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Synthesis and Anticancer Activity of Epipolythiodiketopiperazine Alkaloids

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Abstract

The epipolythiodiketopiperazine (ETP) alkaloids are a highly complex class of natural products with potent anticancer activity. Herein, we report the application of a flexible and scalable synthesis, allowing the construction of dozens of ETP derivatives. The evaluation of these compounds against cancer cell lines in culture allows for the first expansive structure–activity relationship (SAR) to be defined for monomeric and dimeric ETP-containing natural products and their synthetic cognates. Many ETP derivatives demonstrate potent anticancer activity across a broad range of cancer cell lines, and kill cancer cellsviainduction of apoptosis. Several traits thatbode well for the translational potential of the ETP class of natural products includeconcise and efficient synthetic access, potent induction of apoptotic cell death, activity against a wide range of cancer types, and a broad tolerance for modifications at multiple sitesthat should facilitate small-molecule drug development, mechanistic studies, and evaluation *in vivo*.

Introduction

Epipolythiodiketopiperazine (ETP)[1] alkaloids constitute a large (*ca*. 120 members) and diverse family of biologically active secondary metabolites produced by a number of filamentous fungi including those from the *Chaetomium*, *Leptosphaeria*, *Aspergillus*, *Verticillium*, *Penicillium*, and *Pithomyces* genera. These small-molecule natural products are characterized by the incorporation of an intramolecular polysulfide bridge at the α,α'-positions of a *cyclo*-dipeptide (or diketopiperazine – DKP) (Fig. 1). Although mono-, di-, tri-, and tetrasulfide members are naturally occurring, the disulfides are most prevalent.[2] These mycotoxins, containing one ortwo ETP rings, exhibit a wealth of structural diversity[1c] and display a fascinating spectrum of biological activities[1a,3] including antibacterial,[4] anticancer,[3,5] antiviral,[6] antiparasitic, antifungal,[7] antimalarial, immunosuppressive, immunomodulatory,[1b,5a,8] phytotoxic,[9]nematicidal,[10]antiplatelet,[11]and anti-inflammatory effects.[1f]

As a consequence of the intriguing biological activities[3] and the structural diversity[1c]that surrounds the central ETP motif, access to greater quantities of the alkaloids and their analogs is desired; a considerable number of synthetic efforts have thus been directed toward the synthesis of the ETP core[1b,12] and ETP-containing naturally occurring alkaloids.[1f,13,14]Due to the synthetic challenges posed by their complex molecular

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architecture, however, structure–activity relationship (SAR) studies of ETP-containing derivatives are still limited and warrant further exploration.[1a,3]Various ETP alkaloids have been assessed in a diverse array of biological tests, but the non-uniformity of these studies precludes comparative analysis and the inference of meaningful conclusions. As a result, although the polysulfide warhead has been noted to be a critical pharmacophore (*vide infra*), little is known about the influence of the number of sulfur atoms, the stereochemical configuration of the sulfurated centers, or the dimerization state of the carbocyclic structure on the bioactivity of these alkaloids. Investigation of the impact of each of these structural features is crucial to elucidating the mode of action of these compounds, to designing highly potentstructures with suitable physicochemical and biopharmaceutical properties, and to their translation *in vivo* in clinical applications (*e.g.*, biological probes and chemotherapeutic agents).

Several studies have unequivocally demonstrated that the polysulfide bridge plays a key role and is oftensufficient for bioactivity.[1a–b]Indeed, the chemical ablation of the sulfur bridge in naturally occurring alkaloids (*e.g.*, chaetocin A (**4**),[13f] gliotoxin (**1**)[15], sporidesmin[16]) or the synthesis of analogs devoid of sulfur at the α-positions of the *cyclo*-dipeptide[17a] results in biologically inactive compounds. Similarly, reductive *S*-methylation of the epidithiodiketopiperazine motif in a number of natural products (*e.g.*, gliotoxin (**1**),[1b,5c,n,8a] chaetocin A (**4**),[18] scabrosin,[19] sporidesmin,[16,20] T988 B,[21]bionectin C (**8**),[4]and chetracin D[22]) unfailingly results in a dramatic loss of biological activity. Furthermore, a general pattern between the degree of sulfuration and the intensity of the biological response has not been clearly established.[1a–b,3,10,18,21–23] It is ambiguous whether these molecules go through a common mode of action or are converted into a similar active species. Interestingly, epimonothiodiketopiperazine metabolites have been reported to be at least one order of magnitude less active than their congeners.[1b,19,24,25]

At least three pathways of toxicity have been proposed in the literature[1a–c,f,5a] for the ETP-containing products: (1) generation of deleterious reactive oxygen species (ROS) (*e.g.*, superoxide radical anion, hydroxyl radical, hydrogen peroxide) by redox cycling[5f,k–m,26] and induction of significant oxidative stress, DNA strand cleavage, and apoptosis; (2) covalent conjugation and inhibition of cellular proteins by forming mixed disulfides[5a,11,17,20,26b] or catalytic formation of intramolecular disulfide bonds between cysteine residues on proteins[27]; and (3) disruption of the global tertiary structure of proteins containing a Zn^{2+}-binding cysteine-histidine rich protein domain via a zinc ion ejection mechanism.[17,28]

The epidithiodiketopiperazine class of natural products appears to have considerable anticancer potential,[3,5] but definitive conclusions are difficult to draw as an array of these compounds has never been tested against a wide variety of cancer cell lines. The verticillins[5g,i,k,n] and the chaetocins[5d,f,h,l–m,26d,29] both exhibit cytotoxic activity against cancer cell lines in culture and efficacy in *in vivo* tumor models. The true translational potential of the ETP-containing alkaloids, however, will only be defined after a larger number of more detailed cell culture and *in vivo* studies.

Recently, we reported the enantioselective total synthesis of dimeric epidithiodiketopiperazines (+)-12,12'-dideoxyverticillin A (**3**) and (+)-chaetocin A (**4**) as well as higher-order polysulfides (+)-chaetocin C (**5**) and (+)-12,12'-dideoxychetracin A (**6**) relying on a bioinspired approach.[14a,b]Similarly, the development of our Lewis acid-promoted C3-nucleophilic substitution[14c,30] culminated in the concise enantioselective total synthesis of (+)-gliocladin B (**7**)[31]and (+)-12-deoxybionectin A (**10**).[4]Thesesynthetic methodologiesoffer an opportunity to rapidly create multiple derivatives for evaluation

against cancer cell lines in culture; many of these compounds cannot be created by simple and direct chemical modifications of the (scarce or sensitive) parent natural mycotoxins.

With the ultimate goal of identifying ETP derivatives worthy of evaluation *in vivo*, and to aid in chemotherapeutic development through potent but simplified analogs, a large set of structurally diverse natural and synthetic ETP alkaloids were synthesized. Sixty natural alkaloids and their derivatives (Fig. 2) were tested for their ability to induce cell death in two human cancer cell lines, enabling the derivation of a comprehensive SAR. A selection of 25 sulfurated alkaloids with(sub)nanomolar potencies was further evaluated for activityagainst threeadditional humancancer cell lines, allowing for a broad assessment of anticancer effects. Representative compounds from the monomer and dimer classes were shown to induce caspase-dependent apoptosis. The potent and broad anticancer activity of ETP-containing alkaloids suggests that they have considerable translational potential.

Design and synthesis of ETP alkaloids

Overview of synthetic goals

Sulfur atoms have long been recognized as essential to the anticancer activity of ETP natural products; however, a more refined understanding of structure–activity relationships has been lacking. To identify how this and other structural elements within the hexahydropyrroloindoline and diketopiperazine substructures are critical to the anticancer activity, multiple derivatives of both monomeric and dimeric ETP alkaloids were synthesized and evaluated. The general strategy for derivatives was to construct sets of compounds that varied in the substituents about the hexahydropyrroloindolinecarboskeleton and the nature, extent, and configuration of sulfuration. The compounds produced through these synthetic efforts are depicted in Fig. 2.

Design and synthesis of sarcosine-derived monomeric ETP derivatives

In an effort to formulatea SAR for homodimeric tryptophan-derived ETP alkaloids such as chaetocin A (**4**) and verticillin A (**2**), various derivatives of thisalkaloid class were synthesized and evaluated. Specifically, compounds that were differentially substituted at the C3-quaternary stereogenic center were constructed and then elaborated with different types of sulfur motifs. Relying on the versatility of the different synthetic methodologies developed in our group en route to the total synthesis of several naturally occurring monomeric and dimeric ETP alkaloids,[14] compounds **3–7, 10**, and**14**–**67** were concisely and efficiently accessed as described in Schemes 1–3 or according to experimental procedures previously reported by our group.[14a–c,32]

endo-Tetracyclic bromide **54**, prepared from sarcosine L-tryptophan *cyclo*-dipeptide,[14c] was used to access epidithiodiketopiperazines bearing different C3-substituents (Scheme 1). Electrophilic activation using silver(I) tetrafluoroborate in nitroethane and trapping of the transient tertiary benzylic carbocation with the desired nucleophile (*i.e.*, fluoride, *N*-TIPS-pyrrole,[33] anisole, 5-Br-*N*-TIPS-indole) afforded the C3-substituted *endo*-tetracycles **59** and **68–70** in high yields and excellent levels of regio- and stereoselection.[14c] Dihydroxylation of **59** and **68–70** at the C11-methine and C15-methylene positions was achieved with tetra-*n*-butylammonium permanganate (*n*-Bu_4NMnO_4, 4 equiv) in dichloromethane to provide the corresponding diols in moderate to good yields as single diastereomers. The direct double *cis*-thiolation was accomplished in a single step and in good to high yields (47–80%) by exposure of the bis-hemiaminals to trifluoroacetic acid (TFA) in hydrogen sulfide-saturated dichloromethane solution followed by mild aerobic oxidation to access the bridgehead disulfides as β-epimers **26, 30–33** andα-epimers **34–35**.[34]Interestingly, the diastereoselectivities are consistent with the steric bias imposed by the C3-substituents {β:α

ratio= 2:1 (C3-F);4:1 (C3-Br);> 5:1 (C3-pyrrol-3'-yl); > 7:1 (C3-indol-3'-yl); > 10:1 (C3-*p*-MeOPh)}.

As shown in Scheme 2, we also prepared a set of 27 compounds with a modified sulfur motif within the DKP core using the versatile diol **56** as a strategic point of divergence. Relying on our unified and general solution to epidi-, epitri-, and epitetrathiodiketopiperazines[14b] and the innate reactivity differences between the C11 and C15 hemiaminals, chemo- and stereoselective thiolation of diol **56** by treatment with TFA in hydrogen sulfide-saturated dichloromethane solution at 0 °C generated the corresponding thiohemiaminal **48** in 90% yield and in a highly diastereoselective fashion (>10:1 *dr*). Masking of both alcohol and thiol groups as isobutyrates and photoinduced reductive removal of the benzenesulfonyl group gave **51**. The desired degree of sulfidation was eventually accomplished by hydrazinolysis, chemoselective *S*-sulfenylation with chloro(triphenylmethane) sulfane or disulfane followed by hafnium triflate-mediated cyclization to afford (+)-12-deoxybionectin A (**10**) and its epitrithiodiketopiperazine congener **29** in 65 and 47% yield (3-steps), respectively. A similar two-step approach was employed to access benzenesulfonyl-protected epitri- and epitetrathiodiketopiperazines **27** and **28** in 42% and 44% yield, respectively. Ultimately, reduction of the bridgehead disulfide with $NaBH_4$ followed by *in situ S*-methylation afforded (+)-gliocladin B (**7**)[14c,31] and bis(methylthioether) **39** in high yields.

(+)-Gliocladin C (**52**)[31] and several C11-hydroxylated (**57**–**58**) and C11,C12-dehydrogenated (**53**) intermediates were prepared following the procedures previously reported for the synthesis ofthis atypical non-thiolated triketopiperazine.[14c]

Exposure of hemiaminal **56** to benzyl mercaptan and TFA in nitroethane resulted in the formation of the corresponding bis(benzylthioether) (C15β:C15α = 5.7:1) in 80% yield (single diastereomer, C15β). Further derivatization of the indole nitrogen with a *t*-butoxycarbonyl group gave **43** in 69% yield. After masking the indole substituent of the key ETP intermediate **26**, the bridgehead disulfide was reduced with $NaBH_4$ and *S*-methoxymethylated in a single flask. Subsequent *t*-butoxycarbonylremoval with TFA in dichloromethane afforded bis(thioether) **40** in 66% yield over two steps. A similar strategy including the photoinduced reductive removal of the N1-benzenesulfonyl group provided **41** in 55% over three steps. Reduction of the sulfur bridge of ETP **24** with $NaBH_4$ in a mixture of THF and methanol and *in situ* trapping of the resulting thiolates with 2-methoxyethoxy-methyl chloride (MEMCl) led to thioether **47** and bis(thioether) **42** in 19% and 80% yield, respectively.

Further modifications to the sulfur bridge[1b,12c,25] were accomplished by treatment of the corresponding dithiol (obtained from $NaBH_4$reduction of ETP **26**) with 1,1'-thiocarbonyldiimidazole (TCDI) or 1,1'-carbonyldiimidazole (CDI) to afford di- and trithiocarbonates **36** and **37**, respectively. Similarly, thioacetal **38** was accessed directly by double alkylation using diiodomethane. Desulfurization of epidithiodiketopiperazine **26** was realized by treatment with triethylphosphite in THF to give epimonosulfide **25** in 63% yield.[35] The sulfur atoms were also capped with the *S*-acetyl and *S*-methylsulfane functional groupings to afford compounds that are potentially more labile under intracellular conditions. After reduction of the sulfur bridge of epidisulfide **26**, its treatment with an excess of acetyl chloride, methanesulfenyl chloride, or dimethyldisulfide afforded compounds **44, 45** and **46**, respectively, in good yields.

Design and synthesis of *N*-methylalanine-derived monomeric ETP derivatives

We were also interested in investigating the effect of a different substituent at the C15 position of monomers. With this objective in mind, we prepared monomeric ETP-containing products derived from *N*-methyl-L-alanine L-tryptophan *cyclo*-dipeptide (Scheme 3). Synthesis of these derivatives commenced with *endo*-tetracyclic bromide **73**.[14a] Tertiary benzylic bromide **73** also proved to be an excellent substrate for the desired regio- and stereoselective Friedel–Crafts-type coupling[14c] with 5-bromo-1-triisopropylsilylindole (67% over 2 steps) to afford C3-indolyl tetracycle **74**. Allylation of the C3-tertiary benzylic halide using allyltributylstannane under radical conditions[36] followed by hydrogenation of the terminal olefin afforded C3-*n*-propyl tetracycle **75**. These two C3-substituted tetracyclic monomers were subsequently subject to hydroxylation conditions using bis(pyridine) silver(I) permanganate (Pyr_2AgMnO_4) in pyridine. Treatment of the resultant diols with potassium trithiocarbonate and TFA in dichloromethane resulted in rapid formation of the desired monomeric dithiepanethiones **64** and **66** in 63% yield as a 5:1 isomeric mixture, as well as **65** and **67** in 52% and 17% yield, respectively.[14a] Finally, exposure of these compounds to ethanolamine in acetone followed by oxidative workup using potassium triiodide yielded the corresponding epidithiodiketopiperazine analogs **60–63**.

Design and synthesis of dimeric ETP derivatives

The synthesis of the homodimeric DKP and ETP derivatives (Schemes S1 and S2[†]) have been reported previously in the literature en route to the syntheses of (+)-12,12'-dideoxyverticillin A (**3**)[14a] as well as (+)-chaetocins A (**4**) and C (**5**) and (+)-12,12'-dideoxychetracin A (**6**).[14b] The diacetate forms of these epidi- and epitrithiodiketopiperazines (**15**–**16**) were also synthesized and evaluated.[32] In order to explore the effects of N1-sulfonylation on their bioactivity, a variety of related derivatives (**14, 18–19, 21–23**) possessing the sulfonyl group were prepared analogously.[32]

Anticancer activity and structure–activity relationship studies

All 60 synthesized compounds (Fig. 2) were screened for their ability to induce death in two human cancer cell lines: U-937 (leukemic monocyte lymphoma) and HeLa (cervical cancer). Compounds that demonstrated anticancer activity at 1 μM or below were retested in triplicate at a range of compound concentrations to generate logistical dose-response curvesfrom which IC_{50} values were derived.[32]These results are presented in Table 1 and discussed below.

Carboskeleton

In both U-937 and HeLa cells, the homodimers are the most potent compounds {IC_{50} (U-937) ≥ 0.18 nM; IC_{50} (HeLa) ≥ 0.09 nM}, with the N1,N1'-benzenesulfonylated analog (**14**) of (+)-12,12'-dideoxyverticillin A (**3**) showing the best activity {IC_{50} (U-937): 0.18 nM; IC_{50} (HeLa): 0.09 nM}. Monomeric ETP derivatives also show satisfactory activity in both HeLa (IC_{50} ≥ 5.9 nM) and U-937 (IC_{50} ≥ 2.8 nM) human cancer cell lines. Within the N1-benzenesulfonyl monomeric class, various aromatic substituents (indol-3'-yl **26**, *N*-Boc-indol-3'-yl **24**, pyrrol-3'-yl **32**, *p*-MeO-phenyl **33**) are well tolerated at the C3-position and their IC_{50}'s are of the same order of magnitude {IC_{50} (U-937): 2.8–14.8 nM; IC_{50} (HeLa): 22–75 nM}. Although halide substitution at C3 (bromide **30**, fluorides **31** and **35**) results in intermediate activity, C3-fluorinated epidithiodiketopiperazines do not follow the trend we observe with related ETP analogs in this SAR study. It appears that the steric environment

[†]Electronic Supplementary Information (ESI) available: Details for all biological assays as well as experimental procedures, spectroscopic data, and copies of ^{1}H, ^{13}C, and ^{19}F NMR spectra. See DOI: 10.1039/b000000x/

of the C3 position is crucial for biological activity: *n*-alkyl groups at that position (*n*-propyl analogs **61** and **65**) lead to substantially lower potencies than more sterically hindered (hetero)aryl andhalide substituentsorthe C3' quaternary carbon of a second monomeric subunit. The superior activity of the dimers over (hetero)arylated monomers further supports the role of steric crowding at C3.

The difference in activity between the monomers and the dimers was also observed by Numata et *al.* through biological evaluation (growth inhibition in murine P388 leukemia cell line) of the leptosins, a subset of fungal metabolites including structurally diverse dimeric and monomeric ETP natural products.[23] Dimers containing two sulfur bridge groups are one order of magnitude more potent than monomeric C3-(3'-indolyl) analogs and 2 to 3 orders of magnitude more potent than heterodimers bearing a single sulfur bridge. This non-linear increase of biological activity between mono- and dimeric ETP natural products has also been observed in other families (*e.g.*, chetracin,[22] gliocladine,[10]leptosin,[5f,23a]verticillin A (**2**)4) hinting at the possibility of a synergistic effect. It is unclear, however, if pharmacokinetic properties are playing a significant role.

Comparing homodimers (+)-12,12'-dideoxyverticillin A (**3**), (+)-chaetocin A (**4**), (+)-chaetocin C (**5**), and (+)-12,12'-dideoxychetracin A (**6**) head-to-head reveals that the chaetocin-type ETP derivatives are more potent than their non-C15-hydroxylated counterparts {IC_{50} (U-937): 0.75–1.3 nM *vs.* 15.5 nM; IC_{50} (HeLa): 5.6–6.9 nM *vs.* 7.2 nM}. Acetylation of the 17,17'-hydroxyl groups (**15–16**) also results in a reduction of potency (5.8- to 12.9-fold for U-937; 2.0- to 11.1-fold for HeLa). Additionally, methyl substitution at C15 in monomeric alkaloids (Trp–Ala *cyclo*-dipeptides **60** *vs.* **26** and **64** *vs.* **36**) only affects the potency of the compoundsmoderately. In general, the differencein potency between the different types of substituents atC15 is minimal, although it is sensitive to variations of the steric environment. This suggests that this position could be amenable to additional modifications, especially to optimize the pharmacokinetic parameters during drug development.

Our SAR studies also indicate that substitution at N1 and N1' with benzenesulfonyl (**14**) or trifluoroacetyl (**17**) groups does enhance the anticancer activity of the alkaloids, although the magnitude of this effect is variable. In the case of a benzenesulfonyl group (**14**), the potency is dramatically increased {2 orders of magnitude more potent than the corresponding secondary aniline (+)-12,12'-dideoxyverticillin A (**3**)}. The trifluoroacetamide at N1 and N1' (**17***vs.***16**) plays only a minimal role in the anticancer activity, with slightly lower IC_{50} values (1.1-fold for U-937; 2.5-fold for HeLa) for these derivatives. For the monomeric ETP-containing analogs, the N1-benzenesulfonyl substitution generally amplifies the anticancer effect in U-937 cell line (epidisulfide: **26***vs.* **10**; epitrisulfide: **27** *vs.* **29**). The N1 group confers significantly higher chemical stability but also likely affects the pharmacodynamic properties of these ETPs.

Influence of the nature and degree of sulfuration

While prior studies have demonstrated that having no sulfur atoms {naturally occurring (+)-gliocladin C (**52**) and synthetic analogs: opened DKP (**55**),non-oxidized α-positions (**21, 54, 59**), α-hydroxylated (**22–23, 56–58**), α,β-unsaturated (**53**)} results in a complete loss of biological activity, our studies go further and demonstrate that sulfuration at only the tryptophan C_α-position is not sufficient for potent activity {bis(trisulfanes) (**19** and **20**), C11-thioesters (**50–51**), C11-thioether (**49**), and C11-thiols (**47**–**48**)}. This clearly reinforces the necessity of sulfur atoms at the α and α' positions and the importance of the sulfide bridge or sulfur containing bridge for anticancer activity. This is in accordance with the observation made by Numata in the leptosin class, where monomeric and dimeric ETP

alkaloids are 2 to 4 orders of magnitude more potent than heterodimeric metabolites devoid of sulfur bridge.[23] Both amino acid C_α positions appear torequire sulfuration by separate sulfur atoms, although the epimonosulfide (**25**) is not completely inactive.

Sulfur derivatives possessing non-labile alkyl groups {*S*-methylthioethers (**7** and **39**), *S*-(methoxymethyl)thioethers (**40**–**41**), *S*-(2-ethoxyethoxymethyl)thioether (**42** and **47**), *S*-benzylthioether (**43**)} did not display any anticancer activity (IC_{50}> 10 μM). Remarkably, thioacetal **38** retains moderate cytotoxic activity.Interestingly, the degree of sulfuration of the polysulfide bridge {dimers: (+)-chaetocin A (**4**) *vs.* (+)-chaetocin C (**5**) *vs.* (+)-12,12'-dideoxychetracin A (**6**) or **15***vs.* **16**; monomers: **10** *vs.* **29, 26***vs.* **27***vs.* **28**} has no substantial impact on this cell death induction and the IC_{50} values are within the margin of error of each other. It is ambiguous whether these molecules go through a common mode of action or are converted into a similar active species. The different order of polysulfides may have similar biological activity if the rate determining step for their mode of action is after the conversion to a common active intermediate; however, if the rate determining step is the conversion of the bridging polysulfide into this putative species, then one may expect a difference in activity.

In addition to ETP-containing compounds, we found that several monomeric or dimeric derivatives possessing modifications directly on the sulfur bridge serve as competent anticancer agents; IC_{50} values are<10-fold less potent compared to the parents. These include thioacetate (**44**), dithiocarbonate (**37**), trithiocarbonates (**18, 36, 64**), and alkyl disulfides (**45**–**46**). The methyl disulfides would readily be converted to the thiols through reduction or nucleophilic displacement. The data are highly suggestive of a mode of action that involves a common intermediate. In the presence of a reducing cytoplasmic environment combined with the presence of enzymes – hydrolases, carboxylesterases, and lipases[37] – it is reasonable to believe that the methyl disulfides, thioacetates, and thiocarbonates play the role of prodrugs.[38] They may be converted to their corresponding epidisulfide pharmacophores,[1a,26c] which are potentially actively concentrated within the cell via a glutathione-dependent uptake mechanism.[39,40]

Furthermore, the relative stereochemistry of the ETP system seems to be critical since compounds with the sulfur bridge on the α-face of the DKP (**34, 62**–**63, 66**–**67**) are completely inactive.

Efficacy of ETP alkaloids across multiple cancer cell lines

In order to further probe the potential of ETP alkaloids as anticancer agents, 25 derivatives, selected among the most potentfrom the primary screening (Table 1), were tested in cultureagainsta panel of three supplementary human cancer cell lines representing three additional tumor histologies (H460, lung carcinoma; 786-O, renal carcinoma; MCF-7, breast carcinoma).[32]As shown in Table 2, the ETPs retain similar patterns of potency across all of the cell lines. Some compounds, such as **14**, retain higher levels of activity in all of the cell lines as compared to others, such as **17**. Generally, however, U-937 and HeLa are slightly more sensitive to the ETPs than the other three cell lines. In particular,thenon-adherent lymphoma cell line U-937 showsthe highest susceptibility to the ETP class, as evidenced by the generally high potency of both monomers and dimers against this cell line.

The polysulfide dimers (+)-12,12'-dideoxyverticillin A (**3**), (+)-chaetocins A (**4**) and C (**5**), (+)-12,12'-dideoxychetracin A (**6**), **14**, and bisdithiepanethione **18** are the most active compounds across the board. In the case of the N1,N1'-benzenesulfonylated analog of (+)-12,12'-dideoxyverticillin A (**14**), the potency is dramatically increased (2 orders of magnitude more potent than the parent natural product **3** on the five tested cell lines). Our

SAR studies show that the number of sulfur atoms in the bridge for homodimeric ETP derivatives has no significant effect.

Some additional trends are apparent from the data derived from this extended panel of cell lines. First, the degree of sulfuration has a larger impact in some of the adherent cell lines tested (H460, 786-O, and MCF-7), especially in the case of **26**–**28**. This set of compounds shows a 2-fold decrease in activity with each additional sulfur atom in the polysulfide bridge. However, the overall potency of these compounds is still reasonable (*i.e.*, IC_{50}< 300 nM). Interestingly, it appears that lack of substitution at N1 (**10** and **29**) mitigates this effect. This trend is also not observed within the dimers (**4**–**6**), which is again consistent with the lack of substitution at N1. Monomeric ETP derivativeswith weaker activity in our initial assays (**31, 60**, and **64**) were generally less potent in these additional cancer cell lines. Thethree prodrugs of the epidisulfide bridge (bisthioacetate **44**, trithiocarbonate **36**, dithiocarbonate **37**) display lower activity than the corresponding epidisulfide **26**; however, they all showconsistently lower IC_{50} values than the parent epidisulfide **10** (up to 6.7-fold more potent). There were several derivatives which displayed drastically different activity across cell lines; C3-pyrrolyl **32** and C3-*p*-methoxyphenyl **33** epidisulfides displayed a consistent potency (4-fold change in activity, as compared to 6- to 40-fold changes for otherderivatives) in addition to good relative potency in H460 cells, especially when compared to the activity of structurally similar derivatives.

Hemolytic activity of ETP alkaloids

Compounds that induce death in a range of diverse cancer cell lines with single-digit nanomolar potency are valuable, and an emerging application of such compounds is as antibody-drug conjugates (ADC).[41] ADCs use a covalently-bound antibody to selectively target a potent cytotoxin to a tumor site, as exemplified by Seattle Genetics' brentuximab vedotin (an ADC with monomethyl auristatin E) that was recently approved by the FDA for the treatment of Hodgkin's lymphoma and anaplastic large cell lymphoma.[41]Some of the compounds described herein possess traits that suggest potential as ADCs, including ready synthetic access, detailed knowledge of sites that can be modified without loss of activity, and significant potency across a broad panel of cancer cell types. Lack of hemolytic activity is an additional prerequisite for success in the ADC arena, as any hemolysis would be a non-starter for an intravenous drug. Thus the 25 derivatives listed in Table 2 were evaluated for their ability to induce hemolysis in human erythrocytes at concentrations well above their anticancer IC50 values.As shown in Figure S3[32], the compounds show no hemolytic activity. The concentrations at which hemolytic activity was assessed (1 and 10 μM) are, in some cases,over 1000-foldhigher than the IC50 values for cancer cells, indicating significant selectivity.

ETP alkaloids induce caspase-3 dependent apoptotic cell death

Two of the most active compounds from the monomer (**26** and **33**) and dimer (**5** and **14**) classes were further examined for their ability to induce caspase-dependent apoptotic pathway on U-937 cells. Apoptosis is a form of programmed cell death (type I) where cysteine proteases (caspases) are activated and cleave a host of cellular substrates, ultimately resulting in well-defined morphological changes in the cells leading to death. These changes include membrane blebbing, chromatin condensation, and externalization of phosphatidylserine in the plasma membrane.[42]

The induction of apoptosis was first evaluated by the level of phosphatidylserine externalization (detected by FITC-conjugated Annexin V (AnnV)) that occurs prior to the disruption of cell membrane integrity (detected by propidium iodide (PI)).[32] The progression

of cells through the AnnV+/PI- quadrant (lower right, Fig.3a) demonstrates the ability of both monomeric and dimeric ETP-containing derivatives to induce apoptosis.

Another marker of apoptosis is the cleavage patterns of various intracellular proteins. Caspase-3, one of the key apoptotic executioner caspases, is activated from its low-activity zymogen (procaspase-3) at an early stage of apoptosis. This activation can be visualized by Western blot (Fig. 3b) by the cleavage of procaspase-3 (35 kDa) to caspase-3 (12 and 17 kDa). Caspase-3 in turn cleaves one of its cellular substrates, PARP-1.

Treating cells with the four ETP derivatives, followed by Western blotting for procaspase-3/caspase-3 and PARP-1 reveals that all these compounds induce cleavage of procaspase-3 and PARP-1. Combined, the data in Fig.3 indicate that these ETP derivatives induce caspase-dependent apoptotic cell death rather than necrosis.

Conclusions

The implementation of a highly modular and generally applicable strategy for the synthesis of ETP-containing alkaloids has enabled the compilation of 60 derivatives of this class of natural products. This expansive collection of compounds was instrumental in the development of a comprehensive SAR profile. These verticillin- and chaetocin-related compounds were investigated for antitumor activity using five human cancer cell lines. Four sites were targeted for derivatization in this study:N1, C3, C17, and C11/C15 (Fig.4).

Human cancer cell lines were most responsive to variations in functionalization at the C3 and C11/C15 centers while displaying a more modest response to modification at the N1 and C17 positions. In particular, it was found that the compounds were highlypotent only if the diketopiperazines were sulfurated at theC11/C15 sites in a manner consistent with a species capable of being converted to a β-epidisulfide in the biological milieu. Furthermore, the anticancer potencies of this collection of compounds correlate positively with the steric environment at the C3 position, rendering the dimeric ETP alkaloids the most potent with (sub)nanomolar IC_{50}'s against a range of human cancer cell lines. The muted sensitivity of these cell lines to variations in N1 and C17 substitution make these ideal sites for compound optimization. Finally, despite their attenuated activity, the lower molecular-weight monomers may prove to have more optimal pharmacokinetic properties and provide further avenues for molecular modification in the optimization and development of small-molecule drugs.

Notwithstanding the considerable attention it has gained from biologists and chemists, the exact modes of action by which this class of compounds operates have yet to be fully determined. Three mechanistic pathways have been extensively discussed in the literature;[1a–c,f,5a]however, the role of these processes in toxicity is equivocal and many findings can be contradictory.[26b,43]The large collection of compounds described herein will enable a more thorough evaluation of the specific biological targets of the ETP-containing alkaloids, and novel synthetic dimer **14**, in particular, has remarkable potency. Importantly, these compounds are efficiently prepared through concise synthetic routes that affordsubstantial chemical and structural diversity. The implementation of this synthetic discovery platform provides a unique opportunity to study important biological and physiological processes, to validate biological hypotheses, and to discover very promising small-molecules.[44] Additionally, the ETPs would be excellent candidates for ADC therapies,[41] a strategy which requires highly potent compounds and selectively delivers drug to a cellular target.

Cell culture MTS and SRB cytotoxicity assays reveal potent and wide-ranging anticancer activity for 25 natural and unnatural ETP derivatives in the (sub)nanomolar range against a broad variety of cancer types and demonstrate a broad structure–activity relationship:eight sarcosine-derived monomers and five homodimers exhibited potency in the single digit nanomolar range, while chaetocins A (**4**) and C (**5**) as well as verticillin-related compound **14**displayed subnanomolar IC_{50} values. In addition to their activity against cancer cells, the ETPs are not active against human erythrocytes.These compounds are attractive candidates for further studies investigating mode ofaction and exploring translational potential.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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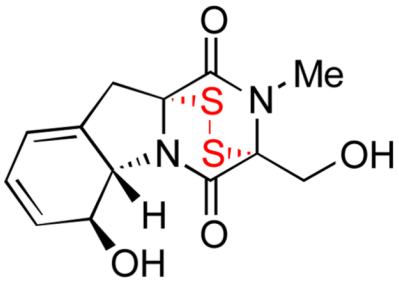
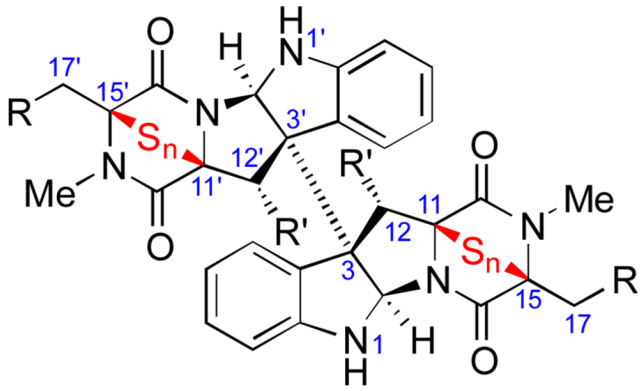
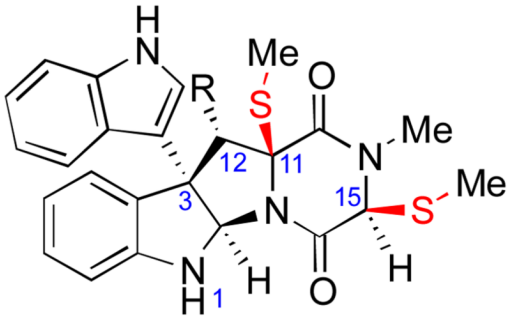


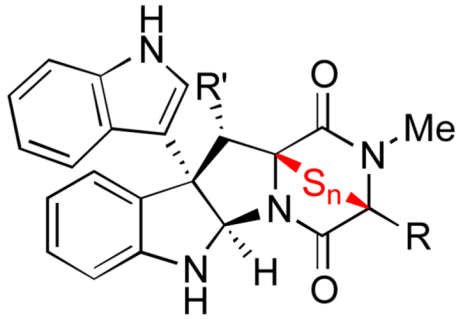
Gliotoxin (**1**)



R=H, R'=OH, n=2 Verticillin A (**2**)
R=H, R'=H, n=2 12,12'-Dideoxyverticillin A (**3**)
R=OH, R'=H, n=2 Chaetocin A (**4**)
R=OH, R'=H, n=3 Chaetocin C (**5**)
R=OH, R'=H, n=4 12,12'-Dideoxychetracin A (**6**)



R=H Gliocladin B (**7**)
R=OH Bionectin C (**8**)



R=H, R'=OH, n=2 Bionectin A (**9**)
R=H, R'=H, n=2 12-Deoxybionectin A (**10**)
R=*i*-Pr, R'=OH, n=3 Leptosin E (**11**)
R=Me, R'=H, n=2 Glioclatine (**12**)
R=Me, R'=OH, n=4 Gliocladine E (**13**)

Fig. 1.
Representative thiodiketopiperazines.

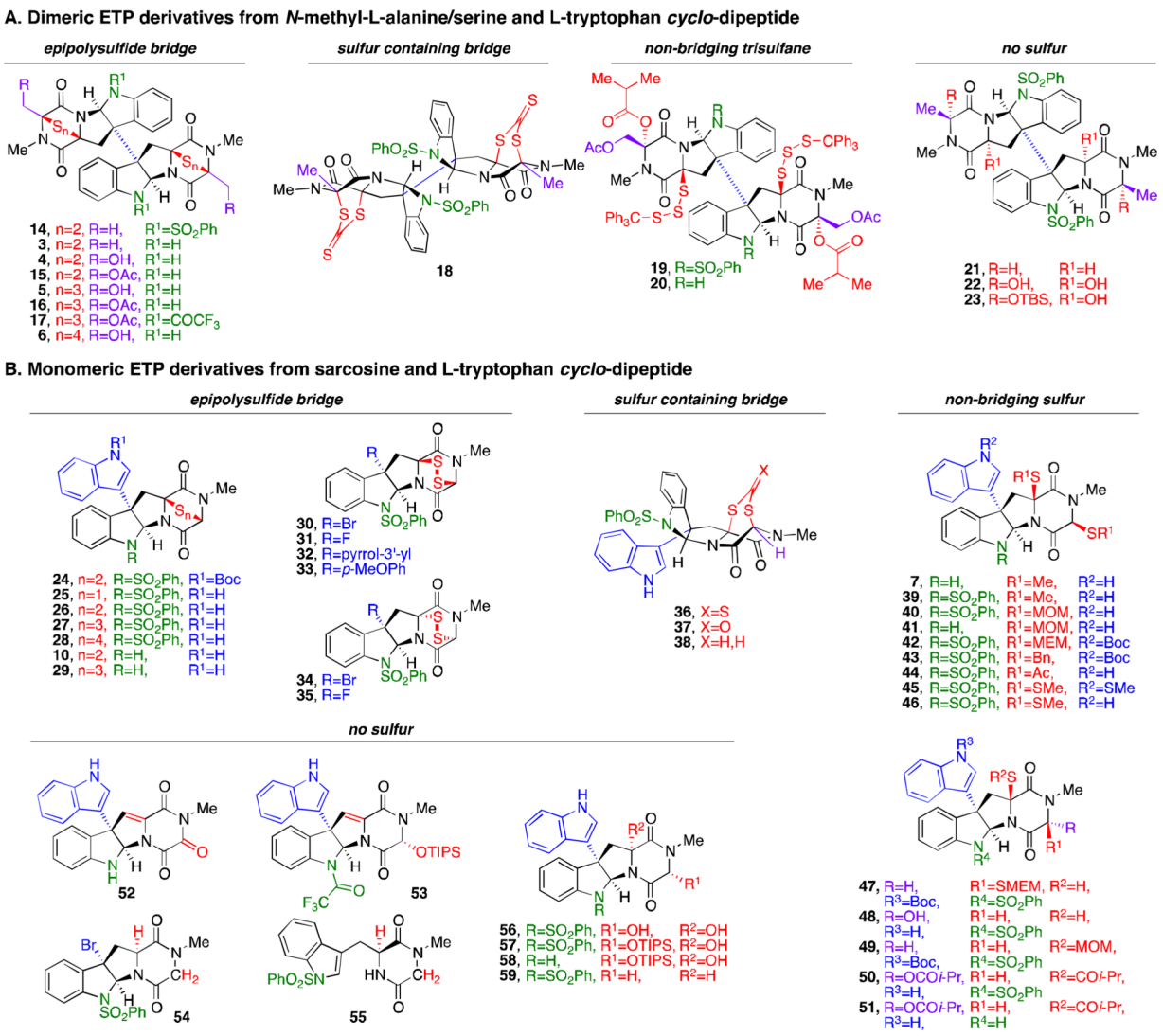


Fig 2.

Molecules synthesized and evaluated for anticancer activity in this study.

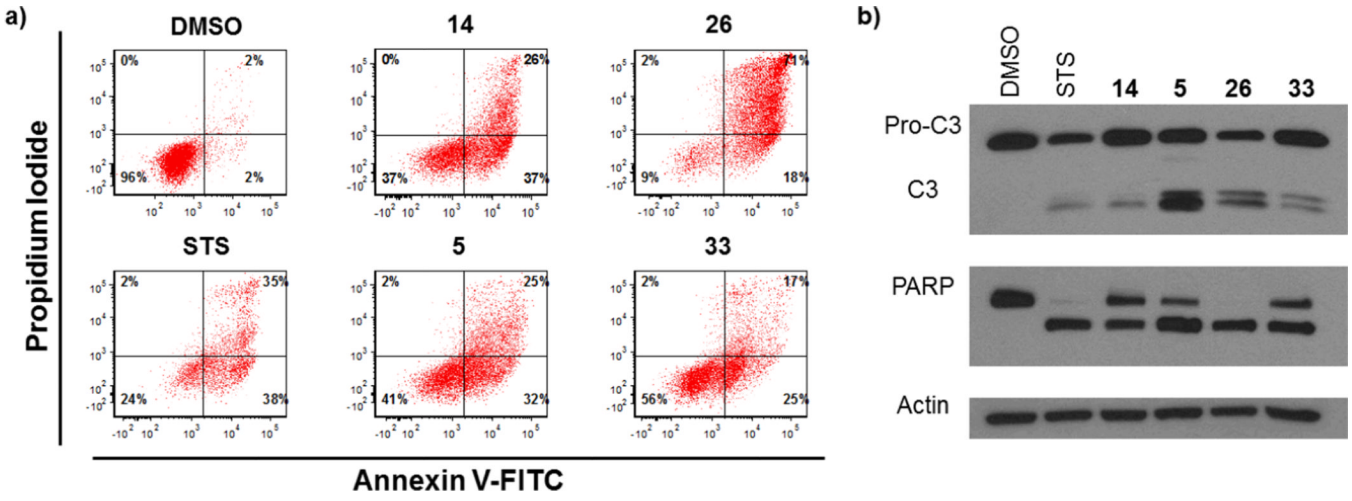


Fig. 3.

ETP derivatives induce caspase-dependent apoptotic cell death.[32] a) Analysis of phosphatidylserine exposure and propidium iodide inclusion at 24 hours in U-937 cells. Compounds were tested at 100-times their 72-hour IC_{50} values [**14** (20 nM), **5** (75 nM), **26** (250 nM), and **33**(500 nM)]. STSwas used at 50 nM as a positive control for apoptosis. b) Western blot analysis of Pro-C3 and PARP-1 cleavage at 24 hours in U-937 cells using β-actin as loading control. Compounds were tested as above, with the exception of STS (100 nM); C3 = caspase-3; ETP = epipolythiodiketopiperazine; FITC = fluorescein isothiocyanate; IC_{50} = half maximal inhibitory concentration;PARP = poly(ADP-ribose) polymerase 1; Pro-C3 = procaspase-3; STS = staurosporine.

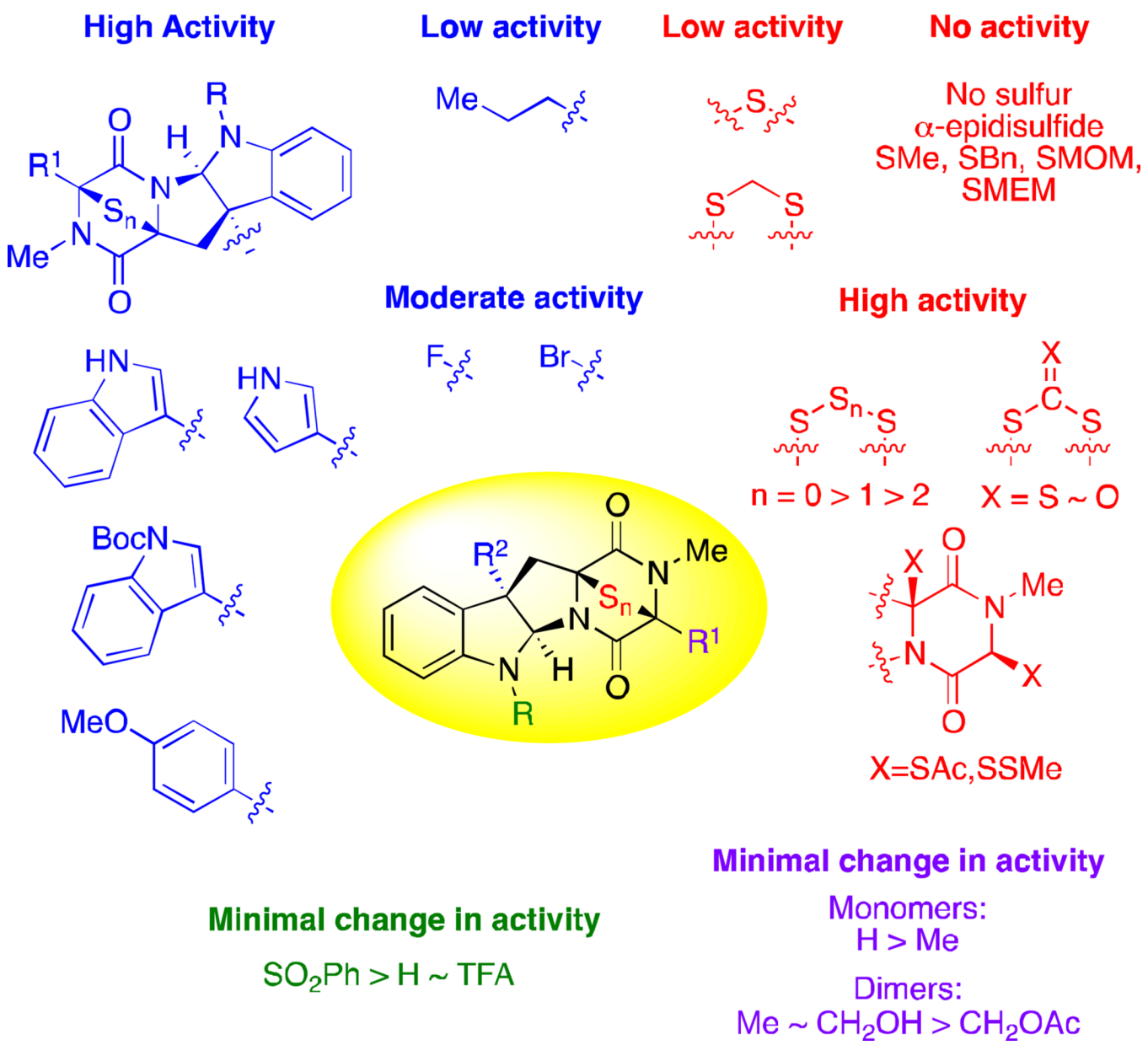
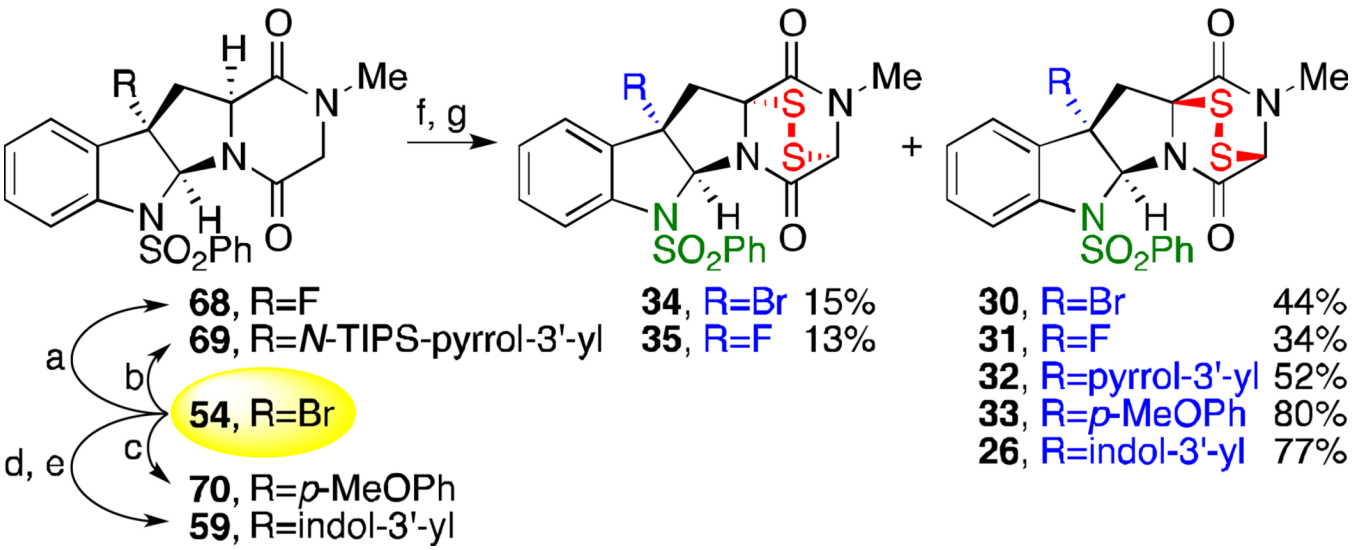
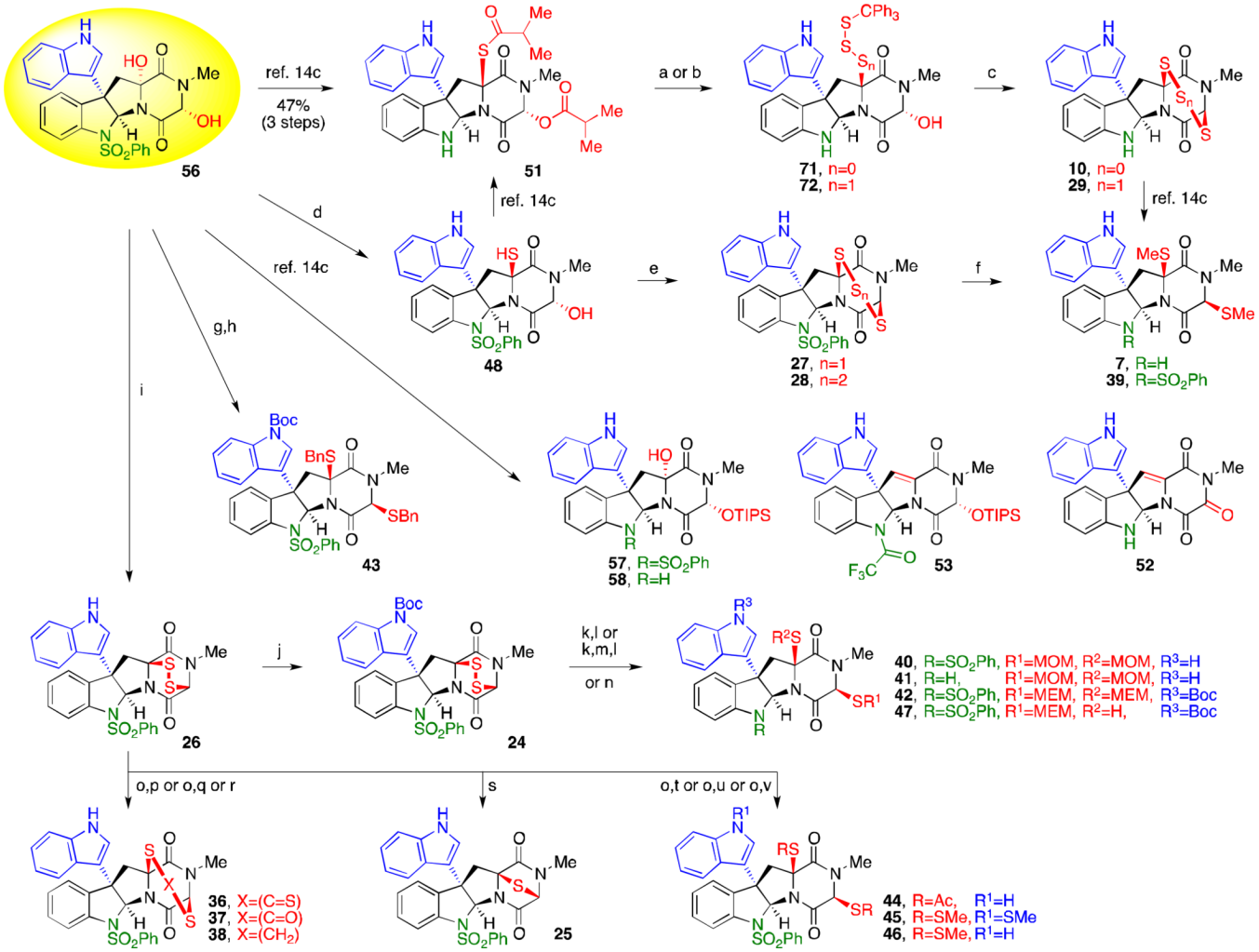


Fig. 4.
Summary of ETP structure–activity relationship.



Scheme 1.

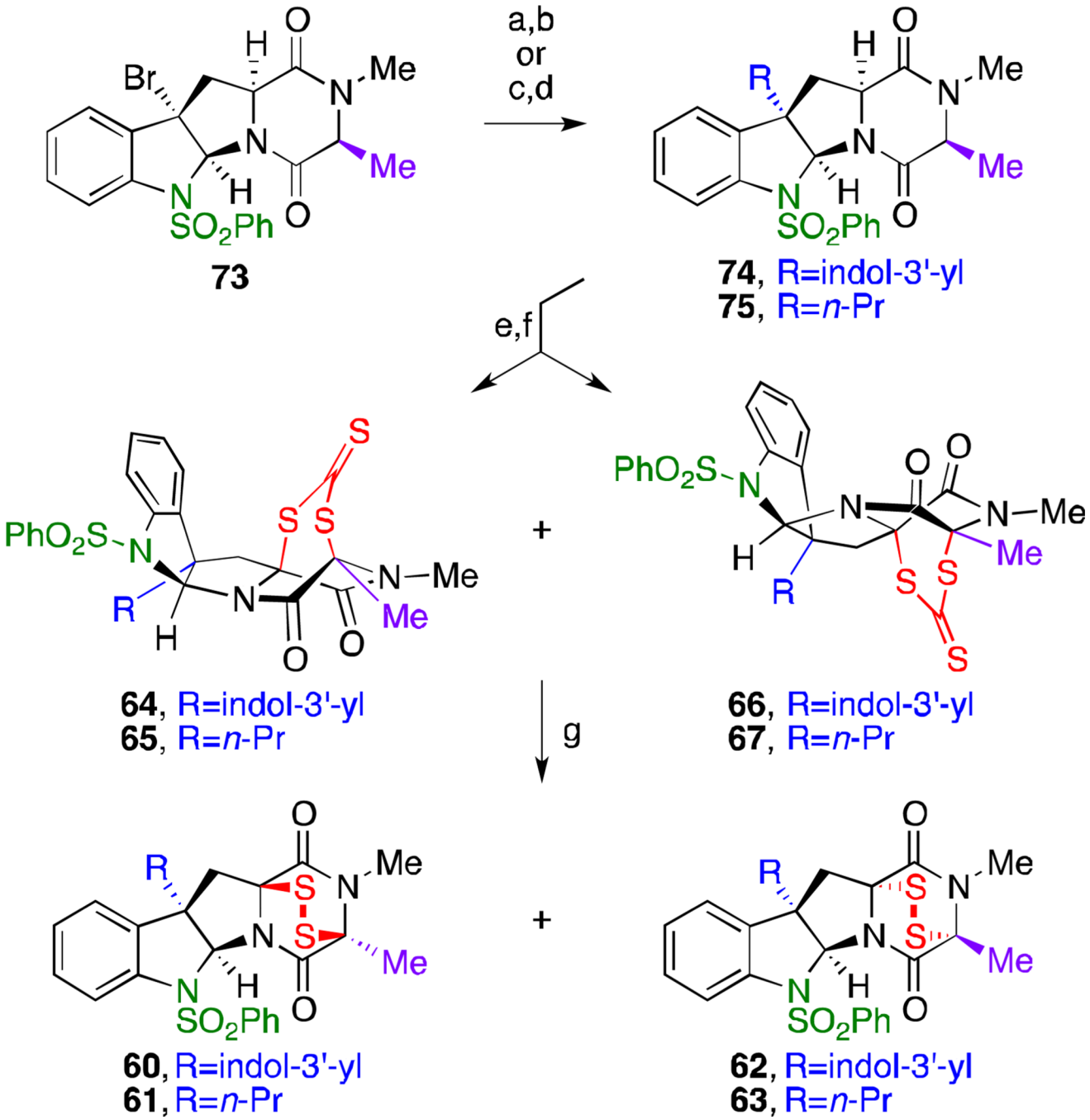
Sarcosine-derived monomeric epidithiodiketopiperazines: modulation of the C3-substituent. Reagents and conditions: (a) $AgBF_4$, DTBMP, $EtNO_2$, 23 °C, 1 h, 90%; (b) *N*-TIPS-pyrrole, $AgBF_4$, DTBMP, $EtNO_2$, 0 °C, 1 h, 72%; (c) Anisole, $AgBF_4$, DTBMP, $EtNO_2$, 0 °C, 1 h, 99%; (d) 5-Br-*N*-TIPS-indole, $AgBF_4$, DTBMP, $EtNO_2$, 0 °C, 1 h; 83%; (e) H_2, Pd/C, NEt_3, MeOH–EtOAc (2:3 v/v), 23 °C, 8 h; $Et_3N{\bullet}3HF$, 23 °C, 13 h, quant.; (f) *n*-Bu_4NMnO_4, CH_2Cl_2, 23 °C, 2 h, 25–52%; (g) H_2S, TFA–$EtNO_2$ (2:3 v/v), 0 to 23 °C, 4 h; O_2, EtOAc, 23 °C, 47–80%; TIPS = triisopropylsilyl; DTBMP = 2,6-di-*tert*-butyl-4-methylpyridine; TFA = trifluoroacetic acid.



Scheme 2.

Sarcosine-derived monomeric polythiodiketopiperazines: modulation of the sulfur bridge. Reagents and conditions: (a) N_2H_4, THF, 0 °C, 1 h; Ph_3CSCl, NEt_3, THF, 0 °C, 90 min, 81% (2-steps); (b) N_2H_4, THF, 0 °C, 1 h; Ph_3CSSCl, Hünig's base, THF, 0 °C, 25 min; (c) $Hf(OTf)_4$, MeCN, 23 °C, 15 min, n=0: 80%, n=1: 47% (3-steps); (d) H_2S, TFA–CH_2Cl_2 (1:9 v/v), 0 to 23 °C, 2 h, 90%, >10:1 *dr*; (e) Ph_3CSS_mCl, Hünig's base, THF, 0 °C, 25 min; $Hf(OTf)_4$, MeCN, 23 °C, 50 min, m=1: 42% (2-steps), m=2: 44% (2-steps); (f) MeI, $NaBH_4$, Pyr, THF, MeOH, 23 °C, 45 min, 80%; (g) BnSH, TFA–$EtNO_2$ (2:3 v/v), 23 °C, 3 h, 80%, 17:3*dr*; (h) Boc_2O, DMAP, MeCN, 23 °C, 3 h, 69%; (i) H_2S, TFA–$EtNO_2$ (3:4 v/v), 0 to 23 °C, 2 h; O_2, EtOAc, 23 °C, 77%, >7:1 *dr*; (j) Boc_2O, DMAP, CH_2Cl_2, 23 °C, 7 h, 81%; (k) $NaBH_4$, THF, MeOH, 23 °C, 2 h; MOMCl, NEt_3, 23 °C, 5 h, 73%; (l) TFA, CH_2Cl_2, 0 to 23 °C, 3 h, 81–91%; (m) hν (350 nm), 1,4-dimethoxynaphthalene, ascorbic acid, sodium ascorbate, H_2O–MeCN (1:4 v/v), 25 °C, 2.5 h, 82%; (n) $NaBH_4$, THF, MeOH, 23 °C, 80 min; MEMCl, NEt_3, 23 °C, 12 h, 80% (**42**) and 19% (**47**); (o) $NaBH_4$, THF, MeOH, 23 °C, 45 min; (p) TCDI, CH_2Cl_2, 23 °C, 22 h, 34% (2-steps); (q) CDI, CH_2Cl_2, 23 °C, 24 h, 8% (2-steps); (r) CH_2I_2, $NaBH_4$, Pyr, THF, MeOH, 0 to 23 °C, 1 h, 46%; (s) $P(OEt)_3$, THF, 23 °C, 6 h, 63%; (t) AcCl, Pyr, CH_2Cl_2, 23 °C, 4 h, 63% (2-steps); (u) MeSCl, Pyr, CH_2Cl_2, 0 to 23 °C, 2 h, 49% (2-steps); (v) $(MeS)_2$, THF, 23 °C, 19 h, 41% (2-steps); TFA = trifluoroacetic acid; Pyr = pyridine; Boc_2O = di-*tert*-butyl dicarbonate; DMAP = 4-(dimethylamino)pyridine; TCDI =1,1'-thiocarbonyldiimidazole; CDI =1,1'-

carbonyldiimidazole;MOMCl = chloromethyl methyl ether; MEMCl = 2-methoxyethoxymethyl chloride.



Scheme 3.

Alanine-derived monomeric polythiodiketopiperazines: modu-lation of the C3-substituent. Reagents and conditions: (a) 5-Br-*N*-TIPS-indole, $AgBF_4$, DTBMP, $EtNO_2$, 0 °C, 1 h, 67%; (b) H_2, Pd/C, NEt_3, MeOH–EtOAc (2:3 v/v), 23 °C, 8 h; $Et_3N{\bullet}3HF$, 23 °C, 13 h, quant.; (c) $AllylSnBu_3$, AIBN, PhH, 80 °C, 5 h, 61%; (d) H_2, Pd/C (5 mol %), CH_2Cl_2, 2 h, 100%; (e) Pyr_2AgMnO_4, Pyr, 23 °C, 4 h, 37% (R=indol-3'-yl), 68% (R=*n*-Pr); (f) K_2CS_3, TFA–CH_2Cl_2 (1:2 v/v), 23 °C, 2.5 h, 63%, 5:1 *dr* (**64:66**), 52% (**65**), 15% (**67**); (g) ethanolamine, acetone, 23°C, 45 min; KI_3, Pyr, CH_2Cl_2, 23°C, 48%, 5:1 *dr* (**60:62**), 70% (**61**), 78% (**63**); TIPS = triisopropyl-silyl;DTBMP = 2,6-di-*tert*-butyl-4-methyl-pyridine; AIBN = α,α′-

azoisobutyronitrile;Pyr_2AgMnO_4 = bis(pyridine) silver(I) permanganate; Pyr = pyridine; TFA = trifluoroacetic acid.

Table 1

Assessment of the 60 ETPs and DKPs from Fig. 2 for anticancer activity against U-937 (hystiocytic lymphoma) and HeLa (cervical carcinoma) human cancer cell lines after a 72-hour exposure.[a]

Cmpd	U-937	HeLa
Dimers with epipolysulfide bridges		
3	15.5 ± 2.9	7.2 ± 3.0
4	0.81 ± 0.15	6.9 ± 2.0
5	0.75 ± 0.13	6.3 ± 0.6
6	1.3 ± 0.5	5.6 ± 1.0
14	0.18 ± 0.06	0.09 ± 0.06
15	4.7 ± 1.3	14.1 ± 7.4
16	9.7 ± 2.1	70 ± 20
17	8.9 ± 2.3	28.1 ± 2.2
18	2.9 ± 2.1	1.1 ± 0.8
Sulfur-containing dimers		
19	>10,000	>10,000
20	>10,000	>10,000
Dimers without sulfur		
21	>1,000	>10,000
22	>1,000	>10,000
23	>1,000	>10,000
Sarcosine-derived monomers with sulfur-containing bridge		
10	17.4 ± 1.1	117 ± 26
24	5.9 ± 1.4	75 ± 10
25	710 ± 134	1476 ± 182
26	2.8 ± 0.3	41.7 ± 5.7
27	36.6 ± 2.4	27.5 ± 9.2
Sarcosine-derived monomers with sulfur-containing bridge (cont.)		
28	8.3 ± 3.3	137 ± 86
29	24.5 ± 7.4	123 ± 29
30	5.0 ± 1.3	44.9 ± 1.3
31	20.7 ± 6.4	1530 ± 440
32	14.8 ± 3.1	26.8 ± 4.8
33	5.0 ± 2.1	22.2 ± 10.9
34	59 ± 23	550 ± 33
35	21.3 ± 8.7	56 ± 21
36	3.8 ± 0.9	33 ± 13
37	5.5 ± 1.3	37 ± 15
38	82 ± 28	465 ± 37
Sarcosine-derived monomers with non-bridging sulfur		
7	>10,000	>10,000
39	>10,000	>10,000
40	>1,000	>1,000
41	>10,000	>10,000
42	>10,000	>10,000
43	>10,000	>10,000
44	4.1 ± 0.5	17.3 ± 4.5
45	14.5 ± 9.1	13.7 ± 2.3
46	20.4 ± 9.0	36.6 ± 3.9
Sarcosine-derived monomers without sulfur		
47	>10,000	>10,000
48	>10,000	>10,000
49	>10,000	>10,000
50	>1,000	>1,000
51	>10,000	>1,000
52	>10,000	>10,000
53	>10,000	>10,000
54	>10,000	>10,000
55	>10,000	>10,000
56	>10,000	>10,000
57	>10,000	>10,000
58	>10,000	>10,000
59	>10,000	>10,000
***N*-Methylalanine-derived monomers with sulfur-containing bridge**		
60	56 ± 23	67 ± 11
61	>1,000	>1,000
62	>1,000	>1,000
63	>1,000	>1,000
64	24 ± 12	25.3 ± 2.0
65	>1,000	>1,000
66	>1,000	>1,000
67	>1,000	>1,000

≤1 nM	1<x≤10 nM	10<x≤50 nM	50<x≤150 nM	150<x≤1,000 nM	>1,000 nM

[a] 72-hour IC_{50} values (in nM) as determined by MTS (U-937) and SRB (HeLa).[32] Error is standard deviation of the mean, n ≥ 3. Cmpd = compound DKP = diketopiperazine ETP = epipolythiodiketopiperazine IC_{50} = half maximal inhibitory concentration MTS = 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*–tetrazolium SRB = sulforhodamine B.

Table 2

Assessment of the top 25 compounds for cytotoxicity in five human cell lines {U-937 (hystiocytic lymphoma), HeLa (cervical carcinoma), H460 (lung carcinoma), 786-O (renal carcinoma), and MCF-7 (breast carcinoma)} after a 72-hour exposure.[a]

Cmpd	U-937	HeLa	H460	786-O	MCF-7
Dimers with epipolysulfide bridges					
3	15.5 ± 2.9	7.2 ± 3.0	42 ± 15	33.5 ± 12.6	28.4 ± 4.5
4	0.81 ± 0.15	6.9 ± 2.0	53 ± 23	26.5 ± 7.7	50 ± 16
5	0.75 ± 0.13	6.3 ± 0.6	26.0 ± 9.3	14.6 ± 0.8	27 ± 11
6	1.3 ± 0.5	5.6 ± 1.0	39.3 ± 41	16.0 ± 2.4	22.5 ± 6.0
14	0.18 ± 0.06	0.09 ± 0.06	1.53 ± 0.85	1.55 ± 0.77	1.65 ± 0.51
15	4.7 ± 1.3	14.1 ± 7.4	140 ± 82	95 ± 23	77 ± 36
16	9.7 ± 2.1	70 ± 20	187 ± 79	204 ± 40	263 ± 98
17	8.9 ± 2.3	28.1 ± 2.2	179 ± 88	189 ± 26	>333
18	2.9 ± 2.1	1.1 ± 0.8	18.5 ± 2.7	14.9 ± 4.4	10.7 ± 8.1
Sarcosine-derived monomers with sulfur-containing bridge					
10	17.4 ± 1.1	117 ± 26	215 ± 100	181 ± 26	156 ± 18
26	2.8 ± 0.3	41.7 ± 5.7	66 ± 19	62.7 ± 7.6	67 ± 19
27	36.6 ± 2.4	27.5 ± 9.2	146.4 ± 6.9	145 ± 10	151 ± 31
28	8.3 ± 3.3	137 ± 86	277 ± 43	344 ± 20	309 ± 31
29	24.5 ± 7.4	123 ± 29	348 ± 110	168 ± 34	217 ± 35
30	5.0 ± 1.3	44.9 ± 1.3	277 ± 44	162 ± 35	152 ± 42
31	20.7 ± 6.4	1530 ± 440	>1000	368 ± 78	218 ± 97
32	14.8 ± 3.1	26.8 ± 4.8	31.8 ± 2.5	66 ± 23	61 ± 11
33	5.0 ± 2.1	22.2 ± 10.9	46.7 ± 5.3	83 ± 18	63 ± 16
35	21.3 ± 8.7	56 ± 21	123 ± 50	64 ± 20	93 ± 34
36	3.8 ± 0.9	33 ± 13	190 ± 61	133 ± 10	163 ± 7
37	5.5 ± 1.3	37 ± 15	119 ± 25	139 ± 3	145 ± 18
Sarcosine-derived monomers with non-bridging sulfur					
44	4.1 ± 0.5	17.3 ± 4.5	149 ± 59	151 ± 7	103 ± 37
45	14.5 ± 9.1	13.7 ± 2.3	252 ± 37	376 ± 120	373 ± 75
***N*-Methylalanine-derived monomers with sulfur-containing bridge**					
60	56 ± 23	67 ± 11	165 ± 65	907 ± 48	327 ± 36
64	24 ± 12	25.3 ± 2.0	222 ± 72	266 ± 85	210 ± 73

≤1 nM	1<x≤10 nM	10<x≤50 nM	50<x≤150 nM	>150 nM

[a] 72-hour IC_{50} values (in nM) as determined by MTS (U-937) and SRB (HeLa, H460, 786-O, and MCF-7).[32] Error is standard deviation of the mean, n ≥ 3 Cmpd = compound IC_{50} = half maximal inhibitory concentration MTS = 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*–tetrazolium SRB = sulforhodamine B.