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N-Terminal Carboxyl and Tetrazole-containing Amides as Adjuvants to Grb2 SH2 Domain Ligand Binding

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Abstract—High affinity binding of peptides to Src homology 2 (SH2) domains, often requires the presence of phosphotyrosyl (pTyr) or pTyr-mimicking moieties in the N-terminal position of the binding ligand. Several reports have shown that N^{α}-acylation of the critical pTyr residue can result in increased SH2 domain binding potency. For Grb2 SH2 domains which recognize pTyr-Xxx-Asn-NH_2 motifs, significant potency enhancement can be incurred by N^{α}-(3-amino)Z derivatization of tripeptides such as pTyr-Ile-Asn-NH_2. Using ligands based on the high affinity pY-Ac_6c-Asn-(naphthylpropylamide) motif, (where Ac_6c = 1-aminocyclohexanecarboxylic acid), additional reports have shown moderate potentiating effects of N^{α}-oxalyl derivatization. The current study examined variations of the N^{α}-oxalyl theme in the context of a Xxx-Ac_6c-Asn-(naphthylpropylamide) platform, where Xxx = the hydrolytically stable pTyr mimetics phosphonomethyl phenylalanine (Pmp) or carboxymethyl phenylalanine (Cmf). The effects of N^{α}-(3-amino)Z derivatization were also investigated for this platform, to ascertain whether the large binding enhancement reported for tripeptides such as pTyr-Ile-Asn-NH_2 could be observed. In ELISA-based extracellular Grb2 SH2 domain binding assays, it was found for the Pmp-based series, that extending the oxalyl carboxyl out by one methylene unit or replacing carboxyl functionality with a tetrazole isostere, resulted in binding potency greater than the parent N^{α}-acetyl-containing compound, with enhancement approximating that observed for the N^{α}-oxalyl derivative. When Cmf was used as the pTyr mimetic, only modest differences in IC_{50} values were observed for the series. Examination of the N^{α}-(3-amino)Z derivatized Pmp-Ac_6c-Asn-(naphthylpropylamide), showed that binding affinity was reduced relative to the parent N^{α}-acetyl analogue, in contrast to the reported significant enhancement of affinity observed with other peptide ligands. Treatment of MDA-453 tumor cells, which are mitogenically driven through erbB-2 tyrosine kinase-dependent pathways, with Pmp-containing inhibitors resulted in growth inhibition, with the N^{α}-oxalyl and N^{α}-malonyl-containing compounds exhibiting IC_{50} values (4.3 and 4.6 μM, respectively) approximately five-fold lower than the parent N^{α}-acetyl-containing compound. Tetrazole and N^{α}-(3-amino)Z-containing inhibitors were from two- to four-fold less potent than these latter analogues in the growth inhibition assays.

Introduction

Protein-tyrosine kinase (PTK)-dependent signal transduction depends on phosphorylation of tyrosyl residues in protein substrates, with subsequent recognition and binding to these newly generated phosphotyrosyl (pTyr)-containing sequences, by pTyr-binding modules such as Src homology 2 (SH2) and phosphotyrosyl binding (PTB) domains.[1,2] Because PTK pathways are involved in the etiology of a variety of cancers,[3] inhibitors of SH2 domain binding may potentially afford attractive new therapeutic approaches by disrupting the continuity of signal transmission.[4] Included among important targets for development of antiproliferative agents, are Grb2 SH2 domains, which have been associated with breast cancer[5] and MET-dependent cancers.[6] For most SH2 domains, high affinity ligand binding depends both on the combined interactions of pTyr residues within well defined pTyr binding pockets, as well as secondary interactions of amino acid residues proximal to this pocket.[7] For Grb2 SH2 domains, an Asn residue in the pY + 2 position, provides critical elements of this recognition. Major design considerations in the development of most Grb2 SH2 domain inhibitors

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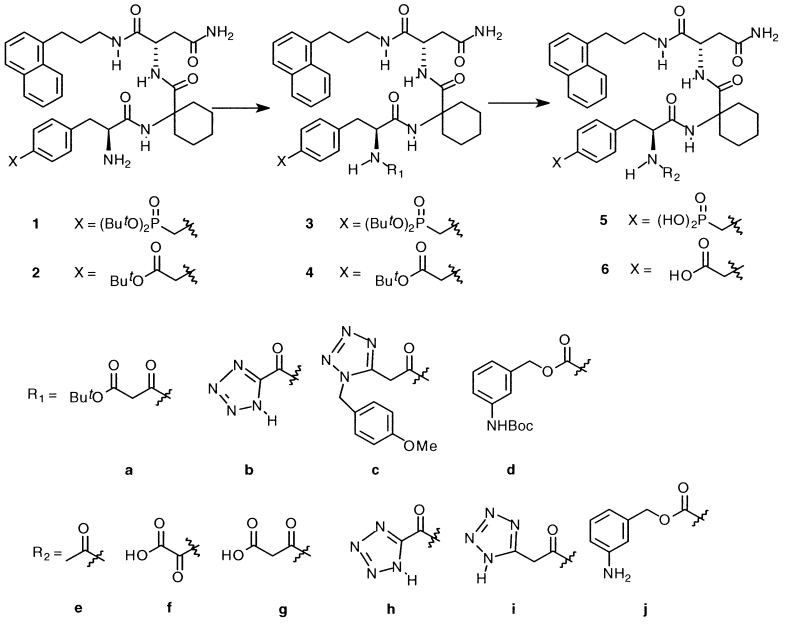
to date, have been predicated on the binding of pTyr-containing peptide leads, with interactions in the pTyr binding pocket being an important component.[8–10] Both X-ray[11–13] and NMR solution structures[14–16] of ligated Grb2 SH2 domain protein have shown that pTyr binding involves key hydrogen bonding between the pTyr phosphoryl oxygens and βC and αA arginine residues (Arg86 and Arg67, respectively).[17] Development of phenyl phosphate mimetics which can engage these critical Arg residues in fashions similar to parent pTyr residues, has been an integral component of efforts to derive SH2 domain inhibitors which are stable to cellular phosphatases.[18–22] To date, a major focus of these efforts has been on substituents at the 4-position[23–31] or 3,4-positions[28,32] of phenylalanine residues. However, studies have also shown that bonding interactions can be enhanced by substituents originating from the tyrosyl α-position.[29,33–37] For Grb2 SH2 domains, binding enhancement of certain N^{α}-substituents has been attributed to interactions with the Arg67 residue.[12,28,38] Potency enhancement through N^{α}-auxiliary functionality is particularly attractive, since it supplements and maintains interactions already provided by phosphate mimicking groups at the phenylalanine 4-position. The N^{α}-oxalyl group is one example of a substituent which both enhances binding potency and potentiates cellular efficacy.[28] To date however, investigations of acidic N^{α}-derivatives in Grb2 SH2 domain inhibitors have not been extensively reported. Therefore, the current study was undertaken to examine a series of oxalyl-related analogues in a Grb2 SH2 domain-binding platform.

Synthesis

Inhibitors were based on an N-terminal 3-(naphth-1-yl)propan-1-yl)-containing tripeptide platform, originally disclosed as providing high affinity Grb2 SH2 domain inhibitors.[39] Two parallel series of analogues (**5** and **6**) bearing phosphonomethyl[40–42] or carboxymethyl[43,44] phosphate mimicking functionalities at the phenylalanyl 4-position, were examined (Scheme 1). Synthesis of both series of naphthyl-containing analogues were accomplished by solution methods, starting from previously reported *tert*-butyl-protected **1**[28] or **2**.[30] Acylation of free α-amino groups of **1** or **2** as described in the Experimental, provided protected intermediates **3a–d** and **4a–c**, respectively (Scheme 1), which after acidic deprotection and purification by HPLC, gave final products **5g–j** and **6g–i**. N^{α}-acetyl analogues **5e** and **6e** and N^{α}-oxalyl analogues **5f** and **6f** have been previously reported.[28,30]

Results and Discussion

Development of Grb2 SH2 domain inhibitors predicated on binding of pTyr-Xxx-Asn'' peptides, is heavily influenced both by interactions within the pTyr binding pocket as well as by interactions afforded by the Asn residue.[21] Previously disclosed N-terminal 3-(naphth-1-yl)propan-1-yl)-containing tripeptide **5e** provides high affinity interactions outside the pTyr-binding pocket.[39] Using **5e** as a model, in prior reports we have investigated binding interactions within the pTyr-binding pocket, through a series of phenylalanine analogues bearing various phosphate-mimicking functionality. Among pTyr mimetics examined phosphonomethyl phenylalanine (Pmp)-containing **5e**[28] and carboxymethylphenylalanine (Cmf)-containing **6e**[30] were the most potent phosphorus and monocarboxy-based inhibitors, examined. A critical component of high affinity binding of these analogues, the interaction of their anionic phosphate mimicking functionality with Arg86 and Arg67 residues, can be augmented by appending oxalyl species at the phenylalanyl N^{α}-position.[28,30] The basis for binding enhancement has been inferred from molecular modelling studies, to potentially reside in



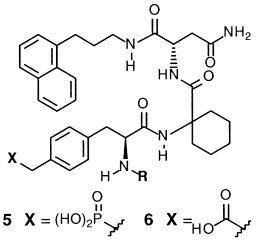
Scheme 1.

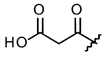
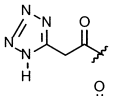
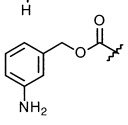
anionic interactions between the oxalyl β-carboxyl group and the positively charged Arg67 guanidino group.[28,30]

Relative Grb2 SH2 domain binding affinities in extracellular ELISA assays

In the current study, N^{α}-oxalyl functionality enhanced binding potency relative to the parent N^{α}-acetyl congener, by approximately three-fold in both the Pmp-containing series (**5e**, $IC_{50} = 0.065\,\mu M$ versus **5f**, $IC_{50} = 0.02\,\mu M$) and the Cmf-containing series (**6e**, $IC_{50} = 9\,\mu M$ versus **6f**, $IC_{50} = 2.7\,\mu M$) (Table 1). Since one objective of the current study was to explore potential binding enhancement afforded by acidic N^{α}-substituents other than the oxalyl group, the N^{α}-oxalyl group was initially replaced by an N^{α}-malonyl substituent, which effectively extended the oxalyl carboxyl out from the N^{α}-position by an additional methylene unit. In the Pmp-series, this change resulted in a binding potency (**5g**, $IC_{50} = 0.03\,\mu M$) which was approximately two-fold higher than the parent N^{α}-acetyl analogue (**5e**), and approximately equipotent to the N^{α}-oxalyl compound (**5f**). In the Cmf series, the N^{α}-malonyl analogue (**6g**, $IC_{50} = 6\,\mu M$) was only slightly more potent than the parent N^{α}-acetyl compound (**6e**), and showed a two-fold loss of potency relative to the N^{α}-oxalyl analogue (**6f**).

Table 1. Inhibition of Grb2 SH2 domain binding[a]



R	No	$IC_{50} \pm$ s.d. (μM)	No	IC_{50} (μM)[b]
	**5e**	0.065±0.008	**6e**	9
	**5f**	0.02±0.004	**6f**	2.7
	**5g**	0.03±0.02	**6g**	6
	**5h**	0.045±0.018	**6h**	6
	**5i**	0.034±0.002	**6i**	4
	**5j**	0.078±0.081		

[a]IC_{50} values were determined using Grb2 SH2 domain fusion protein in an ELISA assay as previously described in ref 45.
[b]Single determinations.

In order to examine structural parameters other than chain length, the effect of replacing the oxalyl carboxyl group by "carboxylic-mimicking" functionality was investigated. Use of tetrazole groups as carboxylic isosteres has been well documented.[45–47] and in the present study, tetrazolyl analogues **5h** ($IC_{50} = 0.045\,\mu M$) and **6h** ($IC_{50} = 6\,\mu M$) were prepared as counterparts of oxalyl-containing **5f** and **6f**, respectively. For Pmp-based **5h**, a higher potency relative to the parent N^{α}-acetyl analogue was observed. Similar relative potency ratios were observed for Cmf-based **6g**. Just as in the oxalyl series, where the effect of chain elongation was examined, similar chain extension was undertaken in the tetrazole series, with compounds **5i** ($IC_{50} = 0.034\,\mu M$) and **6i** ($IC_{50} = 4\,\mu M$) being prepared as tetrazolyl congeners of N^{α}-malonyl analogues **5g** and **6g** respectively. These were found to be approximately equipotent to their malonyl counterparts.

It had been expected that enhancement of potency could potentially be observed for tetrazolyl versus carboxyl analogues in the latter series, due in part to possible cation–π interactions between the Arg67 guanidino group and the tetrazole rings. The experimental observation that tetrazolyl analogues did not exhibit enhanced binding potency relative to their carboxyl counterparts, indicated that cation–π interaction may not have come into play. Nonetheless, it remained of interest to examine the potential utility of cation–π interactions with Arg67 in the context of the naphthyl-containing Pmp-Ac_6c-Asn-(naphthylpropylamide) platform. In this regard, it had previously been reported that the Grb2 SH2 domain binding potency of Xxx-pTyr-Ile-Asn-NH_2 could be enhanced over 100-fold when Xxx = m-aminobenzyloxycarbonyl [(3-amino)Z] as compared to Xxx = Acetyl.[38] By analogy to X-ray studies of a related N-terminal anthranilic acid-derivatized inhibitor,[12] the exceptional enhancement in affinity afforded by the N^{α}-(3-amino)Z group could be partially attributed to π-stacking between the (3-amino)Z aryl ring and the Arg67. Surprisingly, in the current study, the (3-amino)Z-derivatized analogue **5j** ($IC_{50} = 0.078\,\mu M$) exhibited an affinity lower than the parent N^{α}-acetyl analogue (**5e**), indicating the absence of beneficial binding effects.

Inhibition of tumor MDA-453 cell growth

ELISA data presented in Table 1 and discussed above, reflect the ability of inhibitors to block binding of a short pTyr-containing peptide to isolated Grb2 SH2 domain protein. This kind of data is a useful indicator of molecular interactions between the inhibitors and Grb2 SH2 domains, and it provides a basis for comparison of effects on binding induced by structural changes within a series of compounds. However, in physiological contexts, Grb2 SH2 domains exist intracellularly as subunits of larger proteins which bind to pTyr residues that are contained within protein sequences, and which require cell membrane transport of inhibitors before access to the Grb2 protein can be achieved. Additionally, the ultimate aim of these studies is to block Grb2 SH2 domain-dependent mitogenic

signalling. Therefore, in order to more faithfully examine the ability of compounds to inhibit interaction of native Grb2 with cognate p185 $^{erbB-2}$ in whole cells and to block mitogenic signalling, tests were conducted using MDS-453 cells, which are derived from human breast cancer where there is an amplification of erbB-2 gene. These growth inhibition assays (results shown in Table 2) reflect the combined effects of cell membrane transport as well as physiologically relevant inhibition of Grb2 SH2 domain binding. N^{α}-oxalyl **5f** has been reported previously to more potently inhibit intracellular Grb2 SH2 domain binding than does the N^{α}-acetyl parent **5e**.[28] In the current study, **5f** ($IC_{50} = 4.3\,\mu M$) was approximately five-fold more potent than **5e** ($IC_{50} = 23\,\mu M$) as an inhibitor of erbB-2-dependent cell growth. Consistent with extracellular ELISA binding results (Table 1), N^{α}-malonyl derivative **5g** ($IC_{50} = 4.6\,\mu M$) was equi-potent to **5f** in inhibiting cell growth. Tetrazole-containing analogue **5h** ($IC_{50} = 9.6\,\mu M$) was approximately twice as potent as N^{α}-acetyl parent **5e**, while inhibitors **5i** ($IC_{50} = 19.7\,\mu M$) and **5j** ($IC_{50} = 17.0\,\mu M$) were minimally more potent.

Of interest, was a comparison of the relative cellular potencies of tetrazole derivatives (**5h** and **5i**) relative to their carboxyl counterparts (**5f** and **5g**). While being approximately as acidic as carboxyl groups at physiological pH, tetrazole groups are almost 10 times more lipophilic.[48] In previous reports, replacement of carboxylate functionality with tetrazole groups can result either in enhanced potency, as exemplified by CCK-B ligands,[49] or as in the case of phospholipase A(2) antagonists, it can lead to both increased or decreased affinity, depending on the display platform.[50] Such results are exemplary of the general observation, that bioisoterism of the tetrazole moiety with carboxyl functionality, is not uniformly consistent. This is further seen with certain anti-allergic, anti-lipemic and anti-inflammatory agents, where tetrazoles exhibit increased potency relative to their respective carboxylate congeners, while for select oestrogenic acids and anti-arrythmics, lowered potency is observed.[51] The lack of a clearly defined bioisosteric relationship between carboxyl and tetrazole functionality is reflected in the current study, where cellular potency of tetrazole-containing **5h** and **5i** was not enhanced relative to their carboxylic counterparts (**5f** and **5g**), in spite of the fact that **5h** and **5i** would be expected to exhibit greater lipophilicity.

Table 2. Inhibition of MDA-453 tumor cell growth by treatment with phosphonate-containing Grb2 SH2 domain antagonists[a]

No	$IC_{50} \pm$ s.d. (μM)	No	$IC_{50} \pm$ s.d. (μM)
5e	23 ± 2[b]	**5h**	9.6 ± 6.4
5f	4.3 ± 0.8	**5i**	19.7 ± 3.2
5g	4.6 ± 1.4	**5j**	17.0 ± 1.4[b]

[a]IC_{50} values were determined as previously descibed in reference 45, and represent the average of three independent experiments, unless otherwise indicated.
[b]Average of two independent determinations.

Conclusions

Previously it has been shown that for pTyr-containing ligands, derivatization at the tyrosyl N^{α}-position can result in increased Grb2 SH2 domain binding potency, including potentiating effects of N^{α}-oxalyl functionality when incorporated into the naphthylpropylamido tripeptide platform **5**. The primary intent of the current study was to examine variations of the N^{α}-oxalyl theme in the context of both Pmp-containing platform **5** as well as Cmf-containing platform **6**, and also to examine N^{α}-(3-amino)Z derivatization in platform **5**. As with the N^{α}-oxalyl derivative **5f**, it was found that extending the oxalyl carboxyl out by one methylene unit or replacing carboxyl functionality with a tetrazole isostere, resulted in increased potency both in ELISA-based extracellular Grb2 SH2 domain binding assays and erbB-2 tyrosine kinase dependent cell growth assays. Interestingly, in spite of the fact that N^{α}-(3-amino)Z derivatization has been reported to significantly enhance the binding potency of pTyr-containing tripeptides, when applied to the naphthyl-containing platform **5**, this pronounced enhancement was not observed.

Experimental

Cells and cell cultures

Cell lines were obtained from the American Type Culture Collection (Rockville, MD) and the Lombardi Cancer Center, Georgetown University Medical Center. Cells were routinely maintained in improved minimal essential medium (IMEM, Biofluids, Rockville, MD) with 10% fetal bovine serum. Cultures were maintained in a humidified incubator at 37 °C and 5% CO_2.

ELISA assay of Grb2 binding inhibition

A biotinated Shc-derived phosphopeptide (20 ng/mL), was bound to 96-well plate by reaction overnight. Non-specific interactions were inhibited by addition of 5% bovine serum albumin containing TBS. Recombinant purified Grb2 SH2-GST fusion protein, incubated with test compounds at a series of dilutions from 1 mM to 1 μM, were added to each well. Following extensive washing with 0.1% bovine serum albumin in TBS, Grb2 SH2 domain binding was detected using anti-GST antibodies and goat anti-mouse antibody conjugated to alkaline phosphatase. Color reactions were developed using para-nitrophenyl phosphate.

Cell growth inhibition

The effect of Grb2 inhibitors on cell proliferation was determined by direct cell counting. Briefly, 25,000 cells were plated into 24-well plates and Grb2 SH2 domain inhibitors at appropriate concentrations were added and cultured for 8 to 10 days. Cells were collected every other day and counted using a Coulter counter.

General synthetic methods

Fast atom bombardment mass spectra (FABMS) were acquired with a VG Analytical 7070E mass spectrometer under the control of a VG 2035 data system. ^{1}H NMR data were obtained on Bruker AC250 (250 MHz) are reported in ppm relative to TMS and referenced to the solvent in which they were run. Solvents were removed by rotary evaporation under reduced pressure and anhydrous solvents were obtained commercially and used without further drying. Preparative high pressure liquid chromatography (HPLC) was performed using a Waters PrepLC 4000 system with photodiode array detection and an Advantage C_{18} 5μ column (20 mm dia × 250 mm) at a flow rate of 10 mL/min, using a solvent system of A = 0.1% aqueous TFA and B = 0.1% TFA in acetonitrile.

Formation of protected *N*-acyl intermediates 3a,c and d

Preparation of compound 3a. To the solution of **1**[28] (0.066 mmol) in anhydrous DMF (1 mL) was added an activated ester solution formed by reacting mono *tert*-butyl malonate (Aldrich) (11.2 μL 0.073 mmol), 1-hydroxybenzotirazole hydrate (HOBt) (9.9 mg, 0.073 mmol) and 1,3-diisopropylcarboddimide (DIPCDI) (11.2 μL, 0.073 mmol) in anhydrous DMF (1 mL) (10 min). The resulting solution was stirred (overnight) then taken to dryness under high vacuum and purified by silica gel chromatography ($CHCl_3$/EtOAc/MeOH) to provide **3a** as white foam (37 mg, 60% yield). ^{1}H NMR ($CDCl_3$) δ 8.05 (1H, dd, J = 1.95, 7.20 Hz), 7.82 (2H, m), 7.68 (1H, m), 7.51–7.38 (4H, m), 7.36 (2H, d, J = 4.6 Hz), 7.16–7.04 (5H, m), 6.46 (1H, br), 5.37 (1H, br), 4.77 (2H, m), 3.42–3.34 (2H, m), 3.18 (2H, d, J = 1.47 Hz), 3.16–3.10 (3H, m), 3.02 (1H, s), 2.97–2.87 (2H, m), 2.93 (1H, s), 2.53 (1H, dd, J = 5.1, 15.3 Hz), 2.15 (1H, d, J = 5.0 Hz), 2.05–1.62 (6H, m), 1.43 (9H, s), 1.42 (9H, s), 1.40 (9H, s). ($^+$VE, NBA) m/z 920.5 [MH$^+$].

Preparation of compound 3c. Acylation of **1** with 2-(5-(2-methoxybenzyl)tetrazolyl)acetyl chloride (prepared from ethyl 2-(5-(2-methoxybenzyl)tetrazolyl)acetate[52] by initial hydrolysis to the free acid (LiOH) followed by reaction with oxalyl chloride) according to the general procedure, and purification of crude product by silica gel chromatography ($CHCl_3$/MeOH, 20:1) provided **3c** as a foam (62% yield). ^{1}H NMR ($CDCl_3$) δ 8.02 (m, 2H), 7.85 (m, 2H), 7.68 (m, 1H), 7.45 (m, 2H), 7.39–7.07 (m, 9H), 6.88 (s, 2H), 6.83 (s, 1H), 6.41 (brs, 1H), 5.56 (s, 2H), 5.36 (brs, 1H), 4.66 (m, 2H), 3.77 (s, 3H), 3.74 (s, 2H), 3.35 (m, 2H), 3.13–2.84 (m, 5H), 2.92 (d, 2H, J = 18 Hz), 2.47 (dd, 1H, J = 4.9 Hz & 15 Hz), 2.05–1.13 (brm, 12 Hz), 1.41 (s, 9H), 1.41 (s, 9H). FABMS ($^+$VE, NBA) m/z 1008.6 (MH$^+$).

Preparation of compound 3d. Acylation of 1 with 3-*N*-((*tert*-butyloxy)carbonylamino)benzyl-4-nitrophenylcarbonate[38] according to the general procedure, and purification of crude product by silica gel chromatography ($CHCl_3$/EtOAc/MeOH) provided **3d** as a white solid (47% yield). ^{1}H NMR ($CDCl_3$) δ 8.04 (1H, dd, J = 1.90, 7.08 Hz), 7.82 (2H, m), 7.67 (1H, m), 7.58 (1H, m), 7.48–7.15 (9H, m), 7.02–6.99 (2H, d, J = 7.81 Hz), 6.90 (1H, d, J = 7.57 Hz), 6.69 (1H, s), 6.51 (1H, s), 5.65 (1H, s), 5.31 (1H, d, J = 5.1 Hz), 5.07 (1H, d, J = 12.0 Hz), 4.89 (1H, d, J = 12.0 Hz), 7.74 (1H, m), 7.25 (1H, m), 3.37 (2H, m), 3.15–2.69 (8H, m), 2.01–1.65 (8H, m), 1.50 (9H, s), 1.40 (18H, m). FABMS ($^+$VE, NBA) m/z 1027.5 [MH$^+$].

General procedure for formation of protected *N*-acyl intermediates 4a–c14

Preparation of compound 4a. To a solution of amine **2** (0.1 mmol) in DMF (1 mL) was added an active ester solution formed by reacting mono *tert*-butyl malonate (17μ, 0.11 mmol), HOBt (15 mg, 0.11 mmol) and DIPCDI (17μ, 0.11 mmol) in DMF (0.5 mL) (10 min). The resulting solution was stirred (overnight) then taken to dryness under high vacuum and purified by silica gel chromatography (EtOAc then 5% MeOH in EtOAc) to yield **4a** as a syrup (60 mg, 83% yield). ^{1}H NMR ($CDCl_3$) δ 8.12 (d, 1H, J = 6.8 Hz), 8.00–7.86 (m, 2H), 7.80–7.66 (m, 3H), 7.56–7.38 (m, 5H), 7.22 (s, 4H), 6.65 (brs, 1H), 5.74 (brs, 1H), 4.86–4.72 (m, 2H), 3.55 (s, 2H), 3.55–3.35 (m, 2H), 3.26 (s, 2H), 3.25–3.15 (m, 2H), 3.10–2.95 (m, 2H), 2.70 (dd, 1H, J = 3 Hz & 15 Hz), 2.20–1.20 (m, 12H), 1.52 (s, 9H), 1.46 (s, 9H). FABMS ($^+$VE, NBA) m/z 828.6 (MH$^+$).

Preparation of compound 4b. Acylation of **2** with 1-(4-methoxybenzyl)tetrazole-5-carboxylic acid chloride[53] according to the general procedure and purification by silica gel chromatography (EtOAc with MeOH, from 0 to 2.5%) provided **4b** as a syrup (53% yield). ^{1}H NMR ($CDCl_3$) δ 8.44 (d, 1H, J = 7.3 Hz), 8.25 (d, 1H, J = 7.3 Hz), 8.20–8.05 (m, 2H), 7.80–7.30 (m, 10H), 6.94 (d, 2H, J = 8.5 Hz), 6.44 (s, 1H), 6.36 (s, 1H), 5.92 (s, 1H), 5.88 (d, 1H, J = 13 Hz), 5.78 (d, 1H, J = 13 Hz), 5.10–4.95 (m, 1H), 4.78–4.66 (m, 1H), 3.82 (s, 3H), 3.56 (s, 2H), 3.45 (m, 1H), 3.30–3.05 (m, 4H), 2.54 (dd, 1H, J = 9 Hz & 14 Hz), 2.15–0.90 (m, 12H), 1.51 (s, 9H). FABMS ($^+$VE, NBA) m/z 902.9 (MH$^+$).

Preparation of compound 4c. Acylation of **2** with 2-(5-(2-methoxybenzyl)tetrazolyl)acetyl chloride (prepared from ethyl 2-(5-(2-methoxybenzyl)tetrazolyl)acetate[48] by initial hydrolysis to the free acid (LiOH) followed by reaction with oxalyl chloride) according to the general procedure, and purification of crude product by silica gel chromatography (EtOAc with MeOH, from 0 to 20%) provided **4c** as a foam (34% yield).^{1}H NMR ($CDCl_3$) δ 8.07 (d, 1H, J = 7.3 Hz), 7.90–7.80 (m, 3H), 7.74 (d, 1H, J = 6.4 Hz), 7.55–7.30 (m, 10H), 6.92 (d, 2H, J = 8 Hz), 5.60 (2H), 4.90–4.68 (brm, 2H), 3.95–3.75 (m, 3H), 3.82 (s, 3H), 3.55 (s, 2H), 3.45–2.55 (m, 5H, 2.15–1.10 (m, 12H), 1.51 (s, 9H). FABMS ($^+$VE, NBA) m/z 917 (MH$^+$).

Global deprotection and formation of final products 5g–j

Preparation of compound 5g. A solution of **3a** (30 mg, 0.033 mmol) in TFA/H_2O/triethylsilane (TES) (1.9 mL:100 μL : 50 μL) was stirred at room temperature (1 h), then solvent was removed under high vacuum and

residue was purified by preparative HPLC (linear gradient 5 to 50% B over 10 mins, then 50 to 100% B over 15 min: retention time = 16.2 min) to provide product **5g** as a white solid (23 mg, 94% yield). ^{1}H NMR (DMSO-d_6) δ 8.50 (1H, d, J = 7.08 Hz), 8.08 (1H, s), 7.92–7.72 (2H, m), 7.75 (1H, m), 7.49 (3H, m), 7.37 (3H, m), 7.29 (1H, s), 7.15–7.05 3H, s), 6.89 (1H, m), 4.58 (1H, m), 4.39 (1H, m), 3.25. FABMS (−VE, Gly) m/z 714 (M−H).

Preparation of compound 5h. Acylation of **1** with 1-(4-methoxybenzyl)tetrazole-5-carboxylic acid chloride[49] according to the general procedure outlined above and purification by silica gel chromatography ($CHCl_3$:MeOH, 20:1) provided intermediate **3b** as a glass (65% yield) which was directly treated with TFA/H_2O/TES (2 mL:100 μL:50 μL) (room temperature, overnight) as described above and taken to dryness under vacuum. Residue was purified by preparative HPLC (linear gradient 5 to 60% B over 25 min: retention time = 24.8 min) to provide product **5h** as a white solid (6.8 mg, 21% yield). ^{1}H NMR (D_2O) δ 8.43 (brs, 1H), 8.28–8.18 (m, 1H), 8.05–7.90 (m, 3H), 7.85–7.75 (m, 2H), 7.67–7.40 (m, 4H), 7.35–7.20 (m, 6H), 4.76–4.65 (m, 1H), 4.60–4.50 (m, 1H), 3.45–2.8 (m, 10H), 2.1–1.0 (m, 12H). FABMS (−VE, Gly) m/z 760 (M−H).

Preparation of compound 5i. As described in the general procedure for deprotection of **3a** to **5g**, treatment of **3c** (overnight) and purification by preparative HPLC (linear gradient 5 to 60% B over 25 min, then 60 to 100% B over 5 min: retention time = 23.8–27.2 min) provided **5i** as a white solid (43% yield). ^{1}H NMR (DMSO-d_6) δ 8.68 (1, J = 7.3), 8.21 (s, 1H), 8.12–8.06 (μ, 1H), 7.95–7.88 (m, 2H), 7.78–7.72 (m, 2H), 7.56–7.48 (m, 3H), 7.43–7.37 (m, 3H), 7.20–7.08 (m, 4H), 6.94 (brs, 1H), 4.74–4.63 (m, 1H), 4.45–4.35 (m, 1H), 3.94 (d, 1H, J = 16 Hz), 3.82 (d, 1H, J = 16 Hz), 3.25–2.58 (m, 5H), 2.92 (d, 2H, J = 21 Hz), 2.06–1.10 (m, 12 H). FABMS (−VE, Gly) m/z 774 (M−H).

Preparation of compound 5j. As described in the general procedure for deprotection of **3a** to **5g**, treatment of **3d** (1 h) and purification by preparative HPLC (linear gradient from 5 to 50% B over 10 min: retention time = 16.8 min) provided **5j** as a white solid (quantitative). ^{1}H NMR (DMSO-d_6) δ 8.29 (1H, s), 8.08 (1H, m), 7.96 (1H, d, J = 8.05 Hz), 7.89 (1H, m), 7.74 (1H, t, J = 4.40 Hz), 7.61 (1H, d, J = 8.05 Hz), 7.55–7.35 (7H, m), 7.30–7.09 (6H, m), 7.05–6.88 (4H, m), 4.95 (1H, d, J = 12.94 Hz), 4.88 (1H, d, J = 12.94 Hz), 4.40 (2H, m), 3.25–2.98 (5H, m), 2.92 (2H, d, J = 21.24 Hz), 2.70–2.50 93H, m), 2.10–1.10 (12H, m). FABMS (−VE, Gly) m/z 813.7.

General procedure for global deprotection and formation of final products 6g–i.

Preparation of compound 6g. A solution of **4a** (50 mg, 0.076 mmol) in TFA/H_2O/TES (1.9 mL:100 μL; 50 μL) was stirred at room temperature (1 h), then solvent was removed under high vacuum and residue was purified by preparative HPLC (linear gradient 20 to 90% B over 20 min: retention time = 14.7 min) to provide product **6g** as a white solid (25 mg; 46% yield). ^{1}H NMR (DMSO-d_6) δ 8.55 (d, 1H, J = 6.4 Hz), 8.12 (s, 2H), 7.99–7.86 (m, 2H) 7.84–7.75 (m, 1H), 7.60–7.35 (m, 6H), 7.20 (s, 4H), 6.94 (brs, 1H), 4.70–4.60 (m, 2H, 4.50–4.35 (m, 2H), 3.55 (s, 2H), 3.30–3.05 (m, 6H), 2.98–2.84 (m, 1H), 2.08–1.20 (m, 12H). FABMS (−VE, Gly) m/z 714 (M−H).

Preparation of compound 6h. As described in the general procedure for deprotection of **4a** to **6g**, treatment of **4b** (overnight) and purification by preparative HPLC (linear gradient 30 to 50% B over 20 min: retention time = 19.5 min) provided **6h** as a white solid (40% yield). ^{1}H NMR (DMSO-d_6) δ 9.32 (d, 1H, J = 7.3 Hz), 8,48 (s, 1H), 8.16–7.92 (m, 3H), 7.86–7.75 (m, 1H), 7.64–7.38 (m, 6H), 7.32 (d, 2H, J = 7.7 Hz), 7.16 (d, 2H, J = 8.1 Hz), 7.04–6.95 (m, 1H), 5.05–4.90 (m, 1H), 4.50–4.40 (m, 2H), 3.54 (s, 2H), 3.35–2.96 (m, 6H), 2.85–2.68 (m, 1H), 2.15–1.15 (m, 12H). FABMS (−VE, Gly) m/z 724.5 (M−H).

Preparation of compound 6i. As described in the general procedure for deprotection of **4a** to **6g**, treatment of **4c** (1.5 h) and purification by preparative HPLC (linear gradient 20 to 80% B over 20 min: retention time = 14.5 min) provided **6i** as a white solid (32% yield). ^{1}H NMR (DMSO-d_6) δ 8.20–8.10 (m, 1H), 8.05–7.95 (m, 3H), 7.60–7.45 (m, 8H), 7.32–7.16 (m, 4H), 7.04 (brs, 1H), 6.60 (brs, 1H), 4.80–4.70 (m, 1H), 4.65–4.55 (m, 1H), 4.48–4.38 (m, 1H), 3.94 (d, 1H, J = 5.6 Hz), 3.55 (s, 2H), 3.35–2.96 (m, 6H), 2.15–1.15 (m, 12H). FABMS (−VE, Gly) m/z 738 (M−H).

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