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RESEARCH ARTICLE

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Solid-Phase Synthesis of Smac Peptidomimetics Incorporating Triazoloprolines and Biarylalanines

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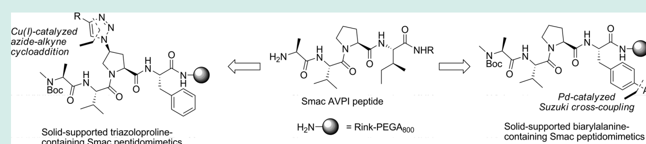
S Supporting Information

ABSTRACT: Apoptotic induction mechanisms are of crucial importance for the general homeostasis of multicellular organisms. In cancer the apoptotic pathways are downregulated, which, at least partly, is due to an abundance of inhibitors of apoptosis proteins (IAPs) that block the apoptotic cascade by deactivating proteolytic caspases. The Smac protein has an antagonistic effect on IAPs, thus providing structural clues for the synthesis of new pro-apoptotic compounds. Herein, we report a solid-phase approach for the synthesis of Smac-derived tetrapeptide libraries. On the basis of a common (*N*-Me)AVPF sequence, peptides incorporating triazoloprolines and biarylalanines were synthesized by means of Cu(I)-catalyzed azide−alkyne cycloaddition and Pd-catalyzed Suzuki cross-coupling reactions. Solid-phase procedures were optimized to high efficiency, thus accessing all products in excellent crude purities and yields (both typically above 90%). The peptides were subjected to biological evaluation in a live/dead cellular assay which revealed that structural decorations on the AVPF sequence indeed are highly important for cytotoxicity toward HeLa cells.



KEYWORDS: solid-phase synthesis, IAP inhibition, Smac, peptidomimetics, Cu(I)-catalyzed azide−alkyne cycloaddition, Suzuki cross-coupling

INTRODUCTION

The apoptotic circuitry ensures the general homeostasis of multicellular organisms via control of cellular proliferation.[1,2] This circuitry is subject to tight regulation through numerous cellular signaling cascades and feed-back mechanisms, where cysteine-aspartic acid proteases (caspases) play a predominant role. In short, two main pathways for controlled cell destruction are generally accepted: an intrinsic (mitochondria-mediated) which typically acts as response to intracellular damage; and an extrinsic which involves the membrane-bound death-receptor ligands and responds to extracellular stimuli such as chemotherapeutic agents.[1] Both pathways converge at the level of activation of caspases 3 and 7 and ultimately lead to cell death.[3] A variety of caspases are implicated in these cellular cascades where they function as degraders of the cellular proteome. One of the hallmarks of cancer is the evasion of natural apoptotic signals, which enable malignant cells to proliferate, even when subjected to radio/chemotherapies, that heavily rely on the cells to engage in their own cellular destruction.[4] This resistance to chemotherapeutic agents is partly mediated by the inhibitors of apoptosis proteins (IAPs) which suppress the activity of caspases through various mechanisms.[2,4−8] IAPs are a group of proteins currently comprising 8 members (XIAP, cIAP-1 and -2, ML-IAP, NAIP, ILP-2, survivin, and Apollon) which are characterized by one or more baculoviral IAP repeats (BIR) but can also incorporate other important domains such as the CARD or RING domains.[9] The mechanism of action of IAPs is still subject to investigation,

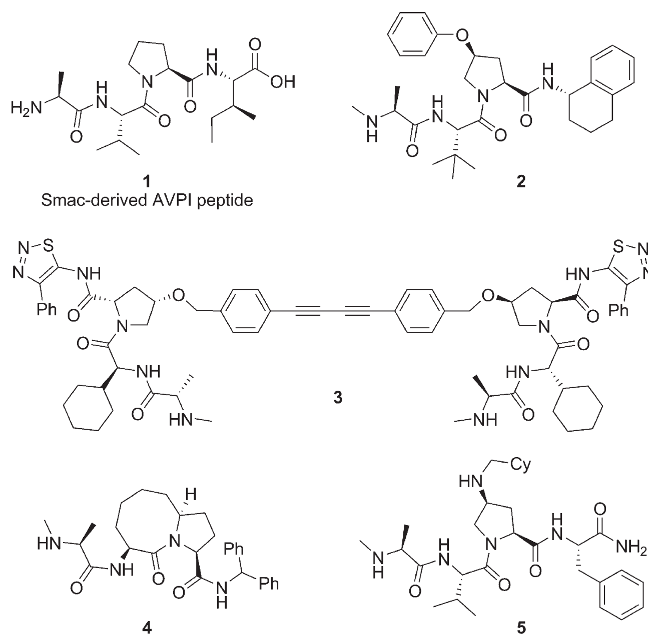


Figure 1. Smac-derived AVPI peptide and selected examples of Smac mimetics.

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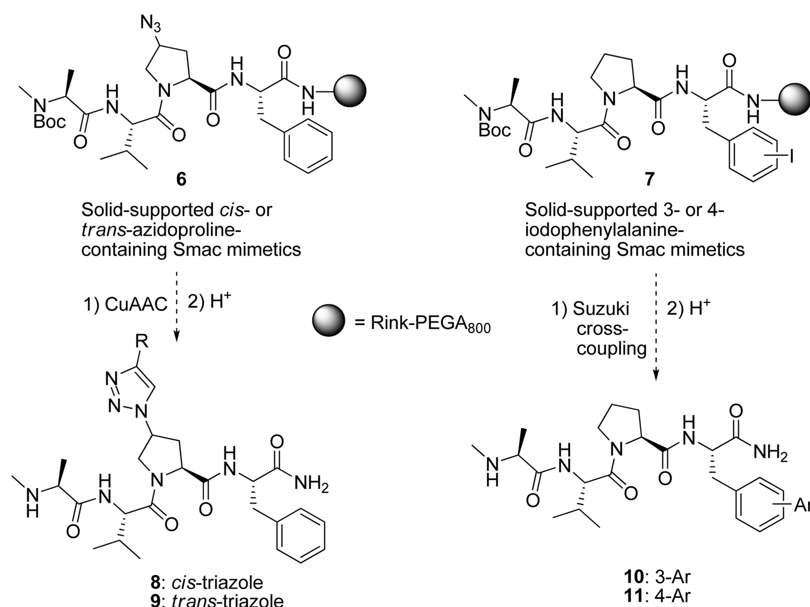
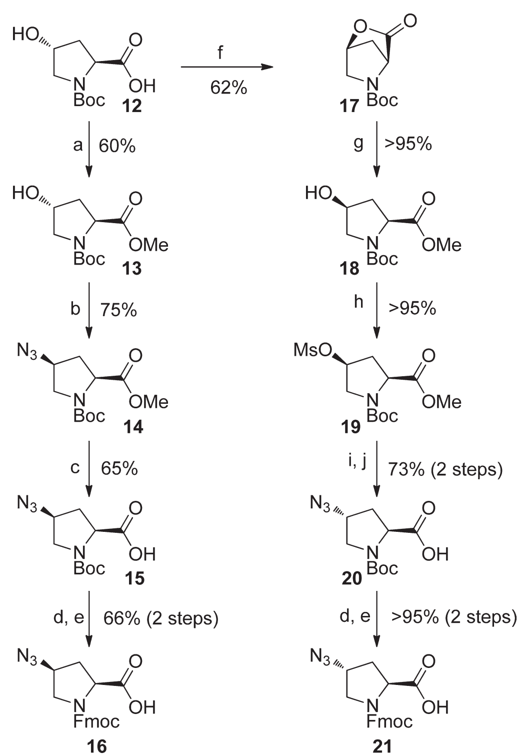


Figure 2. Synthetic strategy for the construction of triazoloproline- and biarylalanine-containing libraries **8**−**11**.

but it is generally accepted that XIAP plays a predominant role in the intrinsic pathway by inhibition of caspase-9 and the downstream executioner caspases 3 and 7.[6] cIAP-1 and -2, on the other hand, have been shown to modulate a range of mechanisms, such as E3 ubiquitin ligase activity.[4,10,11] cIAPs are thus implicated in the death-receptor pathway, but might also function through binding and sequestering of the second mitochondria-derived activator of caspase (Smac) protein,[12,13] which is an endogenous inhibitor of XIAP, and thereby prevent Smac's pro-apoptotic activity.[14]

In the past decade, small molecule antagonistic modulation of IAPs aiming at the re-establishment of normal apoptotic levels has emerged as a promising anticancer strategy.[9,14−16] The discovery of the mode of action of Smac,[17−19] has provided an entry point for synthetic endeavors in the hunt for such molecules.[20,21] Smac is released in the cytosol upon permeabilization of the mitochondrial membrane and antagonizes IAP function by virtue of its *N*-4 terminal (Smac-derived AVPI peptide **1**, Figure 1), both as a monomer and dimer.[9] A multitude of highly potent Smac mimetics, mimicking the action of the *N*-4 terminal residues of the Smac protein have been reported, and at least five IAP-antagonists are currently undergoing clinical trials.[14] The molecular scaffolds typically rely on structural modifications of the AVPI Smac peptide but novel scaffolds have also been reported.[22−28] SAR studies and investigations of the binding groove of XIAP have led to the proposal of a general pharmacophore model of the binding interaction of Smac with XIAP.[15] This model notably suggests the introduction of specific proline substituents and replacement of isoleucine with larger hydrophobic groups to be important structural elements for fine-tuning the biological activity. Not surprisingly, a number of reported Smac peptidomimetics incorporate various types of aromatic functionalities at these positions (**2** and **3**, Figure 1).[14,29] Other investigations, aiming at improved bioavailability through enhanced cell permeability, have focused on the design of constrained peptidomimetics (**4**, Figure 1), where turn-mimicking bicyclic motifs have been incorporated in replacement of the proline residue.[30−35] Interestingly, a number of highly potent polymeric structures mimicking dimeric Smac,

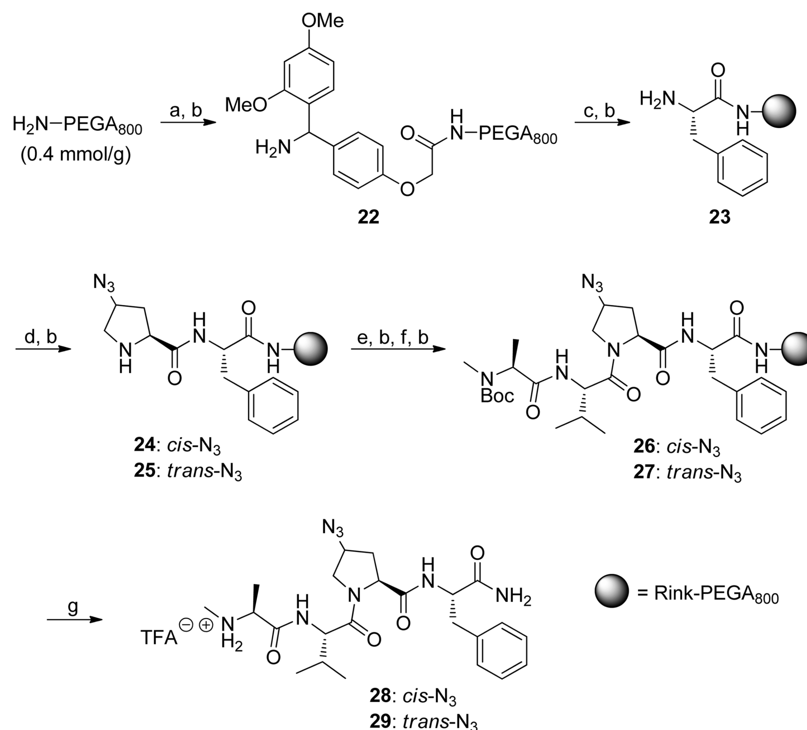
Scheme 1. Synthesis of Fmoc-Protected Azidoproline Building Blocks 16 and 21[a]



[a] Reagents and conditions: (a) Cs_2CO_3, MeI, DMF; (b) DPPA, DEAD, PPh_3, THF; (c) 1 M NaOH (aq), MeOH; (d) 50% TFA (CH_2Cl_2), 0 °C; (e) FmocCl, 10% Na_2CO_3 (aq), MeCN, −20 °C; (f) DIAD, PPh_3, THF, 0 °C; (g) NaN_3, MeOH, 40 °C; (h) MsCl, Et_3N, CH_2Cl_2, 0 °C; (i) NaN_3, DMSO, 80 °C; (j) LiOH (aq), THF.

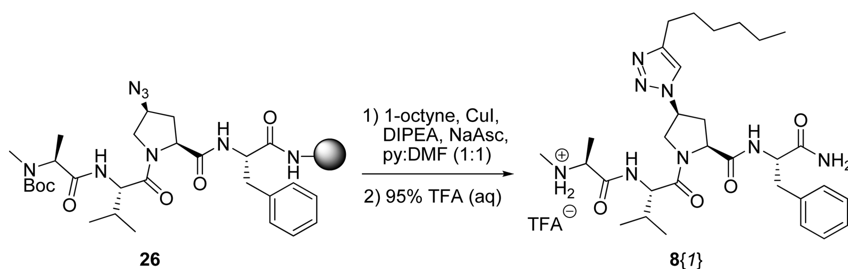
thought to simultaneously bind several IAP BIR-domains, have also been reported.[14,29,36] A number of such bi- or trivalent compounds are connected through aliphatic or aromatic bridging

Scheme 2. Solid-Phase Synthesis of Solid-Supported Azidopeptides 26 and 27[a]



[a] Reagents and conditions: (a) Fmoc-Rink-OH, TBTU, NEM, DMF; (b) 20% Piperidine (DMF), (c) Fmoc-Phe-OH, TBTU, NEM, DMF; (d) **16** or **21**, TBTU, NEM, DMF; (e) Fmoc-Val-OH, TBTU, NEM, DMF; (f) Boc-(*N*-Me)Ala-OH, TBTU, NEM, DMF; (g) 95% TFA (aq).

Table 1. Optimization of Reaction Parameters for the CuAAC Reaction



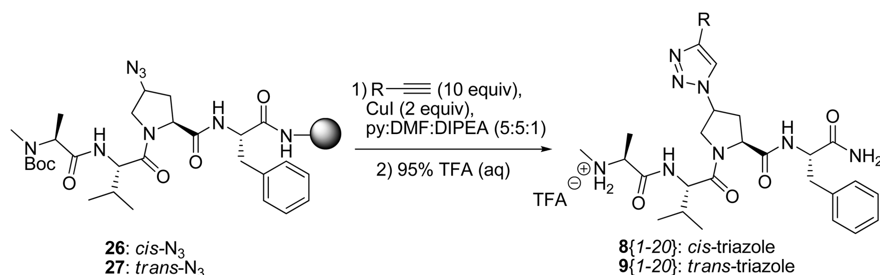
entry	1-octyne (equiv)	CuI (equiv)	DIPEA (equiv)	NaAsc (equiv)	time (h)	purity of **8**{*1*}[a] (%)
1	40	2	50	2	20	0
2	40	2	50	0	20	89
3	40	2	0	0	20	52
4	20	5	50	0	20	71
5	2	5	50	0	48	0
6	10	2	100	0	48	>95

[a] As indicated by RP-HPLC of the crude product (215 nm).

moieties installed on the pyrrolidine ring of the proline residue (**3**, Figure 1).[14]

We have previously transferred the synthesis of a potent Smac peptidomimetic incorporating an alkyl-substitution at the 3-position of the proline residue (**5**, Figure 1) to the solid phase.[37] Along these lines, we herein wish to present a further extension of the strategy to the construction of solid-supported libraries of Smac mimetics, based on a common (*N*-Me)AVPF motif, displaying diverse substitution patterns at the proline and phenylalanine positions. Specifically, we have taken advantage of the Cu(I)-catalyzed azide−alkyne cycloaddition (CuAAC) and the Suzuki cross-coupling reactions to introduce aromatic substituents in the parent scaffold in the form of 3-substituted triazoloprolines and biarylalanines, respectively (Figure 2).

Table 2. Synthesis of Triazoloproline Libraries 8 and 9



entry	R	product, purity (%)[a]
1	hexyl-	8{1}, >95; 9{1}, >95
2	propyl-	8{2}, >95; 9{2}, >95
3	butyl-	8{3}, >95; 9{3}, >95
4	pentyl-	8{4}, >95; 9{4}, >95
5	cyclopropyl-	8{5}, >95; 9{5}, >95
6	cyclopentyl-	8{6}, >95; 9{6}, >95
7	cyclohexyl-	8{7}, >95; 9{7}, >95
8	cyclohexylmethyl-	8{8}, 93; 9{8}, 93
9	phenylthiomethyl-	8{9}, 94; 9{9}, 94
10	phenoxymethyl-	8{10}, 93; 9{10}, 93
11	benzoyloxymethyl-	8{11}, 94; 9{11}, 94
12	phenyl-	8{12}, 93; 9{12}, 93
13	4-phenoxyphenyl-	8{13}, 94; 9{13}, >95
14	3,5-dimethoxyphenyl-	8{14}, >95; 9{14}, >95
15	3,5-difluorophenyl-	8{15}, >95; 9{15}, >95
16	3,4-dichlorophenyl-	8{16}, >95; 9{16}, >95
17	6-methoxynaphtyl-	8{17}, >95; 9{17}, >95
18	2-pyridyl-	8{18}, >95; 9{18}, >95
19	3-thienyl-	8{19}, >95; 9{19}, >95
20	2-carboxyethyl-	8{20}, >95[b]; 9{20}, 71[c]

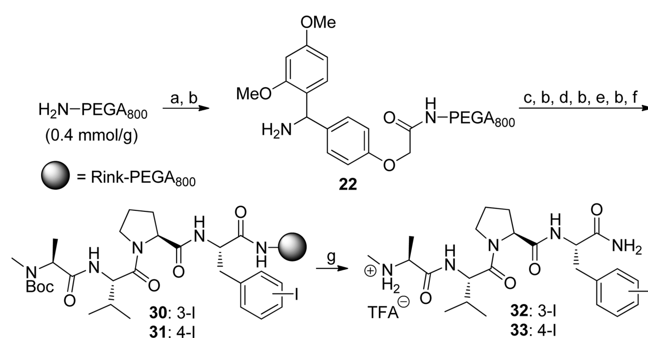
[a] As indicated by RP-HPLC of the crude product (215 nm). [b] Repetitive CuAAC reactions needed for full conversion. [c] Repetitive CuAAC reactions failed to give full conversion.

RESULTS AND DISCUSSION

Synthesis of Triazoloproline Libraries 8 and 9. Originally developed on solid support,[38] CuAAC has now become a widely used reaction in organic synthesis.[39−41] The scope of this robust and regioselective reaction keeps expanding, and 1,4-disubstituted-1,2,3-triazoles now find applications in many areas of organic chemistry, including the synthesis of peptidomimetics and oligonucleotides for biological investigation.[40] Bearing in mind the reported effect of introducing hydrophobic appendages in the vicinity of the pyrrolidine ring, we speculated if we could take advantage of the CuAAC reaction to access Smac mimetic compounds of types **8** and **9** (Figure 2).[42−45] Furthermore, we were interested in correlating the biological activity with the stereochemical orientation of the triazole substituent on the pyrrolidine ring. Along these lines, we envisioned the synthesis of two triazoloproline-containing libraries incorporating *cis*- and *trans*-triazoloproline residues, respectively.

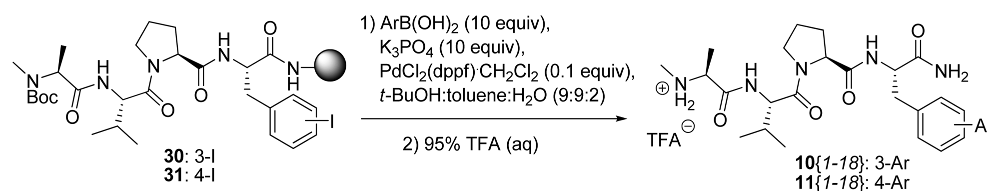
The required building blocks for solid-phase synthesis, Fmoc-*cis*-azidoproline **16** and Fmoc-*trans*-azidoproline **21**, were synthesized (Scheme 1) according to modified literature procedures.[46−50] Starting with Boc-protected *trans*-hydroxyproline **12**, the methyl ester of *cis*-azidoproline **14** was accessed via reaction with methyl iodide followed by direct azidation with DPPA under Mitsunobu

Scheme 3. Solid-Phase Synthesis of Solid-Supported Iodopeptides 30 and 31[a]



[a] Reagents and conditions: (a) Fmoc-Rink-OH, TBTU, NEM, DMF; (b) 20% Piperidine (DMF), (c) Fmoc-(3/4-I)Phe-OH, TBTU, NEM, DMF; (d) Fmoc-Pro-OH, TBTU, NEM, DMF; (e) Fmoc-Val-OH, TBTU, NEM, DMF; (f) Boc-(*N*-Me)Ala-OH, TBTU, NEM, DMF; (g) 95% TFA (aq).

Table 3. Synthesis of Biarylalanine Libraries 10 and 11



entry	Ar	product, purity(%)[a]
1	phenyl-	**10**{*1*}, >95; **11**{*1*}, >95
2	4-hydroxyphenyl-	**10**{*2*}, >95; **11**{*2*}, 94
3	4-methoxyphenyl-	**10**{*3*}, 91; **11**{*3*}, >95
4	4-fluorophenyl-	**10**{*4*}, >95; **11**{*4*}, >95
5	3-methoxyphenyl-	**10**{*5*}, >95; **11**{*5*}, >95
6	3-methylphenyl-	**10**{*6*}, >95; **11**{*6*}, >95
7	3-nitrophenyl-	**10**{*7*}, >95; **11**{*7*}, 90
8	2-methylphenyl-	**10**{*8*}, 94; **11**{*8*}, >95
9	2,4-dimethoxyphenyl-	**10**{*9*}, 91; **11**{*9*}, >95
10	2,6-dimethylphenyl-	**10**{*10*}, 82[b]; **11**{*10*}, 92[b]
11	3,4-dichlorophenyl-	**10**{*11*}, >95; **11**{*11*}, >95
12	3-thienyl-	**10**{*12*}, 88; **11**{*12*}, 83
13	2-benzothienyl-	**10**{*13*}, 90; **11**{*13*}, 90
14	4-pyridyl-	**10**{*14*}, 0[c]; **11**{*14*}, 0[c]
15	6-indolyl-	**10**{*15*}, 0[c]; **11**{*15*}, 0[c]
16	2-thienyl-	**10**{*16*}, 0[c]; **11**{*16*}, 0[c]
17	4-mercaptophenyl-	**10**{*17*}, 0[d]; **11**{*17*}, 0[d]
18	pentafluorophenyl-	**10**{*18*}, 0[d]; **11**{*18*}, 0[d]

[a] As indicated by RP-HPLC of crude product (215 nm). [b] The Suzuki cross-coupling was repeated once to achieve full conversion. [c] Complex reaction mixture. [d] No conversion of starting material.

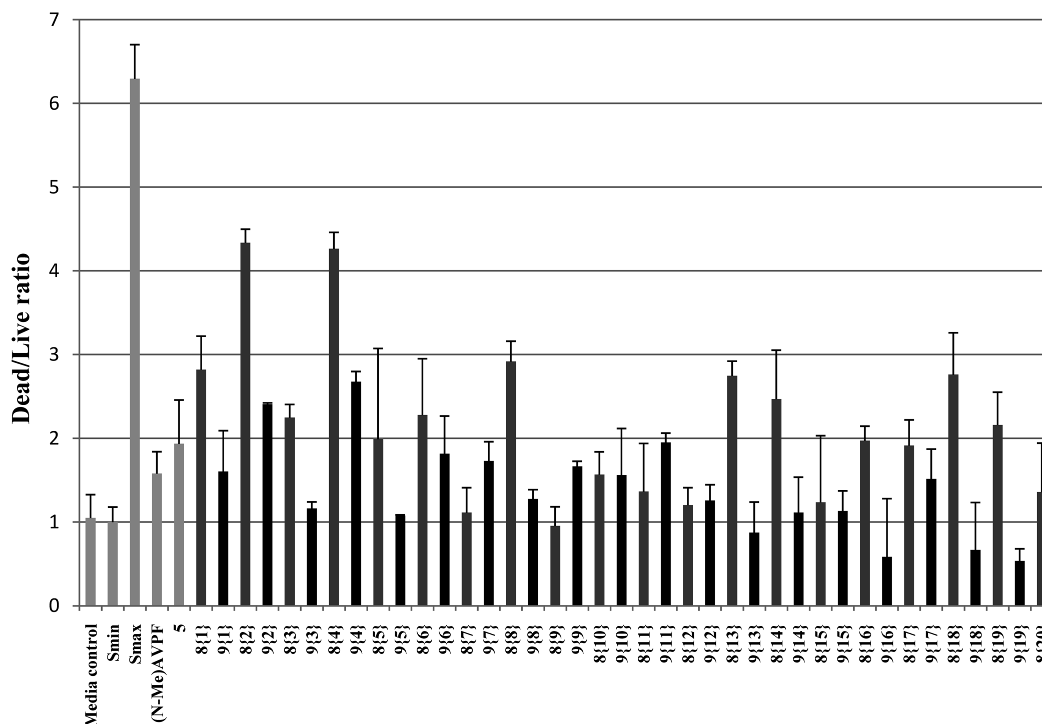
conditions. The ester was then subjected to alkaline hydrolysis to access Boc-protected azidoproline **15**. Acid-mediated Boc deprotection, immediately followed by reaction with FmocCl, yielded the desired Fmoc-*cis*-azidoproline **16** in 20% overall yield. Fmoc-*trans*-azidoproline was also synthesized from *trans*-hydroxyproline **12**. First, *trans*-hydroxyproline **12** was lactonized via intramolecular Mitsunobu esterification to yield bicyclic compound **17**. Then, the bicyclic lactone was ring-opened via treatment with sodium azide in MeOH, affording esterified *cis*-hydroxyproline **18**. This *cis*-hydroxyproline was then converted to Boc-protected *trans*-azidoproline **20** in a 3-step sequence, involving mesylation, S_N2-inversion with sodium azide in DMSO, and hydrolysis with aqueous LiOH. Final conversion of the Boc protecting group to the Fmoc protecting group afforded the desired Fmoc-*trans*-azidoproline **21** in 43% overall yield.

Buildings blocks **16** and **21** were hereafter utilized in the synthesis of support-bound azidoproline-containing tetrapeptides **26** and **27** (Scheme 2). Starting with the amino-functionalized $PEGA_{800}$ resin (0.4 mmol/g),[51] the Rink linker was attached using a TBTU-mediated amide coupling procedure.[52] Consecutive deprotections of the Fmoc protecting group with piperidine, and coupling of amino acids using the TBTU protocol gave resin-bound Boc-protected azides **26** and **27**. For product characterization purposes, both tetrapeptides were released from the solid phase while simultaneously being Boc-deprotected, by acidic treatment with 95% TFA (aq), and were isolated in excellent crude purities (>95%) and yields (>90%).

With solid-supported azides **26** and **27** available, we set out to construct the desired triazoloproline libraries. Initial experiments with standard protocols for solid-phase CuAAC reactions, however, did not yield the desired triazoloproline-containing tetrapeptides in satisfying purities.[38,45,53−55] Consequently, a rapid screen of reaction parameters was therefore performed on solid-supported *cis*-azidopeptide **26**. Reactants, reagent stoichiometries and reaction times were investigated (Table 1). In contrast to our previous work,[54] the use of stoichiometric quantities of sodium ascorbate (NaAsc) proved detrimental for the reaction (Table 1, entry 1). Long reaction times and the use of DIPEA as cosolvent, on the other hand, proved to be crucial for efficient conversion of solid-supported substrate **26** and allowed isolation of the desired triazoloproline **8**{*1*} in excellent purity (Table 1, entry 6).

These optimized reaction conditions were then applied to the synthesis of triazoloproline-containing libraries **8** and **9**, by reaction of azidopeptides **26** and **27** with a range of commercially available and structurally diverse alkynes (Table 2). In general, excellent purities were achieved after cleavage from the solid phase. The reaction with 4-pentynoic acid, however, proved problematic (Table 2, entry 20). The resin had to be subjected to the CuAAC reaction conditions three times before full conversion into *cis*-triazoloproline **8**{*20*} was achieved. Surprisingly, in the case of the related *trans*-azide **27**, the reaction seemed to stop at about 70% conversion and 3−4 repetitive CuAAC reactions failed to provide the desired triazoloproline **9**{*20*} in higher purity.

Chart 1. Screening Data from Live/Dead Cellular STS-Potentiation Assay on Triazoloproline Libraries 8 and 9



With *cis*- and *trans*-triazoloproline libraries **8** and **9**, both containing 20 members, at hand, we set out to investigate the synthesis of the projected biarylalanine libraries **10** and **11**.

Synthesis of Biarylalanine Libraries 10 and 11. The palladium-catalyzed Suzuki cross-coupling reaction has become a powerful method in medicinal chemistry for the construction of aryl−aryl bonds.[56] Although scarcely abundant in natural products, the biaryl motif now constitutes an important structural element for small molecule drug discovery efforts. The Suzuki cross-coupling is an attractive reaction for combinatorial and parallel synthesis. Reaction conditions are generally mild and compatible with most functional groups, and boronic acids are readily available and stable to heat, air, and moisture. Inspired by the potent and selective mimetics made by introducing hydrophobic moieties around the Ile residue of the Smac-derived AVPI sequence, we decided to investigate the Suzuki cross-coupling for the introduction of more lipophilic moieties in the IAP binding groove. Specifically, we desired to examine if we could introduce 3- and 4-iodophenylalanines in our solid-phase synthesis scheme and carry out subsequent Pd-catalyzed cross-coupling with a set of arylboronic acids, thus creating biarylalanine libraries. First, solid-supported aromatic iodides **30** and **31** were easily accessed using commercially available *N*-protected amino acids and standard solid-phase peptide synthesis protocols (Scheme 3).

Several approaches have been reported for Suzuki cross-coupling reactions on solid support.[57−61] After extensive optimization, our preferred method for Suzuki cross-coupling reactions on the PEGA resin involves the use of $Pd(dppf)Cl_2/K_3PO_4$ in an aqueous mixture of *t*-BuOH and toluene at room temperature. Thus, solid-supported aromatic iodides **30** and **31** were subjected to Suzuki cross-coupling with a range of aromatic boronic acids incorporating various electron donating and withdrawing substituents (Table 3). Notably, some boronic acids, such as pentafluorophenylboronic acid and mercaptophenylboronic, did not undergo cross-coupling reactions and only aryl iodides **32** and **33** could be recovered after cleavage from the solid phase (Table 3, entries 17 and 18). Other coupling partners resulted in very complex reaction mixtures (Table 3, entries 14−16). Despite these unsuccessful experiments, all other selected arylboronic acids smoothly underwent the desired cross-coupling (Table 3, entries 1−13) in good to excellent purities (88 to >95%), thus generating the projected 3- and 4-biarylalanine libraries **10** and **11**.

Biological Evaluation of Smac Mimetics. The resulting four Smac mimetic libraries were subjected to biological evaluation. These were first tested for potentiation of the known pro-apoptotic agent staurosporine (STS) in a live/dead fluorometric assay. The assay was conducted on HeLa cells and the results are depicted as a ratio of dead to live cells normalized to treatment with STS alone (100 nM). All peptides were administered at a 30/60 μM concentration along with STS (100 nM).[62] For matters of comparison the peptides were tested along with previously described **5** and (*N*-Me)AVPF peptides (Charts 1 and 2). It must be emphasized that the presented cellular assay is a potentiation assay where cells are concomitantly treated with STS (100 nM, Smin). The STS addition affects the cells by bringing them to the brink of apoptosis, which enables the measurement of a cellular apoptosis response not possible with the Smac mimetics alone. The effect of the STS addition (100 nM, Smin) was examined, and no significant deviation was observed when compared to the media control without STS added. In this way the observed differences in cellular responses are caused by the presence of a given Smac mimetic.

On the basis of the present data set, it was difficult to deduce structure−activity relationships. However, specific trends within the libraries were quite evident. For instance, *cis*-triazoloprolines were generally more potent than their *trans* counterparts.

Chart 2. Screening Data from Live/Dead Cellular STS-Potentiation Assay on Biarylalanine Libraries 10 and 11

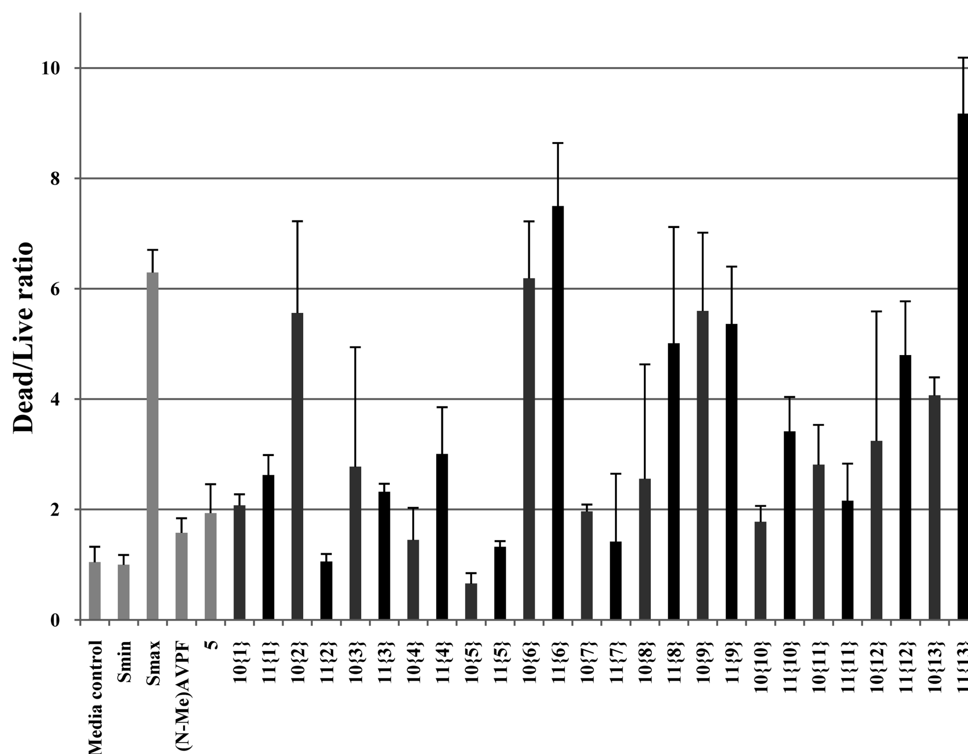


Table 4. Fitted Dose-Response Values for Selected Compounds with and without STS

entry	compound	C^a with STS[b] (μM) (±SD)	C^a without STS (μM) (±SD)
1	**8**{*2*}	12.2 (2.0)	27.4 (2.5)
2	**10**{*6*}	31.8 (2.3)	72.1 (10.7)
3	**11**{*6*}	21.6 (2.7)	95.9 (13.7)
4	**11**{*13*}	2.7 (0.4)	13.5 (0.4)
5	**5**	35.2 (3.9)	106.6 (14.7)
6	STS	2.0 (0.003)	

[a] Concentration needed to reach a dead/live ratio 4-fold higher than STS alone (100 nM). [b] STS concentration: 100 nM.

In general, within triazoloproline libraries **8** and **9**, triazoles derived from aliphatic alkynes appeared more active. In addition, biarylalanine libraries **10** and **11** seemed significantly more biologically active than the triazoloproline libraries. The results of concentration-gradient experiments with and without STS are shown for 4 selected compounds in Table 4. To our delight, when tested along with STS, all peptides proved more active potentiators than our starting hit **5**, with benzothienyl-containing compound **11**{*13*} (Table 4, entry 4) showing very satisfying single-digit potentiating activity (2.7 μM). But more interestingly, when tested without STS, compound **11**{*13*} was shown to possess low micromolar activity (13.5 μM) on its own, indicating a biological mode of action different from sole Smac mimicry.

■ CONCLUSION

There are only few reports on solid-phase organic synthesis strategies for the formation of Smac peptidomimetics. In the present investigation, we have taken advantage of this powerful synthetic tool to generate libraries of structurally modified Smac peptidomimetics. We have developed efficient methodology for the 3-dimensional display of hydrophobic pharmacophore elements in the form of triazoloprolines and biarylalanines, respectively, at the proline and isoleucine positions of the parent Smac AVPI peptide, relying on robust and finely optimized CuAAC and Suzuki cross-coupling reactions. The protocols were optimized to very high efficiency (typical purities >90%) and utilized for the generation of four distinct libraries incorporating *cis*- or *trans*-triazoloproline and 3- or 4-biarylalanine residues. Subsequent biological screening of all compounds revealed promising bioactives from each generic library, thus confirming the relevance of the synthetic approach. The presented biological data must be considered as the results of a preliminary investigation. The specific mode of action of the synthesized peptides cannot be deduced from the performed cell survival assay. However, on the basis of prior art, it is likely that the compounds partly function via IAP inhibition. On-going efforts aim at the synthesis of new AVPI analogues and for their detailed biological evaluation in various assays, including protein binding studies and pull-down experiments with supported AVPI analogues. The present strategy and findings provide an entry for the generation of new Smac peptidomimetics and justify the solid-phase synthesis and screening of larger combinatorial libraries.

■ EXPERIMENTAL PROCEDURES

All experimental procedures are reported in the Supporting Information.

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■ ASSOCIATED CONTENT

Supporting Information. Experimental procedures including general methods, solution-phase and solid-phase synthetic procedures and biological procedures. Analytical data for all building blocks and compounds cleaved from the solid support. Fitted dose-response curves. This material is available free of charge via the Internet at http://pubs.acs.org.

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