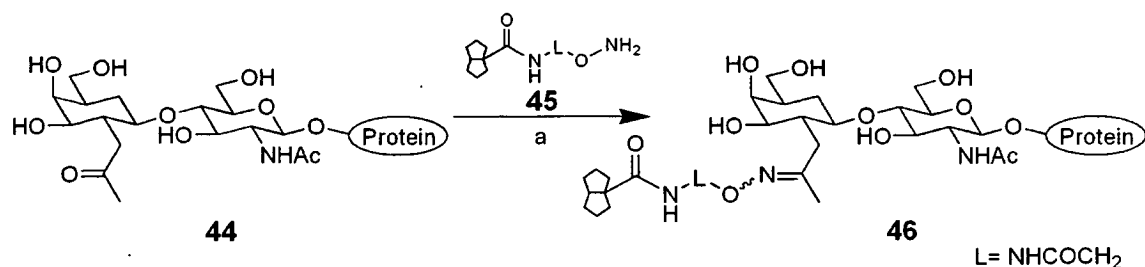
Scheme 1.11: (a) **45**, pH 6.7 buffer (100%).[65]

Oxime formation using a hydroxylamine derived biotin has also been used to couple the ring opened aldehyde form of pyrimido[1,2-α]purin-10(3*H*)one (M1G) **47** (**Figure 1.7**), resulting in the development of a new reagent to measure M1G levels.[66] Lacorn *et al.* have used this oxime based approach to generate a series of biotinylated steroids based on the coupling of **48** (**Figure 1.7**). The biotinylated molecules synthesised were subsequently used in a series of further biochemical investigations.[67]
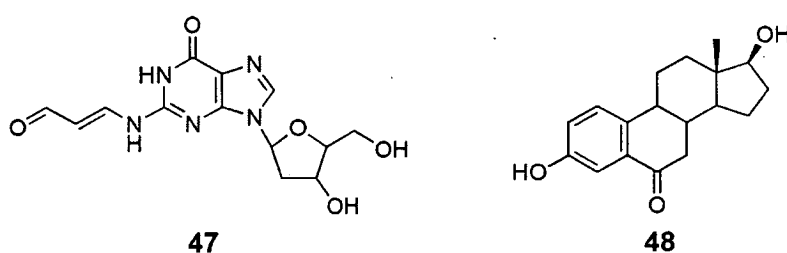
Figure 1.7: Structures of (a) modified M1G **47** and (b) estradiol **48** which have been coupled with biotin-hydroxylamine reagents.[66, 67]

In an analogous coupling approach Danishefsky used a biotinylated hydrazine to attach a tag to the core of a migrastatin analogue.[68] (+)-Migrastatin **49** is a macrolide natural product with antimetastatic properties that was isolated from fermentation broths of *Streptomyces sp.*[69-72] Synthesis of the natural product and a series of analogues led researchers to conclude that the core structure could be dramatically simplified while still retaining biological activity.[68] In an attempt to identify the biological target of this macrocycle, a biotin unit **51** was coupled to the simplified macrocycle **50** in high yield using a Schiff base condensation (**Scheme 1.12**). The molecular probe **52** was then tested for its ability to inhibit tumour cell migration,

showing retention of its activity with only a slight decrease in comparison to the natural product itself.
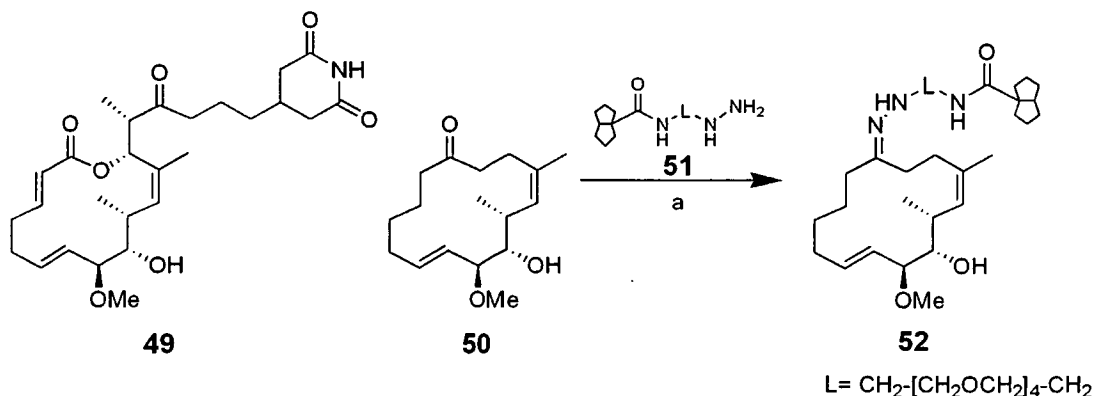
Scheme 1.12: Structure of (+)-migrastatin **49** and coupling conditions (a) **51**, EtOH, 55 °C (75%).[68]

1.3.3.2. Coupling with Biotinylated Hydrazines to form Hydrazides and Hydrazines

Biotinylated hydrazines have also been used with a range of coupling reagents to form hydrazides (**Scheme 1.13; 53→55**). This coupling reaction has been used in a number of studies ranging from carbohydrate and dendrimer chemistry to the development of assays.[73-75] A biotinylated hydrazine **57** has also been condensed onto the reducing end of carbohydrates **56** by reductive amination in the presence of sodium cyanoborohydride to form **58** (**Scheme 1.13**).[76]
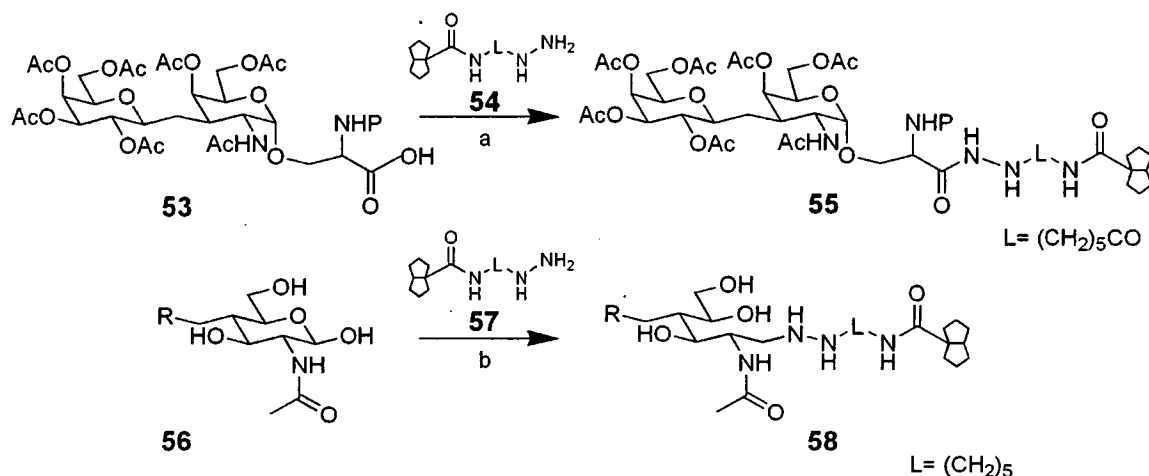
Scheme 1.13: (a) **54**, DCC, HOBT, DMF (68%); (b) **57**, NaCNBH$_3$, DMSO/AcOH, 65 °C (>95 %).[75, 76]

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1.3.4.1 Coupling with Biotinylated Maleimides *via* Nucleophilic Displacement Reactions

Attachment of the reactive maleimide functionality on to a biotinylated linker was developed as a selective method for coupling thiols. The maleimide reagent allows for the selective tagging of cysteine residues within a protein's structure, and has been extensively utilised as a method for introducing biotin into biomolecules.[29]

Spence *et al.* have utilised this coupling reaction in the development of NMR-biosensors **61** using the magnetic properties of xenon.[77] Functionalisation of the xenon cage, cryptophane-A, with a 4 amino acid side chain containing a cysteine unit **59** subsequently allowed the introduction of a biotin moiety **60** (**Scheme 1.14**).[77] This biosensor was used to investigate biosensor/protein interactions to determine the relationship between the molecular composition of the xenon biosensor and the characteristics of protein-bound resonances.[78]

L= CH$_2$-[CH$_2$OCH$_2$]$_2$-CH$_2$NHCOCH$_2$CH$_2$
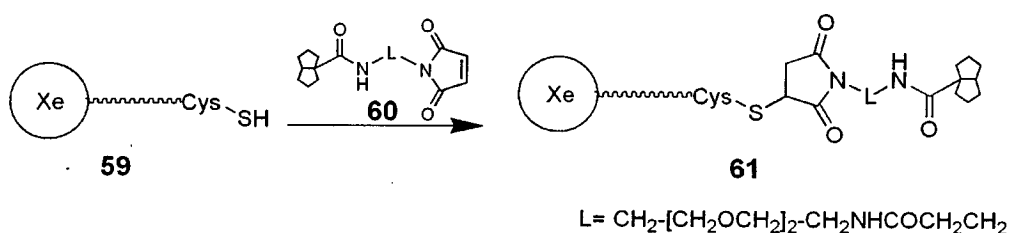
Scheme 1.14: Illustration of biotin maleimide **60** used in the synthesis of biosensor **61** designed to target Xenon.[77]

This maleimide coupling has also been used in the development of a DNA hybridisation biosensor, the final step in the synthetic sequence being the coupling (in high yield) of maleimide-biotin with an oligodeoxynucleotide.[79] In another example, Oba *et al.* coupled a thiol modified analogue of xestoquinone **62** to biotinylated maleimide **63** to form the molecular probe **64**. The probe was then used to investigate the natural product's role as an inhibitor of Ca^{2+} ATPase (**Scheme 1.15**).[80]
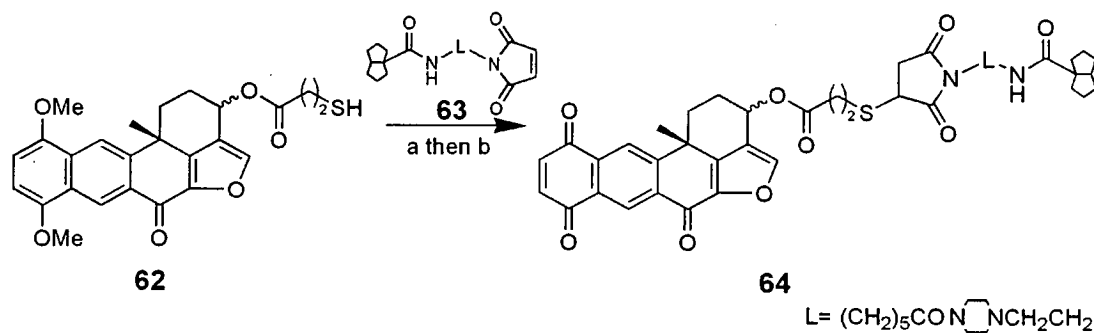
Scheme 1.15: (a) **63**, CH₃CN:H₂O; (b) CAN, CH₃CN (29 % over 2 steps).[80]

1.3.4.2 Coupling with Biotinylated Maleimides *via* Diels-Alder Reactions

In an alternative approach the maleimide functionality has also been used as a reactive dieneophile which can be coupled to a functionalised diene using a Diels-Alder cycloaddition reaction. Saito *et al.* has coupled a number of oligonucleotides incorporating a reactive diene unit to a series of functionalised maleimides.[81] These reactions proceeded *via* a Diels-Alder cycloaddition followed by a [1,3]-H shift and can be carried out under mild conditions with short reaction times (**Scheme 1.16; 65→67**).
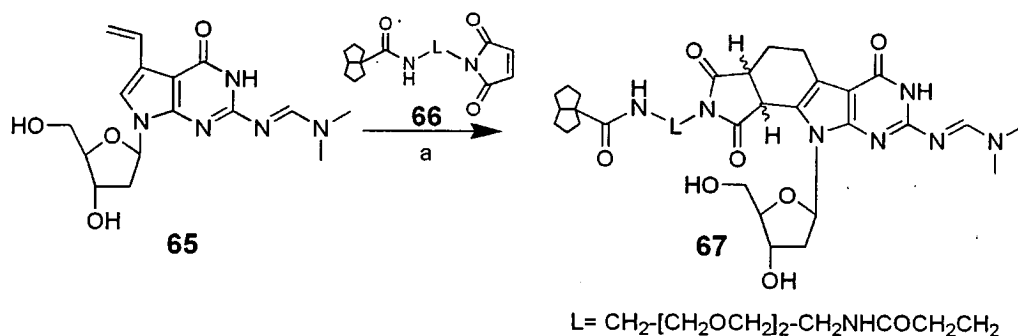
Scheme 1.16: (a) **66**, MeOH, 0 °C (>95 %).[81]

Biotin maleimides have also been used as the dieneophile in the selection of Diels-Alderases from combinatorial RNA libraries.[82, 83] RNA molecules with catalytic properties are termed ribozymes, and some have been shown to accelerate the carbon-carbon bond forming process in the Diels-Alder reaction by up to 20000-fold.[84] Initially this reaction would only proceed when the diene **68** was tethered to

the RNA (R = RNA) (**Scheme 1.17**).[84] However, recent advances have led to the coupling of dieneophiles **69** with dienes **68** (R= alkyl) in the presence of an untethered 49-mer ribozyme catalyst.[85] Under these conditions the reaction undergoes significant rate enhancement and results in the formation of **70** and **71** with enantioselectivities of greater than 95% ee.

L= NHCO(CH$_2$)$_5$
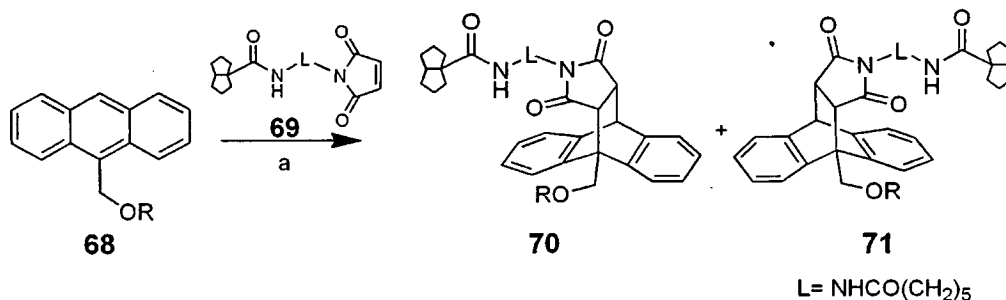
Scheme 1.17: Illustration of the RNA-catalysed coupling of biotin-maleimide **69** with anthracene **68**. (a) **69**, 49-mer ribozyme, pH 7.4 buffer.[85]

1.3.5.1 Coupling with Biotinylated Iodoacteyls *via* Nucleophilic Displacement Reactions

In a complementary approach to that using the malaimide functionality, a biotin moiety containing a reactive iodoacetyl functionality has also been developed. This reagent allows the selective biotinylation of the thiol functionality of cysteine residues within a biomolecule, while also allowing the use of a number of other non-biological nucleophiles.[29]

This iodoacetyl coupling was applied to the biotinylation of a peptide sequence derived from the N-terminal region of the P-selectin glycoprotein ligand 1 (PSGL-1), a major ligand of the selectin family involved in leukocyte adhesion to the vascular endothelium during inflammation.[86] The key peptide was synthesised using Fmoc solid phase synthesis and glycosylated to form **72**. Selective biotinylation of the C-terminal cysteine residue of **72** was then carried out using iodoacetyl biotin **73** to form **74** (**Scheme 1.18**).[87]
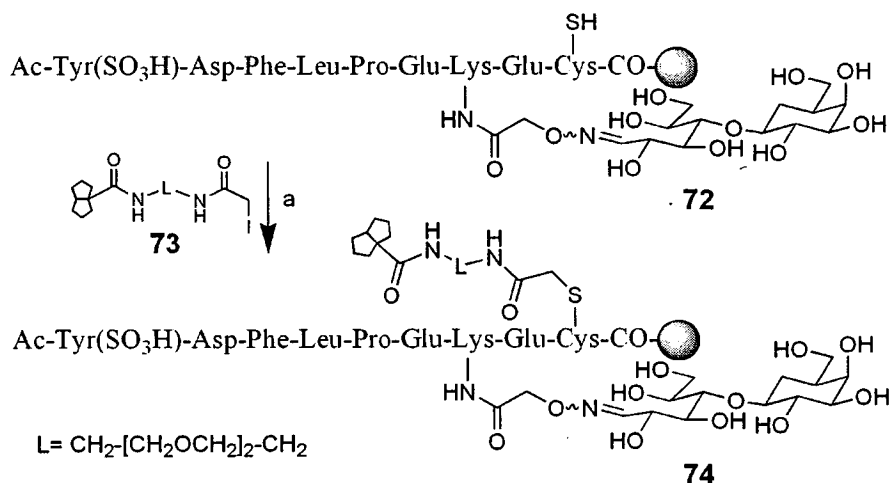
Scheme 1.18: Biotinylation of glycosylated peptide **72** *via* nucleophilic displacement reaction with iodoacetyl biotin **73** (a) **73**, pH 7.3 buffer/CH₃CN (1:1).[87]

The iodoacetyl reactive functionality **76** has also been used in the construction of new biosensors. The probe molecule [*N*-(biotinoyl)-*N*'-(acetyl 4,4'-pyridylpyridinium iodide)] ethylenediamine (BPP+) **77** consists of a biotin molecular recognition fragment coupled to a pyridinium unit and was synthesised in one step from **75** (**Scheme 1.19**).[88] When **77** is added to a solution of poly[lithium 2-methoxy-5-(3'-propyloxysulfonate)-1,4-phenylenevinylene] (Li+-MPSPPV), a weak electrostatic complex forms between the pyridinium and sulfonate groups, which brings the quencher molecule into close proximity with the optically active backbone. This leads to a very efficient fluorescence quenching of Li+-MPS-PPV by BPP+. The addition of avidin, which binds the biotin substructure, has been shown to encapsulate BPP+ and draw it away from Li+-MPS-PPV, resulting in the recovery of emission.[88]
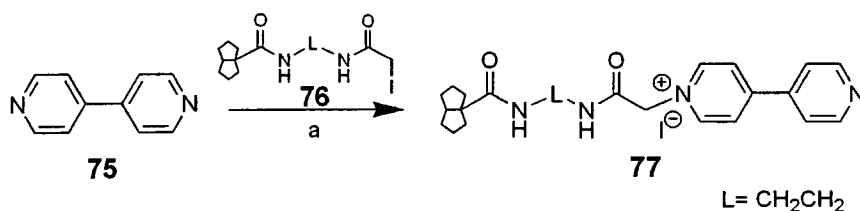
Scheme 1.19: (a) **76**, DMSO (49%).[88]

1.3.6.1 Coupling with Biotinylated Cysteine *via* Native Chemical Ligation

The native chemical ligation developed by Kent *et al.* has been widely used in the synthesis of proteins as the key step in the coupling of peptide fragments of varying sizes.[89] This reaction allows the formation of new peptide bonds by coupling a peptide containing a C-terminal thioester **78** with a second peptide containing an N-terminal cysteine **79**, in the presence of an exogenous thiol catalyst. In a thermodynamically-controlled reversible first step, a transthioesterification occurs to form **80**. This product **80** then rearranges irreversibly under the reaction conditions to form the desired amide bond **81** (**Scheme 1.20**).[90]
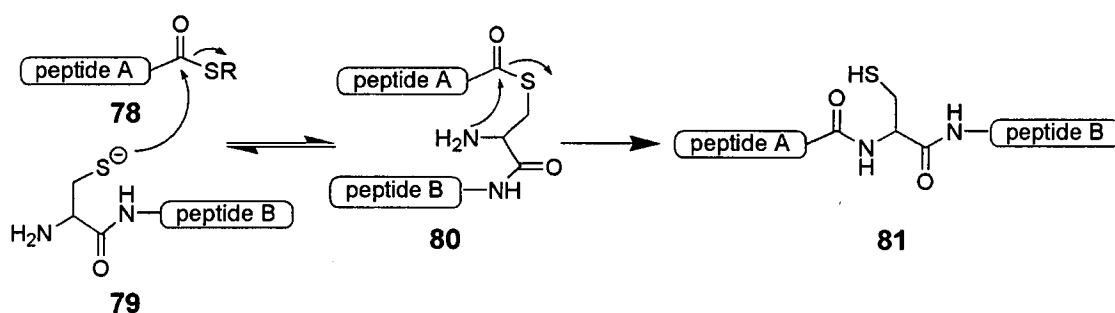
Scheme 1.20: Mechanism of the native chemical ligation.[90]

The native chemical ligation has now been expanded to allow the coupling of a biotinylated-cysteine moiety **84** to proteins, in an intein-directed coupling strategy.[91, 92] Inteins are biochemical markers that can be cleaved tracelessly from the protein under investigation by attack of a nucleophile, thus facilitating the formation of a new amide bond (**Scheme 1.21; 82→85**). The intein unit is displaced, allowing biotinylation to take place site specifically without a residual tag on the biomolecule.

L= (CH$_2$)$_3$NHCO
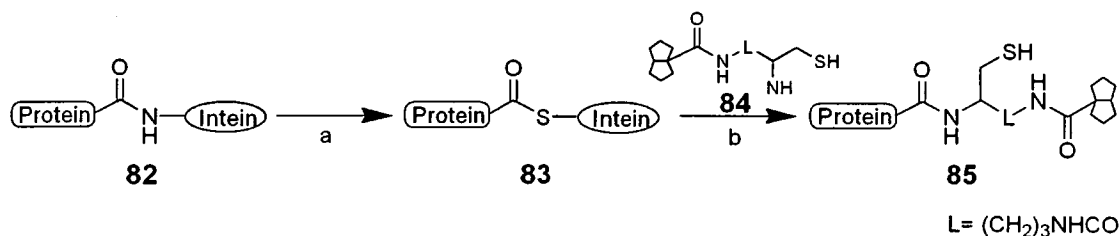
Scheme 1.21: Overview of intein mediated native chemical ligation. (a) Intein-mediated rearrangement; (b) native chemical ligation with cysteine-biotin **84**.[91, 92]

This methodology has been applied both *in vivo* and *in vitro*, with the protein of interest being site specifically biotinylated, without the requirement for a tag that is retained within the final molecule.[93, 94] However, the precursor inteins are bulky, the coupling site is limited to a cysteine residue, and the rates of the ligation reaction is still in the order of hours, making this approach unsuitable for the monitoring of rapid biological processes.

1.3.7.1 Coupling with Biotinylated Alkynes

Azides and alkynes have gained widespread acceptance as biocompatible functionalities over the past few years.[95, 96] This has led to the development of a number of bioconjugate reactions that can be utilised to couple these functionalities in a range of settings including: the Staudinger-Bertozzi ligation; strain promoted cycloaddition; and the copper(I) catalysed Huisgen cycloaddition.[96-98]

Following the 2003 publication from the Cravatt and Sorensen research groups,[99] a number of propargyl biotin reagents have been prepared. Having previously synthesised FR182877,[100-102] a complex natural product which has a number of interesting biological roles, these groups set out to identify the molecule's biological site of action. The key coupling step in the formation of the molecular probes **88** was the copper(I) catalysed Huisgen cycloaddition. This bioorthogonal reaction was used to couple a number of propargyl functionalised tags including biotin and rhodamine **87** to an azido analogue of FR182877 **86** (**Scheme 1.22**).[99]
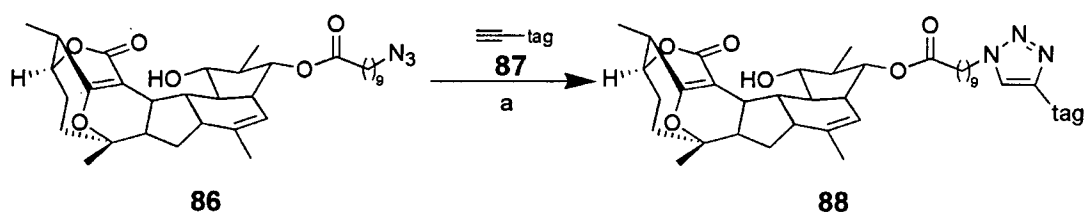
Scheme 1.22: (a) **87**, CuSO$_4$ (10 mol%), NaAsc (20 mol%), ^{t}BuOH:H$_2$O (50 %).[99]

One of the main exponents of propargyl biotin has been Tirrell who has utilised this reagent in a number of examples of protein and cell surface engineering.[103] Co-translational replacement of methionine residues in the outer membrane protein

(Omp) C in _E. coil_ cells with azidohomoalanine **89** (**Figure 1.8**), allows coupling to a biotin-alkyne reagent **90** under 'click' reaction conditions before analysis by Western blotting.[104]

89   **90**
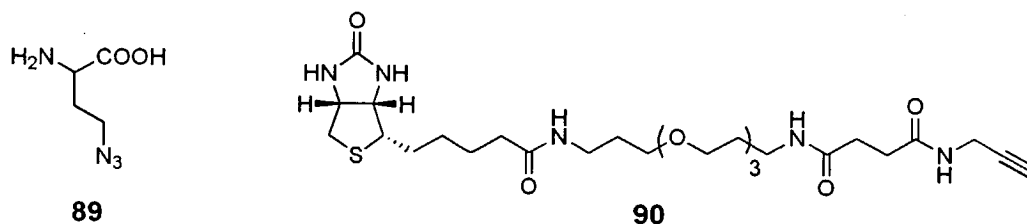
Figure 1.8: Structure of (a) azidohomoalanine **89** and (b) biotin propargylamide **90**.[104]

This tagging methodology has recently been used to investigate protein synthesis upon environmental stimulation.[105] By incorporating an azide modified amino acid during _in vivo_ protein synthesis, the newly formed proteins can be tagged with an analogous propargyl biotin reagent. The tagged proteins can be subsequently purified and identified by tandem MS.[105]

However, the copper(I) catalysed Huisgen cycloaddition has limited applications in biological systems due to the fact that copper(I) is toxic to both bacterial and mammalian cells.[104] Therefore Bertozzi _et al._ set about applying a catalyst free [3+2] cycloaddition to biological systems.[106] This was based on work carried out by Wittig and Krebs in 1961 who found that the reaction of phenyl azide and cyclooctyne "proceeded like an explosion to give a single product", the triazole.[107] Bertozzi subsequently showed that the biotin derivatised cyclooctyne **91** reacts rapidly with a number of azides **92** to form an equal amount of the two possible regioisomers **93** and **94** (**Scheme 1.23**).[106] In addition, they illustrated the formation of the triazole in living cells without the toxicity witnessed under copper(I) catalysed conditions. As a result the development of the strain promoted cyclisation has provided a useful extension of the [3+2] cycloaddition methodology and has been subsequently utilised in a number of biological applications.[108, 109]

L= CH$_2$CH$_2$-[CH$_2$OCH$_2$]$_3$-CH$_2$CH$_2$NHCO-pArCH$_2$
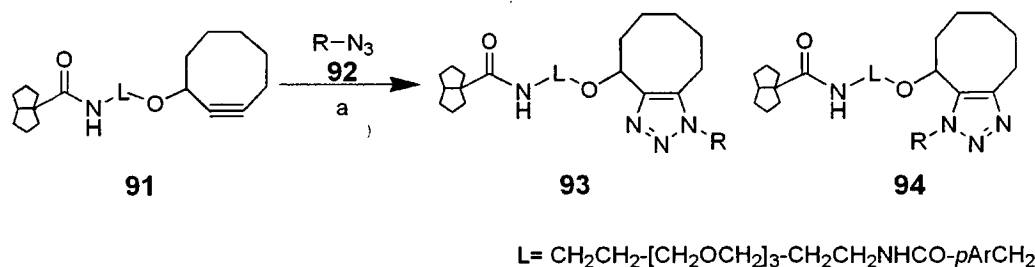
Scheme 1.23: (a) **92**, pH 7.4 buffer.[106]

In an alternative use of the alkyne functionality Arterburn *et al.* have recently synthesised an alkyne derivative of biotin **95** which has been utilised in Pd-catalysed Sonogoshira cross coupling reactions (**Figure 1.9**).[110] This approach provides a contrasting method for biotinylation that does not rely on the formation of amide, ester or thiol functionalities and forms a linkage which cannot be cleaved by endogenous biotinidase enzymes.[111, 112] Although the Sonogashira coupling provides useful new approaches for biotinylation, it has the limitation that it is not readily applicable *in vivo* due to the requirement for copper(I) and palladium(II).
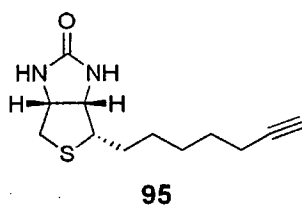
Figure 1.9: Structure of biotin derived alkyne **95**.[110]

1.3.7.2 Coupling with Biotinylated Azides

In an inverse approach to that described in section 1.3.7.1, the coupling of biotin azide with propargyl marked molecules has also been carried out in a number of instances. The first example of a biotin azide was published by Bertozzi and was utilised as a key step in the development of an azido-ELISA assay using the Staudinger ligation as the key bioconjugation reaction.[113, 114]

More recently biotin azides have been utilised by Lin *et al.* in a protein engineering experiment where the maltose binding protein (MBP) was modified with small organic components containing a propargyl moiety **96**.[115] This labelling was

followed by coupling of the protein **96** to the orthogonally reactive azide partner **97** to form **98** (**Scheme 1.24**). This coupling procedure has been extended to the development of microarrays by modification of glass slides with propargyl or azide functionalities.[115] Lin *et al.* have subsequently illustrated the power of the copper(I) catalysed Huisgen cycloaddition for the immobilisation of proteins site specifically onto glass surfaces in the form of microarrays.

96   **98**

L= (CH$_2$)$_3$
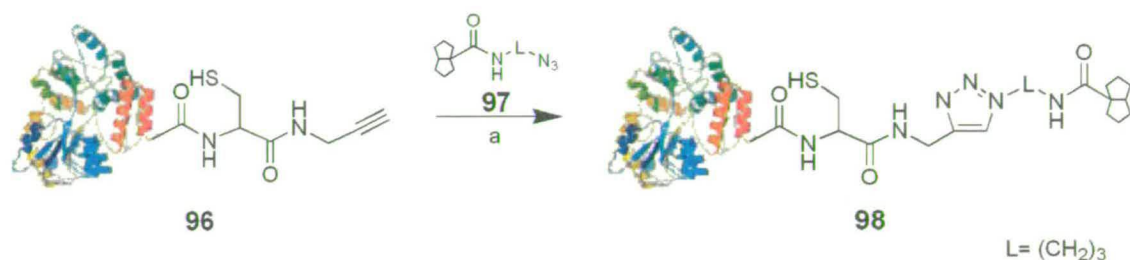
Scheme 1.24: (a) **97**, CuSO$_4$, TCEP, tri(triazolyl)amine.[115]

In an alternative setting, Lahann *et al.* have used a biotin azide as a key reagent in the synthesis of a biofunctional surface which has potential applications in diagnostics, biosensors, and biomedical device coatings.[116] Firstly, a polymer coating containing an alkyne functionality was synthesised using chemical vapour deposition polymerisation **99** (**Scheme 1.25**).[116] The polymer was subsequently coated with a thin film of biotin azide and sodium ascorbate, and dried. This was followed by a patterned stamp which was inked with a solution of copper(II) sulfate and kept in contact with the substrate for 12–18 h. The biotinylated surface **100** was then probed with rhodamine-labelled streptavidin and the product **101** analysed. The result showed that the alkyne groups on the polymer surface are reactive and can be effectively used as anchoring sites for surface modifications.

99   **100**   **101**
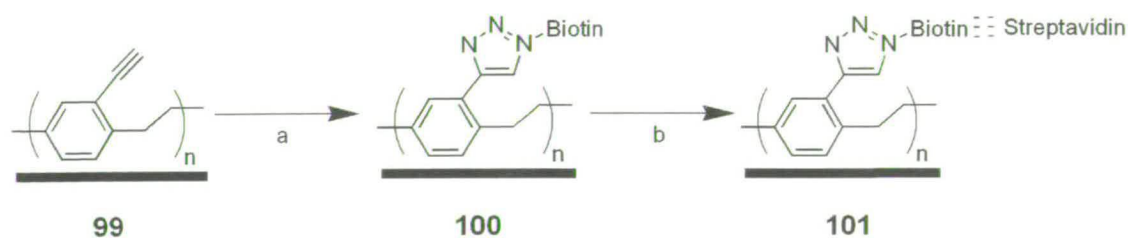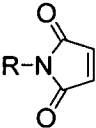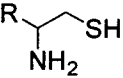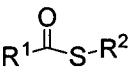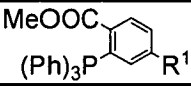
Scheme 1.25: Immobilisation of biotinylated azide on a reactive alkyne polymer. (a) Subsequent coupling occurs when the CuSO$_4$ catalyst is microcontact printed on to a preadsorped layer of biotin azide. (b) Incubation with streptavidin.[116]

1.4 Conclusions

The attachment of biotin to a range of molecular scaffolds is an important synthetic technique which allows the identification, visualisation and localisation of a range of proteins and small molecules. Over the last 30 years a wide range of approaches for the incorporation of biotin into biologically interesting molecules has been developed, facilitating biotinylation with varying degrees of success. However, these reactions have often resulted in low site specificity and poor yields. In addition, these reactions have been restricted to the coupling of a narrow range of functional groups including acids, amines and thiols.

In recent years a number of new strategies, both chemical and biochemical, have made site-specific biotinylation more widely achievable. This has been accomplished by applying new bioconjugate reactions to biotinylation, such as the Staudinger-Bertozzi ligation, the native chemical ligation and the copper(I) catalysed Huisgen cycloaddition. As a result, through careful selection of coupling reactions, reagents and conditions, site-selective biotinylation can now be successfully achieved across a range of substrates in high yields (**see Table 1.1**).

Biotin functionality	Reactive partner	Coupling reaction
R-CO$_2$H	R^1-NH$_2$	Peptide formation
	R^1-NH$_2$	BirA catalysed coupling
	R^1-OH	Esterification
R-CO$_2$X	R^1-NH$_2$	Peptide formation
R-NH$_2$	R^1-CO$_2$H	Peptide formation
	R^1-CHO	Reductive amination
R-NHNH$_2$	R^{1}CHO/R^{1}R^{2}C=O	Schiff Base condensation
R-NHNH$_2$	R^{1}CHO/R^{1}R^{2}C=O	Schiff Base condensation
	R^{1}CHO/R^{1}R^{2}C=O	Reductive amination
	R^1-CO$_2$H	Hydrazide formation
R-N (maleimide)	R^1-SH	Nucleophilic displacement
	(butadiene)	Diels-Alder Reaction
R-C(=O)CH$_2$	R^1-SH/R^1-NH$_2$/R^{1}R^{2}NH	Nucleophilic displacement
R-CH(NH$_2$)-CH$_2$-SH	R^1-C(=O)-S-R^2	Native Chemical Ligation
R-≡	R^1-N$_3$	Copper(I) catalysed or strain promoted Huisgen cycloaddition
R-N$_3$	R^1-≡	Copper(I) catalysed Husigen cycloaddition
	MeOOC-(Ph)$_3$P-R^1	Staudinger-Bertozzi Ligation

Table 1: Table of biotinylation coupling reactions.

2   A Marked Library Approach to Anisomycin

2.1   Anisomycin

Anisomycin **102** was first isolated from the fermentation broths of *Streptomyces griseolus* and *Streptomyces roseochromogenes* by Sobin and Tanner in 1954,[117] while more recently it has been isolated from *Streptomyces sp. SA3079* and *No 638*.[118, 119] Its structure was elucidated in 1965,[120] with its relative stereochemistry being established three years later by NMR studies and X-ray crystallography.[121-123] The absolute stereochemistry was finally confirmed as 2*R*,3*S*,4*S* by chemical correlation with L-tyrosine.[124]
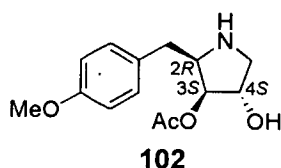
102

Anisomycin was found to exhibit selective and potent activity against pathogenic protozoa and certain strains of fungi. Its antibiotic activity was first tested by Lynch *et al.* in 1954, they discovered that anisomycin inhibited *E. histolytica*, *T. vaginalis*, *T. foetus* and *Candida albicans*.[125] This led to clinical trials for the treatment of amoebic dysentery and vaginitis. However, anisomycin was found to be inactive towards bacteria at medicinally useful concentrations, with *S. aureus*, *S. faecalis* and gram positive organisms all requiring greater than 100 μg per ml for inhibition.

More recently it has been reported that anisomycin has been isolated as an antitumour substance showing *in vitro* cytotoxicity against human tumour lines, such as HBL 100, RAS A and MCF 7 in the nM region.[118, 126] Recent studies have implied that anisomycin may be used in a synergistic fashion with a cyclin-dependent protein kinase inhibitor to kill carcinoma cells.[127] Since its introduction over 15 years ago as a tool for activation of the stress kinase pathway at 'sub-inhibitory' levels, anisomycin has been widely used as a tool in molecular biology.[128-133] However, the cellular target of anisomycin and therefore the precise signalling pathway have yet to be elucidated.

2.2 Signal Transduction

A cell is highly responsive to a wide variety of stimulants. Therefore, understanding how a cell responds to these stimuli and processing of the resultant responses is covered by the field of signal transduction. Signal transduction cascades act as 'molecular circuits' and can detect, amplify and integrate a diverse range of external stimuli thus allowing a wide range of signalling controls within a biological system.[134, 135] This can subsequently generate a series of downstream responses ranging from a change in enzyme activity to an alteration of gene expression (**Figure 2.1**).
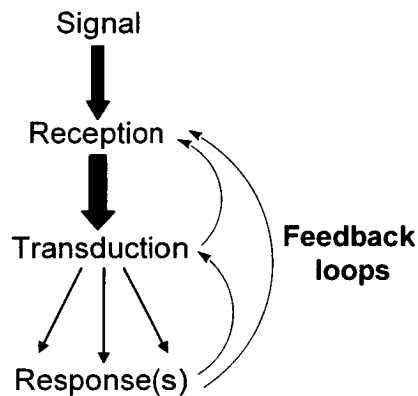
Figure 2.1: An environmental signal such as a hormone or other small molecule is first received by interaction with a cellular component (*e.g.* a cell surface receptor). The information that the signal has arrived is converted to another chemical form, or transduced. The signal is then passed downstream before evoking a response. Feedback pathways control the entire signalling process.

Signal transduction covers a wide range of biochemical processes from the transfer of information from a cell's exterior to interior, to the role played by primary and second messengers in this process. Herein, we will focus on the role played by protein phosphorylation in regulating the signal transduction process.

2.3 Protein Phosphorylation

The modification of a protein by phosphorylation and dephosphorylation of Ser/Thr/Tyr residues by protein kinases and protein phosphatases respectively, can lead to the alteration of cellular function in a vast number of ways; by increasing and decreasing its biological activity, by facilitating or inhibiting its movement between subcellular compartments, or by disrupting protein-protein interactions, to name but a few. Thus, making this regulatory process the most important adopted in eukaryotic cells.[136, 137]

The discovery of the first observed protein kinase activity in 1954 by Kenedy, was subsequently followed by Fischer and Krebs, as well as Wosilait and Sutherland.[138-141] These researchers showed that interconversion of phosphorylase b to phosphorylase a occurred *via* a phosphorylation/dephosphorylation mechanism. Fischer and Krebs also illustrated that the b form could be converted into the a form in the presence of Mg- adenosine triphosphate (ATP) and an enzyme they called phosphorylase kinase, now called a protein kinase (**Figure 2.2**).[138, 140] This was found to occur *via* transfer of the γ-phosphoryl group of ATP to a specific serine residue in phosphorylase b.[142] However, phosphorylase a can be converted back into its b form upon catalysis with a protein releasing enzyme, now termed protein phosphatase.[143]
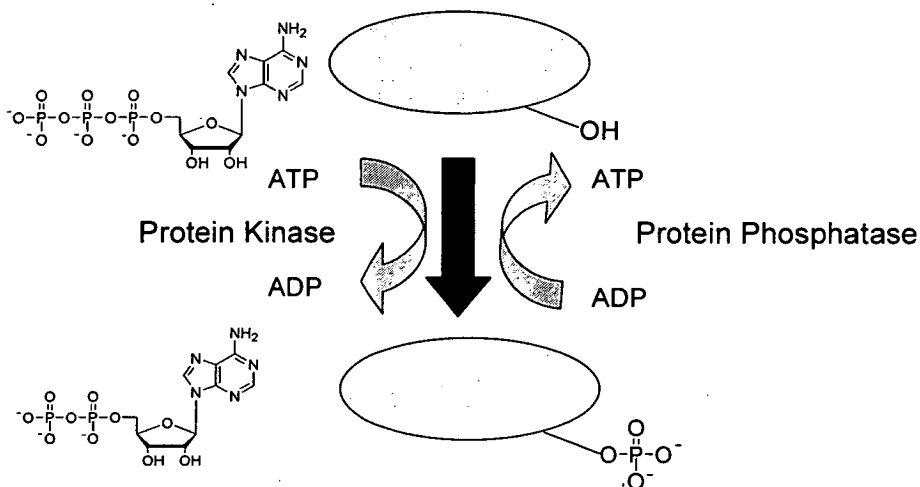
Figure 2.2: Reversible protein phosphorylation: ATP interacts with a protein kinase and a hydroxyl on a protein's backbone, resulting in the phosphorylation of the protein and ADP. In the reverse process a protein phosphatase dephosphorylates the protein in the presence of ADP, forming ATP and the native protein.

For this discovery Fischer and Krebs were awarded the Nobel Prize for Medicine in 1992 'for their discoveries concerning reversible protein phosphorylation as a biological regulatory mechanism'. It is now widely accepted that reversible protein phosphorylation of proteins is a major regulatory process in biological systems. More recently this has expanded into the field of drug discovery, where protein kinase and protein phosphatase inhibitors now account for multi billion dollars expenditure in R&D and are now available in the clinic for the treatment of numerous cancers.[144]

With the publishing of the human genome in 2002, Manning _et al._ undertook the challenge of cataloguing the protein kinase complement of the human genome. They discovered 518 putative protein kinase genes of which 71 had never been reported before, while extending or correcting the protein sequence of a further 56 kinases (**Appendix 1**).[145] This work classified protein kinases primarily by sequence of the catalytic domains, while also utilising known biological function and the structure outside the catalytic site. Therefore, the field of protein phosphorylation continues to develop and be an area of major scientific interest almost half a century after its initial discovery.

2.3.1 Mitogen Activated Protein Kinase (MAPK) Cascade

The first protein kinase cascade was identified back in 1968.[146] However, it was a further 20 years before another example of this type of cascade structure would be identified. An insulin stimulated protein kinase that phosphorylates microtubule-associated protein 2 (MAP2) was identified in 1987 and termed MAP kinase.[147] However, its name was later changed to mitogen activated protein kinase (N.B. still MAP kinase) as a way of emphasising its activation by many mitogens and growth factors. From these beginnings, the study of the mitogen-activated protein kinase (MAPK) pathway has grown rapidly.

It is now recognised that the MAPK superfamily is made up of four main and distinct signalling pathways: the extra cellular signal-regulated protein kinases (ERKs), the c-Jun N-terminal kinases or stress activated protein kinases (JNK/SAPK1), the p38 family of kinases (or SAPK2) and the big MAPKs (BMKs) (**Figure 2.3**).[148,149]
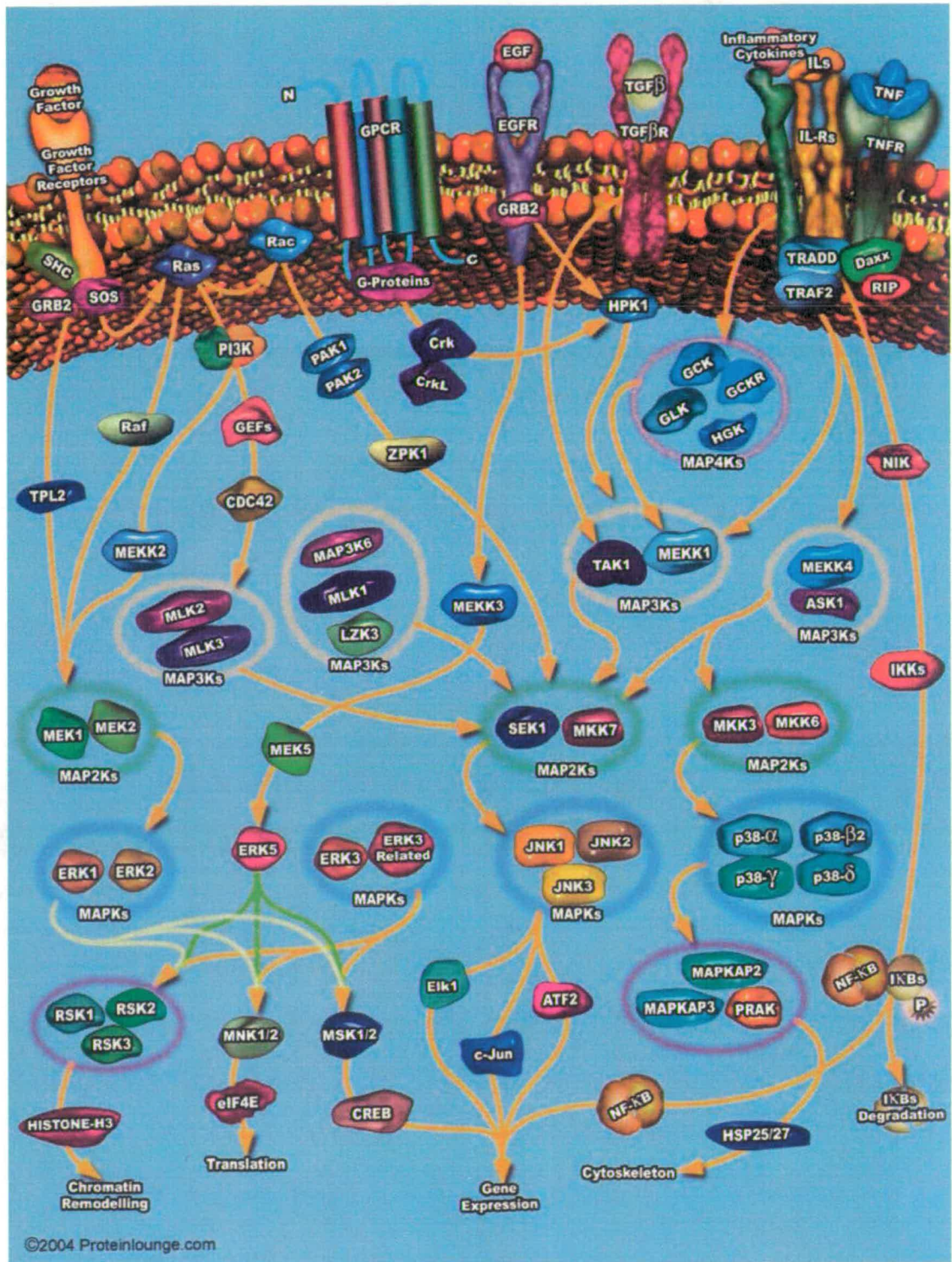
Figure 2.3: The mitogen activated protein kinase (MAPK) pathways; including the ERK, JNK/SAPK1 and p38/SAPK2.[150]

The core unit of the MAPK pathways is a three-membered protein kinase cascade whose components are evolutionary and highly conserved in structure and organisation.[151, 152] In this cascade the MAPKs are phosphorylated and activated by a MAPK kinase (MAPKK/MKK). The MAPKKs are 'dual-specific' kinases that catalyse the phosphorylation of MAPKs at Thr and Tyr sites, specifically targeting a Thr-X-Tyr motif on MAPK (where X is glutamate, proline and glycine for the ERK, JNK and p38 modules, respectively).[153-156] Phosphorylation of the MAPKs, results in a conformational change and a >1000-fold increase in specific activity, so that in effect, MAPKs are inactive unless phosphorylated by their respective upstream kinase.[157-159] The MAPKKs are themselves phosphorylated and activated by a Ser/Thr kinase that acts as a MKK kinase (MAPKKK/MKKK). Upstream of the MAPKKKs additional protein kinases may participate in the signal transduction process along with small GTPases, or cell surface receptors.[158, 160] However, efficient signal transduction by the MAPK cascades requires the components of the cascade to be colocalised within the cell.

The MAPKs themselves represent a family of kinases that can phosphorylate other cytoplasmic proteins and translocate from the cytoplasm to the nucleus to regulate the activity of transcription factors. Hence, the controlled regulation of MAP kinase cascades can result in cell proliferation, differentiation and cell repair/apoptosis.

2.3.2 JNK/SAPK1 Cascade

The JNK protein kinases are encoded by three different genes; *JNK 1* and *JNK 2* are expressed ubiquitously, whereas *JNK 3* has a more limited pattern of expression and is mainly brain-specific.[156] These genes are alternatively spliced to create ten JNK isoforms.[161] Transcripts derived from all three genes encode proteins which are without a COOH-terminal extension to create both 46 and 55 kDa isoforms. However, the functional significance of these spliced variants is unclear.

As the name implies the SAPK1 pathway is activated in response to stresses such as heat shock, UV, alkylating agents, inflammatory cytokins and inhibitors of protein synthesis, resulting in cell cycle arrest or apoptosis.[162] The JNKs are activated by two MKKs, MKK4 [also know as SAPK/ERK kinase (SEK)] and MKK7 (**Figure**

2.4).[131, 163, 164] The MKK7 protein kinase has been described with six distinct isoforms each containing a different NH_2 and COOH termini and has been shown to be primarily activated by cytokines (interleukin (IL) and tumour necrosis factor (TNF)).[149, 165] In contrast, three MKK4 isoforms have been identified and have been shown to be activated by environmental stress. Direct comparison of MKK4 and 7 have shown that although both kinases activate JNK through dual phosphorylation on Thr and Tyr, there are many differences in substrate specificity.

Upstream of the MKKs the network is even more complex. The kinases reported to be operating at this level include MEKK 1-4, the mixed linkage kinases (MLK 1-3), the ASK group (apoptosis signal-regulating kinase 1 and 2) and TAK 1 (transforming growth factor-β-activated kinase).[149, 156] In turn, the MKKKs can be activated by a series of GTP-binding proteins and by germinal centre kinase (GCK).
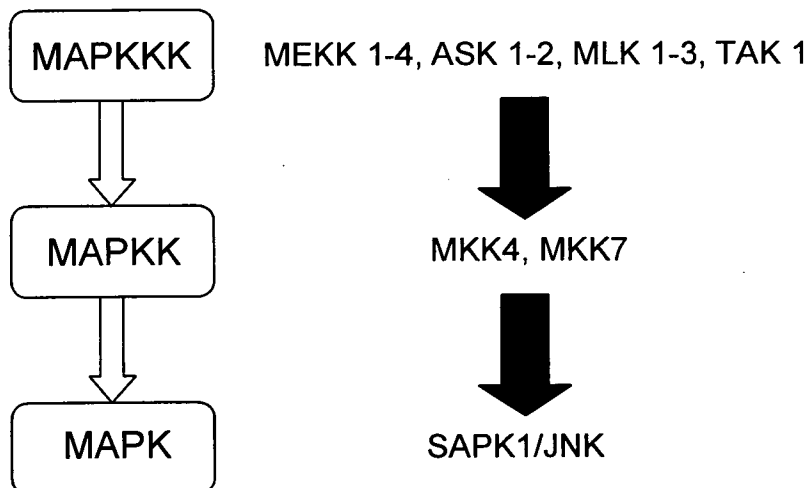
Figure 2.4: The three tier cascade of the SAPK1/JNK pathway, overview of some of the proteins involved at MAPK, MAP2K and MAP3K levels.

Downstream JNKs are activated and translocate across the nuclear membrane.[166] JNK was originally identified as being responsible for the phosphorylation of c-Jun, leading to increased activity of the activator protein-1 (AP-1) transcription factor.[155, 167] The other major substrates for JNK are activating transcription factor-2 (ATF-2), Elk-1, deleted in pancreatic cancer-4 (DPC4) and nuclear factor of activated T cells (NAFT4).[158, 168] This selective focus on transcription factors contrasts with the actions of the ERK and p38 pathways, which phosphorylates targets both inside and

outside the nucleus.[169] Therefore the regulation of transcription factors by JNK helps to regulate gene expression in response to a variety of cellular stimuli including stress events, cytokines and growth factors. Consequently, activation of the JNK cascade generally results in apoptosis, although it has been shown to promote cell survival under certain conditions (*e.g.* in cardiac myocytes after oxidative stress).

2.3.3 p38/SAPK2 Cascade

There are currently four members in the p38 family, p38α, β, γ and δ. The p38α and β genes are ubiquitously expressed.[154] However, the p38γ and δ are differentially expressed with the γ isoform predominant in skeletal muscle,[170] while the δ isoform is enriched in the lung, kidney, pancreas, testis and in the small intestine.[171] Like the JNK/SAPK1 pathway they are activated by most environmental stresses, including heat, osmotic and oxidative stresses, ionising radiation as well as inflammatory cytokines and tumour necrosis factor (TNF) receptor signalling, resulting in cell cycle arrest or apoptosis.[154,172] Certain proinflammatory cytokines, such as IL-1 and TNF-α, also activate the p38 pathway, suggesting a potential role in the regulation of the cellular inflammation response.[173, 174]

Figure 2.5: The three tier cascade of the SAPK2/p38 pathway, overview of some of the proteins involved at MAPK, MAP2K and MAP3K levels.
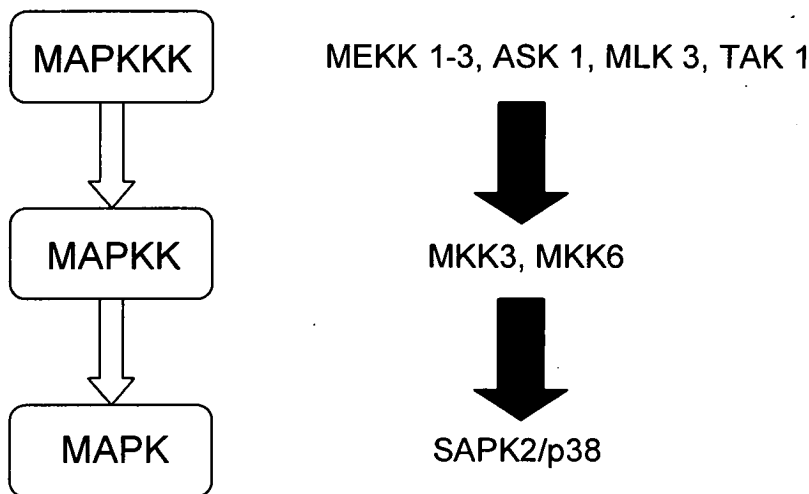
The upstream kinases acting on p38 include MKK3, 4 and 6.[175-178] Specifically, MKK6 which is 80% homologous to the isoform MKK3 can target all four isoforms of p38. In contrast, MKK3 preferentially activates p38α, γ and δ, while MKK4 activates p38α and δ. (**Figure 2.5**).[172, 179] This suggests that substrate selectivity may be a key factor in why each MKK has a distinct function. In turn, greater diversity occurs upstream at the MKKK level, which may be one of the reasons that the p38 pathway can be activated by a wide range of stimuli. At the MKKK level a range of kinases are involved including MTK1-3, MLK3, ASK1 and TAK1.[180-182] However, activation of many of theses kinases also leads to activation of both the p38 and JNK pathways. Above the MKKK level Rac, CDC42 and the Rho small family of GTPases have been found to be potential regulators of the p38 pathway.[157] Consequently, the precise nature of this signal transduction pathway is still under investigation.

Downstream, regulation of p38 MAPKs can lead to the phosphorylation of a large group of regulatory protein kinases, as well as controlling a range of transcription factors. The most studied of the downstream protein kinases is MAPK-activated protein kinase 2 (MAPKAP-K2 or MK2), whose role has been recently reviewed.[172, 179] Moreover, the p38 MAPK can also influence a series of transcription factors which includes AFT1/2, cyclic AMP response element binding proteins (CREBs), Elk-1 and myocyte enhancer factor (MEF) 2A and 2C. Therefore, regulation of the p38 pathway has a profound effect on inflammation, cell growth, cell differentiation, the cell cycle and cell death.

2.3.4 SAPK Regulation

Below the MAPK level the JNK and p38 pathways are two independent units. However, above this level numerous cross-talk mechanisms exist between the two pathways (**Figure 2.6**).[149, 162] MKK 7 and MKK3 specifically activate the JNK and p38 pathways respectively, while MKK 4 has the capacity to activate both the JNK and p38 pathways by phosphorylation of both the threonine and tyrosine phosphorylation sites. The complexity of the pathway is further increased on going to the M3K level where TAK 1, ASK 1 and MLK3 have all been shown to activate both

the MAPK 3/6 and 4/7 cascades.[149, 156, 160, 162] Thus illustrating the intricate network of processes involved in signal transduction.

The regulation of these pathways only becomes more complicated upon introducing the fact that a series of protein phosphates act within the SAPK cascade to dephosphorylate and thus regulate these processes (**Figure 2.6**).[183-185] Protein phosphatases are classified into three groups; Ser/Thr phosphatases, Ser/Thr/Tyr phosphatases and Tyr phosphatases, depending on their phosphoamino acid specificity. As phosphorylation of both Thr/Tyr residues is required in a dual specific manner for SAPK pathway activation, dephosphorylation of either or both amino acid residues is sufficient to inactivate the pathway. This allows negative regulation of the pathway to occur at numerous points throughout the cascade.

Therefore, understanding the overall regulation of the SAPK signal transduction pathways is a complex and challenging biochemical problem that is being studied by a number of researchers around the globe.
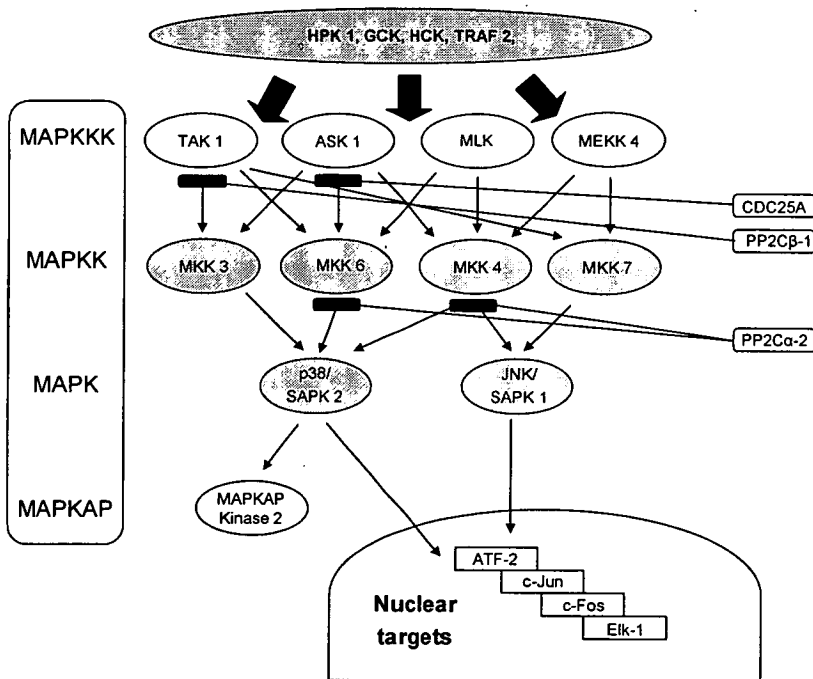
Figure 2.6: The JNK/SAPK1 and p38/SAPK2 pathways, illustrating upstream and downstream targets as well as cross-talk between the pathways and the site of interactions of a number of protein phosphatases.

For the last 15 years anisomycin has been known to strongly activate both stress kinase pathways at a level widely considered to be higher that M3K. Recently Bloem *et al.* have shown that the selective MLK-7 kinase inhibitor DHP-2 inhibits downstream activation of the SAPK pathway when stimulated by UV or anisomycin, but not upon activation by IL-1 or TNF-α.[186] These results suggest that stimulation of the SAPK pathways by anisomycin proceeds through MLK-7. However, above the M3K level there still remains a large amount of uncertainty with regards to both activation and negative regulation of the SAPK pathway. Thus, its precise site of action and therefore precise regulatory mechanism has still to be fully understood.

2.4 Protein Biosynthesis

Protein biosynthesis is the complex multi-step process by which cells build proteins. For the purpose of this thesis the basic processes involved in protein biosynthesis will be touched on as a means of explaining anisomycin's mode of action as an antibacterial antibiotic.

2.4.1 Transcription

Protein biosynthesis starts in the nucleus where the process of transcription catalysed by the enzyme RNA polymerase, converts DNA into RNA.[187, 188] This process can be broken down into three key areas; initiation, elongation and termination.[134]

Transcription begins with RNA polymerase searching for a promoter site or simply promoters within the DNA. This promoter acts as a binding site and initiator for RNA polymerase. As the RNA polymerase binds to the DNA strands they begin to unwind, producing a pair of single strands of DNA (**Figure 2.7**).[187, 188] The RNA polymerase then controls the elongation of one of the two unravelled strands of DNA by complementary base pairing (A-U, C-G).[189] As the RNA polymerase moves along the DNA backbone, the base pairs of the two individual strands recombine behind it and the DNA recoils. This process continues along a single DNA strand until the RNA polymerase reaches a terminator sequence, where transcription ends.[190]

In eukaryotes the newly transcribed RNA forms the short lived pre-messenger RNA (pre-mRNA).[191] This can be further modified by the addition of a 5' cap which

protects it from degradation and a 3' tail which increases its stability and enhances its translational efficiency.[192, 193] Within the structure of the pre-mRNA two different components termed exons and introns exist. The exons code for a specific region of the target protein, while introns do not and are required to be removed before translation can occur. The removal of introns occurs *via* a process called splicing to produce messenger RNA (mRNA).[194, 195]
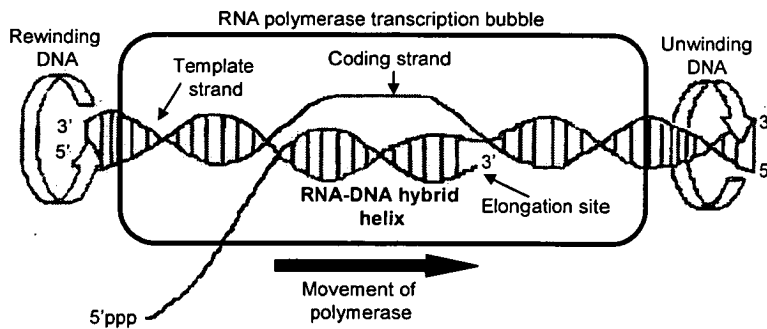
Figure 2.7: Transcription bubble illustrating the elongation of an RNA transcript. Double strand DNA is unwound at the forward end of RNA polymerase and rewound at the rear end. The RNA-DNA hybrid rotates during elongation.

2.4.2 Translation

Transfer RNA (tRNA) is a cloverleaf shaped biomolecule that contains an amino acid attachment site and a template recognition site (**Figure 2.8**).[196] Esterification of an amino acid's carboxyl group with the 2'- or 3'-hydroxyl on the ribose unit at the 3' end of the tRNA chain forms aminoacyl-tRNA. This process is catalysed by aminoacyl-tRNA synthetase and is driven by the cleavage of ATP, forming an 'activated' ester that is used as the building block in protein synthesis.[197]

On reaching the ribosome, the mRNA previously transcribed from DNA begins the next stage in protein synthesis. The mRNA contains a nucleotide sequence called a ribosomal binding site which binds to the 40S ribosomal subunit. The subunit scans the mRNA code starting from the 5' end and, on finding an initiation AUG (methionine) codon, forms an initiation complex comprising aminoacyl-tRNA and a

group of proteins called initiation factors.[198-201] The 60S ribosomal subunit then attaches to the complex forming an 80S ribosome with a mass of 4200 kDa.[202,‡]
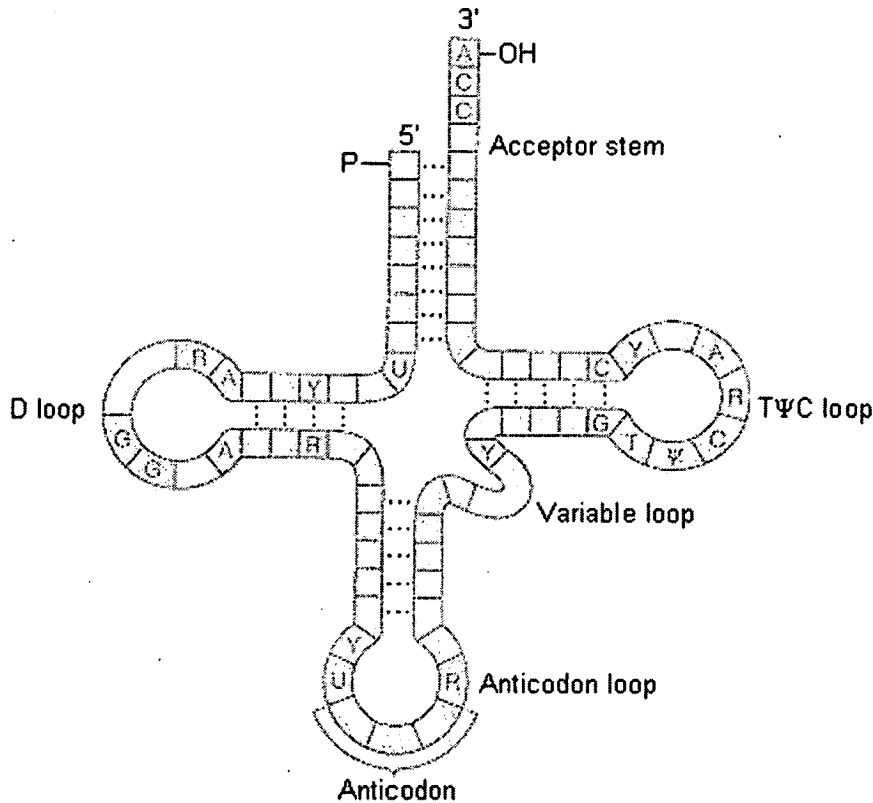
Figure 2.8: Transfer RNA (tRNA) illustrating the cloverleaf secondary structure, the anticodon loop and the 3' acceptor stem which is used to couple to amino acids.

The 80S ribosome is made up of three key sites, an acceptor site (A site), a peptide site (P site) and an exit site (E site).[134] In the first cycle the initiator tRNA linked to methionine sits in the P site, with the A site occupied by an aminoacyl-tRNA. Formation of the peptide bond can now take place, with the amine of the aminoacyl-tRNA attacking the activated ester of the initiator tRNA (**Figure 2.9**, step 2).[203, 204] The newly formed amide is now attached to the tRNA in the A site, with the uncharged tRNA now in the P site.

In order for elongation of the peptide to occur the mRNA must be translocated so that the codon for the next amino acid can be placed in the A site ready for peptide

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‡ The discussion within this section has covered protein biosynthesis in eukaryotic cells. In bacterial cells the process is similar except the 40S, 60S and 80S subunits are replaced with 30S, 50S and 70S subunits respectively.

bond formation. This occurs using the enzyme 'elongation factor G', which leads to the movement of peptidyl-tRNA to the P site, as well as removal of tRNA from the P site *via* the E site.[203, 204] On completion of this step the peptidyl-tRNA is found in the P site with the new aminoacyl-tRNA in the A site and the process is ready to begin again.[205] This sequence of processes continues along the mRNA backbone codon by codon, extending the amino acid chain of the new peptide until a stop codon on the mRNA backbone is reached. At this point a series of proteins called release factors are utilised to terminate the protein synthesis.[206] Although the precise mechanism is still to be fully elucidated it is clear that the release factors act as a Trojan horse, carrying a water molecule into the peptidyl transferase centre. Once at the reactive centre the water molecule is released and hydrolyses the ester linkage of the peptidyl tRNA, leading to the release of the polypeptide which subsequently leaves the ribosome. Finally, the 80S ribosome is dissociated by a ribosome release factor which breaks the ribosome into its individual components (tRNA, mRNA, 40S ribosome and 60S ribosome) ready to be recycled.[206]
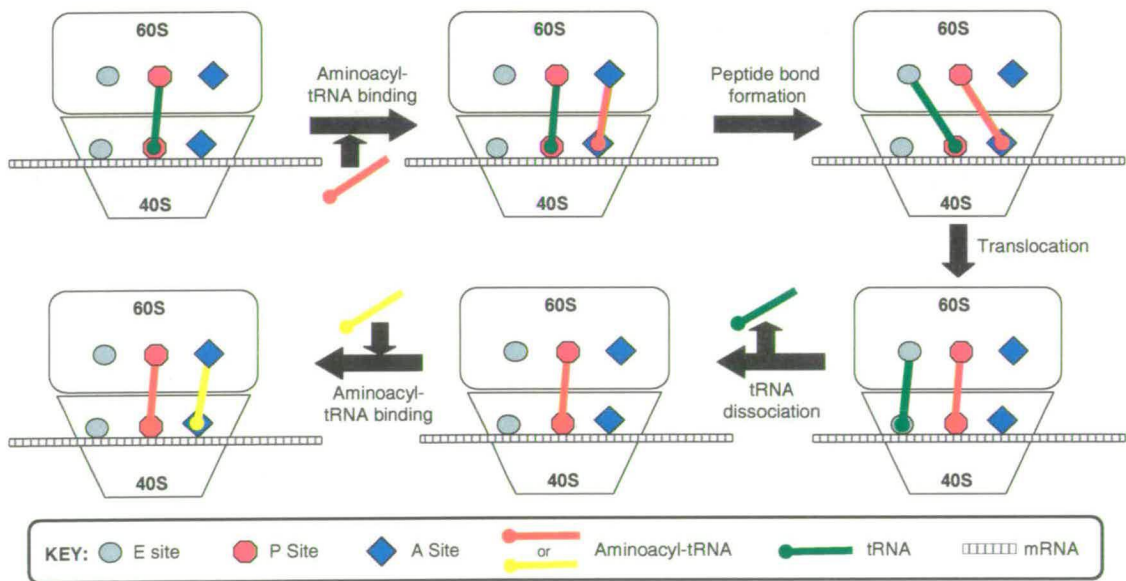
**Figure 2.9:** Mechanism of protein synthesis. The cycle begins with tRNA in the P site. An aminoacyl-tRNA then binds in the A site. With both sites occupied the peptide bond is formed. The tRNA and aminoacyl-tRNA are translocated through the action of elongation factor G, moving the tRNA to the E site and subsequently removing it. A new aminoacyl-tRNA can then occupy the A site and the sequence can begin again.

2.4.3 Protein Synthesis Inhibition

When anisomycin was first discovered it was found to be an antibacterial antibiotic against a range of fungi.[125] During the subsequent decade, research found that anisomycin is a potent, structurally specific and reversible inhibitor of protein and DNA biosynthesis.[207] On administering anisomycin at concentrations that cause 95% inhibition of protein synthesis, RNA synthesis was unaffected. However, DNA synthesis was reduced.[208] This response was believed to be a direct result of the inhibition of protein synthesis.

A series of papers published by Vazquez found that anisomycin inhibits the peptide bond formation step in a range of systems by blocking the peptidyl transferase centre in the 60S ribosomal subunit (**Figure 2.9**, step 2).[209-213] In addition anisomycin also leads to increased levels of native 60S subunits.[214] This effect is explained by the fact that the binding of native 60S subunits to the Met-tRNA-mRNA-40S initiation complex is inhibited.

More recently, crystallographic studies on the peptidyl transferase centre of the 60S ribosomal subunit have further elucidated anisomycin's role.[215] The peptidyl transferase centre in the 60S subunit contains two large hydrophobic crevices which play an important role in interactions with antibiotics. The A-site crevice is found in the peptidyl transferase active site and is the part of the A-site that interacts with amino acid side chains of A-site substrates (**Figure 2.10**). While the second crevice is located at the entrance to the exit tunnel, both crevices are strongly hydrophobic and can effectively bind small molecules.
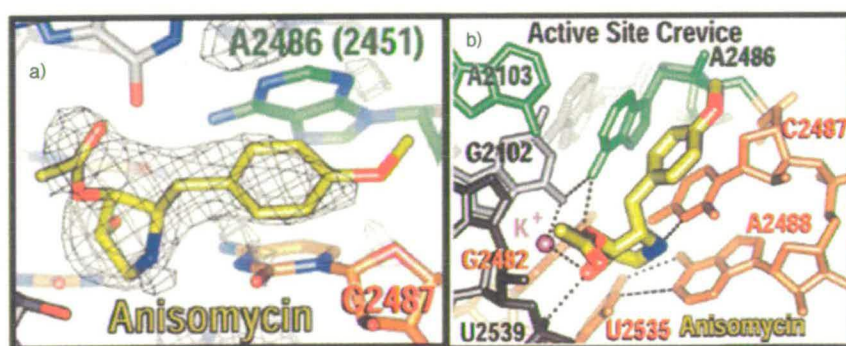
Figure 2.10: Two different angles showing anisomycin's binding site in the 60S ribosomal subunit. Interactions show ribosomal nucleotides that are protected or deprotected from chemical modification are shown in green or whose mutations confer resistance are shown in orange.[215]

Anisomycin was found to insert its aryl moiety into the A-site crevice and stack on to C2487, while its pyrrolidine ring binds into an oblong pocket next to the active site.[215] The NH forms a hydrogen bond to the N3 of C2487 and the hydroxyl group forms a hydrogen bond with U2535. With the *p*-methoxy phenyl group of anisomycin completely filling the hydrophobic crevice that is usually filled by the amino side chain of the A-site bound aminoacyl-tRNA, anisomycin can inhibit protein synthesis. The binding of anisomycin also induces a small conformational change in the peptidyl transferase centre of the ribosome, leading to a loss in non-canonical base pairing and the formation of a buckled base pair.

2.5   Summary of Anisomycin's Biological Role

Since its discovery in 1954 anisomycin has been identified as having a range of biological responses.[117, 125] Initially anisomycin was found to be an antifungal antibiotic for the treatment of amoebic dysentery and vaginitis.[117, 125] However, it was found to be inactive towards bacteria at medicinally useful concentrations.

Anisomycin has found widespread use in studies of protein synthesis both *in vivo* and *in vitro*, as it is a known peptidyl transferase inhibitor, binding to the large ribosomal subunit in eukaryotes.[209-213,215]

More recently it has been used in a number of studies investigating 'fear memories'.[216-218] These studies showed that anisomycin injections into the amygdale or hippocampus of rats after conditioning causes long term effects in contextual freezing. However, the same injections after contextual retrieval have short term reversible effects.[217]

In 1993 anisomycin was reported to show antitumour cytotoxicity *in vitro* against human tumour lines in the nM region.[118, 126] Further studies have implied that anisomycin may be used in a synergistic fashion with a cyclin-dependent protein kinase inhibitor to kill carcinoma cells.[127] Anisomycin has also been found to induce rapid apoptosis in human lymphoid cells in contrast to the delayed apoptosis induced by many other protein synthesis inhibitors that do not activate the SAPK pathways, suggesting an important role of the kinases in anisomycin induced apoptosis in tumour cells.[219-222]

This small pyrrolidine antibiotic has also been utilised as a chemical stimulant for the activation of the SAPK pathways, with a well characterised response downstream of the MAPK level.[128-133] In mammalian cells the ribotoxic stress response involves activation of the JNK and p38 pathways, with subsequent transcriptional induction of immediate early genes such as *c-jun* and *c-fos*. Above this level, the activation of the SAPK pathways and concomitantly anisomycin's mode of activation have yet to be determined. However, inhibition of protein synthesis does not stimulate the SAPK pathways, with efficient kinase activation being achieved at concentrations of anisomycin that inhibit protein synthesis by less than 50%. Therefore, the mode of action whereby anisomycin activates the SAPK pathways and induces it's antitumour activity are both unknown. Consequently, identification of anisomycin's biological target for the activation of the SAPK pathways would provide new information on the regulation of these pathways, while generating a new target for therapeutic agents.

2.6 Synthesis of Anisomycin

Anisomycin **102** and it's deacetyl precursor **106** have been synthesised over 25 times by a number of research groups over the past 35 years.[124, 126, 223-244] Previous work carried out within the Hulme group led to the synthesis of anisomycin in 13 steps with high diastereocontrol and an overall yield of 35% (**Scheme 2.1; 102→105**).[245] A further series of anisomycin analogues were then synthesised using the methodology developed during the initial work, giving a small library of twenty anisomycin based molecules.[246]
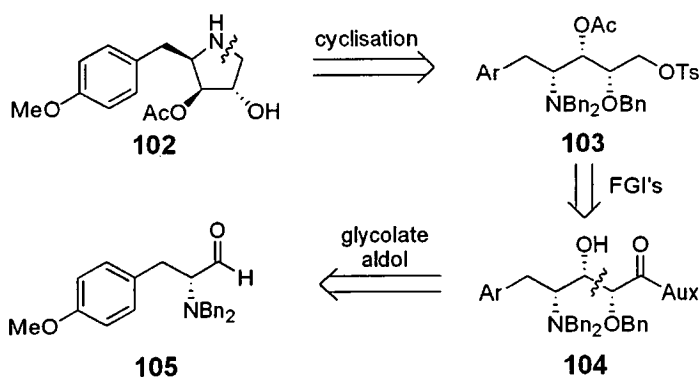
Scheme 2.1: Retrosynthesis approach to anisomycin carried out within the Hulme group.[245]

Although the precise target of anisomycin is unknown, the downstream effects on the SAPK pathways have been well documented.[128-133] The treatment of mammalian, yeast and insect cells with anisomycin is known to strongly activate both the JNK/SAPK1 and the p38/SAPK2 pathway, resulting in phosphorylation of their substrates, such as JNK and MAPKAP-K2 respectively. In order to assess the levels of activation of each compound, the small library of anisomycin analogues was tested for activation of the SAPK pathway. As commercial antibodies against both the phosphorylated and non-phosphorylated states of JNK and MAPKAP-K2 were available, it was simple to assess the activation of these pathways by the different analogues.

The structure activity relationship (SAR) produced gave an insight into the functionality required for activation of the JNK/p38 pathways. These studies showed that the presence of an ester functionality at the C_3 position on the pyrrolidine ring is essential for activity, with both the acetate (anisomycin) and propionate ester shown to be active compounds. The studies also showed that large groups (benzyl) were not tolerated on N_1 and C_4-OH. However, the most interesting discovery was the activity possessed by the C_4-Me **107** and C_4-H **108** analogues. Both of these compounds were found to be active, with the C_4-H analogue being found to show similar levels of activation to that of anisomycin.[246]

106   **107**   **108**
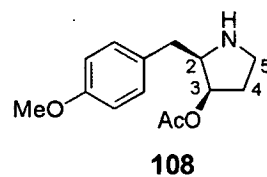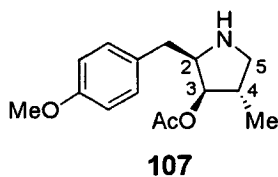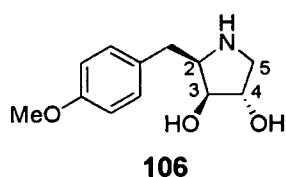
2.7   A Chemical Genetics Approach to Anisomycin

The work carried out on anisomycin can therefore be categorised in a forward chemical genetics sense;[247-249] whereby a small library of analogues have been produced and an initial SAR determined for the SAPK phenotype.[246] However, the biological target of anisomycin has yet to be determined.

Despite rapid advances in the generation and testing of small molecule libraries in recent years, current strategies for the identification of the biological targets of library members in forward chemical genetics screens still require the synthesis of an

appropriate molecular probe. The structure of this molecular probe is typically based on an active library member which has been modified by introduction of a tag, such as a biotin moiety, or a photoactivatable-, radio-, or fluorescent label (**Figure 2.11**).
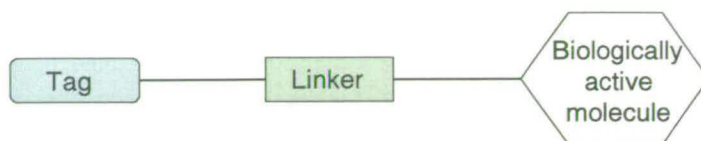
Figure 2.11: Model of a molecular probe comprising tag, linker and biologically active molecule, in this case anisomycin, or an anisomycin analogue.

The synthesis of a series of anisomycin molecular probes was therefore envisaged. Testing of the molecular probes would then take place to assess if activation of the SAPK phenotypic response had been retained, before utilising the molecular probes to identify and isolate the biological target of anisomycin.

However, the synthesis of molecular probes can often be a cumbersome process; the National Institute of Health's drug development timeline (**Figure 2.12**) published in 2002 has allocated a year to go from the initial hit to the successful synthesis of a molecular probe.
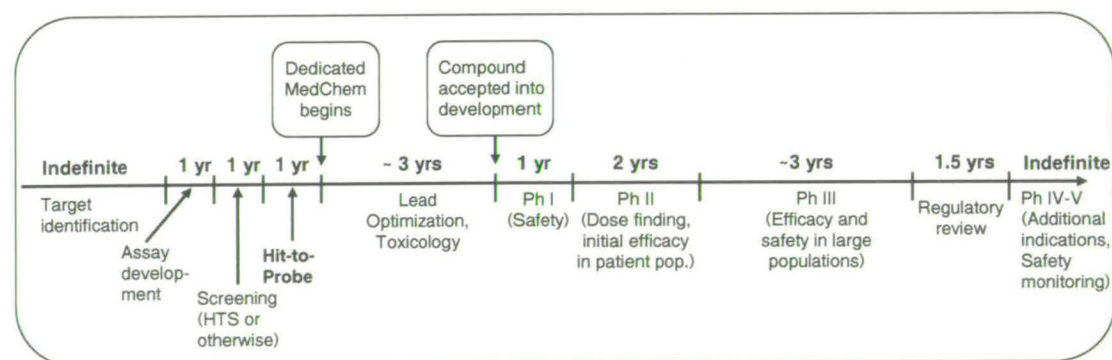
Figure 2.12: National Institute of Health's (NIH's) drug development timeline; illustrating the process involved in drug development from taking an initial target to a marketable drug and the timescales involved.

More often that not, the synthetic route to the active library member may have to be redesigned to allow tagging of the small molecule at various sites around its molecular scaffold (**Figure 2.13**, part 4). Moreover, a number of the molecular

probes synthesised in this manner will have lost their activity due to structural perturbations upon addition of the tag (**Figure 2.13**, part 5). These factors combined can make the synthesis of molecular probes a time consuming and challenging process.
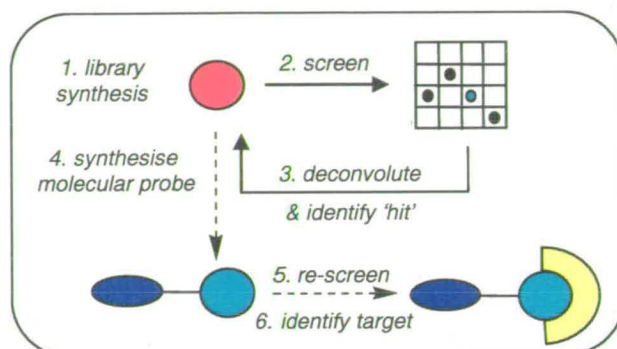
Figure 2.13: Strategies for small molecule library design. Traditional approach involving library synthesis, biological screening, synthesis of molecular probes, re-screening and target identification.

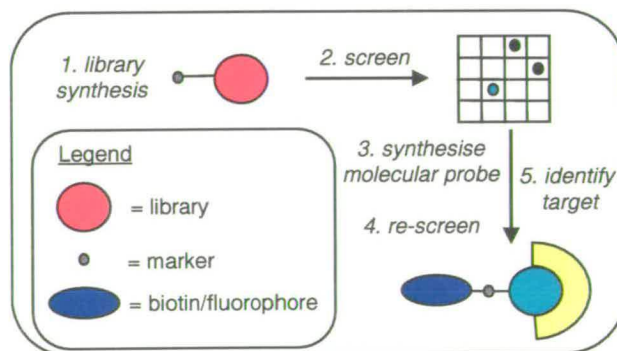Therefore, a more direct approach has been designed which is based on the concept of a marked library, where each individual library member carries a small biocompatible marker which plays no role in the screening process itself, but may be used in the target identification process once screening is complete (**Figure 2.14**).

Figure 2.14: Strategies for small molecule library design. Marked library strategy involving library synthesis incorporating a biocompatible marker, biological screening followed by target identification.

Although this is a new approach to small molecule library design, this concept has precedent in chemical biology, where for example modified amino acids, sugars *etc.* with chemical markers such as azides, alkynes and phosphines *etc.*, have been

incorporated into a range of biomolecules.[95, 96] These markers may then be coupled to orthogonally reactive tags such as biotin, fluorophores, *etc*. utilizing biologically compatible reactions such as the Staudinger-Bertozzi ligation, and the copper(I) catalysed Huisgen 1,3-dipolar cycloaddition, thus allowing the visualization, purification and identification of the biomolecule of interest. In a closely related example to our proposed marked small molecule library strategy, Chang *et al*. have designed a triazine library with a built-in linker containing an amino functionality to facilitate target identification.[250] More recently, Cravatt has generated a natural product analogue library based on a protein-reactive moiety with a pendant alkynyl functionality; allowing subsequent target identification after functionalisation with a fluorescent, and/or biotinylated azide.[99]

2.8   A Marked Library Approach to Anisomycin

We therefore set out to synthesise a focused library of marked compounds based upon the molecular architecture of anisomycin to prove the concept of a marked library strategy and to further investigate the SAR for this small molecule's activation of the SAPK pathways. The previous SAR data indicated that two key sites existed within anisomycin's scaffold that may allow incorporation of the marker; they were the pyrrolidine nitrogen and the phenolic oxygen (**Figure 2.15**).
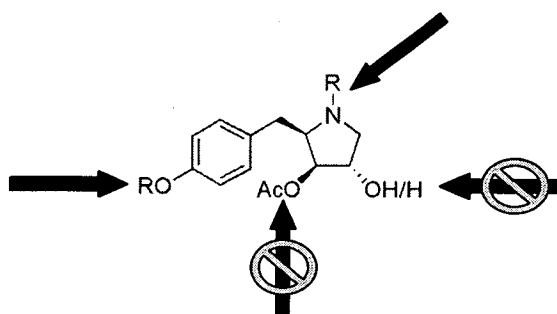
Figure 2.15: Potential points of attachment for 'tags' that have been proposed and/or ruled out based on structure activity relationship data for anisomycin's activation of the SAPK pathways.

It was proposed that adaptation of the previous synthetic route to the C_4-H analogue **108** carried out within the Hulme would allow incorporation of a silyl protecting

group in place of the methyl on the phenolic oxygen. This would allow for selective unveiling of the pyrrolidine nitrogen or the phenolic oxygen and addition of the marker to this functionality later in the synthesis (**Scheme 2.2; 109→112**).
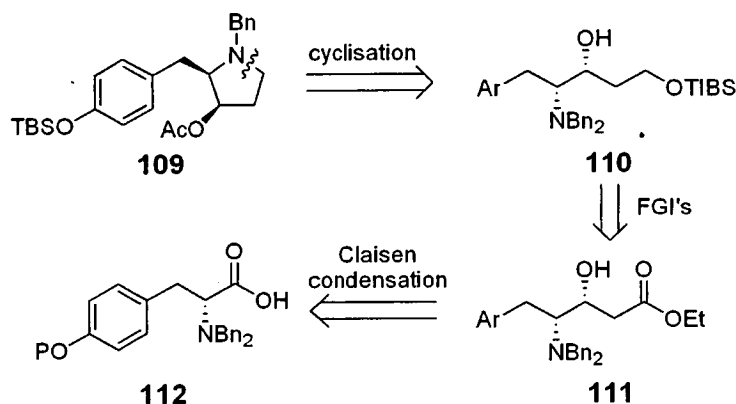
Scheme 2.2: Retrosynthetic analysis of the C_4-H analogue of anisomycin.

2.8.1 A Marked Library Approach to the C_4-H Analogue

Commercially available D-tyrosine **113** was converted to its methyl ester hydrochloride salt **114** quantitatively by refluxing the amino acid in methanol and acetyl chloride (**Scheme 2.3**). The amine **114** was then selectively protected using di-*tert*-butyl dicarbonate in the presence of sodium bicarbonate to give **115** in a quantitative yield. Treatment of **115** with *tert*-butyldimethylsilyl triflate in the presence of 2,6-lutidine gave the TBS-protected phenol **116**. The Boc protecting group was then removed by stirring **116** in TFA overnight, to give the free amine **117** in 100% yield. The amine **117** was then converted to the *N,N*-dibenzylamine **118** by reaction with benzyl bromide and potassium carbonate.
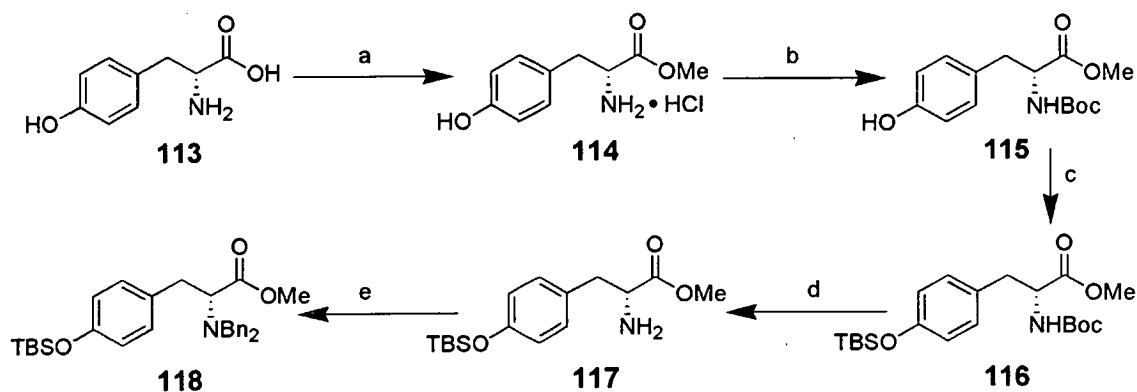
Scheme 2.3: (a) AcCl, MeOH, reflux (100%); (b) Boc$_2$O, NaHCO$_3$, DCM (100%); (c) TBSOTf, 2,6-lutidine, DCM (60%); (d) TFA, DCM (100%); (e) BnBr, K$_2$CO$_3$, CH$_3$CN (71%).

At this point it was believed that lithium hydroxide hydrolysis would successfully yield the functionalised acid required for a Claisen condensation. However, heating a solution of **118** in THF:water with lithium hydroxide gave a mixture of products (**Scheme 2.4; 119** and **120**). Attempts to purify this mixture proved to be problematic and led to a loss of material. Analysis of the crude ^{1}H NMR showed two compounds, the first was the desired acid **119**, while the second was the acid with loss of the TBS protecting group **120**. The integrals of the ^{1}H NMR spectrum showed that **119** was approximately 60%, with the other 40% being the deprotected compound **120**. Attempts to improve the yield of the desired product **119** by altering reaction temperature and time were found to have no effect on the yield. Investigation of the recent literature showed that LiOH had been found to selectively deprotect aryl silyl ethers when stirred in DMF,[251] therefore it is highly conceivable that LiOH would carry out the same reaction in aqueous THF. As a result, it was decided to investigate other routes to the Claisen precursor **119**.
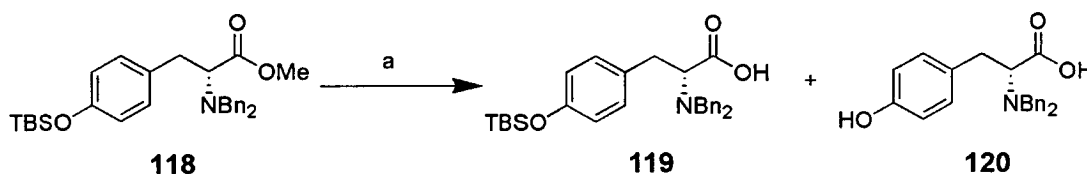
Scheme 2.4: LiOH, reflux, THF: H_2O (95%, **119:120**, and 60:40).

2.8.2 An Alternative Route to the C_4-H Analogue

A new route was therefore developed ensuring that the phenol protection was the final step in the synthesis of the acid **119**, thus removing the possibility of base-induced deprotection of the phenol (**Scheme 2.5**). Once again, starting from D-tyrosine **113**, the methyl ester hydrochloride salt **114** was formed quantitatively. The hydrochloride salt **114** was then converted to the dibenzylamine by refluxing **114**, benzyl bromide and diisopropylethylamine in THF, to give **121** in 85% yield. The ester **121** was then hydrolysed to the acid **120** by heating with lithium hydroxide to give product as a single spot by tlc. Previous studies within the Hulme group have found that when lithium hydroxide was employed as a base for similar hydrolysis reactions it gave the highest yields with the lowest levels of racemisation.[252] The

TBS functionality was then added using two equivalents of TBSOTf to form the di-protected species **122**. This was followed by stirring **122** in acetic acid:THF:water for 8 h to remove the TBS group selectively from the carboxylic acid to give the desired product **119** in 91% yield. Thus giving the tyrosine derived acid **119** required for the Claisen condensation in four high yielding steps.
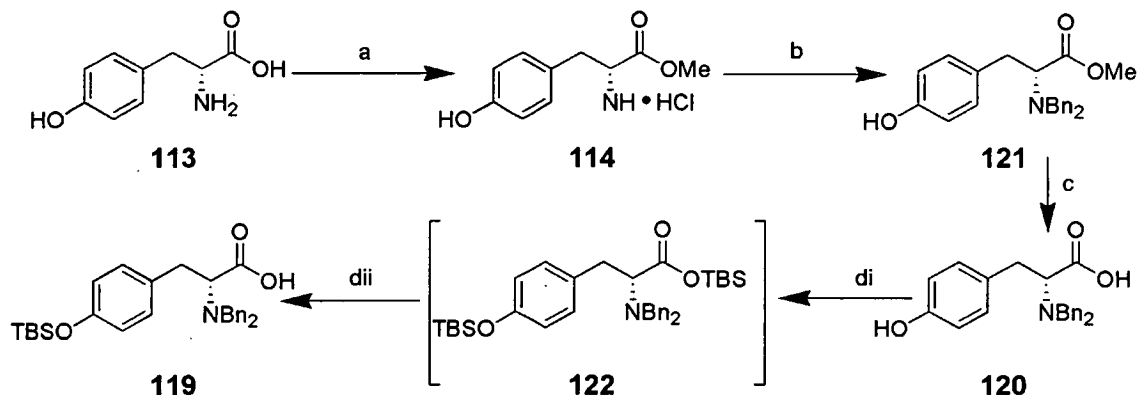

Scheme 2.5: (a) AcCl, MeOH, reflux (100%); (b) BnBr, DIEA, reflux (85%); (c) LiOH, reflux, THF:H_2O (87%); (d) i) TBSOTf, 2,6-lutidine, DCM; ii) AcOH:THF:H_2O [3:1:1] (91%).

The decision to convert the methyl ester to the imidazolide prior to Claisen condensation was based on work carried out by Hoffman.[253] He showed that β-keto esters formed *via* the reaction of α-amino esters with lithium enolates were subject to a loss in enantioselectivity. This was confirmed by work previously carried out within the Hulme group on the Claisen condensation of a serine-derived methyl ester.[252] However, conversion of the methyl ester to the imidazolide was found to reduce racemisation substantially.

The Claisen condensation was initiated by the formation of the imidazolide **123** which was produced *in situ* by reaction of the acid **119** with carbonyldiimidazole (CDI) in THF (**Scheme 2.6**). Meanwhile, the lithium enolate of ethyl acetate was formed by addition of lithium hexamethyldisilazide (LHMDS) in THF to freshly distilled ethyl acetate at -78 °C. The enolate was then cannulated into a solution of the imidazolide at -78 °C. A variety of reaction conditions were investigated, varying the time, temperature, and the species being cannulated. The best yields were found to occur when the reaction was stirred at -78 °C for 40 min after cannulating,

warming to 0 °C over 30 min and stirring at 0 °C for a further 1.5 h. This gave the β-keto ester **124** in 68% yield and allowed the reaction to be carried out on a 5 g scale.
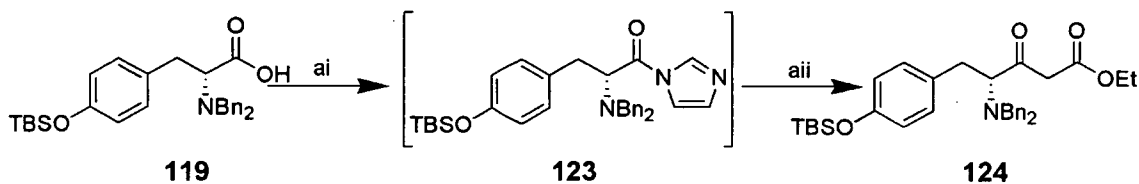
Scheme 2.6: (a) i) CDI, THF; ii) LHMDS, EtOAc → CH₂C(OLi)OEt (68%).

Precedent within the group has also shown that sodium cyanoborohydride is the most effective reagent for the stereoselective reduction of β-keto esters giving >95:5 diastereoselectivity.[252] Therefore, the β-keto ester **124** was treated with sodium cyanoborohydride to give the alcohol **126** in 80% yield, with no reduction of the ester moiety (**Scheme 2.7**).
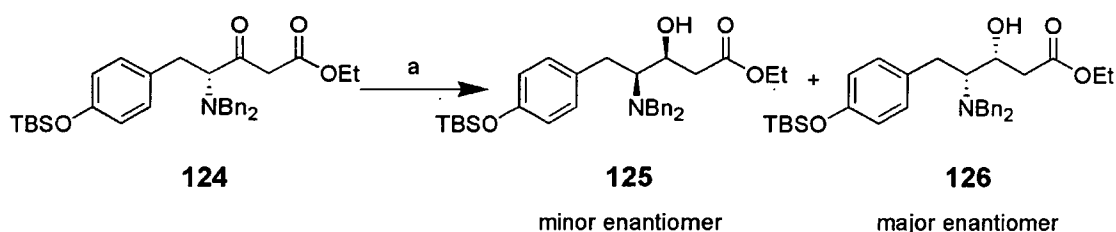
Scheme 2.7: (a) NaCNBH₃, MeOH, AcOH, EtO₂ (80%).

The diastereoselectivity of the sodium cyanoborohydride reduction can be rationalised using a Felkin-Ahn model for the nucleophilic attack of a sp² hybridised centre. Placing the large electronegative dibenzyl protected amine perpendicular to the carbonyl forms the most reactive conformer with best orbital overlap. Therefore, attack by the nucleophile at the Burgi-Dunitz angle of 109° forms the desired product **126** with high stereoselectivly (**Scheme 2.8**).
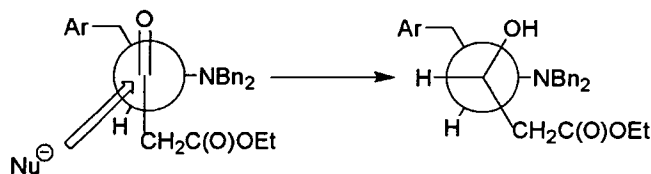
Scheme 2.8: Felkin-Ahn model for the stereoselective reduction of β-keto esters.

At this point a racemic synthesis of the β-hydroxy ester from (±)-tyrosine was carried out so that its enantiopurity could be determined. Analysis was carried out by chiral HPLC which gave two peaks of equivalent area for the racemic compound, and a major and minor peak for the enantiomerically enriched material. The enantiomeric excess of **126** was calculated to be 66% ee, thus showing that the chiral centre of the amino acid had epimerised prior to the Claisen condensation. It was suspected that in this case the use of Hunig's base in the dibenzylation step could have lead to the loss in enantiopurity as it is widely used in aldol type reactions for deprotonation alpha to a carbonyl group.

2.8.3 A Modified Route to the C$_4$-H Analogue

At this point it was decided to investigate the dibenzylation of the hydrochloride salt **114** in detail. Therefore a new dibenzylation strategy was developed utilising a milder base, so that the new reaction conditions could be applied to the previous route to give the desired acid **119**, without a loss in enantiopurity.

Previous work within the group has shown that the dibenzylation of tyrosine, although appearing trivial, is a tricky reaction to carry out in practice, leading to a mixture of mono-, di- and tri-benzylated species in varying yields.[254] The standard method of forming *N,N*-dibenzylamino acids (including serine and threonine derivatives) was to react five equivalents of potassium carbonate with four equivalents of benzyl bromide in acetonitrile. However, on reacting **114** under these conditions not unsurprisingly the tri-benzylated species **127** was formed in high yields (**Table 2.1,** entry 1). Therefore, the number of equivalents of base and benzyl bromide was reduced to 3.0 and 2.1 respectively; leading to a moderate yield of the desired product **121** (**Table 2.1,** entry 2). Various other solvent systems were then tried as the solubility of the starting material in acetonitrile was questioned. However, no improvement was made. On returning to acetonitrile the yields of the reaction were increased on adding the reagents sequentially; base first, followed 30 min later by benzyl bromide. The fully optimised conditions were found to give yields of consistently greater than 75% (**Table 2.1,** entry 5).
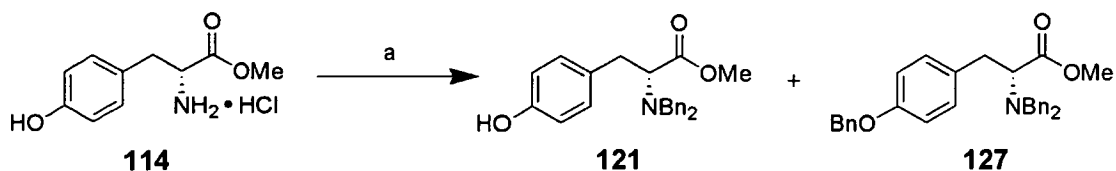
| Entry	Eqv K_2CO_3	Eqv BnBr	Solvent	Yield **121**	Yield **127**
1	5	4	CH_3CN	62	30
2	3	2.1	CH_3CN	61	-
3	3	2.1	DMF	45	-
4	3	2.1	H_2O	20	-
5	3	2.3	CH_3CN	76	-

Table 2.1: Table of results for dibenzylation of **114**. (a) BnBr, K_2CO_3, CH_3CN.

The new benzylation methodology was then applied to the previous synthetic route, giving the acid **119** in four steps from D-tyrosine **113**; the carbon chain was then extended *via* Claisen condensation as previously described (**Scheme 2.9; 119→124**).
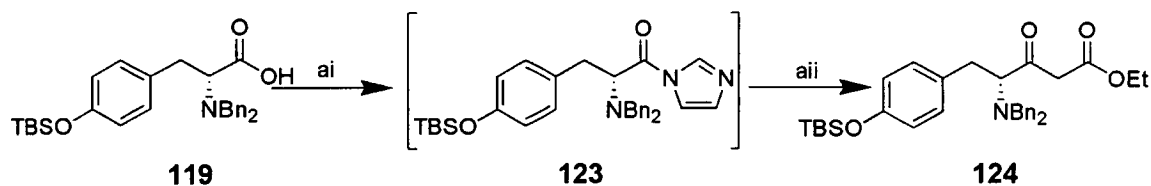
Scheme 2.9: (a) i) CDI, THF; ii) LHMDS, EtOAc → $CH_2C(OLi)OEt$ (79%).

The β-keto ester **124** was then reduced using sodium cyanoborohydride to give the β-hydroxy ester **126** (**Scheme 2.10**). On this occasion analysis by chiral HPLC gave a major peak that was calculated to have an >97% ee (**Apendix 2**). This allowed the conclusion to be drawn that the previously observed epimerisation of the derived amino acid was due to the use of diisopropylamine.
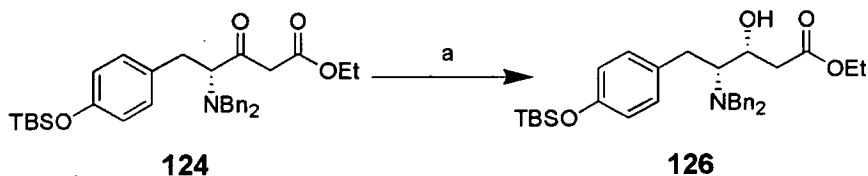
Scheme 2.10: (a) $NaCNBH_3$, MeOH, AcOH, Et_2O (86%).

The β-hydroxy ester **126** was then reduced using lithium aluminium hydride to give the corresponding diol **128** (**Scheme 2.11**). The work-up for this reaction was carried out by quenching with 1M NaOH, dilution with DCM and addition of saturated sodium potassium tartrate which was stirred vigorously overnight in order to remove the aluminium residues and give an optimised yield of 97%.

**126**   **128**
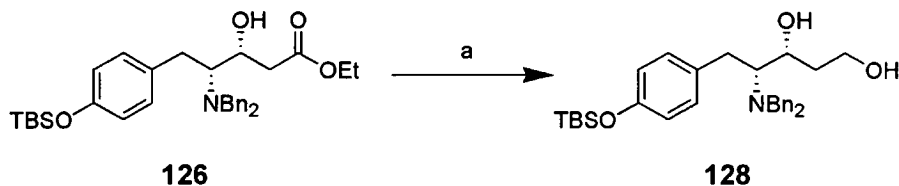
**Scheme 2.11:** (a) LiAlH$_4$, THF (97%).

Pyrrolidinium formation was effected by activation of the primary alcohol **128** with triisopropylbenzenesulfonyl chloride (TIBSCl) and DMAP. This reaction proceeds *via* formation of the TIBS-sulfonate, by selective reaction of TIBSCl with the primary alcohol. The lone pair on the amine then acts as an intramolecular nucleophile attacking the C$_1$ position forming the pyrrolidine ring and ejecting the TIBSO$^-$ as a leaving group in a 5 *exo tet* cyclisation (**Scheme 2.12**). However, these reaction conditions led to a mixture of two salts with different counter ions balancing the tetravalent nitrogen. The compounds were therefore subjected to ion-exchange chromatography on a column that had been pre-treated with 1% hydrochloric acid. This gave the chloride salt **129** in 96 % yield.

128   **129**
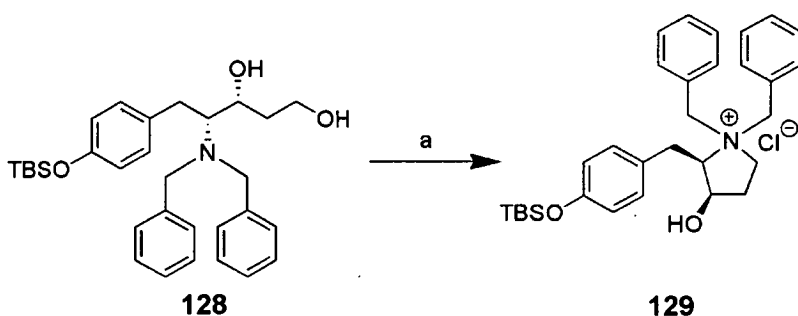
**Scheme 2.12:** (a) i) TIBSCl, DMAP, DCM; ii) Dowex (Cl$^-$) ion-exchange resin (96%).

Analysis of the ^{1}H NMR of **129** proved to be interesting as it contained many unusual signals associated with a dibenzylated amine (**Figure 2.16a**). Under normal

conditions a dibenzylated amine has a large multiplet around 7.30 ppm that integrates for ten protons, and two doublets (3-4 ppm) that correlate to two protons each, with a geminal coupling constant of approximately 14.0 Hz. However, the ^{1}H NMR spectrum of **129** contained two doublets (5.59 and 4.10 ppm, 13.0 Hz) that integrated for one proton each and a multiplet at 4.95 ppm that accounted for the other two benzylic protons. Moreover, the 10 proton aromatic multiplet ranged from 7.6-7.2 ppm. The unusual nature of this spectrum can only be explained by the tetravalent nitrogen attached to the two benzyl groups.
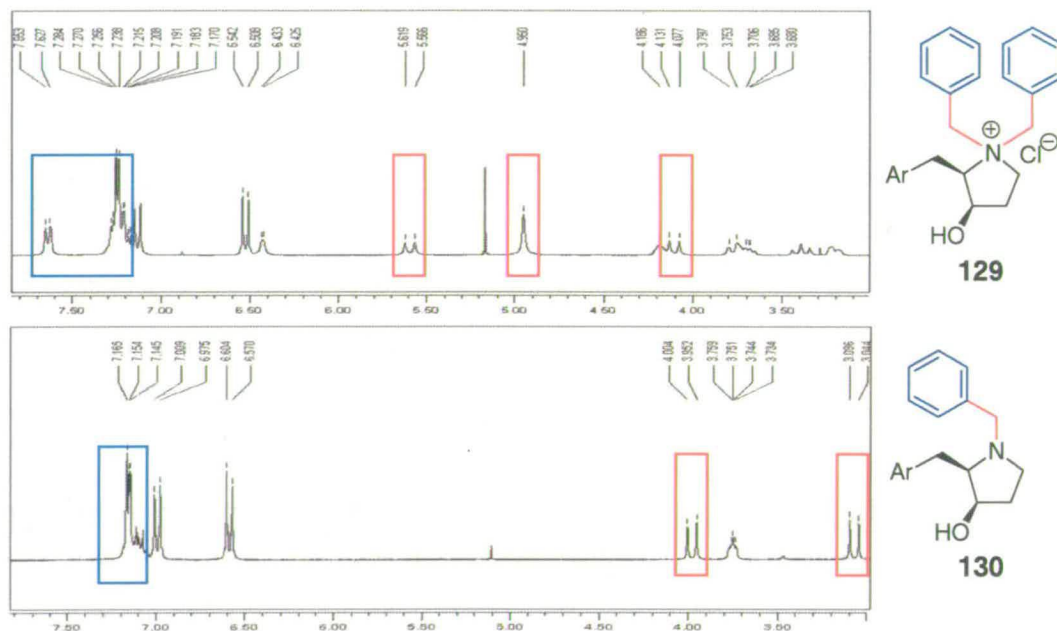
Figure 2.16 : ^{1}H NMR (250 MHz) of **129** and **130**.

Careful hydrogenation of the dibenzyl compound **129** under 1 atm of hydrogen in the presence of 5% Pd/C and potassium carbonate for only 25 min gave the monobenzylated compound **130** as a colourless foam in 89% yield (**Scheme 2.13**). Analysis of the ^{1}H NMR of **130** showed that the signals associated with the benzylic CH$_2$'s group had returned to the more familiar pair of doublets (13.0 Hz), each integrating in this case for a single proton (**Figure 2.16b**).
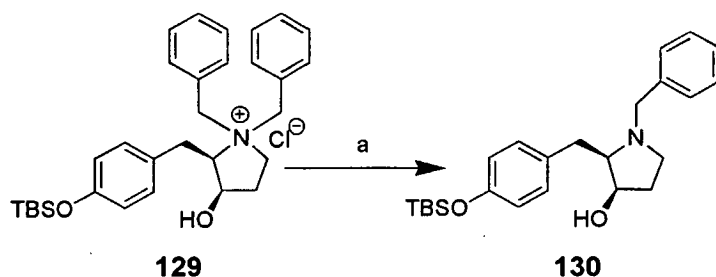
Scheme 2.13: (a) H$_2$, 5% Pd/C, K$_2$CO$_3$, MeOH, 25 min (89%).

Addition of the acetate was then carried out by reaction of the secondary alcohol **130** with acetic anhydride, triethylamine, and a catalytic amount of DMAP, to give **109** in 96% yield after purification by silica gel chromatography (**Scheme 2.14**).
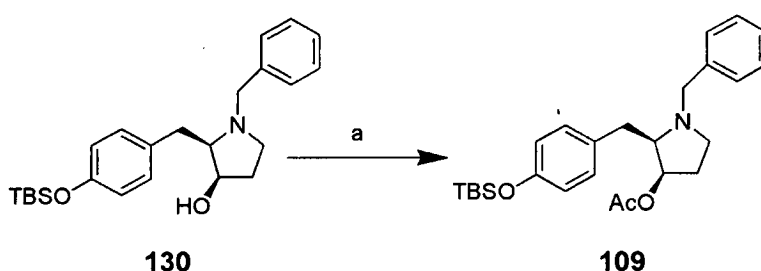
Scheme 2.14: (a) Ac$_2$O, Et$_3$N, DMAP, DCM (96%).

At this point a protecting group switch was carried out by removing the benzyl group and replacing it with a *tert*-butoxy carbamate. This was proposed to remove any complications with the final deprotection step once the C$_4$-H analogue had been marked. Thus, allowing the final synthetic step of the marked library members to be a straightforward deprotection of the amine under mild acid conditions, to give the desired product.

An initial attempt to remove the benzyl protecting group and form the HCl salt *in situ*, was carried out *via* hydrogenation of **109** with Pearlman's catalyst in the presence of ethereal hydrochloric acid (**Scheme 2.15**). This led to the removal of both the benzyl and the silyl protecting groups to give **131**. At this point it was believed that the ethereal hydrochloric acid was facilitating the removal of the TBS protecting group. However, when the reaction was carried out in the absence of the acid, the TBS group was still found to be labile. Altering the solvent from methanol to ethanol was found to be the most suitable solution to this issue, leading to the

selective removal of the benzyl protecting group over the TBS protecting group to give the desired product **132** in high yield (**Scheme 2.15**).
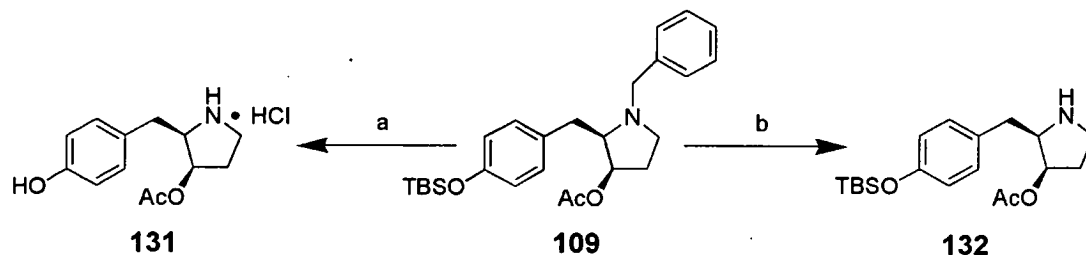
Scheme 2.15: (a) H_2, $Pd(OH)_2$, 1M HCl in Et_2O, MeOH (100%); (b) H_2, $Pd(OH)_2$, EtOH (98%).

The amine **132** was then reprotected by reacting it with di-*tert*-butyl dicarbonate in the presence of triethylamine to give compound **133** (**Scheme 2.16**). Thus, allowing the protecting group switch to take place in near quantitative yield and providing the key advanced stage intermediate required for the synthesis of the C_4-H marked library.
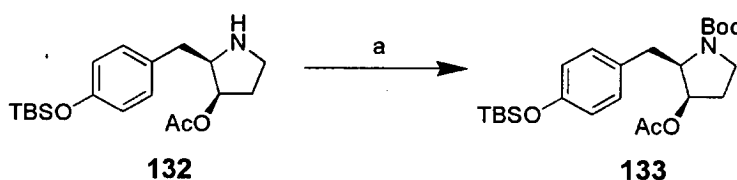
Scheme 2.16: (a) Boc_2O, Et_3N, DCM (97%).

At this point it was decided to make the copper catalysed Huisgen 1,3-dipolar cycloaddition of an azide and a terminal alkyne the key reaction to couple together the tag and the marked library member (discussed in Chapter 3). It was believed that the incorporation of the small, non-polar, bioorthogonal propargyl moiety would cause the least perturbation to the molecular scaffold, while providing the required handle that would allow incorporation of a fluorescent or biotin tag at a later date.

2.8.4 C$_4$-H *O*-Marked Analogues

Careful manipulation of the protecting groups was then carried out in order to obtain the desired marked compound. Removal of the TBS protecting group from **133** was carried out using triethylamine buffered hydrofluoric acid to give phenol **134** in 96% yield (**Scheme 2.17**).
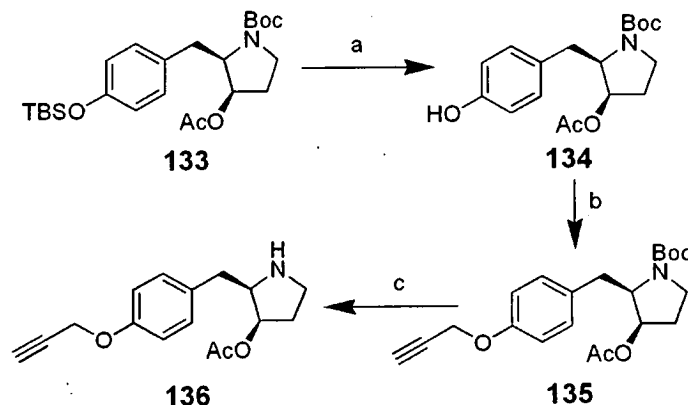
Scheme 2.17: (a) HF•3Et$_3$N, THF (96%); (b) HC≡CCH$_2$Br, K$_2$CO$_3$, DMF (99%); (c) TFA, DCM (97%).

The phenol **134** was converted to the propargyl ether **135** by reaction with potassium carbonate and propargyl bromide in high yield (**Scheme 2.17**), before deprotection with TFA to give the *O*-propargyl compound **136** in three high yielding steps. Incorporation of the propargyl moiety into the molecular scaffold of the anisomycin analogue was confirmed by NMR which showed two distinctive peaks in the proton spectra. A fine doublet at 4.63 ppm with a small coupling constant of 2.4 Hz was shown for the CH$_2$. The signal associated with the CH of the propargyl functionality was a triplet at 2.50 ppm with 2.4 Hz coupling constant (**Appendix 3**). Thus, confirming that marking of the anisomycin analogue's structure had been carried out successfully.
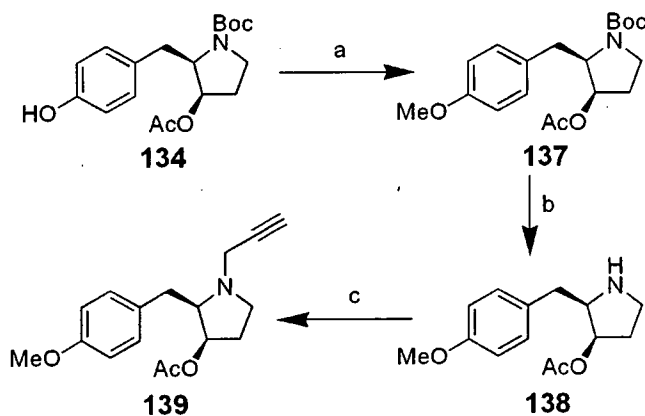
2.8.5 C$_4$-H *N*-Marked Analogues

We then turned our attention to the synthesis of the *N*-marked analogue from **133**, which proved to be rather more complicated. Initial attempts focused on sequential removal of the Boc protecting group, propargylation of the amine and removal of the

silyl protecting group, all which could be carried out in near quantitative yields. ·
However, attempts to methylate the phenol with methyliodide and potassium
carbonate led to quaternisation of the pyrrolidine nitrogen. Manipulation of the
methylation conditions by reducing the volume of methyl iodide to a single
equivalent also led to a complicated mixture of products. It was believed that the
greater nucleophilicity of the pyrrolidine nitrogen still led to the quaternisation of
this centre, thus making this route unfavourable.

It was therefore decided to retain the carbamate as a method of masking the amine
until the phenolic oxygen had been methylated. Consequently, the *N*-propargyl
compound was synthesised from the phenol **134** by reaction with methyl iodide and
potassium carbonate to form **137** (**Scheme 2.18**). The amine **138** was then revealed
quantitatively by removal of the Boc functionality from **137** with TFA. Addition of
the propargyl group under standard conditions (propargyl bromide, potassium
carbonate, DMF) went to completion and could be monitored by tlc and mass
spectrometry. However, isolation of this material proved problematic and led to a
very low yield. Altering the base from potassium carbonate to solid supported
piperidine and therefore removing the aqueous work-up, led to isolation of the
marked propargyl analogue **139**. This was followed by purification on silica gel
chromatography to give **139** in 93%.

Scheme 2.18: (a) MeI, K$_2$CO$_3$, DMF (74%); (b) TFA, DCM (100%); (c) HC≡CCH$_2$Br, piperidinomethyl resin, DCM (93%).

2.9   Anisomycin Marked Analogues

Anisomycin **102** was converted to **140** by reaction of one equivalent of propargyl bromide with anisomycin in the presence of potassium carbonate (**Scheme 2.19**). This proved to be a gratifying result as we were initially concerned about the selectivity of propargyl addition, as this could have occurred at the pyrrolidine nitrogen and/or the C_4OH. A series of NMR experiments were therefore carried out as a means of confirming the connectivity of the propargyl marker to anisomycin's pyrrolidine ring (**Appendix 3**). Using 2D-NMR to interrogate the structure of **140**, the direct proton carbon correlation was confirmed by COSY and HSQC (**Figure 2.17**). The experiments confirmed that the propargyl moiety had been incorporated at only one site within the molecules scaffold, as well as illustrating the very large splitting (0.72 ppm) between the two diastereotopic protons at the C_5 carbon centre.
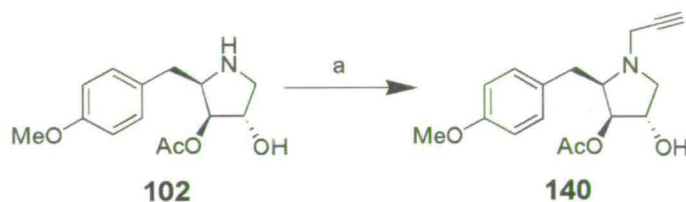
Scheme 2.19: (a) HC≡CCH$_2$Br, K$_2$CO$_3$, DMF (95%).
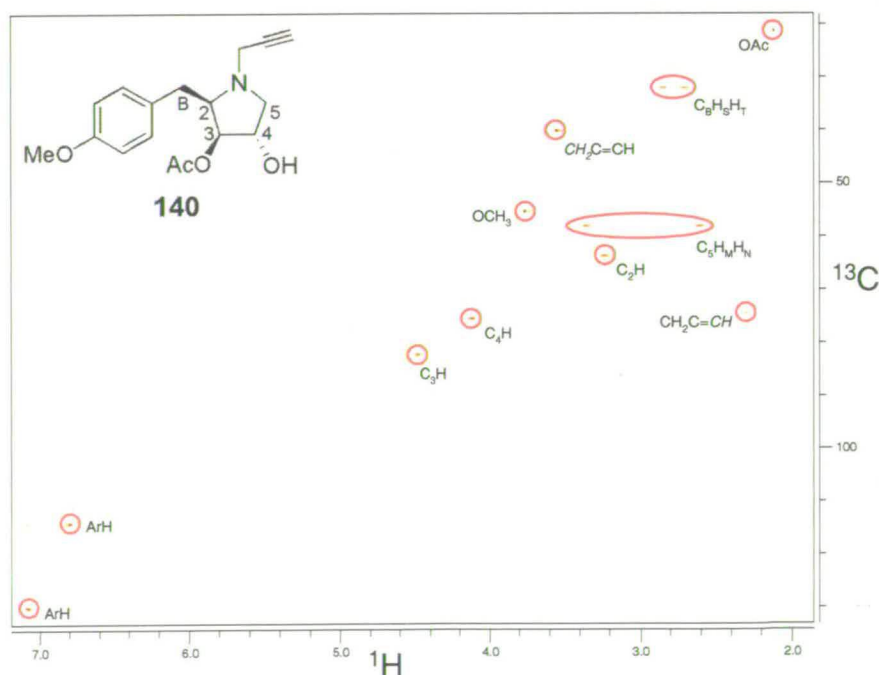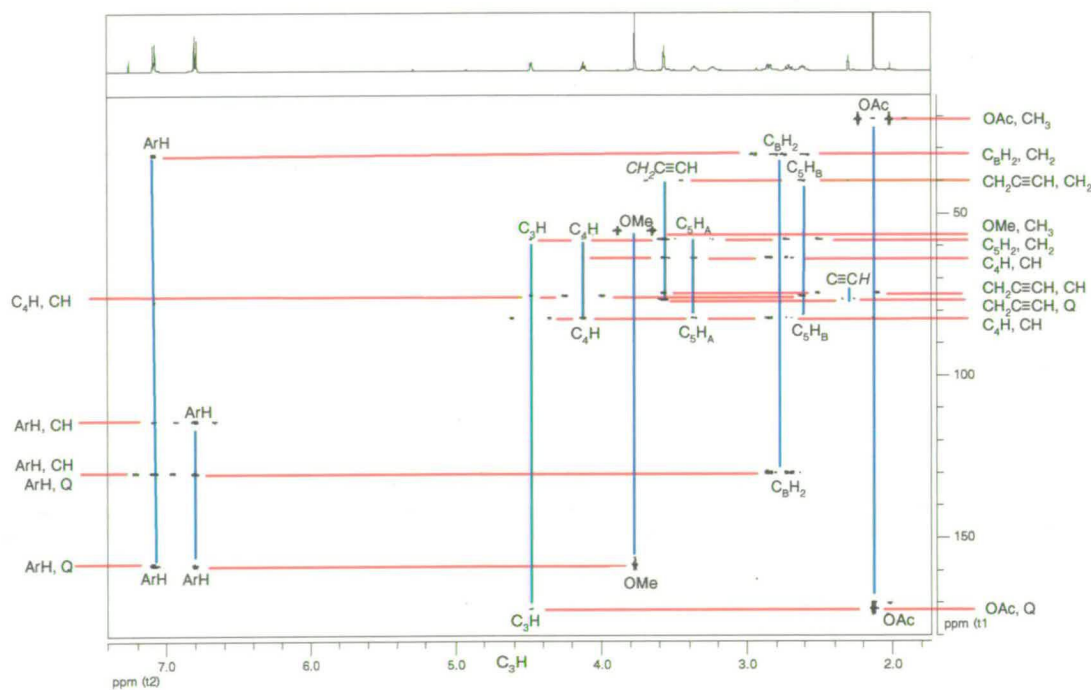

**Figure 2.17:** Fully assigned HSQC spectrum of **140** at 600 MHz.

It was believed that analysis of the HMBC spectrum of compound **140** would confirm the connectivity of the propargyl unit (**Figure 2.18**), as this experiment shows correlations of up to four bonds. A distinct correlation between the C_5H_2 and the CH_2 from the propargyl unit was observed, while there was no apparent correlation from the propargyl unit to the C_4H. This result was confirmed by a 1D-NOE experiment which on irradiation of the propargyl CH_2 brings about enhancement of the C_5H_2, C_2H, and $C_BH_AH_B$ signals, confirming the *N*-propargylation of anisomycin **140**.

Figure 2.18: Fully assigned HMBC spectrum of **140** at 600 MHz.

Having successfully synthesised the propargyl compound **140** it was decided to further probe the SAR of anisomycin by synthesising the mono-*N*-benzyl analogue. Previous results within the group have shown that the di-*N,O*-benzyl compound shows no phenotypic response for SAPK activation. Therefore, testing the *N*-benzyl compound would clarify the allowed positions of substituents around the pyrrolidine ring. Anisomycin **102** was reacted under analogous alkylation conditions, with benzyl bromide and potassium carbonate; purification by column chromatography gave the desired product **141** in high yield (**Scheme 2.20**). As carried out previously,

the regiochemistry of the benzyl group was confirmed by a series of NMR experiments which showed the reaction proceeded exclusively at the pyrrolidine nitrogen.
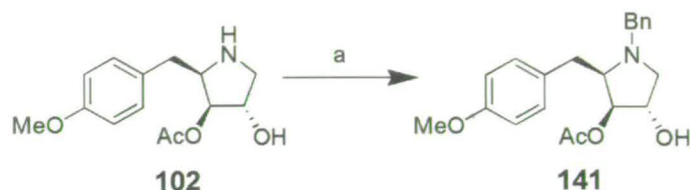

Scheme 2.20: (a) BnBr, K_2CO_3, DMF (89%).

2.10 Introduction to Western Blotting

With the synthesis of a new series of anisomycin analogues complete, the levels of activation of the SAPK pathway were tested to see if the modifications carried out to the structures had led to retention of SAPK phenotype activity.

Human embryonic kidney (HEK) 293 cells were incubated with 9 µl of a 38 mmol stock solution of anisomycin, DMSO or anisomycin analogue for 30 minutes at 37.5 °C. The cells were then lysed with triton cell lysis buffer and the concentration of protein determined by Bradford assay.[255] The lysates (20 mg) were then separated by sodium doceyl sulfate polyacylamide gel electrophoresis (SDS PAGE), before transfer to nitrocellulose membranes (**Figure 2.19,** left hand column).
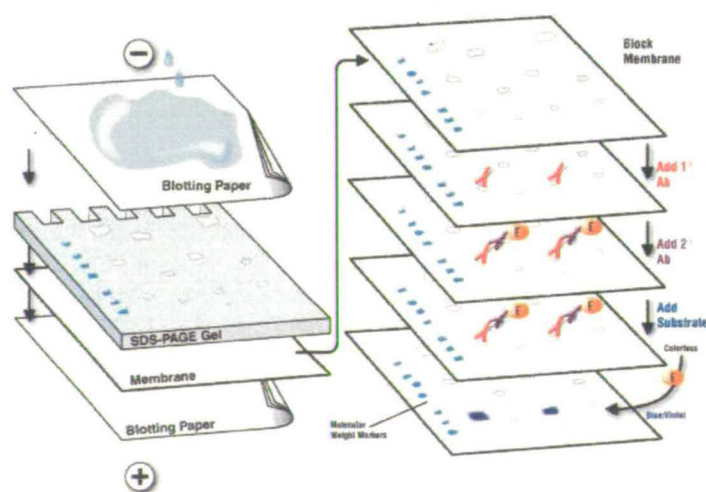
Figure 2.19: Overview of Western Blotting: Separation of cell lysates by SDS-PAGE, transfer of proteins to nitrocellulose membranes (left hand column), blocking of membrane followed by incubation with primary antibody, then incubation with secondary antibody and detection using ECL reagent (right hand column).[256]

The nitrocellulose membranes were then blocked in a milk solution for 1 hour, to prevent non-specific binding (**Figure 2.19**, top of right hand column). The primary antibody solution specific for phosphorylated JNK (pJNK) was then incubated with the membranes for 15 hours, before washing the membranes to remove excess primary antibody. The secondary antibody containing a horseradish peroxidase (HRP) moiety was then added (1 hour) and was followed by washing to remove the excess antibody. Immunoreactive proteins were then visualised using enhanced chemiluminescence (ECL) detection reagent (**Figure 2.20**). ECL is a commercially available protein detection reagent that is based on a cocktail of chemicals but contains a peracid and luminol as the key components.[257] The HRP-conjugated antibody and peracid react to form active complexes, which in turn leads to the oxidation of luminol, and the emission of light. The emission of light is then recorded in a photographic film allowing detection of up to 1 pg of antigen.
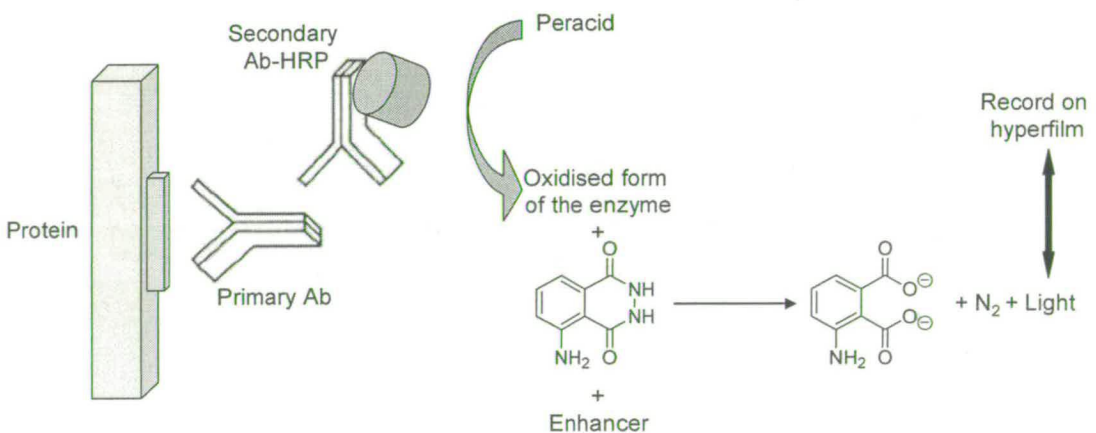
Figure 2.20: Principles of ECL Western blotting.[257]

The membrane can then be stripped of all antibodies and reblotted using an antibody that is equally specific for the phosphorylated and the unphosphorylated form of JNK. This can then be detected using ECL, as described previously. This experiment acts as an internal control by illustrating that even blots that have shown no phosphorylation in the initial experiment contain the unphosphorylated protein. Thus, confirming that JNK was present but unphosphorylated.

2.11 Biological Testing of the Marked Library

The new anisomycin analogues and marked library members were then tested for activation of the JNK/SAPK1 pathway, as described previously.[246, 258] The compounds were analysed in a series of experiments with each blot containing a negative DMSO standard (lane 1) and an anisomycin positive standard (lane 2). The library members could therefore be directly compared to the control lanes on the blots, thus allowing the level of SAPK phenotypic response to be analysed. Moreover, each figure contains an upper blot showing phosphorylated JNK and a lower blot which shows both phosphorylated and unphosphorylated JNK.

Screening of the library of C_4-H anisomycin analogues showed that as expected bulky protecting groups, such as Boc or TBS attached to the core structure gave compounds that had lost their phenotypic SAPK response (**Figure 2.21**, lanes 3-6). However, incorporation of the propargyl marker onto the phenol functionality and removal of the bulk protecting groups in **136** lead to a molecule that activates the SAPK pathway (**Figure 2.21**, lane 7).
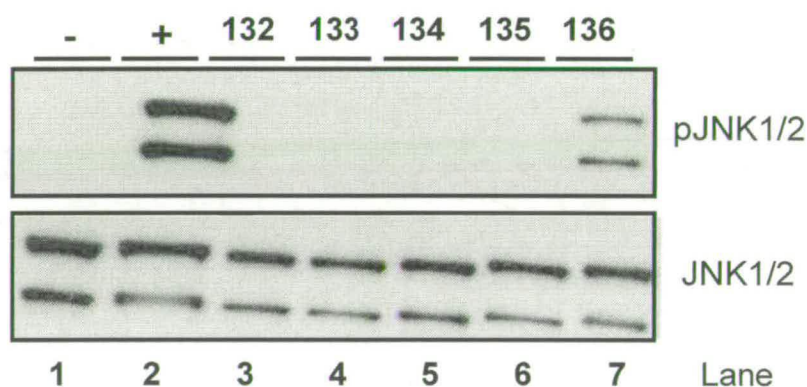
Figure 2.21: Im munoblot assay for the phosphorylation of JNK1/2 upon stimulation by DMSO (negative standard, lane 1), anisomycin **102** (positive standard, lane 2) and C_4-H anisomycin analogues **132** to **136** (lanes 3-7).

In contrast, marking the pyrrolidine nitrogen on the C_4-H analogue turned out to be less fruitful. The marked compound **139** and the protected compound **137** gave no response for SAPK activation (**Figure 2.22**, lanes 9/10 and 5/6 respectively), while the unprotected compound **138** showed similar levels of activation to that of the previous results within the group (**Figure 2.22**, lanes 7/8).
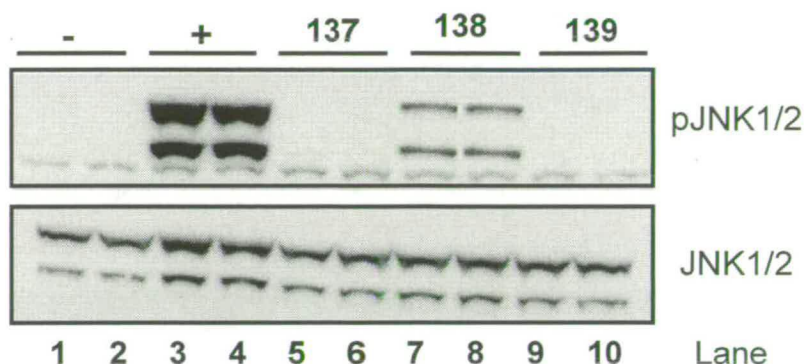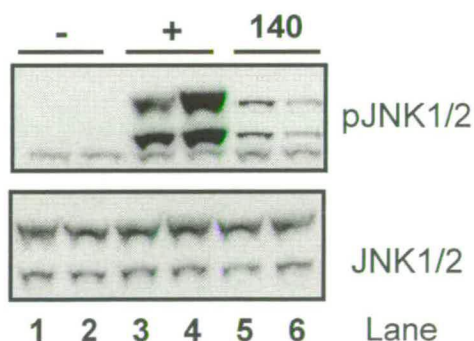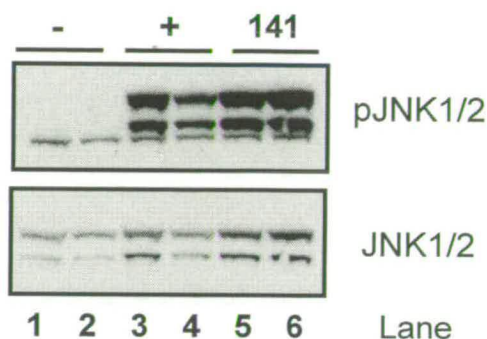
Figure 2.22: Immunoblot assay for the phosphorylation of JNK1/2 upon stimulation by DMSO (negative standard, lanes 1/2), anisomycin **102** (positive standard, lanes 3/4) and C_4-H anisomycin analogues **137** to **139** (lanes 5-10).

Attention then turned to testing the anisomycin based analogues that had been synthesised. Gratifyingly it was found that the *N*-propargyl anisomycin **140** gave a positive result in the SAPK assay (**Figure 2.23**, lanes 5/6). In turn, *N*-benzyl anisomycin **141** showed levels of SAPK activation comparable with that of anisomycin itself (**Figure 2.23**, lanes 5/6). These key results allowed us to conclude that a number of groups could be attached to the pyrrolidine nitrogen of anisomycin while retaining the phenotypic response. Moreover, the activation of the SAPK pathway by *N*-benzyl anisomycin, (in contrast to the lack of SAPK response with that of the dibenzyl anisomycin) illustrates that the C_4 position can only tolerate small structural perturbation (*i.e.* C_4H or C_4Me), in order to retain its biological activity.

Figure 2.23: Immunoblot assay for the phosphorylation of JNK1/2 upon stimulation by DMSO (negative standard, lanes 1/2), anisomycin **102** (positive standard, lanes 3/4) and anisomycin analogues **140** (lanes 5/6).

Figure 2.24: Immunoblot assay for the phosphorylation of JNK1/2 upon stimulation by DMSO (negative standard, lanes 1/2), anisomycin **102** (positive standard, lanes 3/4) and anisomycin analogues **141** (lanes 5/6).

With these promising results in hand the Western Blotting of the two active marked compounds (**136** and **140**) was repeated in order to compare their activity directly with that of deacetylanisomycin **106**, a known activator of the SAPK pathway (**Figure 2.24**). This experiment illustrated that although the marked library members show a weak phenotypic response for phosphorylation of JNK, it can be considered analogous to that of deacetylanisomycin.
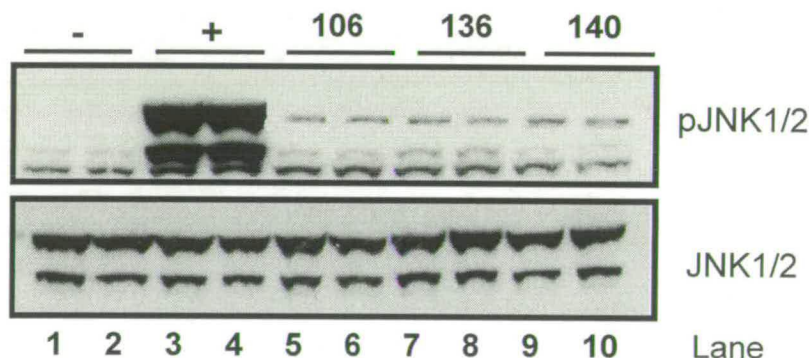

Figure 2.25: Immunoblot assay for the phosphorylation of JNK1/2 upon stimulation by DMSO (negative standard, lanes 1/2), anisomycin **102** (positive standard, lanes 3/4), deacetylanisomycin **106** (lanes 5/6), O-propargyl C_4-H anisomycin analogue **136** (lanes 7/8) and N-propargyl anisomycin analogue **140** (lanes 9/10).

Overall the results of this library study for activation of the SAPK phenotype are in good agreement with the SAR which have previously been determined within the Hulme group.[246] The synthesis of *N*-benzyl **141** and *N*-propargyl anisomycin **140** has confirmed our previous hypothesis that the pyrrolidine nitrogen could be alkylated while retaining biological activity. This provided an excellent contrast to the

dibenzyl compound, which had been previously found to be biologically inactive. It can therefore be concluded that bulky groups are not tolerated on the C$_4$-OH but are allowed on the pyrrolidine nitrogen. However, at the phenolic functionality, addition of the propargyl functionality also led to retention of the phenotypic response. Thus, confirming our initial analysis of anisomycin's SAR, where it was proposed that functionalisation of both the pyrrolidine ring and the phenolic oxygen would lead to retention of biological activity. Therefore, the idea that a small bioorthogonal marker, such as a propargyl group, can be incorporated into a biologically active molecule, whilst retaining activity has been validated.

2.12 Summary

The synthesis of a small library of marked molecules based around the molecular scaffold of the SAPK activator anisomycin, incorporating a bioorthogonal marker into the structure has taken place. Testing of this library has led to a number of biologically active marked library members and the validation of our marked library strategy; as well as developing a greater understanding of anisomycin's SAR.

We therefore envisage the derivatisation of functionalities (alcohols, amines, thiols, *etc*) within a small molecule's scaffold with the attachment of a biocompatible marker. This strategy could then easily be expanded to other functional groups such as carbonyls, amides and esters allowing the incorporation of the marker moiety more generally. Moreover, we imagine the choice of marker being made on a case by case basis with a series of markers and the analogous bioorthogonal reactions readily available.

On completion of the biological screening, active compounds that contain markers could then be coupled to their orthogonal reactive partner to rapidly form molecular probes (see Chapter 3), without the long and often complicated synthesis traditionally associated with their formation. The active probes could then be utilised in the investigation of the biological role of the molecule of interest. We therefore believe that this marked library approach will provide a useful truncation of the small-molecule to molecular probe synthetic process through the incorporation of a biocompatible marker.

3   Anisomycin Based Molecular Probes

3.1   Traditional Molecular Probes

Understanding complex cellular processes requires the detailed analysis of biological processes at the molecular level. This involves the analysis of a wide range of biomolecules from polymers and ions to proteins and metabolites, by tracking the biomolecules of interest within their natural environment. Classically this is done by attaching a tag, such as green fluorescent protein (GFP) (**Figure 3.1**) or biotin, to the species of interest thus allowing visualisation and quantification.[259-261] However, the application of GFP and GFP-like proteins to this task may also cause a series of problems, not least the fact that at 27 kDa, GFP can cause major structural perturbations to the system under investigation. Therefore, over the last decade the attention has turned to an organic chemistry based approach to deliver a series of reactions that can be utilised to couple a wide range of tags (biotin, fluorophores, *etc*) to the biomolecule of interest.[95, 96, 262]
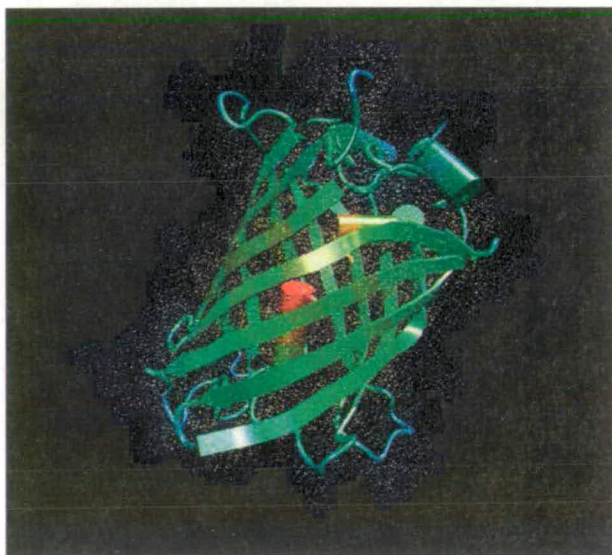
Figure 3.1: Structure of green fluorescent protein (GFP).

3.2 'Click' Chemistry

The terminology 'click chemistry' was first coined by Sharpless *et al.* in 2001 when they used the term to describe their approach to develop a series of powerful, highly selective and reliable reactions which would allow for the rapid modular synthesis of new compounds and libraries.[98]

Using nature's precedent, they set out to synthesise new compounds by joining together two units using a heteroatom linkage (C-X-C). They defined a series of stringent criteria to describe 'click' reactions stating that reactions must be: modular, stereospecific, wide in scope, very high yielding and allow simple product isolation.[97, 98] Moreover, the reaction should have *simple* reaction conditions, using benign solvents, while generating inoffensive byproducts that can be removed without chromatography. Therefore, 'click' reactions can be thought of in terms of being 'spring loaded', with a characteristic high driving force of usually greater than 20 kcal mol^{-1}.[97, 98] With this work Sharpless raised the bar for the modern organic chemist by providing a redesigned toolbox of synthetic reactions that are atom efficient, carried out under green conditions and are very reliable.

3.2.1 Library of 'Click' Reactions

The first generation of 'click' reactions, were based in areas of carbonyl and olefin chemistry.[97, 98] This was due to the widespread availability of these functionalities from commercially available building blocks, thus allowing a large range of reactions to be examined to see if they met the stringent 'click' criteria. This led to the researchers in the Sharpless laboratory developing a series of extremely reliable processes (**Scheme 3.1**) that were the first to be defined as 'click' reactions and include:

- Addition to carbon-carbon multiple bonds, especially oxidative cases such as epoxidation, dihydroxylation, aziridination and certain Michael additions (**Scheme 3.1**; 142→143).

- Nucleophilic substitution chemistry, especially the opening of strained heterocyclic rings such as epoxides, aziridines, cyclic sulfates, aziridinium ions and episulfonium ions (**Scheme 3.1**; **143→144**).

- Cycloaddition reactions of unsaturated species, especially from the 1,3-dipolar cycloaddition family, but also Diels-Alder and hetero-Diels-Alder reactions (**Scheme 3.1**; **142→145**).

- Carbonyl chemistry of the non-aldol type; *i.e.* formation of oximes, hydrazones and aromatic heterocycles (**Scheme 3.1**; **142→147**).
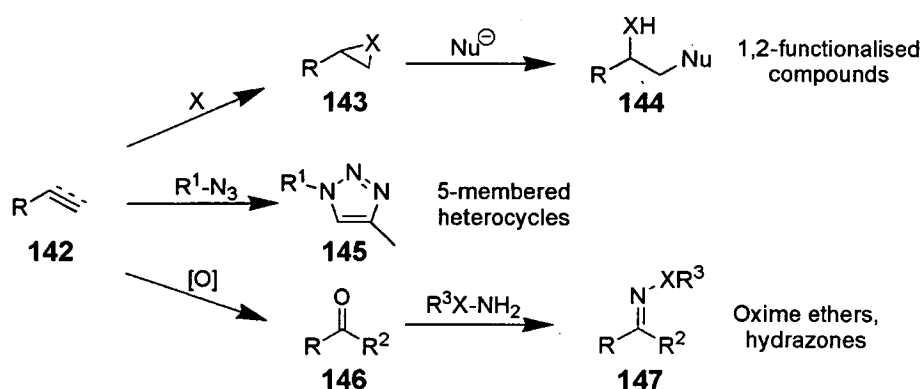
Scheme 3.1: Overview of 'click' chemistry reactions.[97, 98]

Since its inception in 2001 the concept of 'click' chemistry has been utilised across a broad spectrum of chemistry from polymer and materials chemistry to supramolecular and carbohydrate chemistry.[263-267] Thus illustrating the wide range of synthetic areas 'click' chemistry can be applied to.

3.2.2 'Click' Reactions in Water

During the development of 'click' chemistry researchers found that water was the solvent or co-solvent of choice, with reactions proceeding much better that in organic solvents.[97, 98] This is believed to be due to a range of factors including:

- The fact that reactions of organic molecules in aqueous media can have a higher apparent rate constant than the analogous reaction in organic media. This observation has been investigated by a number of groups and many explanations have been offered.[268-270] However, the key factor may be that the free energies of organic molecules are often greater when poorly solvated in water, resulting in an imparted increase in reactivity.[271]

- Two subsets of olefin and acetylene 'click' reactions are oxidation by electrophiles and cycloaddition reactions respectively. These reactions involve either polarisable nuclophile/electrophiles or concerted processes; hence water is not an interfering factor in the reaction.[272]

- Nucleophilic additions to strained heterocyclic rings are favoured by solvents who respond well to a series of hydrogen-bonding situations during the reaction pathway, such as water.

- Water is also an excellent heat-sink, with a high heat capacity for absorbing the large amount of heat given out during 'click' reactions. Therefore making it an ideal solvent for performing these reactions on larger scales.

3.3 Bioconjugate Reactions

With the development of a series of 'click' reactions that could be carried out in aqueous solvents, a number of interdisciplinary scientists working at the interface of chemistry, proteomics and the life sciences have begun to take advantage of this new methodology.[95, 96, 262] Investigations have focused on the use of pairs of biocompatible functionalities, which were inert towards the biological system but could be coupled together using bioconjugate reactions *in situ* (**Table 3.1**). This research has led to the validation of a series of biocompatible markers (**Table 3.1**, column 1 and 2) which include azides, alkynes, phosphines, oximes, hydrazines, ketones, aldehydes and the tetracystine motif.[95, 96, 262]

This approach has been successfully applied to a wide variety of biomolecules including proteins, glycans and lipids (**Table 3.1**, column 4), whereby, a marker has been incorporated into the biomolecule of interest. The markers can then be coupled to their orthogonal partner using a specific bioconjugate reaction, typically at pH 7 with little or no toxic effects, to form their respective ligation product (**Table 3.1**, column 3).

There are a significant number of publications dealing with this topic. However, the main bioconjugate reactions that have been utilised are the Staudinger ligation and the Huisgen cycloaddition. Therefore, an overview to these reactions will be covered in the following sections.

Chemical Reporter	Reactive Partner (R' = Tag)	Ligation Product	Target
Tetracysteine motif	(arsenic reagent structure)	(arsenic ligation product)	Protein
Aldehyde/Ketone	$H_2N-N(H)-C(O)-R^2$	(hydrazone product)	Glycan Protein
	H_2N-O-R^2	(oxime product)	
R–N$_3$ Azide	**Staudinger Ligation** (MeO / Ph$_2$P structure)	(Staudinger product)	Glycan Protein Lipid
	Huisgen cycloaddition $\equiv\!\!-R^1$	(triazole product)	
	Strain promoted cycloaddition (cyclooctyne structure)	(fused triazole product)	
$\equiv\!\!-R^1$ Alkyne	**Huisgen cycloaddition** $R-N_3$	(triazole product)	Protein

Table 3.1: Table of biocompatible markers, their orthogonal partners, the bioconjugate reactions the ligation products and the bimolecular system they have been utilised in.
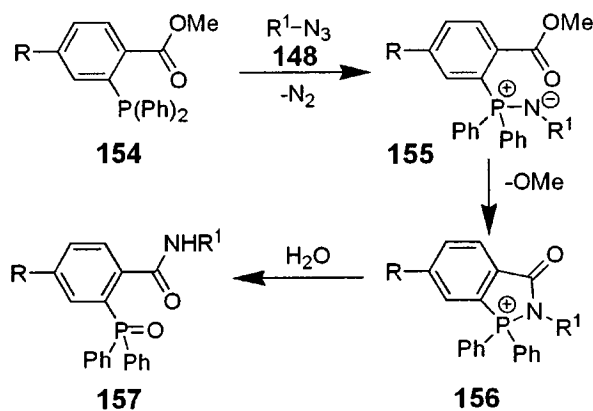
3.3.1 Staudinger Ligation

The Staudinger reduction was first developed in 1919 by Hermann Staudinger and is the reaction of an azide **148** and a phosphine **149** to form an amine and trialkyl or aryl phosphine oxide (**Scheme 3.2**).[273, 274] The reaction proceeds *via* formation of phosphazine intermediate **150**, which immediately loses nitrogen to form an iminophosphorane **151**.[275, 276] In the presence of water the iminophosphorane **151** is spontaneously hydrolysed to a primary amine **153** and the accompanying phosphine oxide **152**.
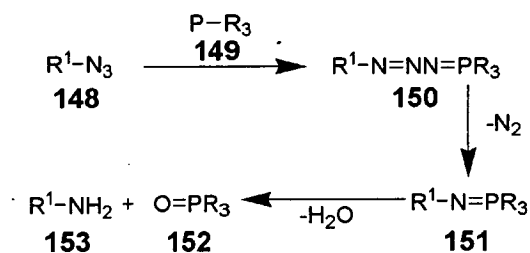
Scheme 3.2: Mechanism of the Staudinger reaction.[274-276]

Likewise, the intermediate in the Staudinger ligation is an iminophosphorane **151** which contains a nucleophilic nitrogen. Work by Vilarrasa showed that this nitrogen can attack an intra- or inter-molecular acyl donor forming an amide, after hydrolysis of the intermediate.[277]


Scheme 3.3: Mechanism of the Staudinger-Bertozzi ligation.[278-280]

In 2000, Bertozzi first reported the modification of the Staudinger ligation (now the Staudinger-Bertozzi ligation) of an organoazide **148** to a specifically functionalised phosphine **154** (**Scheme 3.3**).[278, 279] The reaction proceeds with nucleophilic attack at the organoazide, to form an aza-ylide **155**. The adjacent methylester traps the aza-ylide **156**, which is subsequently hydrolysed to give the new amide **157**.[280]

Subsequently the Staudinger-Bertozzi ligation has been applied to a range of biological substrates including the investigation of synthetic azido-sugars, for attaching substrates to surfaces and for biological labelling.[281-285] Recently, it has now been applied to an enzyme-linked immunosorbent assay (ELISA) to give an azido-ELISA which has been utilised in the screening of different glycosyl transferases (**Scheme 3.4**).[113, 114] Moreover, researchers have begun to move beyond the test tube to investigate the role of biomolecules in living cells, with the Bertozzi research group at the forefront having successfully applied this ligation in living animals, such as mice.[286]
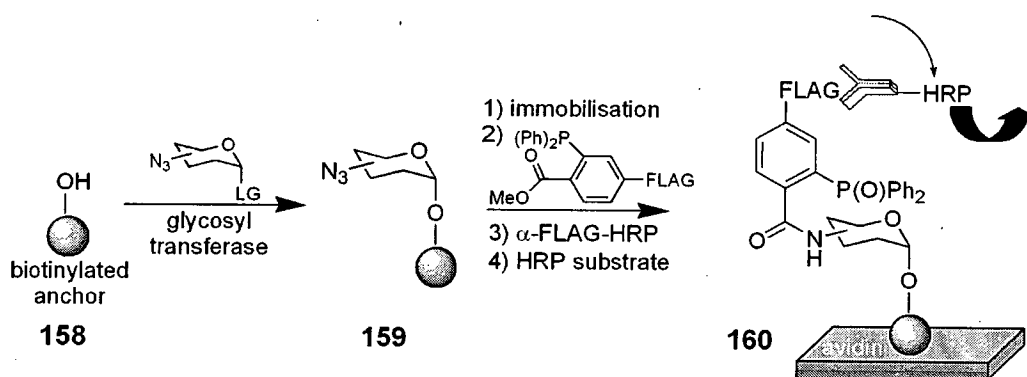
Scheme 3.4: Enzyme-linked immunosorbent assay (ELISA), incorporating the Staudinger-Bertozzi ligation as a new method for screening glycosyl transferase.[113, 114]
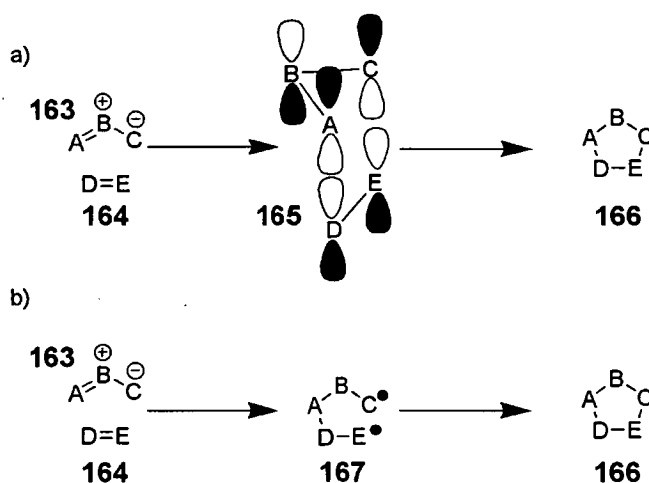
3.3.2 Huisgen Cycloaddition

The 1,3-dipolar cycloaddition of an azide and an alkyne was developed by Rolf Huisgen in the 1960's.[287-289] This reaction occurs between an alkyne dipolarophile **161** and a 1,3-dipole **148** which has a conjugated system of three p orbitals, containing 4 electrons over three atoms. This reaction typically results in a mixture of regioisomers **145:162** with selectivity varying from >95:5 to a 50:50 mixture (**Scheme 3.5**).
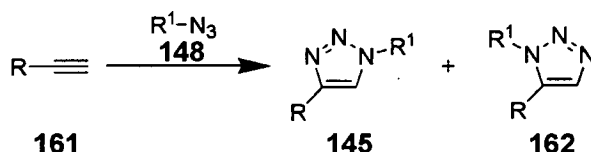
Scheme 3.5: Classical Huisgen 1,3-dipolar cycloaddition forming 1,2- and 1,4-regioisomers as products (**162** and **145** respectively).[287-289]

Studies into the mechanism of the 1,3-dipolar cycloaddition were a contentious issue within the synthetic community for over 20 years. Huisgen proposed a transition state model **165** in which the 4 π-electrons of the dipole **163** interact with the π-bond of the dipolarophile **164** (**Figure 3.6a**).[289, 290] This concerted mechanism was based on an extensive series of experiments investigating kinetic measurements and stereochemical outcomes as well as solvent and substituent effects.[291, 292]


Scheme 3.6: Proposed mechanisms for 1,3-dipolar cycloaddition reaction: (a) Concerted mechanism proposed by Huisgen. (b) Stepwise mechanism proposed by Firestone.[293]

Meanwhile, Firestone passionately believed that these reactions proceeded *via* a singlet diradical intermediate **167** (**Figure 3.6b**).[294-297] However, his argument turned out to have a fatal flaw. If the reaction was to proceed *via* the singlet mechanism the stereochemistry of the product would be scrambled. Unfortunately for Firestone this stereochemical scrambling was not seen under experimental conditions.[298] Therefore, the concerted mechanism was accepted as the valid pathway, a result that was later confirmed by a series of DFT calculations.[293]

The Huisgen 1,3-dipolar cycloaddition reaction is thermodynamically favourable by 30-35 kcal mol^{-1}. However, classically this reaction requires elevated temperatures or pressures to proceed, making these conditions incompatible with living systems.

This problem was overcome by the resultant work of the Sharpless and Meldal research groups who concurrently investigated the catalysis of this reaction.[299, 300] They successfully developed a copper(I)-catalysed variant of the Huisgen 1,3-dipolar cycloaddition that increased the rate of the reaction by 10[6]-fold, formed the 1,4-regioisomer selectively, while only using 10 mol% of the copper catalyst (**Scheme 3.7**). This new variation on the classical 1,3-dipolar cycloaddition rapidly became know as the 'click reaction' as it was believed to be the premier reaction within the 'click' chemistry toolbox.[97, 98, 301]
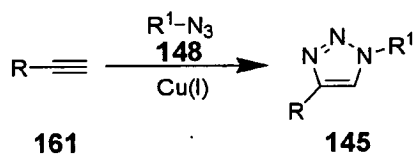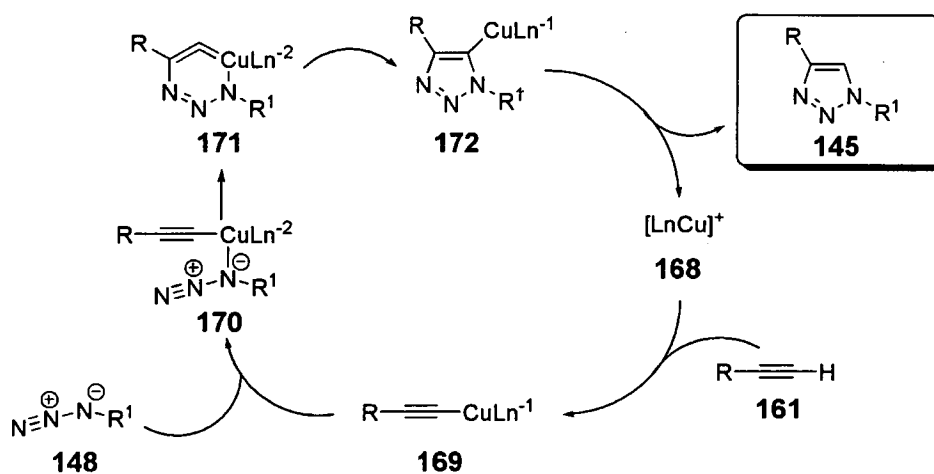

Scheme 3.7: Copper(I)-catalysed Huisgen 1,3-dipolar cycloaddition forming the 1,4-triazole **145** regioselectively.[97, 98, 301]

Investigations into the optimum conditions for the copper(I)-catalysed Huisgen cycloaddition found enormous scope for this reaction. The use of copper(II) salts (0.25-10 mol%) which are subsequently reduced to copper(I) *in situ* by ascorbic acid or sodium ascorbate were found to be the catalytic system of choice. The reaction takes place in 6-36 h in a variety of solvents including ethanol, aqueous *tert*-butyl alcohol and in water alone. In contrast, the direct addition of copper(I) salts required the addition of a single equivalent of a nitrogen base (triethylamine or 2,6-lutidine), acetonitrile as a co-solvent and often led to a range of side products.[97]

Since the first detailed reports were published in 2002 a number of subsequent reaction conditions have been reported. Tirrell *et al.* have utilised copper(I) bromide as the copper source.[302] However, a key factor in using this methodology is the requirement of the copper bromide to be of high purity (>99.999%) and stored under anhydrous conditions. While work within the Hulme research group has shown that using a copper(II) source and reducing it with a mild biological reductant such as glutathione can lead to enhanced reaction rates.[303]

Detailed investigations by Sharpless *et al.* into the mechanism of the copper(I)-catalysed Huisgen cycloaddition are still being carried out. However a mechanism has been proposed on the basis of a detailed series of DFT calculations.[304, 305] The reaction begins with the coordination of the alkyne **161** to the Cu(I) species **168**, displacing a solvent ligand (**Scheme 3.8**). The alkyne is then converted to the acetylide **169**, a process identical to that of the Sonogashira coupling. At this point DFT calculations gave a useful insight into the cycloaddition process, ruling out the two concerted mechanisms *via* transition states (**Figure 3.2; 173** and **174**). Transition state **173** was found to have a higher activation barrier than the uncatalysed reaction, while the activation barrier to transition state **174** was found to be only 2.3 kcal mol^{-1} lower than the reaction without copper. It is therefore clear that neither of the concerted mechanisms could explain the experimental rate enhancement given to this reaction by the addition of copper.


Scheme 3.8: Proposed reaction mechanism for the copper(I)-catalysed Huisgen cycloaddition based on experimental evidence and DFT calculations.[304, 305]
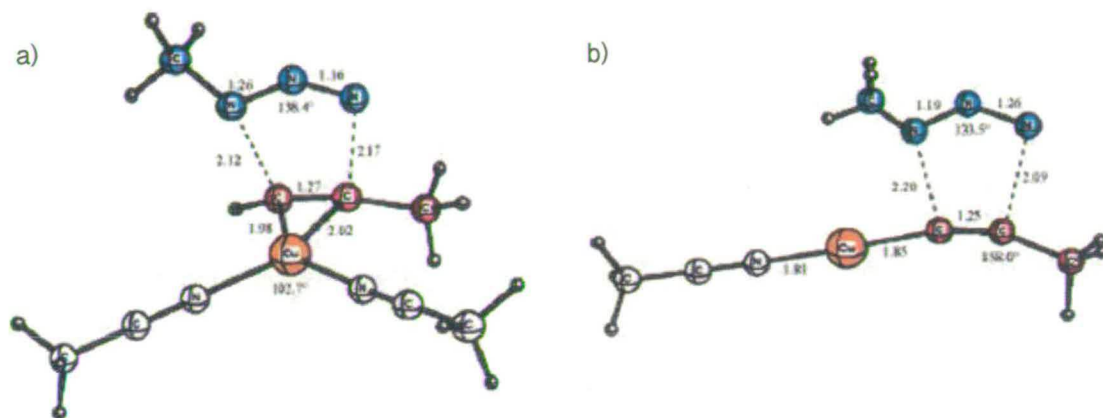
Figure 3.2: Two possible transition states for a concerted mechanism for the copper(I)-catalysed Huisgen cycloaddition. a) **173**; b) **174**.[304, 305]

Having ruled out the concerted processes, they next proposed replacing one of the ligands on the copper by binding the azide moiety **148** to the copper *via* the nitrogen closest to the carbon **170**. From this point a stepwise process can occur with the terminal nitrogen of the azide attacking the C_2 carbon of the acetylide, forming an unusual 6-membered copper metallocycle **171**. This step was found to have an activation barrier of 14.9 kcal mol^{-1} which is significantly lower than the uncatalysed reaction and could explain the 7-8 fold rate enhancement seen for the copper catalysed process. The 6-membered metallocycle **171** then contracts to forms the triazolyl-copper derivative **172**. With water as a ligand the energy barrier for this process is only 3.2 kcal mol^{-1}. Proteolysis of **172** releases the product **145** and regenerates the copper catalyst **168**, thereby completing the catalytic cycle (**Figure 3.8**). These theoretical calculations concur with the experimental findings and give an insight to the rate enhancement copper(I) has on this process, as well as explaining the regiocontrol. However, further experimental work has still to be carried out to confirm these calculations.

As a consequence of the high reliability, mild reaction conditions, high rates and biocompatibility the copper(I)-catalysed Huisgen cycloaddition has become the bioconjugate reaction of choice. In 2003, the group of Schultz showed that unnatural amino acids **175** and **176** could be incorporated into *Saccharomyces Cerevisiae* (**Figure 3.3**).[306] The azide or propargyl markers could then be coupled to an orthogonally marked fluorophore using the copper catalysed cycloaddition.
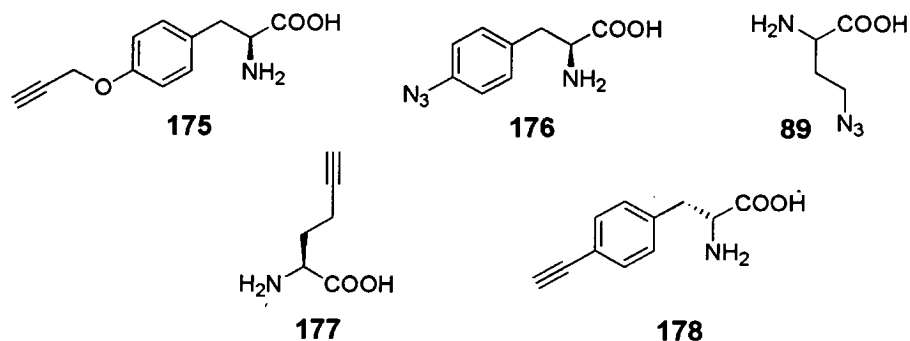
Figure 3.3: A series of marked amino acids that have been incorporated into a protein's structure and used in cell surface engineering studies.[104, 302,306,307]

In a similar manner Tirrell *et al.* showed that they could replace methionine residues into the outer membrane protein (Omp) C in *E. coil* cells with azidohomoalanine **89** (**Figure 3.3**).[104, 302] The cells containing the azide moiety could then be coupled to a biotin-alkyne reagent under 'click' reaction conditions, before analysis by Western blotting. In a further example of cell surface engineering, the same researchers have labelled a series of proteins by incorporating the modified amino acids homopropargylglycine **177** and ethynylphenylalanine **178** into *E. coil* cells (**Figure 3.3**).[307] These modified amino acids have been coupled with an azido-fluorophore to give a fluorescently tagged protein.

In alternative strategy Cravatt *et al.* have used the copper(I)-catalysed Huisgen cycloaddition to carry out activity based protein profiling (ABPP) to interrogate the proteome. ABPP is a new postgenomic method in which affinity labels are used to profile proteins on the basis of their function within biological systems. However, classically the probes are bulky and may inhibit cellular uptake. Therefore, a 'tag free strategy' has been developed, whereby addition of the appropriate biocompatible marker to the probe **180** allows the probe to be used to investigate the proteome, before being coupled to the required tag (**Scheme 3.9**; **179→182**).[308-311] However, one current limitation within this field is that researchers are carrying out profiling where the design of the functional probe relies upon a *known* protein activity, *i.e.* this methodology has yet to be expanded to target identification strategies.
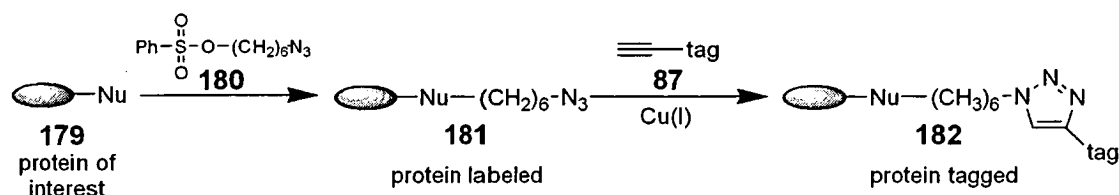
Scheme 3.9: A 'tag free strategy' for affinity based protein profiling (ABPP). Proteins **179** are first labelled with a azido-sulfonate ester **180** to give **181**, then tagged by reaction with an alkyne-tag **87** under copper(I) 'click' chemistry conditions to give **182**.[308-311]

As a result of the wide range of applications within biological chemistry it was believed that the propargyl-azide partnership would give the ideal pair of biocompatible tags to utilise in our marked library strategy. It was expected to be relatively easy to introduce both propargyl and azide functionalities into the small molecule and tag components respectively. Moreover, we have previously shown retention of the SAPK phenotype by anisomycin analogues upon attachment of a propargyl marker (Chapter 2). We therefore set about synthesising a series of molecular probes based on the two active marked library members (**136** and **140**) utilising the copper(I)-catalysed Huisgen cycloaddition as the key coupling reaction.

3.4 Fluorescent Molecular Probes

Fluorescent labels have been commonly utilised as a tool for investigating the mechanism of cellular uptake and intracellular distribution of small molecules, peptides and proteins.[312, 313] This is carried out by attachment of a fluorophore, classically rhodamine **183**, coumarin **184** or dansyl **185**, to the molecule of interest (**Figure 3.4**).[314-316] Therefore, it was decided to develop the fluorescent molecular probe strategy based around the dansyl fluorophore. This was due to a number of factors including the ease in which dansyl's structure could be modified with an azide marker, the small size of the fluorophore and the use of the dansyl moiety in a number of successful studies investigating the cellular location of small molecules and peptides.[312, 313, 317, 318]
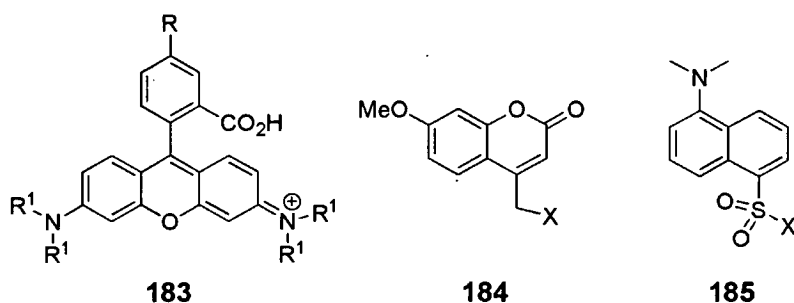
Figure 3.4: Structures of fluorophores rhodamine **183**, coumarin **184** and dansyl **185**.

3.4.1 Synthesis of Fluorescent Molecular Probes

In order to incorporate the azide functionality required to couple the fluorophore to the active marked library member, a short linker section containing an azide had to be appended to the structure of the fluorophore. It was decided to use the shortest possible carbon backbone as the linker, to reduce the unspecific binding of biomolecules within the cell to a minimum and to aid cellular uptake.

The azide linker **187** was successfully synthesised from 2-chloro ethylamine **186** by heating in water in the presence of sodium azide (**Scheme 3.10**).[319] The reaction was quenched with excess potassium hydroxide to give the free amine as a volatile colourless oil isolated in quantitative yield. Analysis of the ^{1}H and ^{13}C NMR spectra showed little alteration from the starting material. However, analysis of the IR

spectrum showed a distinctive stretch at 2100 cm^{-1} characteristic of the azide functionality. Storage of **187** proved to be problematic, even when stored in the freezer at -20 °C, with complete loss of the product occurring after 72 hours. Therefore, the linker was carried through to the next reaction immediately in order to reduce the loss of material.
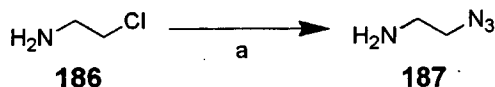
Scheme 3.10: (a) NaN$_3$, H$_2$O, 80 °C (100%).

Coupling of the free amine **187** with the commercially available dansyl chloride **188** occurred readily on stirring the two components in DCM for 2 hours (**Scheme 3.11**). The product **189** was isolated as a golden oil in 98% yield after chromatography. The compound was fully characterised by NMR and retained the distinctive azide peak in the IR spectrum at 2100 cm^{-1}.
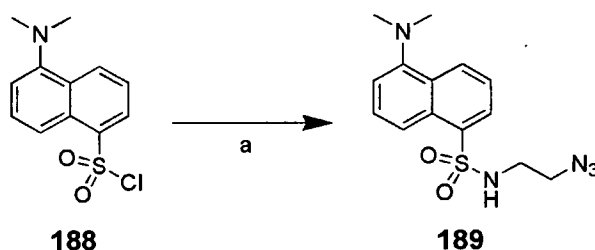
Scheme 3.11: (a) **187**, DCM (98%).

With the desired azido-fluorophore in place, the coupling conditions of the azide and alkynes were investigated. Focusing on the catalytic loading of copper sulfate and the timescale for the reaction; studies found that the reaction proceeded with a range of copper loadings (2-20 mol%) with a two-fold excess of sodium ascorbate reductant (4-40 mol%). However, at lower catalyst loadings the reaction times were found to increase. Therefore, 10 mol% copper(II) sulfate with 20 mol% sodium ascorbate was chosen as our optimum reaction conditions for the 'click' reaction. Investigations also looked at the most favourable solvent for carrying out this reaction. However studies only confirmed previous findings, that the reaction proceeds well in a wide

range of solvents including water:*tert*-butanol (1:1), a range of pH buffers and water.[262, 266, 300]

Facile coupling of the azide **189** and alkyne **136** was carried out in the presence of copper(II) sulfate (10 mol%) and sodium ascorbate (20 mol%) with a distinctive shift to a lower R_f being observed by tlc (**Scheme 3.12**). Purification by column chromatography gave the target material **190** in 77% as a single regioisomer.


189   **190**
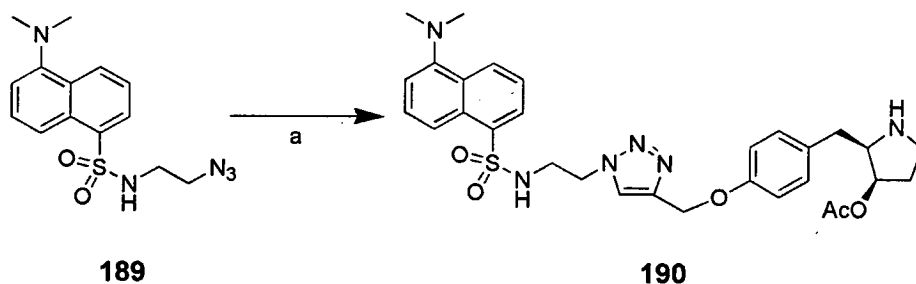
Scheme 3.12: (a) **136**, CuSO₄• 5H₂O (10 mol%), NaAsc (20 mol%), ᵗBuOH:H₂O (1:1) (77%).

An alternative approach was also investigated whereby the click coupling reaction was carried out between **135** and the azido-fluorophore **189**. The Boc protecting group was then removed using TFA to give the desired molecular probe **190** (**Scheme 3.13**). However, no significant synthetic advantage was found in utilising this alternative reaction sequence.

189   **190**
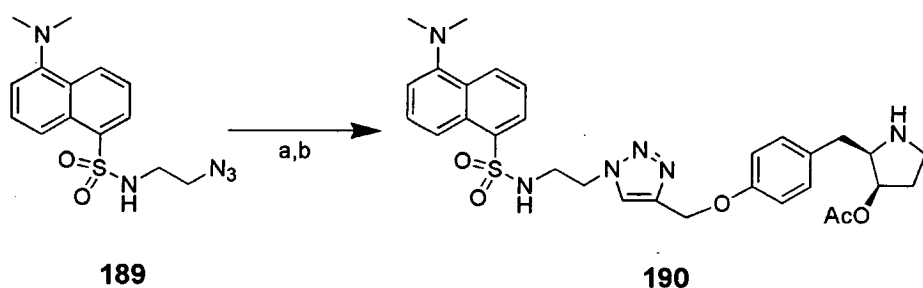
Scheme 3.13: (a) **135**, CuSO₄• 5H₂O (10 mol%), NaAsc (20 mol%), ᵗBuOH:H₂O (1:1) (76%); (b) TFA, DCM (96%).

The molecular probe **190** was rigorously characterised using a series of 1D and 2D NMR experiments. The ^{1}H spectrum showed the removal of the distinctive fine triplet associated with the CH of the propargyl unit, while in the aromatic region the addition of a singlet at 8.06 ppm was thought to account for the CH of the 1,2,3-triazole. Further interrogation of the structure of **190** by COSY clearly showed the correlation between the new $C_{14}H$ and the $C_{16}H_2$. On comparison of the HSQC spectra of the azido-starting material **189** with that of the molecular probe **190** three new peaks were clearly visible within the aromatic region of the spectrum (**Figure 3.5**). Coupling of the anisomycin moiety to the fluorophore would increase the number of peaks in the aromatic region of the HSQC spectra by three, with two peaks associated with the aromatic ring of the marked analogue and a further peak coming from the $C_{14}H$ upon formation of the triazole regioselectively. This spectroscopic evidence confirmed that the click coupling reaction had been carried out successfully, with the fluorescent molecular probe **190** obtained in high yield.
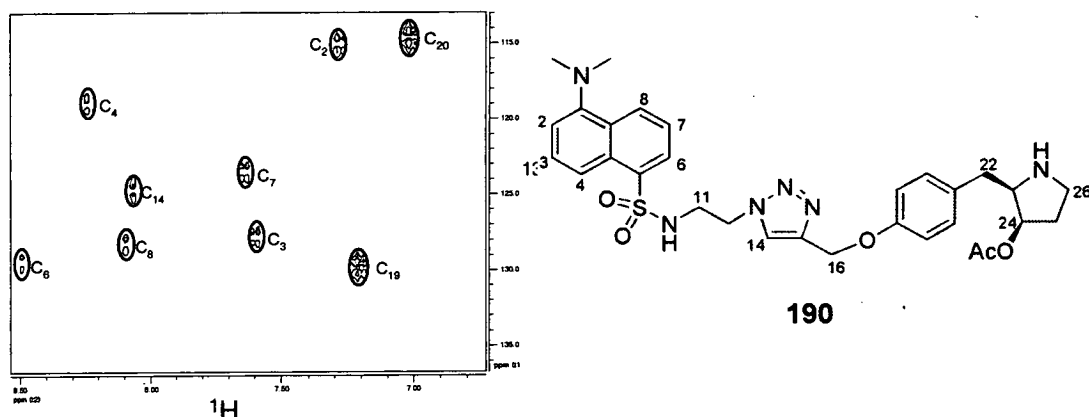

Figure 3.5: Expansion of the aromatic region of the HSQC spectrum of molecular probe **190** (360 MHz, DMSO).

With the synthesis of the first fluorescent molecular probe in place, we turned our attention to the coupling of the *N*-propargyl anisomycin **140**. Utilising the same coupling conditions as used previously, the marked library member **140** was coupled to the fluorescent azide **189** (**Scheme 3.14**). The molecular probe **191** was obtained in 71% yield after purification by column chromatography.
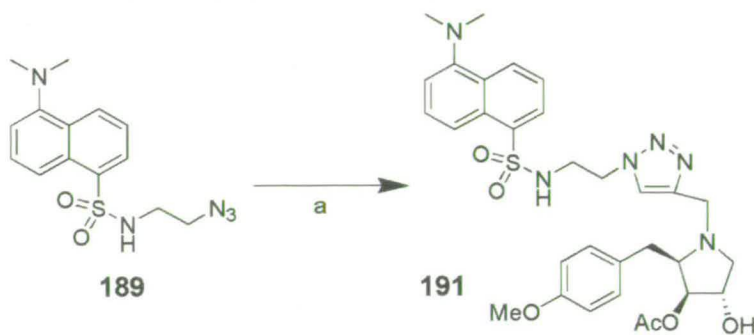
Scheme 3.14: (a) **140**, CuSO$_4 \bullet$ 5H$_2$O (10 mol%), NaAsc (20 mol%), ^{t}BuOH:H$_2$O (1:1) (71%).

Analysis of the ^{1}H spectrum of **191** in methanol showed a well defined aromatic region with all the relevant protons being accounted for. However the C$_{16}$-C$_{22}$ region of the spectrum showed a series of broad peaks with little definition. The same problem was encountered on assigning the broadband ^{13}C NMR and the 2D spectra. It was believed that altering the solvent from methanol to DMSO may help to remove this problem. However, analysis of the spectra in DMSO proved to have the same broad peaks. A series of variable temperature NMR experiments in DMSO were then carried out, recording spectra from 20 °C to 60 °C, but once again this did little to remove the broadening. The broadening of the C$_{16}$-C$_{22}$ region was believed to be associated with two key factors (**Figure 3.6**): the number of nitrogens in the triazole-pyrrolidine core of the molecule; and the hindered rotation that exists around the CH$_2$'s linking the pendant triazole and phenyl groups to the pyrrolidine ring.

191
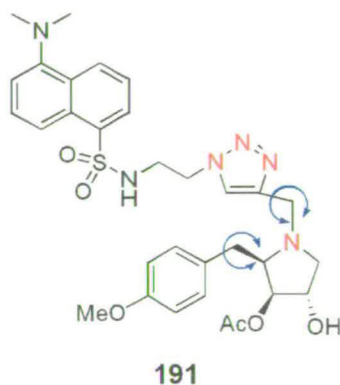
Figure 3.6: Structure of molecular probe **191**. Illustrating the nitrogen dense area within the molecule (red) and the CH$_2$ groups that can undergo hindered rotation (blue) which lead to broadening on the NMR spectra.

The issue was finally resolved upon using deuterated acetonitrile as the solvent for spectroscopic characterisation. Analysis in acetonitrile led to a reduction in broadening that had previously been observed. Examination of the ^{1}H NMR showed much greater definition, while the carbon skeleton could be fully assigned using HSQC (**Figure 3.7**) and HMBC experiments (**Appendix 3**). As with the previous molecular probe the distinctive CH of the 1,2,3-triazole was found to be at 7.57 ppm (^{1}H) and 126.4 ppm (^{13}C). Other distinctive peaks included the large doublet corresponding to the pair of diastereotopic protons of the $C_{18}H_2$ and the pair of diastereotopic protons of the $C_{16}H_2$.
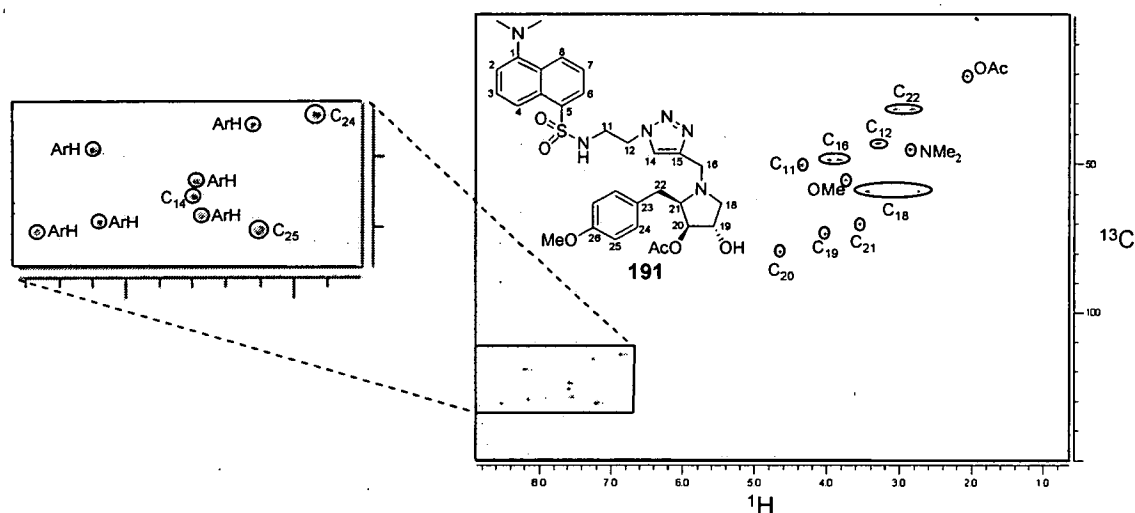
Figure 3.7: HSQC spectrum of **191** acquired at 360 MHz in acetonitrile.

Having synthesised fluorescent molecular probe **191** and confirmed it's structure by NMR spectroscopy investigations went on to focus on its fluorescent properties, to see if coupling of the marked library members had affected its fluorescence. Crude investigations carried out in a UV light box typically used for analysis of tlc's showed that both the azide **189** and the molecular probe **191** had similar fluorescence on visual comparison (**Figure 3.8a**). The fluorescence properties of the probe were then compared with that of the parent dansyl azide **189** by carrying out fluorescence measurements (**Figure 3.8b**). These experiments found that the excitation and emission spectra for dansyl azide **189** and *N*-linked probe **191** were found to be equivalent (λ_{ex} = 345 nm, λ_{em} = 550 nm).
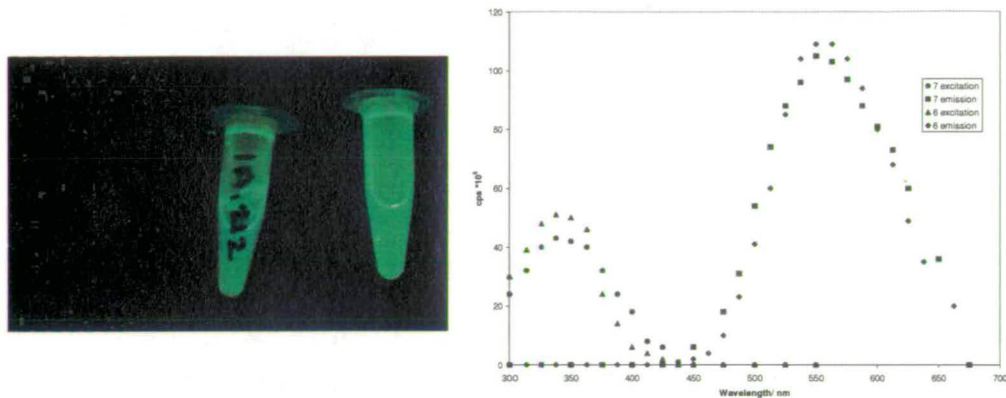
Figure 3.8: (a) Investigation of fluorescence using a UV lightbox: solvent control (left), molecular probe **191** (middle) and dansyl azide **189** (right). (b) Luminescence measurements of **189** and **191** illustrating the excitation and emission spectra.

Thus in one simple step the convertion of active marked library members to functional molecular probes has been successfully carried out, without the need to resort to tedious resynthesis often associated with the synthesis of molecular probes.

3.4.2 Biological Testing of the Fluorescent Molecular Probes

Attention then turned to testing the fluorescent molecular probes to see if their phenotype activity for activation of the SAPK pathway had been retained on coupling of the fluorophore moiety. Immunoblot assays were carried out as described in Chapter 2, probing for the phosphorylation of the protein JNK. The resultant immunoblot assay (**Figure 3.9**) demonstrate that extension of the propargyl ether marker in the C_4-H analogue as the fluorescent probe led to the loss of the SAPK response, when compared to that of the positive anisomycin control.
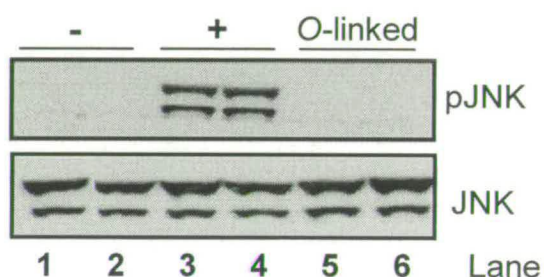
Figure 3.9: Immunoblot assay for the phosphorylation of JNK1/2 and upon stimulation by DMSO (negative control, lanes 1/2), anisomycin **102** (positive control, lanes 3/4) and molecular probe **190** (lane 5/6).

Gratifyingly, formation of the fluorescent molecular probe using the propargyl amine marker of the anisomycin derivative, led to a strong phenotypic response in the SAPK pathway assay, with activation levels comparable to that of the natural product itself (**Figure 3.10**).
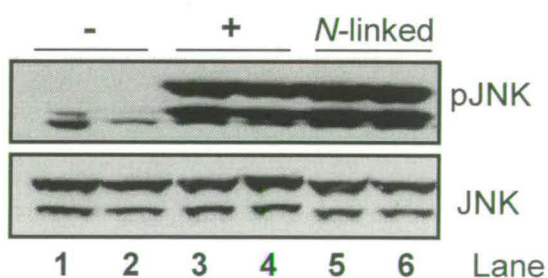
Figure 3.10: Immunoblot assay for the phosphorylation of JNK1/2 upon stimulation by DMSO (negative control, lanes 1/2), anisomycin **102** (positive control, lanes 3/4) and molecular probe **191** (lane 5/6).

3.4.3 Application of the Fluorescent Molecular Probe

Using the active molecular probe, investigations then focused on the cellular uptake and sub-cellular localisation of the fluorophore. Investigations began by screening a range of molecular probe concentrations (mM-nM) as a means of finding an optimal concentration range at which to carry out the more detailed fluorescent experiments. Cellular uptake in HEK293 cells was then investigated at increasing concentrations (22 – 162 μM) of probe. The cells were stimulated for 30 min with the probe, washed with PBS buffer and then the cellular uptake of the fluorophore was analysed by fluorescence-flow cytometry. A distinctive shift in the histograms was observed on comparing the control cells (red) and cells that had been stimulated with molecular probe (green) (**Figure 3.11a**). Moreover, a direct correlation was observed between the level of dansyl fluorescence observed in the cell populations and the initial dosing concentrations (**Figure 3.11b**).
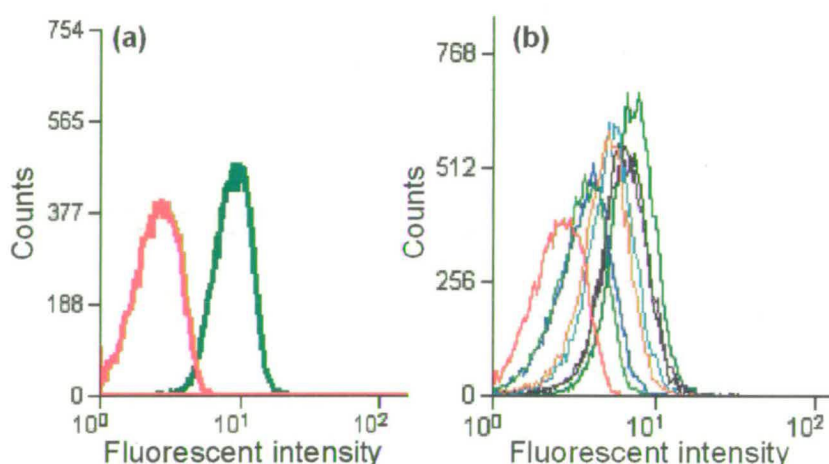
Figure 3.11: Flow cytometry analysis of cellular uptake of molecular probe **191** in HEK293 cells. Cells were incubated with 22-162 μM concentrations of *N*-linked probe **191** for 30 min and were analyzed using a MoFlo FACS instrument with a UV laser. (a) Histograms of unlabeled cells as a control (red) and cell incubated with **191** at 162 μM (green). (b) Histograms illustrating increasing fluorescence intensity with increasing concentrations of **191** (0, 22, 42, 66, 86, 108, 131, 162 μM).

Using the results of the flow cytometry, an optimum concentration (109 μM) for cell stimulation was selected which allowed visualization of the probe uptake, initially at 20-fold magnification (**Figure 3.12**). The results of this experiment showed strong fluorescence of the cells. However, this experiment could not confirm if the

fluorophore had either undergone cellular uptake or had become attached to the cell's surface.
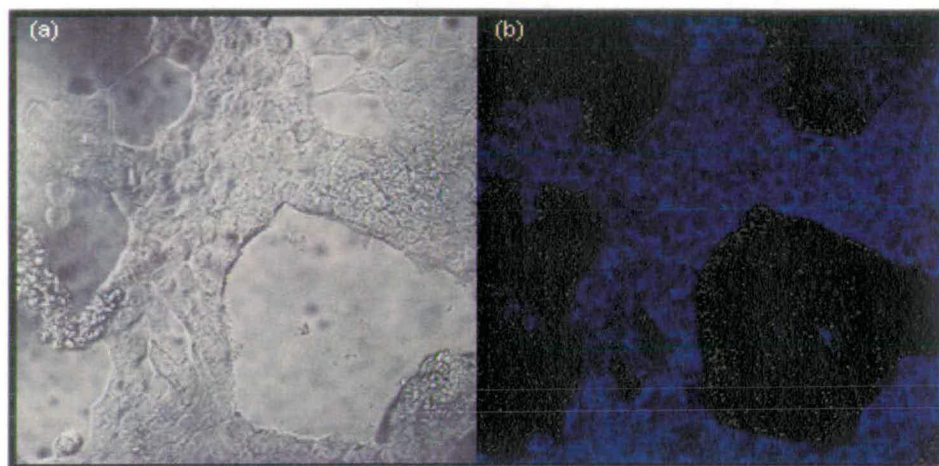
Figure 3.12: Cellular localization and uptake of molecular probe **191**. Cells were incubated for 30 min with 109 µM of **191**, washed with PBS and fixed. Analysis of cells was carried out using a Leica DM IRE2 confocal microscope with a UV laser. (a) Brightfield image of cells at 20-fold magnification. (b) Fluorescent image of cells at 20-fold magnification.

Subsequent experiments using microscopy at 63-fold magnification (**Figure 3.13**) showed a much clearer picture. Comparison of the brightfield image and the fluorescent images showed that the fluorophore was consistently found in the same cellular location, the cytosol (**Figure 3.13**, d-f). Moreover, upon carrying out confocal microscopy and taking a series of slices through the cluster of cells, the images obtained show clearly the uptake of the probe into the cytosol of the HEK293 cells.

As a control experiment, the cells were incubated with the unreactive dansyl azide **189** under the same experimental conditions as used previously. Thus, allowing the specificity of the fluorophore's subcellular location without the anisomycin moiety to be investigated. Analysis of the fluorescent images of the cells at 63-fold magnification showed a distribution of fluorophore throughout the cells, with analysis of the overlay plot confirming these findings (**Figure 3.13**, a-c).
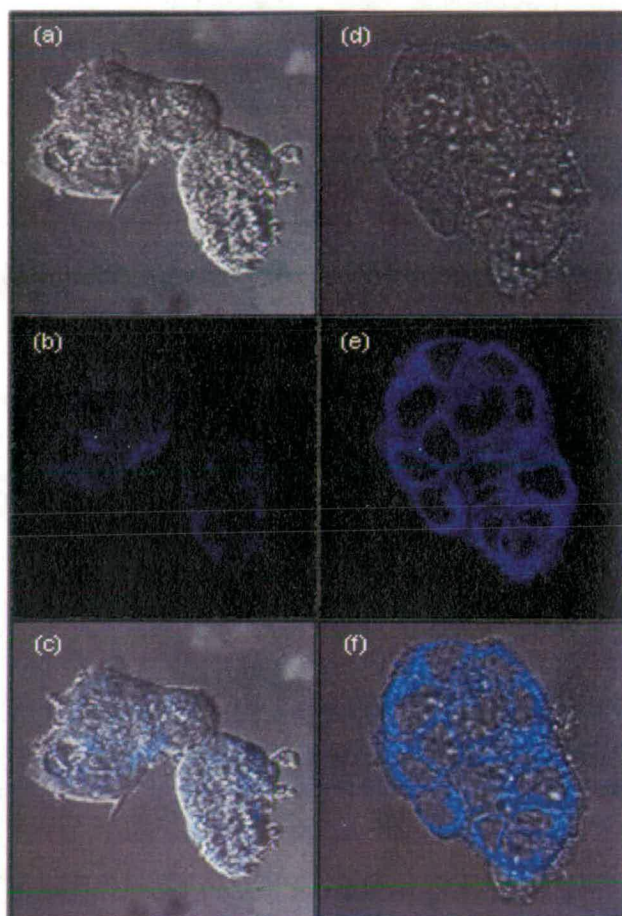
Figure 3.13: Cellular localization and uptake of parent dansyl azide **189** (a-c) and molecular probe **191** (d-f). Cells were incubated for 30 min with 109 μM of **189** or **191**, washed with PBS and fixed. Analysis of cells was carried out using a Leica DM IRE2 confocal microscope. (a and d) Brightfield image of cells at 63-fold magnification. (b and e) Fluorescent image of cells at 63-fold magnification. (c and f) Overlay of brightfield and fluorescent images.

These experiments allowed us to conclude that the binding of the fluorescent molecular probe **191** in the cytosol is directly correlated to the attachment of the anisomycin moiety to the fluorophore. Therefore, it is highly probable that the subcellular target of anisomycin is a cytosolic based protein. Thus, narrowing the field of search by ruling out the family of membrane bound proteins that had been previously postulated as possible targets.

3.5   Biotinylated Molecular Probes

The addition of a biotin tag is one of the methods of choice for identification of a small molecule's binding site.[38, 63, 68] The biotin tag binds to avidin with a K_d of 10^{-15} M, allowing the molecular probe to be attached to an avidin column through biotin.[14] While, at the opposite end of the probe the protein target is covalently attached to the small molecule of interest. This allows the non-specific cell lysates to be removed by washing and the target protein to be eluted and identified.

Investigations then focused on the synthesis of a series of biotinylated molecular probes based on the active marked library members synthesised in Chapter 2. Coupling of the marked library members through the copper(I)-catalysed Huisgen cycloaddition to a series of biotinylated azides would readily give us access to a focused library of molecular probes. Subsequent immunoblot assays of the probes would allow the selection of an active molecular probe to be utilised in the target identification process.

3.5.1 Synthesis of 'Clicked' Biotinylated Molecular Probes

Initial investigations focused on the formation of a biotinylated azide, utilising the short chain-C_2-azide **187** synthesised previously and coupling it to biotin-NHS **192**. The activated ester **192** was commercially obtainable or easily synthesised in one high yielding step from biotin **1**. Coupling of these two components (**187** and **192**) proceeded readily in methanol with triethylamine as a base to give the biotinylated azide **193** in 96% (**Scheme 3.15**). Purification by column chromatography gave a colourless solid which was fully characterised by NMR and showed a distinctive azide stretch at 2103 cm^{-1} upon analysis by IR spectroscopy.
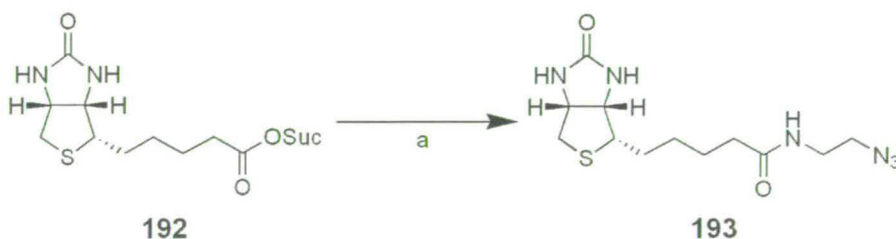

Scheme 3.15: (a) **187**, Et₃N, MeOH (96%).

The synthesis of a series of control probes with structure similarity to that of the anisomycin-based probes but inactive for SAPK pathway activation was envisaged. These control probes could then be used during affinity chromatography to simplify the results by removing non-specific protein binding that may occur on the linker and biotin moieties. The control compound **195** was readily synthesised from the analogous phenol **194** using propargyl bromide in the presence of potassium carbonate (**Scheme 3.16**). Coupling of the propargyl phenol **195** to the biotinylated azide **193** under 'click' reaction conditions gave the desired molecular probe **196** in 86% yield (**Scheme 3.17**).
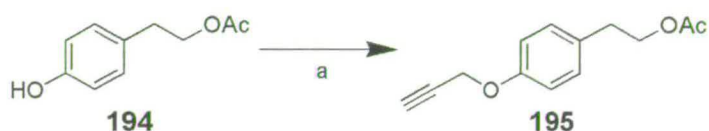
Scheme 3.16: HC≡CCH$_2$Br, K$_2$CO$_3$, DMF (77%).
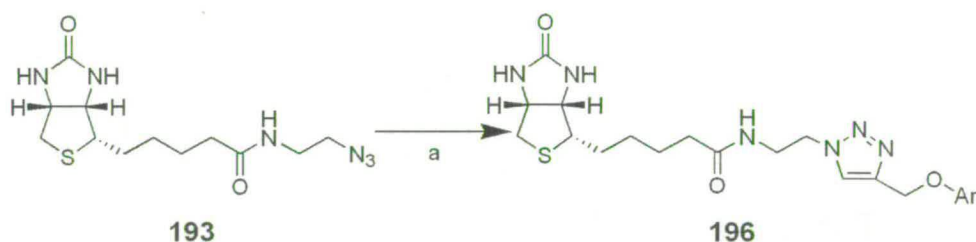
Scheme 3.17: (a) **195**, CuSO$_4$• 5H$_2$O (10 mol%), NaAsc (20 mol%), ^{t}BuOH:H$_2$O (1:1) (86%).

Having synthesised the control molecular probe **196** studies then went on to investigate the coupling of the active marked library members to the biotinylated azide **193**. The reaction of *N*-propargyl anisomycin **140** with **193** proceeded readily to give molecular probe **197** (**Scheme 3.18**).
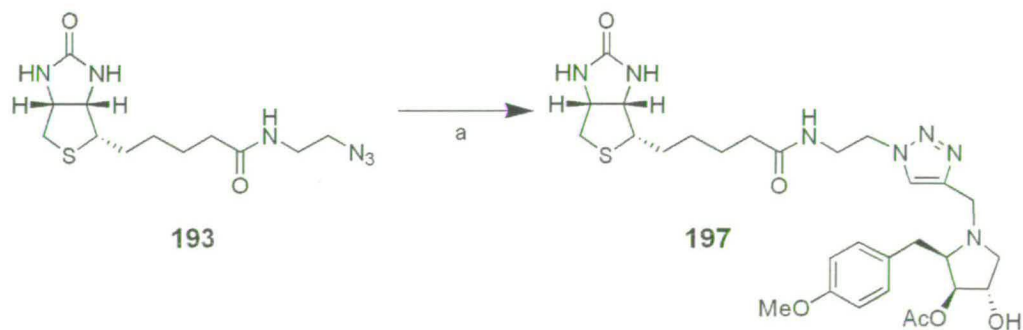

Scheme 3.18: (a) **140**, CuSO$_4$• 5H$_2$O (10 mol%), NaAsc (20 mol%), ^{t}BuOH:H$_2$O (1:1) (71%).

A detailed investigation of the structure of **197** was then carried out using a series of NMR techniques (**Appendix 3**). On comparison of the NMR spectra of the two starting materials **140** and **193** with that of the product **197**, the transfer of signals from each of the components can be clearly observed (**Figure 3.14**). Coupling of the two fragments does however lead to one new peak in the proton spectrum, from the $C_{18}H$ proton of the triazole, which can be clearly seen as a broad singlet at 7.96 ppm. A full assignment of the HSQC spectrum of **197** (**Figure 3.15**) was possible upon comparison with the HSQC spectra of the synthetic precursors and the results of the other 2D NMR experiments undertaken.
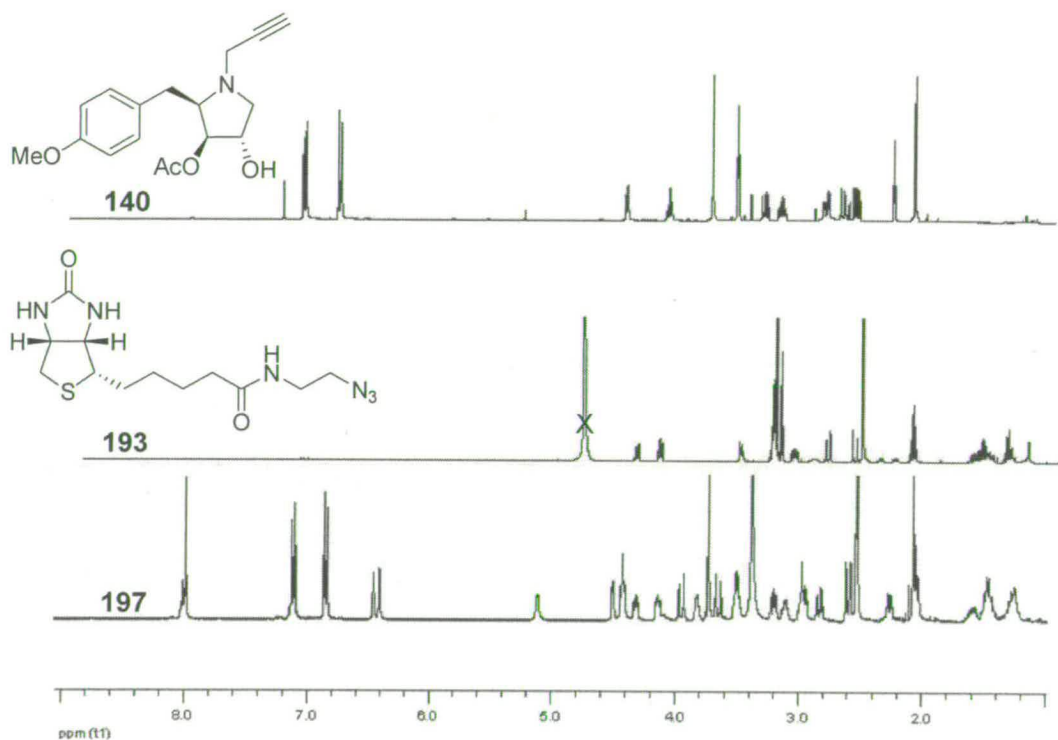
Figure 3.14: NMR stack plot of **140** (top), **193** (middle) and **197** (bottom) at 360 MHz.
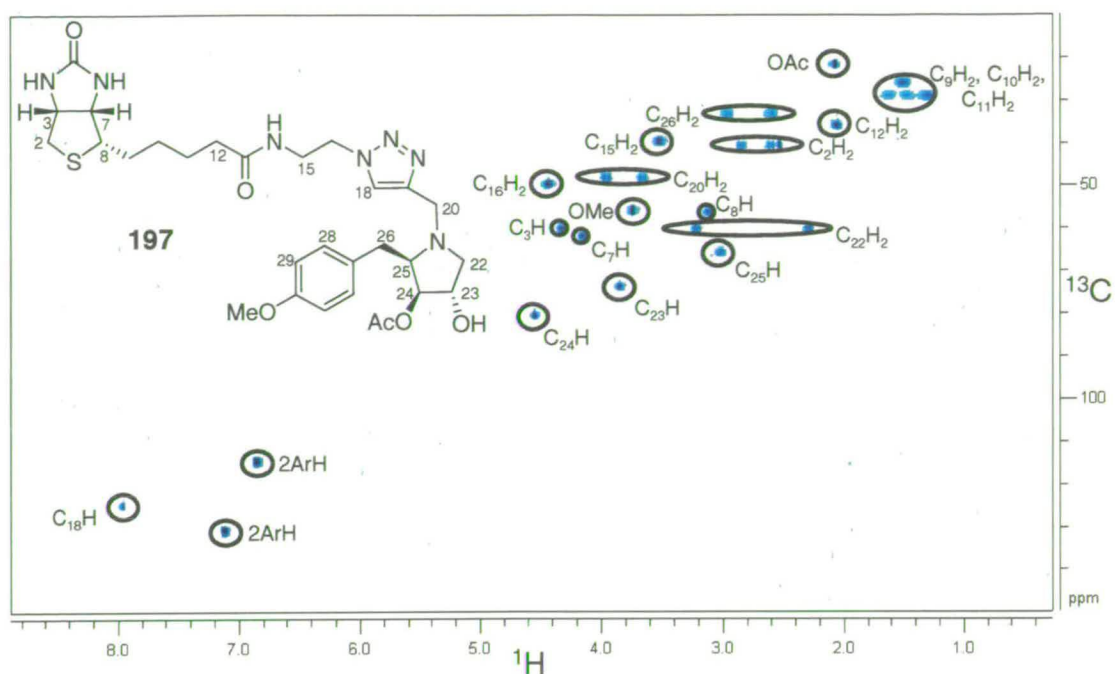
Figure 3.15: Fully assigned HSQC spectrum of **197** at 360 MHz in DMSO.

Our attention then turned to the coupling of the *O*-propargyl marked analogue **136**. Initial synthetic attempts led to the coupling of the two components when monitoring the reaction by tlc. However, purification of the free amine proved to be challenging (**Scheme 3.19**). Upon column chromatography the amine was found to streak on the column, resulting in the isolation of impure product. Attempts to purify the mixture by HPLC and reverse phase column chromatography also proved unsuccessful.
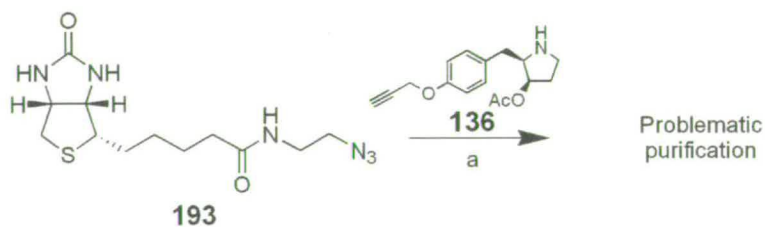
Scheme 3.19: (a) **136**, CuSO$_4$• 5H$_2$O (10 mol%), NaAsc (20 mol%), ^{t}BuOH:H$_2$O (1:1).

However, on coupling the Boc protected marked analogue **135** to the biotinylated azide **193** the product could be isolated cleanly and in high yields (**Scheme 3.20**). Removal of the Boc protecting group under acidic conditions then gave the desired compound **198**.

193   **198**
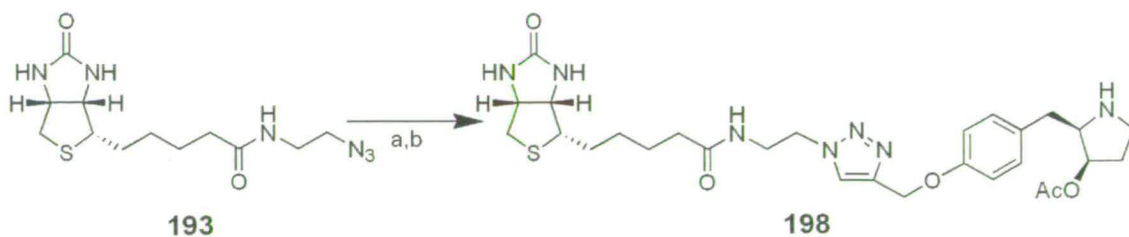
Scheme 3.20: (a) **135**, CuSO$_4$• 5H$_2$O (10 mol%), NaAsc (20 mol%), ^{t}BuOH:H$_2$O (1:1) (70%); (b) TFA, DCM (98%).

With the three molecular probes in hand, it was decided to expand this methodology to explore what role the length of the carbon linker between the biotin tag and the anisomycin analogue had on activation of the SAPK pathways. Starting from 6-amino hexan-1-ol **199** the azide could be readily converted into the desired amino azide linker **201** in two steps. Firstly the alcohol **199** is reacted with thionyl chloride to form the chloride analogue **200**; then the chloride is displaced with sodium azide to give the desired azide **201** in 90% over two steps (**Scheme 3.21**). The azide **201** can then be coupled to biotin-NHS **192** using a procedure analogous to that used for the C$_2$-linker, to give **202** in high yield (**Scheme 3.22**).

199   **200**   **201**
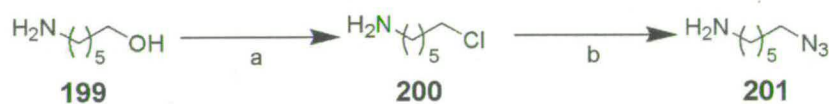
Scheme 3.21: (a) SOCl$_2$, toluene; (b) NaN$_3$, H$_2$O (90% over 2 steps).[320]

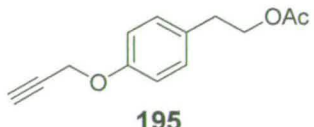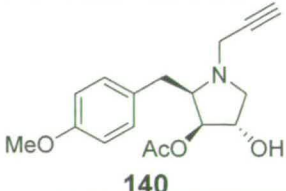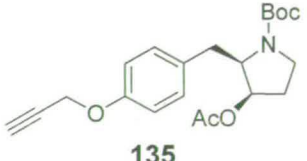**192**   **202**
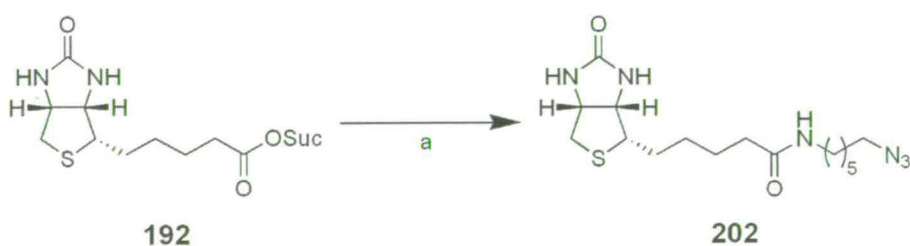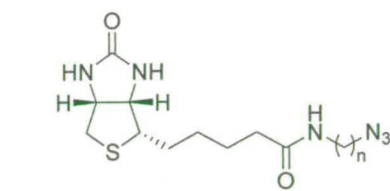
Scheme 3.22: (a) **201**, Et$_3$N, MeOH (89%).

Applying the same 'click' reaction conditions utilised in the previous coupling, the longer azido-C$_6$-biotin **202** was coupled to the control compound **195**, the *N*-propargyl anisomycin **140** and the *O*-propargyl analogue **135**, forming **203**, **204** and

205 respectively, all in high yields. All the molecular probes were characterised by extensive 2D NMR experiments and were found to have very similar spectra to the analogous shorter linker probes. The key differences were found in the alkyl region of the spectra where the C_{16}-C_{19} CH_2's of the linker were found to be overlapping with the C_9-C_{11} CH_2's of the biotin chain.

The synthesis of all the biotinylated molecular probes formed using 'click' chemistry has been summarised (**Table 3.2**). Across the board the coupling of the propargyl marked molecules to the biotinylated azide has occurred in high yields and with high regioselectivity. Thus illustrating the strength of the copper(I)-catalysed Huisgen cycloaddition as a powerful reaction for coupling of two orthogonally marked units.
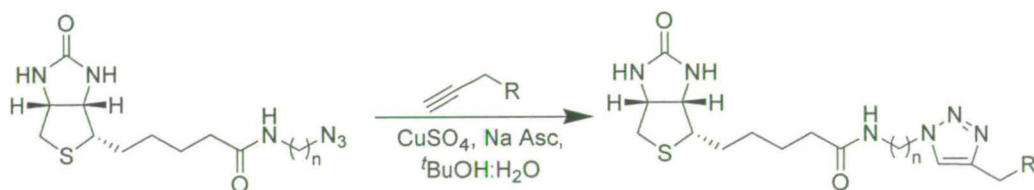

	Click Coupling	
	n = 2 (**193**)	n = 6 (**202**)
control **195**	86% (**196**)	75% (**203**)
N-linked **140**	71% (**197**)	73% (**204**)
O-linked **135**	70% (**198**)	76% (**205**)

Table 3.2: Summary of 'click' coupling reactions used to form a series of biotinylated molecular probes.

## 3.5.2 Synthesis of 'Classical' Biotinylated Molecular Probes

As a contrasting approach to the synthesis of molecular probes, the decision was taken to investigate a more 'classical' approach to the biotinylation of small molecules. Classically, biotin would be attached through the formation of amide or ester bonds. However, in our case this was believed to be an unfeasible strategy as this would generate a number of issues at our proposed coupling sites. At the phenolic functionality, formation of an ester would give a phenolic ester; which are notoriously unstable. In contrast, formation of an amide at the pyrrolidine nitrogen would give a strong bond, however there are no reports in the literature or within the Hulme group that amides [or carbamates] are tolerated for activation of the SAPK phenotype. We therefore decided to apply a different strategy by utilising nucleophilic displacement chemistry as the 'classical' coupling reaction. For this strategy the commercially available iodoacetyl-C_2-biotin **76** (Molecular Probes) was utilised as a direct comparison to our azido-C_2-biotin **193**. Coupling of the phenol control compound **194** to the iodoacetyl biotin **76** occurred readily in the presence of potassium carbonate base upon heating at 80 °C in DMF (**Scheme 3.23**). This gave the desired product **206** in 86% yield after purification by column chromatography. With this initial good result in hand; we set about synthesising the analogous molecular probes based on anisomycin's scaffold.
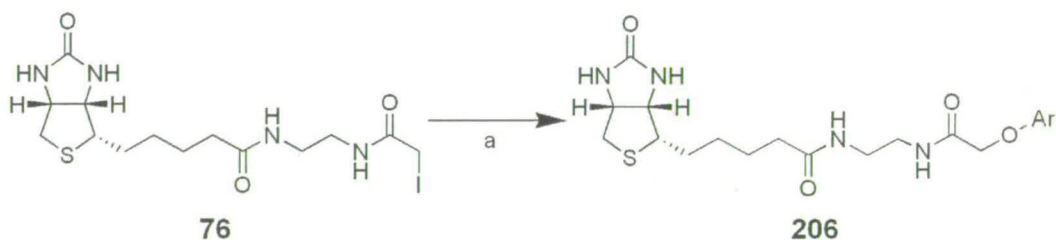
Scheme 3.23: (a) **194**, K_2CO_3, DMF, 80 °C (86%).[320]

Initial attempts focused on applying the successful coupling conditions used for the control compound to the coupling of anisomycin **102** (**Scheme 3.24**). However, the high yields obtained on coupling the control compounds were found to be impossible to reproduce when coupling the more complicated substrate. Heating the two

coupling components **76** and **102** with potassium carbonate in DMF for 5 days led to the isolation of 34% of the target material **207**.
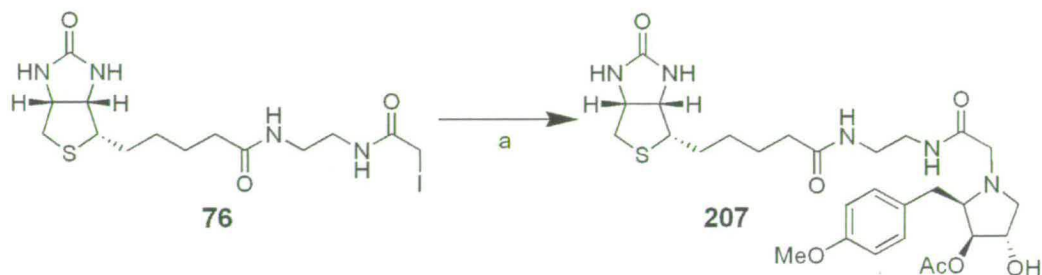
Scheme 3.24: (a) **102**, K₂CO₃, DMF, 80 °C (34%); or **102**, K₂CO₃, Bu₄NI, acetone; or **102**, K₂CO₃, KI, DMF, μwave.

Due to the long timescale of this reaction it was believed that a feasible option was to carry out the reaction in a microwave. The fragments to be coupled **76** and **102** were irradiated in DMF in the presence of potassium carbonate for 5-20 min. However, upon analysis of the crude NMR spectra the loss of the acetate moiety from anisomycin was clearly shown, even after 5 min. Therefore, the base was altered from potassium carbonate, as this was believed to be facilitating the removal of the acetate under microwave conditions, and replaced with triethylamine. However, these alterations did not improve the reaction, with starting materials being recovered in all cases.

We therefore turned our attention back to the original conditions and carried out the coupling of the phenolic analogue **134**, to obtain the molecular probe in 31% yield (**Scheme 3.25**). Removal of the carbamate protecting group occurred in high yield to give the desired molecular probe **208**.
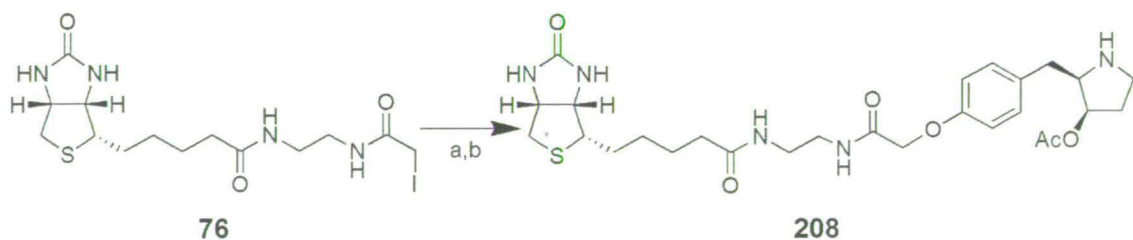
Scheme 3.25: (a) **134**, K₂CO₃, DMF, 80 °C (31%); (b) TFA, DCM (95%).

Having synthesised the classically linked short chain molecular probes our attention therefore moved on to the development of a longer carbon linker; thus allowing a direct comparison to the coupling of the azido-C_6-biotin **202**. Iodo- and chloro-acetyl biotin linkers **210** and **211** were synthesised within the group in three synthetic steps from biotin-NHS **192** and mono-Boc hexane-1,6-diamine **209** (**Scheme 3.26**).
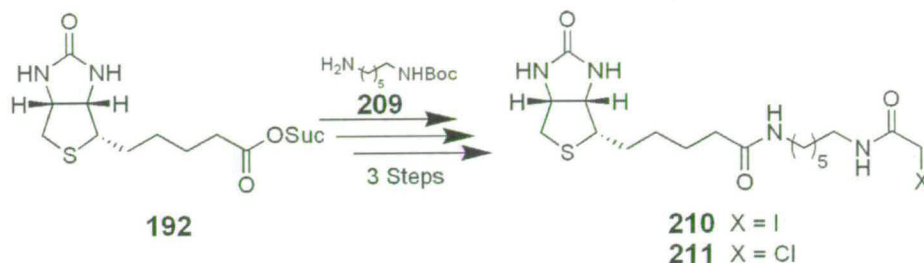
Scheme 3.26: Three step synthesis of iodo/chloroacetyl-C_6-biotin **210/211** from biotin-*N*-succinimidyl ester **192**.[320]

Using the reaction conditions previously optimised iodoacetyl biotin **210** was coupled to the control compound **194** to give **212** in 72%. However, on altering the nucleophile to anisomycin **102** the reaction only gave 35% of **213** after purification by column chromatograph; while in the case of the phenolic analogue **134** no trace of coupled product was isolated. Investigations into the coupling reaction of the chloroacetyl biotin **211** found that it gave analogous results to that of the iodoacetyl reagent **210** in all cases.

The 'classical' coupling results for the nucleophilic displacement reactions are summarised (**Table 3.3**). The data shows that, as with the 'click' couplings, the length of linker does not play a key factor in the reproducibility of the coupling reaction. However, the nature of the nucleophile (either steric or electronic) plays a key role in couplings of this manner. It can be concluded that coupling of simple substrates using this nucleophilic methodology can be achieved in high yield, but on more complex substrates the applicability of this methodology rapidly diminishes.

	Classical Coupling	
	n = 2 (**76**)	n = 6 (**210**)
control **194**	86% (**206**)	72% (**212**)
N-linked **102**	34% (**207**)	35% (**213**)
O-linked **134**	31% (**208**)	N/A

Table 3.3: Summary of 'classical' coupling reactions used to form a series of biotinylated molecular probes.

### 3.5.3 Comparison of 'Click' *v* 'Classical' Coupling Strategies

On comparison of the two coupling strategies a number of distinct advantages were found using the 'click' approach. These included the ease with which the biotinylated azides were synthesised, the high yields and regioselectivities observed across a range of coupling partners and the ease with which the products could be purified. In contrast, the 'classical' approach suffered difficulties from the outset, as the synthesis of the iodoacetyl-C_6-biotin proved problematic. Upon the successful synthesis of the iodoacetyl-C_6-biotin, the coupling reaction to structurally simple coupling partners proceeded in high yield. However, on attempting to couple to more complex molecules this reaction failed to give even moderate yields and purification proved challenging.

The 'classical' approach to the biotinylated molecular probes encountered a range of synthetic issues, in contrast to the straightforward methodology applied to the 'click' substrates, where the coupling partners were easily derivatised with the required azide-alkyne functionality and the coupling proceeded readily. These results further illustrate the power of the copper(I)-catalysed Huisgen cycloaddition, as a key coupling reaction across a broad range of molecular architectures.

3.5.4 Biological Testing of the Biotinylated Molecular Probes

On completing the synthesis of the biotinylated molecular probes, we set out to investigate the response of the SAPK pathways upon stimulation with these molecules. Immunoblot assays were carried out as previously described and tested for the phosphorylation of the protein JNK.[246, 258] The molecular probes were classified and tested according to linker length and the coupling reaction used in their formation. The blots show negative (lanes 1/2) and positive standards (lanes 3/4), followed by the control molecular probe (lanes 5/6), the *N*-linked molecular probe (lanes 7/8) and the *O*-linked molecular probe (lanes 9/10).

Immunoblot assays for the series of probes formed by the 'click' reaction of the azido-C_2-biotin **193** showed that the control probe **196**, as intended, did not activate the SAPK pathway (**Figure 3.16**, lanes 5/6). The *N*-linked molecular probe **197** showed levels of activation analogous to that of anisomycin itself (**Figure 3.16,** lanes 7/8), while the *O*-linked molecule **198** was not found to give any phenotypic SAPK response (**Figure 3.16**, lanes 9/10),
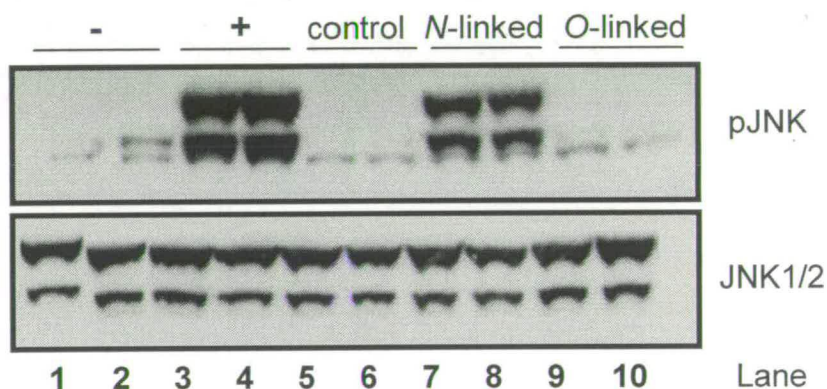
Figure 3.16: Immunoblot assay for the phosphorylation of JNK1/2 and upon stimulation by DMSO (negative standard, lanes 1/2), anisomycin **102** (positive standard, lanes 3/4), control molecular probe **196** (lanes 5/6), *N*-linked molecular probe **197** (lanes 7/8) and *O*-linked molecular probe **198** (lanes 9/10). Molecular probes were formed by the 'click' reaction of the short chain biotinylated azide **193**.

Biological testing of the next series of molecular probes, formed by the 'click' reaction of the long chain-C_6-azide **202** showed an identical pattern for SAPK activation (**Figure 3.17**). Again, the control probe **203** and the *O*-linked probe **205** were found to be inactive (**Figure 3.17**, lanes 5/6 and 9/10 respectively), while the *N*-

linked molecular probe **204** formed by a 'click' reaction of the long chain C$_6$-biotinylated azide **202** shows a strong phenotypic response (**Figure 3.17,** lanes 7/8).

Figure 3.17: Immunoblot assay for the phosphorylation of JNK1/2 and upon stimulation by DMSO (negative standard, lanes 1/2), anisomycin **102** (positive standard, lanes 3/4), control molecular probe **203** (lanes 5/6), *N*-linked molecular probe **204** (lanes 7/8) and *O*-linked molecular probe **205** (lanes 9/10). Molecular probes were formed by the 'click' reaction of the long chain biotinylated azide **202**.

On altering from the 'click' coupled molecular probes to the classically formed probes with the short chain, an identical pattern was found upon testing (**Figure 3.18**). Positive phenotype responses were obtained from the anisomycin standard and the *N*-linked molecular probe **207** (**Figure 3.18,** lanes 7/8). As with the previous cases the control and *O*-linked probes (**206** and **208**) were found to be inactive in testing for phosphorylation of JNK (**Figure 3.18,** lanes 5/6 and 9/10 respectively).
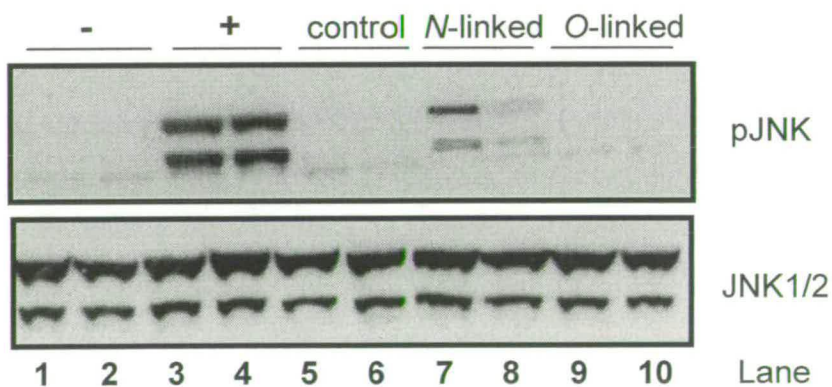
Figure 3.18: Immunoblot assay for the phosphorylation of JNK1/2 and upon stimulation by DMSO (negative standard, lanes 1/2), anisomycin **102** (positive standard, lanes 3/4), control molecular probe **206** (lanes 5/6), *N*-linked molecular probe **207** (lanes 7/8) and *O*-linked molecular probe **208** (lanes 9/10). Molecular probes were formed by the nucleophilic displacement reaction of the short chain biotinylated iodoacetyl **76**.

The final series of long chain classical probes were then tested for the phenotypic SAPK response and were found, reassuringly, to show the same trends as the previous series (**Figure 3.19**). Once again the *N*-linked molecular probe **213** was found to activate the pathway in a comparable level to that of the anisomycin positive standard (**Figure 3.19**, lanes 7/8), while the other molecular probes tested were found to give no response (**Figure 3.19**, lanes 5/6 and 9/10 respectively).
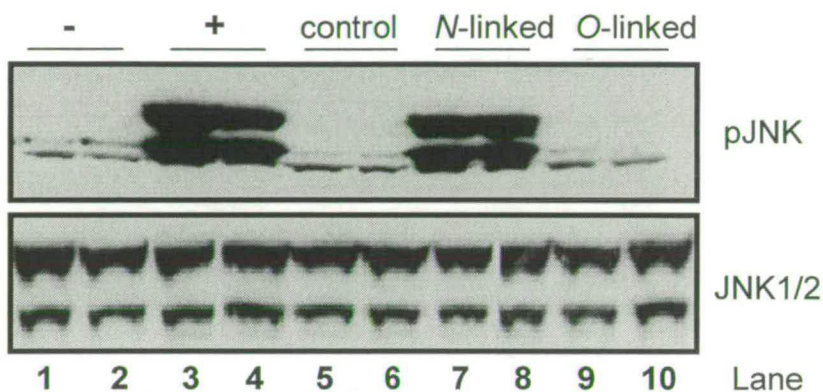
Figure 3.19: Immunoblot assay for the phosphorylation of JNK1/2 and upon stimulation by DMSO (negative standard, lanes 1/2), anisomycin **102** (positive standard, lanes 3/4), control molecular probe **212** (lanes 5/6), *N*-linked molecular probe **213** (lanes 7/8) and DMSO (lanes 9/10). Molecular probes were formed by the nucleophilic displacement reaction of the long chain biotinylated iodoacetyl **210**.

With these results in hand, it can be concluded that we have synthesised four biotinylated molecular probes that active the SAPK pathway. Each active molecular probe was structurally different with varying linker lengths (C_2 and C_6) and was formed using a different coupling strategy ('click' chemistry and 'classical' nucleophilic displacement chemistry). However, two key factors emerged in the synthesis of active molecular probes. Firstly, the structure of the anisomycin moiety must be based solely on the anisomycin core, not the C_4-H analogue. Moreover, the coupling reaction should be carried out at the pyrrolidine nitrogen of anisomycin's molecular scaffold in order to form functionally active molecular probes.

3.5.5 Affinity Chromatography

Having successfully synthesised a number of biotinylated molecular probes, work began on the identification of anisomycin's binding site. Utilising a series of biochemical techniques, a number of preliminary experiments were carried out to find the most applicable method of target identification.

Starting with affinity chromatography using streptavidin-sepharose as the affinity media, the HEK293 cell lysates were precleared to remove all endogenously biotinylated proteins. The molecular probes **196** and **197** were then added to the cell lysates and rotated for 1 h. The excess lysates were removed and the streptavidin-sepharose denatured in SDS. The denatured proteins were separated by SDS-PAGE and stained with Ponceau S. However, analysis of this gel showed a large background response making the detection of individual protein bands impossible.

Our attention then turned to using magnetic Dynabeads® with streptavidin instead of streptavidin-sepharose. It was believed that this alteration would reduce the levels of background response and improve the sensitivity of the experiment. Carrying out the pull-down experiment using the previous conditions but with Dynabeads® led to significant improvements (**Figure 3.20**). Firstly, the large background that had obscured any protein bands during the initial experiment had been removed, while a series of bands distinct from the control lanes (**Figure 3.20**, lanes 1/2) were found in lane 4 (**Figure 3.20**, arrows).
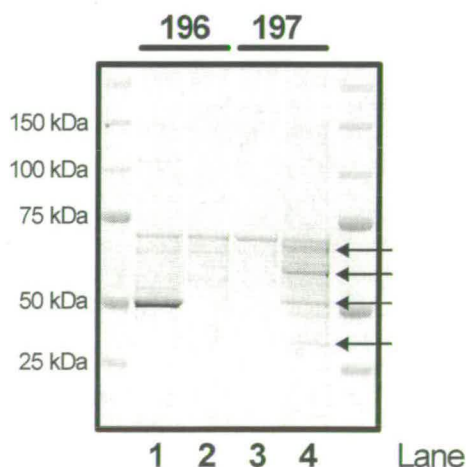
Figure 3.20: SDS-PAGE of cell lysates removed from Dynabeads® and stained with Ponceau S. Lane 1/2: internal standards using the control molecular probe **196**. Lane 3/4: pulldown experiments using active molecular probe **197**.

However, our concern was raised when the bands in lanes 3/4 were found not to match, even though this experiment had been carried out in duplicate. Despite this anomaly the protein bands of interest were cut from the gel, digested in tripsin and analysed by MS-MS. The protein bands were identified as widely expressed protein that had been pulled down in a non-specific manner. However, we believe that the use of the Dynabeads® will lead to an improved experimental procedure for future work.

We therefore turned our attention to using Western blotting and Far Western blotting to identify the biological target of anisomycin. Western blotting was carried out in an identical manner to previous experiments, with HEK293 cell lysates being treated with molecular probes **196**, **197** and **207** for 30 min before lysis. The protein were separated by SDS-PAGE and transferred to nitrocellulose membrane. An avidin-HRP secondary antibody was then incubated with the membranes and immunoreactive protein visualised using ECL, in an attempt to identify the biological target of the molecular probe. However, no distinctive protein bands were identified on comparing the control lanes to the probes **196**, **197** and **207** (**Appendix 4a**). On altering the protein separation conditions from denaturing to non-denaturing, the immunoblot assay resulted in the visualisation of a single protein band across the control and active probe lanes (**Appendix 4b**).

The Far Western blotting experiment took the untreated cell lysates and separated them using SDS-PAGE, before transferring the proteins to nitrocellulose membrane. The membranes were then treated with the molecular probes **196** and **197**, utilising them as a primary antibody. Subsequently incubation with avidin-HRP as a secondary antibody, allowed the immunoreactive proteins to be visualised using ECL. The experiment utilising denaturing conditions led to the visualisation of a large number of proteins, however no bands were found to differ from that of the control lanes (**Appendix 4c**). On utilising native gels to separate the proteins a single protein band was visualised but once again it was found in both the control and active probe lanes (**Appendix 4d**).

At this point in time the library of biotinylated molecular probes was handed over to our collaborators at the MRC Protein Phosphorylation Unit in Dundee to continue the affinity chromatography experiments.

3.6   Solid Supported Anisomycin

Having successfully synthesised a series of biotin and fluorophore derived molecular probes based on coupling anisomycin through its pyrrolidine nitrogen; we next focused on applying our coupling strategy to the synthesis of solid supported anisomycin. The solid support utilised was Affi-gel-10 **214** (Bio-Rad), an agarose based affinity media that contains a neutral 10-atom spacer arm and a reactive *N*-hydroxysuccinimide ester (**Figure 3.21**).[321] It is designed to couple to a primary amino group in both aqueous and non-aqueous systems, and appeared ideal for our purpose.
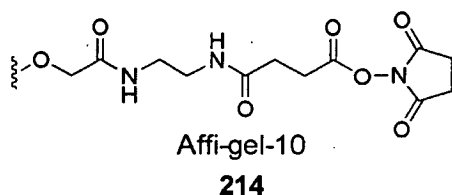
Affi-gel-10
214

Figure 3.21: Structure of Affi-gel-10 **214**.

Coupling of Affi-gel-10 **214** to the short chain azide **187** was carried out readily in methanol in the presence of 5 equivalents of azide to give **215** (**Scheme 3.27**). After extensive washing with methanol and isopropyl alcohol, the introduction of the azide functionality was confirmed by IR spectroscopy which showed an azide stretch at 2109 cm^{-1}.

214   **215**
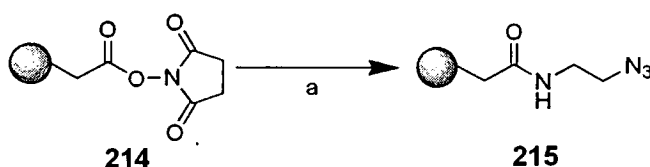
Scheme 3.27: (a) **187**, MeOH (100%).

The functionalised azide **215** was then coupled to *N*-propargyl anisomycin **140** using the copper(I)-catalysed Huisgen cycloaddition reaction in a water:'butanol (1:1) mixture (**Scheme 3.28**). The presence of *N*-propargyl anisomycin **140** was monitored by tlc during the reaction and the reaction stopped upon complete incorporation of

the propargyl moiety. The solid support anisomycin **216** was washed with ^{t}butanol and the organics concentrated to recover trace amounts of unreacted **140**, thus confirming the coupling of the anisomycin to the solid support.
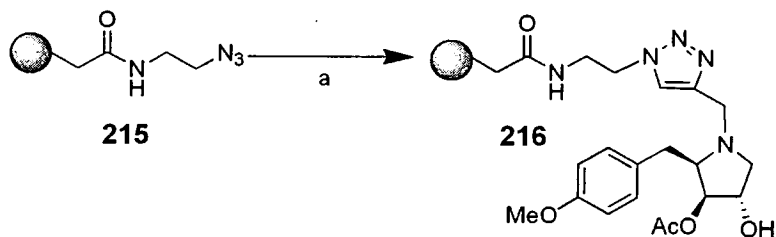
Scheme 3.28: (a) **140**, CuSO$_4$• 5H$_2$O (10 mol%), NaAsc (20 mol%), ^{t}BuOH:H$_2$O (1:1) (98%).

The solid supported anisomycin probe and a sample of the solid supported azide have also been passed onto our collaborators (MRC PPU, Dundee). The solid supported and biotinylated molecular probes are currently being utilised in an attempt to identify anisomycin's subcellular binding partner.

3.7   Summary

The synthesis and characterisation of a number of fluorescent molecular probes based on the active marked library members has been successfully carried out. Immunoblot assay for the activation of the SAPK pathway showed the synthesis of an active fluorescent molecular probe **191**. Subsequent FACS and microscopy investigations into the subcellular location of anisomycin were carried out utilising **191**. These investigations have allowed us to conclude that there is selective uptake of the molecular probe in the cytosol of HEK293 cells.

A series of biotinylated molecular probes (**196-198** and **203-205**) have also been synthesised using the same marked library members. A comparative study of coupling reactions was carried out between the copper(I) catalysed 'click' reaction and a 'classical' nucleophilic displacement. The experimental results overwhelmingly favoured the 'click' reaction, which gave the desired molecular probes in high yields over a range of substrates. In contrast, the 'classical' reaction proved to be slow and unreliable for the coupling of complex substrates.

A distinctive trend emerged during the synthesis of the molecular probes. All the compounds linked through the pyrrolidine nitrogen of anisomycin showed the phenotypic SAPK response. This trend was continued over a wide range of molecular probes whether 'classically' or 'click' coupled, with C_2 or C_6 linkers, with dansyl or biotin. This series of results provides us with new detailed SAR information for anisomycin's activation of the SAPK pathways. Based on this new SAR data the synthesis of a solid supported analogue of anisomycin was readily achieved from commercially available Affigel. The biotinylated and solid supported molecular probes are now being used to identify anisomycin's biological site of action. Consequently, identification of the target protein will lead to a greater understanding of the mechanism of activation of the SAPK pathways and may also generate new therapeutic targets.

Small molecule chemical genetics screens can generate large numbers of active compounds. However current strategies for the development of these "hits" into active molecular probes for further biological investigation are often cumbersome. In the case of anisomycin, it has been demonstrated that the incorporation of a biocompatible propargyl marker into the molecular scaffold can lead to the rapid generation of active molecular probes based on both the dansyl fluorophore and biotin.

Due to the wide range of biocompatible reactions which have been developed over the last few years, there are a number of options for the choice of bioorthogonal markers. This allows the tailoring of this strategy to the small molecule library under investigation. Therefore, future library design using this marked library approach should provide a useful truncation of the small-molecule to molecular probe synthetic process.

4 Steroid Marked Libraries and Molecular Probes

4.1 Steroids

The term steroid covers a large series of biomolecules based on a carbon skeleton arranged in four fused rings. These molecules have a wide range of biological function with common categories including: anabolic steroids which are often used as performance enhancing drugs; corticosteroids which regulate many aspects of metabolism, renal excretion and immune function; sex steroids which include the oestrogens and androgens and phytosterols which are found in a range of plants.[134]

4.1.1 Steroid Nomenclature

The common names of steroids are widely recognised such as cholesterol, testosterone, *etc.* However, systematic nomenclature is becoming more prevalent making familiarity with both nomenclatures increasingly important. The steroid rings are designated as A, B, C and D rings, with the numbering of the carbon skeleton starting at the A ring and working around the backbone sequentially (**Figure 4.1**).[322, 323]

217
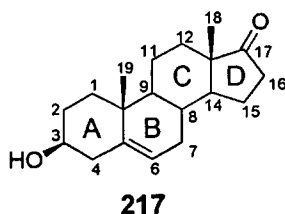
Figure 4.1: General steroid architecture, illustrating the nomenclature used to describe the four fused rings and the numbering of the carbon backbone.[322, 323]

The classification of steroids is based on the number of carbon atoms within the molecule's structure. Steroids with 21 carbon atoms are classified as pregnanes, whereas those with 19 and 18 carbon atoms are classified as androstanes and estranes respectively. Prefixes are often used whereby *nat-* defines the naturally occurring enantiomer, *ent-* the opposite enantiomer and *rac-* the racemic steroid. On top of this the signs α/β indicate the relative stereochemistry with relation to the plane of the

molecule; where a substituent below the plane is termed α and one above the plane β. Therefore, **217** is found under a number of names including dehydroepiandrosterone (DHEA), prasterone and 3β-hydroxy-5-androsten-17-one to name but a few.

4.1.2 Steroid Biosynthesis

Despite the number of steroids that have been identified in plants, animals and humans the vast majority of steroids are derived from the acetyl CoA biosynthetic pathway.[134, 324] The first sequence of reactions in this biosynthetic process involves the synthesis of squalene **225**, the key C_{30} building block in the formation of steroids.   Starting from the small building blocks acetyl and acetoacetyl SCoA (**219** and **218**), condensation and subsequent hydrolysis forms mevalonate **220**, before decarboxylative elimination to form isopentenyl pyrophosphate (IPP) **221** (**Scheme 4.1**).[325-327] Six units of IPP **221** are then condensed resulting in the formation of the C_{30} squalene unit **225**. This occurs in a convergent manner combining three C_5 (isopentenyl) units (**221**+**222**→**223**+**221**→**224**) to give a C_{15} (farnesyl) unit **224** which can then dimerise to give a C_{30} (squalene) unit **225**.[328]
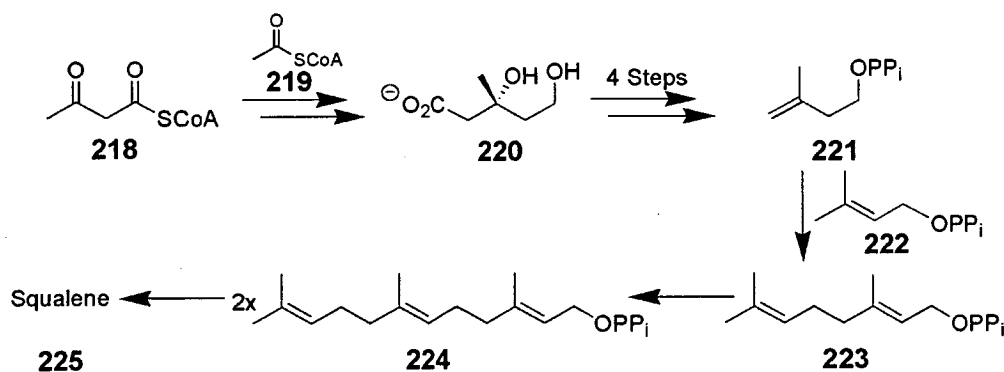
Scheme 4.1: Biosynthesis of squalene **225** the key intermediate in the formation of steroid architecture.[134, 324-328]

The next series of biotransformations leads to the formation of the steroid core, a sequence which begins with the activation of squalene **225** *via* epoxidation of the terminal double bond to give **226** (**Scheme 4.2**).[329-331] A facile cyclisation catalysed by oxidosqualene cyclase then takes place in a remarkable concerted biotransformation which leads to the formation of the tetracyclic steroid core **227**. The carbocation **227**

then rearranges to form lanosterol **228**, which can be converted into cholesterol **229** through a number of biosynthetic steps.[329-331]
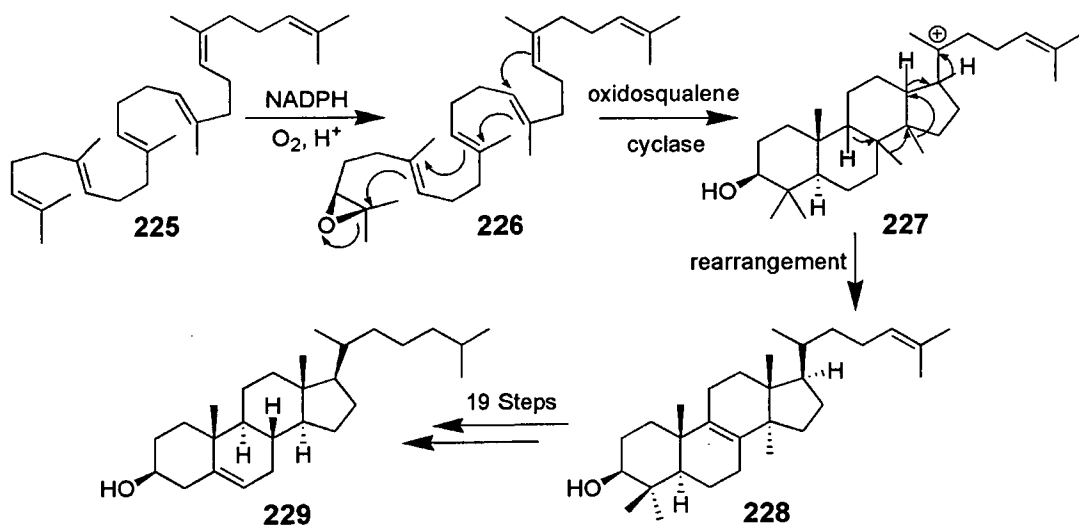
Scheme 4.2: Biosynthesis of cholesterol **229** from squalene **225**; through formation of a terminal expoxide, concerted cyclisation and rearrangement to form the steroid core, before a number of functional group modifications to give cholesterol.[329-331]

Cholesterol **229** is mainly synthesised in the liver, with the intestine also forming significant amounts.[134] In addition it can be obtained from dietary sources; with an adult on a low cholesterol diet typically synthesising about 800 mg of cholesterol per day.[134] Cholesterol has an important biochemical role in the formation and maintenance of cell membranes, the manufacturing of bile and the metabolism of vitamins A, D, E and K, as well as being the key intermediate in the biosynthesis of a wide range of steroids.[332]

The conversion of cholesterol **229** to pregnenolone **230** is accomplished by the cleavage of the cholesterol side chain unit, a process which is catalysed by a cytochrome P450 enzyme termed P450scc, where scc stands for side chain cleavage (**Scheme 4.3**).[333, 334] This enzyme catalyses three distinct sequential reactions on a single active site, firstly the hydroxylation of C_{22}, followed by the hydroxylation of C_{20} and then the cleavage of the C_{20}-C_{22} bond to give **230** and isocaproaldehyde. Each of these individual steps requires a pair of electrons from NADPH, which are passed *via* a flavoprotein to an iron/sulfur protein and on to the P450.[335]

Pregnenolone **230** is then converted to DHEA **217** in a two step process that is catalysed by a single cytochrome enzyme, P450c17.[333, 334] The first step in this process

is the 17α-hydroxylation of the steroidal core to give **231**. This is followed by the cleavage of the C_{17}-C_{20} bond in a lyase reaction, which is stimulated by the presence of cytochrome b_5, forming DHEA **217**. The role of cytochrome b_5 in this process has not been confirmed but experimental data suggest that it acts as an allosteric activator of the lyase reaction, rather than an alternative electron donor.[336] In addition the phosphorylation state of P450c17 is a key factor in regulating the lyase reaction, with phosphorylation of the serine/threonine residues of the cytochrome leading to an increase in lyase activity.[337] It has been proposed that increased phosphorylation results in improved substrate binding and intermediate recognition due to accelerated electron transfer.
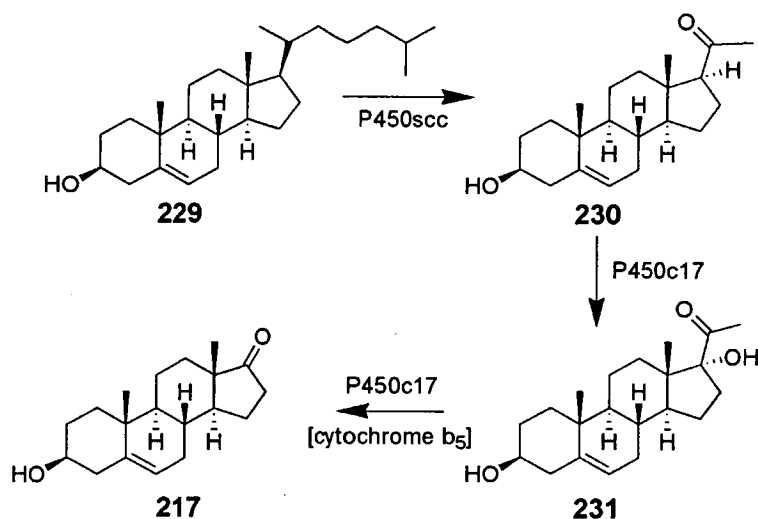
Scheme 4.3: Biosynthetic conversion of cholesterol **229** to DHEA **217** *via* two P450 catalysed oxidative cleavage reactions.[333, 334]

DHEA **217** is the most abundantly produced steroid hormone which plays a number of biological roles, but it can also be converted to a range of downstream steroid metabolites.[338, 339] These include 4-androstene-3,17-diol **232** and 4-androstene-3,17-dione **234** (**Scheme 4.4**), which in turn can be converted into the biologically important steroids testosterone, estrone and estradiol. DHEA **217** can also be sulfated at the C_3-hydroxyl by the enzyme DHEA-sulfotransferase which is expressed in the human liver and adrenals forming dehydroepiandrosterone sulfate (DHEAS) **233**.[339, 340] On the whole DHEAS represents the hydrophilic storage form of the steroid circulating in the blood stream, however only lipophilic DHEA can be converted into

androgens and estrogens intracellularly. Therefore, hydrolysis of the sulfate functionality by a sulfatase enzyme is required before the conversion to the downstream metabolites can occur. Thus differential tissue expression of DHEA, DHEAS, sulfotransferase and sulfatase can regulate the formation of the sex steroids.[339, 340]
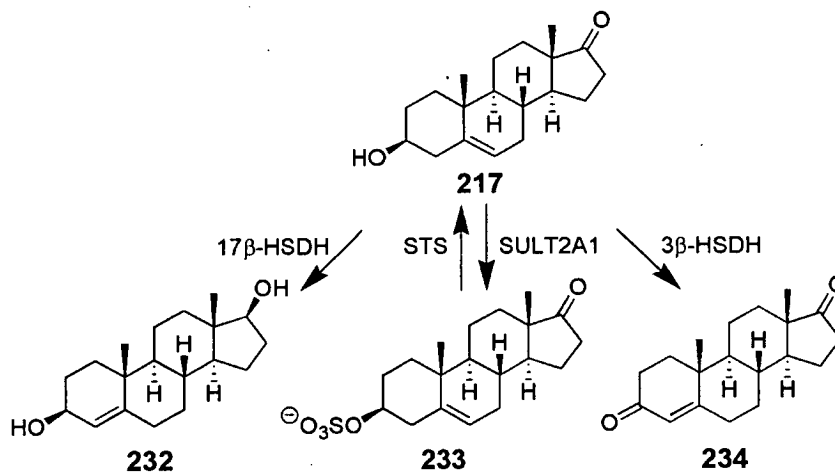
Scheme 4.4: Conversion of DHEA **217** in to downstream metabolites via reduction, sulfation or oxidation to give **232**, **233** and **234** respectively.[338, 339]

4.1.3 Biological Action of DHEA and DHEAS

DHEA and DHEAS are the most abundant steroids in the human circulation and exert a strong age-associated pattern. Serum DHEA and DHEAS levels start to increase from the ages of 6-10 years and peak between 25-35 years of age, a process termed the adrenarche. This is followed by a steady decline of ~1.5% per year with age, with levels finally residing at 10-20% of peak concentrations by the age of 70.[341-343] This age associated decrease in DHEA secretion has been termed the adrenopause and has raised the question of whether some of the increased frailty associated with aging is a consequence of decreased levels of DHEA and DHEAS. Although aging is a complex process that cannot be explained by one theory, a decrease in steroids can play a significant role in the onset of the symptoms of aging.

DHEA and DHEAS exert their biological function by two modes of action; indirectly by conversion to androgens, estrogens or other steroid metabolites (previously mentioned); or directly by interactions with receptors.[339, 340] Baulieu *et al.* were the first to publish the direct action of DHEA as a neurosteroid by showing the synthesis

of DHEA in the central nervous system (CNS), while also demonstrating that DHEAS is found at steady concentration in the brain tissue of rats.[344, 345] These steroids can therefore be described as classical neurosteroids as both the synthesis and biochemical action take place within the CNS. DHEA has been shown to modulate signalling through a number of membrane-bound receptors including the stimulation of *N*-methyl-D-aspartate (NMDA) receptors and as an allosteric antagonist of the γ-aminobutyric acid (GABA) receptor.[346-348] Therefore, DHEA selectively interacts with neurotransmitter receptors which are not classically considered to be steroid receptors. Studies have also tried to link DHEA and DHEAS to depression, as depressed patients have been shown to have significantly higher levels of plasma DHEAS than control patients.[349] Treatment with DHEA has been shown to significantly reduce depression on several scales as well as improving energy levels and motivation.[350] However, once again there is no evidence to confirm if DHEA or one of its downstream metabolites is causing this effect.

In vitro studies have shown that DHEA can modulate immune cell function. Immune responses decline with age, termed immunesenescence. Typically with immunesenescence the serum levels of interleukin 6 (IL-6) increases while the concentration of interleukin 2 (IL-2) decreases.[351, 352] On administration of DHEA the synthesis of IL-2 by T lymphocytes is stimulated, while the production of IL-6 is inhibited, thus reversing some of the effects of the immunesenescence process.[353-355] However, it is possible that immunological effects of DHEA are only mediated indirectly, as a consequence of downstream steroid metabolites.

To date no receptor specific for DHEA has been characterised. However, a number of researchers have recently focused on the theory that steroids may have a distinct binding site within the cell, with studies into DHEA's site of action focussing extensively on membrane bound targets.[356] Research by Dillon *et al.* into the membrane initiated action of DHEA led to the conclusion that the steroid interacts with high affinity binding to G-protein coupled receptors (GPCR). Further studies showed the binding of DHEA to GPCR was functionally linked to an increase in the synthesis of NO, whilst operating through a distinct pathway, separate from other steroid receptors.[357-359] More recently, work from Quon *et al.* showed that DHEA can be used to regulate vasodilatation and vasoconstriction through regulation of the eNOS

and ERK-1 pathways respectively (**Figure 4.2**).[360] These results suggest that DHEA has a high level target within the MAPK cascade, such as a GPCR, that allows regulation of a number of signal transduction pathways.
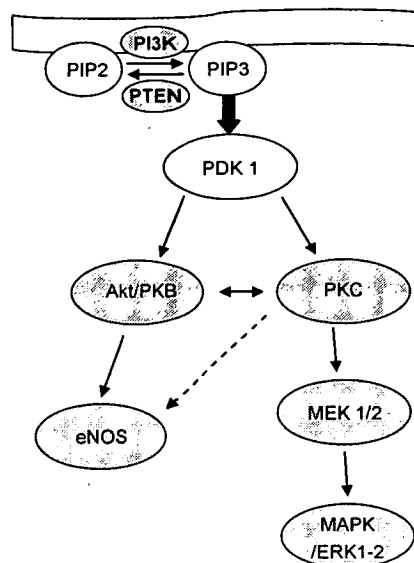
Figure 4.2: The MAPK pathway, illustrating upstream and downstream targets as well as cross-talk between a number of pathways regulated by PDK1, a 'master' kinase.

In contrast, investigations into the site of interaction of DHEAS have yet to be investigated in the same detail, in part due to the widely held belief that the sulfated form of DHEA is biologically inert and functions only as a vehicle for transport of DHEA.[361] Many cells express proteins known as Organic Anion Transporter proteins (OATP), which act as cell surface transporters for sulfated steroids such as DHEAS, allowing them entry to cells.[362-364] However, a recent publication from our collaborators (Prof. J. Lord and Prof. W. Arlt, University of Birmingham) has shown that DHEAS can directly activate neutrophils to generate superoxide.[365] Their preliminary data has shown that this effect can be blocked by bisindoylmaleimide, a protein kinase C (PKC) inhibitor, suggesting that PKC may be the target of DHEAS. Subsequent *in vitro* enzymatic assays have confirmed that DHEAS is able to activate PKC. PKC is known to be able to regulate the generation of superoxide in neutrophils, *via* phosphorylation of key elements of the multimeric enzyme NADPH oxidase. However, other kinases present in neutrophils can also phosphorylate NADPH oxidase

and lie downstream of PKC, namely PKB and MAPK. It has therefore been postulated that DHEAS can enter neutrophils *via* an OATP and then activate NADPH oxidase *via* activation of a protein kinase, which is likely to be PKC, but could also include other targets, *e.g.* MAPK and PKB (**Figure 4.2**).
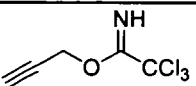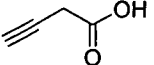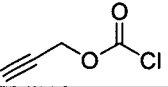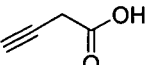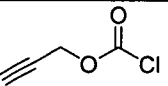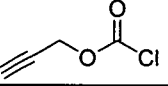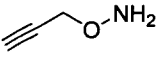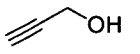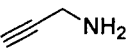
Given the hypothesis that there is a distinct biological target for DHEAS, mediated *via* the PKC/MAPK pathway, the decrease in DHEA(S) with age may play a role in the age-related senescence of cells that are able to respond to this steroid, including immune cells. Other biological responses associated with DHEA(S), such as the control of NMDA receptors, have also been tied to the MAPK pathways with phosphorylation of the receptor thought to play a large part in its mechanism.[366]

The role of DHEA and DHEAS as the key steroids in the formation of hormones has been well defined. However, the role of these steroids as regulators of immunity and signal transduction pathways has yet to be fully defined.[356, 357] Therefore, the identification of the intracellular target(s) of DHEA and DHEAS would lead to a greater understanding of an important signal transduction pathway responsible for the regulation of a wide range of biological functions.

4.2 Strategies for Marking a Range of Functionalities

In order for the marked library strategy to be widely applicable during the synthesis of chemical libraries, incorporation of a biocompatible marker into the parent structure has to be achievable at a variety of functionalities. Using the propargyl marker that proved successful in the case of anisomycin, the literature was searched for a range of alternative reagents that could be used to incorporate the propargyl moiety.

Previously research had focused on using propargyl bromide and coupling it to a range of phenols and amines (**Table 4.1**, row 1 and 5). However, literature precedent also showed that propargyl bromide can be used to couple to a range of alcohols and thiols (**Table 4.1**, row 1 and 8).[367-369] In addition, propargyl alcohol or amine can be coupled to acids, forming the analogous propargyl esters or amides (**Table 4.1**, row 11 and 12).[104, 370-375] While in an inverse approach the propargyl equivalent 3-butyneoic acid can be used to mark alcohols or amines within the parent structure (**Table 4.1**, row 3 and 6).[376-378]

Functionality	Propargyl Marker	Coupling conditions
R−OH	Br	Base, DMF
	O with NH and CCl₃	TfOH, ROH
	OH with O (acid)	DIC, DMAP, DCM
	O–C(=O)–Cl carbonate	Base, DCM
R−NHR¹ or R−NH₂	Br	Base, DMF
	OH with O (acid)	DCC, THF *or* HBTU, DMF
	O–C(=O)–Cl carbonate	Base, DCM
R−SH	Br	K₂CO₃, KI, Acetone
	O–C(=O)–Cl carbonate	BuLi, THF *or* Py, Et₂O
R−C(=O)−R¹	O–NH₂	MeOH
R−COOH	OH	DCC, DMAP, DCM
	NH₂	TBTU, HOBt, NMO, DMF

Table 4.1: Illustrating the range of reagents, marking reactions and conditions for the incorporation of a propargyl marker into a small molecule's scaffold.

Over the past few years propargyl chloroformate (PocCl) has been used infrequently as a method for protecting alcohols and amines within the fields of peptide and carbohydrate chemistry.[379, 380] However, its deprotection conditions utilise tetrathiomolybdate making it an unusual piece of methodology.[381] Nonetheless, the ease with which the Poc protecting group can be coupled with a range of nucleophiles including alcohols, amines and thiols makes it an ideal alternative method to introduce a propargyl marker (**Table 4.1**, row 4, 7 and 9).

With a broad range of coupling to amines, alcohols, thiols and acids available, our focus altered to the marking of carbonyl functionalities. The ideal reaction for the marking of this functionality was believed to be the Schiff base condensation, which in itself is often classed as a 'click' reaction.[97] Coupling of propargyl hydroxylamines has been shown to proceed readily with a range of aldehydes and ketones in high yield (**Table 4.1**, row 10), thus making an ideal reagent for the introduction of the propargyl marker at the carbonyl moiety.[382, 383]

A focused search of the literature has unearthed a range of reagents and reaction conditions which can be used to introduce a propargyl marker into a small molecule's scaffold. Thus illustrating that the concept of a marked small molecule library can be expanded beyond our initial study, to a wide range of biologically interesting molecules.

4.3 Marked Steroids

The role of steroids in the control of biological processes has been well documented. However, the precise signalling interaction of a number of these molecules has still to be fully validated. We therefore set out to apply our marked library strategy to a number of steroids by incorporating a propargyl moiety into the steroidal scaffold. Our initial attention focused on DHEA **217** with incorporation of a propargyl marker proposed at either the C_3-hydroxyl or at the carbonyl functionality (**Figure 4.3**).

217
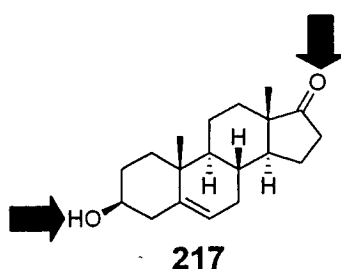
Figure 4.3: Functionalities within DHEA's scaffold which could be used to attach a biocompatible marker.

4.3.1 NMR Assignment

Prior to the incorporation of the propargyl marker, it was decided to investigate the structure of DHEA **217**. In order to fully characterise DHEA, a combination of 2D NMR experiments were used as it was believed that having the fully assigned starting material would facilitate the determination of any products formed.

Analysis of the ^{1}H NMR spectrum obtained showed a distinctive doublet (5.39 ppm) for the C_6H and a triplet of triplets (3.54 ppm) for the C_3HOH (**Figure 4.4**). While the other 26 protons associated with DHEA were clustered together between 2.60 ppm and 0.90 ppm (**Table 4.2**, column 2). In contrast, analysis of the ^{13}C broadband and DEPT spectra obtained allowed the relative assignment (*i.e.* Q, CH$_3$, CH$_2$, CH) to be carried out but not the full structural assignment (**Table 4.2**, column 3).
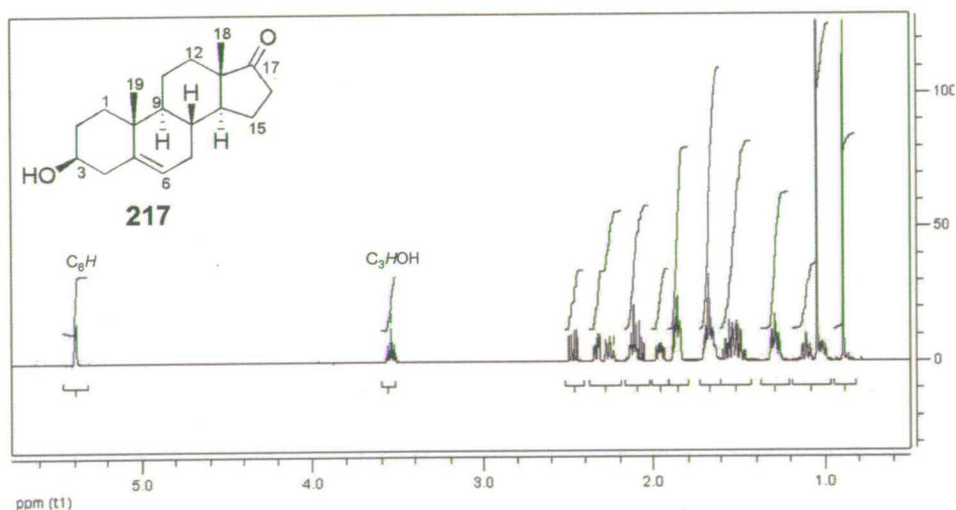
Figure 4.4: ^{1}H NMR of DHEA **217** (CDCl$_3$, 600 MHz)

Correlation of the ^{1}H spectrum with that of the COSY/HSQC/HMBC spectra obtained allowed the assignment of the majority of the structure (**Table 4.2**, column 4). However, determination of three peaks (C_2H_2, C_8H and $C_{12}H_2$) proved problematic as they occurred as four distinct regions in the ^{1}H dimension but were found to lie top of each other at 31.4 ppm in the ^{13}C dimension (**Figure 4.5**).
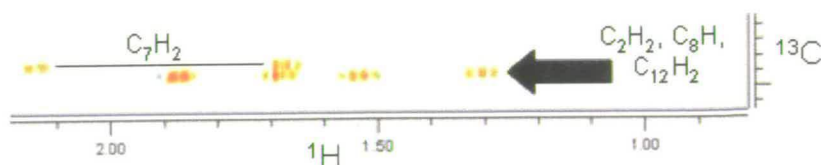
Figure 4.5: Slice of HSQC spectrum of DHEA **217** (CDCl$_3$, 600 MHz).

121
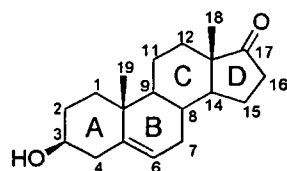
217

	Assignment by ¹H spectrum	Assignment by ¹³C and DEPT spectra	Assignment by COSY, HSQC and HMBC spectra (¹H, ¹³C)	Assignment by HSQC-TOCSY spectrum (¹H, ¹³C)	Fully assigned ¹H spectrum
C_1H_A		CH_2	1.89-1.84, 37.0		1.89-1.84 (m)
C_1H_B		CH_2	1.11, 37.0		1.11 (dt)
C_2H_A		CH_2		1.89-1.84, 31.4	1.89-1.84 (m)
C_2H_B		CH_2		1.57-1.46, 31.4	1.57-1.46 (m)
C_3H	3.54 (tt)	71.5 (CH)	3.54, 71.5		3.54 (tt)
C_4H_A	2.33 (ddd)	CH_2	2.33, 42.1		2.33 (ddd)
C_4H_B	2.29-2.23 (m)	CH_2	2.29-2.23, 42.1		2.29-2.23 (m)
C_5		140.9 (Q)	140.9		-
C_6H	5.39 (d)	120.8 (CH)	5.39, 120.8		5.39 (d)
C_7H_A		CH_2	2..14-2.11, 30.7		2.14-2.11 (m)
C_7H_B		CH_2	1.71-1.63, 30.7		1.71-1.63 (m)
C_8H		CH		1.89-1.84, 31.4	1.89-1.84 (m)
C_9H		CH	1.02, 50.1		1.02 (ddd)
C_{10}		Q	36.5		-
$C_{11}H_A$		CH_2	1.71-1.63, 20.2		1.71-1.63 (m)
$C_{11}H_B$		CH_2	1.57-1.46, 20.2		1.57-1.46 (m)
$C_{12}H_A$		CH_2		1.71-1.63, 31.4	1.71-1.63 (m)
$C_{12}H_B$		CH_2		1.32-1.26, 31.4	1.32-1.26 (m)
C_{13}		Q	47.4		-
$C_{14}H$		CH	1.32-1.26, 51.6		1.32-1.26 (m)
$C_{15}H_A$		CH_2	1.96, 21.8		1.96 (ddd)
$C_{15}H_B$		CH_2	1.57-1.46, 21.8		1.57-1.46 (m)
$C_{16}H_A$	2.47 (dd)	CH_2	2.47, 35.7		2.47 (dd)
$C_{16}H_B$	2.09 (dd)	CH_2	2.09, 35.7		2.09 (dd)
C_{17}		221.2 (Q)	221.2		-
$C_{18}H_3$	0.90 (s)	13.4 (CH_3)	0.90, 13.4		0.90 (s)
$C_{19}H_3$	1.05 (s)	19.3 (CH_3)	1.05, 19.3		1.05 (s)

Table 4.2: Table of NMR data for DHEA **217**, illustrating the NMR experiments used to carry out the full assignment of this molecule.

Attention turned to a series of 1D nOe experiments which it was believed could be used to facilitate the assignment of the C_2H_2, C_8H and $C_{12}H_2$ peaks. Irradiation was carried out at the C_3H, the $C_{18}H_3$, and the $C_{19}H_3$ peaks respectively; as they were located in close proximity to the protons of interest, while also representing well defined signals at which to irradiate (**Figure 4.6**). However, the highly congested nature of the ^1H spectrum in the aliphatic region made it impossible to clarify the assignments using the results of the nOe experiments.
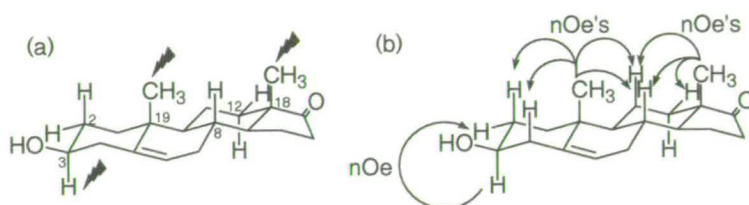
Figure 4.6: Structure of DHEA **217**: (a) showing the signals irradiated during nOe experiments (b) expected nOe signals.

The defining experiment was an HSQC-TOCSY which allowed the structure of DHEA to be fully characterised by NMR (**Appendix 5**). This experiment is a traditional 2D HSQC that allows for this spin to be passed *via* a TOCSY transfer prior to data acquisition. This relays the original proton-carbon correlation peak onto neighbouring protons within the same spin-system, thus producing a ^{13}C-dispersed TOCSY spectrum (**Figure 4.7**).
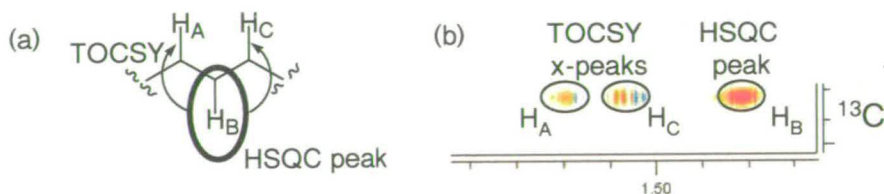
Figure 4.7: (a) Illustration of the spin transfer in an HSQC-TOCSY experiment, and (b) the resultant spectrum with TOCSY cross-peaks in the carbon dimension.

Therefore, correlating all the previously assigned NMR data, the full characterisation of DHEA was completed using the cross peaks from the HSQC-TOCSY spectrum (**Table 4.2**, column 5). Thus, allowing the problematic signals associated with C_2H_2, C_8H and $C_{12}H_2$ to be assigned and the ^1H and ^{13}C spectra of DHEA to be fully

determined (**Figure 4.8**). Gratifyingly the ^{1}H data correlates with the proton sequence of DHEA produced by Keeler; who has recently used DHEA to develop new pulse sequences methodology that can simplifying complex proton spectra.[384]
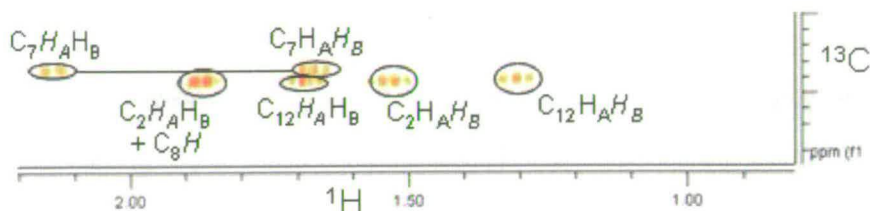
Figure 4.8: Slice of assigned HSQC spectrum of DHEA **217** (CDCl$_3$, 600 MHz).

4.3.2 Marking DHEA

Having fully characterised DHEA **217** attention now focused on the incorporation of a propargyl marker onto the steroidal scaffold. Initial investigations utilised the conditions successfully applied to the propargylation of anisomycin (K$_2$CO$_3$, propargyl bromide) in order to convert DHEA's hydroxyl moiety into the analogous propargyl ether **235**. Unfortunately, under these conditions no coupling reaction was observed upon monitoring by tlc and MS, and after work-up the steroidal starting material was recovered (**Table 4.3**).

Previous work by Pauson *et al.* showed the conversion of DHEA's downstream metabolite cholesterol to analogous propargyl cholesterol.[385] This was carried out by refluxing the steroid in the presence of sodium hydride for 24 h, before addition of propargyl bromide to give the desired functionalised steroid. However, this method proved to be unsuccessful for the propargylation of DHEA (**Table 4.3**, entry 16).

We therefore began a screen of solvents and bases in order to devise a set of conditions that would allow the formation of the propargyl ether (**Table 4.3**). Investigations covered a wide range of organic and inorganic bases over a range of polar and non-polar solvents. However, each time the reaction was carried out only unreacted DHEA was obtained.

Table 4.3: Table of basic conditions for the attempted propargylation of DHEA.
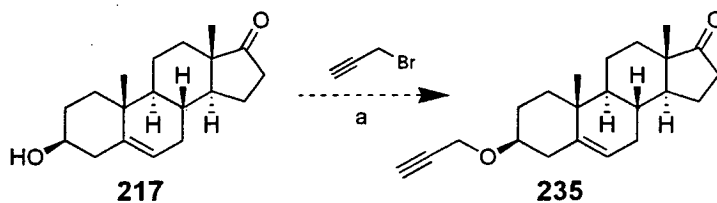
Base	Solvent	Additive/Conditions	217	235
K_2CO_3 (2 eq)	DMF		Quant.	
K_2CO_3 (2 eq)	CH_3CN		Quant.	
pyridine	DMF	imidazole	Quant.	
pyridine	CH_3CN		Quant.	
pyridine	DCM		Quant.	
pyridine	DMF		Quant.	
pyridine	DMF	DMAP	Quant.	
pyridine	DCM	DMAP	Quant.	
Cs_2CO_3 (3 eq)	DMF		Quant.	
Cs_2CO_3 (3 eq)	DCM		Quant.	
2,6-lutidine (2 eq)	DCM		Quant.	
Et_3N (2 eq)	DCM		Quant.	
imidazole (10 eq)	DMF		Quant.	
NaH (2 eq)	DCM		Quant.	
NaH (2 eq)	THF		Quant.	
NaH (2 eq)	DMF	heating at 100 °C	Quant.	
NaH (4 eq)	*p*-xylene	reflux	Quant.	
KO*t*Bu (2.5 eq)	toluene	reflux		Unknown
KO*t*Bu (2.5 eq)	CH_3CN	reflux		Unknown

On altering the base to potassium butoxide a new spot was observed by tlc (**Table 4.3**, entry 18/19). However, this reaction never proceeded to completion and identification of the new spot as the desired product was never possible.

The final attempt to synthesis the propargyl ether was carried out under acidic conditions utilising a method previously developed by Overman to propargylate secondary alcohols.[386, 387] This methodology involved the formation of the propargyl

acetimidate **237** by reacting propargyl alcohol **236** with trichloroacetonitrile. Classically, formation of the acetimidate reagent has been carried out using sodium hydride, but more recently it has been found to proceed more cleanly with DBU as the base.[388] On adding the trichloroacetonitrile to a solution of DBU and propargyl alcohol the solution turned pale orange. After stirring for 2 h the solution was filtered through a plug of silica and concentrated. Analysis of the ^{1}H NMR spectrum showed a broad 1H singlet at 8.15 ppm from the N*H* of the newly formed acetimidate **237**. While, a 2H doublet (4.94 ppm) and a 1H triplet (2.56 ppm) with fine splitting associated with the propargyl functionality were also present in the spectrum, thus confirming the formation of the acetimidate. The acetimidate **237** was rapidly carried through to the next step by addition of DHEA **217** and a catalytic amount of triflic acid in an attempt to form the propargyl ether **235**. However, no reaction between DHEA and the acetimidate was ever observed.
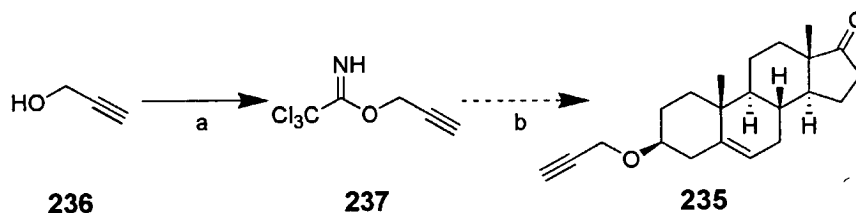
Scheme 4.5: (a) Cl$_3$CC≡N, DBU, DCM (100%); (b) DHEA, TfOH (cat), DCM.

Having exhausted our efforts trying to synthesise the propargyl ether, our attention turned to forming the analogous ester and carbonate as means of introducing the propargyl moiety. Coupling of DHEA **217** to 3-butynoic acid **238** proceeded using EDI and DMAP but the yields were found to be only moderate. However, on altering the coupling agent to DIC the product **239** was isolated in a much more respectable 75% (**Scheme 4.6**). Analysis of the proton spectrum of **239** clearly showed that the triplet of triplets at 3.54 ppm from the C$_3$*H*OH had been replaced by a multiplet at 4.73-4.64 ppm. A singlet (1.98 ppm) was found to correspond to the CH$_2$ of the propargyl moiety, with the corresponding propargyl CH (2.41-2.36 ppm) as part of a 3H multiplet. However, the most surprising finding on interpreting this molecule's spectra was that the CH$_2$ of the propargyl moiety was found to come at 4.0 ppm in the carbon dimension. This was a highly unexpected result but was confirmed by a

number of 2D NMR experiments including HSQC and HSQC-TOCSY.[‡‡] Moreover, this correlated with both the ^{13}C spectrum of 3-butynoic acid which has the CH_2 at 5.1 ppm, as well as a number of literature examples.[389, 390]
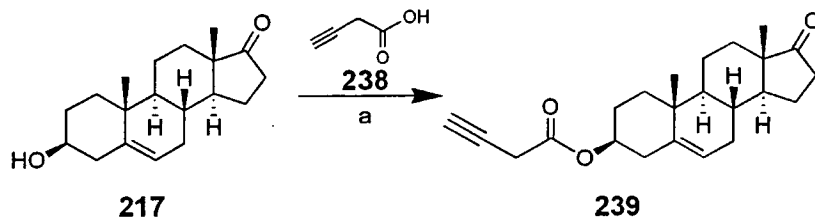
Scheme 4.6: (a) **238**, DIC, DMAP, DCM (75%).

Coupling of propargyl chloroformate **240** to DHEA **217** was found not to proceed when carried out with imidazole in DCM. However, on altering the base from imidazole to pyridine, the reaction was found to give a single spot by tlc (**Scheme 4.7**). Characterisation of this material showed formation of the product **241** in high yield, with the distinctive propargyl peaks being observed at 2.53 ppm (1H, t) and 4.72 (2H, d) with a fine coupling constant (2.5 Hz).
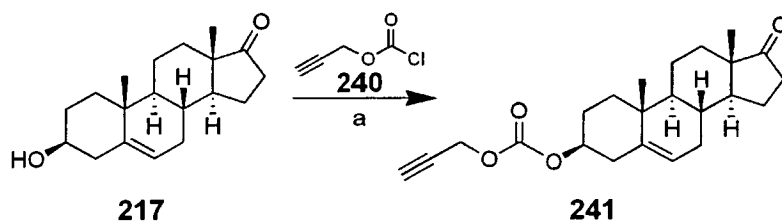
Scheme 4.7: (a) **240**, pyridine, DCM (86%).

Upon slow evaporation of the NMR solvent colourless crystals were formed. The resulting X-ray crystal structure gave an excellent insight into the three dimensional arrangement of the steroid core (**Figure 4.9** and **Appendix 6**). This structure illustrates the chair conformation of the A and C rings, the envelope-like D ring and the flattened B ring due to the presence of the alkene moiety. However, more interesting was the conformation of the propargyl functionality. Analysis of the crystal structure showed that the propargyl unit adopted a near-linear conformation with a bond angle of 173.8°

[‡‡] All steroids were characterised by a combination of 1D and 2D NMR experiments including ^1H, ^{13}C, DEPT, HSQC, HSQC-TOCSY and HMBC.

between the $C_{21}H_2$-$C_{23}H$ atoms. While the angle around the $C_{21}H_2$ of the propargyl was found to be 112.3°, more distorted relative to a typical sp^3 carbon.
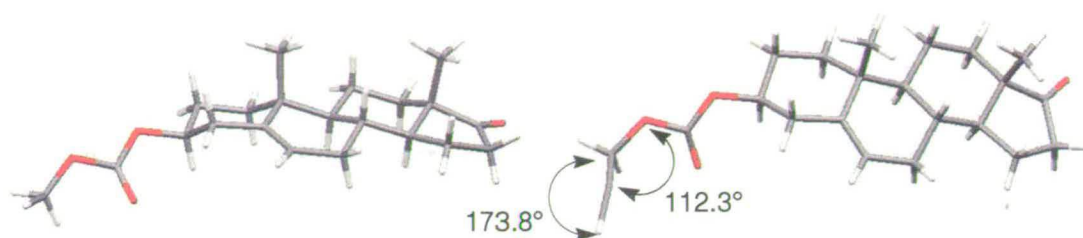
Figure 4.9: X-ray crystal structure of **241**.

Turning our attention to the other potential marking site within DHEA's scaffold, investigations then focused on the synthesis of an appropriate propargyl hydroxylamine. The commercially available *N*-propargyloxy phthalamide **242** was deprotected by stirring with hydrazine monohydrate, to give a colourless precipitate which was removed by filtration. Ethereal hydrochloric acid was then added to the filtrate and a further solid isolated upon filtration. Analysis of the precipitate by NMR showed both the desired product **243** and trace amounts of the 2,3-dihydro-phthalazine-1,4-dione by-product. However, repeating this synthetic process with methyl hydrazine was found to give the desired product **243** in higher yield and as a single compound without contamination (**Scheme 4.8**).

242   **243**
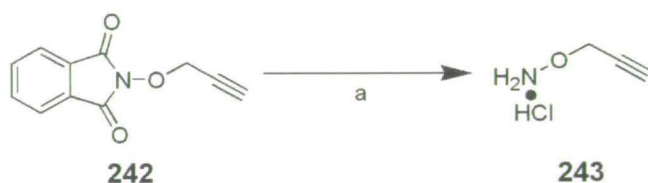
Scheme 4.8: (a) H_2NNHMe, DCM; HCl in Et_2O (80%).

Coupling of the hydroxylamine **243** to DHEA's C_{17}-carbonyl **217** was found to occur readily on stirring the two components in methanol. This gave the desired product **244** in 97% yield after purification by column chromatography. Characterisation of **244** by NMR clearly showed incorporation of the propargyl marker with a 2H doublet at 4.60 ppm and a 1H triplet at 2.42 ppm. Moreover, analysis of the 2D spectra showed a distinctive shift of the $C_{16}H_2$ from 35.7 ppm to 26.1 ppm in the carbon dimension.
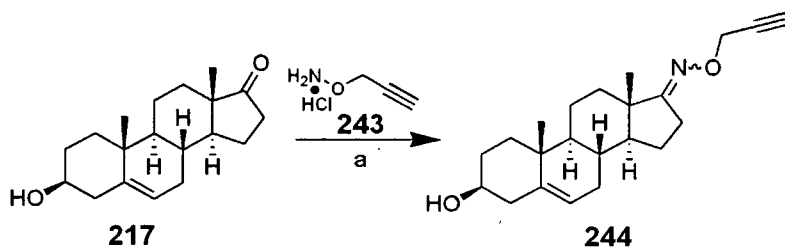
Scheme 4.9: (a) **243**, MeOH (97%).

Expanding on the success of the Schiff base coupling, the development of a short chain hydroxylamine **247** was envisaged. Coupling the protected acid **245** to propargyl amine was carried out readily in the presence of EDI and DMAP to give **246** in high yield (**Scheme 4.10**). The Boc protecting group was then removed under acidic conditions using TFA to give the desired hydroxylamine **247**. However, isolation of this material as its TFA salt proved to be problematic. Therefore, ion exchange to the chloride was carried out using Dowex resin pre-treated with HCl. The newly synthesised hydroxylamine **247** could then be coupled in a Schiff base condensation with DHEA to give **248** in 94% (**Scheme 4.11**). Once again analysis of the NMR spectra obtained showed formation of the oxime with the distinctive shift of the $C_{16}H_2$ signals from 35.7 ppm to 26.2 ppm.
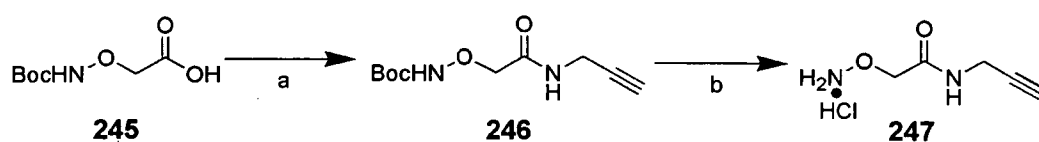
Scheme 4.10: (a) $HC\equiv CCH_2NH_2$, DMAP, EDI, DCM (95%); (b) i) TFA, DCM; ii) Dowex-Cl (91%).
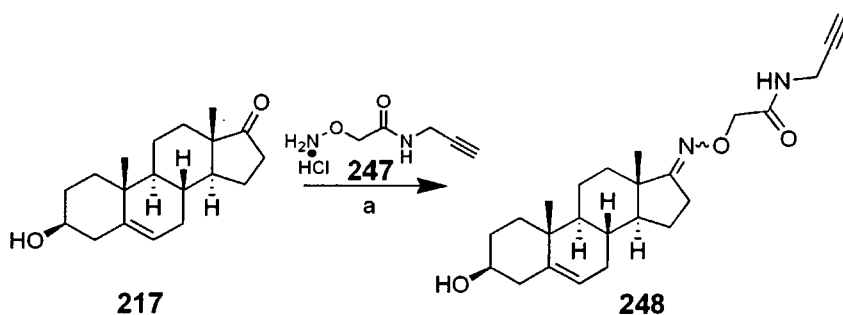
Scheme 4.11: (a) **247**, MeOH (94%).

4.3.3 Marking DHEAS

Having successfully incorporated our biocompatible marker into the structure of DHEA we switched our focus to its downstream metabolite DHEAS. As this molecule is the sulfated form of DHEA, it was proposed that a Schiff base condensation could be exploited to couple propargyl hydroxylamines previously utilised to the ketone of DHEAS.

On coupling commercially available DHEAS **233** to hydroxylamine **243** in methanol, no reaction was observed after 16 h. Initially this was thought to be due to the polarity of the sulfated steroid making it hard to monitor the reaction by tlc (**Scheme 4.12**). However, upon work-up no condensation of the hydroxlamine could be observed by MS or NMR. In addition an identical problem was also found to occur when attempting to couple hydroxylamine **247** to DHEAS (**Scheme 4.12**). Upon examining the literature for synthetic modification of DHEAS only a few examples were found but none involved the formation of oximes or hydrazines. Therefore, it was decided to alter our synthetic approach to tagging DHEAS.

233   **249**
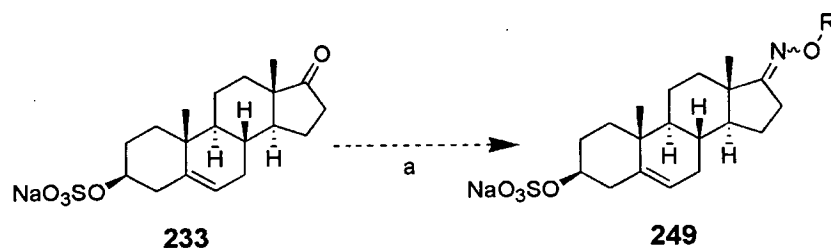
Scheme 4.12: (a) **243** or **247**, MeOH.

Taking the previously synthesised oxime **244**, investigations focused on the sulfation of the C_3-hydroxyl. Using sulfur trioxide trimethylamine complex as the key reagent, the sulfation of the marked steroid proceeded readily in pyridine (**Scheme 4.13**). Analysis of the product **250** by NMR showed a distinct shift from 3.54 ppm to 4.14 ppm of the C_3H signal associated with the sulfation of the hydroxyl. This could be directly contrasted to the analogous spectra of DHEA and DHEAS which show a distinctive shift upon incorporation of the sulfate functionality (**Figure 4.10**). However, the use of pyridine as the reaction solvent led to the formation of the pyridine salt of the sulfated steroid **250**. Treatment of the pyridine salt **250** with

ammonium acetate followed by purification by reverse phase chromatography (5% $NH_4OAc/H_2O \rightarrow MeOH$) gave the desired product as the ammonium salt **251**.
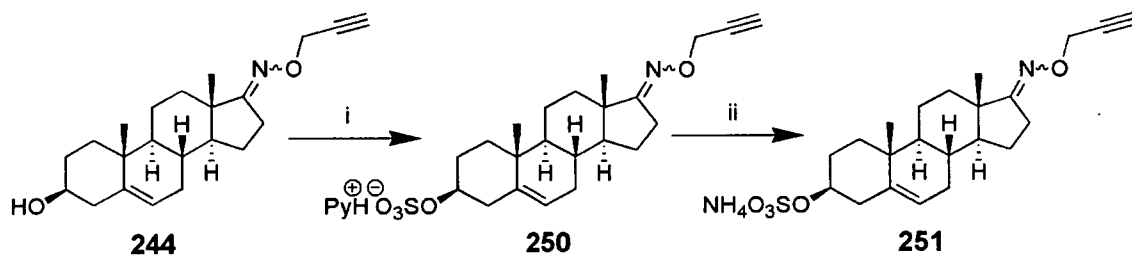
Scheme 4.13: (i) $SO_3 \bullet NMe_3$, pyridine; (ii) 5%NH_4OAc solution (85%).
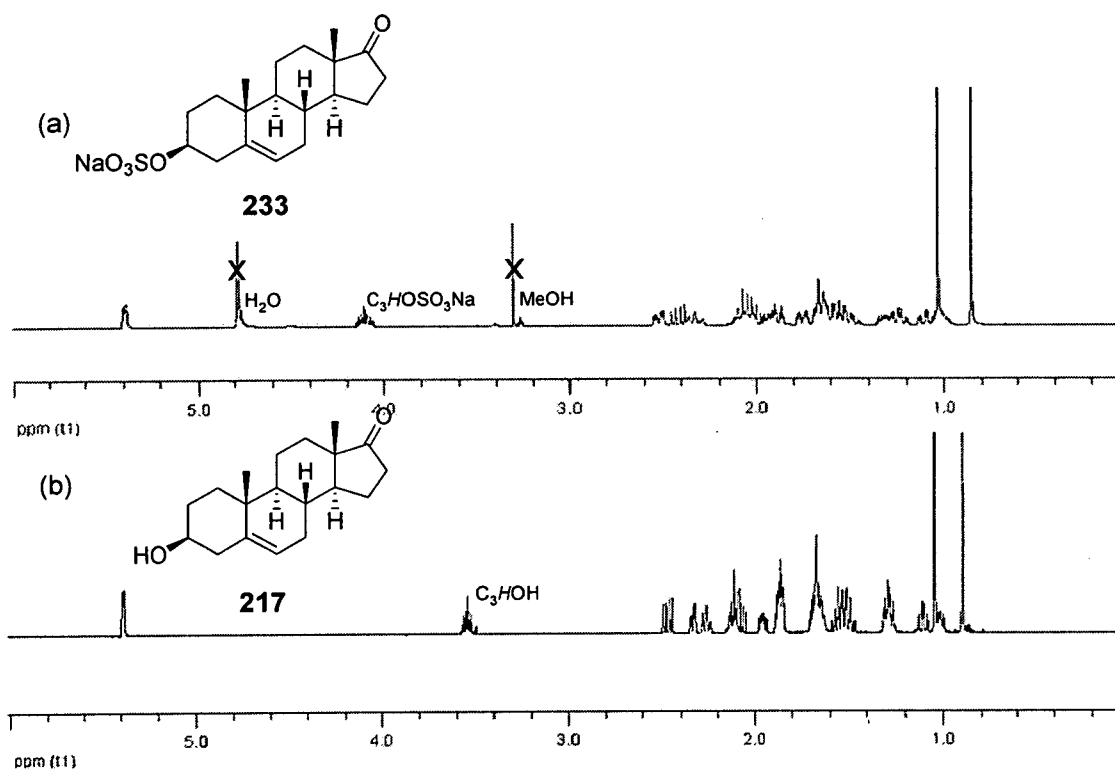
Figure 4.10: ^1H NMR spectra of (a) DHEAS **233** and (b) DHEA **217**: illustrating the characteristic shift of the C_3H upon addition/removal of the sulfate.

Repeating this procedure with the short chain oxime **248** gave the desired sulfated steroid **253** in 96% yield after cation exchange and purification by reverse phase chromatography (**Scheme 4.14**). Sulfation of the C_3-hydroxyl was confirmed by the movement of the C_3H proton from 3.50 ppm to 4.14 ppm, while no loss of the oxime functionality was observed.
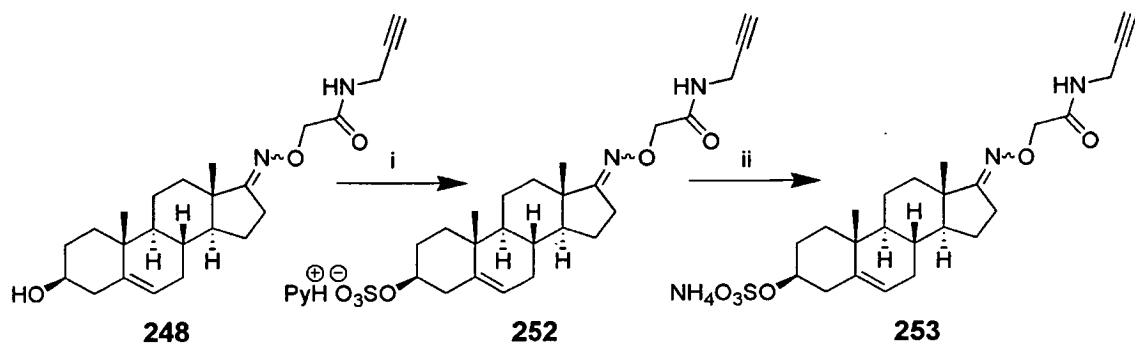
Scheme 4.14: (i) SO$_3$•NMe$_3$, pyridine; (ii) 5%NH$_4$OAc solution (96%).

Therefore, a focused series of marked steroids have been synthesised based on the structure of DHEA and DHEAS. Incorporation of a propargyl marker onto the steroidal core has been carried out by functionalising the C$_3$-hydroxyl as an ester or carbonate, or the C$_{17}$-carbonyl as an oxime.

The application of our marked library strategy to this range of biologically interesting steroids illustrates the scope of functionalities that can be modified with a biocompatible marker, such as a propargyl unit. We therefore expect this marked library concept to be applied to further series of biologically interesting molecules, marking a range of chemical functionalities, through the incorporation of a biocompatible marker.

4.4 Molecular Probe Formation

An initial round of biological testing revealed that **248** showed excellent levels of activity in a chemotaxis assay, resulting in an increase in cell movement in contrast to DHEA and DHEAS.[§§] Therefore, the synthesis of a fluorescent molecular probe based on the scaffold of **248** was envisaged. This probe could then be utilised in subsequent investigations to track the steroid *in vitro*.

The biologically interesting marked steroid **248** was converted to the analogous fluorescent molecular probe **254** in a single step by coupling to dansyl azide **189** (**Scheme 4.15**). Utilising the copper(I) catalysed Huisgen cycloaddition reaction as the

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[§§] Biological testing was carried out in Professor Janet Lord's research group at the University of Birmingham.

coupling step, the standard conditions of 10 mol% copper(II) sulfate and 20 mol% sodium ascorbate were utilised. This reaction proceeded in a spot to spot manner with a distinctive drop in R_f being observed upon coupling of the two components. The product **254** was isolated as a pale green wax in 95% yield.
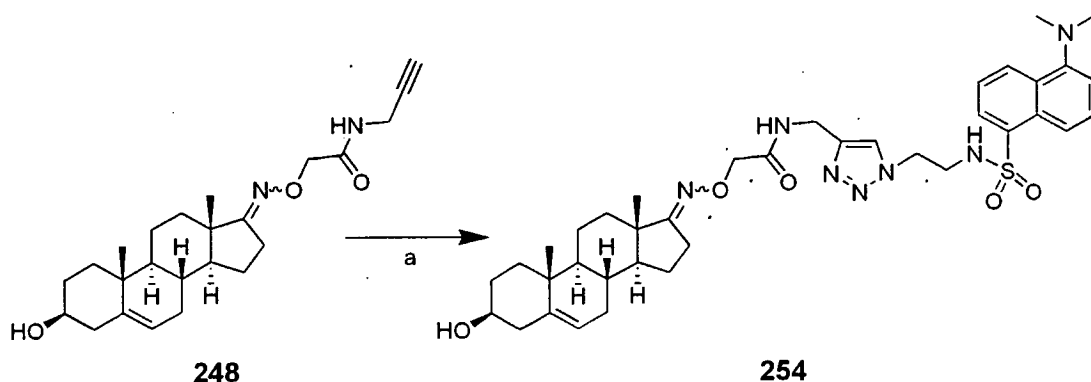
Scheme 4.15: (a) **189**, CuSO$_4$● 5H$_2$O (10 mol%), NaAsc (20 mol%), ^{t}BuOH:H$_2$O (1:1) (95%).

The product **254** was characterised by carrying out a series of 1D and 2D NMR experiments to probe its structure (**Appendix 5**). Comparison of the ^{1}H spectra of the two components (**248** and **189**) with that of the product **254** showed a distinctive transfer of signals, with the fluorophore component giving rise to the aromatic signals, while the steroid moiety gives rise to the aliphatic signals (**Figure 4.11**). A number of distinctive alterations could be observed upon formation of the triazole, with the two peaks associated with the propargyl unit being replaced by a singlet at 7.45 ppm ($C_{27}H$) and a doublet at 4.43 ppm ($C_{25}H_2$). In addition, the CH$_2$ next to the azide has been moved downfield from 3.30 ppm to 4.33 ppm upon formation of the triazole.
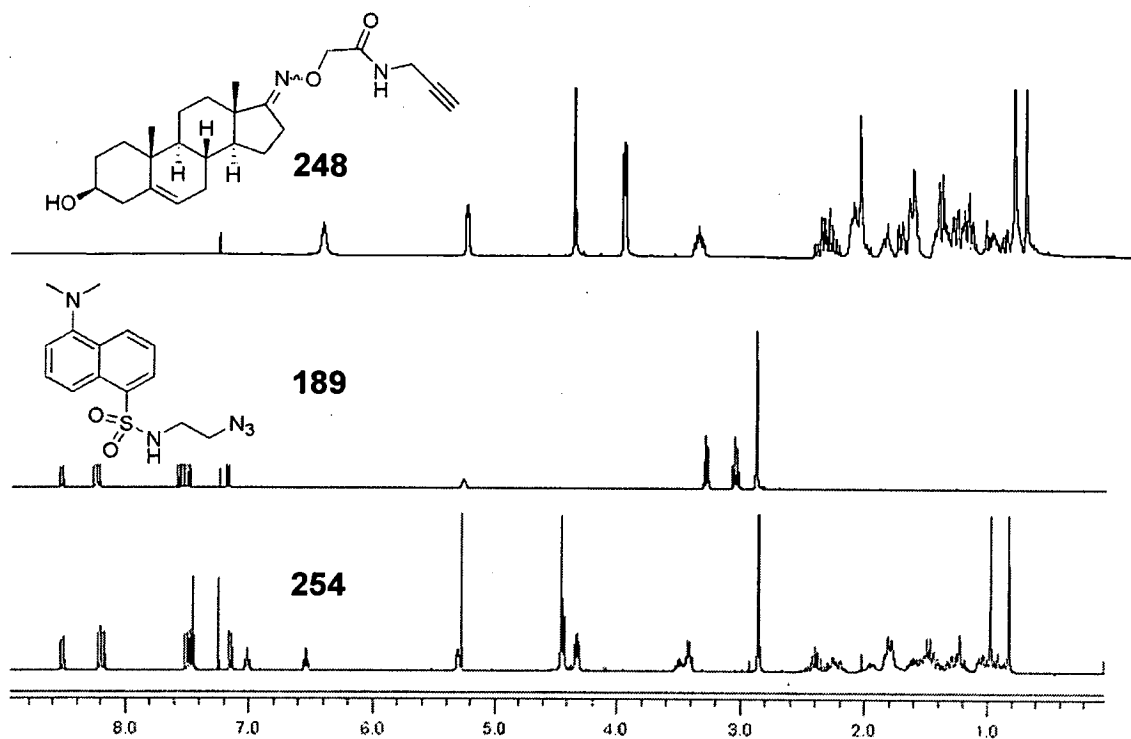
Figure 4.11: NMR stack plot of **248** (top), **189** (middle) and **254** (bottom).

The synthesis of a second molecular probe was then investigated, based on the scaffold of the propargyl carbonate marked steroid **241**. Studies focused on whether the copper(I) catalysed Huisgen cycloaddition could be used in conjunction with the Poc moiety. Initial fears had been raised over the stability of the Poc group in the presence of copper; as a di-molybdenum complex facilitates the removal of the Poc functionality when it is used as a protecting group.[381] However, investigation into the coupling of the propargyl carbonate marked steroid **241** to dansyl azide **189** found that under standard conditions the coupling reaction proceeds in high yield to give the desired product **255** (**Scheme 4.16**).
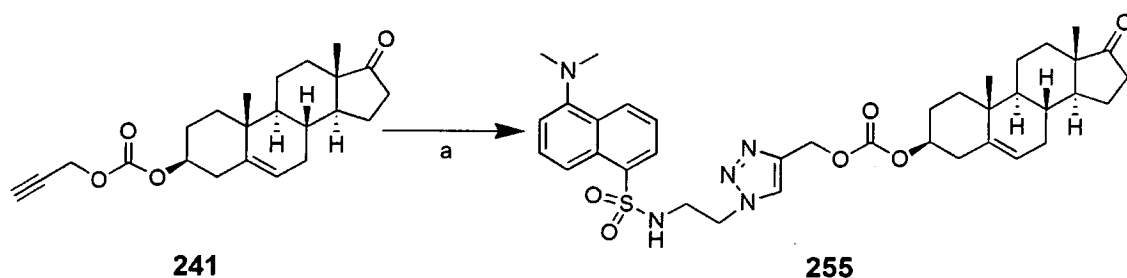
Scheme 4.16: (a) **189**, CuSO$_4$• 5H$_2$O (10 mol%), NaAsc (20 mol%), ^{t}BuOH:H$_2$O (1:1) (81%).

Formation of the molecular probe **255** was confirmed by detailed NMR characterisation (**Appendix 5**). Experiments clearly showed the removal of the propargyl peaks at 4.72 ppm and 2.53 ppm and the introduction of the triazole CH at 7.54 ppm. Upon examining the ^{13}C spectrum, the C_{21} quaternary peak of the carbonate was clearly present at 154.4 ppm, along with the new triazole peaks at 142.3 ppm and 125.2 ppm. Investigations then turned to the full structural assignment of **255**, which was accomplished using HSQC (**Figure 4.12**) and HSQC-TOCSY experiments.
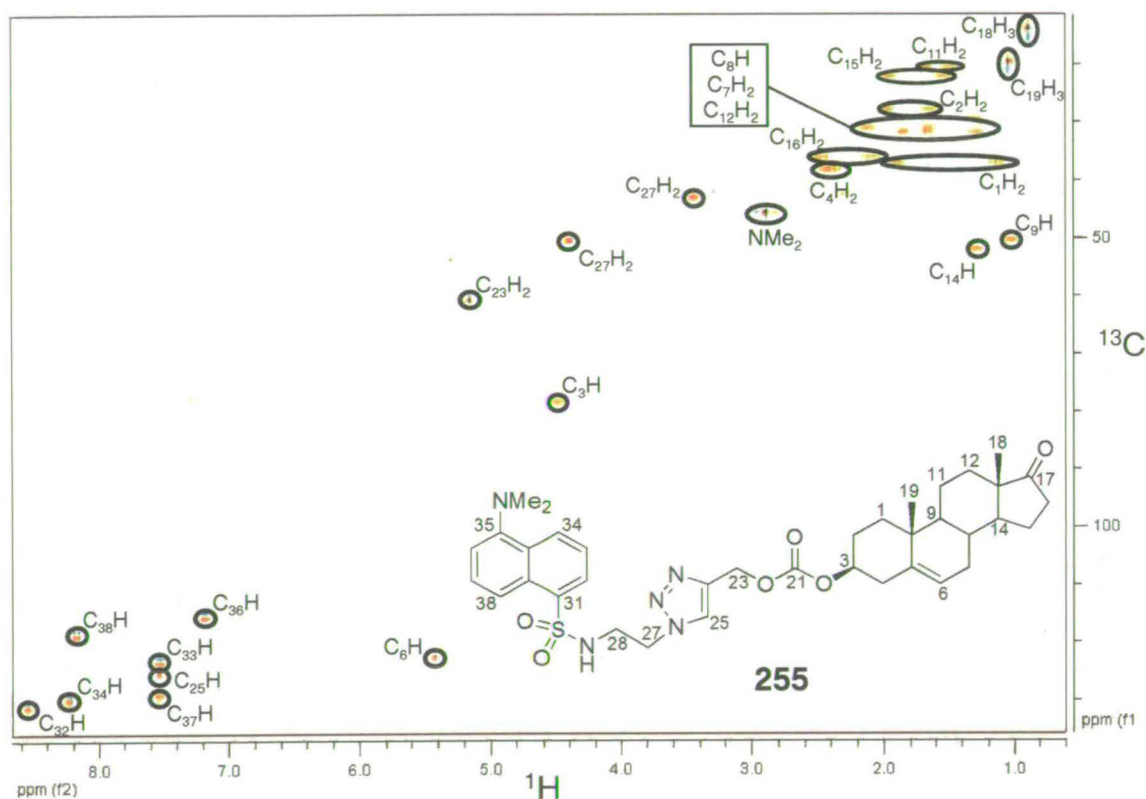
Figure 4.12: Fully assigned HSQC spectrum of **255** at 360 MHz.

It can be concluded that the initial fears regarding the stability of the Poc functionality have been shown to be unfounded. Therefore, the Poc moiety provides a further useful method for introducing a propargyl marker onto the molecular scaffold of biologically interesting molecules.

4.5 Summary

In conclusion, a series of marked steroids based on the scaffold of DHEA and DHEAS has been synthesised. Introduction of the marker component has taken place through the coupling of a number of novel propargyl moieties (acid, hydroxylamine and chloroformate) to either the C_3OH or the C_{17}-carbonyl of the steroidal core. Initial biological screening showed that library member **248** showed interesting biological activity in the chemotaxis assay. As a result, it was readily converted into fluorescent molecular probe **254** which will be utilised in future studies.

Therefore, we have expanded our concept of a marked library to a new range of biologically interesting steroidal molecules. This has allowed us to illustrate that a range of different functional groups can be readily manipulated allowing the attachment of a bioorthogonal propargyl marker. Subsequently, the copper(I) catalysed Huisgen cycloaddition can be utilised as the key coupling step, facilitating the rapid formation of molecular probes. Therefore, the application of a marked library approach has been shown to dramatically condense the small molecule to molecular probe synthetic process across a range of biologically interesting small molecules.

5 Experimental

5.1 General Synthetic Experimental

^{1}H nuclear magnetic resonance (NMR) spectra were recorded at ambient temperature (unless otherwise stated) on Varian Gemini 200 (200 MHz), Bruker AC250 (250 MHz), Bruker DPX360 (360 MHz) and Bruker AVA600 (600 MHz) Fourier transform instruments. The data is presented as follows: chemical shift (in ppm on the δ scale relative to $\delta_{TMS} = 0$), multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad), coupling constant and interpretation. ^{13}C NMR spectra were recorded at ambient temperatures (unless otherwise stated) on Bruker AC250 (62.9 MHz), DPX360 (90.7 MHz) and Bruker AVA600 (151.1 MHz) Fourier transform instruments and were referenced to the solvent carbon peak. The data is presented as follows: chemical shift (in ppm on the δ scale), relative intensity and assignment; and were confirmed by DEPT90 and DEPT135 and/or HSQC and HMBC experiments.

Infra-red spectra were recorded on a Perkin Elmer Paragon 100 FT-IR machine using 0.1 mm sodium chloride solution cells, 5 mm sodium chloride plates or potassium bromide discs. The wavelengths of maximum absorbance (υ_{max}) are quoted in cm^{-1}.

Melting points were determined on a Gallenkamp Electrothermal Melting Point apparatus and are uncorrected.

Optical rotations were measured on an AA-1000 polarimeter with a path length of 1.0 dm at the sodium D line (589 nm) and are reported as follows: $[\alpha]_D$, concentration (c in g/100 cm^3), and solvent. All optical rotations were measured at a temperature of 23 °C.

Fast atom bombardment (FAB) mass spectra were obtained using a Kratos MS50TC mass spectrometer at The University of Edinburgh. Electrospray ionisation (ESI) mass spectra were recorded on a Finnigan LCQ or Micromass Platform instruments

at The University of Edinburgh. The parent ion or relevant fragment is quoted, followed by significant fragments and their percentages.

Chiral high performance liquid chromatography (HPLC) was carried out on a Waters 786 instrument equipped with a Chiracel OD-H column (internal diameter 4.6 mm) and a UV detector. A standard flow rate of 0.5 ml min^{-1} was used. All solvents used for HPLC analysis were vacuum filtered and degassed prior to use.

Luminescence measurements were carried out in Hellma 109.000F-QS 10 mm precision cell using a fluorimeter with phosphorimeter Jobin-Yvon-Horiba Fluoromax-P.

Tlc was performed on Merck 60F$_{245}$ (0.25 mm) silica plates and visualised by ultraviolet (UV) light, ammonium molybdate,[€] potassium permanganate[**] or anisaldehyde stain.[††] Flash column chromatography was carried out on Merck Kieselgel (Merck 9385) under positive pressure by means of a hand pump. Eluent compositions are quoted as percentages.

Reagents were purified by standard techniques. Acetyl chloride, ethyl acetate, acetic anhydride, triethylamine and 2,6-lutidine were distilled from calcium hydride and stored over calcium hydride under nitrogen atmosphere. Tetrahydrofuran (THF) was distilled from sodium metal/benzophenone ketyl and stored under a nitrogen atmosphere. Dichloromethane (DCM) was freshly distilled from calcium hydride and stored under a nitrogen atmosphere. Anisomycin, Dehydroepiandrosterone and Dehydroepiandrosterone sulfate were all purchased from Sigma (Poole, UK). All other reagents were used as supplied from Sigma (Poole, UK), Acros

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[€] Ammonium molybdate stain was prepared by addition of concentrated sulfuric acid (50 ml) to water (950 ml), followed by ammonium molybdate (50 g) and ceric sulfate (3 g). The solution was stirred for 2 h and formed a pale yellow solution.

[**] Potassium permanganate stain was prepared by addition of potassium permanganate (10 g), potassium carbonate (50 g) and sodium hydroxide (40 pellets) to a stirred solution of water (1000 ml). The solution was stirred until all solid had dissolved, forming a purple solution.

[††] Anisaldehyde stain was prepared by slow addition of concentrated sulfuric acid (35 ml) to a stirred solution of ethanol (930 ml), followed by addition of glacial acetic acid (10 ml) and anisaldehyde (2.5 ml) dropwise.

(Loughborough, UK), Novabiochem (Nottingham, UK), Lancaster (Heysham, UK), Molecular Probes (Paisley, UK) and Biorad (Hercules, USA).

All chemical experiments were performed in an inert atmosphere of nitrogen under anhydrous conditions, using oven dried apparatus cooled in a desiccator or flame dried under nitrogen prior to use. Standard techniques for handling air sensitive materials were employed.[391, 392]

5.2 Experimental Procedures

Methyl (2*R*)-2-amino-3-(4'-hydroxyphenyl)-propionate hydrochloride salt[254] 114
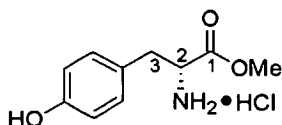
To a stirred solution of methanol (100 ml) at 0 °C, was added the acetyl chloride (5.90 ml, 82.8 mmol). The solution was stirred for 15 min at 0 °C, and then D-tyrosine **113** (5.32 g, 27.3 mmol) was added portionwise to the solution. The resulting solution was heated at reflux for 3 h, before concentration *in vacuo* to give the hydrochloride salt. The salt was recrystallised using methanol to give **114** as a colourless solid (6.32 g, 27.3 mmol, 100%); **mp** 192-193 °C; υ_{max} (neat)/cm^{-1} 4256, 3338, 1744, 1613, 1592, 1515; **^{1}H NMR** δ (200 MHz, CD$_3$OD) 7.07 (2H, d, *J* 8.5, Ar*H*), 6.78 (2H, d, *J* 8.5, Ar*H*), 4.88 (2H, br s, N*H$_2$*), 4.24 (1H, t, *J* 6.6, C$_2$*H*), 3.8 (3H, s, O*Me*), 3.20-3.06 (2H, m, C$_3$*H$_2$*); **^{13}C NMR** δ (62.9 MHz, CD$_3$OD) 168.6 (1C, Q), 156.4 (1C, Q), 129.6 (2C, CH), 123.7 (1C, Q), 115.0 (2C, CH), 51.6 (1C, CH$_3$), 53.5 (1C, CH), 34.7 (1C, CH$_2$); **LRMS** (ESI+) [M+H]$^+$ 196 (100).

All spectroscopic data was in good agreement with that of the literature.[254]

Methyl   (2*R*)-2-*tert*-butoxycarbonylamino-3-(4'-hydroxyphenyl)-propionate[393]
115
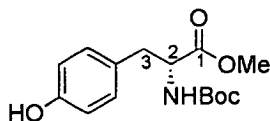
To a solution of ester hydrochloride salt **114** (6.23 g, 27.3 mmol) in ethanol (60 ml) was added sodium hydrogen carbonate (6.88 g, 81.9 mmol) and di-*tert*-butyl dicarbonate (5.95 g, 27.3 mmol). The resulting solution was stirred for 16 h at RT, then filtered and concentrated *in vacuo* to give **115** as a foam (8.07 g, 27.3 mmol, 100%); **R$_f$** [5% MeOH/DCM] = 0.52; **^{1}H NMR** δ (200 MHz, CDCl$_3$) 6.89 (2H, d, *J* 8.2, Ar*H*), 6.74 (2H, d, *J* 8.2, Ar*H*), 4.98 (1H, br s, N*H*), 4.55 (1H, br q, *J* 8.4, C$_2$*H*), 3.71 (3H, s, O*Me*), 3.07-3.00 (2H, m, C$_3$*H$_2$*), 1.43 (9H, s, *'Bu*O); **^{13}C NMR** δ (62.9 MHz, CDCl$_3$) 172.5 (1C, Q), 154.4 (2C, Q), 130.3 (2C, CH), 127.8 (1C, Q), 115.3 (2C, CH), 79.9 (1C, Q), 54.4 (1C, CH), 52.1 (1C, CH$_3$), 37.4 (1C, CH$_2$), 28.2 (3C, CH$_3$).

All spectroscopic data was in good agreement with that of the literature.[393]

**Methyl (2*R*)-2-*tert*-butoxycarbonylamino-3-(4'-*tert*-butyldimethylsilyloxyphenyl)
–propionate 116**
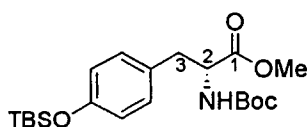
To a solution of the phenol **115** (3.19 g, 10.8 mmol) in DCM (30 ml) at 0 °C was added 2,6-lutidine (2.28 ml, 19.6 mmol), after 5 min TBSOTf (2.31 ml, 11.9 mmol) was added, and the solution stirred for 3 h. The reaction was quenched with saturated aq. sodium hydrogen carbonate (100 ml) and extracted with DCM (3 x 40 ml). The combined organics were washed with brine (40 ml), dried (MgSO$_4$) and concentrated *in vacuo*. The residue was chromatographed on silica gel [2% EtOAc/hexane] to give **116** as a clear oil (2.64 g, 6.45 mmol, 60%); **R$_f$** [2% EtOAc/hexane] = 0.4; [α]$_D$ -32.0 (*c* 0.75, CHCl$_3$); υ_{max} (neat)/cm^{-1} 3438, 1743, 1711, 1609, 1510; **^{1}H NMR** δ (200 MHz, CDCl$_3$) 7.07 (2H, d, *J* 8.4, Ar*H*), 7.00 (2H, d, *J* 8.4, Ar*H*), 4.97 (1H, d, *J* 8.1, N*H*), 4.58-4.50 (1H, m, C$_2$*H*), 3.70 (3H, s, O*Me*), 3.08-2.99 (2H, m, C$_3$*H$_2$*), 1.43 (9H, s, '*Bu*O), 0.98 (9H, s, '*Bu*Si), 0.19 (6H, s, *Me$_2$*Si); **^{13}C NMR** δ (62.9 MHz, CDCl$_3$) 172.3 (1C, Q), 155.0 (1C, Q), 154.6 (1C, Q), 130.1 (2C, CH), 128.5 (1C, Q), 119.9 (2C, CH), 79.8 (1C, Q), 54.4 (1C, CH), 52.0 (1C, CH$_3$), 37.5 (1C, CH$_2$), 28.2 (3C, CH$_3$), 25.5 (3C, CH$_3$), 18.1 (1C, Q), -4.6 (2C, CH$_3$); *m/z* (FAB, NOBA) 409 ([M]$^+$, 65); **HRMS** (FAB, NOBA) (Found: [M+H]$^+$, 409.2280. C$_{21}$H$_{35}$NO$_5$Si requires *m/z*, 409.2285).

Methyl (2*R*)-2-amino-3-(4'-*tert*-butyldimethylsilyloxyphenyl)-propionate 117
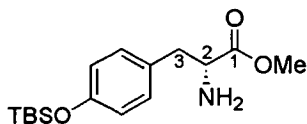
To a solution of Boc-protected ester **116** (460 mg, 1.13 mmol) in DCM (20 ml) was added TFA (0.98 ml) and the solution stirred for 15 h at RT. The reaction was quenched with saturated aq. sodium carbonate (10 ml) until the solution was judged to be alkaline by litmus paper. The organic phase was separated, and the aqueous phase extracted with DCM (3 x 20 ml). The combined organics were dried (MgSO$_4$) and concentrated *in vacuo* to give **117** as an oil (371 mg, 1.20 mmol, 100%); R_f [2% MeOH/DCM] = 0.20; $[\alpha]_D$ +98.8 (*c* 0.84, CHCl$_3$); v_{max} (neat)/cm^{-1} 3211, 1740, 1609, 1510; **^{1}H NMR** δ (250 MHz, CDCl$_3$) 7.05 (2H, d, *J* 8.4, Ar*H*), 6.78 (2H, d, 8.4, Ar*H*), 3.73-3.70 (1H, m, C$_2$*H*), 3.71 (3H, s, O*Me*), 3.03 (1H, dd, *J* 13.6, 5.3, C$_3$*H$_S$H$_T$*), 2.84 (1H, dd, *J* 13.6, 7.6, C$_3$H$_S$*H$_T$*), 1.83-1.81 (2H, m, N*H$_2$*), 0.98 (9H, s, '*Bu*Si), 0.19 (6H, s, *Me$_2$*Si); **^{13}C NMR** δ (62.9 MHz, CDCl$_3$) 175.2 (1C, Q), 154.4 (1C, Q), 130.1 (2C, CH), 129.5 (1C, Q), 120.0 (2C, CH), 55.8 (1C, CH), 51.8 (1C, CH$_3$), 40.1 (1C, CH$_2$), 25.5 (3C, CH$_3$), 18.1 (1C, Q), -4.6 (2C, CH$_3$); *m/z* (FAB, NOBA) 310 ([M+H]$^+$, 30%), 250 (38), 221 (100); **HRMS** (FAB, NOBA) (Found: [M+H]$^+$, 310.1841. C$_{16}$H$_{28}$NO$_3$Si requires *m/z*, 310.1839).

Methyl (2R)-3-(4'-*tert*-butyldimethylsilyloxyphenyl)-2-dibenzylamino-propionate 118
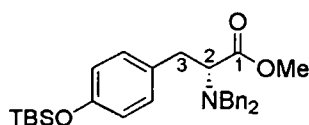
To a solution of amino ester **117** (300 mg, 970 μmol) in acetonitrile (15 ml) was added potassium carbonate (669 mg, 4.85 mmol) and benzyl bromide (460 μl, 3.88 mmol). The mixture was stirred for 96 h at RT. Water (40 ml) was added to the solution and the aqueous phase extracted with EtOAc (3 x 30 ml). The organic extract was dried (MgSO$_4$) and concentrated *in vacuo*. The residue was chromatographed on silica gel [10% EtOAc/hexane] to give **118** as a yellow oil (338 mg, 690 μmol, 71%); R_f [25% EtOAc/hexane] = 0.80; v_{max} (neat)/cm^{-1} 1741, 1608, 1509; **^{1}H NMR** δ (250 MHz, CDCl$_3$) 7.35-7.26 (10H, m, Ar*H*), 6.92 (2H, d, *J* 8.3, Ar*H*), 6.79 (2H, d, *J* 8.3, Ar*H*), 4.01 (2H, d, *J* 14.0, NC*H$_X$*H$_Y$Ph x 2), 3.78 (3H, s, O*Me*), 3.70 (1H, t, *J* 8.3, C$_2$*H*), 3.59 (2H, d, *J* 14.0, NCH$_X$*H$_Y$*Ph x 2), 3.20 (1H, dd, *J* 14.0, 8.3, C$_3$*H$_S$*H$_T$), 3.10 (1H, dd, *J* 14.0, 8.3, C$_3$H$_S$*H$_T$*), 1.07 (9H, s, *^{t}Bu*Si), 0.28 (6H, s, *Me$_2$*Si); **^{13}C NMR** δ (62.9 MHz, CDCl$_3$) 172.7 (1C, Q), 154.0 (1C, Q), 139.2 (2C, Q), 130.6 (1C, Q), 130.2 (2C, CH), 128.6 (4C, CH), 128.0 (4C, CH), 126.8 (2C, CH), 119.6 (2C, CH), 62.2 (1C, CH), 54.2 (2C, CH$_2$), 50.9 (1C, CH$_3$), 34.8 (1C, CH$_2$), 25.6 (3C, CH$_3$), 18.2 (1C, Q), -4.5 (2C, CH$_3$); *m/z* (FAB, NOBA) 490 ([M+H]$^+$, 74%), 430 (80), 266 (81), 91 (100); **HRMS** (FAB, NOBA) (Found: [M+H]$^+$, 490.2785. C$_{30}$H$_{40}$NO$_3$Si requires *m/z*, 490.2778).

Methyl (2*R*)-2-dibenzylamino-3-(4'-hydroxyphenyl)-propionate 121
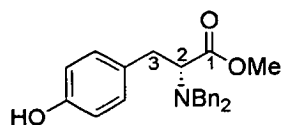
To a solution of the hydrochloride salt **114** (12.8 g, 55.2 mmol) in acetonitrile (400 ml) was added potassium carbonate (22.9 g, 166 mmol) followed by benzyl bromide (15.1 ml, 127 mmol), and the solution stirred for 48 h at RT. The reaction was quenched by addition of water (100 ml). The organics were separated and the aqueous extracted with ethyl acetate (3 x 100 ml). The combined organics were washed with brine (100 ml), dried (Na_2SO_4), and concentrated *in vacuo* to give a yellow oil. The oil was chromatographed on silica gel [10% EtOAc/hexane] to give the product **121** as a clear oil (16.1 g, 42.3 mmol, 76 %); **R$_f$** [30% EtOAc/hexane] = 0.50; [α]$_D$ +75.2 (*c* 1.25, CHCl$_3$); υ$_{max}$ (neat)/cm^{-1} 3411, 1708, 1614, 1514; **^{1}H NMR** δ (250 MHz, CDCl$_3$) 7.17-7.08 (10H, m, Ar*H*), 6.75 (2H, d, *J* 8.5, Ar*H*), 6.61 (2H, d, *J* 8.5, Ar*H*), 5.95 (1H, br s, O*H*), 3.84 (2H, d, *J* 14.0, NC*H$_X$*H$_Y$Ph x 2), 3.60 (3H, s, O*Me*), 3.53 (1H, t, *J* 7.8, C$_2$*H*), 3.43 (2H, d, *J* 14.0, NCH$_X$*H$_Y$*Ph x 2), 2.95 (1H, dd, *J* 14.0, 7.8, C$_3$*H$_S$*H$_T$), 2.81 (1H, dd, *J* 14.0, 7.8, C$_3$H$_S$*H$_T$*); **^{13}C NMR** δ (62.9 MHz, CDCl$_3$) 173.2 (1C, Q), 154.1 (1C, Q), 139.0 (2C, Q), 130.3 (2C, CH), 129.7 (1C, Q), 128.5 (4C, CH), 128.0 (4C, CH), 126.8 (2C, CH), 114.9 (2C, CH), 62.5 (1C, CH), 54.3 (2C, CH$_2$), 51.1 (1C, CH$_3$), 34.7 (1C, CH$_2$); ***m/z*** (FAB, NOBA) 398 ([M+Na]$^+$, 25%), 376 ([M+H]$^+$, 85%), 316 (73), 268 (98), 91 (100); **HRMS** (FAB, NOBA) (Found: [M+H]$^+$, 376.1912. C$_{24}$H$_{26}$NO$_3$ requires *m/z*, 376.1913).

(2*R*)-2-Dibenzylamino-3-(4'-hydroxyphenyl)-propanoic acid 120
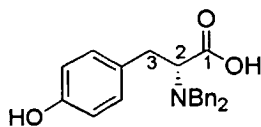
To a solution of ester **121** (16.1 g, 42.3 mmol) in THF:H_2O (200 ml, 4:1) was added lithium hydroxide (9.03 g, 215 mmol) and the solution heated at 82 °C for 48 h. The reaction was quenched by addition of 1N HCl until the solution was judged to be pH 2 by litmus paper. The aqueous phase was extracted with DCM (3 x 100 ml). The combined organics were washed with water (100 ml), brine (100 ml), dried (Na_2SO_4) and concentrated *in vacuo* to give **120** as a yellow foam (14.9 g, 41.4 mmol, 96%); R_f [30% EtOAc/hexane] = 0.13; $[\alpha]_D$ +52.1 (*c* 3.55, EtOH); υ_{max} (neat)/cm^{-1} 3407, 1731, 1614, 1515; **^{1}H NMR** δ (250 MHz, CD_3OD) 7.56-7.44 (10H, m, Ar*H*), 7.13 (2H, d, *J* 8.5, Ar*H*), 6.95 (2H, d, *J* 8.5, Ar*H*), 4.16 (2H, d, *J* 13.8, NC*H*$_X$H$_Y$Ph x 2), 3.94 (2H, d, *J* 13.8, NCH$_X$*H*$_Y$Ph x 2), 3.86 (1H, dd, *J* 8.4, 6.8, C$_2$*H*), 3.33 (1H, dd, *J* 14.2, 6.8, C$_3$*H*$_S$H$_T$), 3.17 (1H, dd, *J* 14.2, 8.4, C$_3$H$_S$*H*$_T$); **^{13}C NMR** δ (62.9 MHz, $CDCl_3$) 173.2 (1C, Q), 155.0 (1C, Q), 137.7 (2C, Q), 129.5 (2C, CH), 128.4 (1C, Q), 128.1 (4C, CH), 127.4 (4C, CH), 126.4 (2C, CH), 114.1 (2C, CH), 62.5 (1C, CH), 53.6 (2C, CH_2), 33.4 (1C, CH_2); *m/z* (FAB, NOBA) 384 ($[M+Na]^+$, 63%), 362 ($[M+H]^+$, 100%), 316 (49), 254 (73), 91 (72); **HRMS** (FAB, NOBA) (Found: $[M+H]^+$, 362.1756. $C_{23}H_{24}NO_3$ requires *m/z*, 362.1756).

(2R)-3-(4'-*tert*-Butyldimethylsilyloxyphenyl)-2-dibenzylamino-propanoic acid 119
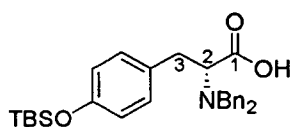
To a solution of phenol **120** (14.9 g, 41.4 mmol) in CH_2Cl_2 (200 ml) at 0 °C was added 2,6-lutidine (19.6ml, 168 mmol) and the solution stirred for 30 min. TBSOTf (19.8 ml, 86.3 mmol) was added to the solution and stirred for a further 3.5 h. The reaction was poured onto iced 1M phosphoric acid (50 ml) and the aqueous extracted with CH_2Cl_2 (3 x 100 ml). The combined organics were washed with water (100 ml), brine (100 ml), dried (Na_2SO_4) and concentrated *in vacuo* to give the di-TBS protected compound **122**.

The crude material was dissolved in acetic acid:THF:H_2O (200 ml, 3:1:1) and stirred for 6 h. The reaction was quenched by addition of sodium hydrogen carbonate (~35 g), diluted with water (200 ml) and extracted with EtOAc (5 x 100 ml). The combined organics were washed with brine (100 ml), dried (Na_2SO_4) and concentrated *in vacuo* to give **119** as a foam (15.2 g, 32.1 mmol, 78%); R_f [30% EtOAc/hexane] = 0.28; $[\alpha]_D$ +23.4 (*c* 3.40, $CHCl_3$); υ_{max} (neat)/cm^{-1} 3407, 1731, 1614, 1514; **^{1}H NMR** δ (250 MHz, $CDCl_3$) 9.50 (1H, br s, CO_2H), 7.22-7.07 (10H, m, Ar*H*), 6.88 (2H, d, *J* 8.5, Ar*H*), 6.67 (2H, d, *J* 8.5, Ar*H*), 3.70 (2H, d, *J* 13.6, NC*H*$_X$H$_Y$Ph x 2), 3.68 (2H, d, *J* 13.6, NCH$_X$*H*$_Y$Ph x 2), 3.61 (1H, dd, *J* 9.1, 5.8, C$_2$*H*), 3.15 (1H, dd, *J* 14.4, 5.8, C$_3$*H*$_S$H$_T$), 2.90 (1H, dd, *J* 14.4, 9.1, C$_3$H$_S$*H*$_T$), 0.89 (9H, s, *Bu*Si), 0.12 (6H, s, *Me*$_2$Si); **^{13}C NMR** δ (62.9 MHz, $CDCl_3$) 175.7 (1C, Q), 154.2 (1C, Q), 137.7 (2C, Q), 130.6 (1C, Q), 130.2 (2C, CH), 128.8 (4C, CH), 128.3 (4C, Q), 127.4 (2C, CH), 119.9 (2C, CH), 62.5 (1C, CH), 54.2 (2C, CH_2), 33.2 (1C, CH_2), 25.6 (3C, CH_3), 18.1 (1C, Q), -4.5 (2C, CH_3); *m/z* (FAB, NOBA) 498 ([M+Na]$^+$, 85%), 476 ([M+H]$^+$, 89%), 430 (84), 91 (100); **HRMS** (FAB, NOBA) (Found: [M+H]$^+$, 476.2618. $C_{29}H_{38}NO_3Si$ requires *m/z*, 476.2621).

Ethyl (4*R*)-5-(4'-*tert*-butyldimethylsilyloxyphenyl)-4-dibenzylamino-3-oxo-pentanoate 124
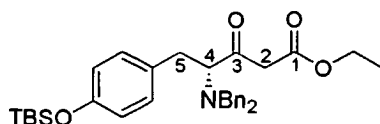
To a solution of acid **119** (7.27 g, 15.3 mmol) in THF (50 ml) was added carbonyl diimidazole (7.44 g, 45.9 mmol) and the solution stirred for 2 h, before cooling to -78 °C. Meanwhile, a stirred solution of ethyl acetate (5.21 ml, 53.6 mmol) was cooled to -78 °C, LiHMDS (54.0 ml, 1.0 M in THF) was added and the solution stirred for 20 min. The enolate was then transferred *via* cannula to the imidazolide **123** and the solution stirred at -78 °C for 40 min. The reaction was warmed to 0 °C over 30 min, and stirred for a further 1 h at 0 °C. The reaction was quenched by addition of 1% HCl (15 ml) and diluted with DCM (50 ml). The organic layer was separated and the aqueous phase extracted with DCM (3 x 50 ml). The combined organics were washed sequentially with water (50 ml) and brine (50 ml), then dried (Na$_2$SO$_4$), and concentrated *in vacuo*. The residue was chromatographed on silica gel [8% EtOAc/hexane] to give **124** as a clear oil (6.66 g, 12.2 mmol, 79 %); **R$_f$** [30% EtOAc/hexane] = 0.83; [α]$_D$ +12.7 (*c* 1.10, CHCl$_3$); υ$_{max}$ (neat)/cm^{-1} 1745, 1717 1608, 1509; **¹H NMR** δ (250 MHz, CDCl$_3$) 7.34-7.03 (10H, m, Ar*H*), 7.01 (2H, d, *J* 8.6, Ar*H*), 6.74 (2H, d, *J* 8.6, Ar*H*), 4.03 (2H, q, *J* 7.1, OC*H$_2$*CH$_3$), 3.85 (2H, d, *J* 14.0, NC*H$_X$*H$_Y$Ph x 2), 3.68 (1H, d, *J* 15.6, C$_2$*H$_E$*H$_F$), 3.60 (1H, dd, *J* 9.1, 3.9, C$_4$*H*), 3.56 (2H, d, *J* 14.0 NCH$_X$*H$_Y$*Ph x 2), 3.37 (1H, d, *J* 15.6, C$_2$H$_E$*H$_F$*), 3.14 (1H, dd, *J* 14.0, 9.1, C$_5$*H$_S$*H$_T$), 2.81 (1H, dd, *J* 14.0, 3.9, C$_5$H$_S$*H$_T$*), 1.12 (3H, t, *J* 7.1, OCH$_2$*CH$_3$*), 1.01 (9H, s, *^{t}Bu*Si), 0.20 (6H, s, *Me$_2$*Si); **¹³C NMR** δ (62.9 MHz, CDCl$_3$) 202.5 (1C, Q), 167.1 (1C, Q), 153.8 (1C, Q), 138.7 (2C, Q), 131.5 (1C, Q), 131.0 (2C, CH), 128.9 (4C, CH), 128.5 (4C, CH), 127.2 (2C, CH), 119.8 (2C, CH), 68.2 (1C, CH), 60.9 (1C, CH$_2$), 54.4 (2C, CH$_2$), 46.7 (1C, CH$_2$), 27.7 (1C, CH$_2$), 25.6 (3C, CH$_3$), 18.0 (1C, Q), 14.0 (1C, CH$_3$), -4.6 (2C, CH$_3$); **m/z** (FAB, THIOG) 546 ([M+H]⁺, 37%), 430 (85), 221 (71), 91 (100); **HRMS** (FAB, NOBA) (Found: [M+H]⁺, 546.3042. C$_{33}$H$_{44}$NO$_4$Si requires *m/z*, 546.3038).

## Ethyl (3*R*,4*R*)-5-(4'-*tert*-butyldimethylsilyloxyphenyl)-4-dibenzylamino-3-hydroxy-pentanoate 126
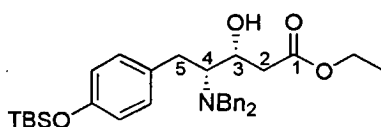
To a solution of β-keto ester **124** (6.66 g, 12.2 mmol), in ether (100 ml), was added methanol (20 ml) and the solution adjusted to pH 4 by addition of a few drops of acetic acid. Sodium cyanoborohydride (11.5 g, 183 mmol) was added to the solution at 0 °C, the solution was allowed to warm to RT and stirred for 19 h. The solution was quenched by addition of saturated aq. ammonium chloride (40 ml). The organic phase was separated and the aqueous extracted with DCM (3 x 40 ml). The combined organics were washed with brine (40 ml), dried (Na_2SO_4) and concentrated *in vacuo*. The residue was chromatographed on silica gel [8% EtOAc/hexane] to give **126** as a clear oil (5.77 g, 10.5 mmol, 86%); R_f [10% EtOAc/hexane] = 0.28; $[\alpha]_D$ -30.2 (*c* 3.05, $CHCl_3$); υ_{max} (neat)/cm^{-1} 3453, 1732, 1607, 1509; **^{1}H NMR** δ (250 MHz, $CDCl_3$) 7.41-7.30 (10H, m Ar*H*), 7.11 (2H, d, *J* 8.5, Ar*H*), 6.85 (2H, d, *J* 8.5, Ar*H*), 4.25 (1H, br s, O*H*), 4.18 (2H, d, *J* 13.7, NC*H*$_X$H$_Y$Ph x 2), 4.09 (2H, q, *J* 7.2, OC*H*$_2$CH$_3$), 4.05-4.02 (1H, m, C$_3$*H*), 3.48 (2H, d, *J* 13.7, NCH$_X$*H*$_Y$Ph x 2), 3.14 (1H, m, C$_4$*H*), 2.85-2.75 (2H, m, C$_5$*H*$_2$), 2.40 (1H, dd, *J* 15.7, 9.3, C$_2$*H*$_E$H$_F$), 2.18 (1H, dd, *J* 15.7, 2.7, C$_2$H$_E$*H*$_F$), 1.26 (3H, t, *J* 7.2, OCH$_2$C*H*$_3$), 1.06 (9H, s, ^{t}*Bu*Si), 0.28 (6H, s, *Me*$_2$Si); **^{13}C NMR** δ (62.9 MHz, $CDCl_3$) 172.5 (1C, Q), 153.9 (1C, Q), 139.0 (2C, Q), 132.4 (1C, Q), 129.9 (2C, CH), 128.9 (4C, CH), 128.3 (4C, CH), 127.1 (2C, CH), 120.1 (2C, CH), 67.8 (1C, CH), 63.0 (1C, CH), 60.4 (1C, CH$_2$), 54.2 (2C, CH$_2$), 39.7 (1C, CH$_2$), 30.1 (1C, CH$_2$), 25.6 (3C, CH$_3$), 18.1 (1C, Q), 14.0 (1C, CH$_3$), -4.6 (2C, CH$_3$); *m/z* (FAB, NOBA) 570 ([M+Na]$^+$, 82%), 548 ([M+H]$^+$, 98%), 430 (100), 91 (96); **HRMS** (FAB, NOBA) (Found: [M+H]$^+$, 548.3191. $C_{33}H_{46}NO_4Si$ requires *m/z*, 548.3196); **Chiral HPLC** (*R* enantiomer) R_t = 5.30 min, (*S* enantiomer) R_t = 6.26 min [2% ethanol/hexane], 97% ee.

(3R,4R)-5-(4'-*tert*-Butyldimethylsilyloxyphenyl)-4-dibenzylamino-3-hydroxy-pentan-1-ol 128
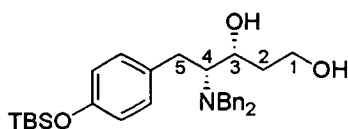
A solution of ester **126** (5.77 g, 10.5 mmol) in THF (100 ml) was cooled to -78 °C and lithium aluminium hydride (53.0 ml, 52.5 mmol, 1.0 M in THF) added. The solution was stirred at -78 °C for 6 h, then allowed to warm to RT over 1 h. The reaction was quenched by addition of 1 M sodium hydroxide (30 ml), diluted with DCM (30 ml) and saturated aq. sodium potassium tartrate (30 ml) and stirred for 16 h. The organic phase was separated and the aqueous phase extracted with DCM (3 x 80 ml). The combined organics were dried (Na_2SO_4) and concentrated *in vacuo*. The residue was chromatographed on silica gel [30% EtOAc/hexane] to give **128** as a clear oil (5.17 g, 10.2 mmol, 97%); R_f [30% EtOAc/hexane] = 0.39; $[\alpha]_D$ -28.3 (*c* 2.40, $CHCl_3$); υ_{max} (neat)/cm^{-1} 3376, 1607, 1509; **^{1}H NMR** δ (250 MHz, $CDCl_3$) 7.41-7.24 (10H, m, Ar*H*), 7.12 (2H, d, *J* 8.4, Ar*H*), 6.88 (2H, d, *J* 8.4, Ar*H*), 4.88 (1H, br s, O*H*), 3.95 (2H, d, *J* 13.2, NC*H$_X$*H$_Y$Ph x 2), 3.84 (1H, td, *J* 2.7, 9.0, C$_3$*H*), 3.73-3.69 (2H, m, C$_1$*H$_2$*OH), 3.45 (2H, d, *J* 13.2, NCH$_X$*H$_Y$*Ph x 2), 3.10 (1H, dd, *J* 14.2, 6.5, C$_5$*H$_S$*H$_T$), 2.99-2.94 (2H, m, C$_4$*H, O*H*), 2.65 (1H, dd, *J* 14.2, 6.5, C$_5$H$_S$*H$_T$*), 1.66-1.64 (1H, m, C$_2$*H$_E$*H$_F$), 1.36-1.31 (1H, m, C$_2$H$_E$*H$_F$*), 1.06 (9H, s, *^{t}Bu*Si), 0.27 (6H, s, *Me$_2$*Si); **^{13}C NMR** δ (62.9 MHz, $CDCl_3$) 154.0 (1C, Q), 138.5 (2C, Q), 132.5 (1C, Q), 129.9 (2C, CH), 128.9 (4C, CH), 128.4 (4C, CH), 127.2 (2C, CH), 120.2 (2C, CH), 70.4 (1C, CH), 63.8 (1C, CH), 61.1 (1C, CH$_2$), 53.6 (2C, CH$_2$), 35.6 (1C, CH$_2$), 31.3 (1C, CH$_2$), 25.6 (3C, CH$_3$), 18.1 (1C, Q), -4.6 (2C, CH$_3$); *m/z* (FAB, THIOG) 506 ([M+H]$^+$, 9%), 430 (12), 284 (35), 221 (55), 91 (100); **HRMS** (FAB, THIOG) (Found: [M+H]$^+$, 506.3091. $C_{31}H_{44}NO_3Si$ requires *m/z*, 506.3091).

(2*R*,3*R*)-2-(4'-*tert*-Butyldimethylsilyloxybenzyl)-1,1-dibenzyl-3-hydroxy-pyrrolidinium chloride 129
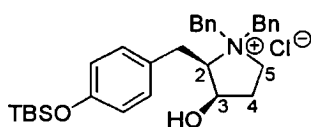
To a solution of alcohol **128** (5.17 g, 10.2 mmol) in DCM (100 ml) at 0 °C was added DMAP (2.05 g, 16.8 mmol) and trisopropylbenzene sulfonyl chloride (3.40 g, 11.2 mmol). The solution was stirred for 19 h before being diluted with DCM (50 ml) and water (50 ml). The organic phase was separated and washed with 1% HCl (2 x 20 ml) then dried (Na$_2$SO$_4$) and concentrated *in vacuo*. The residue was chromatographed on silica gel [5% MeOH/DCM] to give a colourless foam. The salt was subjected to ion exchange chromatography [Dowex Cl$^-$; prepared by treating Dowex resin with methanol, then 1% HCl, followed by flushing with methanol until the eluent returned to pH 7] eluting with methanol to give the chloride salt **129** as an amorphous solid (5.14 g, 9.82 mmol, 96%); **R**$_f$ [5% MeOH/DCM] = 0.12; [α]$_D$ -42.1 (*c* 1.50, CHCl$_3$); υ$_{max}$ (neat)/cm^{-1} 3397, 1607, 1508; **^{1}H NMR** δ (250 MHz, CDCl$_3$) 7.78-7.29 (10H, m, Ar*H*), 7.26 (2H, d, *J* 8.5, Ar*H*), 6.65 (2H, d, *J* 8.5, Ar*H*), 6.56 (1H, br s, O*H*), 5.73 (1H, d, *J* 13.4, NC*H*$_X$H$_Y$Ph x 1), 5.08 (2H, m, NCH$_X$*H*$_Y$Ph x 2), 4.32 (1H, br s, C$_3$*H*), 4.23 (1H, d, *J* 13.4, NC*H*$_X$H$_Y$Ph x 1), 3.91 (1H, br d, *J* 11.1, C*H*$_S$H$_T$Ar), 3.84-3.81 (1H, m, C$_5$*H*$_M$H$_N$), 3.53 (1H, br d, *J* 11.1, CH$_S$*H*$_T$Ar), 3.33 (1H, br t, *J* 11.1, C$_2$*H*), 3.04 (1H, td, 11.1, 8.7, C$_5$H$_M$*H*$_N$), 2.58 (1H, td, *J* 14.1, 7.9, C$_4$*H*$_E$H$_F$), 1.94-1.87 (1H, m, C$_4$H$_E$*H*$_F$), 0.98 (9H, s, ^{t}*Bu*Si), 0.17 (6H, s, *Me*$_2$Si); **^{13}C NMR** δ (62.9 MHz, CDCl$_3$) 154.4 (1C, Q), 133.4 (2C, CH), 133.0 (2C, CH), 130.5 (3C, CH), 130.2 (1C, CH), 129.2 (2C, CH), 129.0 (2C, CH), 128.3 (1C, Q), 127.9 (1C, Q), 127.3 (1C, Q), 120.1 (2C, CH), 76.1 (1C, CH), 67.6 (1C, CH), 62.2 (1C, CH$_2$), 61.6 (1C, CH$_2$), 55.6 (1C, CH$_2$), 31.2 (1C, CH$_2$), 27.4 (1C, CH$_2$), 25.5 (3C, CH$_3$), 17.9 (1C, Q), -4.6 (2C, Q); *m/z* (FAB, NOBA) 488 ([M]$^+$, 100%), 396 (21), 307 (14), 91 (75); **HRMS** (FAB, NOBA) (Found: [M]$^+$, 488.2978. C$_{31}$H$_{42}$NO$_2$Si requires *m/z*, 488.2985).

## (2R,3R)-1-Benzyl-2-(4'-*tert*-butyldimethylsilyloxybenzyl)-3-hydroxy-pyrrolidine 130
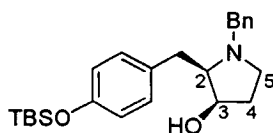
To a solution of the chloride salt **129** (4.32 g, 8.25 mmol) in methanol (40 ml) was added 5% Pd/C (300 mg) and potassium carbonate (3.42 g, 24.8 mmol). The mixture was exposed to a hydrogen atmosphere and stirred vigorously for 20 min. The suspension was filtered through a pad of celite and concentrated under reduced pressure. The residue was dissolved in DCM (15 ml) and washed with water (15 ml). The organic phase was separated and the aqueous phase extracted with DCM (3 x 15 ml). The combined organic phases were dried (Na_2SO_4) and concentrated *in vacuo* to give **130** as an oil (2.90 g, 7.30 mmol, 89%); **R_f** [5% MeOH/DCM] = 0.12; **[α]$_D$** -64.3 (*c* 2.10, CHCl$_3$); υ_{max} (neat)/cm^{-1} 3397, 1607, 1508; **^{1}H NMR** δ (250 MHz, CDCl$_3$) 7.35-7.26 (5H, m, Ar*H*), 7.19 (2H, d, *J* 8.5, Ar*H*), 6.78 (2H, d, *J* 8.5, Ar*H*), 4.17 (1H, d, *J* 13.0, NC*H*$_X$H$_Y$Ph), 3.96-3.92 (1H, m, C$_3$*H*), 3.26 (1H, d, *J* 13.0, NCH$_X$*H*$_Y$Ph), 3.05 (1H, td, *J* 9.2, 3.9, C$_2$*H*), 2.90-2.93 (2H, m, C*H*$_2$Ar), 2.50 (1H, td, *J* 7.1, 3.9, C$_5$*H*$_M$H$_N$), 2.15 (1H, td, *J* 9.6, 7.1, C$_5$H$_M$*H*$_N$), 2.01-1.97 (1H, m, C$_4$*H*$_E$H$_F$), 1.71-1.68 (1H, m, C$_4$H$_E$*H*$_F$), 0.98 (9H, s, *^{t}Bu*Si), 0.19 (6H, s, *Me$_2$*Si); **^{13}C NMR** δ (62.9 MHz, CDCl$_3$) 153.8 (1C, Q), 138.3 (1C, Q), 131.8 (1C, Q), 130.1 (2C, CH), 128.8 (2C, CH), 128.2 (2C, CH), 127.0 (1C, CH), 119.9 (2C, CH), 71.9 (1C, CH), 70.8 (1C, CH), 57.6 (1C, CH$_2$), 51.4 (1C, CH$_2$), 32.8 (1C, CH$_2$), 32.1 (1C, CH$_2$), 25.6 (3C, CH$_3$), 18.1 (1C, Q), -4.5 (2C, CH$_3$); **m/z** (FAB, NOBA) 398 ([M+H]$^+$, 100%), 221 (61), 176 (90), 91 (99); **HRMS** (FAB, NOBA) (Found: [M+H]$^+$, 398.2511. C$_{24}$H$_{36}$NO$_2$Si requires *m/z*, 398.2515).

(2R,3R)-3-Acetoxy-1-benzyl-2-(4'-*tert*-butyldimethylsilyloxybenzyl)-pyrrolidine 109
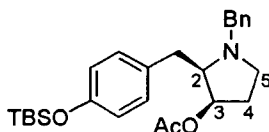

To a solution of the alcohol **130** (2.90 g, 7.30 mmol) in DCM (100 ml) was added a catalytic amount of DMAP, freshly distilled acetic anhydride (1.38 ml, 14.6 mmol) and triethylamine (2.02 ml, 14.6 mmol). The solution was stirred for 18 h at RT and then quenched by addition of saturated aq. sodium bicarbonate solution (40 ml). The organic phase was separated and the aqueous phase extracted with DCM (3 x 40 ml). The combined organics were dried (Na_2SO_4) and concentrated *in vacuo.* The residue was chromatographed on silica gel [30% EtOAc/hexane] to give **109** as an oil (3.11 g, 7.08 mmol, 96%); R_f [30% EtOAc/hexane] = 0.62; $[\alpha]_D$ -93.1 (*c* 2.90, $CHCl_3$); υ_{max} (neat)/cm^{-1} 1738, 1608, 1509; **^{1}H NMR** δ (250 MHz, $CDCl_3$) 7.35-7.18 (5H, m, Ar*H*), 7.04 (2H, d, *J* 8.4, Ar*H*), 6.75 (2H, d, *J* 8.4, Ar*H*), 5.02-4.98 (1H, m, C_3H), 4.05 (1H, d, *J* 13.1, NC*H*$_X$H$_Y$Ph), 3.33 (1H, d, *J* 13.1, NCH$_X$*H*$_Y$Ph), 3.00-2.75 (4H, m, C*H$_2$*Ar, C_5H_MH$_N$, C_2H), 2.23-2.13 (2H, m, C_4H_EH$_F$, C_5H$_M$*H$_N$*), 2.07 (3H, s, O*Ac*), 1.72-1.70 (1H, m, C_4H$_E$*H$_F$*), 0.97 (9H, s, ^{t}*Bu*Si), 0.18 (6H, s, *Me$_2$*Si); **^{13}C NMR** δ (62.9 MHz, $CDCl_3$) 170.4 (1C, Q), 153.8 (1C, Q), 138.1 (1C, Q), 131.6 (1C, Q), 129.6 (2C, CH), 129.0 (2C, CH), 128.1 (2C, CH), 126.9 (1C, CH), 119.9 (2C, CH), 74.6 (1C, CH), 68.0 (1C, CH), 58.2 (1C, CH_2), 51.5 (1C, CH_2), 33.4 (1C, CH_2), 30.5 (1C, CH_2), 25.6 (3C, CH_3), 21.1 (1C, CH_3), 18.1 (1C, Q), -4.6 (2C, CH_3); *m/z* (FAB, NOBA) 540 ([M+H]$^+$, 100%), 380 (51), 218 (96), 91 (90); **HRMS** (FAB, NOBA) (Found: [M+H]$^+$, 440.2630. $C_{26}H_{38}NO_3Si$ requires *m/z*, 440.2621).

## (2*R*,3*R*)-3-Acetoxy-2-(4'-hyroxy-benzyl)-pyrrolidine hydrochloride 131
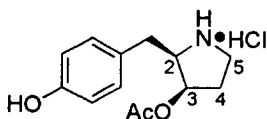
To a solution of monobenzylamine **109** (54.0 mg, 120 μmol) in EtOH (5 ml) was added 1M ethereal hydrochloric acid (0.24 ml) and Pearlman's catalyst (20% Pd(OH)$_2$/C; 54.0 mg). The solution was exposed to a hydrogen atmosphere (1 atm) and stirred vigorously for 2.5 h. The solution was filtered through a pad of Celite (prewashed with MeOH, DCM) and concentrated *in vacuo* to give **131** as a colourless solid (33.0 mg, 120 μmol, 100%); **R$_f$** [10% MeOH/DCM] = 0.15; [α]$_D$ +5.00 (*c* 1.60, EtOH); υ$_{max}$ (neat)/cm^{-1} 3376, 2950, 1739, 1614, 1517; **^{1}H NMR** δ (360 MHz, CD$_3$OD) 7.16 (2H, d, *J* 8.5, Ar*H*), 6.81 (2H, d, *J* 8.5, Ar*H*), 5.45 (1H, br s, C$_3$*H*), 3.93 (1H, br s, C$_2$*H*), 3.60-3.39 (2H, m, C$_5$*H$_2$*), 3.07 (1H, dd, *J* 14.1, 6.1, C*H$_S$*H$_T$Ar), 2.95 (1H, dd, *J* 14.1, 8.6, CH$_S$*H$_T$*Ar), 2.42-2.31 (H, m, C$_4$*H$_E$*H$_F$), 2.20 (3H, s, O*Ac*), 2.20-2.13 (1H, m, C$_4$H$_E$*H$_F$*); **^{13}C NMR** δ (62.9 MHz, CD$_3$OD) 171.6 (1C, Q), 158.2 (1C, Q), 131.3 (2C, CH), 127.9 (1C, Q), 117.2 (2C, CH), 74.7 (1C, CH), 66.5 (1C, CH), 44.7 (1C, CH$_2$), 33.0 (1C, CH$_2$), 32.2 (1C, CH$_2$), 21.2 (1C, CH$_3$); **m/z** (FAB, NOBA) 235 ([M]$^+$, 62%), 176 (86), 128 (74), 107 (51); **HRMS** (FAB, NOBA) (Found: [M+H]$^+$, 236.1284. C$_{13}$H$_{18}$NO$_3$Si requires *m/z*, 236.1287).

(2*R*,3*R*)-3-Acetoxy-2-(4'-*tert*-butyldimethylsilyloxybenzyl)-pyrrolidine 132
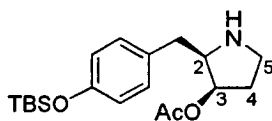
A solution of monobenzylamine **109** (1.01 g, 2.29 mmol) and Pearlman's catalyst (20% Pd(OH)$_2$/C; 300 mg) in EtOH (15 ml) was exposed to a hydrogen atmosphere (1 atm) and stirred vigorously for 2.5 h. The solution was filtered through a pad of Celite (prewashed with EtOH, DCM) and concentrated *in vacuo* to give **132** as an oil (785 mg, 2.25 mmol, 98%); **R$_f$** [5% MeOH/DCM] = 0.16; [α]$_D$ -42.3 (*c* 1.30, CHCl$_3$); υ$_{max}$ (neat)/cm^{-1} 2930, 1742, 1608, 1510; **^{1}H NMR** δ (250 MHz, CDCl$_3$) 7.05 (2H, d, *J* 8.4, Ar*H*), 6.75 (2H, d, *J* 8.4, Ar*H*), 5.12 (1H, br s, C$_3$*H*), 3.86 (1H, br s, C$_2$*H*), 3.33-3.23 (2H, m, C$_5$*H$_2$*), 3.07-2.89 (2H, m, C*H$_2$*Ar), 2.23-2.17 (H, m, C$_4$*H$_E$H$_F$*), 2.17 (3H, s, O*Ac*), 2.13-1.92 (1H, m, C$_4$H$_E$*H$_F$*), 0.93 (9H, s, '*Bu*Si), 0.17 (6H, s, *Me$_2$*Si); **^{13}C NMR** δ (62.9 MHz, CDCl$_3$) 170.5 (1C, Q), 154.4 (1C, Q), 130.8 (1C, Q), 129.8 (2C, CH), 120.3 (2C, CH), 74.4 (1C, CH), 64.4 (1C, CH), 43.8 (1C, CH$_2$), 33.9 (1C, CH$_2$), 32.6 (1C, CH$_2$), 25.8 (3C, CH$_3$), 21.3 (1C, CH$_3$), 18.3 (1C, Q), -4.3 (2C, CH$_3$); **m/z** (FAB, NOBA) 350 ([M+H]$^+$, 100%), 290 (34), 221 (60); **HRMS** (FAB, NOBA) (Found: [M+H]$^+$, 350.2154. C$_{19}$H$_{32}$NO$_3$Si requires *m/z*, 350.2152).

(2*R*,3*R*)-3-Acetoxy-1-*tert*-butoxycarbonylamino-2-(4'-*tert*-butyldimethylsilyloxy benzyl)-pyrrolidine 133
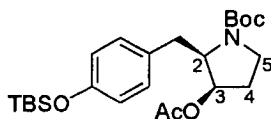
To a solution of amine **132** (726 mg, 2.08 mmol) in DCM (15 ml) was added triethylamine (456 µl, 3.27 mmol) and the solution cooled to 0 °C. Di-*tert*-butyl dicarbonate (713 mg, 3.27 mmol) was added and the solution stirred for 5 min, before warming to RT and stirring for 15 h. The reaction was quenched with saturated aq. sodium bicarbonate (30 ml) and diluted with DCM (50 ml). The organic phase was separated and washed with 1% HCl (20 ml) then dried (Na_2SO_4) and concentrated *in vacuo*. The residue was chromatographed on silica gel [2% MeOH/DCM] to give **133** as an oil (903 mg, 2.01 mmol, 97%); R_f [5% MeOH/DCM] = 0.68; $[\alpha]_D$ +3.50 (*c* 2.00, CHCl$_3$); υ_{max} (neat)/cm^{-1} 1743, 1697, 1608, 1509; **^{1}H NMR** δ (360 MHz, CDCl$_3$, 323 K) 7.06 (2H, d, *J* 8.4, Ar*H*), 6.73 (2H, d, *J* 8.4, Ar*H*), 5.13 (1H, br q, *J* 6.7 C$_3$*H*), 4.29-4.24 (1H, m, C$_2$*H*), 3.45-3.40 (1H, m, C$_5$H$_M$*H$_N$*), 3.34-3.27 (1H, m, C$_5$H$_M$*H$_N$*), 2.94 (1H, br d, C*H$_S$*H$_T$Ar), 2.86-2.80 (1h, dd, *J* 13.8, 8.3, CH$_S$*H$_T$*Ar), 2.08-2.04 (1H, m, C$_4$*H$_E$*H$_F$), 2.01 (3H, s, O*Ac*), 1.83-1.76 (1H, m, C$_4$H$_E$*H$_F$*), 1.47 (9H, s, *'Bu*O) 0.99 (9H, s, *'Bu*Si), 0.19 (6H, s, *Me$_2$*Si); **^{13}C NMR** δ (90.7 MHz, CDCl$_3$, 323 K) 170.8 (1C, Q), 155.1 (1C, Q), 154.6 (1C, Q), 131.8 (1C, Q), 130.9 (2C, CH), 120.5 (2C, CH), 80.4 (1C, Q), 73.9 (1C, CH), 59.6 (1C, CH), 42.0 (1C, CH$_2$), 34.1 (1C, CH$_2$), 33.1 (1C, CH$_2$), 29.1 (3C, CH$_3$), 26.3 (3C, CH$_3$), 21.6 (1C, CH$_3$), 18.9 (1C, Q), -4.4 (2C, CH$_3$); *m/z* (FAB, NOBA) 450 ([M+H]$^+$, 9%), 221 (83), 91 (22); **HRMS** (FAB, NOBA) (Found: [M+H]$^+$, 450.2671. $C_{24}H_{40}NO_5Si$ requires *m/z*, 450.2676).

(2*R*,3*R*)-3-Acetoxy-1-*tert*-butoxycarbonylamino-2-(4'-hydroxy-benzyl)-pyrrolidine 134
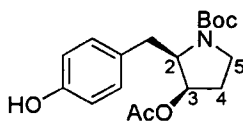
To a solution of the silyl ether **133** (572 mg, 1.27 mmol) in THF (15 ml) at 0 °C was added triethylamine trihydrofluoride (1.02 ml, 6.35 mmol), the solution was stirred for 5 min at 0 °C, before warming to RT and stirring for 2 h. The reaction was quenched with saturated aq. sodium bicarbonate (30 ml) and diluted with EtOAc (50 ml). The organic phase was separated and washed with brine (20 ml) then dried (Na$_2$SO$_4$) and concentrated *in vacuo*. The residue was chromatographed on silica gel [5% MeOH/DCM] to give **134** as an oil (415 mg, 1.23 mmol, 96%); **R$_f$** [5% MeOH/DCM] = 0.25; [α]$_D$ +9.30 (*c* 0.80, CHCl$_3$); υ$_{max}$ (neat)/cm^{-1} 3350, 1741, 1666, 1615, 1515; **^{1}H NMR** δ (360 MHz, CDCl$_3$, 323 K) 7.02 (2H, d, *J* 8.2, Ar*H*), 6.73 (2H, d, *J* 8.2, Ar*H*), 5.13 (1H, br q, *J* 6.8, C$_3$*H*), 4.26 (1H, br q, *J* 6.8, C$_2$*H*), 3.48-3.41 (1H, m, C$_5$*H*$_M$H$_N$), 3.36-3.29 (1H, m, C$_5$H$_M$*H*$_N$), 2.91 (1H, br s, C*H*$_S$H$_T$Ar), 2.80 (1H, dd, *J* 13.7, 8.1, CH$_S$*H*$_T$Ar), 2.09-2.04 (1H, m, C$_4$*H*$_E$H$_F$), 2.02 (3H, s, O*Ac*), 1.85-1.62 (1H, m, C$_4$H$_E$*H*$_F$), 1.45 (9H, s, *'Bu*O); **^{13}C NMR** δ (90.7 MHz, CDCl$_3$, 323 K) 170.8 (1C, Q), 155.4 (1C, Q), 155.3 (1C, Q), 130.9 (2C, CH), 130.4 (1C, Q), 115.8 (2C, CH), 80.6 (1C, Q), 73.9 (1C, CH), 59.8 (1C, CH), 43.6 (1C, CH$_2$), 34.6 (1C, CH$_2$), 29.4 (1C, CH$_2$), 29.0 (3C, CH$_3$), 21.2 (1C, CH$_3$); *m/z* (FAB, THIOG) 336 ([M+H]$^+$, 50%), 176 (70); **HRMS** (FAB, THIOG) (Found: [M+H]$^+$, 336.1810. C$_{18}$H$_{26}$NO$_5$ requires *m/z*, 336.1811).

## (2*R*,3*R*)-3-Acetoxy-1-*tert*-butoxycarbonylamino-2-(4'-prop-2-ynyloxybenzyl) pyrrolidine 135
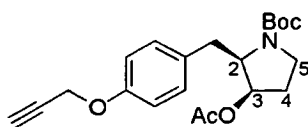
To a solution of the phenol **134** (27.0 mg, 80.0 μmol) in DMF (2 ml) was added potassium carbonate (17.0 mg, 120 μmol) and propargyl bromide (15.0 μl, 120 μmol), the solution was stirred at RT for 12 h, before quenching with water (5ml) and diluting with ether (10 ml). The organic phase was separated and washed with brine (10 ml) then dried (Na_2SO_4) and concentrated *in vacuo*. The residue was chromatographed on silica gel [2% MeOH/DCM] to give **135** as an oil (30.0 mg, 80.0 μmol, 100%); $\mathbf{R_f}$ [5% MeOH/DCM] = 0.70; [α]$_D$ +9.00 (*c* 1.50, CHCl$_3$); υ$_{max}$ (neat)/cm^{-1} 3286, 1739, 1692, 1610, 1510; **^{1}H NMR** δ (360 MHz, CDCl$_3$, 318 K) 7.20 (2H, d, *J* 8.6, Ar*H*), 6.89 (2H, d, *J* 8.6, Ar*H*), 5.13 (1H, br q, *J* 6.5, C$_3$*H*), 4.66 (1H, d, *J* 2.4, HC≡CC*H$_2$*) 4.3 (1H, ddd, *J* 8.2, 6.5, 4.7, C$_2$*H*), 3.48-3.41 (1H, m, C$_5$*H$_M$*H$_N$), 3.36-3.31 (1H, m, C$_5$H$_M$*H$_N$*), 2.96 (1H, br s, C*H$_S$*H$_T$Ar), 2.86-2.80 (1H, dd, *J* 13.8, 8.2, CH$_S$*H$_T$*Ar), 2.49 (1H, t, *J* 2.4, *H*C≡CCH$_2$), 2.08-2.04 (1H, m, C$_4$*H$_E$*H$_F$), 1.99 (3H, s, O*Ac*), 1.84-1.78 (1H, m, C$_4$H$_E$*H$_F$*), 1.45 (9H, s, '*Bu*O); **^{13}C NMR** δ (90.7 MHz, CDCl$_3$, 318 K) 170.2 (1C, Q), 156.4 (1C, Q), 154.6 (1C, Q), 131.9 (1C, Q), 130.6 (2C, CH), 115.1 (2C, CH), 79.9 (1C, Q), 79.0 (1C, Q), 75.4 (1C, CH), 73.4 (1C, CH), 59.4 (1C, CH), 56.2 (1C, CH$_2$), 42.0 (1C, CH$_2$), 34.4 (1C, CH$_2$), 33.1 (1C, CH$_2$), 28.6 (3C, CH$_3$), 21.0 (1C, CH$_3$); **m/z** (FAB, THIOG) 374 ([M+H]$^+$, 8%), 318 (100), 274 (39), 214 (61), 91 (93); **HRMS** (FAB, THIOG) (Found: [M+H]$^+$, 374.1966. C$_{21}$H$_{28}$NO$_5$ requires *m/z*, 374.1968).

## (2*R*,3*R*)- 3-Acetoxy-2-(4'-prop-2-ynyloxybenzyl)-pyrrolidine 136
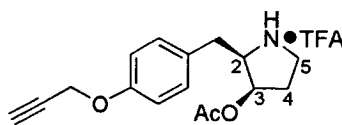
To a solution of carbamate **135** (30.0 mg, 80.0 µmol) in DCM (2 ml) was added TFA (0.20 ml, 0.80 mmol) and the solution stirred for 15 h at RT. The solution was quenched with saturated aq. sodium bicarbonate (5 ml). The organic phase was separated and the aqueous phase extracted with DCM (3 x 10 ml).The combined organic phases were dried (Na$_2$SO$_4$) and concentrated *in vacuo* to give **136** as an oil (18.0 mg, 70.0 µmol, 87%); **R$_f$** [5% MeOH/DCM] = 0.11; [α]$_D$ -24.2 (*c* 0.95, CHCl$_3$); υ$_{max}$ (neat)/cm^{-1} 3282, 2925, 1730, 1610, 1510; **^{1}H NMR** δ (360 MHz, CDCl$_3$, 323 K) 7.09 (2H, d, *J* 8.7, Ar*H*), 6.90 (2H, d, *J* 8.7, Ar*H*), 5.29-5.27 (1H, m, C$_3$*H*), 4.63 (2H, d, *J* 2.4, HC≡CC*H$_2$*), 3.85-3.80 (1H, m, C$_2$*H*), 3.45-3.32 (2H, m, C$_5$*H$_2$*), 3.05-2.99 (2H, m, C*H$_2$*Ar), 2.50 (1H, t, *J* 2.4, *H*C≡CCH$_2$), 2.29-2.17 (2H, m, C$_4$*H$_2$*), 2.18 (3H, s, O*Ac*); **^{13}C NMR** δ (90.7 MHz, CDCl$_3$, 323 K) 169.9 (1C, Q), 157.3 (1C, Q), 130.0 (2C, CH), 128.2 (1C, Q), 115.9 (2C, CH), 78.7 (1C, Q), 75.8 (1C, CH), 72.6 (1C, CH), 64.5 (1C, CH), 56.2 (1C, CH$_2$), 43.2 (1C, CH$_2$), 31.8 (1C, CH$_2$), 33.3 (1C, CH$_2$), 20.7 (1C, CH$_3$); *m/z* (FAB, THIOG) 274 ([M+H]$^+$, 87%), 214 (57); **HRMS** (FAB, THIOG) (Found: [M+H]$^+$, 274.1441. C$_{16}$H$_{19}$NO$_3$ requires *m/z*, 274.1443).

## (2R,3R)-3-Acetoxy-1-*tert*-butoxycarbonylamino-2-(4'-methoxybenzyl)-pyrrolidine 137
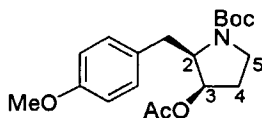
To a solution of phenol **134** (300 mg, 0.90 mmol) in DMF (5 ml) was added K_2CO_3 (618 mg, 4.48 mmol) and methyl iodide (279 µl, 4.48 mmol), the solution was stirred for 15 h at RT. The solution was quenched with water (5 ml) and DCM (10 ml) added. The organic phase was separated and the aqueous phase extracted with DCM (3 x 10 ml). The combined organic phases were dried (Na_2SO_4) and concentrated *in vacuo* to give **137** as an oil (230 mg, 0.66 mmol, 74%); R_f [5% MeOH/DCM] = 0.76; $[\alpha]_D$ +10.5 (*c* 1.00, CHCl$_3$); υ_{max} (neat)/cm^{-1} 1741, 1692, 1612, 1512; **^{1}H NMR** δ (360 MHz, CDCl$_3$, 323 K) 7.12 (2H, d, *J* 8.6, Ar*H*), 6.81 (2H, d, *J* 8.6, Ar*H*), 5.13 (1H, br q, *J* 6.6 C$_3$*H*), 4.26 (1H, ddd, *J* 8.1, 6.6, 4.5, C$_2$*H*), 3.79 (3H, s, O*Me*), 3.47-3.42 (1H, m, C$_5$*H*$_M$H$_N$), 3.36-3.29 (1H, m, C$_5$H$_M$*H*$_N$), 2.95 (1H, br s, C*H*$_S$H$_T$Ar), 2.84 (1H, dd, *J* 13.8, 8.1, CH$_S$*H*$_T$Ar), 2.08-2.01 (1H, m, C$_4$*H*$_E$H$_F$), 2.00 (3H, s, O*Ac*), 1.83-1.77 (1H, m, C$_4$H$_E$*H*$_F$), 1.46 (9H, s, ^tBuO); **^{13}C NMR** δ (90.7 MHz, CDCl$_3$, 323 K) 170.2 (1C, Q), 158.5 (1C, Q), 154.7 (1C, Q), 131.0 (1C, Q), 130.6 (2C, CH), 114.1 (2C, CH), 79.9 (1C, Q), 73.6 (1C, CH), 59.6 (1C, CH), 55.5 (1C, CH$_3$), 43.6 (1C, CH$_2$), 34.1 (1C, CH$_2$), 29.4 (1C, CH$_2$), 28.6 (3C, CH$_3$), 21.0 (1C, CH$_3$); *m/z* (FAB, THIOG) 351 ([M+H]$^+$, 18%), 350 ([M]$^+$, 38%), 294 (66), 190 (59), 122 (56), 91 (58); **HRMS** (FAB, THIOG) (Found: [M+H]$^+$, 350.1965. C$_{19}$H$_{28}$NO$_5$ requires *m/z*, 335.1968).

(2*R*,3*R*)-3-Acetoxy -2-(4'-methoxybenzyl)-pyrrolidine[254] 138
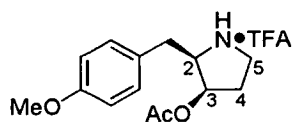
To a solution of carbamate **137** (230 mg, 0.66 mmol) in DCM (15 ml) was added trifluoroacetic acid (1.00 ml, 6.60 mmol), the solution was stirred for 7 h at RT. The reaction was concentrated *in vacuo* to give **138** as a foam (239 mg, 0.66 mmol, 100%); **R$_f$** [5% MeOH/DCM] = 0.09; [α]$_D$ +18.7 (*c* 1.10, CH$_3$OH); υ$_{max}$ (neat)/cm^{-1} 3004, 1750, 1614, 1516; **^{1}H NMR** δ (360 MHz, CD$_3$OD) 7.19 (2H, d, *J* 8.7, Ar*H*), 6.89 (2H, d, *J* 8.7, Ar*H*), 5.30 (1H, t, *J* 3.6, C$_3$*H*), 3.92-3.87 (1H, m, C$_2$*H*), 3.76 (3H, s, O*Me*), 3.51-3.33 (2H, m, C$_5$*H$_2$*), 3.07 (1H, dd, *J* 14.3, 6.7, C*H$_S$*H$_T$Ar), 2.95 (1H, dd, *J*, 14.3, 8.6, CH$_S$*H$_T$*Ar), 2.40-2.29 (1H, m, C$_4$*H$_E$*H$_F$), 2.16 (3H, s, O*Ac*), 2.20-2.13 (1H, m, C$_4$H$_E$*H$_F$*); **^{13}C NMR** δ (90.7 MHz, CD$_3$OD) 170.2 (1C, Q), 160.5 (1C, Q), 130.9 (2C, CH), 128.9 (1C, Q), 115.5 (2C, CH), 74.3 (1C, CH), 65.9 (1C, CH), 55.6 (1C, CH$_3$), 44.2 (1C, CH$_2$), 32.6 (1C, CH$_2$), 31.8 (1C, CH$_2$), 20.7 (1C, CH$_3$); *m/z* (FAB, THIOG) 499 ([2M+H]$^+$, 22.1%), 250 ([M+H]$^+$, 98%), 190 (70), 121 (74), 91 (43); **HRMS** (FAB, NOBA) (Found: [M+H]$^+$, 250.1442. C$_{14}$H$_{20}$NO$_3$ requires *m/z*, 250.1443).

All spectroscopic data was in good agreement with that of the literature.[254]

(2R,3R)-3-Acetoxy -2-(4'-methoxybenzyl)-1-prop-2-ynyl-pyrrolidine 139
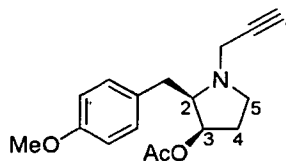
To a solution of the amine **138** (54.4 mg, 150 μmol) in DCM (4 ml) at 0 °C was added piperidinomethyl resin (173 mg, 450 μmol) and propargyl bromide (65.0 μl, 300 μmol), the solution was stirred for 6 h at RT. The reaction was filtered through a cotton wool plug, washing with DCM (15 ml). The organics were concentrated *in vacuo* to give an oil. The residue was chromatographed on silica gel [3% MeOH/DCM] to give **139** as an oil (40.5 mg, 140 μmol, 93%); R_f [5% MeOH/DCM] = 0.44; $[\alpha]_D$ -1.05 (*c* 0.95, CHCl$_3$); υ_{max} (neat)/cm^{-1} 3282, 2961, 1733, 1612, 1512; ^{1}**H NMR** δ (360 MHz, CDCl$_3$) 7.07 (2H, d, *J* 8.6, Ar*H*), 6.81 (2H, d, *J* 8.6, Ar*H*), 4.92 (1H, ddd, *J* 6.7, 4.6, 2.0, C$_3$*H*), 3.78 (3H, s, O*Me*), 3.61 (2H, dd, *J* 2.3, 1.1, HC≡CC*H$_2$*), 3.10 (1H, ddd, *J* 12.8, 9.1, 3.7, C$_5$*H$_M$*H$_N$), 2.96-2.88 (1H, m, C$_2$*H*), 2.86-2.81 (1H, m, C*H$_S$*H$_T$Ar), 2.74-2.64 (2H, m, CH$_S$*H$_T$*Ar, C$_5$H$_M$*H$_N$*), 2.26 (1H, t, *J* 2.3, *H*C≡CCH$_2$), 2.25-2.15 (1H, m, C$_4$*H$_E$*H$_F$), 2.12 (3H, s, O*Ac*), 1.81-1.72 (1H, m, C$_4$H$_E$*H$_F$*); ^{13}**C NMR** δ (90.7 MHz, CDCl$_3$) 170.7 (1C, Q), 158.2 (1C, Q), 130.6 (1C, Q), 129.9 (2C, CH), 114.0 (2C, CH), 77.4 (1C, Q), 74.7 (1C, CH), 74.0 (1C, CH), 65.7 (1C, CH), 55.4 (1C, CH$_3$), 50.2 (1C, CH$_2$), 40.2 (1C, CH$_2$), 32.5 (1C, CH$_2$), 30.7 (1C, CH$_2$), 21.4 (1C, CH$_3$); *m/z* (FAB, NOBA) 288 ([M+H]$^+$, 59%), 228 (48), 121 (66), 106 (54), 91 (51); **HRMS** (FAB, NOBA) (Found: [M+H]$^+$, 288.1604. C$_{17}$H$_{22}$NO$_3$ requires *m/z*, 288.1600).
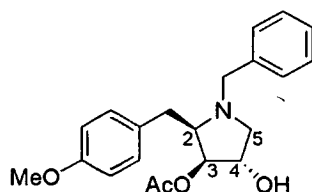
**(2*R*,3*S*,4*S*)-3-Acetoxy-4-hydroxy-2-(4'-methoxybenzyl)-1-prop-2-ynyl-pyrrolidine 140**
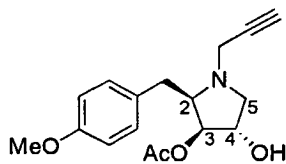
To a solution of anisomycin **102** (20.0 mg, 80.0 µmol) in DMF (2 ml) was added potassium carbonate (11.0 mg, 0.08 mmol) and propargyl bromide (12.0 µl, 0. 80.0 µmol), the solution was stirred at RT for 9 h. The solution was then concentrated *in vacuo*, and the residue was purified by flash chromatography [5% MeOH/DCM] to give **140** as an oil (22.0 mg, 70.0 µmol, 95%); R_f [5% MeOH/DCM] = 0.22; $[\alpha]_D$ -116 (*c* 0.90, CHCl$_3$); υ_{max} (neat)/cm^{-1} 3305, 3019, 1726, 1613, 1513; ^{1}**H NMR** δ (360 MHz, CDCl$_3$) 7.10 (2H, d, *J* 8.6, Ar*H*), 6.81 (2H, d, *J* 8.6, Ar*H*), 4.47 (1H, dd, *J* 5.6, 1.5, C$_3$*H*), 4.13 (1H, td, *J* 6.8, 1.5, C$_4$*H*), 3.79 (3H, s, O*Me*), 3.58 (2H, t, *J* 2.3, HC≡CC*H$_2$*), 3.36 (1H, dd, *J* 9.9, 7.1 C$_5$*H$_M$H$_N$*), 3.22 (1H, qn, *J* 5.2, C$_2$*H*), 2.86 (1H, dd, *J* 13.3, 4.7, C*H$_S$*H$_T$Ar), 2.71 (1H, dd, *J* 13.3, 10.3, CH$_S$*H$_T$*Ar), 2.62 (1H, dd, *J* 9.9, 6.7, C$_5$H$_M$*H$_N$*), 2.32 (1H, t, *J* 2.3, *H*C≡CCH$_2$), 2.14 (3H, s, O*Ac*); ^{13}**C NMR** δ (90.7 MHz, CDCl$_3$) 172.0 (1C, Q), 158.2 (1C, Q), 130.2 (1C, Q), 130.0 (2C, CH), 114.0 (2C, CH), 82.5 (1C, CH), 76.7 (1C, Q), 75.7 (1C, CH), 74.5 (1C, CH), 63.6 (1C, CH), 58.0 (1C, CH$_2$), 55.4 (1C, CH$_3$), 40.0 (1C, CH$_2$), 32.1 (1C, CH$_2$), 21.3 (1C, CH$_3$); *m/z* (FAB, THIOG) 304 ([M+H]$^+$, 67%), 244 (42), 121 (100), 91 (88); **HRMS** (FAB, THIOG) (Found: [M+H]$^+$, 304.1540. C$_{17}$H$_{22}$NO$_4$ requires *m/z*, 304.1549).

(2*R*,5*R*,4*S*)-3-Acetoxy-1-benzyl-4-hydroxy-2-(4'-methoxybenzyl)-pyrrolidine 141

To a solution of anisomycin **102** (20.0 mg, 80.0 µmol) in DMF (1 ml) was added potassium carbonate (11.0 mg, 80.0 µmol) and benzyl bromide (10.0 µl, 80.0 µmol), the solution was stirred at RT for 4 h. The solution was then concentrated *in vacuo*, and the residual colourless solid was purified by flash chromatography [4% MeOH/ DCM] to give **141** as an oil (25.0 mg, 70.0 µmol, 89%); R_f [5% MeOH/DCM] = 0.33; $[\alpha]_D$ -98.0 (*c* 1.00, CHCl$_3$); υ_{max} (neat)/cm^{-1} 3399, 3053, 1730, 1612, 1512; ^{1}H NMR δ (360 MHz, CD$_3$OD) 7.42-7.35 (5H, m, Ar*H*), 7.14 (2H, d, *J* 8.4, Ar*H*), 6.87 (2H, d, *J* 8.4, Ar*H*), 4.81 (1H, br d, *J* 3.0, C$_3$*H*), 4.15 (1H, d, *J* 12.6, NC*H*$_X$H$_Y$Ph), 4.11-4.09 (1H, m, C$_4$*H*), 3.79 (3H, s, O*Me*), 3.67 (1H, d, *J* 12.6, NCH$_X$*H*$_Y$Ph), (1H, m, C$_2$*H*), 3.36-3.34 (1H, m, C$_5$*H*$_M$H$_N$), 2.91 (1H, dd, *J* 13.5, 5.3, C*H*$_S$H$_T$Ar), 2.83 (1H, dd, *J* 13.5, 9.8, CH$_S$*H*$_T$Ar), 2.58-2.56 (1H, m, C$_5$H$_M$*H*$_N$), 2.14 (3H, s, O*Ac*); ^{13}C NMR δ (90.7 MHz, CD$_3$OD) 171.8 (1C, Q), 160.9 (1C, Q), 131.2 (1C, Q), 130.9 (2C, CH), 130.8 (1C, Q), 130.7 (2C, CH), 129.6 (2C, CH), 129.0 (1C, CH), 115.0 (2C, CH), 80.8 (1C, CH), 74.5 (1C, CH), 68.7 (1C, CH), 60.8 (1C, CH$_2$), 60.5 (1C, CH$_2$), 55.6 (1C, CH$_3$), 33.7 (1C, CH$_2$), 20.9 (1C, CH$_3$); *m/z* (FAB, THIOG) 356 ([M+H]$^+$, 90%), 296 (63), 91 (95); **HRMS** (FAB, NOBA) (Found: [M+H]$^+$, 356.1861. C$_{21}$H$_{26}$NO$_4$ requires *m/z*, 356.1862).

2-Azido-1-ethylamine[319] 187

$$H_2N \overset{1}{\frown} \overset{2}{\smile} N_3$$
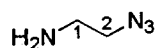
To a solution of 2-chloro-1-ethylamine **186** (500 mg, 4.31 mmol) in water (5 ml) was added sodium azide (840 mg, 12.9 mmol) and the reaction mixture heated at 80 °C for 15 h. The solution was basified with KOH (solid) and extracted with ether. The organics were dried and concentrated to give **187** as a volatile colourless oil (371 mg, 4.31 mmol, 100%); v_{max} (neat)/cm^{-1} 3375, 2104; **^{1}H NMR** δ (360 MHz, CDCl$_3$) 3.30 (2H, t, *J* 5.7, C*H$_2$*), 2.79-2.74 (2H, m, C*H$_2$*), 1.43 (2H, s, N*H$_2$*); **^{13}C NMR** δ (90.7 MHz, CDCl$_3$) 54.2 (1C, CH$_2$), 40.9 (1C, CH$_2$); ***m/z*** (ESI+) 194 ([2M+H]$^+$).

All spectroscopic data was in good agreement with that of the literature.[319]

Safety in the Handling of Sodium Azide and other Azides:[394] Sodium azide is toxic (LD$_{50}$ oral (rats) = 27 mgkg^{-1}) and can be absorbed through the skin. It decomposes explosively upon heating to above 275 °C; hence its use in airbags in the automotive industry.

Sodium azide reacts vigorously with CS$_2$, bromine, nitric acid, dimethyl sulfate, and a series of heavy metals, including copper and lead. In reaction with water or Brønsted acids the highly toxic and explosive hydrogen azide is released. It has been reported that sodium azide and polymer-bound azide reagents form explosive di- and triazidomethane with CH$_2$Cl$_2$ and CHCl$_3$, respectively.

Heavy-metal azides that are highly explosive under pressure or shock are formed when solutions of NaN$_3$ or HN$_3$ vapours come into contact with heavy metals or their salts. Heavy-metal azides can accumulate under certain circumstances, for example, in metal pipelines and on the metal components of diverse equipment (rotary evaporators, freeze drying equipment, cooling traps, water baths, waste pipes), and thus lead to violent explosions. Some organic and other covalent azides are classified as toxic and highly explosive, and appropriate safety measures must be taken at all times.

2-Azido-1-*N*-dansyl-ethylamine 189
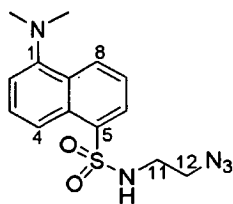
To a solution of 2-azido-1-ethylamine **187** (371 mg, 4.31 mmol) in DCM (7 ml) was added dansyl chloride **188** (225 mg, 840 μmol) and the reaction mixture was stirred for 2 h at RT. The solution was concentrated *in vacuo*, and the residue was purified by flash chromatography [25% EtOAc/hexane] to give **189** as a pale yellow oil (260 mg, 820 μmol, 98%); R_f [30% EtOAc/hexane] = 0.27; λ_{max} (H_2O)/nm 345; υ_{max} (neat)/cm^{-1} 3301, 2942, 2103, 1318; **^{1}H NMR** δ (360 MHz, CDCl$_3$) 8.57 (1H, br d, *J* 8.5, C$_6$*H*), 8.29 (1H, br d, *J* 8.6, C$_4$*H*), 8.26 (1H, dd, *J* 7.3, 1.3, C$_8$*H*), 7.59 (1H, dd, *J* 8.6, 7.6, C$_3$*H*), 7.53 (1H, dd, *J* 8.5, 7.3 C$_7$*H*), 7.20 (1H, br d, *J* 7.6, C$_2$*H*), 5.28 (1H, t, *J* 5.9, N$_{10}$*H*), 3.30 (2H, t, *J* 5.7, C$_{12}$*H$_2$*), 3.06 (2H, q, *J* 6.1, C$_{11}$*H$_2$*), 2.90 (6H, s, N*Me$_2$*); **^{13}C NMR** δ (90.7 MHz, CDCl$_3$) 152.1 (1C, *Ar*, Q), 134.6 (1C, *Ar*, Q), 130.9 (1C, C$_6$H, CH), 130.0 (1C, *Ar*, Q), 129.7 (1C, C$_8$H, CH), 129.6 (1C, *Ar*, Q), 128.8 (1C, C$_3$H, CH), 123.3 (1C, C$_7$H, CH), 118.7 (1C, C$_4$H, CH), 115.5 (1C, C$_2$H, CH), 51.0 (1C, *C$_{12}$*H$_2$, CH$_2$), 45.5 (2C, N*Me$_2$*, CH$_3$), 42.5 (1C, C$_{11}$*H$_2$*, CH$_2$); ***m/z*** (FAB, NOBA) 319 ([M]$^+$, 90%), 170 (100); **HRMS** (FAB, THIOG) (Found: [M+H]$^+$, 320.1184. C$_{14}$H$_{18}$N$_5$O$_2$S requires *m/z*, 320.1181).

O-Linked Boc C(4)-H anisomycin dansyl molecular probe 190a
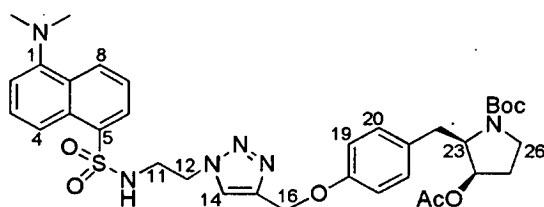
To propargyl ether **135** (45.0 mg, 120 μmol) in 'BuOH:H$_2$O (3 ml, 1:1) was added dansyl azide **189** (40.0 mg, 130 μmol), followed by copper(II) sulfate (3.0 mg, 10 mol%) and sodium ascorbate solution (22.0 μl, 1 M solution, 20 mol%). After 4 hours stirring at RT the reaction was complete by tlc. The solution was concentrated *in vacuo*, and the residue was purified by flash chromatography [60% to 100% EtOAc/hexane] to give **190a** as a foam (68.0 mg, 92.0 μmol, 76%); **R$_f$** [40%EtOAc/hexane] = 0.11; ^{1}**H NMR** δ (360 MHz, CDCl$_3$, 323 K) 8.58 (1H, br d, *J* 8.5, Ar*H*), 8.24 (2H, d, *J* 7.6, Ar*H*), 7.27 (2H, t, *J* 7.5, Ar*H*); 7.46 (1H, br s, C$_{14}$*H*), 7.19(1H, d, *J* 7.5, Ar*H*), 7.11 (2H, d, *J* 8.5, Ar*H*), 6.87 (2H, d, *J* 8.5, Ar*H*), 5.68 (1H, t, *J* 3.8, N$_{10}$*H*), 5.14-5.08 (3H, m, C$_{16}$*H$_2$*, C$_{24}$*H*), 4.38 (2H, t, *J* 5.6, C$_{12}$*H$_2$*), 4.29-4.23 (1H, m, C$_{23}$*H*), 3.49-3.42 (3H, m, C$_{26}$*H$_M$*H$_N$, C$_{11}$*H$_2$*), 3.36-3.29 (1H, m, C$_{26}$H$_M$*H$_N$*), 3.00-2.91 (1H, m, C$_{22}$*H$_S$*H$_T$), 2.90 (6H, s, N*Me$_2$*), 2.80 (1H, dd, *J* 13.7, 8.5, C$_{22}$H$_S$*H$_T$*), 2.10-2.01 (1H, m, C$_{25}$*H$_E$*H$_F$), 1.95 (3H, s, O*Ac*), 1.87-1.75 (1H, m, C$_{25}$H$_E$*H$_F$*), 1.45 (9H, s, '*Bu*O); ^{13}**C NMR** δ (90.7 MHz, CDCl$_3$, 323 K) 172.1 (1C, *C*O'Bu, Q), 170.3 (1C, O*Ac*, Q), 156.9 (1C, *Ar*, Q), 152.1 (1C, *Ar*, Q), 144.4 (1C, *C$_{15}$*, Q), 134.9 (1C, *Ar*, Q), 131.6 (1C, *Ar*, Q), 130.9 (1C, *Ar*H, CH), 130.6 (2C, *Ar*H, CH), 130.2 (1C, *Ar*, Q), 129.8 (1C, *Ar*, Q), 129.7 (1C, *Ar*H, CH), 128.8 (1C, Ar*H*, CH), 124.2 (1C, *C$_{14}$*H, CH), 123.4 (1C, Ar*H*, CH), 119.1 (1C, Ar*H*, CH), 115.7 (1C, Ar*H*, CH), 115.2 (2C, Ar*H*, CH), 79.9 (1C, '*Bu*, Q), 73.5 (1C, *C$_{24}$*H, CH), 62.2 (1C, *C$_{16}$*H$_2$, CH$_2$), 59.5 (1C, *C$_{23}$*H, CH), 50.3 (1C, *C$_{12}$*H$_2$, CH$_2$), 45.6 (2C, N*Me$_2$*, CH$_3$), 43.1 (1C, *C$_{26}$*H$_2$, CH$_2$), 42.9 (1C, *C$_{11}$*H$_2$, CH$_2$), 34.4 (1C, *C$_{22}$*H$_2$, CH$_2$), 28.7 (3C, '*Bu*O,CH$_3$), 28.7 (1C, *C$_{25}$*H$_2$, CH$_2$), 20.9 (1C, O*Ac*, CH$_3$); ***m/z*** (FAB, NOBA) 693 ([M+H]$^+$, 35%), 91(56); **HRMS** (FAB, NOBA) (Found: [M+H]$^+$, 693.3071. C$_{35}$H$_{45}$N$_6$O$_7$S requires *m/z*, 693.3071).

O-Linked C(4)-H anisomycin dansyl molecular probe 190
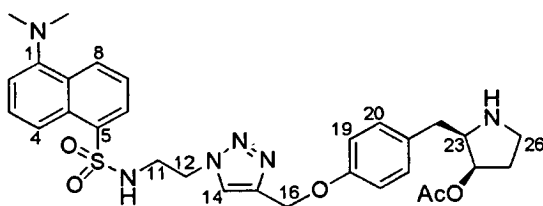
**Method 1:** To propargyl ether **136** (32.6 mg, 120 μmol) in 'BuOH:H_2O (3 ml, 1:1) was added dansyl azide **189** (40.0 mg, 130 μmol), followed by copper(II) sulfate (3.0 mg, 10 mol%) and sodium ascorbate solution (22.0 μl, 1 M solution, 20 mol%). After 4 hours stirring at RT the reaction was complete by tlc. The solution was concentrated *in vacuo*, and the residue was purified by flash chromatography [5% to 10% MeOH/ DCM] to give **190** as a foam (54.5 mg, 90.0 μmol, 77%).

Method 2: To a solution of carbamate **190a** (68.0 mg, 92.0 μmol) in DCM (5 ml) was added trifluoroacetic acid (500 μl), the solution was stirred for 4 h at RT. The reaction was concentrated *in vacuo* to give **190** as an oil (63.0 mg, 89.0 μmol, 96%); **R_f** [5%MeOH/DCM] = 0.24; λ_{max} (H_2O)/nm 345; **^{1}H NMR** δ (360 MHz, DMSO) 8.47 (1H, br d, *J* 8.5, C_6H), 8.27 (1H, t, *J* 5.8, N_9H), 8.22 (1H, br d, *J* 8.7, C_4H), 8.26 (1H, d, *J* 7.9, C_8H), 8.06 (1H, s, $C_{14}H$), 7.65-7.56 (2H, m, C_3H, C_7H), 7.27 (1H, d, *J* 7.4, C_2H), 7.20 (2H, d, *J* 8.6, Ar*H*), 7.00 (2H, d, *J* 8.6, Ar*H*), 5.16 (1H, t, *J* 3.8, $C_{24}H$), 5.03 (2H, s, $C_{16}H_2$), 4.41 (2H, t, *J* 5.9, $C_{12}H_2$), 3.89-3.81 (1H, m, $C_{23}H$), 3.34-3.24 (2H, m, $C_{26}H_2$), 3.26 (2H, br q, *J* 5.8, $C_{11}H_2$), 2.98-2.88 (2H, m, $C_{22}H_2$), 2.84 (6H, s, N*Me₂*), 2.30-2.19 (1H, m, $C_{25}H_EH_F$), 2.12 (3H, s, O*Ac*), 2.05-1.98 (1H, m, $C_{25}H_EH_F$); **^{13}C NMR** δ (90.7 MHz, DMSO) 169.7 (1C, O*Ac*, Q), 157.2 (1C, *Ar*, Q), 151.2 (1C, *Ar*, Q), 142.5 (1C, *C₁₅*, Q), 135.5 (1C, *Ar*, Q), 130.1 (2C, *Ar*H, CH), 129.7 (1C, *Ar*H, CH), 129.1 (1C, *Ar*, Q), 129.0 (1C, *Ar*, Q), 128.8 (1C, *Ar*, Q), 128.5 (1C, Ar*H*, CH), 128.1 (1C, Ar*H*, CH), 125.0 (1C, *C₁₄*H, CH), 123.8 (1C, Ar*H*, CH), 119.3 (1C, Ar*H*, CH), 115.4 (1C, Ar*H*, CH), 114.9 (2C, *Ar*H, CH), 72.9 (1C, *C₂₄*H, CH), 63.5 (1C, *C₂₃*H, CH), 61.1 (1C, *C₁₆*H₂, CH₂), 49.4 (1C, *C₁₁*H₂, CH₂), 45.2 (2C, N*Me₂*, CH₃), 42.8 and 42.5 (2C, *C₁₂*H₂ + *C₂₆*H₂, CH₂), 31.1 (1C, *C₂₂*H₂, CH₂), 30.6 (1C, *C₂₅*H₂, CH₂), 20.9 (1C, O*Ac*, CH₃); **m/z** (FAB, NOBA) 593 ([M+H]$^+$, 43%), 91(52); **HRMS** (FAB, NOBA) (Found: [M+H]$^+$, 593.2548. $C_{30}H_{37}N_6O_5S$ requires *m/z*, 593.2546).

N-Linked anisomycin dansyl molecular probe 191
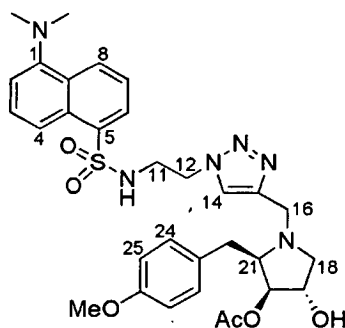
To propargyl amine **140** (27.0 mg, 90.0 μmol) in ^{t}BuOH:H$_2$O (2 ml, 1:1) was added dansyl azide **189** (26.0 mg, 80.0 μmol), followed by copper(II) sulfate (2.0 mg, 10 mol%) and sodium ascorbate solution (20.0 μl, 1 M solution, 20 mol%). After 5 hours the reaction was complete by tlc. The solution was concentrated *in vacuo*, and the residue was purified by flash chromatography [5% to 10% MeOH/DCM] to give **191** as a foam (40.0 mg, 60.0 μmol, 71%); **R$_f$** [10%MeOH/DCM] = 0.34; λ_{max} (H$_2$O)/nm 345; **^{1}H NMR** δ (360 Hz, CD$_3$CN) 8.54 (1H, d, *J* 8.5, Ar*H*), 8.19 (1H, d, *J* 8.7, Ar*H*), 8.16 (1H, dd, *J* 7.3, 1.3 Ar*H*), 7.59-7.54 (3H, m, 2Ar*H*, C$_{14}$*H*), 7.25 (1H, d, *J* 7.6, Ar*H*), 7.21 (2H, d, *J* 8.5, Ar*H*), 6.87 (2H, d, *J* 8.5, Ar*H*), 4.66 (1H, br s, C$_{20}$*H*), 4.35 (2H, q, *J* 5.0, C$_{11}$*H$_2$*), 4.03 (1H, br s, C$_{19}$*H*), 3.97 (1H, br d, *J* 13.6, C$_{16}$*H$_X$*H$_Y$), 3.86-3.84 (1H, m, C$_{16}$H$_X$*H$_Y$*), 3.76 (3H, s, O*Me*), 3.56-3.54 (1H, m, C$_{21}$*H*), 3.47-3.43 (1H, m, C$_{18}$*H$_M$*H$_N$), 3.30 (2H, br t, *J* 6.1, C$_{12}$*H$_2$*), 3.05 (1H, dd, *J* 13.4, 5.7, C$_{22}$H$_S$H$_T$), 2.86 (6H, s, N*Me$_2$*), 2.85 (1H, m, C$_{18}$H$_M$*H$_N$*), 2.84 (1H, dd, *J* 7.2, 5.7, C$_{22}$H$_S$*H$_T$*), 2.07 (3H, s, O*Ac*); **^{13}C NMR** δ (90.7 MHz, CD$_3$CN) 170.9 (1C, OAc, Q), 159.4 (1C, Ar, Q), 152.6 (1C, *Ar*, Q), 141.6 (1C, *C$_{14}$*, Q), 136.1 (1C, *Ar*, Q), 131.2 (2C, *Ar*H, CH), 130.6 (1C, *Ar*, Q), 130.3 (1C, *Ar*, Q), 130.2 (1C, *Ar*, Q), 130.1(1C, *Ar*, Q), 130.0 (1C, ArH, CH), 129.2 (1C, ArH, CH), 126.4 (1C, ArH, CH), 124.4 (1C, ArH, CH), 119.9 (1C, ArH, CH), 116.2 (1C, ArH, CH), 114.9 (2C, *Ar*H, CH), 79.7 (1C, *C$_{20}$*H, CH), 73.9 (1C, *C$_{19}$*H, CH), 67.7 (1C, *C$_{21}$*H, CH), 59.6 (1C, *C$_{18}$*H$_2$, CH$_2$), 55.8 (1C, O*Me*, CH$_3$), 50.9 (1C, *C$_{11}$*H$_2$, CH$_2$), 49.0 (1C, *C$_{16}$*H$_2$, CH$_2$), 45.7 (2C, N*Me$_2$*, CH$_3$), 43.6 (1C, *C$_{12}$*H$_2$, CH$_2$), 32.2 (1C, *C$_{22}$*H$_2$, CH$_2$), 21.1 (1C, O*Ac*, CH$_3$); *m/z* (FAB, NOBA) 623 ([M+H]$^+$, 60%), 501 (46%), 91 (68%); **HRMS** (FAB, NOBA) (Found: [M+H]$^+$, 623.2655. C$_{31}$H$_{39}$N$_6$O$_6$S requires *m/z*, 623.2652).

N-Biotinyl-2-azido-1-ethylamine 193
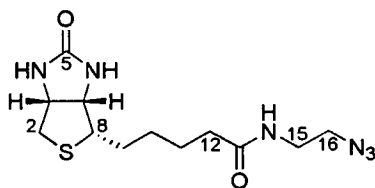
To a solution of biotin-NHS **192** (60.0 mg, 170 μmol) in methanol (2 ml) was added triethylamine (73.0 μl, 530 μmol) and 1-azido-ethylamine **187** (90.0 mg, 1.06 mmol), and the solution stirred at RT for 6 h. The solution was concentrated *in vacuo*, and the residue was purified by flash chromatography [8% to 12 % MeOH/DCM] to give **193** as a colourless solid (48.0 mg, 160 μmol, 94 %); R_f [8% MeOH/DCM] = 0.19; υ_{max} (neat)/cm^{-1} 3286, 2103, 1694, 1651; ^{1}**H NMR** δ (360 MHz, DMSO) 8.06 (1H, t, *J* 5.8, $N_{14}H$), 6.44 (1H, br s, N_6H), 6.38 (1H, br s, N_4H), 4.31-4.28 (1H, m, C_3H), 4.16-4.12 (1H, m, C_7H), 3.32 (2H, t, *J* 5.8, $C_{16}H_2$), 3.23 (2H, t, *J* 5.8, $C_{15}H_2$), 3.09-3.07 (1H, m, C_8H), 2.87 (1H, dd, *J* 12.5, 5.1, $C_2H_AH_B$), 2.56 (1H, d, *J* 12.5, $C_2H_AH_B$), 2.07 (2H, t, *J* 7.4, $C_{12}H_2$), 1.52-1.28 (6H, m, C_9H_2, $C_{10}H_2$, $C_{11}H_2$); ^{13}**C NMR** δ (90.7 MHz, DMSO) 172.7 (1C, C_{13}, Q), 163.0 (1C, C_5, Q), 61.2 (1C, C_7, CH), 59.4 (1C, C_3, CH), 55.7 (1C, C_8, CH), 50.2 (1C, C_{16}, CH$_2$), 40.1 (1C, C_2, CH$_2$), 38.4 (1C, C_{15}, CH$_2$), 35.4 (1C, C_{12}, CH$_2$), 28.4 (1C, C_{10}, CH$_2$), 28.3 (1C, C_9, CH$_2$), 25.4 (1C, C_{11}, CH$_2$); *m/z* (FAB, THIOG) 313 ([M+H]$^+$, 48%), 45 (100); **HRMS** (FAB, THIOG) (Found: [M+H]$^+$, 313.1445. $C_{12}H_{21}N_6O_2S$ requires *m/z*, 313.1447).

Acetic acid 2-(4-propargyl-phenyl)-ethyl ester 195
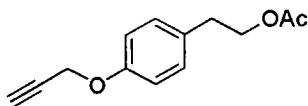
To a solution of phenol **194** (80.0 mg, 440 μmol) in DMF (4 ml) was added potassium carbonate (125 mg, 0.88 mmol) and propargyl bromide (200 μl, 1.60 mmol), the solution was stirred at RT for 30 h. The reaction was quenched by addition of water (10 ml) and diluted with ether (20 ml). The organic phase was separated and the aqueous phase extracted with ether (3 x 20 ml). The combined organics were washed with brine (10 ml), dried (Na_2SO_4) and concentrated *in vacuo*. The product **195** was obtained as an oil (75.0 mg, 340 μmol, 77%); R_f [20% EtOAc/ hexane] = 0.43; υ_{max} (neat)/cm^{-1} 3286, 1735, 1610, 1510; **^{1}H NMR** δ (360 MHz, CDCl$_3$) 7.16 (2H, d, *J* 8.7, Ar*H*), 6.92 (2H, d, *J* 8.7, Ar*H*), 4.67 (2H, d, *J* 2.4, HC≡CC*H$_2$*), 4.24 (2H, t, *J* 7.1, C*H$_2$*), 2.89 (2H, t, *J* 7.1, C*H$_2$*), 2.53 (1H, t, *J* 2.4, *H*C≡CCH$_2$), 2.04 (3H, s, O*Ac*); **^{13}C NMR** δ (90.7 MHz, CDCl$_3$) 170.9 (1C, Q), 156.0 (1C, Q), 130.6 (1C, Q), 129.7 (2C, CH), 114.7 (2C, CH), 78.4 (1C, Q), 75.3 (1C, CH), 64.9 (1C, CH$_2$), 55.6 (1C, CH$_2$), 34.0 (1C, CH$_2$), 20.8 (1C, CH$_3$); *m/z* (FAB, THIOG) 219 ([M+H]$^+$, 20%), 159 (79), 120 (23), 91 (92); **HRMS** (FAB, THIOG) (Found: [M+H]$^+$, 219.1023. C$_{13}$H$_{15}$O$_3$ requires *m/z*, 219.1021).

1-(*N*-Biotinyl-2-aminoeth-1-yl)-4-[4'-(2''-acetoxyeth-1''-yl)phenyloxymethyl]-1*H*-[1,2,3] triazole 196
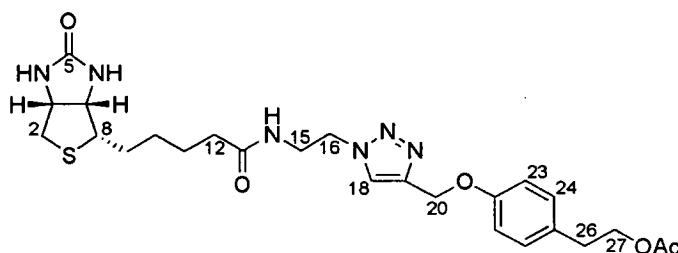

To biotinylated azide **193** (30.0 mg, 90.0 µmol) in H_2O:*t*BuOH (2 ml, 1:1) was added **195** (24.0 mg, 100 µmol), followed by copper(II) sulfate (2.00 mg, 10 mol%) and sodium ascorbate solution (20.0 µl, 1 M solution, 20 mol%). The solution was stirred for 15 h at RT. The solution was concentrated *in vacuo*, and the residue was purified by flash chromatography [8% to 15% MeOH/DCM] to give **196** as a colourless solid (41.0 mg, 77.4 µmol, 86%); R_f [20% MeOH/DCM] = 0.30; ^{1}**H NMR** δ (600 MHz, DMSO) 8.18 (1H, s, $C_{18}H$), 8.00 (1H, t, *J* 5.9, $N_{14}H$), 7.17 (2H, d, *J* 8.6, Ar*H*), 6.96 (2H, d, *J* 8.6, Ar*H*), 6.44 (1H, br s, N_6H), 6.38 (1H, br s, N_4H), 5.09 (2H, s, $C_{20}H_2$), 4.42 (2H, t, *J* 5.9, $C_{16}H_2$), 4.28-4.27 (1H, m, C_3H), 4.16 (2H, t, *J* 7.0, $C_{27}H_2$), 4.14-4.09 (1H, m, C_7H), 3.48 (2H, br q, *J* 5.9, $C_{15}H_2$), 3.10-3.05 (1H, m, C_8H), 2.81 (2H, t, *J* 7.0, $C_{26}H_2$), 2.79 (1H, dd, *J* 12.2, 5.2, $C_2H_AH_B$), 2.54 (1H, d, *J* 12.2, $C_2H_AH_B$), 2.03 (2H, t, *J* 7.4, $C_{12}H_2$), 1.98 (3H, s, O*Ac*), 1.56-0.86 (6H, m, C_9H_2, $C_{10}H_2$, $C_{11}H_2$); ^{13}**C NMR** δ (151.1 MHz, DMSO) 173.5 (1C, C_{13}, Q), 171.2 (1C, C_{29}, Q), 163.6 (1C, C_5, Q), 157.6 (1C, *Ar*, Q), 143.4 (1C, C_{19}, Q), 131.0 (1C, *Ar*, Q), 130.6 (2C, *Ar*H, CH), 125.6 (1H, C_{18}, CH), 115.5 (2C, *Ar*H, CH), 65.6 (1C, C_{27}, CH_2), 62.1 (1C, C_{20}, CH_2), 61.9 (1C, C_7, CH), 60.2 (1C, C_3, CH), 56.3 (1C, C_8, CH), 49.8 (1C, C_{16}, CH_2), 40.8 (1C, C_2, CH_2), 38.7 (1C, C_{15}, CH_2), 36.0 (1C, C_{12}, CH_2), 34.4 (1C, C_{26}, CH_2), 29.3 (1C, C_{10}, CH_2), 28.0 (1C, C_9, CH_2), 26.0 (1C, C_{11}, CH_2), 21.6 (1C, C_{30}, CH_3); ***m/z*** (FAB, THIOG) 531 ([M+H]$^+$, 27%), 270 (22) 227 (16), 45 (100); **HRMS** (FAB, THIOG) (Found: [M+H]$^+$, 531.2390. $C_{25}H_{35}N_6O_5S$ requires *m/z*, 531.2387).

C$_2$-Click *N*-linked anisomycin biotin molecular probe 197
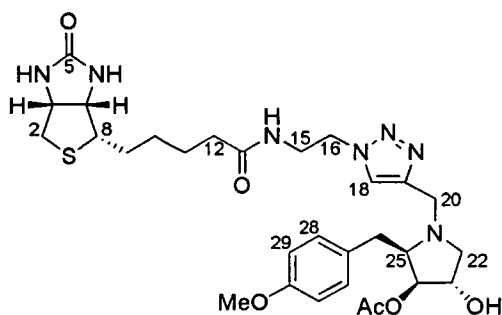
To biotinylated azide **193** (27.0 mg, 90.0 µmol) in H$_2$O:^{t}BuOH (2 ml, 1:1) was added *N*-propargyl anisomycin **140** (17.0 mg, 50 µmol), followed by copper(II) sulfate (2.0 mg, 10 mol%) and sodium ascorbate solution (20.0 µl, 1 M solution, 20 mol%). The solution was stirred for 15 h at RT. The solution was concentrated *in vacuo*, and the residue was purified by flash chromatography [8% to 15% MeOH/DCM] to give **197** as a colourless solid (22.0 mg, 35.5 µmol, 71%); **R$_f$** [20% MeOH/DCM] = 0.35; ^{1}**H NMR** δ (360 MHz, DMSO) 7.98 (1H, t, *J* 5.4, N$_{14}$*H*), 7.96 (1H, s, C$_{18}$*H*), 7.11 (2H, d, *J* 8.6, Ar*H*), 6.82 (2H, d, *J* 8.6, Ar*H*), 6.43 (1H, br s, N$_6$*H*), 6.38 (1H, br s, N$_4$*H*), 5.10 (1H, d, J 3.9, O*H*), 4.48 (1H, dd, *J* 4.5, 1.8, C$_{24}$*H*), 4.39 (2H, t, *J* 6.0, C$_{16}$*H$_2$*), 4.32-4.28 (1H, m, C$_3$*H*), 4.15-4.10 (1H, m, C$_7$*H*), 3.92 (1H, d, *J* 14.1, C$_{20}$H$_X$H$_Y$), 3.81-3.78 (1H, m, C$_{23}$*H*), 3.71 (3H, s, O*Me*), 3.63 (1H, d, *J* 14.1, C$_{20}$H$_X$*H$_Y$*), 3.48 (2H, br q, *J* 5.8, C$_{15}$*H$_2$*), 3.18 (1H, dd, *J* 10.3, 6.2, C$_{22}$*H$_M$*H$_N$), 3.09 (1H, ddd, *J* 8.2, 6.1, 4.5, C$_8$*H*), 3.01-2.93 (2H, m, C$_{25}$*H*, C$_{26}$*H$_S$*H$_T$), 2.81 (1H, dd, *J* 12.4, 5.0, C$_2$*H$_A$*H$_B$), 2.60-2.53 (2H, m, C$_2$H$_A$*H$_B$*, C$_{26}$H$_S$*H$_T$*), 2.24 (1H, dd, *J* 10.3, 4.5, C$_{22}$H$_M$*H$_N$*), 2.04 (3H, s, O*Ac*), 2.02 (2H, t, *J* 7.4, C$_{12}$*H$_2$*), 1.48-0.80 (6H, m, C$_9$*H$_2$*, C$_{10}$*H$_2$*, C$_{11}$*H$_2$*); ^{13}**C NMR** δ (90.7 MHz, DMSO) 173.5 (1C, *C$_{13}$*, Q), 169.7 (1C, O*Ac*, Q), 162.7 (1C, *C$_5$*, Q), 157.5 (1C, *Ar*, Q), 143.0 (1C, *C$_{19}$*, Q), 131.0 (1C, *Ar*, Q), 129.8 (2C, *Ar*H, CH), 123.8 (1C, *C$_{18}$*, CH), 113.7 (2C, *Ar*H, CH), 79.5 (1C, *C$_{24}$*, CH), 72.9 (1C, *C$_{23}$*, CH), 64.8 (1C, *C$_{25}$*, CH), 61.0 (1C, *C$_7$*, CH), 59.2 (1C, *C$_3$*, CH), 55.4 (1C, *C$_{22}$*, CH$_2$), 49.9 (2C, CH, CH$_3$, *C$_8$*, *C$_{32}$*), 48.7 (1C, *C$_{16}$*, CH$_2$), 47.0 (1C, *C$_{20}$*, CH$_2$), 39.8 (1C, *C$_2$*, CH$_2$), 38.7 (1C, *C$_{15}$*, CH$_2$), 35.0 (1C, *C$_{12}$*, CH$_2$), 32.2 (1C, *C$_{26}$*, CH$_2$), 28.1 (1C, *C$_{10}$*, CH$_2$), 28.0 (1C, *C$_9$*, CH$_2$), 25.1 (1C, *C$_{11}$*, CH$_2$), 20.8 (1C, O*Ac*, CH$_3$); *m/z* (FAB, THIOG) 615 ([M+H]$^+$, 19%); **HRMS** (FAB, THIOG) (Found: [M+H]$^+$, 616.2916. C$_{29}$H$_{42}$N$_7$O$_6$S requires *m/z*, 616.2917).

C_2-Click *O*-linked BocC(4)-H anisomycin biotin molecular probe **198a**
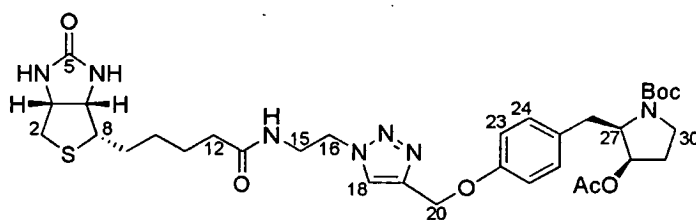
To biotinylated azide **193** (50.0 mg, 160 μmol) in H_2O:*^{t}BuOH* (3 ml, 1:1) was added *O*-propargyl analogue **135** (60.0 mg, 170 μmol), followed by copper(II) sulfate (3.5 mg, 10 mol%) and sodium ascorbate solution (37.0 μl, 1 M solution, 20 mol%). The solution was stirred for 15 h at RT. The solution was concentrated *in vacuo*, and the residue was purified by flash chromatography [5% to 15% MeOH/DCM] to give **198a** as a colourless oil (76.0 mg, 111 μmol, 70%); R_f [10% MeOH/DCM] = 0.19; **^{1}H NMR** δ (360 MHz, CDCl$_3$, 323 K) 7.71 (1H, s, C$_{18}$*H*), 7.11 (2H, d, *J* 8.4, Ar*H*), 6.89 (2H, d, *J* 8.4, Ar*H*), 5.14 (2H, s, C$_{20}$*H$_2$*), 5.10 (1H, br q, *J* 7.0, C$_{28}$*H*), 4.54-4.57 (3H, m, C$_3$*H*, C$_{16}$*H$_2$*), 4.36-4.31 (1H, m, C$_7$*H*), 4.26-4.21 (1H, m, C$_{27}$*H*), 3.70-3.76 (2H, q, *J* 5.8, C$_{15}$*H$_2$*), 3.48-3.41 (1H, m, C$_{30}$*H$_M$*H$_N$), 3.35-3.28 (1H, m, C$_{30}$H$_M$*H$_N$*), 3.16-3.11 (1H, m, C$_8$*H*), 3.03-2.96 (1H, m, C$_{26}$*H$_S$*H$_T$), 2.89 (1H, dd, *J* 13.0, 4.9, C$_2$*H$_A$*H$_B$), 2.80 (1H, dd, *J* 13.8, 8.6, C$_{26}$H$_S$*H$_T$*), 2.70 (1H, d, *J* 13.0, C$_2$H$_A$*H$_B$*), 2.18 (2H, t, *J* 6.5, C$_{12}$*H$_2$*), 2.09-2.01 (1H, m, C$_{29}$*H$_E$*H$_F$), 1.97 (3H, s, O*Ac*), 1.85-1.76 (1H, m, C$_{29}$H$_E$*H$_F$*), 1.72-1.38 (6H, m, C$_9$*H$_2$*, C$_{10}$*H$_2$*, C$_{11}$*H$_2$*), 1.45 (9H, s, *^{t}BuO*); **^{13}C NMR** δ (90.7 MHz, CDCl$_3$, 323 K) 174.3 (1C, *CO^{t}Bu*, Q), 172.7 (1C, *C$_{13}$*, Q), 170.3 (1C, O*Ac*, Q), 156.9 (1C, *C$_5$*, Q), 155.5 (1C, *Ar*, Q), 144.3 (1C, *C$_{19}$*, Q), 131.7 (1C, *Ar*, Q), 130.7 (2C, *Ar*H, CH), 124.1 (1C, *C$_{18}$*, CH), 115.1 (2C, *Ar*H, CH), 80.0 (1C, *^{t}Bu*, Q), 73.5 (1C, *C$_{28}$*, CH), 62.3 (1C, *C$_7$*, CH), 62.2 (1C, *C$_{20}$*, CH$_2$), 60.7 (1C, *C$_3$*, CH), 59.6 (1C, *C$_{27}$*, CH), 55.7 (1C, *C$_8$*, CH), 49.8 (1C, *C$_{16}$*, CH$_2$), 43.7 (1C, *C$_{30}$*, CH$_2$), 40.6 (1C, *C$_2$*, CH$_2$), 39.5 (1C, *C$_{15}$*, CH$_2$), 35.8 (1C, *C$_{12}$*, CH$_2$), 34.3 (1C, *C$_{26}$*, CH$_2$), 29.8 (1C, *C$_{29}$*, CH$_2$), 28.7 (3C, *^{t}Bu*, CH$_3$), 27.9 (1C, *C$_{10}$*, CH$_2$), 27.8 (1C, *C$_9$*, CH$_2$), 25.1 (1C, *C$_{11}$*, CH$_2$), 20.0 (1C, O*Ac*, CH$_3$); **m/z** (FAB, THIOG) 686 ([M+H]$^+$, 16%), 586 (60); **HRMS** (FAB, THIOG) (Found: [M+H]$^+$, 668.3336. C$_{33}$H$_{48}$N$_7$O$_7$S requires *m/z*, 686.3336).

C_2-Click *O*-linked C(4)-H anisomycin biotin molecular probe 198
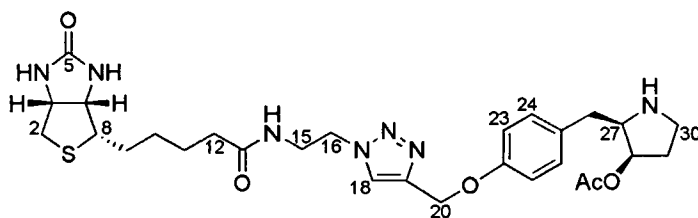
To a solution of carbamate **198a** (25.0 mg, 36.0 µmol) in DCM (2 ml) was added trifluoroacetic acid (27.0 µl, 360 µmol), the solution was stirred for 4 h at RT. The reaction was concentrated *in vacuo* to give **198** (23.0 mg, 32.9 µmol, 91%) as a colourless foam; R_f [20% MeOH/DCM] = 0.16; **^{1}H NMR** δ (360 MHz, DMSO) 8.19 (1H, s, $C_{18}H$), 8.01 (1H, t, *J* 5.6, $N_{14}H$), 7.20 (2H, d, *J* 8.6, Ar*H*), 7.02 (2H, d, *J* 8.6, Ar*H*), 6.76 (1H, br s, N_6H), 6.44 (1H, br s, N_4H), 5.16 (1H, t, *J* 3.7, $C_{28}H$), 5.10 (2H, s, $C_{20}H_2$), 4.42 (2H, t, *J* 6.0, $C_{16}H_2$), 4.31-4.28 (1H, m, C_3H), 4.13-4.10 (1H, m, C_7H), 3.87-3.84 (1H, m, $C_{27}H$), 3.49 (2H, q, *J* 5.8, $C_{15}H_2$), 3.33-3.23 (2H, m, $C_{30}H_2$), 3.11-3.06 (1H, m, C_8H), 2.98-2.85 (2H, m, $C_{26}H_2$), 2.80 (1H, dd, *J* 12.4, 5.0, $C_2H_AH_B$), 2.57 (1H, d, *J* 12.4, $C_2H_AH_B$), 2.30-2.20 (1H, m, $C_{29}H_EH_F$), 2.12 (3H, s, O*Ac*), 2.03 (2H, t, *J* 7.4, $C_{12}H_2$), 2.01-1.97 (1H, m, $C_{29}H_EH_F$), 1.64-1.21 (6H, m, C_9H_2, $C_{10}H_2$, $C_{11}H_2$); **^{13}C NMR** δ (90.7 MHz, DMSO) 172.1 (1C, C_{13}, Q), 169.3 (1C, O*Ac*, Q), 162.1 (1C, C_5, Q), 157.1 (1C, *Ar*, Q), 142.1 (1C, C_{19}, Q), 129.7 (2C, *Ar*H, CH), 128.3 (1C, *Ar*, Q), 124.5 (1C, C_{18}, CH), 114.5 (2C, *Ar*H, CH), 72.5 (1C, C_{28}, CH), 63.0 (1C, C_{27}, CH), 60.7 (1C, C_{20}, CH2), 60.7 (1C, C_7, CH), 58.9 (1C, C_3, CH), 55.1 (1C, C_8, CH), 48.7 (1C, C_{16}, CH2), 42.5 (1C, C_{30}, CH2), 39.6 (1C, C_2, CH2), 38.5 (1C, C_{15}, CH2), 34.9 (1C, C_{12}, CH2), 30.7 (1C, C_{26}, CH2), 30.1 (1C, C_{29}, CH2), 27.9 (1C, C_{10}, CH2), 27.7 (1C, C_9, CH2), 24.8 (1C, C_{11}, CH2), 20.5 (1C, O*Ac*, CH3); *m/z* (FAB, THIOG) 586 ([M+H]$^+$, 29%); **HRMS** (FAB, THIOG) (Found: [M+H]$^+$, 586.2812. $C_{28}H_{40}N_7O_5S$ requires *m/z*, 586.2812).

6-Azido-1-hexylamine[320] 201

$$H_2N \overset{1}{\diagdown} \diagup \diagdown \diagup \diagdown \overset{6}{\diagup} N_3$$
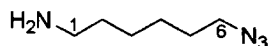
A solution of 6-amino-hexan-1-ol **199** (1.17 g, 10.0 mmol) and $SOCl_2$ (3.28 ml, 45.0 mmol) in toluene (10 ml) was heated at reflux for 1 h. The solvent was removed *in vacuo* and the intermediate 6-chloro-1-hexylamine **200** was obtained as a hygroscopic solid; *m/z* (ESI+, MeOH) 136 ([M+H]$^+$, 100), 138 ([M+H]$^+$, 33). To a solution of sodium azide (1.95 g, 30.0 mmol) in water (10 ml) was added 6-chloro-1-hexylamine **200** and the reaction was stirred at 90 °C for 2 h. The solution was basified (pH 12-14) with KOH (solid), extracted with DCM, dried ($MgSO_4$) and concentrated *in vacuo*. The product **201** was obtained as a colourless solid (1.28g, 9.01 mmol, 90% over two steps); ^{1}**H NMR** δ (360 MHz, DMSO) 3.40-3.20 (4H, m, C*H$_2$*NH$_2$, C*H$_2$*N$_3$), 1.75-1.45 (4H, m, 2CH$_2$), 1.41-1.22 (4H, m, 2CH$_2$); ^{13}**C NMR** δ (90.6 MHz, DMSO) 51.0 (1C, CH$_2$), 39.4 (1C, CH$_2$), 29.6 (1C, CH$_2$), 29.5 (1C, CH$_2$), 27.2 (1C, CH$_2$), 26.8 (1C, CH$_2$); *m/z* (ESI+, MeOH) 143.0 ([M+H]$^+$, 100%); **HRMS** (FAB, NOBA) (Found: [M+H]$^+$, 143.1297. $C_6H_{15}N_4$ requires *m/z*, 143.1297).

N-Biotinyl-6-azido-1-hexylamine 202
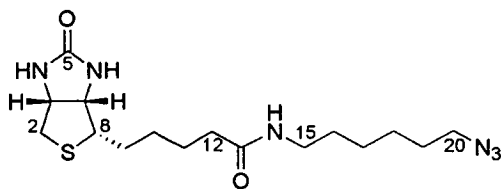
To a solution of 6-azido-1-hexylamine **201** (316 mg, 1.46 mmol) and triethylamine (202 µl, 1.46 mmol) in methanol (20 ml) was slowly added a solution of biotin-NHS **192** (500 mg, 1.46 mmol) in methanol (10 ml). The reaction was stirred for 15 h at RT. The solution was concentrated *in vacuo*, and the residue was purified by flash chromatography [5% MeOH/DCM] to give **202** as a pale yellow solid (479 mg, 1.30 mmol, 89%); R_f [10% MeOH/DCM] = 0.11; υ_{max} (neat)/cm^{-1} 3301, 2099, 1704, 1638; ^{1}**H NMR** δ (360 MHz, DMSO) 7.76 (1H, t, *J* 5.5, N$_{14}$*H*), 6.44 (1H, br s, N$_6$*H*), 6.38 (1H, br s, N$_4$*H*), 4.32-4.29 (1H, m, C$_3$*H*), 4.15-4.11 (1H, m, C$_7$*H*), 3.31 (2H, t, *J* 6.9, C$_{20}$*H$_2$*), 3.12-3.06 (1H, m, C$_8$*H*), 3.01 (2H, dt, *J* 12.5, 6.7, C$_{15}$*H$_2$*), 2.82 (1H, dd, *J* 12.5, 5.1, C$_2$*H$_A$*H$_B$), 2.58 (1H, d, *J* 12.5, C$_2$H$_A$*H$_B$*), 2.04 (2H, t, *J* 7.4, C$_{12}$*H$_2$*), 1.54-1.25 (14H, m, C$_9$*H$_2$*, C$_{10}$*H$_2$*, C$_{11}$*H$_2$*, C$_{16}$*H$_2$*, C$_{17}$*H$_2$*, C$_{18}$*H$_2$*, C$_{19}$*H$_2$*,); ^{13}**C NMR** δ (90.7 MHz, DMSO) 172.1 (1C, *C$_{13}$*, Q), 163.0 (1C, *C$_5$*, Q), 61.3 (1C, *C$_7$*, CH), 59.4 (1C, *C$_3$*, CH), 55.7 (1C, *C$_8$*, CH), 50.8 (1C, *C$_{20}$*, CH$_2$), 39.9 (1C, *C$_2$*, CH$_2$), 38.5 (1C, *C$_{15}$*, CH$_2$), 35.5 (1C, *C$_{12}$*, CH$_2$), 29.3, 28.5, 28.3, 26.2, 26.1, 25.6, 25.5 (7C, *C$_9$*, *C$_{10}$*, *C$_{11}$*, *C$_{16}$*, *C$_{17}$*, *C$_{18}$*, *C$_{19}$*, CH$_2$); ***m/z*** (FAB, THIOG) 369 ([M+H]$^+$, 58%), 258 (46); **HRMS** (FAB, THIOG) (Found: [M+H]$^+$, 369.2074. C$_{16}$H$_{29}$N$_6$O$_2$S requires *m/z*, 369.2073).

1-(*N*-Biotinyl-6-aminohex-1-yl)-4-[4'-(2''-acetoxyeth-1''-yl)phenyloxymethyl]-1*H*-[1,2,3] triazole 203
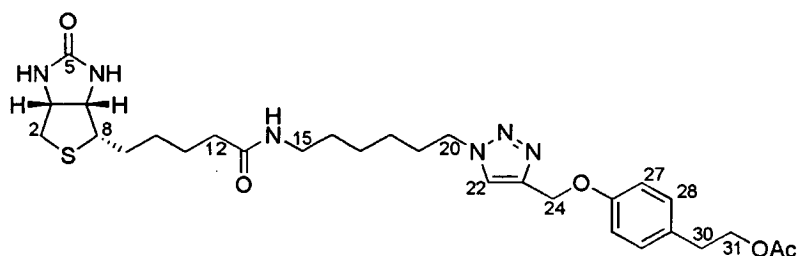
To biotinylated azide **202** (50.0 mg, 140 μmol) in H_2O:'BuOH (5 ml, 1:1) was added *O*-propargyl analogue **195** (30.0 mg, 140 μmol), followed by copper(II) sulfate (4.0 mg, 15 mol%) and sodium ascorbate solution (28.0 μl, 1 M solution, 20 mol%). The solution was stirred for 24 h at RT. The solution was concentrated *in vacuo*, and the residue was purified by flash chromatography [2% to 5% MeOH/DCM] to give **203** as a colourless solid (62.0 mg, 105 μmol, 75%); **R$_f$** [10% MeOH/DCM] = 0.08; **^{1}H NMR** δ (360 MHz, DMSO) 8.30 (1H, s, $C_{22}H$), 7.72 (1H, br s, $N_{14}H$), 7.16 (2H, d, *J* 8.5, Ar*H*), 6.95 (2H, d, *J* 8.5, Ar*H*), 6.40 (1H, br s, N_6H), 6.35 (1H, br s, N_4H), 5.09 (2H, s, $C_{24}H_2$), 4.34 (2H, t, *J* 7.1, $C_{20}H_2$), 4.32-4.30 (1H, m, C_3H), 4.15 (2H, t, *J* 6.9, $C_{31}H_2$), 4.14-4.10 (1H, m, C_7H), 3.12-3.06 (1H, m, C_8H), 3.01 (2H, dt, *J* 12.5, 6.7, $C_{15}H_2$), 2.81 (2H, t, *J* 6.9, $C_{30}H_2$), 2.80 (1H, dd, *J* 12.2, 5.1, $C_2H_AH_B$), 2.58 (1H, d, *J* 12.2, $C_2H_AH_B$), 2.04 (2H, t, *J* 7.3, $C_{12}H_2$), 1.97 (3H, s, O*Ac*), 1.80 (2H, qn, *J* 6.9, $C_{19}H_2$), 1.66-1.16 (12H, m, C_9H_2, $C_{10}H_2$, $C_{11}H_2$, $C_{16}H_2$, $C_{17}H_2$, $C_{18}H_2$); **^{13}C NMR** δ (90.7 MHz, DMSO) 171.7 (1C, C_{13}, Q), 170.1 (1C, O*Ac*, Q), 162.6 (1C, C_5, Q), 156.6 (1C, Ar, Q), 142.5 (1C, C_{23}, Q), 130.2 (1C, Ar, Q), 129.6 (2C, *Ar*H, CH), 124.2 (1C, C_{22}, CH), 114.5 (2C, *Ar*H, CH), 64.4 (1C, C_{31}, CH_2), 61.0 (1C, C_{24}, CH_2), 60.8 (1C, C_7, CH), 59.0 (1C, C_3, CH), 55.3 (1C, C_8, CH), 49.0 (1C, C_{20}, CH_2), 39.6 (1C, C_2, CH_2), 38.1 (1C, C_{15}, CH_2), 35.0 (1C, C_{12}, CH_2), 33.2 (1C, C_{30}, CH_2), 29.4 (1C, C_{19}, CH_2), 28.8, 28.0, 25.8, 25.7, 25.2, 25.1 (6C, C_9, C_{10}, C_{11}, C_{16}, C_{17}, C_{18}, CH_2), 20.5 (1C, O*Ac*, CH_3); **m/z** (FAB, THIOG) 587 ([M+H]$^+$, 18%), 91 (78); **HRMS** (FAB, THIOG) (Found: [M+H]$^+$, 587.3020. $C_{29}H_{43}N_6O_5S$ requires *m/z*, 587.3016).

C$_6$-Click *N*-linked anisomycin biotin molecular probe 204
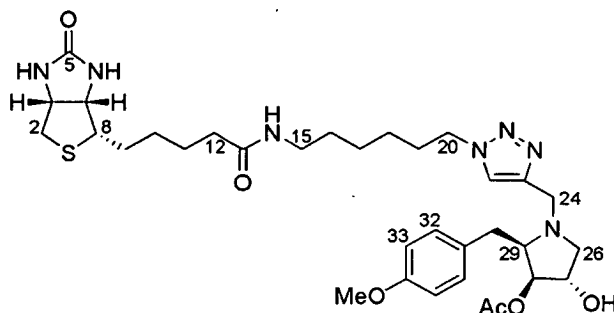
To biotinylated azide **202** (47.0 mg, 130 µmol) in H$_2$O:^{t}BuOH (3 ml, 1:1) was added *N*-propargyl anisomycin **140** (42.0 mg, 140 µmol), followed by copper(II) sulfate (3.0 mg, 10 mol%) and sodium ascorbate solution (28.0 µl, 1 M solution, 20 mol%). The solution was stirred for 15 h at RT. The solution was concentrated *in vacuo*, and the residue was purified by flash chromatography [8% to 15% MeOH/DCM] to give **204** as a colourless solid (64.0 mg, 95.0 µmol, 73%); **R$_f$** [20% MeOH/DCM] = 0.35; **^{1}H NMR** δ (360 MHz, 323 K, DMSO) 8.13 (1H, br s, C$_{22}$*H*), 7.58 (1H, t, *J* 5.6, N$_{14}$*H*), 7.09 (2H, d, *J* 8.6, Ar*H*), 6.83 (2H, d, *J* 8.6, Ar*H*), 6.26 (1H, br s, N$_6$*H*), 6.23 (1H, br s, N$_4$*H*), 5.06 (1H, br s, O*H*), 4.58 (1H, dd, *J* 4.9, 2.1, C$_{28}$*H*), 4.33 (2H, t, *J* 7.0, C$_{20}$*H$_2$*), 4.32-4.28 (1H, m, C$_3$*H*), 4.15-4.12 (1H, m, C$_7$*H*), 3.98 (1H, br d, *J* 13.9, C$_{24}$*H$_X$H$_Y$*), 3.90-3.86 (1H, m, C$_{27}$*H*), 3.75 (1H, br d, *J* 13.9, C$_{24}$H$_X$*H$_Y$*), 3.72 (3H, s, O*Me*), 3.25-3.10 (2H, m, C$_{26}$*H$_M$*H$_N$, C$_{29}$*H*), 3.08 (1H, ddd, *J* 8.1, 6.6, 4.8, C$_8$*H*), 2.98 (2H, br q, *J* 6.6, C$_{15}$*H*), 2.91 (1H, dd, *J 13.3, 4.6,* C$_{30}$H$_S$*H$_T$*), 2.83 (1H, dd, *J* 12.4, 5.1, C$_2$*H$_A$*H$_B$*), 2.60 (1H, d, *J 13.3,* C$_{30}$H$_S$*H$_T$*), 2.81 (1H, d, *J* 12.4, C$_2$H$_A$*H$_B$*), 2.41-2.32 (1H, m, C$_{26}$H$_M$*H$_N$*), 2.05 (2H, t, *J* 7.3, C$_{12}$*H$_2$*), 2.04 (3H, s, O*Ac*), 1.82 (2H, qn, *J* 7.0, C$_{19}$*H$_2$*), 1.70-1.20 (12H, m, C$_9$*H$_2$*, C$_{10}$*H$_2$*, C$_{11}$*H$_2$*,C$_{16}$*H$_2$*, C$_{17}$*H$_2$*, C$_{18}$*H$_2$*,); **^{13}C NMR** δ (90.7 MHz, DMSO) 171.8 (1C, *C$_{13}$*, Q), 169.5 (1C, O*Ac*, Q), 162.6 (1C, *C$_5$*, Q), 157.6 (1C, Ar, Q), 142.3 (1C, *C$_{23}$*, Q), 130.4 (1C, Ar, Q), 129.6 (2C, *Ar*H, CH), 123.4 (1C, *C$_{22}$*, CH), 113.5 (2C, *Ar*H, CH), 78.9 (1C, *C$_{28}$*, CH), 72.3 (1C, *C$_{27}$*, CH), 64.7 (1C, *C$_{29}$*, CH), 60.9 (1C, *C$_7$*, CH), 59.0 (1C, *C$_3$*, CH), 58.8 (1C, *C$_{26}$*, CH$_2$), 55.0 (1C, *C$_8$*, CH), 54.7 (1C, O*Me*, CH$_3$), 49.0 (1C, *C$_{20}$*, CH$_2$), 47.3 (1C, *C$_{24}$*, CH$_2$), 39.5 (1C, *C$_2$*, CH$_2$), 38.0 (1C, *C$_{15}$*, CH$_2$), 34.9 (1C, *C$_{12}$*, CH$_2$), 31.9 (1C, *C$_{30}$*, CH$_2$), 29.4 (1C, *C$_{19}$*, CH$_2$), 28.6, 27.9, 27.8, 25.5, 25.2, 25.0 (6H, *C$_9$, C$_{10}$, C$_{11}$, C$_{16}$, C$_{17}$, C$_{18}$,*

CH_2), 20.4 (1C, O*Ac*, CH_3); ***m/z*** (FAB, THIOG) 672 ([M+H]$^+$, 38%), 91 (88); **HRMS** (FAB, THIOG) (Found: [M+H]$^+$, 672.3540. $C_{33}H_{50}N_7O_6S$ requires *m/z*, 672.3543).

C_6-Click *O*-linked Boc C(4)-H anisomycin biotin molecular probe 205a
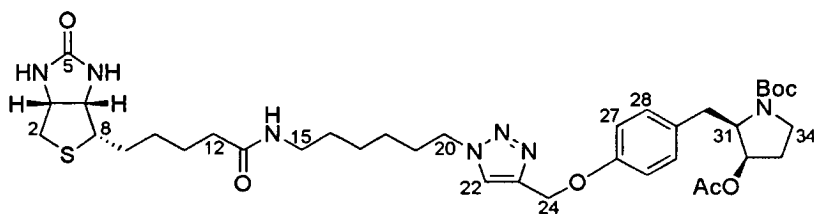
To biotinylated azide **202** (41.0 mg, 111 μmol) in H_2O:^{t}BuOH (2 ml, 1:1) was added *O*-propargyl analogue **135** (45.0 mg, 120 μmol), followed by copper(II) sulfate (3.0 mg, 10 mol%) and sodium ascorbate solution (25.0 μl, 1 M solution, 20 mol%). The solution was stirred for 18 h at RT. The solution was concentrated *in vacuo*, and the residue was purified by flash chromatography [5% to 15% MeOH/DCM] to give **205a** as a colourless oil (62.0 mg, 84.0 μmol, 76%); **R$_f$** [10% MeOH/DCM] = 0.24; **^{1}H NMR** δ (360 MHz, CDCl$_3$, 323 K) 7.60 (1H, s, C$_{22}$*H*), 7.10 (2H, d, *J* 8.5, Ar*H*), 6.88 (2H, d, *J* 8.5, Ar*H*), 5.16 (2H, s, C$_{24}$*H$_2$*), 5.11 (1H, dt, *J* 6.5, 14.1, C$_{32}$*H*), 4.52-4.46 (1H, m, C$_3$*H*), 4.34 (2H, t, *J* 7.0, C$_{20}$*H$_2$*), 4.33-4.29 (1H, m, C$_7$*H*), 4.29-4.21 (1H, m, C$_{31}$*H*), 3.47-3.27 (2H, m, C$_{34}$*H$_2$*), 3.25-3.14 (3H, m, C$_8$*H*, C$_{15}$*H$_2$*), 3.01-3.92 (1H, m, C$_{30}$*H$_S$H$_T$*), 2.89 (1H, dd, *J* 12.6, 4.3, C$_2$*H$_A$*H$_B$), 2.81 (1H, dd, *J* 13.8, 8.4 C$_{30}$H$_S$*H$_T$*), 2.72 (1H, d, *J* 12.6, C$_2$H$_A$*H$_B$*), 2.19 (2H, t, *J* 6.9, C$_{12}$*H$_2$*), 2.09-2.00 (1H, m, C$_{33}$*H$_E$*H$_F$), 1.97 (3H, s, O*Ac*), 1.91 (2H, br t, *J* 6.6, C$_{19}$*H*), 1.84-1.74 (1H, m, C$_{33}$H$_E$*H$_F$*), 1.71-1.56 (4H, m, C$_9$*H$_2$*, C$_{11}$*H$_2$*), 1.44 (9H, s, ^{t}*Bu*O), 1.49-1.27 (8H, m, C$_{10}$*H$_2$*, C$_{16}$*H$_2$*, C$_{17}$*H$_2$*, C$_{18}$*H$_2$*); **^{13}C NMR** δ (90.7 MHz, CDCl$_3$, 323 K) 173.1 (1C, *C$_{13}$*, Q), 170.7 (1C, CO^t*Bu*, Q), 169.7 (1C, O*Ac*, Q), 162.1 (1C, *C$_5$*, Q), 156.8 (1C, Ar, Q), 144.0 (1C, *C$_{23}$*, Q), 131.4 (1C, Ar, Q), 130.3 (2C, *Ar*H, CH), 122.5 (1C, *C$_{22}$*, CH), 114.7 (2C, *Ar*H, CH), 79.7 (1C, ^{t}*Bu*, Q), 73.0 (1C, *C$_{32}$*, CH), 62.1 (1C, *C$_{24}$*, CH$_2$), 61.8 (1C, *C$_7$*, CH), 60.2 (1C, *C$_3$*, CH), 59.1 (1C, *C$_{31}$*, CH), 55.4 (1C, *C$_8$*, CH), 50.1 (1C, *C$_{20}$*, CH$_2$), 43.0 (1C, *C$_{34}$*, CH$_2$), 40.3 (1C, *C$_2$*, CH$_2$), 39.1 (1C, *C$_{15}$*, CH$_2$), 35.8 (1C, *C$_{12}$*, CH$_2$), 33.9 (1C, *C$_{30}$*, CH$_2$), 29.8 (1C, *C$_{19}$*, CH$_2$), 29.4, 29.2, 28.5, 28.3, 28.3, 28.1, 25.8, 25.4 (10C, *C$_9$*, *C$_{10}$*, *C$_{11}$*, *C$_{16}$*, *C$_{17}$*, *C$_{18}$*, *C$_{33}$* all CH$_2$'s and ^{t}*Bu* CH$_3$'s), 20.7 (1C, O*Ac*, CH$_3$); **m/z** (FAB, THIOG) 742 ([M+H]$^+$, 20%), 642 (57), 91 (61); **HRMS** (FAB, THIOG) (Found: [M+H]$^+$, 742.3960. C$_{37}$H$_{56}$N$_7$O$_7$S requires *m/z*, 742.3962).

C$_6$-Click *O*-linkedC(4)-H anisomycin biotin molecular probe 205
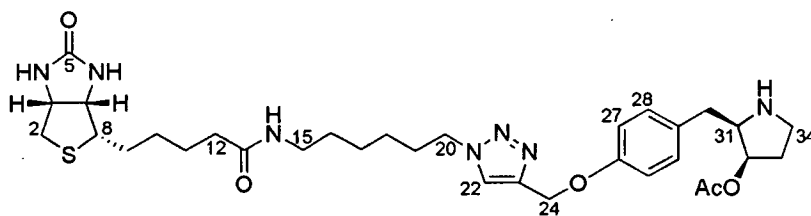
To a solution of carbamate **205a** (41.0 mg, 55.0 µmol) in DCM (2 ml) was added trifluoroacetic acid (41.0 µl, 550 µmol), the solution was stirred for 4 h at RT. The reaction was concentrated *in vacuo* to give **205** (40.0 mg, 52.9 µmol, 96%) as a colourless foam; **R$_f$** [20% MeOH/DCM] = 0.38; **^{1}H NMR** δ (360 MHz, DMSO) 8.23 (1H, s, C$_{22}$*H*), 7.76 (1H, t, *J* 5.6, N$_{14}$*H*), 7.19 (2H, d, *J* 8.6, Ar*H*), 7.00 (2H, d, *J* 8.6, Ar*H*), 6.44 (1H, br s, N$_6$*H*), 6.38 (1H, br s, N$_4$*H*), 5.16 (1H, t, *J* 3.8, C$_{32}$*H*), 5.10 (2H, s, C$_{24}$*H$_2$*), 4.42 (2H, t, *J* 7.0, C$_{20}$*H$_2$*), 4.31-4.28 (1H, m, C$_3$*H*), 4.13-4.10 (1H, m, C$_7$*H*), 3.87-3.81 (1H, m, C$_{31}$*H*), 3.34-3.22 (2H, m, C$_{34}$*H$_2$*), 3.08 (1H, ddd, 8.5, 6.1, 4.8, C$_8$*H*), 2.99 (2H, br q, *J* 6.0, C$_{15}$*H$_2$*), 3.33-3.23 (2H, m, C$_{30}$*H$_2$*), 2.80 (1H, dd, *J* 12.5, 5.1, C$_2$*H$_A$*H$_B$*), 2.57 (1H, d, *J* 12.5, C$_2$H$_A$*H$_B$*), 2.30-2.18 (1H, m, C$_{33}$*H$_E$*H$_F$*), 2.12 (3H, s, O*Ac*), 2.03 (2H, t, *J* 7.3, C$_{12}$*H$_2$*), 2.01-1.97 (1H, m, C$_{33}$H$_E$*H$_F$*), 1.80 (2H, qn, *J* 7.1, C$_{19}$*H*), 1.65-1.19 (12H, m, C$_9$*H$_2$*, C$_{10}$*H$_2$*, C$_{11}$*H$_2$*, C$_{16}$*H$_2$*, C$_{17}$*H$_2$*, C$_{18}$*H$_2$*); **^{13}C NMR** δ (90.7 MHz, DMSO) 172.0 (1C, C$_{13}$, Q), 169.8 (1C, O*Ac*, Q), 162.9 (1C, C$_5$, Q), 157.3 (1C, Ar, Q), 142.9 (1C, C$_{23}$, Q), 130.3 (2C, *Ar*H, CH), 128.8 (1C, Ar, Q), 124.8 (1C, C$_{22}$, CH), 115.1 (2C, *Ar*H, CH), 73.0 (1C, C$_{32}$, CH), 63.5 (1C, C$_{31}$, CH), 61.2 (1C, C$_7$, CH), 61.2 (1C, C$_{24}$, CH$_2$), 59.4 (1C, C$_3$, CH), 55.7 (1C, C$_8$, CH), 49.5 (1C, C$_{20}$, CH$_2$), 42.8 (1C, C$_{34}$, CH$_2$), 40.0 (1C, C$_2$, CH$_2$), 38.4 (1C, C$_{15}$, CH$_2$), 35.5 (1C, C$_{12}$, CH$_2$), 31.1 (1C, C$_{30}$, CH$_2$), 30.6 (1C, C$_{33}$, CH$_2$), 29.9 (1C, C$_{19}$, CH$_2$), 29.1, 28.5, 28.3, 26.0, 25.7, 25.6 (6H, C$_9$, C$_{10}$, C$_{11}$, C$_{16}$, C$_{17}$, C$_{18}$, CH$_2$), 21.0 (1C, O*Ac*, CH$_3$); **m/z** (FAB, THIOG) 642 ([M+H]$^+$, 30%), 91 (69); **HRMS** (FAB, THIOG) (Found: [M+H]$^+$, 642.3437. C$_{32}$H$_{48}$N$_7$O$_5$S requires *m/z*, 642.3438).

2'-*N*-Biotinylamino-eth-1-yl   4'-(2''-acetoxyeth-1''-yl)phenyloxy-acetamide[320]
206
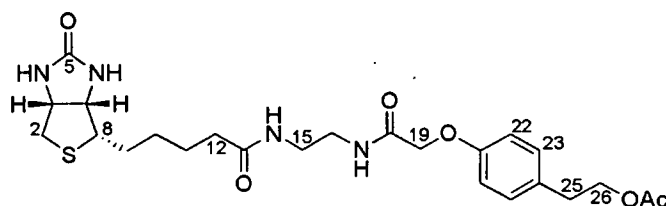
To biotinylated iodoacetamide **76** (20.0 mg, 40.0 μmol) in DMF (1.5 ml) was added **194** (10.0 mg, 40.0 μmol), followed by K_2CO_3 (6.00 mg, 40.0 μmol). The solution was stirred for 48 h at 80 °C. The solution was concentrated *in vacuo*, and the residue was purified by flash chromatography [10% MeOH/DCM] to give **206** as a colourless solid (17.0 mg, 34.4 μmol, 86%); $\mathbf{R_f}$ [10% MeOH/DCM] = 0.30; **^{1}H NMR** δ (360 MHz, DMSO) 8.12 (1H, t, *J* 5.2, $N_{17}H$), 7.87 (1H, t, *J* 5.2, $N_{14}H$), 7.17 (2H, d, *J* 8.7, Ar*H*), 6.89 (2H, d, *J* 8.7, Ar*H*), 6.42 (1H, br s, N_6H), 6.36 (1H, br s, N_4H), 4.42 (2H, s, $C_{19}H_2$), 4.30-4.27 (1H, m, C_3H), 4.15 (2H, t, *J* 7.0, $C_{26}H_2$), 4.12-4.08 (1H, m, C_7H), 3.15 (4H, dt, *J* 9.9, 5.6, $C_{15}H_2$, $C_{16}H_2$), 3.10-3.04 (1H, m, C_8H), 2.81 (2H, t, *J* 7.0, $C_{25}H_2$), 2.80 (1H, dd, *J* 12.3, 5.2, $C_2H_AH_B$), 2.56 (1H, d, *J* 12.3, $C_2H_AH_B$), 2.04 (2H, t, *J* 7.3, $C_{12}H_2$), 1.97 (3H, s, O*Ac*), 1.64-1.23 (6H, m, C_9H_2, $C_{10}H_2$, $C_{11}H_2$); **^{13}C NMR** δ (90.7 MHz, DMSO) 172.3 (1C, C_{13}, Q), 170.2 (1C, O*Ac*, Q), 167.8 (1C, C_{18}, Q), 162.6 (1C, C_5, Q), 156.6 (1C, Ar, Q), 130.4 (1C, Ar, Q), 129.5 (2C, *Ar*H, CH), 114.4 (2C, *Ar*H, CH), 66.7 (1C, C_{19}, CH_2), 64.2 (1C, C_{26}, CH_2), 60.7 (1C, C_7, CH), 59.0 (1C, C_3, CH), 55.1 (1C, C_8, CH), 39.6 (1C, C_2, CH_2), 38.3 (1C, C_{16}, CH_2), 37.8 (1C, C_{15}, CH_2), 34.9 (1C, C_{12}, CH_2), 33.2 (1C, C_{25}, CH_2), 28.0 (1C, C_{10}, CH_2), 27.8 (1C, C_9, CH_2), 24.9 (1C, C_{11}, CH_2), 20.5 (1C, O*Ac*, CH_3); ***m/z*** (FAB, THIOG) 507 ([M+H]$^+$, 29%), 91 (75); **HRMS** (FAB, THIOG) (Found: [M+H]$^+$, 507.2281. $C_{24}H_{35}N_4O_6S$ requires *m/z*, 507.2277).

C$_2$-Classical *N*-linked anisomycin biotin molecular probe 207
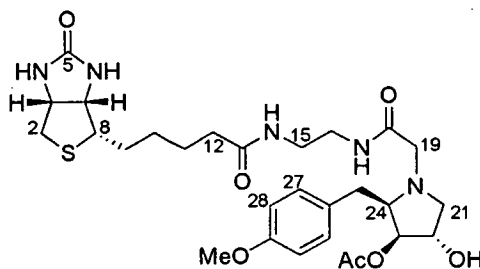
To biotinylated iodoacetamide **76** (25.0 mg, 55.0 μmol) in DMF (5 ml) was added anisomycin **102** (15.0 mg, 55.0 μmol), followed by K$_2$CO$_3$ (7.60 mg, 55.0 μmol). The solution was stirred for 48 h at 80 °C. The solution was concentrated *in vacuo*, and the residue was purified by flash chromatography [5% to 15% MeOH/DCM] to give **207** as a colourless solid (11.0 mg, 18.7 μmol, 34%); **R$_f$** [14% MeOH/DCM] = 0.33; **^{1}H NMR** δ (360 MHz, DMSO) 7.87 (2H, br s, N$_{14}$*H*, N$_{17}$*H*), 7.08 (2H, d, *J* 8.6, Ar*H*), 6.81 (2H, d, *J* 8.6, Ar*H*), 6.43 (1H, br s, N$_6$*H*), 6.38 (1H, br s, N$_4$*H*), 4.60 (1H, dd, *J* 4.2, 1.5, C$_{23}$*H*), 4.31-4.28 (1H, m, C$_3$*H*), 4.13-4.09 (1H, m, C$_7$*H*), 3.95-3.90 (1H, m, C$_{22}$*H*), 3.70 (3H, s, O*Me*), 3.49-3.35 (2H, m, C$_{19}$*H$_X$*H$_Y$, C$_{21}$*H$_M$*H$_N$), 3.17-3.05 (5H, m, C$_8$*H*, C$_{15}$*H$_2$*, C$_{16}$*H$_2$*,), 3.00-2.85 (3H, m, C$_{19}$H$_X$*H$_Y$*, C$_{21}$H$_M$*H$_N$*), 2.80 (1H, dd, *J* 12.5, 5.0, C$_2$*H$_A$*H$_B$), 2.77-2.71 (1H, m, C$_{25}$*H$_S$*H$_T$), 2.66-2.60 (1H, m, C$_{25}$H$_S$*H$_T$*), 2.57 (1H, d, *J* 12.5, C$_2$H$_A$*H$_B$*), 2.08 (3H, s, O*Ac*), 2.06 (2H, t, *J* 7.6, C$_{12}$*H$_2$*), 1.76-1.22 (6H, m, C$_9$*H$_2$*, C$_{10}$*H$_2$*, C$_{11}$*H$_2$*); **^{13}C NMR** δ (151.1 MHz, DMSO) 172.5 (1C, *C$_{13}$*, Q), 171.1 (1C, *C$_{18}$*, Q), 169.7 (1C, O*Ac*, Q), 162.8 (1C, *C$_5$*, Q), 157.7 (1C, *Ar*, Q), 130.2 (1C, *Ar*, Q), 129.7 (2C, *Ar*H, CH), 113.5 (2C, *Ar*H, CH), 78.5 (1C, *C$_{23}$*, CH), 72.5 (1C, *C$_{22}$*, CH), 69.6 (1C, *C$_{24}$*, CH), 60.8 (1C, *C$_7$*, CH), 60.8 (1C, *C$_{21}$*, CH$_2$), 59.0 (1C, *C$_3$*, CH), 58.2 (1C, *C$_{19}$*, CH$_2$), 55.2 (1C, *C$_8$*, CH), 54.7 (1C, O*Me*, CH$_3$), 39.5 (1C, *C$_2$*, CH$_2$), 38.2 (1C, *C$_{16}$*, CH$_2$), 37.8 (1C, *C$_{15}$*, CH$_2$), 35.1 (1C, *C$_{12}$*, CH$_2$), 32.4 (1C, *C$_{25}$*, CH$_2$), 28.2 (1C, *C$_{10}$*, CH$_2$), 27.8 (1C, *C$_9$*, CH$_2$), 25.0 (1C, *C$_{11}$*, CH$_2$), 20.7 (1C, O*Ac*, CH$_3$); ***m/z*** (FAB, THIOG) 592 ([M+H]$^+$, 52%), 91 (100); **HRMS** (FAB, THIOG) (Found: [M+H]$^+$, 592.2804. C$_{28}$H$_{42}$N$_5$O$_7$S requires *m/z*, 592.2805).

C_2-Classical *O*-linked Boc C(4)-H anisomycin biotin molecular probe **208a**
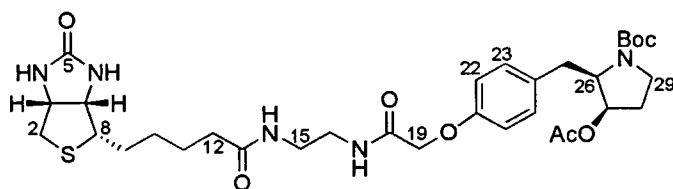
To biotinylated iodoacetamide **76** (50.0 mg, 111 μmol) in DMF (2.5 ml) was added **134** (37.0 mg, 111 μmol), followed by K_2CO_3 (18.0 mg, 130 μmol). The solution was stirred for 48 h at 80 °C. The solution was concentrated *in vacuo*, and the residue was purified by flash chromatography [5% to 15% MeOH/DCM] to give **208a** as a colourless solid (23.0 mg, 34.0 μmol, 31%); R_f [20% MeOH/DCM] = 0.27; ^{1}**H NMR** δ (600 MHz, CDCl$_3$, 323 K) 7.23 (1H, br t, *J* 4.6, N$_{17}$*H*), 7.14 (2H, d, *J* 8.0, Ar*H*), 6.85 (2H, d, *J* 8.0, Ar*H*), 6.80 (1H, br s, N$_{14}$*H*), 6.35 (1H, br s, N$_6$*H*), 5.45 (1H, br s, N$_4$*H*), 5.11 (1H, br q, *J* 6.6, C$_{27}$*H*), 4.52-4.45 (3H, br s, C$_3$*H*, C$_{19}$*H$_2$*), 4.31-4.29 (1H, m, C$_7$*H*), 4.24 (1H, br q, *J* 6.1, C$_{26}$H), 3.48-3.43 (5H, m, C$_{15}$*H$_2$*, C$_{16}$*H$_2$*, C$_{29}$*H$_M$*H$_N$), 3.35-3.31 (1H, m, C$_{29}$H$_M$*H$_N$*) 3.14-3.12 (1H, m, C$_8$*H*), 2.95-2.92 (1H, m, C$_{25}$*H$_S$*H$_T$), 2.89 (1H, dd, *J* 12.7, 4.4, C$_2$*H$_A$*H$_B$), 2.82 (1H, dd, 13.5, 5.5, C$_{25}$H$_S$*H$_T$*), 2.71 (1H, d, *J* 12.7, C$_2$H$_A$*H$_B$*), 2.21 (2H, br q, *J* 6.5, C$_{12}$*H$_2$*), 2.10-2.05 (1H, m, C$_{28}$*H$_E$*H$_F$), 2.00 (3H, s, O*Ac*), 1.82-1.65 (5H, m, C$_9$*H$_2$*, C$_{11}$*H$_2$*, C$_{28}$H$_E$*H$_F$*), 1.46 (9H, s, ^{t}*Bu*O), 1.30-1.27 (2H, m, C$_{10}$*H$_2$*); ^{13}**C NMR** δ (151.1 MHz, CDCl$_3$, 323 K) 174.3 (1C, *CO^{t}Bu*, Q), 174.2 (1C, *C$_{13}$*, Q), 170.3 (1C, O*Ac*, Q), 169.9 (1C, *C$_{18}$*, Q), 164.1 (1C, *C$_5$*, Q), 156.2 (1C, *Ar*, Q), 132.7 (1C, *Ar*, Q), 130.9 (2C, *Ar*H, CH), 114.9 (2C, *Ar*H, CH), 80.1 (1C, ^{t}*Bu*, Q), 73.3 (1C, *C$_{27}$*, CH), 67.7 (1C, *C$_{19}$*, CH$_2$), 61.9 (1C, *C$_7$*, CH), 60.4 (1C, *C$_3$*, CH), 59.5 (1C, *C$_{26}$*, CH), 55.7 (1C, *C$_8$*, CH), 43.3 (1C, *C$_{29}$*, CH$_2$), 40.7 (1C, *C$_2$*, CH$_2$), 39.6 (1C, *C$_{16}$*, CH$_2$), 39.6 (1C, *C$_{15}$*, CH$_2$), 35.9 (1C, *C$_{12}$*, CH$_2$), 34.4 (1C, *C$_{25}$*, CH$_2$), 29.8 (1C, *C$_{10}$*, CH$_2$), 29.0 (1C, *C$_{28}$*, CH$_2$), 28.4 (3C, ^{t}*Bu*, CH$_3$), 28.4 (1C, *C$_9$*, CH$_2$), 25.5 (1C, *C$_{11}$*, CH$_2$), 21.0 (1C, O*Ac*, CH$_3$); **m/z** (FAB, THIOG) 662 ([M+H]$^+$, 3%), 562 (27) 91 (22); **HRMS** (FAB, THIOG) (Found: [M+H]$^+$, 662.3229. $C_{32}H_{48}N_5O_8S$ requires *m/z*, 662.3224).

C$_2$-Classical *O*-linked C(4)-H anisomycin biotin molecular probe 208
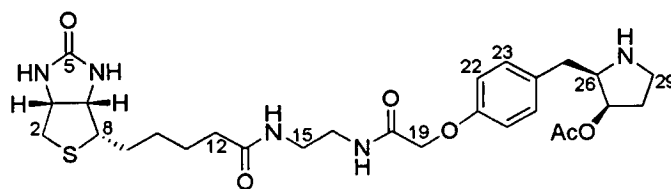
To a solution of carbamate **208a** (19.0 mg, 31.0 µmol) in DCM (2 ml) was added trifluoroacetic acid (23.0 µl, 310 µmol), the solution was stirred for 3 h at RT. The reaction was concentrated *in vacuo* to give **208** (20.0 mg, 29.0 µmol, 94%) as a colourless foam; **R$_f$** [20% MeOH/DCM] = 0.36; **^{1}H NMR** δ (360 MHz, DMSO) 8.16 (1H, t, *J* 5.3, N$_{17}$*H*), 7.90 (1H, t, *J* 5.3, N$_{14}$*H*), 7.19 (2H, d, *J* 8.6, Ar*H*), 6.93 (2H, d, *J* 8.6, Ar*H*), 6.45 (1H, br s, N$_6$*H*), 6.38 (1H, br s, N$_4$*H*), 5.15 (1H, t, *J* 3.7, C$_{27}$*H*), 4.43 (2H, s, C$_{19}$*H$_2$*), 4.31-4.28 (1H, m, C$_3$*H*), 4.14-4.10 (1H, m, C$_7$*H*), 3.88-3.82 (1H, m, C$_{26}$*H*), 3.33-3.23 (2H, m, C$_{29}$*H$_2$*), 3.20-3.10 (4H, m, C$_{15}$*H$_2$*, C$_{16}$*H$_2$*), 3.08 (1H, ddd, *J* 8.7, 6.2, 4.5, C$_8$*H*), 2.98-2.86 (2H, m, C$_{25}$*H$_2$*), 2.80 (1H, dd, *J* 12.4, 5.0, C$_2$*H$_A$*H$_B$), 2.57 (1H, d, *J* 12.4, C$_2$*H$_A$H$_B$*), 2.29-2.18 (1H, m, C$_{28}$*H$_E$H$_F$*), 2.12 (3H, s, O*Ac*), 2.05 (2H, t, *J* 7.4, C$_{12}$*H$_2$*), 2.02-1.97 (1H, m, C$_{28}$H$_E$*H$_F$*), 1.65-1.25 (6H, m, C$_9$*H$_2$*, C$_{10}$*H$_2$*, C$_{11}$*H$_2$*); **^{13}C NMR** δ (90.7 MHz, DMSO) 172.2 (1C, *C$_{13}$*, Q), 169.4 (1C, O*Ac*, Q), 167.7 (1C, *C$_{18}$*, Q), 162.5 (1C, *C$_5$*, Q), 156.4 (1C, *Ar*, Q), 129.7 (2C, *Ar*H, CH), 128.9 (1C, *Ar*, Q), 114.8 (2C, *Ar*H, CH), 72.6 (1C, *C$_{27}$*, CH), 66.8 (1C, *C$_{19}$*, CH$_2$), 63.1 (1C, *C$_{26}$*, CH), 60.8 (1C, *C$_7$*, CH), 59.0 (1C, *C$_3$*, CH), 55.2 (1C, *C$_8$*, CH), 42.5 (1C, *C$_{29}$*, CH$_2$), 39.6 (1C, *C$_2$*, CH$_2$), 38.3 (1C, *C$_{16}$*, CH$_2$), 37.8 (1C, *C$_{15}$*, CH$_2$), 35.0 (1C, *C$_{12}$*, CH$_2$), 30.8 (1C, *C$_{25}$*, CH$_2$), 30.2 (1C, *C$_{28}$*, CH$_2$), 28.1 (1C, *C$_{10}$*, CH$_2$), 27.9 (1C, *C$_9$*, CH$_2$), 25.0 (1C, *C$_{11}$*, CH$_2$), 20.6 (1C, O*Ac*, CH$_3$); ***m/z*** (FAB, THIOG) 562 ([M+H]$^+$, 80%), 91 (100); **HRMS** (FAB, NOBA) (Found: [M+H]$^+$, 562.2705. C$_{27}$H$_{40}$N$_5$O$_6$S requires *m/z*, 562.2699).

6'-_N_-Biotinylamino-hex-1-yl 4'-(2''-acetoxyeth-1''-yl)phenyloxy-acetamide[320] 212
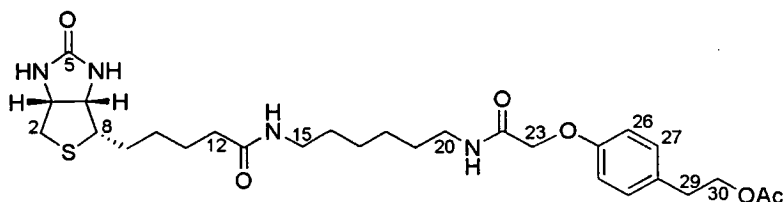
To biotinylated iodoacetamide **210** (20.0 mg, 47.0 μmol) in DMF (5.0 ml) was added **194** (8.60 mg, 47.0 μmol), followed by K_2CO_3 (6.60 mg, 47.0 μmol). The solution was stirred for 48 h at 80 °C. The solution was concentrated _in vacuo_, and the residue was purified by flash chromatography [5% to 15% MeOH/DCM] to give **212** as a colourless solid (20.0 mg, 33.8 μmol, 72%); **R**$_f$ [10% MeOH/DCM] = 0.16; ^{1}**H NMR** δ (600 MHz, DMSO) 8.03 (1H, t, _J_ 5.7, $N_{21}H$), 7.72 (1H, t, _J_ 5.7, $N_{14}H$), 7.17 (2H, d, _J_ 8.5, Ar_H_), 6.88 (2H, d, _J_ 8.5, Ar_H_), 6.41 (1H, br s, N_6H), 6.35 (1H, br s, N_4H), 4.42 (2H, s, $C_{23}H_2$), 4.31-4.29 (1H, m, C_3H), 4.15 (2H, t, _J_ 6.9, $C_{30}H_2$), 4.13-4.11 (1H, m, C_7H), 3.10 (2H, br q, _J_ 6.8, $C_{20}H_2$), 3.09-3.07 (1H, m, C_8H), 3.00 (2H, br q, _J_ 6.7, $C_{15}H_2$), 2.60 (1H, dd, _J_ 12.5, 5.0, $C_2H_AH_B$), 2.81(2H, t, _J_ 6.7, $C_{29}H_2$), 2.57 (1H, d, _J_ 12.5, $C_2H_AH_B$), 2.04 (2H, t, _J_ 7.4, $C_{12}H_2$), 1.97 (3H, s, O_Ac_), 1.64-1.21 (14H, m, C_9H_2, $C_{10}H_2$, $C_{11}H_2$, $C_{16}H_2$, $C_{17}H_2$, $C_{18}H_2$, $C_{19}H_2$); ^{13}**C NMR** δ (151.5 MHz, DMSO) 171.6 (1C, C_{13}, Q), 170.1 (1C, O_Ac_, Q), 167.3 (1C, C_{22}, Q), 162.5 (1C, C_5, Q), 156.2 (1C, Ar, Q), 130.4 (1C, Ar, Q), 129.7 (2C, _Ar_H, CH), 114.5 (2C, _Ar_H, CH), 66.9 (1C, C_{23}, CH_2), 64.4 (1C, C_{30}, CH_2), 60.9 (1C, C_7, CH), 59.0 (1C, C_3, CH), 55.2 (1C, C_8, CH), 39.7 (1C, C_2, CH_2), 38.1 (1C, C_{15}, CH_2), 38.0 (1C, C_{20}, CH_2), 35.1 (1C, C_{12}, CH_2), 33.3 (1C, C_{29}, CH_2), 29.0, 28.9, 28.8, 28.1, 27.9, 25.9, 25.2 (7C, C_9, C_{10}, C_{11}, C_{16}, C_{17}, C_{18}, C_{19}, CH_2), 20.6 (1C, O_Ac_, CH_3); **_m/z_** (FAB, THIOG) 563 ([M+H]$^+$, 49%), 91 (70); **HRMS** (FAB, NOBA) (Found: [M+H]$^+$, 563.2907. $C_{28}H_{43}N_4O_6S$ requires _m/z_, 563.2903).

C_6-Classical *N*-linked anisomycin biotin molecular probe[320] 213
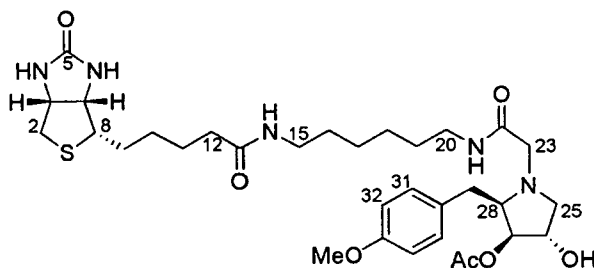
To biotinylated iodoacetamide **210** (20.0 mg, 47.0 μmol) in DMF (5.0 ml) was added **102** (12.6 mg, 47.0 μmol), followed by K_2CO_3 (6.60 mg, 47.0 μmol). The solution was stirred for 48 h at 80 °C. The solution was concentrated *in vacuo*, and the residue was purified by flash chromatography [5% to 15% MeOH/DCM] to give **213** as a colourless solid (11.0 mg, 16.5 μmol, 35%); R_f [10% MeOH/DCM] = 0.19; **¹H NMR** δ (360 MHz, DMSO) 7.74 (1H, t, *J* 5.6, $N_{14}H$), 7.68 (1H, t, *J* 5.6, $N_{21}H$), 7.06 (2H, d, *J* 8.6, Ar*H*), 6.80 (2H, d, *J* 8.6, Ar*H*), 6.43 (1H, br s, N_6H), 6.36 (1H, br s, N_4H), 5.29 (1H, d, *J* 4.8, O*H*), 4.59 (1H, dd, *J* 4.9, 2.2, $C_{27}H$), 4.32-4.28 (1H, m, C_3H), 4.14-4.10 (1H, m, C_7H), 3.89-3.85 (1H, m, $C_{26}H$), 3.70 (3H, s, O*Me*), 3.42-3.38 (1H, m, $C_{25}H_M H_N$) 3.26 (1H, d, *J* 15.9, $C_{23}H_X H_Y$), 3.19-3.13 (1H, m, $C_{28}H$), 3.11-2.98 (5H, m, C_8H, $C_{15}H_2$, $C_{20}H_2$,), 2.97 (1H, d, *J* 15.9, $C_{23}H_X H_Y$), 2.81 (1H, dd, *J* 12.3, 5.1, $C_2H_A H_B$), 2.75-2.72 (1H, m, $C_{29}H_S H_T$), 2.59-2.57 (1H, m, $C_{29}H_S H_T$), 2.57 (1H, d, *J* 12.3, $C_2H_A H_B$), 2.31 (1H, dd, *J* 10.4, 3.5, $C_{25}H_M H_N$), 2.06 (3H, s, O*Ac*), 2.04 (2H, t, *J* 7.4, $C_{12}H_2$), 1.49-1.25 (14H, m, C_9H_2, $C_{10}H_2$, $C_{11}H_2$, $C_{16}H_2$, $C_{17}H_2$, $C_{18}H_2$, $C_{19}H_2$); **¹³C NMR** δ (90.7 MHz, DMSO) 171.7 (1C, C_{13}, Q), 169.9 (1C, C_{22}, Q), 169.6 (1C, O*Ac*, Q), 162.5 (1C, C_5, Q), 157.4 (1C, *Ar*, Q), 130.7 (1C, *Ar*, Q), 129.7 (2C, *Ar*H, CH), 113.6 (2C, *Ar*H, CH), 78.9 (1C, C_{27}, CH), 72.9 (1C, C_{26}, CH), 65.6 (1C, C_{28}, CH), 60.8 (1C, C_7, CH), 60.1 (1C, C_{25}, CH₂), 59.0 (1C, C_3, CH), 57.1 (1C, C_{23}, CH₂), 55.2 (1C, C_8, CH), 54.7 (1C, O*Me*, CH₃), 39.7 (1C, C_2, CH₂), 38.2 (1C, C_{20}, CH₂), 37.9 (1C, C_{15}, CH₂), 35.0 (1C, C_{12}, CH₂), 32.8 (1C, C_{29}, CH₂), 29.2, 28.9, 28.0, 27.8, 25.9, 25.5, 25.2 (7C, C_9, C_{10}, C_{11}, C_{16}, C_{17}, C_{18}, C_{19}, CH₂), 20.9 (1C, O*Ac*, CH₃); *m/z* (FAB, NOBA) 648 ([M+H]⁺, 5%), 307 (50), 77 (70); **HRMS** (FAB, THIOG) (Found: [M+H]⁺, 648.3435. $C_{32}H_{50}N_5O_7S$ requires *m/z*, 648.3431).

N-Affi-gel-2-azido-1-ethylamine 215
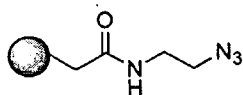
To a solution of Affi-gel-10 **214** (25.0 ml, 375 µmol) in methanol (50 ml) was added 1-azido-ethylamine **187** (500 mg, 5.81 mmol) and the solution shaken for 48 h. The solution was filtered and the matrix washed with methanol (200 ml). The matrix **215** was stored in isopropylalcohol; ν_{max} (neat)/cm^{-1} 3416, 2919, 2114, 1648.

Solid supported anisomycin 216
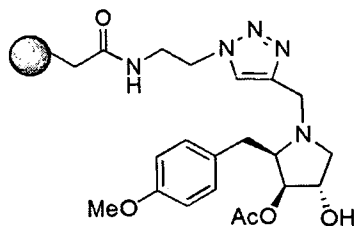
To azide **215** (380 μmol) in H$_2$O:^{t}BuOH (80 ml, 1:1) was added *N*-propargyl anisomycin **140** (109 mg, 360 μmol), followed by copper(II) sulfate (8.0 mg, 10 mol%) and sodium ascorbate solution (40.0 μl, 1 M solution, 20 mol%). The solution was shaken for 15 h at RT. The reaction was monitored by tlc probing for the reaction of *N*-propargyl anisomycin. Upon completion of the reaction by tlc, the solution was filtered and the matrix washed with isopropylalcohol (200 ml). The filtrate was concentrated *in vacuo*, and the residue analysed by NMR and found to be unreacted *N*-propargyl anisomycin (~1 mg). The matrix **216** was stored in a solution of isopropylalcohol.

3β-Hydroxy-androst-5-en-17-one or Dehydroepiandrosterone (DHEA)[384, 395] 217
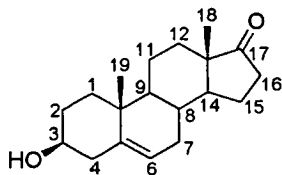
R_f [40% EtOAc/hexane] = 0.37; **¹H NMR** δ (360 MHz, CDCl$_3$) 5.39 (1H, d, J 5.2, C$_6$$H$), 3.54 (1H, tt, J 11.2, 4.1, C$_3$$H$), 2.47 (1H, dd, J 19.3, 8.7, C$_{16}$$H_SH_T$), 2.33 (1H, ddd, J 13.0, 4.9, 2.3, C$_4$$H_EH_F$), 2.29-2.23 (1H, m, C$_4H_E$$H_F$), 2.14-2.11 (1H, m, C$_7$$H_GH_H$), 2.09 (1H, dd, J 19.3, 9.5, C$_{16}$H$_S$$H_T$), 1.96 (1H, ddd, 12.8, 8.7, 6.0, C$_{15}$$H_QH_R$), 1.89-1.84 (3H, m, C$_1$$H_AH_B$, C$_2$$H_CH_D$, C$_8$$H$), 1.71-1.63 (4H, m, C$_7H_G$$H_H$, C$_{11}$$H_KH_L$, C$_{12}$$H_MH_N$, O$H$), 1.57-1.46 (3H, m, C$_2H_C$$H_D$, C$_{11}H_K$$H_L$, C$_{15}H_Q$$H_R$), 1.32-1.26 (2H, m, C$_{12}H_M$$H_N$, C$_{14}$$H$), 1.11 (1H, dt, J 13.8, 4.0, C$_1$H$_A$$H_B$), 1.05 (3H, s, C$_{19}$$H_3$), 1.02 (1H, ddd, J 12.3, 10.6, 5.3, C$_9$$H$), 0.90 (3H, s, C$_{18}$$H_3$); **¹³C NMR** δ (151.2 MHz, CDCl$_3$) 221.2 (1C, C_{17}, Q), 140.9 (1C, C_5, Q), 120.8 (1C, C_6H, CH), 71.5 (1C, C_3H, CH), 51.6 (1C, C_{14}H, CH), 50.1 (1C, C_9H, CH), 47.4 (1C, C_{13}, Q), 42.1 (1C, C_4H$_2$, CH$_2$), 37.0 (1C, C_1H$_2$, CH$_2$), 36.5 (1C, C_{10}, Q), 35.7 (1C, C_{16}H$_2$, CH$_2$), 31.4 (3C, C_2H$_2$, C_8H, C_{12}H$_2$, 2CH$_2$, CH), 30.7 (1C, C_7H$_2$, CH$_2$), 21.8 (1C, C_{15}H$_2$, CH$_2$), 20.2 (1C, C_{11}H$_2$, CH$_2$), 19.3 (1C, C_{19}H$_3$, CH$_3$), 13.4 (1C, C_{18}H$_3$, CH$_3$).

All spectroscopic data was in good agreement with that of the literature.[384, 395]

5-Androsten-3β-ol-17-one sulfate sodium salt or Dehydroepiandrosterone-3β-sulfate sodium salt (DHEAS) 233
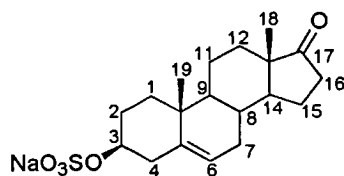
R_f [20% MeOH/DCM] = 0.35; ^{1}**H NMR** δ (360 MHz, CD$_3$OD) 5.40 (1H, d, J 5.2, C_6H), 4.11 (1H, tt, J 11.5, 4.8, C_3H), 2.53 (1H, ddd, J 13.3, 5.0, 2.3, $C_4H_EH_F$), 2.42 (1H, dd, J 19.1, 8.6, $C_{16}H_SH_T$), 2.37-2.29 (1H, m, $C_4H_EH_F$), 2.13-2.01 (2H, m, $C_7H_GH_H$, $C_2H_CH_D$), 2.04 (1H, dd, J 19.1, 9.1, $C_{16}H_SH_T$), 1.96 (1H, ddd, 12.2, 9.1, 5.7, $C_{15}H_QH_R$), 1.88 (1H, dt, J 13.5, 3.5, $C_1H_AH_B$), 1.76 (1H, ddd, J 12.9, 4.1, 2.8, $C_{12}H_MH_N$), 1.70-1.48 (6H, m, $C_2H_CH_D$, $C_7H_GH_H$, C_8H, $C_{15}H_QH_R$, $C_{11}H_2$), 1.31 (1H, ddd, J 12.8, 10.6, 5.7, $C_{14}H$), 1.24 (1H, td, J 12.9, 4.1, $C_{12}H_MH_N$), 1.09 (1H, td, J 13.5, 3.8, $C_1H_AH_B$), 1.03 (3H, s, $C_{19}H_3$), 0.99 (1H, ddd, J 12.4, 4.8, 1.8, C_9H), 0.86 (3H, s, $C_{18}H_3$); ^{13}**C NMR** δ (90.7 MHz, CD$_3$OD) 223.9 (1C, C_{17}, Q), 141.7 (1C, C_5, Q), 122.7 (1C, C_6H, CH), 79.6 (1C, C_3H, CH), 53.0 (1C, C_{14}H, CH), 51.7 (1C, C_9H, CH), 47.1 (1C, C_{13}, Q), 40.3 (1C, C_4H$_2$, CH$_2$), 38.3 (1C, C_1H$_2$, CH$_2$), 37.8 (1C, C_{10}, Q), 36.7 (1C, C_{16}H$_2$, CH$_2$), 32.7 (1C, C_8H, CH), 32.6 (1C, C_{12}H$_2$, CH$_2$), 31.8 (1C, C_7H$_2$, CH$_2$), 29.9 (1C, C_2H$_2$, CH$_2$), 22.8 (1C, C_{15}H$_2$, CH$_2$), 21.4 (1C, C_{11}H$_2$, CH$_2$), 19.8 (1C, C_{19}H$_3$, CH$_3$), 13.9 (1C, C_{18}H$_3$, CH$_3$).

3β-(But-3'-ynoyloxy)-androst-5-en-17-one 239
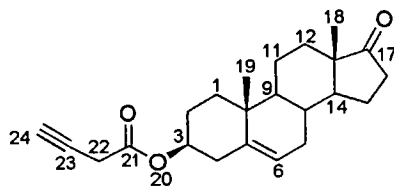
To a solution of but-3-ynoic acid **238** (34.0 mg, 400 μmol) in DCM (15 ml) at 0 °C was added DMAP (35.0 mg, 290 μmol), DIC (64.0 μl, 400 μmol) and DHEA **217** (100 mg, 360 μmol) and the solution stirred for 1 h. The reaction was then warmed to RT and stirred for a further 48 h, giving a pale yellow solution. The reaction was quenched with 1% HCl (15 ml) and the aqueous extracted (3 x 20 ml). The combined organics were washed with brine (20 ml), dried (MgSO$_4$) and concentrated *in vacuo*. The residue was chromatographed on silica gel [35% EtOAc/hexane] to give **239** as a colourless solid (95.0 mg, 270 μmol, 75%); **R$_f$** [40% EtOAc/hexane] = 0.67; υ$_{max}$ (neat)/cm^{-1} 1736, 1701, 1524, 1022; [α]$_D$ +21.9 (*c* 1.14, CHCl$_3$); **^{1}H NMR** δ (360 MHz, CDCl$_3$) 5.41 (1H, d, *J* 4.9, C$_6$*H*), 4.73-4.64 (1H, m, C$_3$*H*), 2.47 (1H, dd, *J* 19.2, 9.0, C$_{16}$*H$_S$*H$_T$), 2.41-2.36 (3H, br d, *J* 7.9, C$_4$*H$_2$*, C$_{24}$H), 2.16-2.08 (1H, m, C$_7$*H$_G$*H$_H$), 2.07 (1H, dd, *J* 19.2, 9.0, C$_{16}$H$_S$*H$_T$*), 1.98 (2H, s, C$_{22}$*H$_2$*), 1.97-1.81 (4H, m, C$_1$*H$_A$*H$_B$, C$_2$*H$_C$*H$_D$, C$_8$*H*, C$_{15}$*H$_Q$*H$_R$), 1.71-1.63 (4H, m, C$_2$H$_C$*H$_D$*, C$_7$H$_G$*H$_H$*, C$_{11}$*H$_K$*H$_L$, C$_{12}$*H$_M$*H$_L$), 1.60-1.39 (2H, m, C$_{15}$H$_Q$*H$_R$*, C$_{11}$H$_K$*H$_L$*), 1.32-1.24 (2H, m, C$_{12}$H$_M$*H$_N$*, C$_{14}$*H*), 1.14 (1H, td, *J* 13.4, 4.0, C$_1$H$_A$*H$_B$*), 1.04 (3H, s, C$_{19}$*H$_3$*), 1.02 (1H, ddd, *J* 12.4, 4.8, 1.9, C$_9$*H*), 0.89 (3H, s, C$_{18}$*H$_3$*); **^{13}C NMR** δ (90.7 MHz, CDCl$_3$) 221.2 (1C, *C$_{17}$*, Q), 153.3 (1C, *C$_{21}$*, Q), 139.7 (1C, *C$_5$*, Q), 122.4 (1C, *C$_6$*H, CH), 85.4 (1C, *C$_{23}$*, Q), 75.6 (1C, *C$_3$*H, CH), 72.9 (1C, *C$_{24}$*H, CH), 51.9 (1C, *C$_{14}$*H, CH), 50.3 (1C, *C$_9$*H, CH), 47.7 (1C, *C$_{13}$*, Q), 38.0 (1C, *C$_4$*H$_2$, CH$_2$), 37.0 (1C, *C$_1$*H$_2$, CH$_2$), 36.9 (1C, *C$_{10}$*, Q), 36.0 (1C, *C$_{16}$*H$_2$, CH$_2$), 31.6 (1C, *C$_8$*H, CH), 31.6 (1C, *C$_{12}$*H$_2$, CH$_2$), 30.9 (1C, *C$_7$*H$_2$, CH$_2$), 27.7 (1C, *C$_2$*H$_2$, CH$_2$), 22.8 (1C, *C$_{15}$*H$_2$, CH$_2$), 21.1 (1C, *C$_{11}$*H$_2$, CH$_2$), 19.5 (1C, *C$_{19}$*H$_3$, CH$_3$), 13.7 (1C, *C$_{18}$*H$_3$, CH$_3$), 4.0 (1C, *C$_{22}$*H$_2$, CH$_2$); **m/z** (FAB, THIOG) 355 ([M+H]$^+$, 10%), 272 (45); **HRMS** (FAB, THIOG) (Found: [M+H]$^+$, 355.2274. C$_{23}$H$_{31}$O$_3$ requires *m/z*, 355.2273).

3β-(Prop-2'-ynloxycarbonyloxy)-androst-5-en-17-one 241
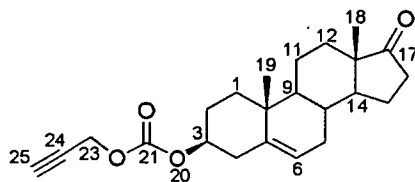
To a solution of DHEA **217** (100 mg, 360 μmol) in DCM (10 ml) was added propargyl chloroformate **240** (70.0 μl, 700 μmol) and pyridine (100 μl, 1.20 mmol) and the solution stirred for 14 h at RT. The reaction was quenched with 1% HCl (15 ml) and the aqueous extracted (3 x 30 ml). The combined organics were washed with saturated aq. copper sulfate solution (20 ml), water (20 ml), brine (15 ml), dried (MgSO$_4$) and concentrated *in vacuo*. The residue was chromatographed on silica gel [30% EtOAc/hexane] to give **241** as a colourless solid (90.0 mg, 260 μmol, 74%); **R$_f$** [40% EtOAc/hexane] = 0.61; υ_{max} (neat)/cm^{-1} 1737, 1557, 1022; [α]$_D$ +2.79 (*c* 1.10, CHCl$_3$); **^{1}H NMR** δ (360 MHz, CDCl$_3$) 5.43 (1H, d, *J* 5.2, C$_6$*H*), 4.72 (2H, d, *J* 2.5, C$_{23}$*H$_2$*), 4.51 (1H, tt, *J* 11.4, 4.8, C$_3$*H*), 2.53 (1H, t, *J* 2.5, C$_{25}$*H*), 2.46 (1H, dd, *J* 19.2, 8.1, C$_{16}$H$_S$H$_T$), 2.46-2.35 (2H, m, C$_4$*H$_2$*), 2.15-2.10 (1H, m, C$_7$H$_E$H$_F$), 2.08 (1H, dd, *J* 19.2, 9.1, C$_{16}$H$_S$*H$_T$*), 2.00-1.91 (2H, m, C$_2$*H$_C$*H$_D$, C$_{15}$*H$_Q$*H$_R$), 1.88 (1H, t, *J* 3.5, C$_1$*H$_A$*H$_B$), 1.85 (1H, ddt, *J* 12.7, 2.8, 1.3, C$_8$*H*), 1.72-1.60 (4H, m, C$_2$H$_C$*H$_D$*, C$_7$H$_E$*H$_F$*, C$_{11}$*H$_K$*H$_L$, C$_{12}$*H$_M$*H$_N$), 1.58-1.48 (1H, m, C$_{15}$H$_Q$*H$_R$*), 1.47 (1H, td, *J* 13.5, 4.1, C$_{11}$H$_K$*H$_L$*), 1.33-1.24 (2H, m, C$_{12}$H$_M$*H$_N$*, C$_{14}$*H*), 1.14 (1H, td, *J* 13.7, 3.5, C$_1$H$_A$*H$_B$*), 1.04 (3H, s, C$_{19}$*H$_3$*), 1.01 (1H, ddd, *J* 10.4, 4.7, 1.9, C$_9$*H*), 0.88 (3H, s, C$_{18}$*H$_3$*); **^{13}C NMR** δ (90.7 MHz, CDCl$_3$) 221.3 (1C, *C$_{17}$*, Q), 154.0 (1C, *C$_{21}$*, Q), 139.6 (1C, *C$_5$*, Q), 122.5 (1C, *C$_6$*H, CH), 78.5 (1C, *C$_3$*H, CH), 76.9 (1C, *C$_{24}$*, Q), 75.7 (1C, *C$_{25}$*, CH), 55.2 (1C, *C$_{23}$*H$_2$, CH$_2$), 51.8 (1C, *C$_{14}$*H, CH), 50.2 (1C, *C$_9$*H, CH), 47.7 (1C, *C$_{13}$*, Q), 38.0 (1C, *C$_4$*H$_2$, CH$_2$), 36.9 (1C, *C$_1$*H$_2$, CH$_2$), 36.8 (1C, *C$_{10}$*, Q), 36.0 (1C, *C$_{16}$*H$_2$, CH$_2$), 31.6 (1C, *C$_8$*H, CH), 31.5 (1C, *C$_{12}$*H$_2$, CH$_2$), 30.9 (1C, *C$_7$*H$_2$, CH$_2$), 27.7 (1C, *C$_2$*H$_2$, CH$_2$), 22.0 (1C, *C$_{15}$*H$_2$, CH$_2$), 20.5 (1C, *C$_{11}$*H$_2$, CH$_2$), 19.5 (1C, *C$_{19}$*H$_3$, CH$_3$), 13.7 (1C, *C$_{18}$*H$_3$, CH$_3$); **m/z** (FAB, THIOG) 371 ([M+H]$^+$, 5%), 271 (80); **HRMS** (FAB, THIOG) (Found: [M+H]$^+$, 371.2221. C$_{23}$H$_{31}$O$_4$ requires *m/z*, 371.2222).

O-Propargyl-hydroxylamine hydrochloride[396] 243
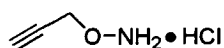
To a solution of *N*-(propargyloxy)phthalimide **242** (1.40 g, 6.97 mmol) in DCM (30 ml) was added methylhydrazine (371 µl, 6.97 mmol) and the solution stirred for 24 h at RT. A colourless precipitate was removed by filtration, washed with DCM and the solid discarded. Ethereal hydrochloric acid (15 ml) was added to the organic layer, leading to the precipitation of a colourless solid. The solid was removed by filtration and washed with ether before recrystalysing from methanol. The product **243** was obtained as a colourless solid (600 mg, 5.58 mmol, 80%); υ_{max} (thin film)/cm^{-1} 3255, 1523, 1025; **^{1}H NMR** δ (360 MHz, DMSO) 11.50-10.80 (2H, m, N*H$_2$*), 4.78 (2H, d, *J* 2.4, C*H$_2$*), 3.90 (1H, t, *J* 2.4, C*H*); **^{13}C NMR** δ (90.7 MHz, DMSO) 81.3 (1C, Q), 76.6 (1C, CH), 61.8 (1C, CH$_2$); *m/z* (FAB, NOBA) 72 ([M+H]$^+$, 76%), 46 (100); **HRMS** (EI, +) (Found: [M]$^+$, 71.0372. C$_3$H$_5$NO requires *m/z*, 71.0371).

All spectroscopic data was in good agreement with that of the literature.[396]

3β-Hydroxy-androst-5-en-17-(*O*-prop-2-ynyl)-oxime 244
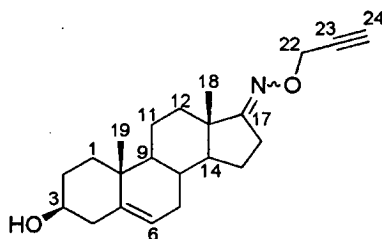
To a solution of DHEA **217** (500 mg, 1.70 mmol) in methanol (40 ml) was added hydroxylamine **243** (194 mg, 1.80 mmol). The reaction was stirred for 48 h at RT before being concentrated *in vacuo*. The resultant solid was dissolved in DCM (20 ml), washed with saturated aq. Na_2CO_3 solution (20 ml) and the aqueous extracted (3 x 30 ml). The combined organics were washed with brine (20 ml), dried ($MgSO_4$) and concentrated *in vacuo*. The product **244** was obtained as a colourless foam (563 mg, 1.65 mmol, 97%); R_f [40% EtOAc/hexane] = 0.45; υ_{max} (neat)/cm^{-1} 3398, 2360, 1045; $[\alpha]_D$ -51.7 (*c* 0.89, CHCl₃); **^{1}H NMR** δ (360 MHz, CDCl₃) 5.33 (1H, d, *J* 5.2, C₆*H*), 4.60 (2H, d, *J* 2.4, C₂₂*H₂*), 3.50 (1H, tt, *J* 11.1, 4.4, C₃*H*), 2.50 (1H, ddd, *J* 19.1, 9.1, 1.9, C₁₆H$_S$H$_T$), 2.45-2.40 (1H, m, C₁₆H$_S$*H$_T$*), 2.42 (1H, t, *J* 2.4, C₂₄*H*), 2.29 (1H, ddd, *J* 13.1, 5.2, 1.9, C₄*H$_E$*H$_F$), 2.23 (1H, dd, *J* 11.1, 1.9, C₄H$_E$*H$_F$*), 2.10-1.93 (2H, m, C₇*H$_G$*H$_H$, C₁₂*H$_M$*H$_N$), 1.87-1.73 (3H, m, C₁*H₄*H$_B$, C₂*H$_C$*H$_D$, C₁₅*H$_Q$*H$_R$), 1.69-1.41 (6H, m, C₂H$_C$*H$_D$*, C₇H$_G$*H$_H$* C₈*H*, C₁₁*H₂*, C₁₂H$_M$*H$_N$*), 1.34 (1H, tt, *J* 12.7, 9.1, C₁₅H$_Q$*H$_R$*), 1.16 (1H, ddd, *J* 10.3, 6.0, 4.4, C₁₄*H*), 1.09 (1H, dd, *J* 13.2, 3.7, C₁*H₄*H$_B$), 1.01 (3H, s, C₁₉*H₃*), 1.04-0.96 (1H, m, C₉*H*), 0.91 (3H, s, C₁₈*H₃*); **^{13}C NMR** δ (90.7 MHz, CDCl₃) 172.0 (1C, *C₁₇*, Q), 141.1 (1C, *C₅*, Q), 121.2 (1C, *C₆*H, CH), 80.3 (1C, *C₂₃*, Q), 74.1 (1C, *C₂₄*H, CH), 71.6 (1C, *C₃*H, CH), 60.9 (1C, *C₂₂*H₂, CH₂), 54.2 (1C, *C₁₄*H, CH), 50.3 (1C, *C₉*H, CH), 44.2 (1C, *C₁₃*, Q), 42.3 (1C, *C₄*H₂, CH₂), 37.3 (1C, *C₁*H₂, CH₂), 36.7 (1C, *C₁₀*, Q), 34.0 (1C, *C₁₂*H₂, CH₂), 31.6 (1C, *C₂*H₂, CH₂), 31.4 (1C, *C₇*H₂, CH₂), 31.0 (1C, *C₈*H, CH), 26.1 (1C, *C₁₆*H₂, CH₂), 23.4 (1C, *C₁₅*H₂, CH₂), 20.7 (1C, *C₁₁*H₂, CH₂), 19.5 (1C, *C₁₉*H₃, CH₃), 17.1 (1C, *C₁₈*H₃, CH₃); **m/z** (FAB, THIOG) 342 ([M+H]$^+$, 33%); **HRMS** (FAB, THIOG) (Found: [M+H]$^+$, 342.2436. $C_{22}H_{32}NO_2$ requires *m/z*, 342.2433).

tert-Butoxycarbonylaminooxy-acetic acid[383] 245
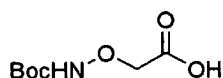
To a solution of aminooxy-acetic acid (500 mg, 4.60 mmol) in dioxane/H_2O (20 ml, 1:1) was added Et_3N (805 µl, 5.80 mmol) and di-*tert*-butyl dicarbonate (1.11 g, 5.10 mmol) and the solution stirred for 7 h at RT. The reaction was quenched with 1% HCl (30 ml) and the aqueous extracted with DCM (3x 30 ml). The combined organics were washed with brine (30 ml), dried ($MgSO_4$) and concentrated *in vacuo*. The product **245** was obtained as a colourless solid (530 mg, 2.50 mmol, 55%); R_f [5% MeOH/DCM] = 0.43; **¹H NMR** δ (360 MHz, $CDCl_3$) 4.26 (2H, s, CH_2), 1.63 (9H, s, 'BuO); **¹³C NMR** δ (90.7 MHz, DMSO) 170.5 (1C, Q), 156.8 (1C, Q), 80.4 (1C, Q), 72.4 (1C, CH_2), 28.3 (3C, CH_3); ***m/z*** (FAB, THIOG) 192 ([M+H]$^+$, 51%), 91 (49); **HRMS** (FAB, THIOG) (Found: [M+H]$^+$, 192.0869. $C_7H_{14}NO_5$ requires *m/z*, 192.0872).

All spectroscopic data was in good agreement with that of the literature.[383]

2-(*tert*-Butoxycarbonylaminooxy)-*N*-prop-2-ynyl-acetamide[397] 246
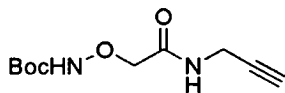
To a solution of acid **245** (1.27 g, 6.60 mmol) in DCM (40 ml) at 0 °C was added DMAP (891 mg, 7.30 mmol) and DIC (1.14 ml, 7.30 mmol), followed by propargyl amine (679 µl, 9.90 mmol). The reaction was stirred for 1 h at 0 °C, before warming to room temperature and stirring for a further 20 h. The reaction was quenched with 1% HCl (70 ml) and the aqueous extracted with DCM (3x 50 ml). The combined organics were washed with brine (60 ml), dried (MgSO$_4$) and concentrated *in vacuo*. The product **246** was obtained as a pale brown wax (1.25 g, 5.48 mmol, 83%); **R$_f$** [10% MeOH/DCM] = 0.44; υ_{max} (neat)/cm^{-1} 3299, 1725, 1666; **^{1}H NMR** δ (360 MHz, CDCl$_3$) 8.69-8.57 (1H, m, N*H*), 8.56-8.40 (1H, m, N*H*), 4.28 (2H, s, C*H$_2$*C=O), 4.02 (2H, dd, *J* 5.4, 2.5, NHC*H$_2$*), 2.18 (1H, t, *J* 2.5, C*H*), 1.42 (9H, s, ′BuO); **^{13}C NMR** δ (90.7 MHz, CDCl$_3$) 169.6 (1C, Q), 158.3 (1C, Q), 83.2 (1C, Q), 79.5 (1C, Q), 76.1 (1C, CH$_2$), 71.7 (1C, CH), 28.9 (1C, CH$_2$), 28.4 (3C, CH$_3$); ***m/z*** (FAB, THIOG) 229 ([M+H]$^+$, 25%), 129 (66); **HRMS** (FAB, THIOG) (Found: [M+H]$^+$, 229.1186. C$_{10}$H$_{17}$N$_2$O$_4$ requires *m/z*, 229.1188).

^{1}H spectroscopic data was in good agreement with that of the literature.[397]

2-Aminooxy-*N*-prop-2-ynyl-acetamide hydrochloride 247
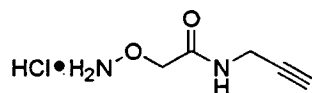
To a solution of carbonate **246** (650 mg, 2.85 mmol) in DCM (40 ml) was added TFA (10 ml). The reaction was stirred for 19 h at RT before being concentrated *in vacuo* to give a golden oil. The salt was subjected to ion exchange chromatography [Dowex Cl⁻; prepared by treating Dowex resin with methanol, then 1% HCl, followed by flushing with methanol until the eluent returned to pH 7] eluting with methanol to give the chloride salt. The residue was chromatographed on silica gel [10% MeOH/DCM] to give **247** as a golden oil (350 mg, 2.71 mmol, 95%); R_f [10% MeOH/DCM] = 0.16; υ_{max} (neat)/cm⁻¹ 3293, 3080, 1668; **¹H NMR** δ (360 MHz, CD$_3$OD) 4.37 (2H, s, C*H$_2$*C=O), 4.04 (2H, d, *J* 2.6, NHC*H$_2$*), 2.60 (1H, t, *J* 2.6, C*H*); **¹³C NMR** δ (90.7 MHz, CD$_3$OD) 171.4 (1C, Q), 80.7 (1C, Q), 74.4 (1C, CH$_2$), 73.0 (1C, CH), 29.6 (1C, CH$_2$); *m/z* (FAB, THIOG) 130 ([M+H]⁺, 59%), 129 ([M]⁺, 67%); **HRMS** (FAB, THIOG) (Found: [M]⁺, 129.0668. C$_5$H$_9$N$_2$O$_2$ requires *m/z*, 129.0664).

3β-Hydroxy-androst-5-en-17-(O-[-N-prop-2-ynyl]acetamidyl)-oxime 248
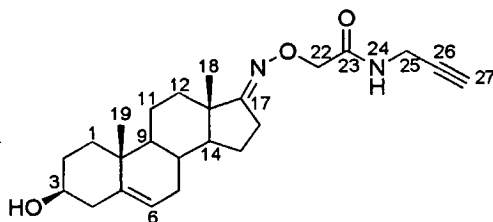
To a solution of DHEA **217** (100 mg, 350 µmol) in methanol (10 ml) was added hydroxylamine **247** (96.0 mg, 890 µmol). The reaction was stirred for 48 h at RT before being concentrated *in vacuo*. The resultant solid was dissolved in DCM (10 ml), washed with saturated aq. Na_2CO_3 solution (10 ml) and the aqueous extracted (3 x 15 ml). The combined organics were washed with brine (10 ml), dried ($MgSO_4$) and concentrated *in vacuo*. The residue was chromatographed on silica gel [50% to 70% EtOAc/hexane] to give **248** as a colourless solid (131 mg, 330 µmol, 94%); R_f [40% EtOAc/hexane] = 0.14; υ_{max} (neat)/cm^{-1} 3398, 3304, 1664, 1528, 1061; $[\alpha]_D$ -46.5 (*c* 0.99, CHCl$_3$); **^{1}H NMR** δ (360 MHz, CDCl$_3$) 6.45 (1H, br t, N$_{24}$H), 5.31 (1H, d, *J* 5.2, C$_6$H), 4.45 (2H, s, C$_{22}$H$_2$), 4.05 (2H, dd, *J* 5.4, 2.5, C$_{25}$H$_2$), 3.47 (1H, tt, *J* 11.1, 4.6, C$_3$H), 2.53 (1H, dd, *J* 19.3, 7.7, C$_{16}$H$_S$H$_T$), 2.43 (1H, dt, *J* 19.3, 8.7, C$_{16}$H$_S$H$_T$), 2.29-2.16 (2H, m, C$_4$H$_2$), 2.21 (1H, t, *J* 2.5, C$_{27}$H), 2.02 (1H, ddd, *J* 12.6, 5.2, 2.6, C$_7$H$_G$H$_H$), 1.90 (1H, dt, *J* 12.0, 3.3, C$_{12}$H$_M$H$_N$), 1.84-1.76 (3H, m, C$_1$H$_A$H$_B$, C$_2$H$_C$H$_D$, C$_{15}$H$_Q$H$_R$), 1.65-1.44 (5H, m, C$_2$H$_C$H$_D$, C$_7$H$_G$H$_H$, C$_8$H, C$_{11}$H$_2$), 1.42-1.32 (2H, m, C$_{12}$H$_M$H$_N$, C$_{15}$H$_Q$H$_R$), 1.15 (1H, ddd, *J* 10.3, 6.1, 4.4, C$_{14}$H), 1.11 (1H, dd, *J* 13.3, 3.3, C$_1$H$_A$H$_B$), 0.99 (3H, s, C$_{19}$H$_3$), 1.03-0.93 (1H, m, C$_9$H), 0.90 (3H, s, C$_{18}$H$_3$); **^{13}C NMR** δ (90.7 MHz, CDCl$_3$) 173.1 (1C, C$_{17}$, Q), 170.2 (1C, C$_{23}$, Q), 141.2 (1C, C$_5$, Q), 120.9 (1C, C$_6$H, CH), 79.4 (1C, C$_{26}$, Q), 72.5 (1C, C$_{22}$H$_2$, CH$_2$), 71.7 (1C, C$_3$H, CH), 71.5 (1C, C$_{27}$H, CH), 54.2 (1C, C$_{14}$H, CH), 50.3 (1C, C$_9$H, CH), 44.4 (1C, C$_{13}$, Q), 42.2 (1C, C$_4$H$_2$, CH$_2$), 37.3 (1C, C$_1$H$_2$, CH$_2$), 36.7 (1C, C$_{10}$, Q), 34.0 (1C, C$_{12}$H$_2$, CH$_2$), 31.6 (1C, C$_2$H$_2$, CH$_2$), 31.4 (1C, C$_7$H$_2$, CH$_2$), 31.4 (1C, C$_8$H, CH), 28.7 (1C, C$_{25}$H$_2$, CH$_2$), 26.2 (1C, C$_{16}$H$_2$, CH$_2$), 23.4 (1C, C$_{15}$H$_2$, CH$_2$), 20.6 (1C, C$_{11}$H$_2$, CH$_2$), 19.5 (1C, C$_{19}$H$_3$, CH$_3$), 17.1 (1C, C$_{18}$H$_3$, CH$_3$); **m/z** (FAB, THIOG) 399 ([M+H]$^+$, 33%), 268 (32); **HRMS** (FAB, NOBA) (Found: [M+H]$^+$, 399.2650. C$_{24}$H$_{35}$N$_2$O$_3$ requires *m/z*, 399.2648).

3β-Sulfonyloxy-androst-5-en-17-(*O*-prop-2-ynyl)-oxime ammonium salt 251
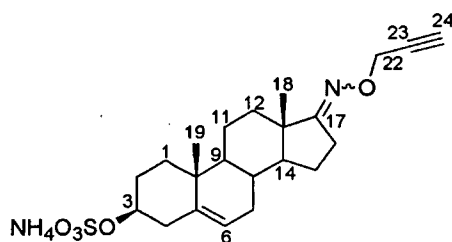
To oxime **244** (50.0 mg, 150 µmol) in pyridine (1 ml) was added sulfur trioxide trimethylamine complex (32.0 mg, 230 µmol). The reaction was stirred for 14 h at RT before being quenched with 5% NH_4OAc solution (2 ml) and the aqueous layer extracted with DCM (2 x 10 ml). The aqueous layer was then purified on a reverse phase C_{18} column, initially washing (6 x 7 ml, 2:1, H_2O:5% NH_4OAc) before eluting the product with methanol. The fractions containing the product were then combined and concentrated *in vacuo*. The product **251** was obtained as a colourless solid (56.0 mg, 128 µmol, 85%); R_f [20% MeOH/DCM] = 0.45; $[α]_D$ -24.5 (*c* 1.02, MeOH); **¹H NMR** δ (360 MHz, CD_3OD) 5.41 (1H, d, *J* 5.2, C_6H), 4.56 (2H, d, *J* 2.4, $C_{22}H_2$), 4.14 (1H, tt, *J* 11.5, 4.9, C_3H), 2.79 (1H, t, *J* 2.4, $C_{24}H$), 2.55 (1H, ddd, *J* 13.1, 4.9, 2.2, $C_4H_EH_F$), 2.49 (1H, dd, *J* 9.0, 1.6, $C_{16}H_SH_T$), 2.44 (1H, t, *J* 9.0, $C_{16}H_SH_T$), 2.39-2.32 (1H, m, $C_4H_EH_F$), 2.10-2.05 (2H, m, $C_7H_GH_H$, $C_2H_CH_D$), 2.02-1.90 (2H, m, $C_1H_AH_B$, $C_{12}H_MH_N$), 1.86-1.78 (1H, m, $C_{15}H_QH_R$), 1.72-1.51 (5H, m, $C_2H_CH_D$, $C_7H_GH_H$, C_8H, $C_{11}H_2$), 1.48-1.34 (2H, m, $C_{12}H_MH_N$, $C_{15}H_QH_R$), 1.24-1.02 (3H, m, $C_1H_AH_B$, C_9H, $C_{14}H$), 1.07 (3H, s, $C_{19}H_3$), 0.94 (3H, s, $C_{18}H_3$); **¹³C NMR** δ (90.7 MHz, CD_3OD) 173.5 (1C, C_{17}, Q), 141.7 (1C, C_5, Q), 122.9 (1C, C_6H, CH), 81.1 (1C, C_{23}, Q), 79.7 (1C, C_3H, CH), 75.2 (1C, $C_{24}H$, CH), 61.6 (1C, $C_{22}H_2$, CH_2), 55.6 (1C, $C_{14}H$, CH), 51.7 (1C, C_9H, CH), 45.3 (1C, C_{13}, Q), 40.4 (1C, C_4H_2, CH_2), 38.4 (1C, C_1H_2, CH_2), 37.8 (1C, C_{10}, Q), 35.2 (1C, $C_{12}H_2$, CH_2), 32.6 (1C, C_8H, CH), 32.4 (1C, C_7H_2, CH_2), 30.0 (1C, C_2H_2, CH_2), 26.9 (1C, $C_{16}H_2$, CH_2), 24.2 (1C, $C_{15}H_2$, CH_2), 21.7 (1C, $C_{11}H_2$, CH_2), 19.8 (1C, $C_{19}H_3$, CH_3), 17.4 (1C, $C_{18}H_3$, CH_3); ***m/z*** (FAB, NOBA) 422 ([M+H]⁺, 32%), 330 (16), 324 (16), 255 (40); **HRMS** (FAB, NOBA) (Found: [M+Na]⁺, 444.1823. $C_{22}H_{31}NO_5SNa$ requires *m/z* 444.1821).

3β-Sulfonyloxy-androst-5-en-17-(*O*-[-*N*-prop-2-ynyl]acetamidyl)-oxime 253
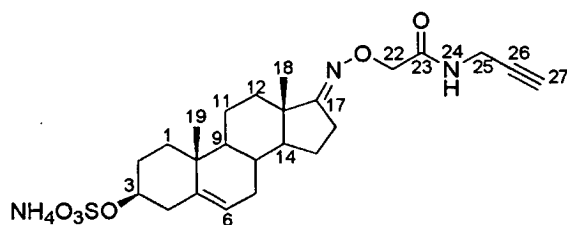
To oxime **248** (100 mg, 250 μmol) in pyridine (2 ml) was added sulfur trioxide trimethylamine complex (56.0 mg, 400 μmol). The reaction was stirred for 24 h at RT before being quenched with 5% NH_4OAc solution (4 ml) and the aqueous extracted with DCM (2 x 10 ml). The aqueous layer was then purified on a reverse phase C_{18} column, initially washing (6 x 7 ml, 2:1, H_2O:5% NH_4OAc) before eluting the product with methanol. The fractions containing the product were then combined and concentrated *in vacuo*. The product **253** was obtained as a colourless solid (123 mg, 240 μmol, 96%); R_f [20% MeOH/DCM] = 0.32; $[α]_D$ -23.0 (*c* 1.22, MeOH); **¹H NMR** δ (360 MHz, CD_3OD) 5.41 (1H, d, *J* 5.0, C_6H), 4.43 (2H, s, $C_{22}H_2$), 4.14 (1H, tt, *J* 11.4, 4.7, C_3H), 4.02 (2H, d, *J* 2.5, $C_{25}H_2$), 2.64 (1H, ddd, *J* 19.3, 7.3, 1.6, $C_{16}H_SH_T$), 2.58 (1H, t, *J* 2.5, $C_{27}H$), 2.56-2.50 (2H, m, $C_{16}H_SH_T$, $C_4H_EH_F$), 2.36 (1H, br t, *J* 11.4, $C_4H_EH_F$), 2.11-2.05 (2H, m, $C_2H_CH_D$, $C_7H_GH_H$,), 1.92 (2H, dt, *J* 12.8, 3.2, $C_1H_AH_B$, $C_{12}H_MH_N$), 1.88-1.81 (1H, m, $C_{15}H_QH_R$), 1.70-1.53 (5H, m, $C_2H_CH_D$, $C_7H_GH_H$, C_8H, $C_{11}H_2$), 1.51-1.36 (2H, m, $C_{12}H_MH_N$, $C_{15}H_QH_R$), 1.29-1.09 (2H, m, $C_1H_AH_B$, $C_{14}H$), 1.06 (3H, s, $C_{19}H_3$), 1.05-1.02 (1H, m, C_9H), 0.95 (3H, s, $C_{18}H_3$); **¹³C NMR** δ (90.7 MHz, CD_3OD) 174.3 (1C, C_{17}, Q), 172.6 (1C, C_{23}, Q), 141.7 (1C, C_5, Q), 122.8 (1C, C_6H, CH), 80.6 (1C, C_{26}, Q), 79.7 (1C, C_3H, CH), 73.2 (1C, $C_{22}H_2$, CH_2), 72.0 (1C, $C_{27}H$, CH), 55.5 (1C, $C_{14}H$, CH), 51.7 (1C, C_9H, CH), 45.4 (1C, C_{13}, Q), 40.4 (1C, C_4H_2, CH_2), 38.4 (1C, C_1H_2, CH_2,), 37.8 (1C, C_{10}, Q), 35.1 (1C, $C_{12}H_2$, CH_2), 32.6 (1C, C_8H, CH), 32.4 (1C, C_7H_2, CH_2), 29.9 (1C, C_2H_2, CH_2), 29.1 (1C, $C_{25}H_2$, CH_2), 26.9 (1C, $C_{16}H_2$, CH_2), 24.3 (1C, $C_{15}H_2$, CH_2), 21.6 (1C, $C_{11}H_2$, CH_2), 19.7 (1C, $C_{19}H_3$, CH_3), 17.4 (1C, $C_{18}H_3$, CH_3); *m/z* (FAB, THIOG) 479 ([M+H]⁺, 11%); **HRMS** (FAB, THIOG) (Found: [M]⁺, 479.2211. $C_{24}H_{35}N_2O_6S$ requires *m/z*, 479.2216).

1-(*N*-Dansyl-2-aminoeth-1-yl)-4-(3''β-Hydroxy-androst-5''-en-17''-(*O*-[-*N*-methyl]acetamidyl)-oxime-1*H*-[1,2,3] triazole 254
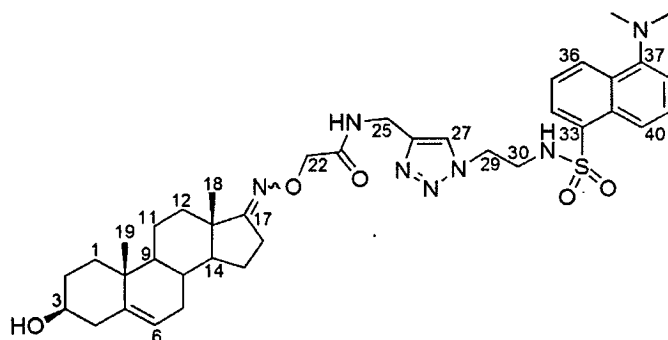
To dansyl azide **189** (25.0 mg, 63.0 µmol) in H_2O:'BuOH (3 ml, 1:1) was added oxime **248** (23.0 mg, 70.0 µmol), followed by copper(II) sulfate (2.0 mg, 10 mol%) and sodium ascorbate solution (20.0 µl, 1 M solution, 20 mol%). The solution was stirred for 15 h at RT. The solution was concentrated *in vacuo*, and the residue was purified by flash chromatography [5% MeOH/DCM] to give **254** as a pale green wax (43.0 mg, 60.0 µmol, 95%); R_f [5% MeOH/DCM] = 0.16; λ_{max} (H_2O)/nm 342; ^{1}**H NMR** δ (360 MHz, CDCl$_3$) 8.52 (1H, d, *J* 8.5, C$_{34}$*H*), 8.21 (1H, dd, *J* 7.3, 1.3, C$_{36}$*H*), 8.20 (1H, br d, *J* 8.7, C$_{40}$*H*), 7.52-7.44 (2H, m, C$_{35}$*H*, C$_{39}$*H*), 7.45 (1H, s, C$_{27}$*H*), 7.15 (1H, d, *J* 7.6, C$_{38}$*H*), 7.01 (1H, t, *J* 5.9, N$_{31}$*H*), 6.54 (1H, t, *J* 6.3, N$_{24}$*H*), 5.30 (1H, d, *J* 5.2, C$_6$*H*), 4.45 (2H, s, C$_{22}$*H$_2$*), 4.43 (2H, d, *J* 6.3, C$_{25}$*H$_2$*), 4.33 (2H, t, *J* 5.5, C$_{29}$*H$_2$*), 3.53-3.44 (1H, m, C$_3$*H*), 3.42 (2H, q, *J* 6.2, C$_{30}$*H$_2$*), 2.86 (6H, s, N*Me$_2$*), 2.48-2.32 (2H, m, C$_{16}$*H$_2$*), 2.30-2.16 (2H, m, C$_4$*H$_2$*), 1.95 (1H, ddd, *J* 11.9, 5.1, 2.4, C$_7$H$_G$*H$_H$*), 1.85-1.77 (3H, m, C$_1$*H$_A$*H$_B$, C$_2$H$_C$*H$_D$*, C$_{12}$*H$_M$*H$_N$), 1.66-1.40 (6H, m, C$_2$H$_C$*H$_D$*, C$_7$H$_G$*H$_H$*, C$_8$*H*, C$_{11}$*H$_2$*, C$_{15}$H$_Q$*H$_R$*), 1.33-1.19 (2H, m, C$_{12}$H$_M$*H$_N$*, C$_{15}$H$_Q$*H$_R$*), 1.09-1.00 (2H, m, C$_1$H$_A$*H$_B$*, C$_{14}$*H*), 0.97 (3H, s, C$_{19}$*H$_3$*), 0.97-0.91 (1H, m, C$_9$*H*), 0.82 (3H, s, C$_{18}$*H$_3$*); ^{13}**C NMR** δ (90.7 MHz, CDCl$_3$) 174.0 (1C, *C$_{17}$*, Q), 171.6 (1C, *C$_{23}$*, Q), 153.0 (1C, *Ar*, Q), 145.4 (1C, *C$_{26}$*, Q), 142.0 (1C, *C$_5$*, Q), 135.6 (1C, *Ar*, Q), 131.8 (1C, *C$_{34}$*H, CH), 130.9 (1C, *Ar*, Q), 130.5 (1C, *C$_{36}$*H, CH), 130.4 (1C, *Ar*, Q), 129.6 (1C, *C$_{39}$*H, CH), 124.8 (1C, *C$_{27}$*H, CH), 124.1 (1C, *C$_{35}$*H, CH), 122.0 (1C, *C$_6$*H, CH), 119.7 (1C, *C$_{40}$*H, CH), 116.4 (1C, *C$_{38}$*H, CH,), 73.5 (1C, *C$_{22}$*H$_2$, CH$_2$), 72.6 (1C, *C$_3$*H, CH), 54.9 (1C, *C$_{14}$*H, CH), 51.4 (1C, *C$_{29}$*H$_2$, CH$_2$), 51.1 (1C, *C$_9$*H, CH), 46.4 (2C, N*Me$_2$*, CH$_3$), 45.2 (1C, *C$_{13}$*, Q), 43.5 (1C, *C$_{30}$*H$_2$, CH$_2$), 43.2 (1C, *C$_4$*H$_2$, CH$_2$),

38.2 (1C, C_1H_2, CH_2), 37.6 (1C, C_{10}, Q), 35.1 (1C, $C_{12}H_2$, CH_2), 34.8 (1C, $C_{25}H_2$, CH_2), 32.6 (1C, C_2H_2, CH_2), 32.2 (1C, C_7H_2, CH_2), 32.2 (1C, C_8H, CH), 27.1 (1C, $C_{16}H_2$, CH_2), 24.2 (1C, $C_{15}H_2$, CH_2), 21.5 (1C, $C_{11}H_2$, CH_2), 20.4 (1C, $C_{19}H_3$, CH_3), 18.0 (1C, $C_{18}H_3$, CH_3); *m/z* (FAB, THIOG) 718 ([M+H]$^+$, 27%), 433 (26), 217 (48), 91 (100); **HRMS** (FAB, THIOG) (Found: [M+H]$^+$, 718.3746. $C_{38}H_{52}N_7O_5S$ requires *m/z*, 718.3751).

1-(*N*-Dansyl-2-aminoeth-1-yl)-4-(3'β-(methyloxycarbonyloxy)-androst-5'-en-17'-one-1*H*-[1,2,3] triazole 255
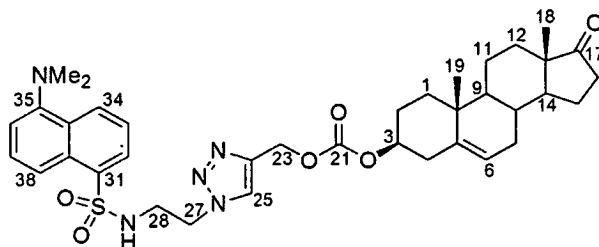
To dansyl azide **189** (19.0 mg, 54.0 µmol) in H_2O:^{t}BuOH (4 ml, 1:1) was added oxime **241** (20.0 mg, 60.0 µmol), followed by copper(II) sulfate (2.0 mg, 10 mol%) and sodium ascorbate solution (20.0 µl, 1 M solution, 20 mol%). The solution was stirred for 17 h at RT. The solution was concentrated in vacuo, and the residue was purified by flash chromatography [5% MeOH/DCM] to give **255** as a pale green wax (30.0 mg, 44.0 µmol, 81%); **R$_f$** [5% MeOH/DCM] = 0.45; λ_{max} (H_2O)/nm 341; **^{1}H NMR** δ (360 MHz, CDCl$_3$) 8.56 (1H, d, *J* 8.5, C$_{32}$*H*), 8.24 (1H, dd, *J* 7.3, 1.0, C$_{34}$*H*), 8.18 (1H, d, *J* 8.5, C$_{38}$*H*), 7.56-7.51 (2H, m, C$_{33}$*H*, C$_{37}$*H*), 7.54 (1H, s, C$_{25}$*H*), 7.19 (1H, br d, *J* 7.5, C$_{36}$*H*), 5.66 (1H, t, *J* 6.2, N$_{29}$*H*), 5.43 (1H, d, *J* 5.0, C$_6$*H*), 5.17 (2H, s, C$_{23}$*H$_2$*), 4.49 (1H, tt, *J* 11.3, 5.0, C$_3$*H*), 4.40 (2H, t, *J* 5.6, C$_{27}$*H$_2$*), 3.44 (2H, q, *J* 6.1, C$_{28}$*H$_2$*), 2.89 (6H, s, N*Me$_2$*), 2.46 (1H, dd, *J* 19.2, 9.1, C$_{16}$H$_S$H$_T$), 2.44-2.31 (2H, m, C$_4$*H$_2$*), 2.14-2.07 (1H, m, C$_7$*H$_E$*H$_F$), 2.08 (1H, dd, *J* 19.2, 9.1, C$_{16}$H$_S$*H$_T$*), 1.99-1.90 (2H, m, C$_2$H$_C$*H$_D$*, C$_{15}$H$_Q$*H$_R$*), 1.88-1.86 (1H, m, C$_1$*H$_A$*H$_B$), 1.83 (1H, br t, *J* 3.3, C$_8$*H*), 1.72-1.60 (4H, m, C$_2$H$_C$*H$_D$*, C$_7$H$_E$*H$_F$*, C$_{11}$*H$_K$*H$_L$, C$_{12}$*H$_M$*H$_N$), 1.58-1.50 (1H, m, C$_{15}$H$_Q$*H$_R$*), 1.48-1.43 (1H, m, C$_{11}$H$_K$*H$_L$*), 1.33-1.26 (2H, m, C$_{12}$H$_M$*H$_N$*, C$_{14}$*H*), 1.14 (1H, td, *J* 13.5, 3.4, C$_1$H$_A$*H$_B$*), 1.03 (3H, s, C$_{19}$*H$_3$*), 1.06-0.98 (1H, m, C$_9$*H*), 0.89 (3H, s, C$_{18}$*H$_3$*); **^{13}C NMR** δ (90.7 MHz, CDCl$_3$) 221.3 (1C, *C$_{17}$*, Q), 154.4 (1C, *C$_{21}$*, Q), 152.3 (1C, *Ar*, Q), 142.3 (1C, *C$_{24}$*, Q), 139.7 (1C, *C$_5$*, Q), 134.4 (1C, *Ar*, Q), 131.1 (1C, *C$_{32}$*H, CH), 130.1 (1C, *Ar* Q), 129.7 (1C, *C$_{34}$*H, CH), 129.6 (1C, *Ar*, Q), 128.9 (1C, *C$_{37}$*H, CH), 125.2 (1C, *C$_{25}$*H, CH), 123.3 (1C, *C$_{33}$*H, CH), 122.4 (1C, *C$_6$*H, CH), 118.6 (1C, *C$_{38}$*H, CH), 115.6 (1C, *C$_{36}$*H, CH), 78.2 (1C, *C$_3$*H, CH), 60.6 (1C, *C$_{23}$*H$_2$, CH$_2$), 51.9 (1C, *C$_{14}$*H, CH), 50.4 (1C, *C$_{27}$*H$_2$, CH$_2$), 50.3 (1C, *C$_9$*H, CH), 47.7 (1C, *C$_{13}$*, Q), 45.6 (2C, N*Me$_2$*, CH$_3$), 42.8 (1C, *C$_{28}$*H$_2$, CH$_2$), 38.1 (1C, *C$_4$*H$_2$, CH$_2$), 36.9

(1C, C_1H$_2$, CH$_2$), 36.8 (1C, C_{10}, Q), 36.0 (1C, C_{16}H$_2$, CH$_2$), 31.6 (1C, C_8H, CH), 31.5 (1C, C_{12}H$_2$, CH$_2$), 30.9 (1C, C_7H$_2$, CH$_2$), 27.8 (1C, C_2H$_2$, CH$_2$), 22.0 (1C, C_{15}H$_2$, CH$_2$), 20.5 (1C, C_{11}H$_2$, CH$_2$), 19.5 (1C, C_{19}H$_3$, CH$_3$), 13.7 (1C, C_{18}H$_3$, CH$_3$); *m/z* (FAB, THIOG) 690 ([M+H]$^+$, 14%), 226 (82), 91 (84); **HRMS** (FAB, THIOG) (Found: [M+H]$^+$, 690.3325. C$_{37}$H$_{48}$N$_5$O$_6$S requires *m/z*, 690.3325).

5.3 General Biological Experimental

'Complete' Protease Inhibitor tablet was obtained from Roche (Lewes, Sussex, UK), cell culture media from Gibco (Paisley, UK), precast Bis-Tris gradient SDS-polyacylamide gels, running buffer and transfer buffer from Invitrogen (Paisley, UK) and enhanced chemiluminescence (ECL) reagents from Amersham (Bucks, UK). Phospho-specific antibodies that recognize JNK1/2 phosphorylated at Thr183, or unphosphorylated JNK1/2 were purchased from Biosource (Nivelles, Belgium), while horseradish peroxidase-conjugated secondary antibodies were from Pierce (Cheshire, UK).

WesternBlotting

Cell culture and stimulation

Human embryonic kidney (HEK) 293 cells were cultured at 37 °C, 95% air / 5% CO_2, in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine. Anisomycin was dissolved at 10 mg/ml (38 mmol l^{-1}) in DMSO, while the other compounds were dissolved in DMSO at 38 mmol l^{-1}. Cells (9 ml of cell culture) were incubated with the library members by addition of 9 µl of anisomycin solution, anisomycin analogue in DMSO, or DMSO as a control.

Cell lysis

After stimulation for 30 min the media was aspirated and the cells lysed in 50 mM Tris/HCl, pH 7.5, containing 1 mM EDTA, 1 mM EGTA, 1 mM sodium orthovanadate, 10 mM sodium β-glycerophosphate, 5 mM sodium pyrophosphate, 50 mM sodium fluoride, 0.27 M sucrose, 1% (v/v) Triton X-100, 0.1 % (v/v) 2-mercaptoethanol and 'Complete' protease inhibitor cocktail (one tablet per 50 ml). Lysates were centrifuged at 13000 g for 10 min at 4 °C and the supernatants (termed 'cell extract') were removed. Protein concentrations were determined according to the method of Bradford.[255]

Immunoblotting[246, 258]

Samples were denatured in SDS, run on polyacylamide gels and transferred to nitrocellulose membranes. The membranes were incubated for 1 h at room temperature in 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.2% (v/v) Tween and 5% (w/v) skimmed milk powder. Primary antibodies were added to 10 ml of the previous buffer and incubated at 4 °C overnight. The membranes were then washed four times with buffer (5 min per wash) to remove the excess primary antibody. The membranes were then incubated with the secondary antibody at room temperature for 1 h. After washing six times with buffer to remove the excess secondary antibody (5 min per wash), immunoreactive proteins were visualized _via_ enhanced chemiluminescence reagent according to the manufacturer's instructions.

FACS analysis

Cells (cultured as above, 350 μl of cell culture) were incubated with molecular probe 191 (22-162 μM). After stimulation for 30 min the media was aspirated and the cells washed with PBS buffer (350 μl). The PBS buffer was aspirated, replaced with tripsin (200 μl) and the cells incubated for 5 min. Media (200 μl) was added to the cells and the combined solution was centrifuged at 1000 g for 4 min. The media was aspirated and the cells re-suspended in PBS - 2% serum (200 μl). The cells were analyzed on a DakoCytomation MoFlo MLS high speed sorter using a multi-line UV laser (350-360 nm).

Confocal microscopy

Cells (cultured as above, 1.5 ml of cell culture) were incubated with molecular probe 191 (109 μM) or dansyl azide 189 (109 μM) as a control. After stimulation for 30 min the media was aspirated and the cells washed with PBS buffer (1.5 ml). The cells were fixed using 4% paraformaldehyde in PBS (1.2 ml). Confocal microscopy experiments were carried out using a Leica DM IRE2 microscope with a UV laser; cells were analyzed at 20-fold and 63-fold magnification.

REFERENCES

[1] D. E. Metzeler, 'Biochemistry: The Chemical Reactions of Living Cells', Harcourt, 2001, and references therein.

[2] J. E. Cronan, *Journal of Biological Chemistry*, 1990, **265**, 10327-10333.

[3] A. Chapman-Smith and J. E. Cronan, *Journal of Nutrition*, 1999, **129**, 477S-484S.

[4] A. Chapman-Smith and J. E. Cronan, *Trends in Biochemical Sciences*, 1999, **24**, 359-363.

[5] A. Leondelrio, D. Leclerc, B. Akerman, N. Wakamatsu and R. A. Gravel, *Proceedings of the National Academy of Sciences of the United States of America*, 1995, **92**, 4626-4630.

[6] Y. Suzuki, Y. Aoki, Y. Ishida, Y. Chiba, A. Iwamatsu, T. Kishino, N. Niikawa, Y. Matsubara and K. Narisawa, *Nature Genetics*, 1994, **8**, 122-128.

[7] A. Leondelrio and R. A. Gravel, *Journal of Biological Chemistry*, 1994, **269**, 22964-22968.

[8] J. E. Cronan and G. L. Waldrop, *Progress in Lipid Research*, 2002, **41**, 407-435.

[9] P. V. Attwood and J. C. Wallace, *Accounts of Chemical Research*, 2002, **35**, 113-120.

[10] R. N. Perham, *Annual Review of Biochemistry*, 2000, **69**, 961-1004.

[11] M. Howarth, K. Takao, Y. Hayashi and A. Y. Ting, *Proceedings of the National Academy of Sciences of the United States of America*, 2005, **102**, 7583-7588.

[12] S. Bhuniya, S. M. Park and B. H. Kim, *Organic Letters*, 2005, **7**, 1741-1744.

[13] O. C. Boerman, F. G. van Schaijk, W. J. G. Oyen and F. H. M. Corstens, *Journal of Nuclear Medicine*, 2003, **44**, 400-411.

[14] E. A. Bayer and M. Wilchek, *Journal of Chromatography*, 1990, **510**, 3-11.

[15] L. Pugliese, A. Coda, M. Malcovati and M. Bolognesi, *Journal of Molecular Biology*, 1993, **231**, 698-710.

[16] A. Pahler, W. A. Hendrickson, M. A. Kolks, C. E. Argarana and C. R. Cantor, *Journal of Biological Chemistry*, 1987, **262**, 13933-13937.

[17] L. A. Klumb, V. Chu and P. S. Stayton, *Biochemistry*, 1998, **37**, 7657-7663.

[18] A. Chilkoti and P. S. Stayton, *Journal of the American Chemical Society*, 1995, **117**, 10622-10628.

[19] B. A. Katz, *Journal of Molecular Biology*, 1997, **274**, 776-800.

[20] S. Freitag, I. L. Trong, L. Klumb, P. S. Stayton and R. E. Stenkamp, *Protein Science*, 1997, **6**, 1157-1166.

[21] K. Kaiser, M. Marek, T. Haselgrubler, H. Schindler and H. J. Gruber, *Bioconjugate Chemistry*, 1997, **8**, 545-551.

[22] Pierce, 'Avidin-Biotin Products', Pierce Biotechnology Inc., 2005.

[23] M. D. Savage, G. Mattson, S. Desai, G. W. Nielander, S. Morgensen and E. J. Conklin, 'Avidin-Biotin Chemistry: A Handbook', Pierce, 1992.

[24] M. Shimkus, J. Levy and T. Herman, *Proceedings of the National Academy of Sciences of the United States of America*, 1985, **82**, 2593-2597.

[25] W. J. Jahng, C. David, N. Nesnas, K. Nakanishi and R. R. Rando, *Biochemistry*, 2003, **42**, 6159-6168.

26   U. Kempin, Y. Kanaoka and Y. Hatanaka, *Heterocycles*, 1998, **49**, 465-468.

27   K. Fang, M. Hashimoto, S. Jockusch, N. J. Turro and K. Nakanishi, *Journal of the American Chemical Society*, 1998, **120**, 8543-8544.

28   J. Olejnik, S. Sonar, E. Krzymanskaolejnik and K. J. Rothschild, *Proceedings of the National Academy of Sciences of the United States of America*, 1995, **92**, 7590-7594.

29   M. Brinkley, *Bioconjugate Chemistry*, 1992, **3**, 2-13.

30   G. Means and R. Feeney, *Bioconjugate Chemistry*, 1990, **1**, 2-12.

31   M. Wilchek and E. A. Bayer, *Trends in biochemical sciences* 1989, **14**, 408-412.

32   W. A. Volkert and T. J. Hoffman, *Chemical Reviews*, 1999, **99**, 2269-2292.

33   D. S. Wilbur, *Bioconjugate Chemistry*, 1992, **3**, 433-470.

34   F. Albericio, *Current Opinion in Chemical Biology*, 2004, **8**, 211-221.

35   S. Y. Han and Y. A. Kim, *Tetrahedron*, 2004, **60**, 2447-2467.

36   D. I. Angus, M. J. Kiefel and M. von Itzstein, *Bioorganic & Medicinal Chemistry*, 2000, **8**, 2709-2718.

37   T. Ikunaga, H. Ikeda and A. Ueno, *Chemistry-A European Journal*, 1999, **5**, 2698-2704.

38   N. Nesnas, R. R. Rando and K. Nakanishi, *Tetrahedron*, 2002, **58**, 6577-6584.

39   M. Schelhaas, E. Nagele, N. Kuder, B. Bader, J. Kuhlmann, A. Wittinghofer and H. Waldmann, *Chemistry-A European Journal*, 1999, **5**, 1239-1252.

40   M. B. Parrott and M. A. Barry, *Biochemical and Biophysical Research Communications*, 2001, **281**, 993-1000.

41   M. B. Parrott and M. A. Barry, *Molecular Therapy*, 2000, **1**, 96-104.

42   J. E. Cronan and K. E. Reed, in 'Biotinylation of proteins in vivo: A useful posttranslational modification for protein analysis', 2000.

43   S. Duffy, K. L. Tsao and D. S. Waugh, *Analytical Biochemistry*, 1998, **262**, 122-128.

44   K. E. Reed and J. E. Cronan, *Journal of Biological Chemistry*, 1991, **266**, 11425-11428.

45   E. Schwarz, D. Oesterhelt, H. Reinke, K. Beyreuther and P. Dimroth, *Journal of Biological Chemistry*, 1988, **263**, 9640-9645.

46   P. A. Smith, B. C. Tripp, E. A. DiBlasio-Smith, Z. J. Lu, E. R. LaVallie and J. M. McCoy, *Nucleic Acids Research*, 1998, **26**, 1414-1420.

47   J. Tucker and R. Grisshammer, *Biochemical Journal*, 1996, **317**, 891-899.

48   K. L. Tsao, B. DeBarbieri, H. Michel and D. S. Waugh, *Gene*, 1996, **169**, 59-64.

49   H. Tatsumi, S. Fukuda, M. Kikuchi and Y. Koyama, *Analytical Biochemistry*, 1996, **243**, 176-180.

50   D. Beckett, E. Kovaleva and P. J. Schatz, *Protein Science*, 1999, **8**, 921-929.

51   P. J. Schatz, *Bio-Technology*, 1993, **11**, 1138-1143.

52   E. de Boer, P. Rodriguez, E. Bonte, J. Krijgsveld, E. Katsantoni, A. Heck, F. Grosveld and J. Strouboulis, *Proceedings of the National Academy of Sciences of the United States of America*, 2003, **100**, 7480-7485.

53   I. Chen and A. Y. Ting, *Current Opinion in Biotechnology*, 2005, **16**, 35-40.

54   E. Strachan, A. K. Mallia, J. M. Cox, B. Antharavally, S. Desai, L. Sykaluk, V. O'Sullivan and P. A. Bell, *Journal of Molecular Recognition*, 2004, **17**, 268-276.

55   J. V. Staros, *Biochemistry*, 1982, **21**, 3950-3955.

56   S. B. Scheurer, C. Roesli, D. Neri and G. Elia, *Proteomics*, 2005, **5**, 3035-3039.

57   S. L. Hussey, S. S. Muddana and B. R. Peterson, *Journal of the American Chemical Society*, 2003, **125**, 3692-3693.

58   S. A. Kalovidouris, C. I. Gama, L. W. Lee and L. C. Hsieh-Wilson, *Journal of the American Chemical Society*, 2005, **127**, 1340-1341.

59   T. Honda, T. Janosik, Y. Honda, J. Han, K. T. Liby, C. R. Williams, R. D. Couch, A. C. Anderson, M. B. Sporn and G. W. Gribble, *Journal of Medicinal Chemistry*, 2004, **47**, 4923-4932.

60   W. Frick, A. Bauer-Schafer, J. Bauer, F. Girbig, D. Corsiero, H. Heuer and W. Kramer, *Bioorganic & Medicinal Chemistry*, 2003, **11**, 1639-1642.

61   T. Sambaiah, K. Y. King, S. C. Tsay, N. W. Mei, S. Hakimclahi, Y. K. Lai, C. H. Lieu and J. R. Hwu, *European Journal of Medicinal Chemistry*, 2002, **37**, 349-353.

62   M. S. Shearman, D. Beher, E. E. Clarke, H. D. Lewis, T. Harrison, P. Hunt, A. Nadin, A. L. Smith, G. Stevenson and J. L. Castro, *Biochemistry*, 2000, **39**, 8698-8704.

63   Y. M. Li, M. Xu, M. T. Lai, Q. Huang, J. L. Castro, J. DiMuzio-Mower, T. Harrison, C. Lellis, J. L. Nadin, J. G. Neduvelil, R. B. Register, M. K. Sardana, M. S. Shearman, A. L. Smith, X. P. Shi, K. C. Yin, J. A. Shafer and S. J. Gardell, *Nature*, 2000, **405**, 689-694.

64   M. Daghish, L. Hennig, M. Findeisen, S. Giesa, F. Schumer, H. Hennig, A. G. Beck-Sickinger and P. Welzel, *Angewandte Chemie-International Edition*, 2002, **41**, 2293-2297.

65   N. Khidekel, S. Arndt, N. Lamarre-Vincent, A. Lippert, K. G. Poulin-Kerstien, B. Ramakrishnan, P. K. Qasba and L. C. Hsieh-Wilson, *Journal of the American Chemical Society*, 2003, **125**, 16162-16163.

66   Y. C. Jeong, R. Sangaiah, J. Nakamura, B. F. Pachkowski, A. Ranasinghe, A. Gold, L. M. Ball and J. A. Swenberg, *Chemical Research in Toxicology*, 2005, **18**, 51-60.

67   M. Lacorn, K. Fleischer, S. Willig, S. Gremmel, H. Steinhart and R. Claus, *Journal of Immunological Methods*, 2005, **297**, 225-236.

68   C. Gaul, J. T. Njardarson, D. Shan, D. C. Dorn, K. D. Wu, W. P. Tong, X. Y. Huang, M. A. S. Moore and S. J. Danishefsky, *Journal of the American Chemical Society*, 2004, **126**, 11326-11337.

69   E. J. Woo, C. M. Starks, J. R. Carney, R. Arslanian, L. Cadapan, S. Zavala and P. Licari, *Journal of Antibiotics*, 2002, **55**, 141-146.

70   H. Nakamura, Y. Takahashi, H. Naganawa, K. Nakae, M. Imoto, M. Shiro, K. Matsumura, H. Watanabe and T. Kitahara, *Journal of Antibiotics*, 2002, **55**, 442-444.

71   K. Nakae, Y. Yoshimoto, M. Ueda, T. Sawa, Y. Takahashi, H. Naganawa, T. Takeuchi and M. Imoto, *Journal of Antibiotics*, 2000, **53**, 1228-1230.

72   K. Nakae, Y. Yoshimoto, T. Sawa, Y. Homma, M. Hamada, T. Takeuchi and M. Imoto, *Journal of Antibiotics*, 2000, **53**, 1130-1136.

73   T. Korpimaki, J. Rosenberg, P. Virtanen, T. Karskela, U. Lamminmaki, M. Tuomola, M. Vehniainen and P. Saviranta, *Journal of Agricultural and Food Chemistry*, 2002, **50**, 4194-4201.

74   M. G. Baek and R. Roy, *Bioorganic & Medicinal Chemistry*, 2001, **9**, 3005-3011.

75   S. Bay, O. Berthier-Vergnes, V. Biberovic and D. Cantacuzene, *Carbohydrate Research*, 1997, **303**, 25-31.

76   C. H. Grün, S. J. van Vliet, W. E. C. M. Schiphorst, C. M. C. Bank, S. Meyer, I. van Die and Y. van Kooyk, *Analytical Biochemistry*, 2006, **354**, 54–63.

77   M. M. Spence, E. J. Ruiz, S. M. Rubin, T. J. Lowery, N. Winssinger, P. G. Schultz, D. E. Wemmer and A. Pines, *Journal of the American Chemical Society*, 2004, **126**, 15287-15294.

78   T. J. Lowery, S. Garcia, L. Chavez, E. J. Ruiz, T. Wu, T. Brotin, J. P. Dutasta, D. S. King, P. G. Schultz, A. Pines and D. E. Wemmer, *ChemBioChem*, 2006, **7**, 65-73.

79   A. Okamoto, K. Tanabe, T. Inasaki and I. Saito, *Angewandte Chemie-International Edition*, 2003, **42**, 2502-2504.

80   M. Nakamura, T. Kakuda, Y. Oba, M. Ojika and H. Nakamura, *Bioorganic & Medicinal Chemistry*, 2003, **11**, 3077-3082.

81   A. Okamoto, T. Taiji, K. Tainaka and. I. Saito, *Bioorganic & Medicinal Chemistry Letters*, 2002, **12**, 1895-1896.

82   K. E. Jaeger and M. T. Reetz, *Current Opinion in Chemical Biology*, 2000, **4**, 68-73.

83   A. Jaschke and B. Seelig, *Current Opinion in Chemical Biology*, 2000, **4**, 257-262.

84   B. Seelig and A. Jaschke, *Chemistry & Biology*, 1999, **6**, 167-176.

85   B. Seelig, S. Keiper, F. Stuhlmann and A. Jaschke, *Angewandte Chemie-International Edition*, 2000, **39**, 4576-4579.

86   O. Spertini, A. S. Cordey, N. Monai, L. Giuffre and M. Schapira, *Journal of Cell Biology*, 1996, **135**, 523-531.

87   P. Durieux, J. Fernandez-Carneado and G. Tuchscherer, *Tetrahedron Letters*, 2001, **42**, 2297-2299.

88   S. J. Dwight, B. S. Gaylord, J. W. Hong and G. C. Bazan, *Journal of the American Chemical Society*, 2004, **126**, 16850-16859.

89   P. E. Dawson, T. W. Muir, I. Clark-Lewis and S. B. H. Kent, *Science*, 1994, **266**, 776-779.

90   P. E. Dawson and S. B. H. Kent, *Annual Review of Biochemistry*, 2000, **69**, 923-960.

91   R. Y. P. Lue, G. Y. J. Chen, Y. Hu, Q. Zhu and S. Q. Yao, *Journal of the American Chemical Society*, 2004, **126**, 1055-1062.

92   M. L. Lesaicherre, R. Y. P. Lue, G. Y. J. Chen, Q. Zhu and S. Q. Yao, *Journal of the American Chemical Society*, 2002, **124**, 8768-8769.

93   D. S. Y. Yeo, R. Srinivasan, G. Y. J. Chen and S. Q. Yao, *Chemistry-A European Journal*, 2004, **10**, 4664-4672.

94   L. P. Tan, R. Y. P. Lue, G. Y. J. Chen and S. Q. Yao, *Bioorganic & Medicinal Chemistry Letters*, 2004, **14**, 6067-6070.

95   P. F. van Swieten, M. A. Leeuwenburgh, B. M. Kessler and H. S. Overkleeft, *Organic & Biomolecular Chemistry*, 2005, **3**, 20-27.

96   J. A. Prescher and C. R. Bertozzi, *Nature Chemical Biology*, 2005, **1**, 13-21.

97   H. C. Kolb and K. B. Sharpless, *Drug Discovery Today*, 2003, **8**, 1128-1137.

98   H. C. Kolb, M. G. Finn and K. B. Sharpless, *Angewandte Chemie-International Edition*, 2001, **40**, 2004-2021.

99   G. C. Adam, C. D. Vanderwal, E. J. Sorensen and B. F. Cravatt, *Angewandte Chemie-International Edition*, 2003, **42**, 5480-5484.

100   C. D. Vanderwal, D. A. Vosburg, S. Weiler and E. J. Sorensen, *Journal of the American Chemical Society*, 2003, **125**, 5393-5407.

101   D. A. Vosburg, C. D. Vanderwal and E. J. Sorensen, *Journal of the American Chemical Society*, 2002, **124**, 4552-4553.

102   C. D. Vanderwal, D. A. Vosburg and E. J. Sorensen, *Organic Letters*, 2001, **3**, 4307-4310.

103   A. J. Link, M. L. Mock and D. A. Tirrell, *Current Opinion in Biotechnology*, 2003, **14**, 603-609.

104   A. J. Link and D. A. Tirrell, *Journal of the American Chemical Society*, 2003, **125**, 11164-11165.

105   D. C. Dieterich, A. J. Link, J. Graumann, D. A. Tirrell and E. M. Schuman, *Proceedings of the National Academy of Sciences of the United States of America*, 2006, **103**, 9482-9487.

106   N. J. Agard, J. A. Prescher and C. R. Bertozzi, *Journal of the American Chemical Society*, 2004, **126**, 15046-15047.

107   G. Wittig and A. Krebs, *Chemische Berichte-Recueil*, 1961, **94**, 3260-3275.

108   A. J. Link, M. K. S. Vink, N. J. Agard, J. A. Prescher, C. R. Bertozzi and D. A. Tirrell, *Proceedings of the National Academy of Sciences of the United States of America*, 2006, **103**, 10180-10185.

109   J. M. Baskin, N. J. Agard, J. A. Prescher, A. Lo and C. R. Bertozzi, *Glycobiology*, 2005, **15**, 1201-1202.

110   C. Corona, B. K. Bryant and J. B. Arterburn, *Organic Letters*, 2006, **8**, 1883-1886.

111   A. Bogusiewcz, N. I. Mock and D. M. Mock, *Analytical Biochemistry*, 2004, **331**, 260-266.

112   R. Singh and E. K. Maloney, *Analytical Biochemistry*, 2002, **304**, 147-156.

113   H. C. Hang, C. Yu, M. R. Pratt and C. R. Bertozzi, *Journal of the American Chemical Society*, 2004, **126**, 6-7.

114   D. J. Vocadlo and C. R. Bertozzi, *Angewandte Chemie-International Edition*, 2004, **43**, 5338-5342.

115   P. C. Lin, S. H. Ueng, M. C. Tseng, J. L. Ko, K. T. Huang, S. C. Yu, A. K. Adak, Y. J. Chen and C. C. Lin, *Angewandte Chemie-International Edition*, 2006, **45**, 4286-4290.

116   H. Nandivada, H. Y. Chen, L. Bondarenko and J. Lahann, *Angewandte Chemie-International Edition*, 2006, **45**, 3360-3363.

117   B. A. Sobin and F. W. Tanner, *Journal of the American Chemical Society*, 1954, **76**, 4053-4053.

118   Y. Hosoya, T. Kameyama, H. Naganawa, Y. Okami and T. Takeuchi, *Journal of Antibiotics*, 1993, **46**, 1300-1302.

119   S. Ishida, O. Yamada, F. Futatsuya, K. Ito, H. Yamamoto and K. Munakata, *Proc. Int. Congr. IAMS 1st* 1974, 641.

[120] J. J. Beereboom, K. Butler, F. C. Pennington and I. A. Solomons, *Journal of Organic Chemistry*, 1965, **30**, 2334-2342.

[121] J. P. Schaefer and P. J. Wheatley, *Journal of Organic Chemistry*, 1968, **33**, 166-169.

[122] K. Butler, *Journal of Organic Chemistry*, 1968, **33**, 2136-2141.

[123] J. P. Schaefer and P. J. Wheatley, *Chemical Communications*, 1967, 578-579.

[124] C. M. Wong, *Canadian Journal of Chemistry*, 1968, **46**, 1101-1104.

[125] J. E. Lynch, A. R. English, H. Bauck and H. Deligianis, *Antibiotics and Chemotherapy*, 1954, **4**, 844-848.

[126] O. Schwardt, U. Veith, C. Gaspard and V. Jager, *Synthesis-Stuttgart*, 1999, 1473-1490.

[127] S. Ruller, C. Stahl, G. Kohler, B. Eickhoff, J. Breder, M. Schlaak and J. van der Bosch, *Clinical Cancer Research*, 1999, **5**, 2714-2725.

[128] D. Chen, S. B. Waters, K. H. Holt and J. E. Pessin, *Journal of Biological Chemistry*, 1996, **271**, 6328-6332.

[129] C. A. Hazzalin, E. Cano, A. Cuenda, M. J. Barratt, P. Cohen and L. C. Mahadevan, *Current Biology*, 1996, **6**, 1028-1031.

[130] E. Cano, Y. N. Doza, R. BenLevy, P. Cohen and L. C. Mahadevan, *Oncogene*, 1996, **12**, 805-812.

[131] R. Meier, J. Rouse, A. Cuenda, A. R. Nebreda and P. Cohen, *European Journal of Biochemistry*, 1996, **236**, 796-805.

[132] J. M. Kyriakis, P. Banerjee, E. Nikolakaki, T. A. Dai, E. A. Rubie, M. F. Ahmad, J. Avruch and J. R. Woodgett, *Nature*, 1994, **369**, 156-160.

[133] E. Cano, C. A. Hazzalin and L. C. Mahadevan, *Molecular and Cellular Biology*, 1994, **14**, 7352-7362.

[134] J. M. Berg, J. L. Tymoczko and L. Stryer, 'Biochemistry', W. H. Freeman and Company, 2002.

[135] T. Pawson, *Nature*, 1995, **373**, 573-580.

[136] P. Cohen, *Nature Cell Biology*, 2002, **4**, E127-E130.

[137] P. Cohen, *European Journal of Biochemistry*, 2001, **268**, 5001-5010.

[138] E. G. Krebs and E. H. Fischer, *Biochimica et Biophysica Acta*, 1956, **20**, 150-157.

[139] E. W. Sutherland and W. D. Wosilait, *Nature*, 1955, **175**, 169-170.

[140] E. H. Fischer and E. G. Krebs, *Journal of Biological Chemistry*, 1955, **216**, 121-132.

[141] G. Burnett and E. P. Kennedy, *Journal of Biological Chemistry*, 1954, **211**, 969-980.

[142] E. H. Fischer, D. J. Graves, E. R. S. Crittenden and E. G. Krebs, *Journal of Biological Chemistry*, 1959, **234**, 1698-1704.

[143] T. S. Ingebritsen and P. Cohen, *European Journal of Biochemistry*, 1983, **132**, 255-261.

[144] P. Cohen, *Nature Reviews Drug Discovery*, 2002, **1**, 309-315.

[145] G. Manning, D. B. Whyte, R. Martinez, T. Hunter and S. Sudarsanam, *Science*, 2002, **298**, 1912-1934.

[146] D. A. Walsh, J. P. Perkins and E. G. Krebs, *Journal of Biological Chemistry*, 1968, **243**, 3763-3765.

[147] L. B. Ray and T. W. Sturgill, *Proceedings of the National Academy of Sciences of the United States of America*, 1987, **84**, 1502-1506.

148   M. J. Robinson and M. H. Cobb, *Current Opinion in Cell Biology*, 1997, **9**, 180-186.

149   Z. Chen, T. B. Gibson, F. Robinson, L. Silvestro, G. Pearson, B. E. Xu, A. Wright, C. Vanderbilt and M. H. Cobb, *Chemical Reviews*, 2001, **101**, 2449-2476.

150   Calbiochem, 'MAPK Family Pathways', Calbiochem, 2005.

151   J. M. Kyriakis and J. Avruch, *Physiological Reviews*, 2001, **81**, 807-869.

152   T. S. Lewis, P. S. Shapiro and N. G. Ahn, in 'Signal Transduction Through MAP Kinase Cascades', 1998.

153   H. Kishimoto, K. Nakagawa, T. Watanabe, D. Kitagawa, H. Momose, J. Seo, G. Nishitai, N. Shimizu, S. Ohata, S. Tanemura, S. Asaka, T. Goto, H. Fukushi, H. Yoshida, A. Suzuki, T. Sasaki, T. Wada, J. M. Penninger, H. Nishina and T. Katada, *Journal of Biological Chemistry*, 2003, **278**, 16595-16601.

154   Y. Shi and M. Gaestel, *Biological Chemistry*, 2002, **383**, 1519-1536.

155   L. F. Chang and M. Karin, *Nature*, 2001, **410**, 37-40.

156   R. J. Davis, *Cell*, 2000, **103**, 239-252.

157   K. J. Cowan and K. B. Storey, *Journal of Experimental Biology*, 2003, **206**, 1107-1115.

158   J. R. Woodgett, 'Protein Kinase Functions', Oxford University Press, 2000.

159   P. Cohen, *Trends in Cell Biology*, 1997, **7**, 353-361.

160   G. L. Johnson, H. G. Dohlman and L. M. Graves, *Current Opinion in Chemical Biology*, 2005, **9**, 325-331.

161   S. Gupta, T. Barrett, A. J. Whitmarsh, J. Cavanagh, H. K. Sluss, B. Derijard and R. J. Davis, *EMBO Journal*, 1996, **15**, 2760-2770.

162   L. A. Tibbles and J. R. Woodgett, *Cellular and Molecular Life Sciences*, 1999, **55**, 1230-1254.

163   J. Lisnock, P. Griffin, J. Calaycay, B. Frantz, J. Parsons, S. J. O'Keefe and P. LoGrasso, *Biochemistry*, 2000, **39**, 3141-3148.

164   S. Lawler, Y. Fleming, M. Goedert and P. Cohen, *Current Biology*, 1998, **8**, 1387-1390.

165   C. Tournier, A. J. Whitmarsh, J. Cavanagh, T. Barrett and R. J. Davis, *Molecular and Cellular Biology*, 1999, **19**, 1569-1581.

166   M. Cavigelli, F. Dolfi, F. X. Claret and M. Karin, *EMBO Journal*, 1995, **14**, 5957-5964.

167   E. Shaulian and M. Karin, *Nature Cell Biology*, 2002, **4**, E131-E136.

168   R. J. Davis, *Molecular Reproduction and Development*, 1995, **42**, 459-467.

169   S. H. Yang, A. D. Sharrocks and A. J. Whitmarsh, *Gene*, 2003, **320**, 3-21.

170   C. Lechner, M. A. Zahalka, J. F. Giot, N. P. H. Moller and A. Ullrich, *Proceedings of the National Academy of Sciences of the United States of America*, 1996, **93**, 4355-4359.

171   M. C. T. Hu, Y. P. Wang, A. Mikhail, W. R. Qiu and T. H. Tan, *Journal of Biological Chemistry*, 1999, **274**, 7095-7102.

172   J. Yang, Y. N. Yu and P. J. Duerksen-Hughes, *Mutation Research-Reviews in Mutation Research*, 2003, **543**, 31-58.

173   E. P. McDermott and L. A. J. O'Neill, *Journal of Biological Chemistry*, 2002, **277**, 7808-7815.

174 Y. L. Zu, J. F. Qi, A. Gilchrist, G. A. Fernandez, D. Vazquez-Abad, D. L. Kreutzer, C. K. Huang and R. I. Sha'afi, *Journal of Immunology*, 1998, **160**, 1982-1989.

175 B. Stein, H. Brady, M. X. Yang, D. B. Young and M. S. Barbosa, *Journal of Biological Chemistry*, 1996, **271**, 11427-11433.

176 A. Cuenda, G. Alonso, N. Morrice, M. Jones, R. Meier, P. Cohen and A. R. Nebreda, *EMBO Journal*, 1996, **15**, 4156-4164.

177 J. Han, J. D. Lee, L. Bibbs and R. J. Ulevitch, *Science*, 1994, **265**, 808-811.

178 B. Derijard, J. Raingeaud, T. Barrett, I. H. Wu, J. H. Han, R. J. Ulevitch and R. J. Davis, *Science*, 1995, **267**, 682-685.

179 K. Ono and J. H. Han, *Cellular Signalling*, 2000, **12**, 1-13.

180 C. Wang, L. Deng, M. Hong, G. R. Akkaraju, J. Inoue and Z. J. J. Chen, *Nature*, 2001, **412**, 346-351.

181 K. Sayama, Y. Hanakawa, Y. Shirakata, K. Yamasaki, Y. Sawada, L. Sun, K. Yamanishi, H. Ichijo and K. Hashimoto, *Journal of Biological Chemistry*, 2001, **276**, 999-1004.

182 L. A. Tibbles, Y. L. Ing, F. Kiefer, J. Chan, N. Iscove, J. R. Woodgett and N. J. Lassam, *EMBO Journal*, 1996, **15**, 7026-7035.

183 A. Farooq and M. M. Zhou, *Cellular Signalling*, 2004, **16**, 769-779.

184 S. Tamura, M. Hanada, M. Ohnishi, K. Katsura, M. Sasaki and T. Kobayashi, *European Journal of Biochemistry*, 2002, **269**, 1060-1066.

185 S. M. Keyse, *Current Opinion in Cell Biology*, 2000, **12**, 186-192.

186 X. S. Wang, M. M. Mader, J. E. Toth, X. H. Yu, N. J. Jin, R. M. Campbell, J. K. Smallwood, M. E. Christe, A. Chatterjee, T. Goodson, C. J. Vlahos, W. F. Matter and L. J. Bloem, *Journal of Biological Chemistry*, 2005, **280**, 19298-19305.

187 A. Travers, *Cell*, 1974, **3**, 97-104.

188 J. M. Saucier and J. C. Wang, *Nature-New Biology*, 1972, **239**, 167-170.

189 U. Maitra and J. Hurwitz, *Proceedings of the National Academy of Sciences of the United States of America*, 1965, **54**, 815-822.

190 R. H. Burdon, 'Progress in Nucleic Acid Research and Molecular Biology', ed. I. N. Davidson and W. E. Cohn, Academic Press, 1971.

191 M. Soller, *Cellular and Molecular Life Sciences*, 2006, **63**, 796-819.

192 M. G. Gu and C. D. Lima, *Current Opinion in Structural Biology*, 2005, **15**, 99-106.

193 D. L. Bentley, *Current Opinion in Cell Biology*, 2005, **17**, 251-256.

194 E. P. Geidusch and R. Haselkor, *Annual Review of Biochemistry*, 1969, **38**, 647-676.

195 G. Brawermann, *Annual Review of Biochemistry*, 1974, **43**, 621-642.

196 K. L. Ewalt and P. Schimmel, 'Protein biosynthesis: tRNA synthetases', ed. W. J. Lennarz and M. D. Lane, Elsevier, 2004.

197 V. Marquez and K. H. Nierhaus, ' tRNA and synthetases: tRNA: structure and function', ed. V. Marquez and K. H. Nierhaus, Wiley-VCH 2004.

198 S. M. Heywood, *Proceedings of the National Academy of Sciences of the United States of America*, 1970, **67**, 1782-1788.

199 J. A. Steitz, *Nature*, 1969, **224**, 957-964.

200 R. N. Bryan, D. H. Gelfand and M. Hayashi, *Nature*, 1969, **224**, 1019.

201   T. V. Pestova and C. U. T. Hellen, *Cellular and Molecular Life Sciences*, 2000, **57**, 651-674.

202   M. Cannon, R. Krug and W. Gilbert, *Journal of Molecular Biology*, 1963, **7**, 360.

203   B. T. Wimberly, 'Ribosome structure.' ed. W. J. Lennarz and M. D. Lane, Elsevier, 2004.

204   J. L. Woolford, 'Ribosome assembly.' ed. W. J. Lennarz and M. D. Lane, Elsevier, 2004.

205   S. S. Thach and R. E. Thach, *Proceedings of the National Academy of Sciences of the United States of America*, 1971, **68**, 1791-1795.

206   N. Koloteva-Levin and M. F. Tuite, 'Translation termination and ribosome recycling. ' ed. W. J. Lennarz and M. D. Lane, Elsevier, 2004.

207   A. P. Grollman, *Journal of Biological Chemistry*, 1967, **242**, 3226-3232.

208   D. Vasquez, *FEBS Lett. Suppl.*, 1974, **40**, S63-S84.

209   A. Jimenez and D. Vazquez, *European Journal of Biochemistry*, 1975, **54**, 483-492.

210   A. Jimenez, L. Sanchez and D. Vazquez, *Biochimica et Biophysica Acta*, 1975, **383**, 427-434.

211   M. Barbacid and D. Vazquez, *Journal of Molecular Biology*, 1974, **84**, 603-623.

212   L. Carrasco and D. Vazquez, *Journal of Antibiotics*, 1972, **25**, 732-737.

213   E. Battaner and D. Vazquez, *Biochimica et Biophysica Acta*, 1971, **254**, 316-330.

214   W. J. Vanvenrooi, J. Vaneenbergen and A. P. M. Janssen, *Biochemistry*, 1977, **16**, 2343-2348.

215   J. L. Hansen, P. B. Moore and T. A. Steitz, *Journal of Molecular Biology*, 2003, **330**, 1061-1075.

216   N. Naghdi, N. Majlessi and T. Bozorgmehr, *Behavioural Brain Research*, 2005, **156**, 263-268.

217   K. M. Lattal and T. Abel, *Proceedings of the National Academy of Sciences of the United States of America*, 2004, **101**, 4667-4672.

218   K. Nader, G. E. Schafe and J. E. Le Doux, *Nature*, 2000, **406**, 722-726.

219   K. H. Lee, S. Nishimura, S. Matsunaga, N. Fusetani, H. Ichijo, S. Horinouchi and M. Yoshida, *Bioscience, Biotechnology and Biochemistry*, 2006, **70**, 161-171.

220   K. H. Lee, S. Nishimura, S. Matsunaga, N. Fusetani, S. Horinouchi and M. Yoshida, *Cancer Science*, 2005, **96**, 357-364.

221   A. J. Polverino and S. D. Patterson, *Journal of Biological Chemistry*, 1997, **272**, 7013-7021.

222   S. K. Kochi and R. J. Collier, *Experimental Cell Research*, 1993, **208**, 296-302.

223   S. H. Shi, S. R. Zhu, S. W. Gerritz, K. Esposito, R. Padmanabha, W. Y. Li, J. J. Herbst, H. Wong, Y. Z. Shu, K. S. Lam and M. J. Sofia, *Bioorganic & Medicinal Chemistry Letters*, 2005, **15**, 4151-4154.

224   J. H. Kim, M. J. Curtis-Long, W. D. Seo, Y. B. Ryu, M. S. Yang and K. H. Park, *Journal of Organic Chemistry*, 2005, **70**, 4082-4087.

225   S. Hirner and P. Somfai, *Synlett*, 2005, 3099-3102.

226   M. Ono, S. Tanikawa, K. Suzuki and H. Akita, *Tetrahedron*, 2004, **60**, 10187-10195.

227   M. Y. Chang, S. T. Chen and N. C. Chang, *Heterocycles*, 2003, **60**, 1203-1209.

228   S. Chandrasekhar, T. Ramachandar and M. V. Reddy, *Synthesis-Stuttgart*, 2002, 1867-1870.

229   P. Delair, E. Brot, A. Kanazawa and A. E. Greene, *Journal of Organic Chemistry*, 1999, **64**, 1383-1386.

230   U. Veith, O. Schwardt and V. Jager, *Synlett*, 1996, 1181-1183.

231   S. H. Kang and H. W. Choi, *Chemical Communications*, 1996, 1521-1522.

232   H. Yoda, T. Nakajima, H. Yamazaki and K. Takabe, *Heterocycles*, 1995, **41**, 2423-2426.

233   N. Ikota, *Heterocycles*, 1995, **41**, 983-994.

234   R. Ballini, E. Marcantoni and M. Petrini, *Journal of Organic Chemistry*, 1992, **57**, 1316-1318.

235   H. Takahata, Y. Banba, M. Tajima and T. Momose, *Journal of Organic Chemistry*, 1991, **56**, 240-245.

236   S. Jegham and B. C. Das, *Tetrahedron Letters*, 1988, **29**, 4419-4422.

237   H. H. Baer and M. Zamkanei, *Journal of Organic Chemistry*, 1988, **53**, 4786-4789.

238   T. Shono and N. Kise, *Chemistry Letters*, 1987, 697-700.

239   A. I. Meyers and B. Dupre, *Heterocycles*, 1987, **25**, 113-116.

240   H. Iida, N. Yamazaki and C. Kibayashi, *Journal of Organic Chemistry*, 1986, **51**, 1069-1073.

241   J. G. Buchanan, K. A. Maclean, R. H. Wightman and H. Paulsen, *Journal of the Chemical Society-Perkin Transactions 1*, 1985, 1463-1470.

242   J. P. H. Verheyden, A. C. Richardson, R. S. Bhatt, B. D. Grant, W. L. Fitch and J. G. Moffatt, *Pure and Applied Chemistry*, 1978, **50**, 1363-1383.

243   I. Felner and K. Schenker, *Helvetica Chimica Acta*, 1970, **53**, 754-763.

244   S. Oida and E. Ohki, *Chemical & Pharmaceutical Bulletin*, 1969, **17**, 1405-1421.

245   A. N. Hulme and E. M. Rosser, *Organic Letters*, 2002, **4**, 265-267.

246   E. M. Rosser, S. Morton, K. S. Ashton, P. Cohen and A. N. Hulme, *Organic & Biomolecular Chemistry*, 2004, **2**, 142-149.

247   D. R. Spring, *Chemical Society Reviews*, 2005, **34**, 472-482.

248   S. L. Schreiber, *Bioorganic & Medicinal Chemistry*, 1998, **6**, 1127-1152.

249   D. P. Walsh and Y. T. Chang, *Chemical Reviews*, 2006, **106** 2476-2530.

250   S. M. Khersonsky and Y. T. Chang, *ChemBioChem*, 2004, **5**, 903-908.

251   S. V. Ankala and G. Fenteany, *Tetrahedron Letters*, 2002, **43**, 4729-4732.

252   K. S. Curley, 'Stereoselective Reactions of *N,N*-Dibenzyl Protected Synthons', PhD Thesis, University of Edinburgh, Edinburgh, 2002.

253   R. V. Hoffman and J. H. Tao, *Journal of Organic Chemistry*, 1997, **62**, 2292-2297.

254   E. M. Rosser, 'The Synthesis and Biological Evaluation of the Pyrrolidine Antibiotic Anisomycin', University of Edinburgh, Edinburgh, 2003.

255   M. M. Bradford, *Analytical Biochemistry*, 1976, **72**, 248-254.

256   Chemicon, 'Introduction to Antibodies - Protein (Western) Blotting', Chemicon, 2004.

257 GE Healthcare, 'ECL Western blotting detection reagents and analysis system', GE Healthcare, 2006.

258 S. Morton, R. J. Davis, A. McLaren and P. Cohen, *EMBO Journal*, 2003, **22**, 3876-3886.

259 J. Lippincott-Schwartz and G. H. Patterson, *Science*, 2003, **300**, 87-91.

260 J. Zhang, R. E. Campbell, A. Y. Ting and R. Y. Tsien, *Nature Reviews Molecular Cell Biology*, 2002, **3**, 906-918.

261 R. Y. Tsien, *Annual Review of Biochemistry*, 1998, **67**, 509-544.

262 R. Breinbauer and M. Kohn, *ChemBioChem*, 2003, **4**, 1147-1149.

263 W. R. Dichtel, O. S. Miljanic, J. M. Spruell, J. R. Heath and J. F. Stoddart, *Journal of the American Chemical Society*, 2006, **128**, 10388-10390.

264 P. Wu, A. K. Feldman, A. K. Nugent, C. J. Hawker, A. Scheel, B. Voit, J. Pyun, J. M. J. Frechet, K. B. Sharpless and V. V. Fokin, *Angewandte Chemie-International Edition*, 2004, **43**, 3928-3932.

265 H. N. Lin and C. T. Walsh, *Journal of the American Chemical Society*, 2004, **126**, 13998-14003.

266 B. H. M. Kuijpers, S. Groothuys, A. R. Keereweer, P. Quaedflieg, R. H. Blaauw, F. L. van Delft and F. Rutjes, *Organic Letters*, 2004, **6**, 3123-3126.

267 B. Helms, J. L. Mynar, C. J. Hawker and J. M. J. Frechet, *Journal of the American Chemical Society*, 2004, **126**, 15020-15021.

268 C. J. Li and T. H. Chan, 'Organic Reactions in Aqueous Media', Wiley, 1997.

269 B. Cornils and W. A. Herrmann, 'Aqueous Phase Organometallic Reactions', Wiely-VCH, 1998.

270 P. A. Grieco, 'Organic Synthesis in Water', Blackie, 1998.

271 J. J. Gajewski, *Accounts of Chemical Research*, 1997, **30**, 219-225.

272 D. van Mersbergen, J. W. Wijnen and J. Engberts, *Journal of Organic Chemistry*, 1998, **63**, 8801-8805.

273 H. Staudinger and J. Meyer, *Helvetica Chimica Acta*, 1919, **2**, 635-646.

274 Y. G. Gololobov and L. F. Kasukhin, *Tetrahedron*, 1992, **48**, 1353-1406.

275 M. Alajarin, C. Conesa and H. S. Rzepa, *Journal of the Chemical Society-Perkin Transactions 2*, 1999, 1811-1814.

276 D. E. Shalev, S. M. Chiacchiera, A. E. Radkowsky and E. M. Kosower, *Journal of Organic Chemistry*, 1996, **61**, 1689-1701.

277 I. Bosch, A. Gonzalez, F. Urpi and J. Vilarrasa, *Journal of Organic Chemistry*, 1996, **61**, 5638-5643.

278 K. L. Kiick, E. Saxon, D. A. Tirrell and C. R. Bertozzi, *Proceedings of the National Academy if Sciences of the United States of America*, 2002, **99**, 19-24.

279 E. Saxon and C. R. Bertozzi, *Science*, 2000, **287**, 2007-2010.

280 E. Saxon, J. I. Armstrong and C. R. Bertozzi, *Organic Letters*, 2000, **2**, 2141-2143.

281 M. C. Bryan, F. Fazio, H. K. Lee, C. Y. Huang, A. Chang, M. D. Best, D. A. Calarese, C. Blixt, J. C. Paulson, D. Burton, I. A. Wilson and C. H. Wong, *Journal of the American Chemical Society*, 2004, **126**, 8640-8641.

282 C. C. Y. Wang, T. S. Seo, Z. M. Li, H. Ruparel and J. Y. Ju, *Bioconjugate Chemistry*, 2003, **14**, 697-701.

283 G. A. Lemieux, C. L. de Graffenried and C. R. Bertozzi, *Journal of the American Chemical Society*, 2003, **125**, 4708-4709.

284   M. Kohn, R. Wacker, C. Peters, H. Schroder, L. Soulere, R. Breinbauer, C. M. Niemeyer and H. Waldmann, *Angewandte Chemie-International Edition*, 2003, **42**, 5830-5834.

285   E. Saxon, S. J. Luchansky, H. C. Hang, C. Yu, S. C. Lee and C. R. Bertozzi, *Journal of the American Chemical Society*, 2002, **124**, 14893-14902.

286   J. A. Prescher, D. H. Dube and C. R. Bertozzi, *Nature*, 2004, **430**, 873-877.

287   R. Huisgen, R. Knorr, L. Mobius and G. Szeimies, *Chemische Berichte-Recueil*, 1965, **98**, 4014-4021.

288   R. Huisgen, *Angewandte Chemie-International Edition*, 1963, **2**, 565-632.

289   R. Huisgen, *Angewandte Chemie-International Edition*, 1963, **2**, 633-696.

290   R. Huisgen, *Angewandte Chemie-International Edition*, 1968, **7**, 321-328.

291   R. Huisgen, *Journal of Organic Chemistry*, 1976, **41**, 403-419.

292   R. Huisgen, *Journal of Organic Chemistry*, 1968, **33**, 2291-2297.

293   K. N. Houk, J. Gonzalez and Y. Li, *Accounts of Chemical Research*, 1995, **28**, 81-90.

294   R. A. Firestone, *Tetrahedron*, 1977, **33**, 3009-3039.

295   R. A. Firestone, *Journal of Organic Chemistry*, 1972, **37**, 2181-2191.

296   R. A. Firestone, *Journal of the Chemical Society A-Inorganic Physical Theoretical*, 1970, 1570-1575.

297   R. A. Firestone, *Journal of Organic Chemistry*, 1968, **33**, 2285-2290.

298   K. N. Houk, R. A. Firestone, L. L. Munchausen, P. H. Mueller, B. H. Arison and L. A. Garcia, *Journal of the American Chemical Society*, 1985, **107**, 7227-7228.

299   C. W. Tornoe, C. Christensen and M. Meldal, *Journal of Organic Chemistry*, 2002, **67**, 3057-3064.

300   V. V. Rostovtsev, L. G. Green, V. V. Fokin and K. B. Sharpless, *Angewandte Chemie-International Edition*, 2002, **41**, 2596-2599.

301   V. D. Bock, H. Hiemstra and J. H. van Maarseveen, *European Journal of Organic Chemistry*, 2006, 51–68.

302   A. J. Link, M. K. S. Vink and D. A. Tirrell, *Journal of the American Chemical Society*, 2004, **126**, 10598-10602.

303   A. N. Hulme and R. F. H. Viguier, *Journal of the American Chemical Society*, 2006, **128**, 11370-11371.

304   F. Himo, T. Lovell, R. Hilgraf, V. V. Rostovtsev, L. Noodleman, K. B. Sharpless and V. V. Fokin, *Journal of the American Chemical Society*, 2005, **127**, 210-216.

305   V. O. Rodionov, V. V. Fokin and M. G. Finn, *Angewandte Chemie-International Edition*, 2005, **44**, 2210-2215.

306   A. Deiters, T. A. Cropp, M. Mukherji, J. W. Chin, J. C. Anderson and P. G. Schultz, *Journal of the American Chemical Society*, 2003, **125**, 11782-11783.

307   K. E. Beatty, F. Xie, Q. Wang and D. A. Tirrell, *Journal of the American Chemical Society*, 2005, **127**, 14150-14151.

308   A. E. Speers and B. F. Cravatt, *ChemBioChem*, 2004, **5**, 41-47.

309   A. E. Speers and B. F. Cravatt, *Chemistry & Biology*, 2004, **11**, 535-546.

310   A. Saghatelian, N. Jessani, A. Joseph, M. Humphrey and B. F. Cravatt, *Proceedings of the National Academy of Sciences of the United States of America*, 2004, **101**, 10000-10005.

311   A. E. Speers, G. C. Adam and B. F. Cravatt, *Journal of the American Chemical Society*, 2003, **125**, 4686-4687.

312   S. Fuchs, H. Otto, S. Jehle, P. Henklein and A. D. Schluter, *Chemical Communications*, 2005, 1830-1832.

313   H. Kakuta, Y. Koiso, K. Nagasawa and Y. Hashimoto, *Bioorganic & Medicinal Chemistry Letters*, 2003, **13**, 83-86.

314   M. D. Alexander, M. D. Burkart, M. S. Leonard, P. Portonovo, B. Liang, X. Ding, M. M. Joullie, B. M. Gulledge, J. B. Aggen, A. R. Chamberlin, J. Sandler, W. Fenical, J. Cui, S. J. Gharpure, A. Polosukhin, H. R. Zhang, P. A. Evans, A. D. Richardson, M. K. Harper, C. M. Ireland, B. G. Vong, T. P. Brady, E. A. Theodorakis and J. J. La Clair, *ChemBioChem*, 2006, **7**, 409-416.

315   R. M. Sanchez-Martin, M. Muzerelle, N. Chitkul, S. E. How, S. Mittoo and M. Bradley, *ChemBioChem*, 2005, **6**, 1341-1345.

316   R. Srinivasan, S. Q. Yao and D. S. Y. Yeo, *Combinatorial Chemistry & High Throughput Screening*, 2004, **7**, 597-604.

317   H. H. Szeto, P. W. Schiller, K. Zhao and G. Luo, *FASEB J.*, 2005, **19**, 118-120.

318   S. J. Slater, C. Ho and C. D. Stubbs, *Chemistry and Physics of Lipids*, 2002, **116**, 75-91.

319   A. Benalil, B. Carboni and M. Vaultier, *Tetrahedron*, 1991, **47**, 8177-8194.

320   R. F. H. Viguier, 'Unpublished work', Edinburgh, 2005.

321   BioRad, 'Affigel affinity matrix', BioRad, 2005.

322   G. P. Moss, *European Journal of Biochemistry*, 1989, **186**, 429-458.

323   I. A. Rose, K. R. Hanson, K. D. Wilkinson and M. J. Wimmer, *Proceedings of the National Academy of Sciences of the United States of America-Biological Sciences*, 1980, **77**, 2439-2441.

324   J. Mann, 'Chemical Aspects of Biosynthesis', ed. S. G. Davies, Oxford University Press, 2001.

325   A. F. Wagner and K. Folkers, *Advances in Enzymology and Related Subjects of Biochemistry*, 1961, **23**, 471-483.

326   F. Lynen, H. Eggerer, U. Henning and I. Kessel, *Angewandte Chemie-International Edition*, 1958, **70**, 738-742.

327   S. Chaykin, J. Law, A. H. Phillips, T. T. Tchen and K. Bloch, *Proceedings of the National Academy of Sciences of the United States of America*, 1958, **44**, 998-1004.

328   G. J. Schroepfer, *Annual Review of Biochemistry*, 1982, **51**, 555-585.

329   R. A. Yoder and J. N. Johnston, *Chemical Reviews*, 2005, **105**, 4730-4756.

330   D. M. Harrison, *Natural Product Reports*, 1985, **2**, 525-560.

331   J. W. Cornforth and G. Popjak, *British Medical Bulletin*, 1958, **14**, 221-226.

332   W. L. Miller, *Molecular and Cellular Endocrinology*, 2002, **198**, 7-14.

333   W. L. Miller, *Endocrine Reviews*, 1988, **9**, 295-318.

334   R. J. Auchus, *Seminars in Reproductive Medicine*, 2004, **22**, 281-288.

335   W. L. Miller, R. J. Auchus and D. H. Geller, *Steroids*, 1997, **62**, 133-142.

336   R. J. Auchus, T. C. Lee and W. L. Miller, *Journal of Biological Chemistry*, 1998, **273**, 3158-3165.

337 L. H. Zhang, H. Rodriguez, S. Ohno and W. L. Miller, *Proceedings of the National Academy of Sciences of the United States of America*, 1995, **92**, 10619-10623.

338 P. D. Kroboth, F. S. Salek, A. L. Pittenger, T. J. Fabian and R. F. Frye, *Journal of Clinical Pharmacology*, 1999, **39**, 327-348.

339 B. Allolio and W. Arlt, *Trends in Endocrinology and Metabolism*, 2002, **13**, 288-294.

340 E. E. Baulieu, *Journal of Clinical Endocrinology and Metabolism*, 1996, **81**, 3147-3151.

341 N. Orentreich, J. L. Brind, J. H. Vogelman, R. Andres and H. Baldwin, *Journal of Clinical Endocrinology and Metabolism*, 1992, **75**, 1002-1004.

342 N. Orentreich, J. L. Brind, R. L. Rizer and J. H. Vogelman, *Journal of Clinical Endocrinology and Metabolism*, 1984, **59**, 551-555.

343 C. J. Migeon, A. R. Keller, B. Lawrence and T. H. Shepard, *Journal of Clinical Endocrinology and Metabolism*, 1957, **17**, 1051-1062.

344 P. Robel and E. E. Baulieu, in 'Dehydroepiandrosterone (DHEA) is a neuroactive neurosteroid', 1995.

345 C. Corpechot, P. Robel, M. Axelson, J. Sjovall and E. E. Baulieu, *Proceedings of the National Academy of Sciences of the United States of America-Biological Sciences*, 1981, **78**, 4704-4707.

346 R. Bergeron, C. de Montigny and G. Debonnel, *Journal of Neuroscience*, 1996, **16**, 1193-1202.

347 S. Demirgoren, M. D. Majewska, C. E. Spivak and E. D. London, *Neuroscience*, 1991, **45**, 127-135.

348 M. D. Majewska, S. Demirgoren, C. E. Spivak and E. D. London, *Brain Research*, 1990, **526**, 143-146.

349 M. Takebayashi, A. Kagaya, Y. Uchitomi, A. Kugaya, M. Muraoka, N. Yokota, J. Horiguchi and S. Yamawaki, *Journal of Neural Transmission*, 1998, **105**, 537-542.

350 O. M. Wolkowitz, V. I. Reus, A. Keebler, N. Nelson, M. Friedland, L. Brizendine and E. Roberts, *American Journal of Psychiatry*, 1999, **156**, 646-649.

351 D. G. Young, G. Skibinski, J. I. Mason and K. James, *Clinical and Experimental Immunology*, 1999, **117**, 476-481.

352 R. H. Straub, L. Konecna, S. Hrach, G. Rothe, M. Kreutz, J. Scholmerich, W. Falk and B. Lang, *Journal of Clinical Endocrinology and Metabolism*, 1998, **83**, 2012-2017.

353 A. D. Delpedro, M. J. Barjavel, Z. Mamdouh and O. Bakouche, *Journal of Interferon and Cytokine Research*, 1998, **18**, 125-135.

354 J. A. McLachlan, C. D. Serkin and O. Bakouche, *Journal of Immunology*, 1996, **156**, 328-335.

355 R. A. Daynes, D. J. Dudley and B. A. Araneo, *European Journal of Immunology*, 1990, **20**, 793-802.

356 S. J. Webb, T. E. Geoghegan, R. A. Prough and K. K. M. Miller, *Drug Metabolism Reviews*, 2006, **38**, 89-116.

357 R. L. Widstrom and J. S. Dillon, *Seminars in Reproductive Medicine*, 2004, **22**, 289-298.

358 D. M. Liu and J. S. Dillon, *Steroids*, 2004, **69**, 279-289.

359 D. M. Liu and J. S. Dillon, *Journal of Biological Chemistry*, 2002, **277**, 21379-21388.

360 G. Formoso, H. Chen, J.-A. Kim, M. Montagnani, A. Consoli and M. J. Quon, *Molecular Endocrinology*, 2006, **20**, 1153-1163.

361 S. Reuter and D. Mayer, *Journal of Steroid Biochemistry and Molecular Biology*, 1995, **54**, 227-235.

362 M. Hasegawa, H. Kusuhara, H. Endou and Y. Sugiyama, *Journal of Pharmacology and Experimental Therapeutics*, 2003, **305**, 1087-1097.

363 F. Zhou, N. P. Illsley and G. You, *European Journal of Pharmaceutical Sciences*, 2006, **27**, 518-523.

364 L. Dalla Valle, V. Toffolo, A. Nardi, C. Fiore, P. Bernante, R. Di Liddo, P. P. Parnigotto and L. Colombo, *Journal of Endocrinology* 2006, **190**, 129-139.

365 S. K. Butcher, V. Killampalli, D. Lascelles, K. Wang, E. K. Alpar and J. M. Lord, *Aging Cell*, 2005, **4**, 319-324.

366 J. J. Haddad, *Progress in Neurobiology*, 2005, **77**, 252-282.

367 Y. Chen, Q. Zhang, B. Zhang, P. Xia, Y. Xia, Z. Y. Yang, N. Kilgore, C. Wild, S. L. Morris-Natschke and K. H. Lee, *Bioorganic & Medicinal Chemistry*, 2004, **12**, 6383-6387.

368 Y. F. Xiong, H. J. Xia and H. W. Moore, *Journal of Organic Chemistry*, 1995, **60**, 6460-6467.

369 J. Griffiths and J. A. Murphy, *Tetrahedron*, 1992, **48**, 5543-5556.

370 B. M. Trost and M. T. Rudd, *Journal of the American Chemical Society*, 2005, **127**, 4763-4776.

371 I. Ott, K. Schmidt, B. Kircher, P. Schumacher, T. Wiglenda and R. Gust, *Journal of Medicinal Chemistry*, 2005, **48**, 622-629.

372 M. A. Arrica and T. Wirth, *European Journal of Organic Chemistry*, 2005, 395-403.

373 L. Zhu, V. M. Lynch and E. V. Anslyn, *Tetrahedron*, 2004, **60**, 7267-7275.

374 Y. Yamamoto, K. Kinpara, T. Saigoku, H. Nishiyama and K. Itoh, *Organic & Biomolecular Chemistry*, 2004, **2**, 1287-1294.

375 S. Punna, S. Meunier and M. G. Finn, *Organic Letters*, 2004, **6**, 2777-2779.

376 T. P. Curran, R. S. H. Yoon and B. R. Volk, *Journal of Organometallic Chemistry*, 2004, **689**, 4837-4847.

377 C. Y. Wu, C. F. Chang, J. S. Y. Chen, C. H. Wong and C. H. Lin, *Angewandte Chemie-International Edition*, 2003, **42**, 4661-4664.

378 A. Casaschi, R. Grigg, J. M. Sansano, D. Wilson and J. Redpath, *Tetrahedron*, 2000, **56**, 7541-7551.

379 P. R. Sridhar and S. Chandrasekaran, *Organic Letters*, 2002, **4**, 4731-4733.

380 R. G. Bhat, S. Sinha and S. Chandrasekaran, *Chemical Communications*, 2002, 812-813.

381 R. Ramesh, R. G. Bhat and S. Chandrasekaran, *Journal of Organic Chemistry*, 2005, **70**, 837-840.

382 A. Cerri, N. Almirante, P. Barassi, A. Benicchio, G. Fedrizzi, P. Ferrari, R. Micheletti, L. Quadri, E. Ragg, R. Rossi, M. Santagostino, A. Schiavone, F. Serra, M. P. Zappavigna and P. Melloni, *Journal of Medicinal Chemistry*, 2000, **43**, 2332-2349.

383 H. Ide, K. Akamatsu, Y. Kimura, K. Michiue, K. Makino, A. Asaeda, Y. Takamori and K. Kubo, *Biochemistry*, 1993, **32**, 8276-8283.

384   J. Keeler, R. A. E. Edden and M. J. Thrippleton, *Journal of Magnetic Resonance*, 2005, **174**, 97–109.

385   J. A. Dunn and P. L. Pauson, *Journal of Organometallic Chemistry*, 1991, **419**, 383-389.

386   L. E. Overman, L. A. Clizbe, R. L. Freerks and C. K. Marlowe, *Journal of the American Chemical Society*, 1981, **103**, 2807-2815.

387   L. E. Overman, C. K. Marlowe and L. A. Clizbe, *Tetrahedron Letters*, 1979, 599-600.

388   A. G. Jamieson and A. Sutherland, *Organic & Biomolecular Chemistry*, 2005, **3**, 735-736.

389   P. Wipf and P. D. G. Coish, *Journal of Organic Chemistry*, 1999, **64**, 5053-5061.

390   Sigma-Aldrich, in 'NMR of 3-butynoic acid ', 2005.

391   M. Casey, J. Leonard, B. Lygo and G. Proctor, 'Advanced Practical Organic Chemistry', Blackie, 1990.

392   D. A. Perrin and W. L. F. Amarego, 'Purification of Labotatory Chemicals', Pergamon Press, 1988.

393   D. A. Quagliato, P. M. Andrae and E. M. Matelan, *Journal of Organic Chemistry*, 2000, **65**, 5037-5042.

394   S. Brase, C. Gil, K. Knepper and V. Zimmermann, *Angewandte Chemie-International Edition*, 2005, **44**, 5188-5240.

395   H. Li, H. M. Liu, W. Geb, L. Huanga and L. Shan, *Steroids*, 2005, **70**, 970–973.

396   H. Tecle, S. D. Barrett, D. J. Lauffer, C. Augelli-Szafran, M. R. Brann, M. J. Callahan, B. W. Caprathe, R. E. Davis, P. D. Doyle, D. Eubanks, W. Lipiniski, T. Mirzadegan, W. H. Moos, D. W. Moreland, C. B. Nelson, M. R. Pavia, C. Raby, R. D. Schwarz, C. J. Spencer, A. J. Thomas and J. C. Jaen, *Journal of Medicinal Chemistry*, 1998, **41**, 2524-2536.

397   E. Trevisiol, E. Defrancq, J. Lhomme, A. Laayoun and P. Cros, *European Journal of Organic Chemistry*, 2000, 211-217.

ABBREVIATIONS

Ac	Acetyl
ABPP	Affinity Based Protein Profiling
AD	Alzheimer's Disease
ADP	Adenosine DiPhosphate
AMP	Adenosine MonoPhosphate
AP	Activator Protein
aq.	aqueous
Ar	Aryl
ASK	Apoptosis Signal-regulating Kinase
Asc	Ascorbate
ATF	Activating Transcription Factor
ATP	Adenosine TriPhosphate
atm	atmosphere
Aux	chiral Auxiliary
BCP	Biotin Carrier Protein
BMK	Big Mitogen activated protein Kinase
Bn	Benzyl
Boc	*t*-Butyloxycarbonyl
BPL	Biotin Protein Ligase
CAN	Ceric Ammonium Nitrate
CDI	CarbonylDiImidazole
CNS	Central Nervous System
COSY	COrrelation SpectroscopY
CoA	Coenzyme A
CREB	Cyclic AMP Response Element Binding
Da	Dalton
DBU	Diaza-1,3-Bicyclo[5.4.0]Undecane
DCC	DiCyclohexylCarbodiimide
DCM	DiChloroMethane
DEPT	Distortionless Enhancement Polarisation Transfer
DFT	Density Functional Theory

DHEA	DeHydroEpiAndrosterone
DHEAS	DeHydroEpiAndrosterone Sulfate
DIC	DiIsopropylCarbodiimide
DIEA	DiIsopropylEthylAmine
DMAP	DiMethylAminoPyridine
DMEM	Dulbecco's Modified Eagle's Medium
DMF	DiMethylFormamide
DMSO	DiMethylSulphOxide
DNA	DeoxyriboNucleic Acid
DPC4	Deleted in Pancreatic Cancer
ECL	Enhanced ChemiLuminescence
EDC or EDI	1-Ethyl-3-(-3-Dimethylaminopropyl)-Carbodimide
ee	enantiomeric excess
ELISA	Enzyme-Linked ImmunoSorbent Assay
eNOS	endothelial Nitric Oxide Synthases
ERK	Extra cellular signal-Regulated protein Kinase
ESI	ElectroSpray Ionisation
Et	Ethyl
FAB	Fast Atom Bombardment
GABA	γ-AminoButyric Acid
GCK	Germinal Centre Kinase
GFP	Green Fluorescent Protein
GTP	Guanidine TriPhosphate
h	hours
HEK	Human Embryonic Kidney
HMBC	Heteronuclear Multiple Bond Correlation
HOBt	HydrOxyBenzotriazole
HPLC	High Performance Liquid Chromatography
HRP	HorseRadish Peroxidase
HSDH	HydroxySteroid DeHydrogenase
HSQC	Heteronuclear Single-Quantum Coherence
Il	InterLeukin

IR	Infra Red
JNK	c-Jun N-terminal Kinase
LG	Leaving Group
LiHMDS	Lithium HexaMethylDiSilazide
Ln	Ligand
MAP	Mitogen Activated Protein
MAPK	Mitogen Activated Protein Kinase
MAP2K	Mitogen Activated Protein Kinase Kinase (or MAPKK or MKK)
MAP3K	Mitogen Activated Protein Kinase Kinase Kinase (or MAPKKK or MKKK)
MAPKAP-K2	Mitogen Activated Protein Kinase-Activated Protein Kinase 2 (or MK2)
MBP	Maltose Binding Protein
mp	melting point
Me	Methyl
MEF	Myocyte Enhancer Factor
min	minutes
MLK	Mixed Linkage Kinase
ml	millilitre
MRC PPU	Medical Research Council Protein Phosphorylation Unit
MS	Mass Spectrometry
NADP	Nicotinamide Adenine Dinucleotide Phosphate
NHS	*N*-Hydroxy Succinamide
NIH	National Institute of Health
nm	nanometer
NMDA	*N*-Methyl-D-Aspartate
NMR	Nuclear Magnetic Resonance
NOBA	3-NitrO Benzyl Alcohol
nOe	nuclear Overhauser effect
Nu	Nucleophile
OMP	Outer Membrane Protein
P450	cytochrome P450

PBS	Phospho-Buffered Saline
PDK	Phosphoinositide-Dependent protein Kinase
PEG	PolyEthylene Glycol
Ph	Phenyl
PI3K	PhosphoInositide 3-Kinases
pJNK	phosphorylated c-Jun N-terminal Kinase
PKC	Protein Kinase C
P_i	monophosphate
PP_i	diphosphate
Poc	Propargyloxycarbonyl
ppm	parts per million
Py	Pyridine
Quant	Quantitative
R&D	Research and Development
RNA	RiboNucleic Acid
RT	Room Temperature
SAPK	Stress Activated Protein Kinase
SAR	Structure Activity Relationship
scc	side chain cleavage
SDS PAGE	Sodium Dodecyl Sulfate PolyAcylamide Gel Electrophoresis
SULT	SULfoTransferases
Suc	Succinimide
TAK	Transforming growth factor-β-Activated Kinase
TBSOTf	*t*-ButyldimethylSilyl Trifluoromethane sulfonate
TBS	*t*-ButyldimethylSilyl
TBTU	*O*-BenzoTriazol-1-yl-*N,N,N',N'*-TetramethylUronium tetrafluoroborate
TCEP	Tris(CarboxyEthyl)-Phosphine
Tf	TriFluoromethanesulfonate, TriFlate
TFA	TriFluoroacetic Acid
THF	TetraHydroFuran
THIOG	THIOGlycerol
TIBSCl	TriIsopropylBenzeneSulfonyl Chloride

TIBS	TriIsopropylBenzeneSulfonyl
tlc	thin layer chromatography
TNF	Tumour Necrosis Factor
TOCSY	TOtal Correlated SpectroscopY
Ts	*p*-Toluene Sulfonyl, ToSyl
UV	Ultra Violet

APPENDICES
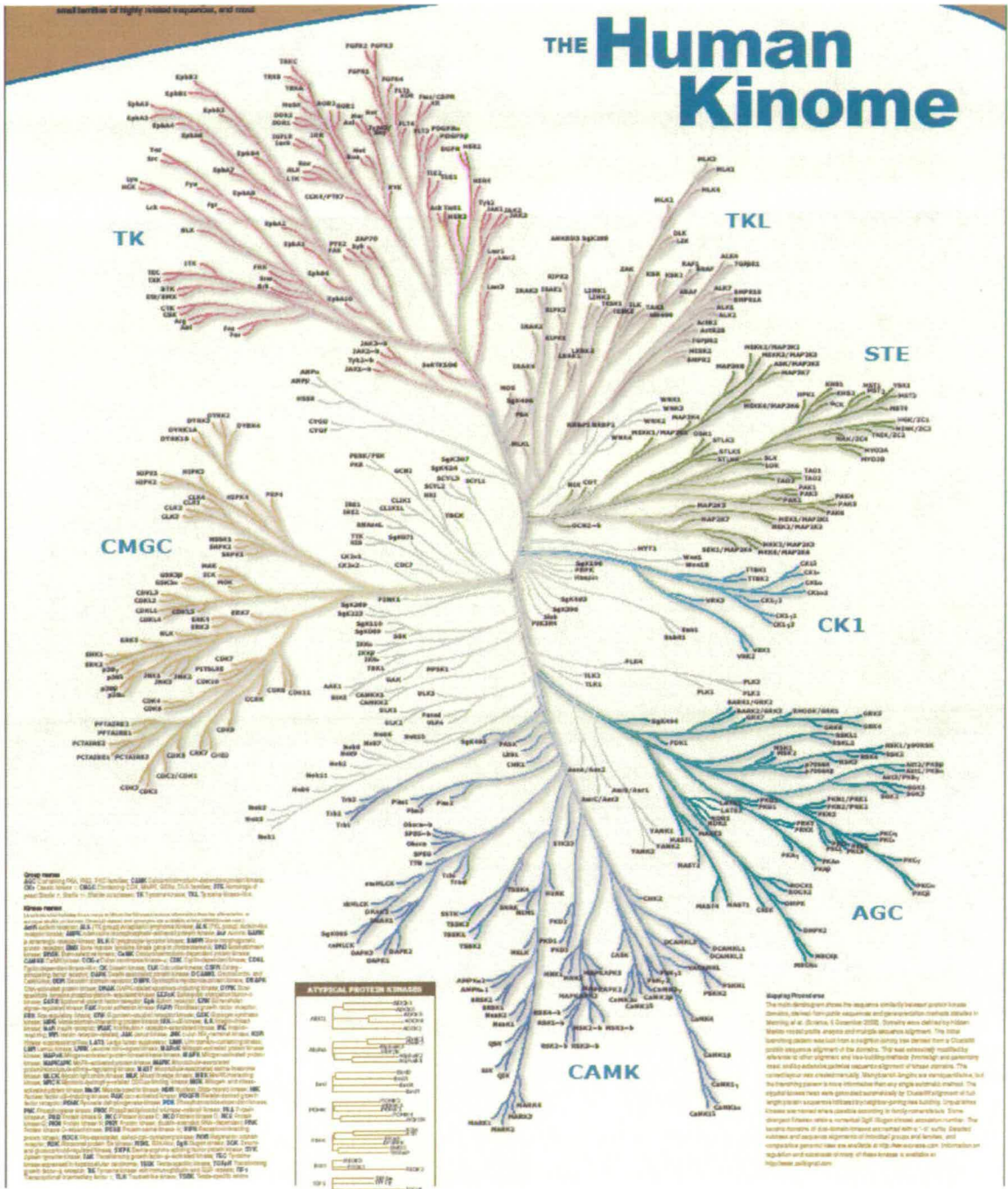
Schematic diagram of the human kinome.[145]
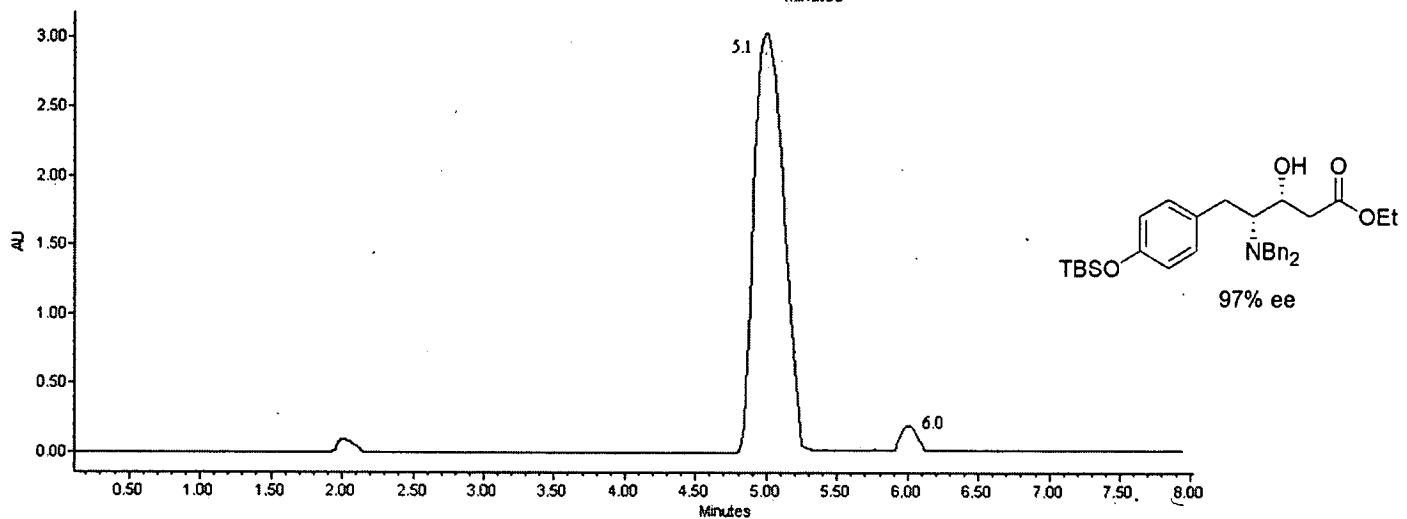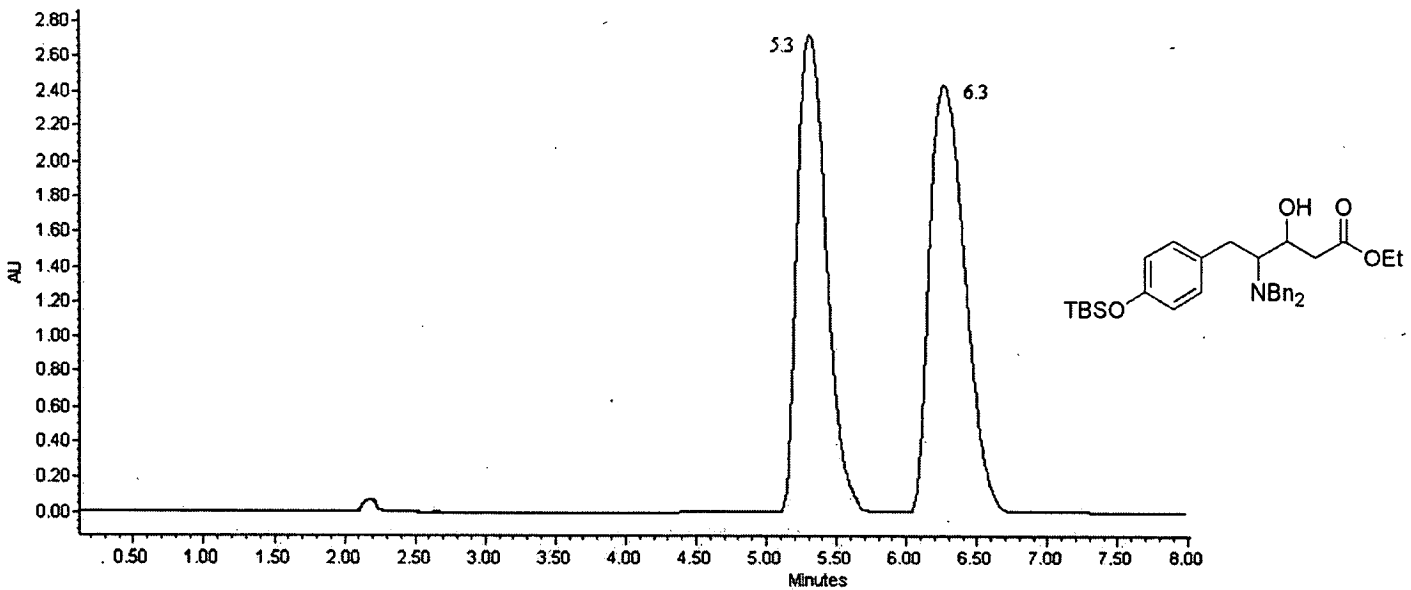
Chiral HPLC traces of racemic β-keto ester and **126** (2% EtOH/hexane).

(2R,3R)- 3-Acetoxy-2-(4'-prop-2-ynyloxybenzyl)-pyrrolidine 136
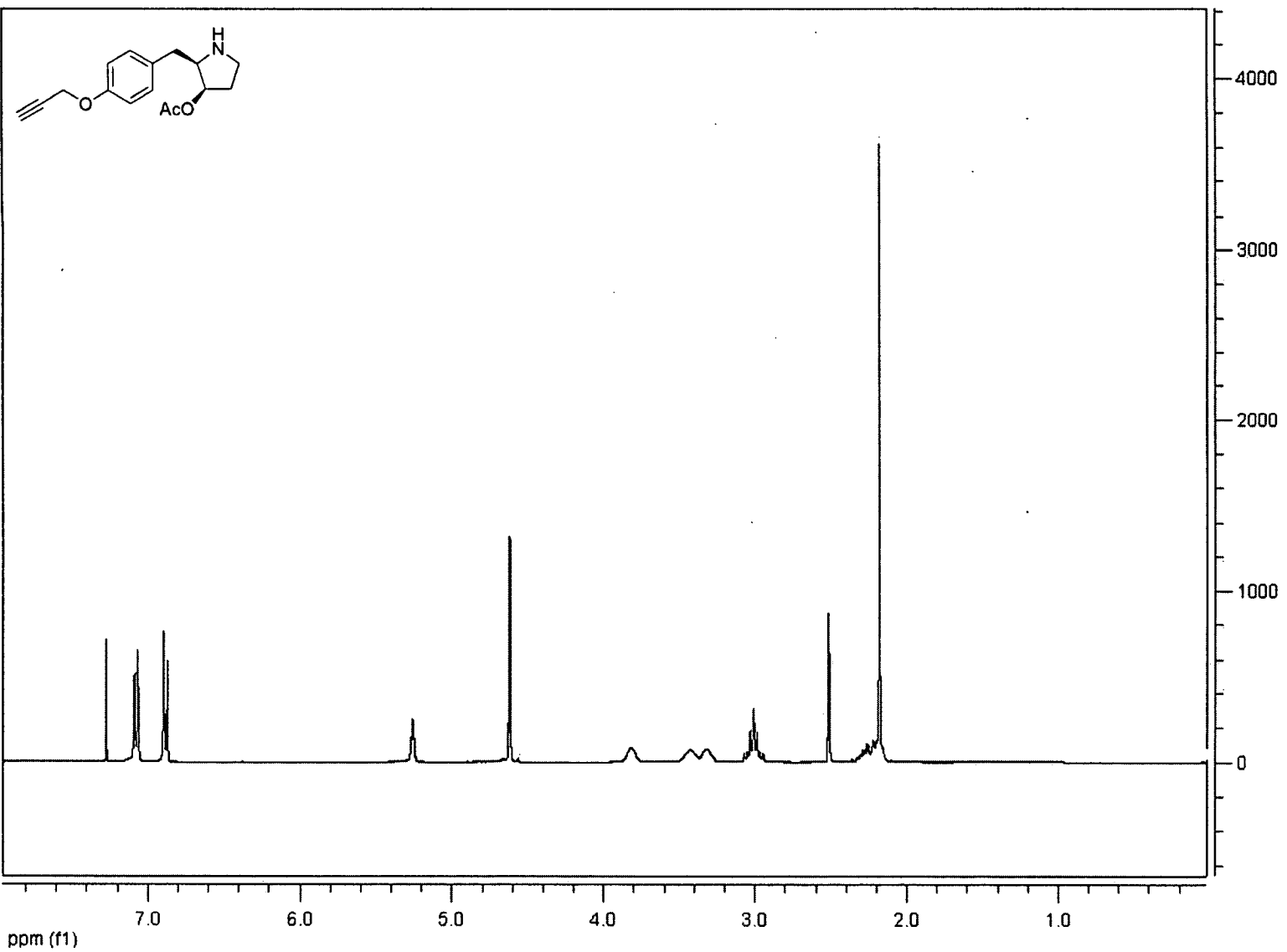
ppm (f1)

(2R,3R)-3-Acetoxy-2-(4'-prop-2-ynyloxybenzyl)-pyrrolidine 136

ppm (f1)
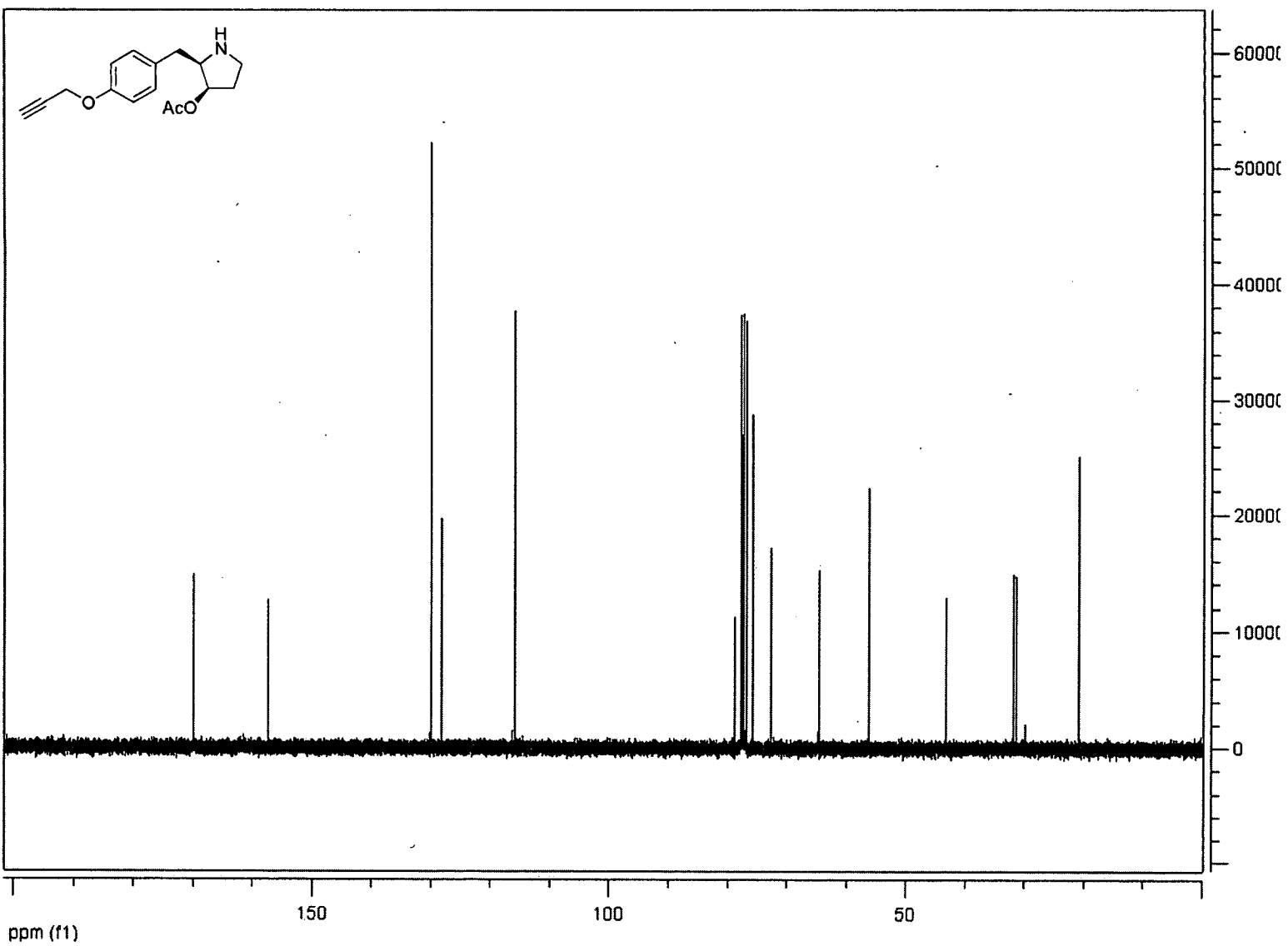
(2R,3S,4S)-3-Acetoxy-4-hydroxy-2-(4'-methoxybenzyl)-1-prop-2-ynyl pyrrolidine 140
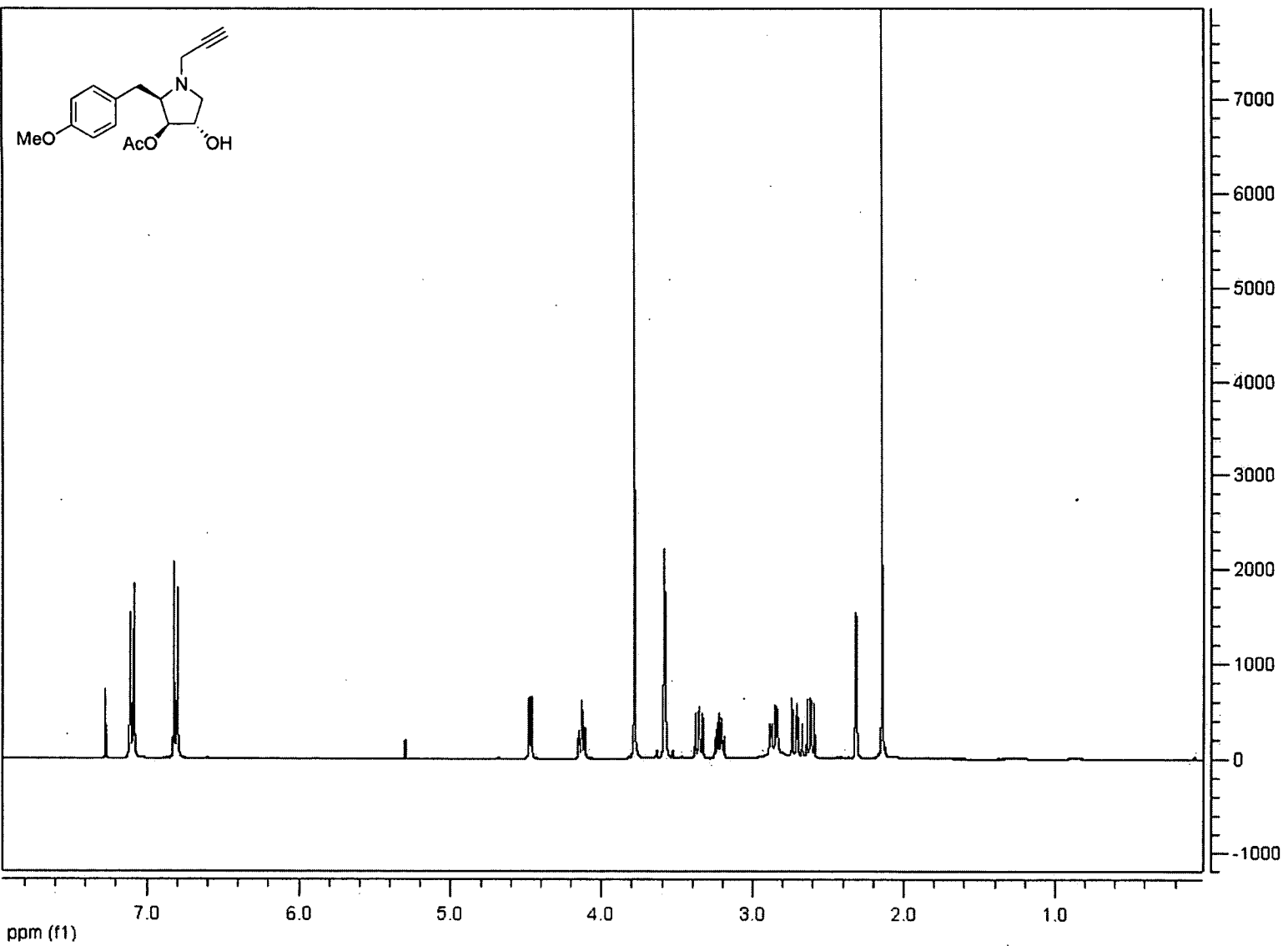
(2R,3S,4S)-3-Acetoxy-4-hydroxy-2-(4'-methoxybenzyl)-1-prop-2-ynyl pyrrolidine 140
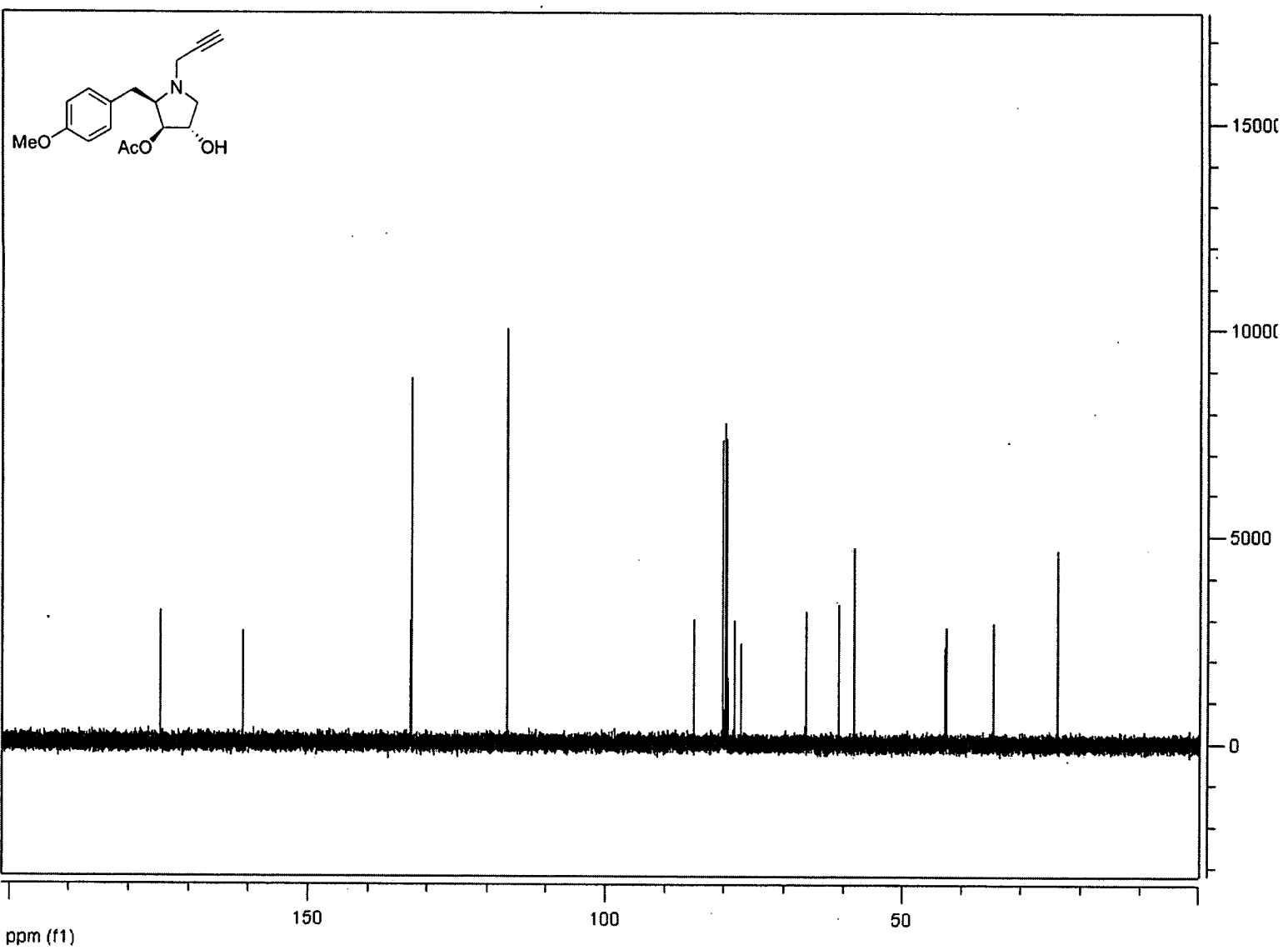
N-Linked anisomycin dansyl molecular probe 191
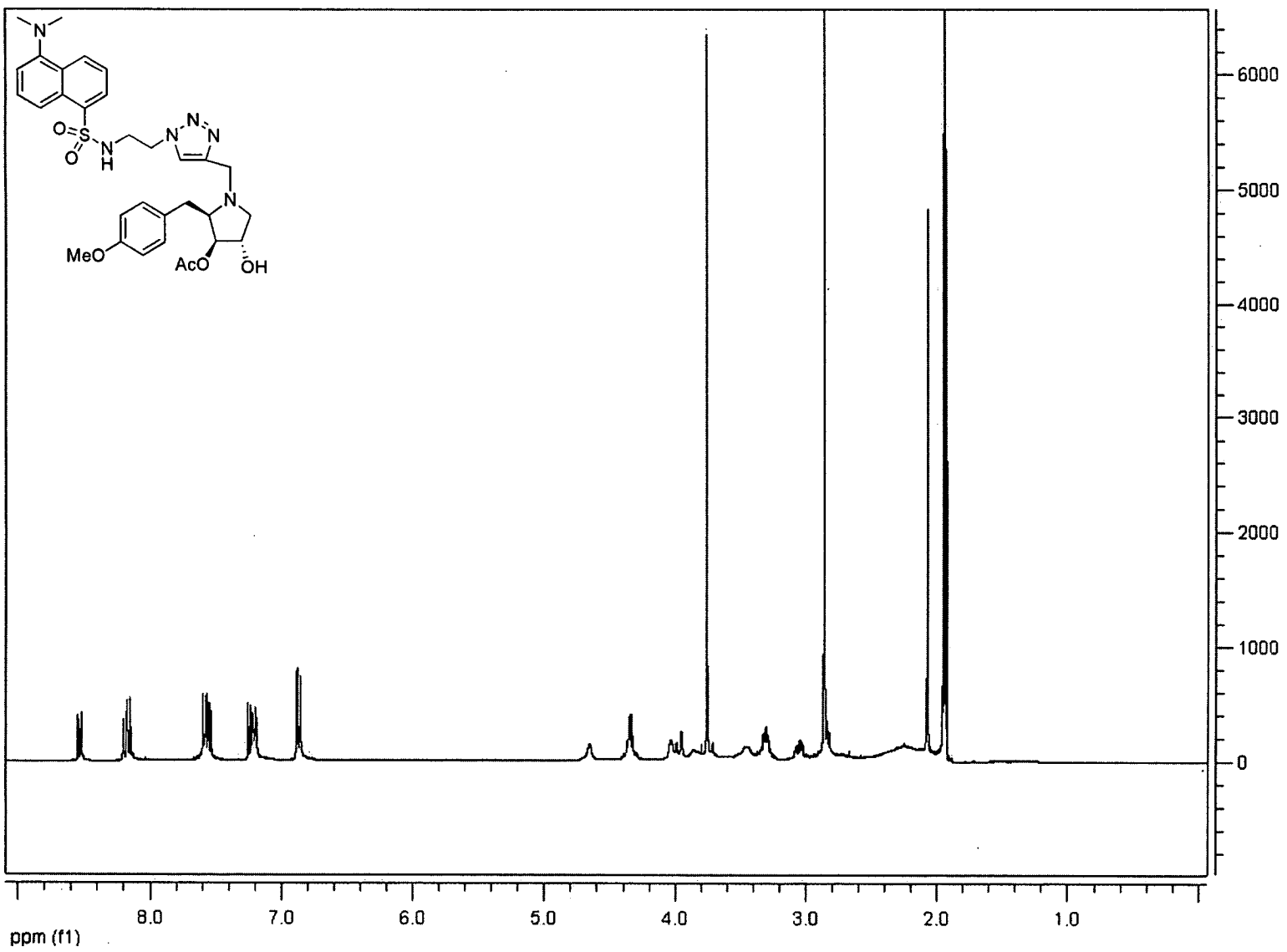
ppm (f1)

C₂-Click *N*-linked anisomycin biotin molecular probe 197

ppm (f1)

C$_2$-Click *N*-linked anisomycin biotin molecular probe 197

ppm (f1)
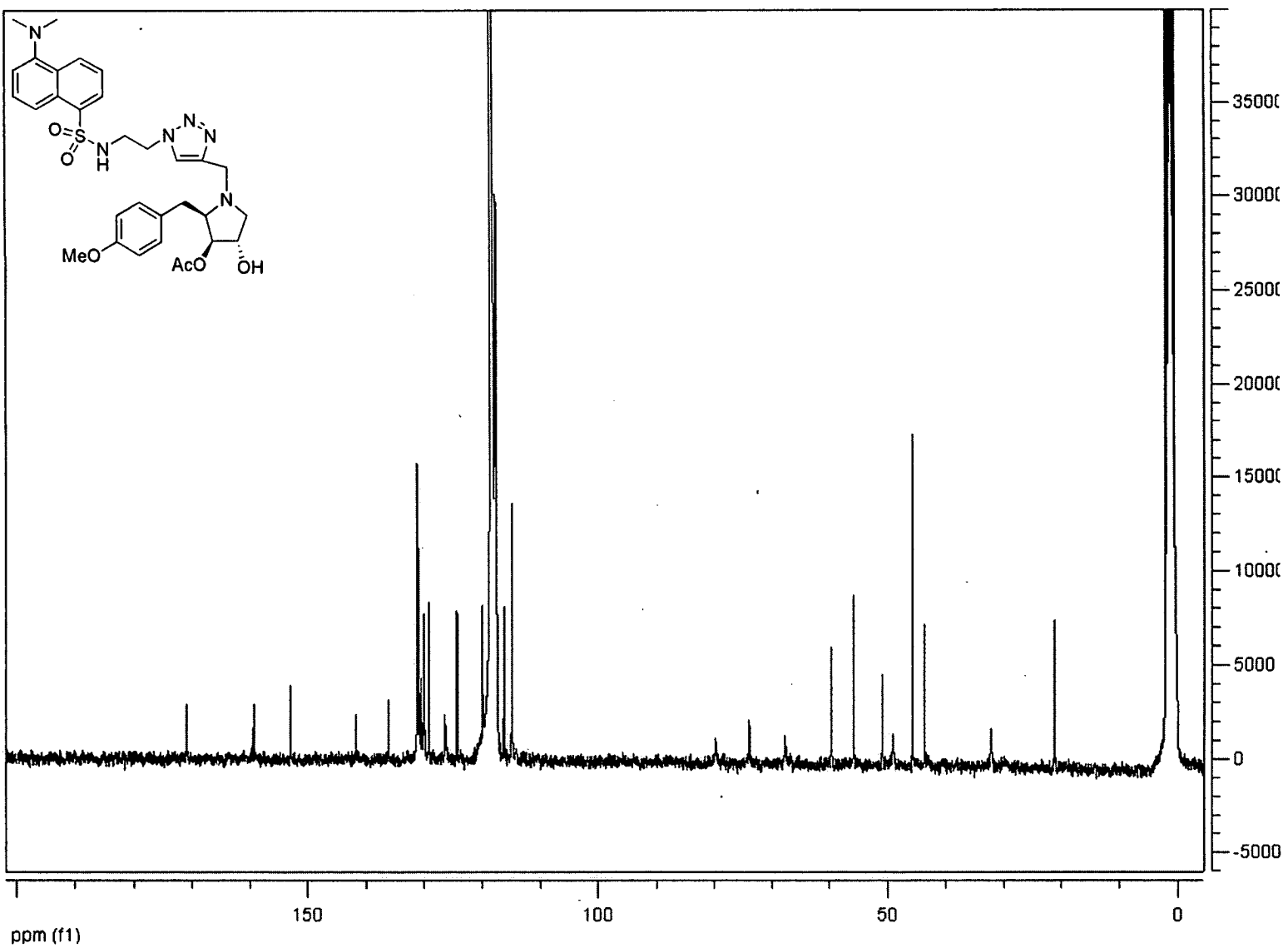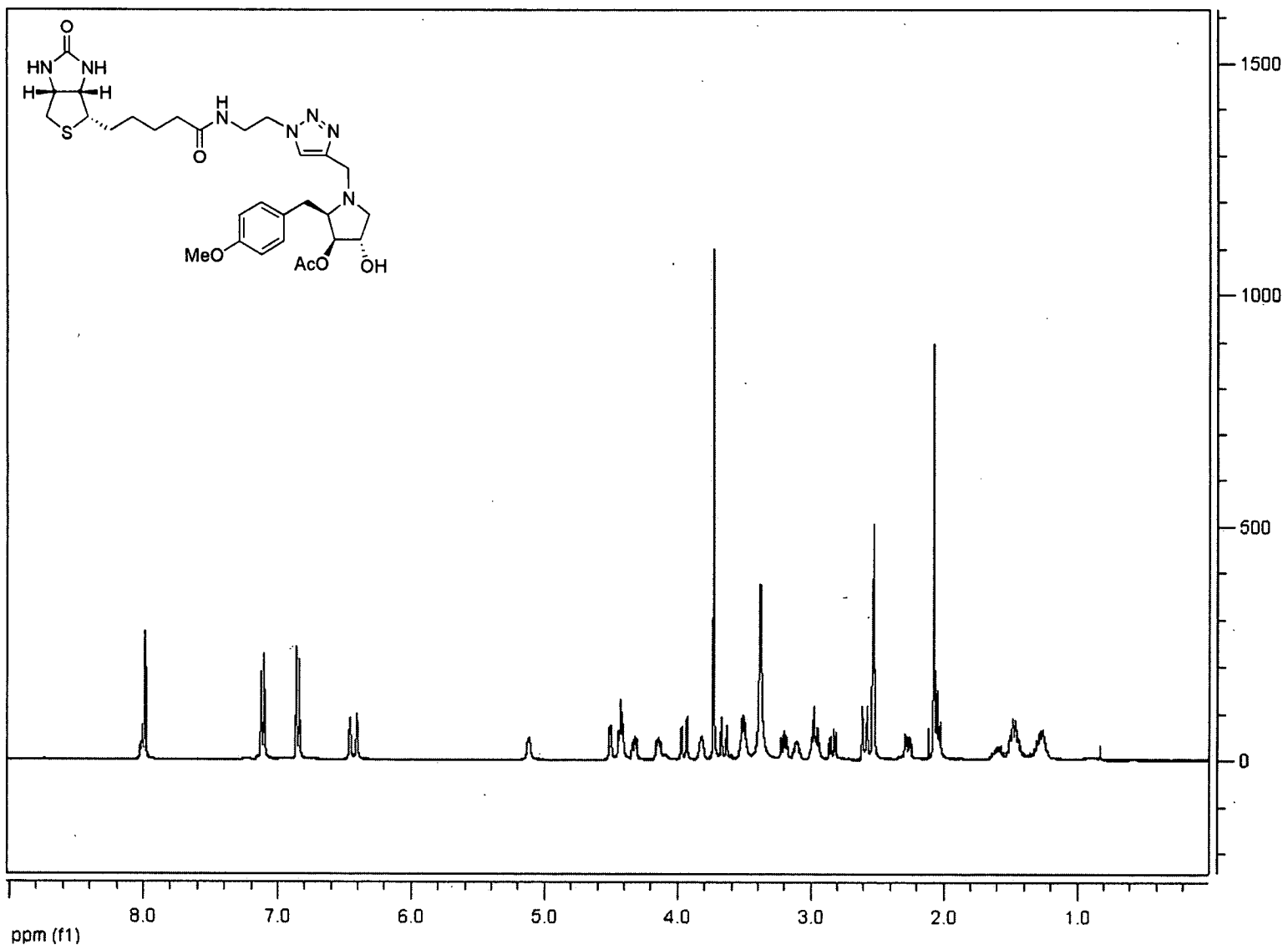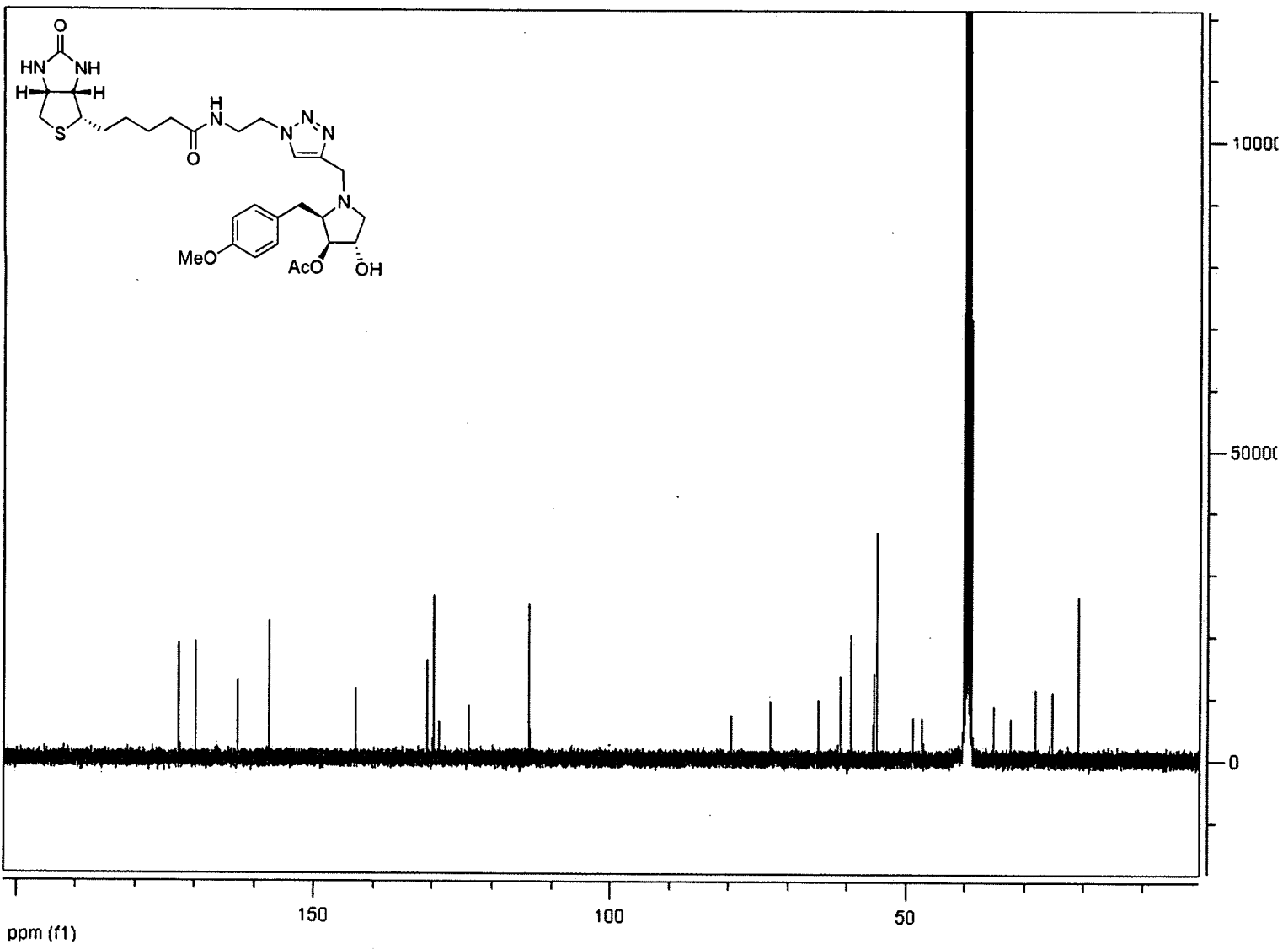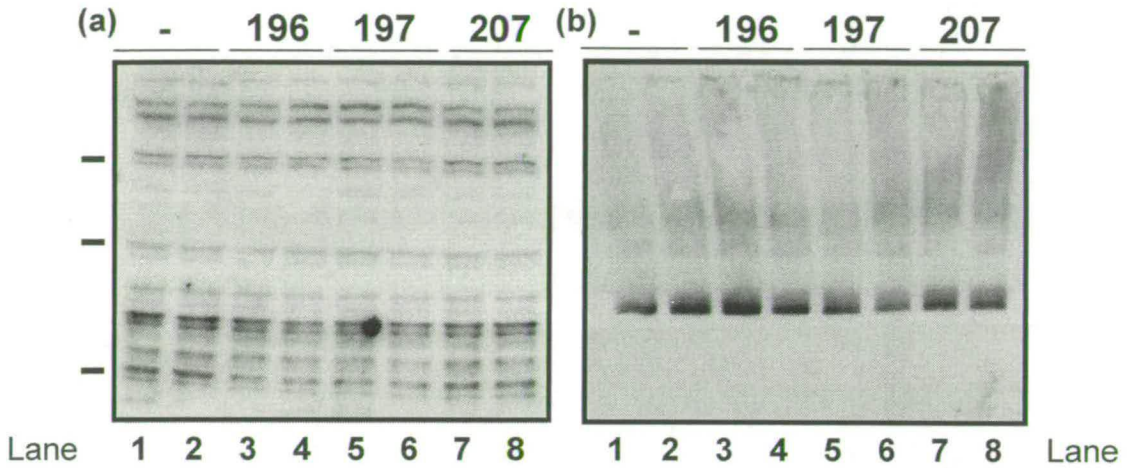
Western Blotting: HEK293 cells were exposed to DMSO (Lane 1/2), **196** (Lane 2/3), **197** (Lane 4/5), or **207** (Lane 6/7), each dissolved in DMSO. The cells were lysed and an aliquot (20 µg of lysate protein) was separated by SDS (a) or native gel (b) electrophoresis. The proteins were transferred to a nitrocellulose membrane and immunoblotted with an antibody that recognized biotin (avidin-HRP), before identifying the immunoreactive proteins using ECL.
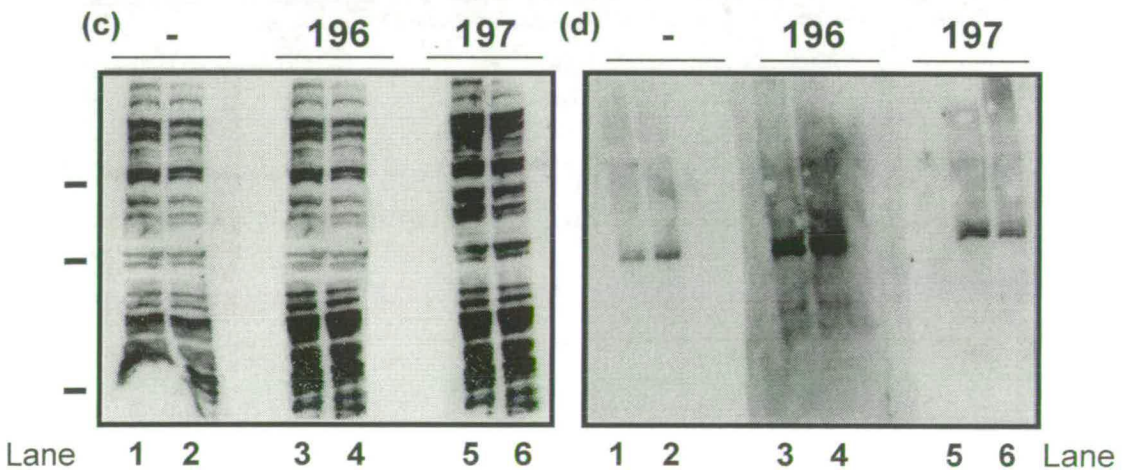
Far Western Blotting: HEK293 cells were lysed and an aliquot (20 µg of lysate protein) was separated by SDS (c) or native gel (d) electrophoresis. The proteins were transferred to a nitrocellulose membrane and incubated with DMSO (Lane 1/2), **196** (Lane 2/3), **197** (Lane 4/5), each dissolved in DMSO. The membranes were then immunoblotted with an antibody that recognized biotin (avidin-HRP), before identifying the immunoreactive proteins using ECL.

Dehydroepiandrosterone (DHEA) 217
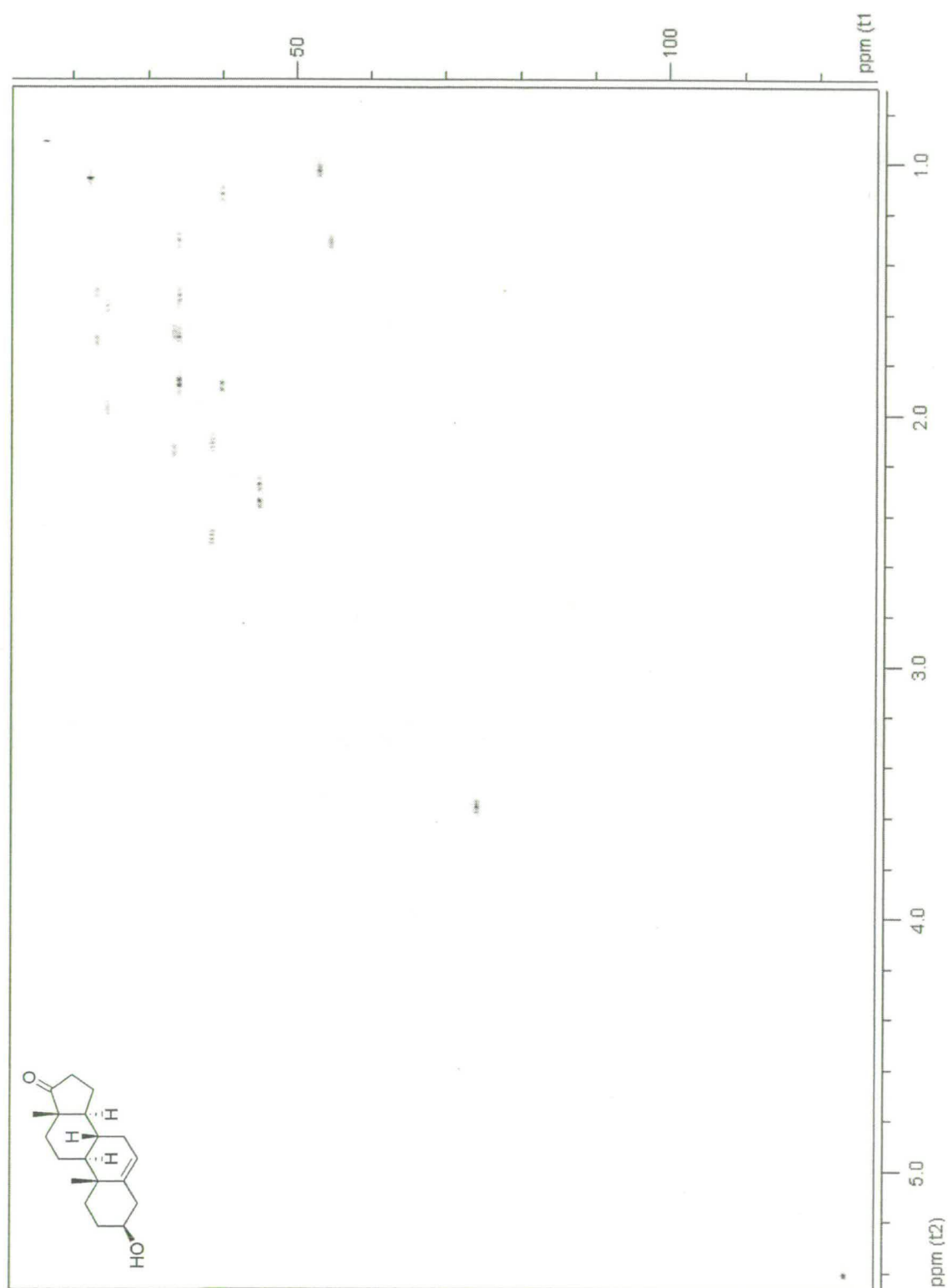
Dehydroepiandrosterone (DHEA) 217
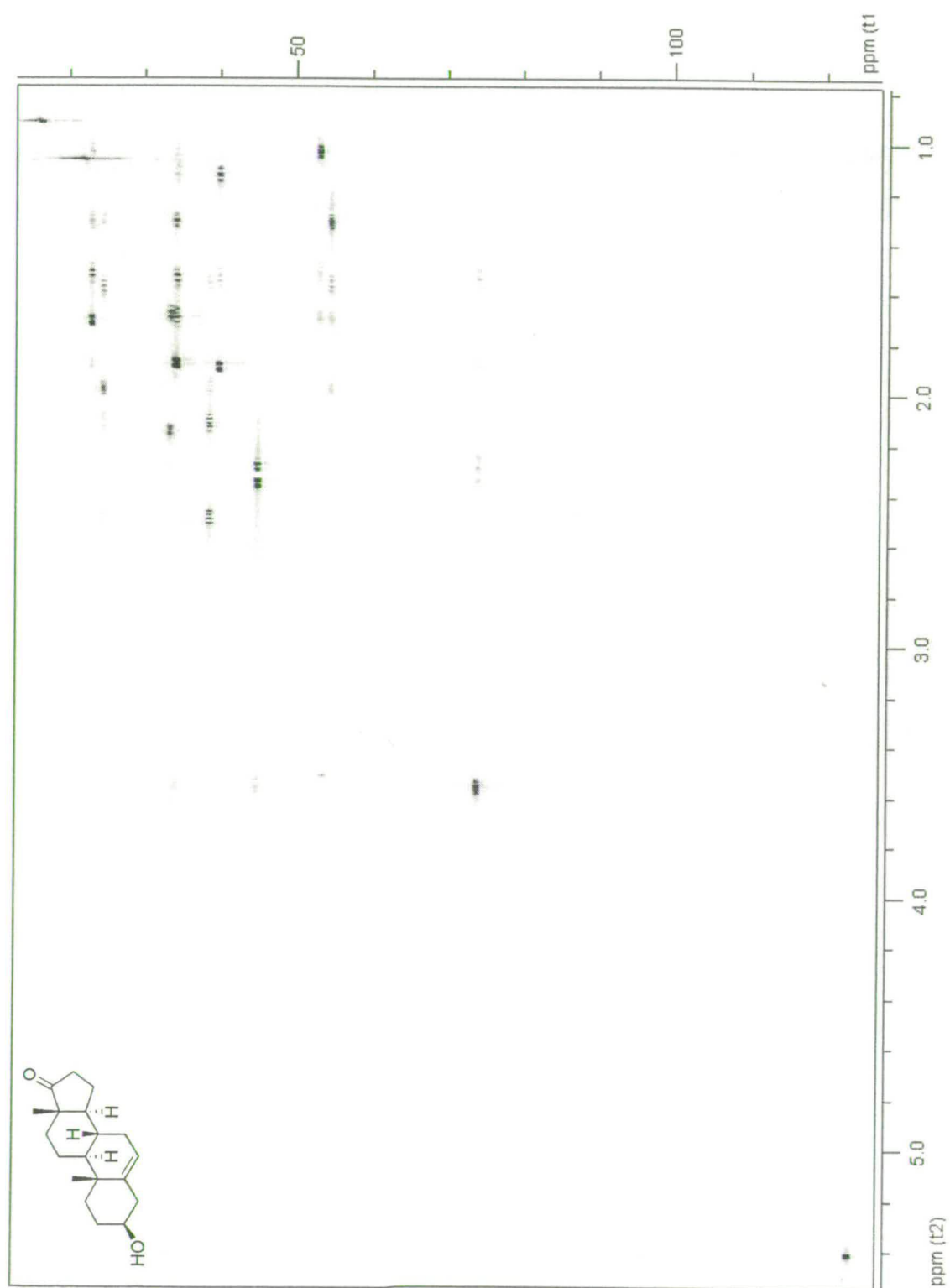
1-(*N*-Dansyl-2-aminoeth-1-yl)-4-(3'β-Hydroxy-androst-5'-en-17'-(*O*-[-*N*-methyl])
acetamidyl)-oxime-1*H*-[1,2,3] triazole 254

ppm (f1)
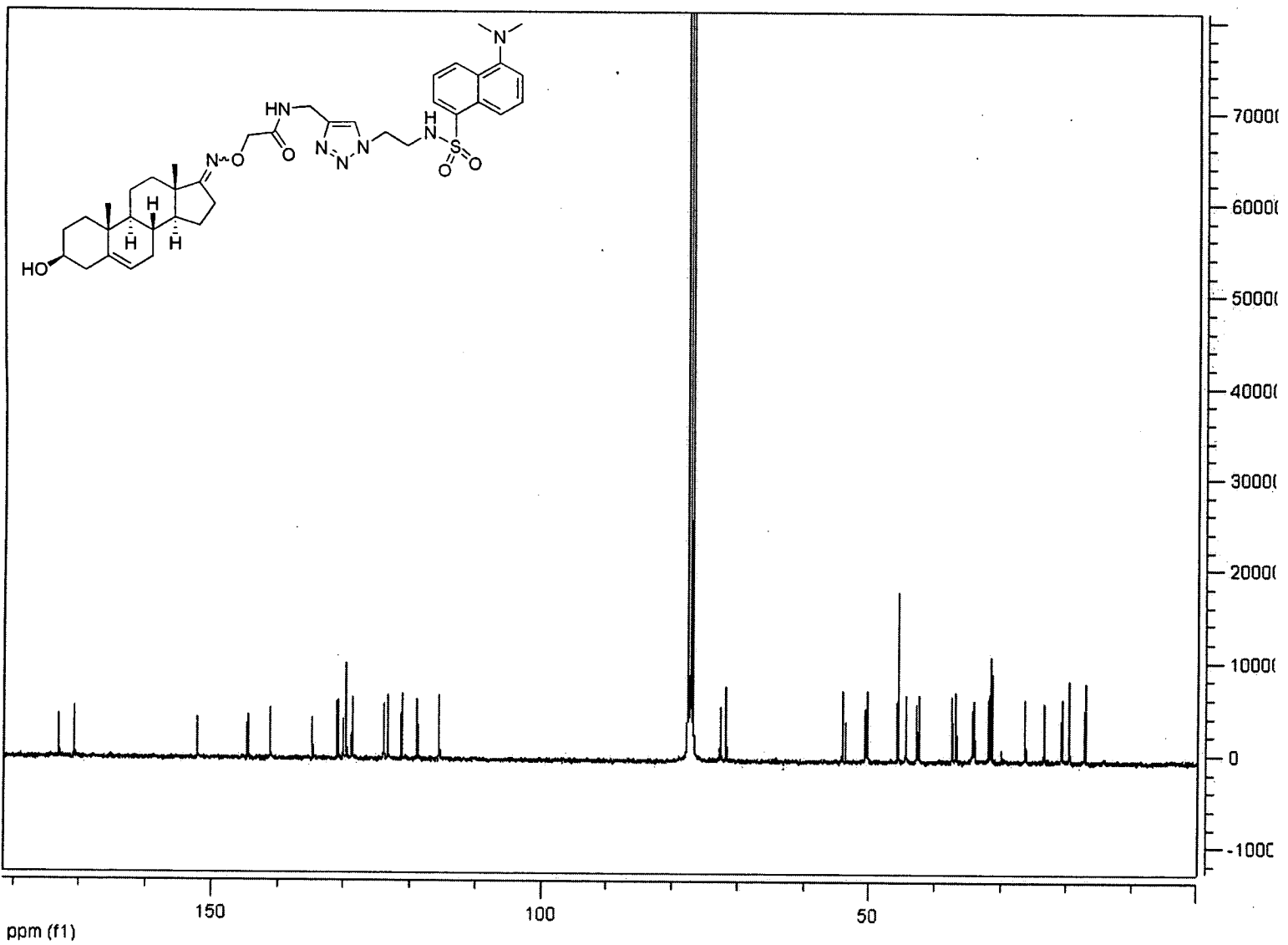
1-(*N*-Dansyl-2-aminoeth-1-yl)-4-(3'β-Hydroxy-androst-5'-en-17'-(*O*-[-*N*-methyl] acetamidyl)-oxime-1*H*-[1,2,3] triazole 254

1-(N-Dansyl-2-aminoeth-1-yl)-4-(3'β-(methyloxycarbonyloxy)-androst-5'-en-17'-one-1H-[1,2,3] triazole 255
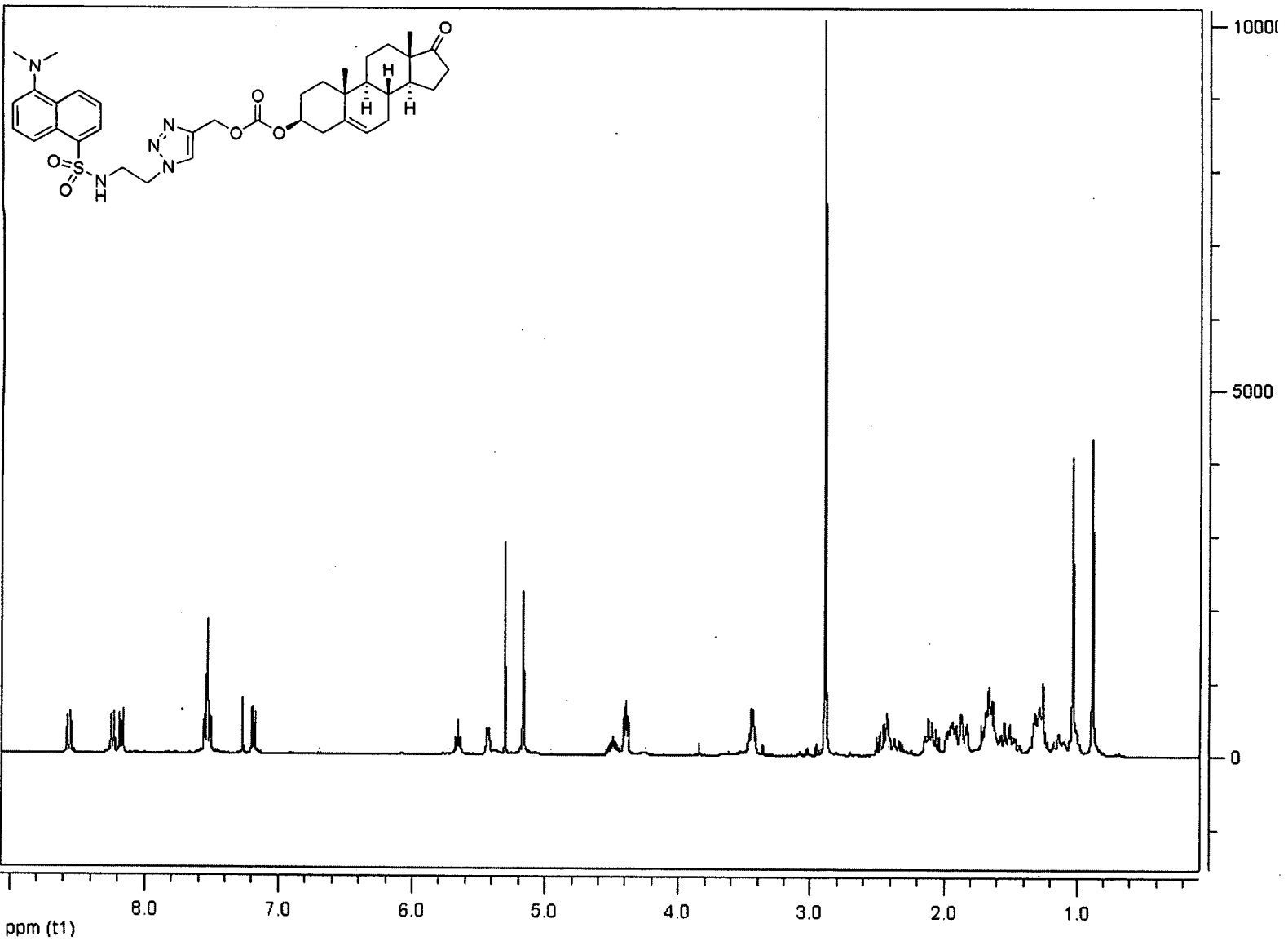
ppm (t1)

1-(*N*-Dansyl-2-aminoeth-1-yl)-4-(3'β-(methyloxycarbonyloxy)-androst-5'-en-
17'-one-1*H*-[1,2,3] triazole 255

ppm (f1)
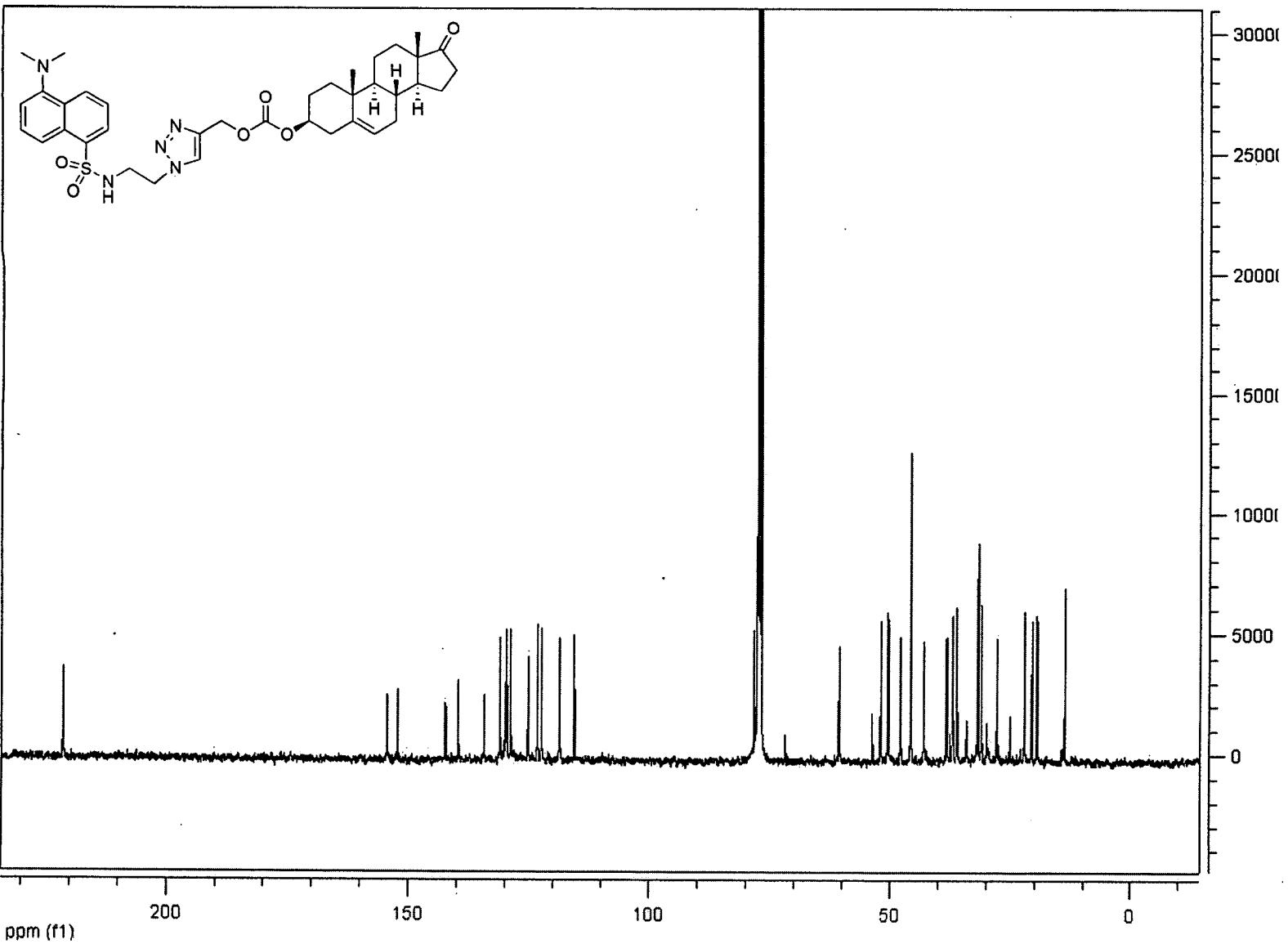
Part A: CRYSTAL DATA	
Empirical formula	$C_{23}H_{30}O_4$
Formula weight	370.47
Wavelength	0.71073 Å
Temperature	150(2) K
Crystal system	Monoclinic
Space group	P21
Unit cell dimension	a = 5.9268(2) Å alpha = 90 deg.
	b = 10.1731(4) Å beta = 93.990(2) deg.
	c = 16.5161(6) Å gamma = 90 deg.
Volume	993.41(6) Å^3
Number of reflections for cell	4185 (2 < theta < 28 deg.)
Z	2
Density (calculated)	1.239 Mg/m^3
Absorption coefficient	0.083 mm^{-1}
F(OOO)	400
Part B: DATA COLLECTION	
Crystal description	colourless block
Crystal size	0.49 x 0.45 x 0.24 mm
Instrument	Bruker Smart Apex CCD
Theta range for data collection	2.35 to 30.39 deg.
Index ranges	-8<=h<=8, -11<=k<=13, -21<=l<=23
Reflections collected	11941
Independent reflections	4892 [R(int) = 0.0582]
Scan type	omega and phi
Absorption correction	Multiscan (Tmin= 0.2695,Tax=0.8614)

Table 1: Crystal data and structure refinement for **241**.

247

Part C: SOLUTION AND REFINEMENT	
Solution	direct (SIR92)
Refinement type	Full-matrix least-squares on F^2
Program used for refinement	SHELXL-97
Hydrogen atom placement	Geom
Hydrogen atom treatment	riding
Data/Restraints/Parameters	4892/1/244
Goodness-of-fit on F^2	1.050
Conventional R [F>4sigma(F)]	R1 = 0.0574 [3472 data]
Weighted R (F^2 and all data)	wR2 = 0.1585
Absolute structure parameter	-0.2(14)
Final maximum delta/sigma	0.001
Weighting scheme	calc w=1/[\s^2^(Fo^2^)+(0.0836P)^2^+0.0000 P] where P=(Fo^2^+2Fc^2^)/3
Largest diff. peak and hole	0.321 and -0.384 e. Å^{-3}

	x	y	Z	U(eq)
C(1)	4761(4)	1010(3)	5786(2)	27(1)
C(2)	5180(4)	527(3)	4938(2)	29(1)
C(3)	6431(4)	1574(3)	4505(2)	26(1)
C(4)	8701(4)	1861(3)	4951(2)	28(1)
C(5)	8381(4)	2282(3)	5820(2)	23(1)
C(6)	9327(4)	3372(2)	6112(2)	27(1)
C(7)	9143(5)	3890(3)	6954(2)	30(1)
C(8)	8216(4)	2871(2)	7524(2)	23(1)
C(9)	6226(4)	2103(2)	7089(2)	24(1)
C(10)	6937(4)	1371(2)	6314(2)	22(1)
C(11)	5055(4)	1183(3)	7669(2)	30(1)
C(12)	4351(4)	1877(3)	8439(2)	33(1)
C(13)	6383(4)	2562(3)	8868(2)	25(1)
C(14)	7383(4)	3520(2)	8275(2)	25(1)
C(15)	8973(5)	4393(3)	8818(2)	31(1)
C(16)	7588(5)	4619(3)	9560(2)	33(1)
C(17)	5917(4)	3482(3)	9558(2)	29(1)
O(17)	4486(3)	3342(2)	10043(1)	41(1)
C(18)	8116(4)	1567(3)	9245(2)	30(1)
C(19)	8316(4)	120 3)	6530(2)	29(1)
C(20)	6806(4)	2007(3)	3118(2)	28(1)
C(21)	6747(5)	2270(3)	1707(2)	41(1)
C(22)	8982(5)	2745(3)	1516(2)	32(1)
C(23)	10689(5)	3122(3)	1291(2)	37(1)
O(24)	6753(3)	1089(2)	3686(1)	29(1)
O(25)	6812(4)	3169(2)	3213(1)	44(1)
O(26)	6862(3)	1391(2)	2403(1)	34(1)

Table 2: Atomic coordinates (x 10^4) and equivalent isotropic displacement parameters (Å^2 x 10^3) for XXX. U(eq) is defined as one third of the trace of the orthogonalized Uij tensor.

BOND	LENGTH Å
C(1)-C(2)	1.522(4)
C(1)-C(10)	1.549(3)
C(2)-C(3)	1.507(4)
C(3)-O(24)	1.463(3)
C(3)-C(4)	1.517(3)
C(4)-C(5)	1.521(3)
C(5)-C(6)	1.319(4)
C(5)-C(10)	1.534(3)
C(6)-C(7)	1.499(4)
C(7)-C(8)	1.527(3)
C(8)-C(14)	1.519(3)
C(8)-C(9)	1.548(3)
C(9)-C(11)	1.540(3)
C(9)-C(10)	1.563(3)
C(10)-C(19)	1.541(3)
C(11)-C(12)	1.537(4)
C(12)-C(13)	1.523(4)
C(13)-C(17)	1.514(3)
C(13)-C(14)	1.531(3)
C(13)-C(18)	1.542(4)
C(14)-C(15)	1.537(4)
C(15)-C(16)	1.538(4)
C(16)-C(17)	1.523(4)
C(17)-O(17)	1.215(3)
C(20)-O(25)	1.193(3)
C(20)-O(24)	1.327(3)
C(20)-O(26)	1.338(3)
C(21)-O(26)	1.455(3)
C(21)-C(22)	1.465(4)
C(22)-C(23)	1.167(4)

Table 3: Bond lengths for **241**.

BOND	ANGLES	BOND	ANGLES
C(2)-C(1)-C(10)	114.3(2)	C(12)-C(11)-C(9)	113.3(2)
C(3)-C(2)-C(1)	109.0(2)	C(13)-C(12)-C(11)	110.1(19)
O(24)-C(3)-C(2)	107.4(2)	C(17)-C(13)-C(12)	117.0(19)
O(24)-C(3)-C(4)	110.2(19)	C(17)-C(13)-C(14)	100.7(2)
C(2)-C(3)-C(4)	110.7(2)	C(12)-C(13)-C(14)	108.8(2)
C(3)-C(4)-C(5)	110.3(18)	C(17)-C(13)-C(18)	104.7(2)
C(6)-C(5)-C(4)	120.2(2)	C(12)-C(13)-C(18)	111.8(2)
C(6)-C(5)-C(10)	123.5(2)	C(14)-C(13)-C(18)	113.5(18)
C(4)-C(5)-C(10)	116.3(2)	C(8)-C(14)-C(13)	114.1(2)
C(5)-C(6)-C(7)	125.3(2)	C(8)-C(14)-C(15)	120.5(19)
C(6)-C(7)-C(8)	112.7(2)	C(13)-C(14)-C(15)	104.1(2)
C(14)-C(8)-C(7)	111.0(2)	C(14)-C(15)-C(16)	102.2(2)
C(14)-C(8)-C(9)	108.6(17)	C(17)-C(16)-C(15)	105.5(2)
C(7)-C(8)-C(9)	110.6(2)	O(17)-C(17)-C(13)	126.6(3)
C(11)-C(9)-C(8)	112.3(2)	O(17)-C(17)-C(16)	124.8(2)
C(11)-C(9)-C(10)	112.3(2)	C(13)-C(17)-C(16)	108.6(2)
C(8)-C(9)-C(10)	112.4(17)	O(25)-C(20)-O(24)	127.1(3)
C(5)-C(10)-C(19)	108.4(18)	O(25)-C(20)-O(26)	125.5(3)
C(5)-C(10)-C(1)	108.5(19)	O(24)-C(20)-O(26)	107.3(2)
C(19)-C(10)-C(1)	110.0(2)	O(26)-C(21)-C(22)	112.3(2)
C(5)-C(10)-C(9)	109.9(2)	C(23)-C(22)-C(21)	173.8(3)
C(19)-C(10)-C(9)	111.9(2)	C(20)-O(24)-C(3)	115.3(2)
C(1)-C(10)-C(9)	108.2(17)	C(20)-O(26)-C(21)	114.0(2)

Table 4: Bond angles for **241**.

	U11	U22	U33	U23	U13	U12
C(1)	24(1)	31(1)	26(1)	-3(1)	5(1)	-5(1)
C(2)	26(1)	33(2)	27(1)	-2(1)	4(1)	-4(1)
C(3)	31(1)	22(1)	27(1)	-5(1)	9(1)	3(1)
C(4)	27(1)	27(1)	31(1)	-1(1)	10(1)	-2(1)
C(5)	19(1)	21(1)	30(1)	4(1)	7(1)	3(1)
C(6)	30(1)	24(1)	29(1)	6(1)	9 1)	-4(1)
C(7)	41(1)	23(1)	28(2)	-2(1)	8(1)	-7(1)
C(8)	22(1)	20(1)	27(1)	-1(1)	5(1)	-3(1)
C(9)	19(1)	26(1)	27(1)	0(1)	5(1)	1(1)
C(10)	20(1)	21(1)	26(1)	1(1)	7(1)	0(1)
C(11)	27(1)	34(2)	30(1)	-3(1)	8(1)	-11(1)
C(12)	25(1)	47(2)	29(1)	-3(1)	9(1)	-7(1)
C(13)	25(1)	26(1)	27(1)	1(1)	9(1)	1(1)
C(14)	26(1)	21(1)	28(1)	2(1)	7(1)	2(1)
C(15)	41(1)	22(1)	33(2)	-3(1)	8(1)	-4(1)
C(16)	43(1)	23(1)	32(2)	-2(1)	6(1)	5(1)
C(17)	28(1)	37(2)	22(1)	-2(1)	1(1)	8(1)
O(17)	37(1)	58(2)	31(1)	-5(1)	13(1)	4(1)
C(18)	36(1)	26(1)	28(1)	3(1)	6(1)	1(1)
C(19)	30(1)	21(1)	35(2)	1(1)	9(1)	2(1)
C(20)	27(1)	29(2)	30(1)	0(1)	9(1)	2(1)
C(21)	34(1)	51(2)	36(2)	10(1)	5(1)	-2(1)
C(22)	38(1)	27(1)	32(2)	-1(1)	4(1)	3(1)
C(23)	36(1)	31(2)	45(2)	1(1)	8(1)	4(1)
O(24)	42(1)	22(1)	25(1)	-1(1)	9(1)	1(1)
O(25)	67(1)	25(1)	41(1)	4(1)	18(1)	9(1)
O(26)	44(1)	33(1)	27(1)	0(1)	9(1)	-6(1)

Table 5: Anisotropic displacement parameters (Å^2 x 10^3) for **241**. The anisotropic displacement factor exponent takes the form: $-2 \pi^2 [h^2 a^{*2} U11 + ... + 2 h k a^* b^* U12]$.

	x	y	z	U(eq)
H(1A)	3942	318	6069	32
H(1B)	3770	1794	5738	32
H(2A)	6085	-292	4973	34
H(2B)	3720	334	4633	34
H(3)	5504	2396	4468	32
H(4A)	9478	2568	4667	33
H(4B)	9661	1065	4954	33
H(6)	10195	3870	5759	33
H(7A)	8139	4669	6931	36
H(7B)	10658	14176	7177	36
H(8)	9449	2237	7694	28
H(9)	5077	2773	6898	28
H(11A)	3694	801	7378	36
H(11B)	6093	451	7829	36
H(12A)	3156	2531	8291	40
H(12B)	3729	1225	8808	40
H(14)	6104	4103	8076	30
H(15A)	9298	5232	8546	38
H(15B)	10415	3938	8973	38
H(16A)	8585	4621	10066	39
H(16B)	6778	5470	9513	39
H(18A)	8504	932	8831	445
H(18B)	7462	1102	9693	45
H(18C)	9484	2034	9451	45
H(19A)	7417	-474	6846	43
H(19B)	9709	358	6851	43
H(19C)	8698	-322	6030	43
H(21A)	5778	3032	1820	49
H(21B)	6037	1802	1229	49
H(23)	12078	3429	1107	45

Table 6: Hydrogen coordinates (x 10^4) and isotropic displacement parameters (A^2 x 10^3) for **241**.

Marked small molecule libraries: a truncated approach to molecular probe design†

Iain A. Inverarity and Alison N. Hulme*
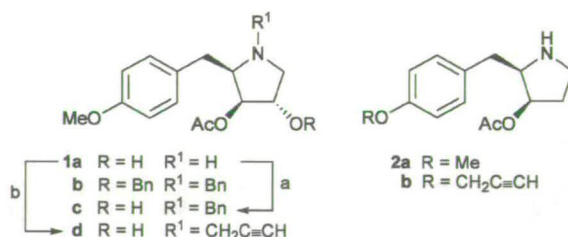
A truncated approach to the design of molecular probes from small molecule libraries is outlined, based upon the incorporation of a bioorthogonal marker. The applicability of this strategy to small molecule chemical genetics screens has been demonstrated using analogues of the known stress activated protein kinase (SAPK) pathway activator, anisomycin. Compounds marked with a propargyl group have shown activation of the SAPK pathways comparable to that induced by their parent structures, as demonstrated by immunoblot assays against the downstream target JNK1/2. The considerable advantages of this new approach to molecular probe design have been illustrated through the rapid development of a functionally active fluorescent molecular probe, through coupling of the marked analogues to fluorescent azides using the copper(I)-catalyzed Huisgen 1,3-dipolar cycloaddition reaction. Active molecular probes generated in this study were used to investigate cellular uptake through FACS analysis and confocal microscopy.

Introduction

Chemical tools to alter the functions of gene-products with temporal and spatial control in tissue culture cells, or animals, provide a highly attractive alternative to traditional genetic approaches when studying basic cell biology.[1] Chemical genetics screens frequently make use of large compound libraries which are screened for activity and, following 'hit' identification, strategies for gene-product target identification may be pursued.[2] Frequently the target identification process may require a tedious resynthesis and/or redesign of the synthetic strategy for the library member.[3] Hence, there are as yet only a handful of examples of target identification, even though a number of groups world-wide have carried out such chemical genetics screens.[4] Indeed, it is in carrying out the target identification stage of such a screen in our study of the activation of the stress activated protein kinase (SAPK) pathways by both the *Streptomyces* sp. metabolite, anisomycin **1a** (Scheme 1),[5] and a small library of anisomycin analogues,[6] that we have encountered these challenges at first hand.

Anisomycin was first isolated from the fermentation broths of *Streptomyces griseolus* and *S. roseochromogenes* in 1954,[7] while more recently it has been isolated from *Streptomyces* sp. SA3079 and No. 638.[8] Its structure was elucidated in 1965,[9] whilst its relative stereochemistry was established three years later by NMR studies and X-ray crystallography.[10] Anisomycin was found to exhibit selective and potent activity against pathogenic protozoa and certain strains of fungi as well as inhibiting *Entamoeba histolytica*, *Trichomonas vaginalis*, *Tritrichomonas foetus* and *Candida albicans*.[11] This led to clinical trials for the treatment of amoebic dysentery and vaginitis. However, anisomycin was found to be

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† Electronic supplementary information (ESI) available: Preparation of compounds **2b** from **3**, NMR spectra of key compounds. See DOI: 10.1039/b616494c

Scheme 1   *Reagents and conditions*: (a) BnBr, K$_2$CO$_3$, DMF (89%); (b) HC≡CCH$_2$Br, K$_2$CO$_3$, DMF (95%).

inactive towards bacteria at medicinally useful concentrations, with *Staphylococcus aureus*, *Streptomyces faecalis* and gram positive organisms all requiring greater than 100 µg per ml of cell culture for inhibition. More recently it was reported that anisomycin had been identified as an antitumour substance showing *in vitro* cytotoxicity against human tumour lines, such as mammalian cell lines HBL 100, RAS A and MCF 7 in the nM region.[8,12] Recent studies have implied that anisomycin may be used in a synergistic fashion with a cyclin-dependent protein kinase inhibitor to kill carcinoma cells.[13]

Anisomycin has found widespread use by the biochemical and medical communities in studies of protein synthesis both *in vivo* and *in vitro*, as it is a known peptidyl transferase inhibitor, binding to the 60S ribosomal subunit in eukaryotes.[14] More recently it has been utilized at 'sub-inhibitory' concentrations, as a chemical stimulant for the activation of the SAPK pathways (Fig. 1).[15] The stress kinase pathways are a sub-section of the mitogen activated protein kinase (MAPK) pathways, and play a vital role in the intracellular signaling which results from a range of stressors including: oxidative stress; inflammatory cytokines; UV radiation; heat; and chemical stimulants.[16] However, the cellular target of anisomycin and therefore its precise mode of activation of this signaling pathway have yet to be elucidated.

Despite rapid advances in the generation and testing of small molecule libraries in recent years, current strategies for the
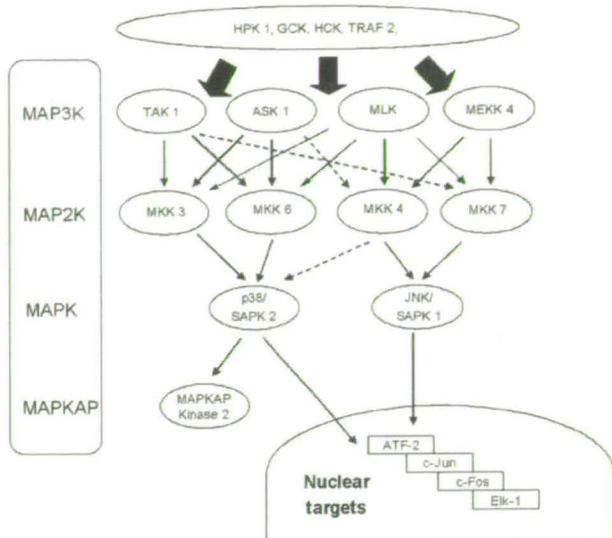
Fig. 1 Stress activated protein kinase (SAPK) pathways.

identification of the biological targets of library members in forward chemical genetics screens still require the synthesis of an appropriate molecular probe. The structure of this molecular probe is typically based on an active library member which has been modified by introduction of a tag, such as a biotin moiety, or a photoactivatable-, radio-, or fluorescent-label [Fig. 2(a)].[17] In a number of instances, this molecular probe-based approach has allowed the identification of the small molecule's site of

Fig. 2 Strategies for small molecule library design: (a) traditional approach involves library synthesis, biological screening, redesign of synthesis to form molecular probes, re-screening and target identification; (b) marked library strategy involves library synthesis incorporating a bio-compatible marker, biological screening, rapid molecular probe formation, re-screening followed by target identification.

interaction.[18] However, the synthesis of molecular probes can often be a cumbersome process: more often that not, the synthetic route to the active library member may have to be redesigned to allow tagging of the small molecule at various sites around its molecular scaffold [Fig. 2(a), part 4]. Moreover, a number of the molecular probes synthesized in this manner will have lost their activity due to structural perturbations upon addition of the tag [Fig. 2(a), part 5]. These factors combined can make the synthesis of molecular probes a time consuming and challenging process.

We have therefore designed a more direct approach which is based on the concept of a marked library, where each individual library member carries a small biocompatible marker which plays no role in the screening process itself, but may be used in the target identification process once screening is complete [Fig. 2(b)]. Although this is a new approach to small molecule library design, this concept has precedent in chemical biology, where, for example, modified amino acids, sugars, *etc.*, with chemical markers such as azides, alkynes and phosphines, *etc.*, have been incorporated into a range of biomolecules.[19] These markers may then be coupled to orthogonally reactive tags such as biotin, fluorophores, *etc.*, utilizing biologically compatible reactions such as the Staudinger–Bertozzi ligation, and the Huisgen 1,3-dipolar cycloaddition, thus allowing the visualization,[20] purification[21] and identification of the biomolecule of interest. This strategy has also been used by Cravatt and co-workers using activity-based protein profiling (ABPP) to interrogate the proteome, where the design of the functional probe relies upon a *known* protein activity.[22] In a closely related example to our proposed marked small molecule library strategy, Chang and Khersonsky have designed a triazine library with a built-in linker containing an amino functionality to facilitate target identification.[23] More recently, Cravatt's group has generated a natural product analogue library based on a protein-reactive moiety with a pendant alkynyl functionality; allowing subsequent target identification after functionalization with a fluorescent, and/or biotinylated azide.[24] In this paper, we demonstrate an analogous strategy which incorporates a functionally inert marker directly onto the small molecule library members; we also demonstrate the efficacy of this approach to the synthesis of molecular probes through the rapid generation of a biologically active fluorescent probe. We believe that adoption of this 'marked library' approach may address the problem of molecular probe synthesis in harmony with current molecular screening strategies; and thus offers the potential for acceleration of the hit-to-target identification process.

Results and discussion

Over the past few years 1,3-dipolar cycloaddition reactions, notably the Huisgen cycloaddition, have enjoyed a renaissance, due to the introduction of the copper(I)-catalyzed variant (click reaction) developed by Sharpless and co-workers.[25] Following the recent explosion of interest in this reaction as applied to complex biological systems,[19] and the validation of both the propargyl and azide components of this reaction in a number of settings,[19] we chose to base our marker around a propargyl group. This marker was particularly attractive due to the commercial availability of a wide range of reactive precursors, including propargyl bromide, propargylamine, propargyl chloroformate, but-3-ynoic acid. This diverse collection of chemical reactivities
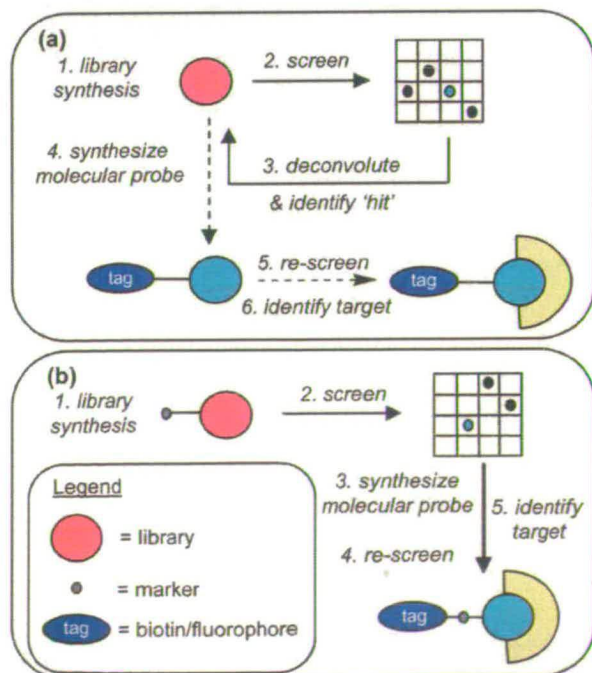
allows the ready functionalization of a range of groups in the small molecule and the tailoring of the resultant functionalities to known structure activity relationship (SAR) constraints. In order to demonstrate the compatibility of the propargyl marker with a small molecule library in a forwards chemical genetics screen, we set out to synthesize a series of marked anisomycin analogues. Using the known phenotypic response to activation of the SAPK pathways, phosphorylation of the downstream protein kinase c-Jun N-terminal kinase (JNK), we would then be able to validate the addition of a propargyl marker to the small molecule scaffold in these screens.

Previous studies into the SAR profile of anisomycin **1a** indicated that the phenolic oxygen had potential as the site of attachment of the key propargyl marker,[6,12] since minor structural variation at this position was possible whilst still retaining activity. However, in order to validate the marked library strategy more thoroughly, we wished to investigate alternative sites of attachment of the propargyl marker to the small molecule core. The di-benzyl analogue **1b**, an intermediate in previous synthetic studies, has been shown to be inactive in assays for SAPK pathway activation.[6] Nonetheless, the pyrrolidine nitrogen presents an attractive target for functionalization either as an amine, or amide derivative. Thus, in order to investigate more closely the reasons for the loss of activity by the di-benzyl analogue **1b**, we synthesized the mono-benzyl analogue **1c** (Scheme 1). This new analogue gave strong activation of the SAPK pathways, suggesting that the loss of activity in **1b** was due to functionalization of the C(4)-OH position, rather than of the pyrrolidine nitrogen. We these results in hand, we focused our attention on two potential marking sites within the molecule: the phenolic oxygen and the pyrrolidine nitrogen. Access to the O-propargyl C(4)-H analogue **2b** was envisaged through modification of our previous synthetic route to the C(4)-H series.[6] Thus, protected amino acid **4** was readily accessed from D-tyrosine **3** in four steps (Scheme 2). Chain extension via a Claisen condensation, followed by stereoselective reduction gave β-hydroxy ester **5**. Reduction of the ester with LiAlH₄, followed by selective activation of the primary alcohol with the hindered reagent triisopropylbenzene sulfonyl chloride (TIBSCl), resulted in cyclization to give pyrrolidinium salt **6**. Careful manipulation of the protecting groups to give key intermediate **7**, allowed for a highly efficient end-sequence of selective formation of the propargyl ether, before a final Boc-deprotection of the pyrrolidine nitrogen to give **2b**. The required N-propargyl analogue **1d** was very readily synthesized from anisomcin **1a** using a single equivalent of propargyl bromide in the presence of potassium carbonate in almost quantitative yield (Scheme 1).

Although the precise target of anisomycin is unknown, the downstream effects on the SAPK pathways have been well documented.[16] Treatment of mammalian, yeast and insect cells with anisomycin **1a** is known to strongly activate both the JNK/SAPK1 and the p38/SAPK2 pathways, resulting in phosphorylation of their respective substrates, including JNK and MAPKAP-K2. In order to assess the relative levels of activation by marked compounds, they were screened using an immunoblot assay for phosphorylation of JNK1/2 in HEK-293 cells. The activation levels induced by these compounds were scaled against anisomycin, deacetylanisomycin, and DMSO (strong and moderate activators, and control respectively). Selected results of these assays are shown in Fig. 3; which demonstrates that the level
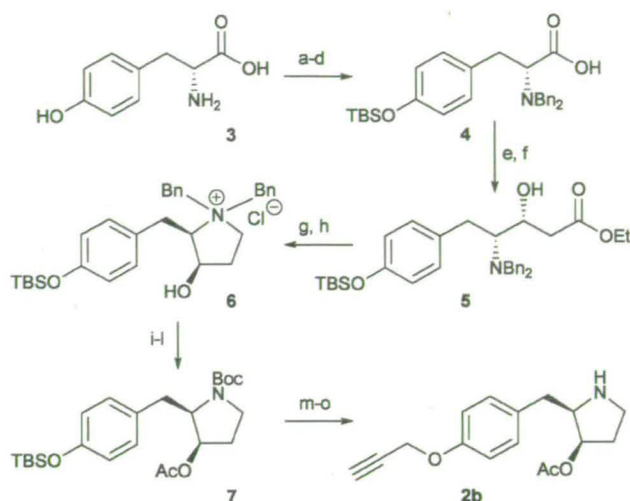
Scheme 2 *Reagents and conditions*: (a) AcCl, MeOH, reflux (100%); (b) BnBr, K₂CO₃, MeCN (91%); (c) LiOH, THF–H₂O (4 : 1), reflux (87%); (d) i) TBSOTf, 2,6-lutidine, CH₂Cl₂; ii) AcOH–THF–H₂O (3 : 1 : 1) (90%); (e) i) CDI, THF; ii) CH₂=C(OLi)OEt (71%); (f) NaCNBH₃, MeOH, AcOH, Et₂O (80%); (g) LiAlH₄, THF (91%); (h) i) TIBSCl, DMAP, CH₂Cl₂; ii) Dowex (Cl⁻) ion-exchange resin (92%); (i) H₂, 5% Pd/C, K₂CO₃, MeOH (80%); (j) Ac₂O, Et₃N, DMAP, CH₂Cl₂ (94%); (k) H₂, EtOH, Pd(OH)₂ (95%); (l) Boc₂O, Et₃N, CH₂Cl₂ (89%); (m) HF·3Et₃N, THF (94%); (n) HC≡CCH₂Br, K₂CO₃, DMF (99%); (o) TFA, CH₂Cl₂ (97%).
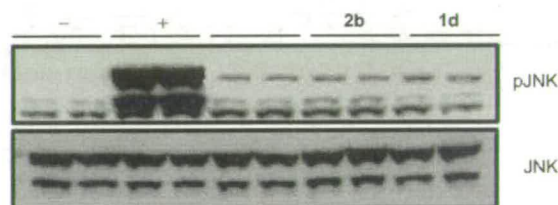
Fig. 3 Effect of anisomycin and its analogues on the phosphorylation of JNK1/2 isoforms in HEK-293 cells. The cells were exposed to DMSO (lanes 1 and 2), anisomycin **1a** (lanes 3 and 4), deacetylanisomycin (lanes 5 and 6), or the anisomycin analogues **2b** (lanes 7 and 8) and **1d** (lanes 9 and 10), each dissolved in DMSO. The cells were lysed and an aliquot (20 μg of lysate protein) was denatured in SDS, subjected to electrophoresis on a 10% polyacrylamide gel, transferred to a nitrocellulose membrane and immunoblotted with an antibody that recognized JNK1/2 phosphorylated at Thr183, or with an antibody that recognizes phosphorylated and unphosphorylated JNK1/2 equally well.‡

of JNK1/2 activation by the marked analogues **1d** and **2b** is comparable with that induced by deacetylanisomycin. Thus we have successfully exchanged the benzyl group of **1c** for a propargyl group in **1d**, and the methyl group in **2a** for a propargyl group in **2b**, whilst retaining comparable activity; hence validating the hypothesis that a chemical marker can be incorporated into a small molecule library.

The next step in our abbreviated small molecule to molecular probe synthetic process [Fig. 2(b)] is the direct conversion of an active marked library member to a fully functional probe. We

‡ The immunoblots show two distinct bands per lane due to phosphorylation of the 46 kDa and the 54 kDa spliced variants of JNK1/2.
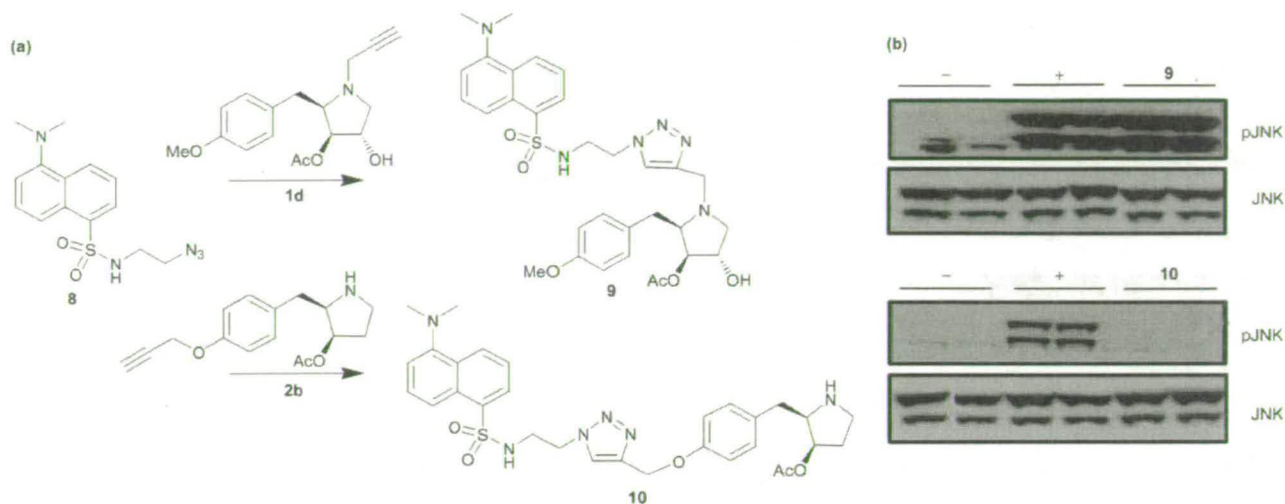
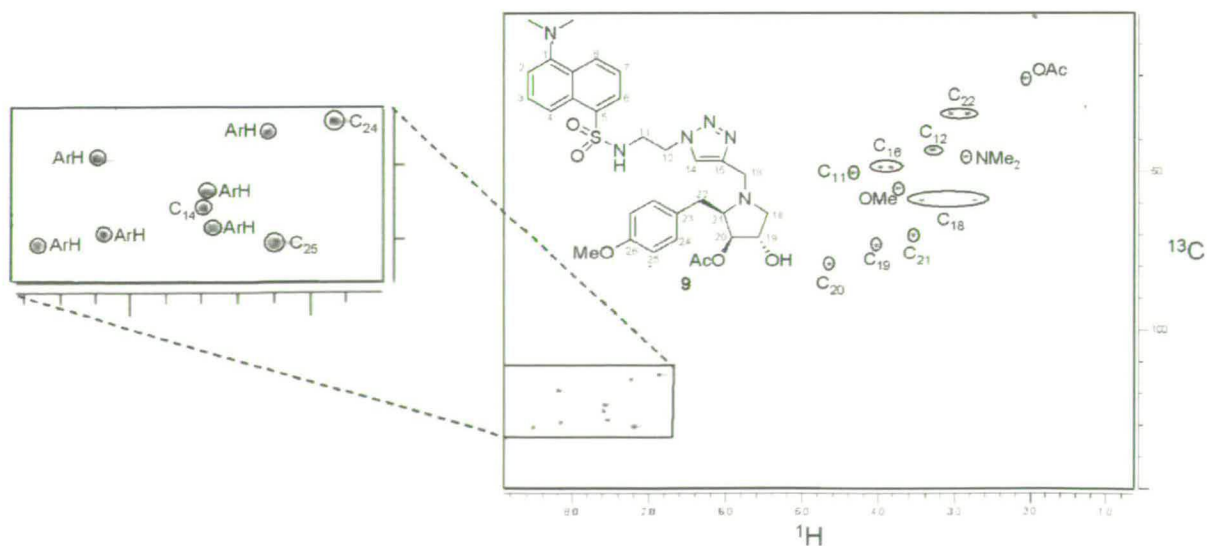Fig. 4 (a) *Reagents and conditions*: $CuSO_4 \cdot 5H_2O$ (10 mol%), NaAsc (1M aq, 20 mol%), 'BuOH–H_2O (1 : 1), rt, 4–5 h (1d → 9, 71%; 2b → 10, 77%); (b) effect of molecular probes 9 and 10 on the phosphorylation of JNK1/2 isoforms in HEK-293 cells. The cells were exposed to DMSO (lanes 1 and 2), anisomycin 1a (lanes 3 and 4), and the molecular probe 9 or 10 (lanes 5 and 6), each dissolved in DMSO. Immunoblot assays were conducted as described in Fig. 3.

chose the dansyl fluorophore due to the ease with which it could be synthetically modified to give a reactive azide partner,[26] and its widespread use in small molecule target investigation.[27] To this end, library members 1d and 2b were both readily converted to the corresponding fluorescent molecular probes through a copper(I)-catalyzed Huisgen 1,3-dipolar cycloaddition reaction with 2-azido-1-N-dansylethylamine 8 [Fig. 4(a)].[28] Rigorous characterization was then carried out on the fluorescent molecular probes 9 and 10, utilizing a series of 2D NMR experiments including COSY, HSQC and HMBC to fully assign the structure of these molecules. The COSY spectrum showed that, as expected, a single regioisomer had been obtained from the copper(I)-catalyzed Huisigen cycloaddition; whereas the HSQC and HMBC experiments allowed full assignment of the carbon backbone. The HSQC (Fig. 5) also illustrated the formation of the triazole, with a distinctive CH signal [at 7.57 (^{1}H), 126.4 (^{13}C) ppm] corresponding

to the 5-position of the triazole. The fluorescence properties of the probes were then compared with that of the parent dansyl azide 8, and were found to be equivalent (8: λ_{ex} = 345, λ_{em} = 550 nm). Thus, in one simple step, we have successfully converted our active marked library members to functional molecular probes.

These fluorescent probes (9 and 10) were then screened for activation of the SAPK1 pathway in HEK-293 cells, using an immunoblot assay for phosphorylation of JNK as for the original small molecule library. The resultant immunoblot assays [Fig. 4(b)] demonstrate that extension of the propargyl ether marker in the C(4)-H analogue 2b as the fluorescent probe 10 led to the loss of the phenotypic SAPK response. In contrast, formation of fluorescent molecular probe 9, using the propargyl amine marker of anisomycin derivative 1d, led to a strong phenotypic response in the SAPK pathway assay, with activation levels comparable to that of the natural product itself.

Fig. 5 HSQC spectrum of molecular probe 9 in CD_3CN at 360 MHz.

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The use of fluorescent probe **9** as a means to determine anisomycin's site of interaction within HEK-293 cells was assessed by two means. In the first instance, HEK-293 cells were incubated with increasing concentrations (22–162 μM) of probe **9** for 30 min. Fluorescence-activated cell sorting (FACS) was used to assess probe uptake, and a direct correlation was observed between the level of dansyl fluorescence observed in the cell populations and the initial dosing concentrations (Fig. 6). Using the results of FACS sorting, an optimum concentration for cell stimulation was selected (109 μmol) which allowed visualization of the probe uptake, initially at 20-fold magnification and subsequently at 63-fold magnification (Fig. 7). In a preliminary experiment, HEK-293 cells were incubated with dansyl azide **8** for 30 min and then washed with phosphate buffered saline (PBS) buffer. A series of c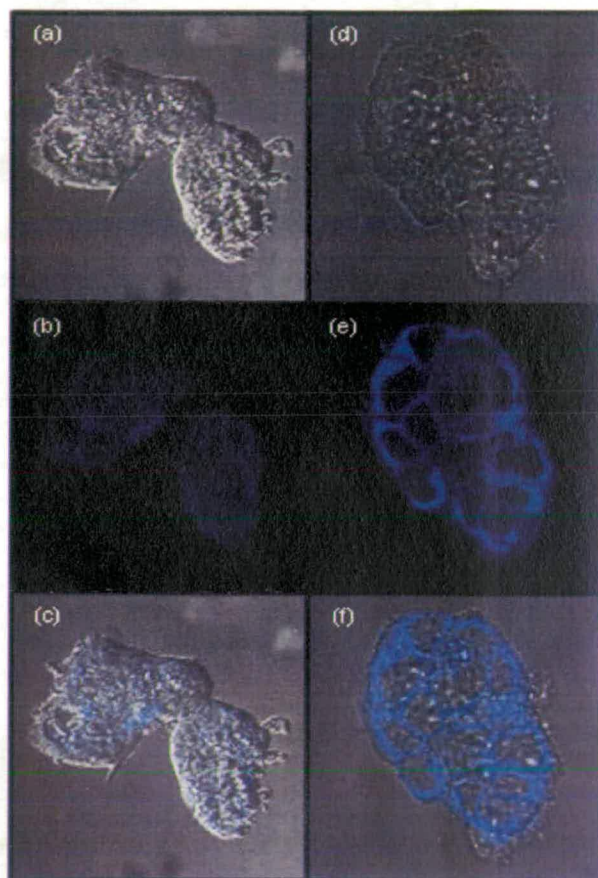onfocal optical sections at 63-fold magnification showed the fluorescent azide to be distributed evenly throughout the cell, indicating that the dansyl azide itself was cell-permeable and that it was not expected to confer any inherent cellular distribution on the fully-formed probe **9**. In contrast, confocal optical sections at 63-fold magnification through a group of HEK-293 cells incubated with fluorescent probe **9** showed diffuse intracellular staining throughout the cytosol. Whilst these preliminary studies must be viewed with caution, since the incorporation of fluorescent labels can themselves lead to a distortion of the intracellular distribution of any particular small molecule,[27a] they suggest a cytosolic distribution of the biological target of this molecular probe. This hypothesis is in good agreement with the results of recent studies which suggest that anisomycin-induced activation of the SAPK pathways might be through the cytosolic MAP3K protein MLK7,[29] suggesting a target at the MAP3K level or above. The application of a range of functional molecular probes generated using this strategy should allow us to interrogate the biological function of this interesting small molecule.

Fig. 7 Cellular localization and uptake of parent dansyl azide **8** (a–c) and molecular probe **9** (d–f). Cells were incubated for 30 min with 109 μM of **8** or **9**, washed with PBS and fixed. Analysis of cells was carried out using a Leica DM IRE2 confocal microscope. (a and d) Brightfield image of cells at 63-fold magnification; (b and e) fluorescent image of cells at 63-fold magnification; (c and f) overlay of brightfield and fluorescent images.

Fig. 6 FACS analysis of cellular uptake of molecular probe **9** in HEK-293 cells. Cells were incubated with 22–162 μM of molecular probe for 30 min and were analyzed using a MoFlo FACS instrument with a UV laser. (a) Histograms of unlabeled cells as a control (red) and cells incubated with **9** at 162 μM (green); (b) histograms illustrating increasing fluorescence intensity with increasing concentrations of **9** (0, 22, 42, 66, 86, 108, 131, 162 μM).

Conclusions

Small molecule chemical genetics screens can generate large numbers of active compounds; however, current strategies for the development of these 'hits' into active molecular probes for further biological investigation are often cumbersome. In this study we have demonstrated that the incorporation of a biocompatible propargyl marker into the molecular scaffold of library members can lead to the rapid generation of active molecular probes. Given the range of robust, biocompatible reactions which have been developed in the past five years, there are a number of options for the choice of bioorthogonal markers (alkynes, azides, phosphines, *etc.*) which allow tailoring of this strategy to the small molecule library under investigation. As a result, this approach should allow the rapid coupling of a diverse range of functional tags, such as fluorophores, NMR spin labels and biotin to any individual marked library member. Therefore, future library design using this marked library approach should provide a useful truncation of the small molecule to the molecular probe synthetic process.

Experimental

General methods

All reactions involving air- or water-sensitive reagents were carried out under an atmosphere of argon using flame- or oven-dried

glassware. Unless otherwise noted, starting materials and reagents were obtained from commercial suppliers and were used without further purification. THF was distilled from Na-benzophenone ketyl immediately prior to use. CH_2Cl_2, 2,6-lutidine, ethyl acetate, acetyl chloride, acetic anhydride and Et_3N were distilled from calcium hydride. Anhydrous methanol, DMF, and acetonitrile were used as supplied. Unless otherwise indicated, organic extracts were dried over anhydrous sodium sulfate and concentrated under reduced pressure using a rotary evaporator. Purification by flash column chromatography was carried out using Merck Kieselgel 60 silica gel as the stationary phase. Chiral high performance liquid chromatography (HPLC) was carried out on a Waters 786 instrument equipped with a Chiracel OD-H column (internal diameter 4.6 mm) and a UV detector. A standard flow rate of $0.5 \text{ cm}^3 \text{ min}^{-1}$ was used. All solvents used for HPLC analysis were vacuum filtered and degassed prior to use. IR spectra were measured on a Perkin-Elmer Paragon 1000 FT-IR spectrometer as thin films unless otherwise stated. ^1H and ^{13}C NMR spectra were measured on Varian Gemini 200, Bruker AC250, Bruker DPX360 instruments; J values are in Hz. Melting points were determined on a Gallenkamp Electrothermal Melting Point apparatus and are uncorrected. Optical rotations were measured on an AA-1000 polarimeter with a path length of 1.0 dm, at the sodium D-line at room temperature. Fast atom bombardment (FAB) mass spectra were obtained using a Kratos MS50TC mass spectrometer at The University of Edinburgh. Luminescence measurements were carried out in Hellma 109.000F-QS 10 mm precision cell using a fluorimeter with phosphorimeter Jobin-Yvon-Horiba Fluoromax-P.

'Complete' Protease Inhibitor tablets were obtained from Roche (Lewes, Sussex, UK), cell culture media from Gibco (Paisley, UK), precast Bis-Tris gradient SDS-polyacrylamide gels, running buffer and transfer buffer from Invitrogen (Paisley, UK) and enhanced chemiluminescence (ECL) reagents from Amersham (Bucks, UK). Phospho-specific antibodies that recognize JNK1/2 phosphorylated at Thr183, or unphosphorylated JNK1/2 were purchased from Biosource (Nivelles, Belgium), while horseradish peroxidase-conjugated secondary antibodies were from Pierce (Cheshire, UK).

Chemical synthesis

(2R,3S,4S)-3-Acetoxy-1-benzyl-4-hydroxy-2-(4'-methoxybenzyl)-pyrrolidine 1c. To a solution of anisomycin **1a** (20.0 mg, 80.0 μmol) in DMF (1 ml) was added potassium carbonate (11.0 mg, 80.0 μmol) and benzyl bromide (10.0 μl, 80.0 μmol), the solution was stirred at room temperature for 4 h. The solution was then concentrated *in vacuo*, and the residual colorless solid was purified by flash chromatography (4% $MeOH/CH_2Cl_2$) to give **1c** as an oil (25.0 mg, 70.0 μmol, 89%); R_f (5% $MeOH/CH_2Cl_2$) = 0.33; $[a]_D$ −98.0 (c 1.00, $CHCl_3$); v_{max} (neat)/cm^{-1} 3399, 3053, 1730, 1612, 1512; ^1H NMR δ (360 MHz, CD_3OD) 7.42–7.35 (5H, m, ArH), 7.14 (2H, d, J 8.4, ArH), 6.87 (2H, d, J 8.4, ArH), 4.81 (1H, br d, J 3.0, C_3H), 4.15 (1H, d, J 12.6, NCH$_X$H$_Y$Ph), 4.11–4.09 (1H, m, C_4H), 3.79 (3H, s, OMe), 3.67 (1H, d, J 12.6, NCH$_X$H$_Y$Ph), (1H, m, C_2H), 3.36–3.34 (1H, m, $C_5H_MH_N$), 2.91 (1H, dd, J 13.5, 5.3, CH_SH_TAr), 2.83 (1H, dd, J 13.5, 9.8, CH_SH_TAr), 2.58–2.56 (1H, m, $C_5H_MH_N$), 2.14 (3H, s, OAc); ^{13}C NMR δ (90.7 MHz, CD_3OD) 171.8 (1C, Q), 160.9 (1C, Q), 131.2 (1C, Q), 130.9 (2C, CH), 130.8 (1C, Q), 130.7 (2C, CH), 129.6

(2C, CH), 129.0 (1C, CH), 115.0 (2C, CH), 80.8 (1C, CH), 74.5 (1C, CH), 68.7 (1C, CH), 60.8 (1C, CH_2), 60.5 (1C, CH_2), 55.6 (1C, CH_3), 33.7 (1C, CH_2), 20.9 (1C, CH_3); m/z (FAB, THIOG) 356 ([M + H]$^+$, 90%), 296 (63), 91 (95); HRMS (FAB, NOBA) (Found: [M + H]$^+$, 356.1861. $C_{21}H_{26}NO_4$ requires m/z, 356.1862).

(2R,3S,4S)-3-Acetoxy-4-hydroxy-2-(4'-methoxybenzyl)-1-propargylpyrrolidine 1d. To a solution of anisomycin **1a** (20.0 mg, 80.0 μmol) in DMF (2 ml) was added potassium carbonate (11.0 mg, 80.0 μmol) and propargyl bromide (12.0 μl, 80.0 μmol), the solution was stirred at room temperature for 9 h. The solution was then concentrated *in vacuo*, and the residue was purified by flash chromatography (5% $MeOH/CH_2Cl_2$) to give **1d** as an oil (22.0 mg, 70.0 μmol, 95%); R_f (5% $MeOH/CH_2Cl_2$) = 0.22; $[a]_D$ −116 (c 0.90, $CHCl_3$); v_{max} (neat)/cm^{-1} 3305, 3019, 1726, 1613, 1513; ^1H NMR δ (360 MHz, $CDCl_3$) 7.10 (2H, d, J 8.6, ArH), 6.81 (2H, d, J 8.6, ArH), 4.47 (1H, dd, J 5.6, 1.5, C_3H), 4.13 (1H, td, J 6.8, 1.5, C_4H), 3.79 (3H, s, OMe), 3.58 (2H, t, J 2.3, HC≡CCH_2), 3.36 (1H, dd, J 9.9, 7.1, $C_5H_MH_N$), 3.22 (1H, qn, J 5.2 C_2H), 2.86 (1H, dd, J 13.3, 4.7, CH_SH_TAr), 2.71 (1H, dd, J 13.3, 10.3, CH_SH_TAr), 2.62 (1H, dd, J 9.9, 6.7, $C_5H_MH_N$), 2.32 (1H, t, J 2.3, HC≡CCH_2), 2.14 (3H, s, OAc); ^{13}C NMR δ (90.7 MHz, $CDCl_3$) 172.0 (1C, Q), 158.2 (1C, Q), 130.2 (1C, Q), 130.0 (2C, CH), 114.0 (2C, CH), 82.5 (1C, CH), 76.7 (1C, Q), 75.7 (1C, CH), 74.5 (1C, CH), 63.6 (1C, CH), 58.0 (1C, CH_2), 55.4 (1C, CH_3), 40.0 (1C, CH_2), 32.1 (1C, CH_2), 21.3 (1C, CH_3); m/z (FAB, THIOG) 304 ([M + H]$^+$, 67%), 244 (42), 121 (100), 91 (88); HRMS (FAB, THIOG) (Found: [M + H]$^+$, 304.1540. $C_{17}H_{22}NO_4$ requires m/z, 304.1549).

(2R,3R)-3-Acetoxy-2-(4'-propargyloxybenzyl)-pyrrolidine 2b. See ESI† for the preparation of **2b**; R_f (5% $MeOH/CH_2Cl_2$) = 0.11; $[a]_D$ −24.2 (c 0.95, $CHCl_3$); v_{max} (neat)/cm^{-1} 3282, 2925, 1730, 1610, 1510; ^1H NMR δ (360 MHz, $CDCl_3$, 323 K) 7.09 (2H, d, J 8.7, ArH), 6.90 (2H, d, J 8.7, ArH), 5.29–5.27 (1H, m, C_3H), 4.63 (2H, d, J 2.4, HC≡CCH_2), 3.85–3.80 (1H, m, C_2H), 3.45–3.32 (2H, m, C_5H_2), 3.05–2.99 (2H, m, CH_2Ar), 2.50 (1H, t, J 2.4, HC≡CCH_2), 2.29–2.17 (2H, m, C_4H_2), 2.18 (3H, s, OAc); ^{13}C NMR δ (90.7 MHz, $CDCl_3$, 323 K) 169.9 (1C, Q), 157.3 (1C, Q), 130.0 (2C, CH), 128.2 (1C, Q), 115.9 (2C, CH), 78.7 (1C, Q), 75.8 (1C, CH), 72.6 (1C, CH), 64.5 (1C, CH), 56.2 (1C, CH_2), 43.2 (1C, CH_2), 31.8 (1C, CH_2), 33.3 (1C, CH_2), 20.7 (1C, CH_3); m/z (FAB, THIOG) 274 [M + H]$^+$, (87%), 214 (57); HRMS (FAB, THIOG) (Found: [M + H]$^+$, 274.1441. $C_{16}H_{19}NO_3$ requires m/z, 274.1443).

2-Azido-1-ethylamine. To a solution of 2-chloro-1-ethylamine (500 mg, 4.31 mmol) in water (5 ml) was added sodium azide (840 mg, 12.9 mmol) and the reaction mixture was heated at 80 °C for 15 h. The solution was basified with KOH (solid) and extracted with diethyl ether. The organics were dried and concentrated to give a volatile colourless oil (371 mg, 4.31 mmol, 100%); v_{max} (neat)/cm^{-1} 3375, 2104; ^1H NMR δ (360 MHz, $CDCl_3$) 3.30 (2H, t, J 5.7, CH_2,), 2.79–2.74 (2H, m, CH_2), 1.43 (2H, s, NH_2); ^{13}C NMR δ (90.7 MHz, $CDCl_3$) 54.2 (1C, CH_2), 40.9 (1C, CH_2); m/z (ESI+) 194 ([2M + H]$^+$). Spectroscopic data in good agreement with the literature.[30]

2-Azido-1-N-dansylethylamine 8. To a solution of 2-azido-1-ethylamine (371 mg, 4.31 mmol) in CH_2Cl_2 (7 ml) was added dansyl chloride (225 mg, 840 μmol) and the reaction mixture was stirred

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for 2 h. The solution was concentrated *in vacuo*, and the residue was purified by flash chromatography (25% EtOAc/hexane) to give **8** as a pale yellow oil (260 mg, 820 μmol, 98%); R_f (30% EtOAc/hexane) = 0.27; λ_{max} (H$_2$O)/nm 345 nm; ν_{max} (neat)/cm^{-1} 3301, 2942, 2103, 1318; ^{1}H NMR δ (360 MHz, CDCl$_3$) 8.57 (1H, br d, *J* 8.5, Ar*H*), 8.29 (1H, br d, *J* 8.6, Ar*H*), 8.26 (1H, dd, *J* 7.3, 1.3, Ar*H*), 7.59 (1H, dd, *J* 8.6, 7.6, Ar*H*), 7.53 (1H, dd, *J* 8.5, 7.3 Ar*H*), 7.20 (1H, br d, *J* 7.6, Ar*H*), 5.28 (1H, t, *J* 5.9, N*H*), 3.30 (2H, t, *J* 5.7, C*H$_2$*), 3.06 (2H, q, *J* 6.1, C*H$_2$*), 2.90 (6H, s, N*Me$_2$*); ^{13}C NMR δ (90.7 MHz, CDCl$_3$) 152.1 (1C, Q), 134.6 (1C, Q), 130.9 (1C, CH), 130.0 (1C, Q), 129.7 (1C, CH), 129.6 (1C, Q), 128.8 (1C, CH), 123.3 (1C, CH), 118.7 (1C, CH), 115.5 (1C, CH), 51.0 (1C, CH$_2$), 45.5 (2C, CH$_3$), 42.5 (1C, CH$_2$); *m/z* (FAB, NOBA) 319 ([M]$^+$, 90%), 170 (100); HRMS (FAB, THIOG) (Found: [M + H]$^+$, 320.1184. C$_{14}$H$_{18}$N$_5$O$_2$S requires *m/z*, 320.1181).

N-Linked dansyl molecular probe 9. To propargyl amine **1d** (27.0 mg, 90.0 μmol) in ^{t}BuOH–H$_2$O (2 ml, 1 : 1) was added dansyl azide **8** (26.0 mg, 80.0 μmol), followed by copper(II) sulfate (2.0 mg, 10 mol%) and sodium ascorbate solution (20.0 μl, 1 M solution, 20 mol%). After 5 h the reaction was complete by TLC. The solution was concentrated *in vacuo*, and the residue was purified by flash chromatography (5 to 10% MeOH/CH$_2$Cl$_2$) to give **9** as a foam (40.0 mg, 60.0 μmol, 71%); R_f (10% MeOH/CH$_2$Cl$_2$) = 0.34; λ_{max} (H$_2$O)/nm 345 nm; ^{1}H NMR δ (360 MHz, CD$_3$CN) 8.54 (1H, d, *J* 8.5, Ar*H*), 8.19 (1H, d, *J* 8.7, Ar*H*), 8.16 (1H, dd, *J* 7.3, 1.3 Ar*H*), 7.59–7.54 (3H, m, 2Ar*H* + C*H*), 7.25 (1H, d, *J* 7.6, Ar*H*), 7.21 (2H, d, *J* 8.5, Ar*H*), 6.87 (2H, d, *J* 8.5, Ar*H*), 4.66 (1H, br s, C*H*), 4.35 (2H, q, *J* 5.0, C*H$_2$*), 4.03 (1H, br s, C*H*), 3.97 (1H, br d, *J* 13.6, C*H$_A$H$_B$*), 3.86–3.84 (1H, m, CH$_A$*H$_B$*), 3.76 (3H, s, O*Me*), 3.56–3.54 (1H, m, C*H*), 3.47–3.43 (1H, m, C*H$_A$H$_B$*), 3.30 (2H, br t, *J* 6.1, C*H$_2$*), 3.05 (1H, dd, *J* 13.4, 5.7, C*H$_A$H$_B$*), 2.86 (6H, s, N*Me$_2$*), 2.85 (1H, m, CH$_A$*H$_B$*), 2.84 (1H, dd, *J* 7.2, 5.7, C*H$_A$H$_B$*), 2.07 (3H, s, O*Ac*); ^{13}C NMR δ (90.7 MHz, CD$_3$CN) 170.9 (1C, Q), 159.4 (1C, Q), 152.6 (1C, Q), 141.6 (1C, Q), 136.1 (1C, Q), 131.2 (2C, CH), 130.6 (1C, Q), 130.3 (1C, Q), 130.2 (1C, Q), 130.1 (1C, Q), 130.0 (1C, CH), 129.2 (1C, CH), 126.4 (1C, CH), 124.4 (1C, CH), 119.9 (1C, CH), 116.2 (1C, CH), 114.9 (2C, CH), 79.7 (1C, CH), 73.9 (1C, CH), 67.7 (1C, CH), 59.6 (1C, CH$_2$), 55.8 (1C, CH$_3$), 50.9 (1C, CH$_2$), 49.0 (1C, CH$_2$), 45.7 (2C, CH$_3$), 43.6 (1C, CH$_2$), 32.2 (1C, CH$_2$), 21.1 (1C, CH$_3$); *m/z* (FAB, NOBA) 623 ([M + H]$^+$, 60%), 501 (46), 91 (68); HRMS (FAB, NOBA) (Found: [M + H]$^+$, 623.2655. C$_{31}$H$_{39}$N$_6$O$_6$S requires *m/z*, 623.2652).

O-Linked dansyl molecular probe 10. To propargyl ether **2b** (32.6 mg, 120 μmol) in ^{t}BuOH–H$_2$O (3 ml, 1 : 1) was added dansyl azide **6** (40.0 mg, 130 μmol), followed by copper(II) sulfate (3.0 mg, 10 mol%) and sodium ascorbate solution (25.0 μl, 1 M solution, 20 mol%). After 4 h the reaction was complete by TLC. The solution was concentrated *in vacuo*, and the residue was purified by flash chromatography (5 to 10% MeOH/CH$_2$Cl$_2$) to give **10** as a foam (54.5 mg, 90.0 μmol, 77%); R_f (5% MeOH/CH$_2$Cl$_2$) = 0.24; ^{1}H NMR δ (360 MHz, DMSO) 8.47 (1H, br d, *J* 8.5, Ar*H*), 8.27 (1H, t, *J* 5.8, N*H*), 8.22 (1H, br d, *J* 8.7, Ar*H*), 8.26 (1H, d, *J* 7.9, Ar*H*), 8.06 (1H, s, C*H*), 7.65–7.56 (2H, m, Ar*H*), 7.27 (1H, d, *J* 7.4, Ar*H*), 7.20 (2H, d, *J* 8.6, Ar*H*), 7.00 (2H, d, *J* 8.6, Ar*H*), 5.16 (1H, t, *J* 3.8, C*H*), 5.03 (2H, s, C*H$_2$*), 4.41 (2H, t, *J* 5.9, C*H$_2$*), 3.89–3.81 (1H, m, C*H*), 3.34–3.24 (2H, m, C*H$_2$*), 3.26 (2H, br q, *J* 5.8, C*H$_2$*), 2.98–2.88 (2H, m, C*H$_2$*), 2.84 (6H, s, N*Me$_2$*), 2.30–2.19 (1H, m, C*H$_A$H$_B$*), 2.12 (3H, s, O*Ac*), 2.05–1.98 (1H, m,

C$_{25}$H$_A$*H$_B$*); ^{13}C NMR δ (62.9 MHz, DMSO) 169.7 (1C, Q), 157.2 (1C, Q), 151.2 (1C, Q), 142.5 (1C, Q), 135.5 (1C, Q), 130.1 (2C, CH), 129.7 (1C, CH), 129.1 (1C, Q), 129.0 (1C, Q), 128.8 (1C, Q), 128.5 (1C, CH), 128.1 (1C, CH), 125.0 (1C, CH), 123.8 (1C, CH), 119.3 (1C, CH), 115.4 (1C, CH), 114.9 (2C, CH), 72.9 (1C, CH), 63.5 (1C, CH), 61.1 (1C, CH$_2$), 49.4 (1C, CH$_2$), 45.2 (2C, CH$_3$), 42.8 (1C, CH$_2$), 42.5 (1C, CH$_2$), 31.1 (1C, CH$_2$), 30.6 (1C, CH$_2$), 20.9 (1C, CH$_3$); *m/z* (FAB, NOBA) 593 ([M + H]$^+$, 43%), 91 (52); HRMS (FAB, NOBA) (Found: [M + H]$^+$, 593.2548. C$_{30}$H$_{37}$N$_6$O$_5$S requires *m/z*, 593.2546).

Immunoblot assays[6,16b,31]

Cell culture and stimulation. Human embryonic kidney (HEK) 293 cells were cultured at 37 °C, 95% air/5% CO$_2$, in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum, 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 2 mM L-glutamine. Anisomycin was dissolved at 10 mg ml^{-1} (38 mM) in DMSO, while the other compounds were dissolved in DMSO at 38 mM. Cells (9 ml of cell culture) were incubated with the library members by addition of 9 μl of anisomycin solution, anisomycin analogue in DMSO, or DMSO as a control.

Cell lysis. After stimulation for 30 min the media was aspirated and the cells lysed in 50 mM Tris/HCl, pH 7.5, containing 1 mM EDTA, 1 mM EGTA, 1 mM sodium orthovanadate, 10 mM sodium β-glycerophosphate, 5 mM sodium pyrophosphate, 50 mM sodium fluoride, 0.27 M sucrose, 1% (v/v) Triton X-100, 0.1% (v/v) 2-mercaptoethanol and 'Complete' protease inhibitor cocktail (one tablet per 50 ml). Lysates were centrifuged at 13 000 g for 10 min at 4 °C and the supernatants (termed 'cell extract') were removed. Protein concentrations were determined according to the method of Bradford.[32]

Immunoblotting. Samples were denatured in SDS, run on polyacylamide gels and transferred to nitrocellulose membranes. The membranes were incubated for 1 h at room temperature in 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.2% (v/v) Tween and 5% (w/v) skimmed milk powder. Primary antibodies were added to 10 ml of the previous buffer and incubated at 4 °C overnight. The membranes were then washed four times with buffer (5 min per wash) to remove the excess primary antibody. The membranes were then incubated with the secondary antibody at room temperature for 1 h. After washing six times with buffer to remove the excess secondary antibody (5 min per wash), immunoreactive proteins were visualized *via* enhanced chemiluminescence reagent according to the manufacturer's instructions.

FACS analysis. Cells (cultured as above, 350 μl of cell culture) were incubated with molecular probe **9** (22–162 μM). After stimulation for 30 min the media was aspirated and the cells washed with PBS buffer (350 μl). The PBS buffer was aspirated, replaced with tripsin (200 μl) and the cells incubated for 5 min. Media (200 μl) was added to the cells and the combined solution was centrifuged at 1000 g for 4 min. The media was aspirated and the cells re-suspended in PBS – 2% serum (200 μl). The cells were analyzed on a DakoCytomation MoFlo MLS high speed sorter using a multi-line UV laser (350–360 nm).

Confocal microscopy. Cells (cultured as above, 1.5 ml of cell culture) were incubated with molecular probe **9** (109 μM). After

stimulation for 30 min the media was aspirated and the cells washed with PBS buffer (1.5 ml). The cells were fixed using 4% paraformaldehyde in PBS (1.2 ml). Confocal microscopy experiments were carried out using a Leica DM IRE2 microscope with a UV laser; cells were analyzed at 20-fold and 63-fold magnification.

Acknowledgements

References and notes

1 S. L. Schreiber, Bio. Med. Chem., 1998, 6, 1127–1152; D. R. Spring, Chem. Soc. Rev., 2005, 34, 472–482; Y.-T. Chang and D. P. Walsh, Chem. Rev., 2006, 106, 2479–2530.

2 D. S. Tan, M. A. Foley, B. R. Stockwell, M. D. Shair and S. L. Schreiber, J. Am. Chem. Soc., 1999, 121, 9073–9087; S. M. Khersonsky and Y.-T. Chang, Comb. Chem. High Throughput Screening, 2004, 7, 645–652; H. Luesch, T. Y. H. Wu, P. Ren, N. S. Gray, P. G. Schultz and F. Supek, Chem. Biol., 2005, 12, 55–63; R. A. Butcher, B. S. Bhullar, E. O. Perlstein, G. Marsischky, J. LaBaer and S. L. Schreiber, Nat. Chem. Biol., 2006, 2, 103–109.

3 For recent examples of redesign strategies in the synthesis of molecular probes, see: H. Fuwa, K. Hiromoto, Y. Takahashi, S. Yokoshima, T. Kan, T. Fukuyama, T. Iwatsubo, T. Tomita and H. Natsugari, Bioorg. Med. Chem. Lett., 2006, 16, 4184–4189; A. B. Smith, III, P. V. Rucker, I. Brouard, B. S. Freeze, S. Xia and S. B. Horwitz, Org. Lett., 2005, 7, 5199–5202; J.-Y. Le Brazidec, A. Kamal, D. Busch, L. Thao, L. Zhang, G. Timony, R. Grecko, K. Trent, R. Lough, T. Salazar, S. Khan, F. Burrows and M. F. Boehm, J. Med. Chem., 2004, 47, 3865–3873.

4 L. Burdine and T. Kodadek, Chem. Biol., 2004, 11, 593–597; G. P. Tochtrop and R. W. King, Comb. Chem. High Throughput Screening, 2004, 7, 677–688.

5 E. M. Rosser and A. N. Hulme, Org. Lett., 2002, 4, 265–267.

6 E. M. Rosser, K. S. Ashton, S. Morton, P. Cohen and A. N. Hulme, Org. Biomol. Chem., 2004, 2, 142–149.

7 B. A. Sobin and F. W. Tanner, Jr., J. Am. Chem. Soc., 1954, 76, 4053.

8 Y. Hosoya, T. Kameyama, H. Naganawa, Y. Okami and T. Takeuchi, J. Antibiot., 1993, 46, 1300–1302; S. Ishida, O. Yamada, F. Futatsuya, K. Ito, H. Yamamoto and K. Munakata, Proc. Int. Congr. IAMS 1st, 1974, 3, 641.

9 J. J. Beereboom, K. Butler, F. C. Pennington and I. A. Solomons, J. Org. Chem., 1965, 30, 2334–2342.

10 J. P. Schaefer and P. J. Wheatley, J. Org. Chem., 1968, 33, 166–169; J. P. Schaefer and P. J. Wheatley, Chem. Commun. (London), 1967, 578–579; K. Butler, J. Org. Chem., 1968, 33, 2136–2141.

11 J. E. Lynch, A. R. English, H. Bauck and H. Deigiania, Antibiot. Chemother., 1954, 4, 844–848.

12 O. Schwartdt, U. Veith, C. Gaspard and V. Jager, Synthesis, 1999, 1473–1490.

13 J. van der Bosch, S. Rueller and M. Schlaak, Ger. Offen., DE 97-19744676, 1999.

14 J. L. Hansen, P. B. Moore and T. A. Steitz, J. Mol. Biol., 2003, 330, 1061–1075.

15 E. Cano, Y. Doza, C. Hazzalin and L. Mahadevan, Mol. Cell. Biol., 1994, 14, 7352–7362; E. Cano, Y. Doza, R. Ben-Levy, P. Cohen and L. Mahadevan, Onocogene, 1996, 12, 805–812; J. Kyriakis, P. Banerjee, E. Nikolakaki, T. Dai, E. Rubie, M. Ahmad, J. Avruch and J. Woodgett, Nature, 1994, 369, 156–160; D. Chen, S. B. Waters, K. H. Holt and J. E.

Pessin, J. Biol. Chem., 1996, 271, 6328–6332; C. A. Hazzalin, E. Cano, A. Cuenda, M. J. Barratt, P. Cohen and L. Mahadevan, Curr. Biol., 1996, 6, 1028–1031; R. Meier, J. Rouse, A. Cuenda, A. R. Nebreda and P. Cohen, Eur. J. Biochem., 1996, 236, 796–805.

16 (a) Z. Chen, T. Beers, F. Robinson, L. Silvestro, G. Pearson, B. Xu, A. Wright, C. Vanderbilt and M. H. Cobb, Chem. Rev., 2001, 101, 2449–2476; L. A. Tibbles and J. R. Woodgett, Cell. Mol. Life Sci., 1999, 55, 1230–1254; (b) S. Morton, R. J. Davis, A. McLaren and P. Cohen, EMBO J., 2003, 22, 3876–3886.

17 B. R. Peterson and S. S. Muddana, Org. Lett., 2004, 6, 1409–1412; N. Nesnas, R. R. Rando and K. Nakanishi, Tetrahedron, 2002, 58, 6577–6584; J. L. Musachio and J. R. Lever, Bioconjugate Chem., 1992, 3, 167–175; M. D. Alexander, M. D. Burkart, M. S. Leonard, P. Portonovo, B. Liang, X. Ding, M. M. Joullié, B. M. Gulledge, J. B. Aggen, A. R. Chamberlin, J. Sandler, W. Fenical, J. Cui, S. J. Gharpure, A. Polosukhin, H.-R. Zhang, P. A. Evans, A. D. Richardson, M. K. Harper, C. M. Ireland, B. G. Vong, T. P. Brady, E. A. Theodorakis and J. J. La Clair, ChemBioChem, 2006, 7, 409–416.

18 Y. M. Li, M. Xu, M. T. Lai, Q. Huang, J. L. Castro, J. DiMuzio-Mower, T. Harrison, C. Lellis, A. Nadin, J. G. Neduvelil, R. B. Register, M. K. Sardana, M. S. Shearman, A. L. Smith, X. P. Shi, K. C. Yin, J. A. Shafer and S. J. Gardell, Nature, 2000, 405, 689–694; W. J. Jahng, C. David, N. Nesnas, K. Nakanishi and R. R. Rando, Biochemistry, 2003, 42, 6159–6168; W. Frick, A. Bauer-Schafer, J. Bauer, F. Girbig, D. Corsiero, H. Heuer and W. Kramer, Bio. Med. Chem., 2003, 11, 1639–1642; J. J. Siekierka, S. H. Y. Hung, M. Poe, C. S. Lin and N. H. Sigal, Nature, 1989, 341, 755–757; M. W. Harding, A. Galat, D. E. Uehling and S. L. Schreiber, Nature, 1989, 341, 758–760.

19 J. A. Prescher and C. R. Bertozzi, Nat. Chem. Biol., 2005, 1, 13–21; P. F. van Swieten, M. A. Leeuwenburgh, B. M. Kessler and H. S. Overkleeft, Org. Biomol. Chem., 2005, 3, 20–27.

20 K. E. Beatty, F. Xie, Q. Wang and D. A. Tirrell, J. Am. Chem. Soc., 2005, 127, 14150–14151; A. Deiters, T. A. Cropp, M. Mukherji, J. W. Chin, J. C. Anderson and P. G. Schultz, J. Am. Chem. Soc., 2003, 125, 11782–11783; M.-L. Tsao, F. Tian and P. G. Schultz, ChemBioChem, 2005, 6, 2147–2149.

21 J. A. Link, M. K. S. Vink and D. A. Tirrell, J. Am. Chem. Soc., 2004, 126, 10598–10602; E. Saxon and C. R. Bertozzi, Science, 2000, 287, 2007–2010.

22 A. E. Spears and B. F. Cravatt, Chem. Biol., 2004, 11, 535–546; A. E. Spears and B. F. Cravatt, ChemBioChem, 2004, 5, 41–47; A. E. Spears, G. C. Adam and B. F. Cravatt, J. Am. Chem. Soc., 2003, 125, 4686–4687.

23 Y.-T. Chang and S. M. Khersonsky, ChemBioChem, 2004, 5, 903–908.

24 M. J. Evans, A. Saghatelian, E. J. Soernsen and B. F. Cravatt, Nat. Biotechnol., 2005, 23, 1303–1307.

25 H. C. Kolb, M. A. Finn and K. B. Sharpless, Angew. Chem., Int. Ed., 2001, 40, 2004–2021; H. C. Kolb and K. B. Sharpless, Drug Discovery Today, 2003, 8, 1128–1137; F. Himo, T. Lovell, R. Hilgraf, V. V. Rostovtsev, L. Noodleman, K. B. Sharpless and V. V. Fokin, J. Am. Chem. Soc., 2005, 127, 210–216.

26 Low molecular weight azides can be explosive and caution should be exercised when handling them. For a review covering the synthesis and reactivity of azides, see: S. Bräse, C. Gil, K. Knepper and V. Zimmermann, Angew. Chem., Int. Ed., 2005, 44, 5188–5240.

27 (a) H. H. Szeto, P. W. Schiller, K. Zhao and G. Luo, FASEB J., 2005, 19, 118–120; (b) S. Fuchs, H. Otto, S. Jehle, P. Henkleinc and A. D. Schlüter, Chem. Commun., 2005, 1830–1832; H. Kakuta, Y. Koiso, K. Nagasawa and Y. Hashimoto, Bioorg. Med. Chem. Lett., 2003, 13, 83–86; S. J. Slater, C. Ho and C. D. Stubbs, Chem. Phys. Lipids, 2002, 116, 75–91.

28 Dansyl azide 6 was synthesized from dansyl chloride by reaction with azidoethylamine, using the method of Schultz: ref. 20.

29 X. Wang, M. M. Mader, J. E. Toth, X. Yu, N. Jin, R. M. Campbell, J. K. Smallwood, M. E. Christe, A. Chatterjee, T. Goodson, Jr., C. J. Vlahos, W. F. Matter and L. J. Bloem, J. Biol. Chem., 2005, 280, 19 298–19 305.

30 A. Benalil, B. Carboni and M. Vaultier, Tetrahedron, 1991, 47, 8177–8194.

31 I. V. Klevernic, M. J. Stafford, N. Morrice, M. Peggie, S. Morton and P. Cohen, Biochem. J., 2006, 394, 365–373.

32 M. M. Bradford, Anal. Biochem., 1976, 72, 248–254.

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