Marked small molecule libraries: a truncated approach to molecular probe design†

Iain A. Inverarity and Alison N. Hulme*

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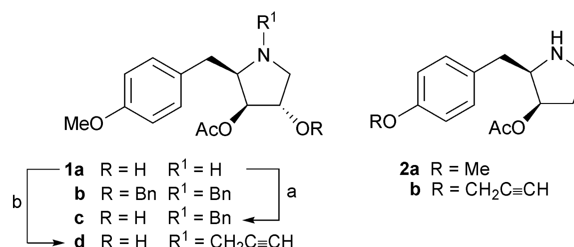
A truncated approach to the design of molecular probes from small molecule libraries is outlined, based upon the incorporation of a bioorthogonal marker. The applicability of this strategy to small molecule chemical genetics screens has been demonstrated using analogues of the known stress activated 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 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]



Scheme 1 *Reagents and conditions*: (a) BnBr, K_2CO_3, DMF (89%); (b) $HC{\equiv}CCH_2Br$, K_2CO_3, DMF (95%).

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

School of Chemistry, The University of Edinburgh, West Mains Road, Edinburgh, UK EH9 3JJ. E-mail: Alison.Hulme@ed.ac.uk; Fax: 0131 650 4743; Tel: 0131 650 4711

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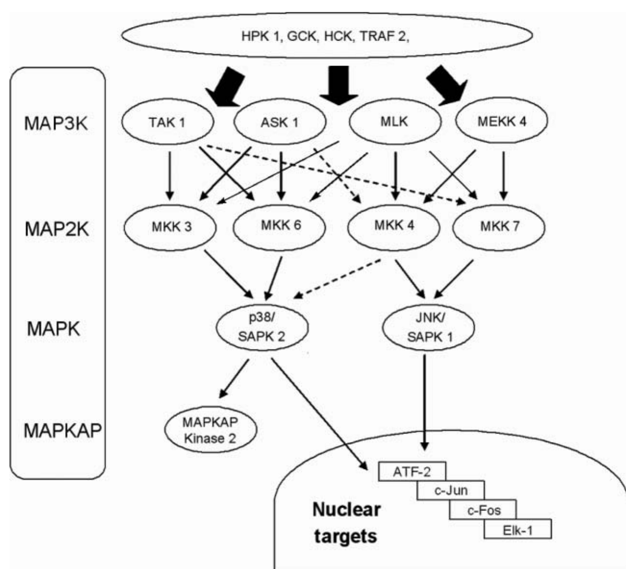


Fig. 1 Stress activated protein kinase (SAPK) pathways.

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 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.

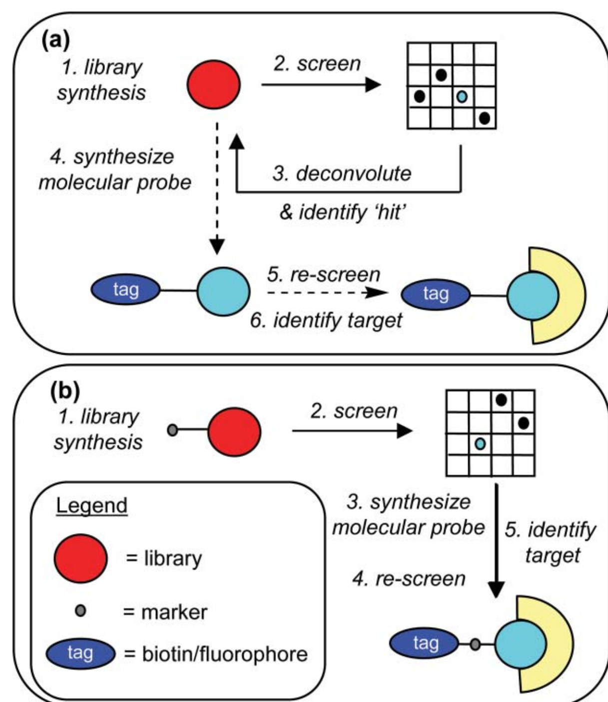


Fig. 2 Strategies for small molecule library design: (a) traditional approach involves library synthesis, biological screening, redesign of synthesis to form molecular probes, re-screening and target identification; (b) marked library strategy involves library synthesis incorporating a biocompatible marker, biological screening, rapid molecular probe formation, re-screening followed by target identification.

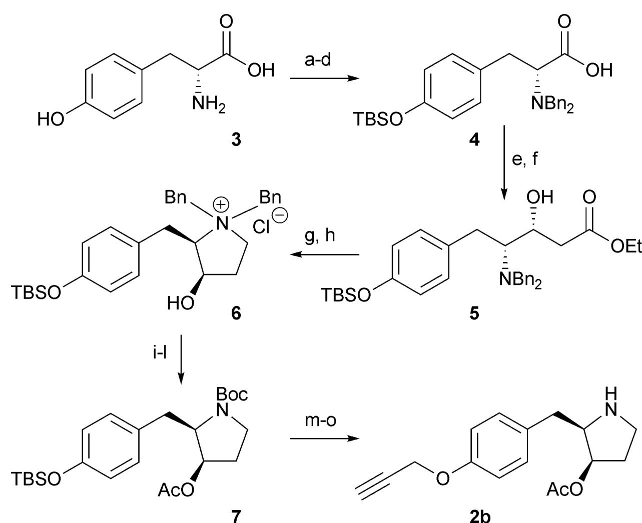
We have therefore designed a more direct approach which is based on the concept of a marked library, where each individual library member carries a small biocompatible marker which plays no role in the screening process itself, but may be used in the target identification process once screening is complete [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 $LiAlH_4$, 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).



Scheme 2 *Reagents and conditions*: (a) AcCl, MeOH, reflux (100%); (b) BnBr, K_2CO_3, MeCN (91%); (c) LiOH, THF–H_2O (4 : 1), reflux (87%); (d) i) TBSOTf, 2,6-lutidine, CH_2Cl_2; ii) AcOH–THF–H_2O (3 : 1 : 1) (90%); (e) i) CDI, THF; ii) CH_2=C(OLi)OEt (71%); (f) $NaCNBH_3$, MeOH, AcOH, Et_2O (80%); (g) $LiAlH_4$, THF (91%); (h) i) TIBSCl, DMAP, CH_2Cl_2; ii) Dowex (Cl^-) ion-exchange resin (92%); (i) H_2, 5% Pd/C, K_2CO_3, MeOH (80%); (j) Ac_2O, Et_3N, DMAP, CH_2Cl_2 (94%); (k) H_2, EtOH, $Pd(OH)_2$ (95%); (l) Boc_2O, Et_3N, CH_2Cl_2 (89%); (m) HF·3Et_3N, THF (94%); (n) HC≡CCH_2Br, K_2CO_3, DMF (99%); (o) TFA, CH_2Cl_2 (97%).

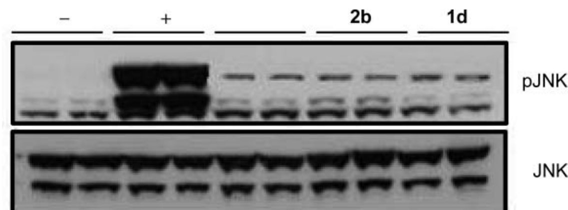


Fig. 3 Effect of anisomycin and its analogues on the phosphorylation of JNK1/2 isoforms in HEK-293 cells. The cells were exposed to DMSO (lanes 1 and 2), anisomycin **1a** (lanes 3 and 4), deacetylanisomycin (lanes 5 and 6), or the anisomycin analogues **2b** (lanes 7 and 8) and **1d** (lanes 9 and 10), 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.‡

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 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.

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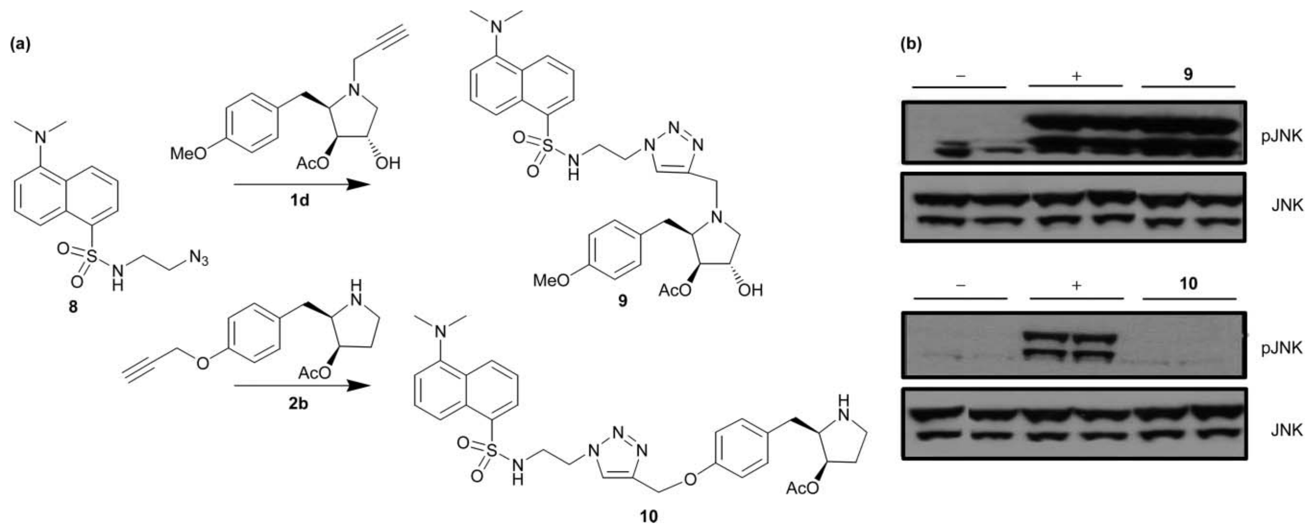

Fig. 4 (a) *Reagents and conditions*: $CuSO_4·5H_2O$ (10 mol%), NaAsc (1M aq, 20 mol%), $^tBuOH–H_2O$ (1 : 1), rt, 4–5 h (**1d** → **9**, 71%; **2b** → **10**, 77%); (b) effect of molecular probes **9** and **10** on the phosphorylation of JNK1/2 isoforms in HEK-293 cells. The cells were exposed to DMSO (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**: $\lambda_{ex} = 345$, $\lambda_{em} = 550$ 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.

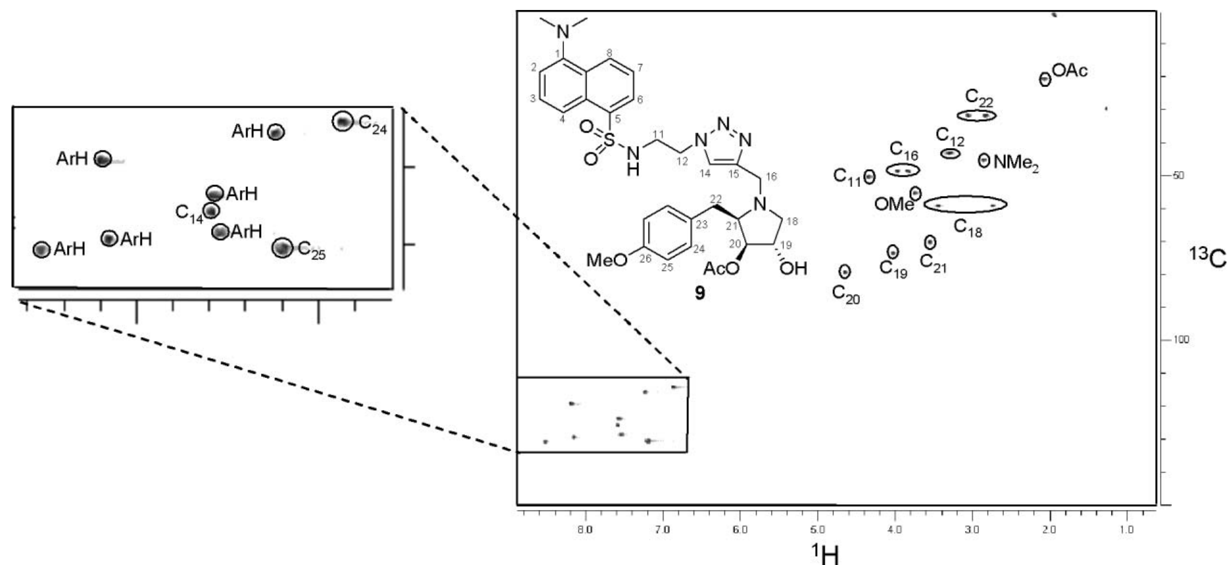

Fig. 5 HSQC spectrum of molecular probe **9** in CD_3CN at 360 MHz.

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 confocal 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.

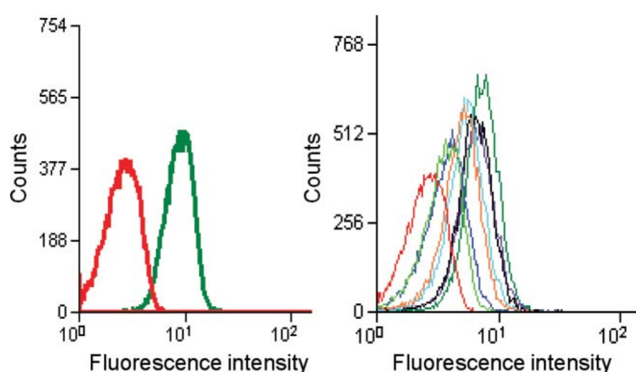

Fig. 6 FACS analysis of cellular uptake of molecular probe **9** in HEK-293 cells. Cells were incubated with 22–162 μM of molecular probe for 30 min and were analyzed using a MoFlo FACS instrument with a UV laser. (a) Histograms of unlabeled cells as a control (red) and cells incubated with **9** at 162 μM (green); (b) histograms illustrating increasing fluorescence intensity with increasing concentrations of **9** (0, 22, 42, 66, 86, 108, 131, 162 μM).

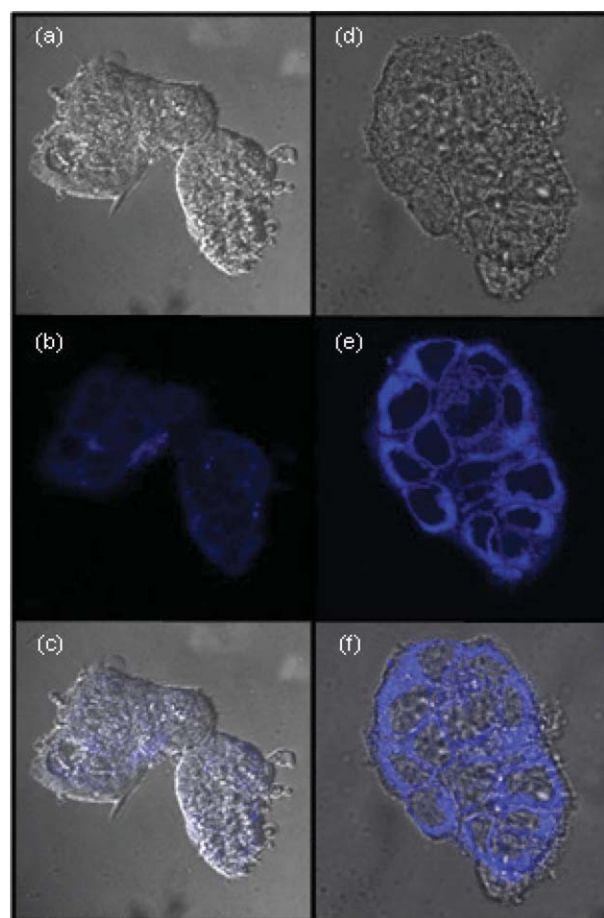

Fig. 7 Cellular localization and uptake of parent dansyl azide **8** (a–c) and molecular probe **9** (d–f). Cells were incubated for 30 min with 109 μM of **8** or **9**, washed with PBS and fixed. Analysis of cells was carried out using a Leica DM IRE2 confocal microscope. (a and d) Brightfield image 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.

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

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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 cm^3 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

(2*R*,3*S*,4*S*)-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; $[\alpha]_D$ −98.0 (*c* 1.00, $CHCl_3$); ν_{max} (neat)/cm^{-1} 3399, 3053, 1730, 1612, 1512; ^1H NMR δ (360 MHz, CD_3OD) 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_3H), 4.15 (1H, d, *J* 12.6, NCH_XH_YPh), 4.11–4.09 (1H, m, C_4H), 3.79 (3H, s, O*Me*), 3.67 (1H, d, *J* 12.6, NCH_XH_YPh), (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, O*Ac*); ^{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).

(2*R*,3*S*,4*S*)-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; $[\alpha]_D$ −116 (*c* 0.90, $CHCl_3$); ν_{max} (neat)/cm^{-1} 3305, 3019, 1726, 1613, 1513; ^1H 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_3H), 4.13 (1H, td, *J* 6.8, 1.5, C_4H), 3.79 (3H, s, O*Me*), 3.58 (2H, t, *J* 2.3, $HC{\equiv}CCH_2$), 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, $HC{\equiv}CCH_2$), 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*,3*R*)-3-Acetoxy-2-(4′-propargyloxybenzyl)-pyrrolidine 2b. See ESI† for the preparation of **2b**; R_f (5% MeOH/CH_2Cl_2) = 0.11; $[\alpha]_D$ −24.2 (*c* 0.95, $CHCl_3$); ν_{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, Ar*H*), 6.90 (2H, d, *J* 8.7, Ar*H*), 5.29–5.27 (1H, m, C_3H), 4.63 (2H, d, *J* 2.4, $HC{\equiv}CCH_2$), 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, $HC{\equiv}CCH_2$), 2.29–2.17 (2H, m, C_4H_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).

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%); ν_{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

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_2O)/nm 345 nm; ν_{max} (neat)/cm^{-1} 3301, 2942, 2103, 1318; ^1H 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, CH_2), 3.06 (2H, q, *J* 6.1, CH_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. $C_{14}H_{18}N_5O_2S$ 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_2O (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_2Cl_2) to give **9** as a foam (40.0 mg, 60.0 µmol, 71%); R_f (10% MeOH/CH_2Cl_2) = 0.34; λ_{max} (H_2O)/nm 345 nm; ^1H NMR δ (360 MHz, CD_3CN) 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, CH_2), 4.03 (1H, br s, C*H*), 3.97 (1H, br d, *J* 13.6, CH_AH_B), 3.86–3.84 (1H, m, CH_AH_B), 3.76 (3H, s, O*Me*), 3.56–3.54 (1H, m, C*H*), 3.47–3.43 (1H, m, CH_AH_B), 3.30 (2H, br t, *J* 6.1, CH_2), 3.05 (1H, dd, *J* 13.4, 5.7, CH_AH_B), 2.86 (6H, s, N*Me*$_2$), 2.85 (1H, m, CH_AH_B), 2.84 (1H, dd, *J* 7.2, 5.7, CH_AH_B), 2.07 (3H, s, O*Ac*); ^{13}C NMR δ (90.7 MHz, CD_3CN) 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. $C_{31}H_{39}N_6O_6S$ requires *m*/*z*, 623.2652).

***O*-Linked dansyl molecular probe 10.** To propargyl ether **2b** (32.6 mg, 120 µmol) in ^{t}BuOH–H_2O (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_2Cl_2) to give **10** as a foam (54.5 mg, 90.0 µmol, 77%); R_f (5% MeOH/CH_2Cl_2) = 0.24; ^1H 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, CH_2), 4.41 (2H, t, *J* 5.9, CH_2), 3.89–3.81 (1H, m, C*H*), 3.34–3.24 (2H, m, CH_2), 3.26 (2H, br q, *J* 5.8, CH_2), 2.98–2.88 (2H, m, CH_2), 2.84 (6H, s, N*Me*$_2$), 2.30–2.19 (1H, m, CH_AH_B), 2.12 (3H, s, O*Ac*), 2.05–1.98 (1H, m, $C_{25}H_AH_B$); ^{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. $C_{30}H_{37}N_6O_5S$ 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

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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.

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