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Chemical Synthesis of Cell-Permeable Apoptotic Peptides from *in Vivo* Produced Proteins

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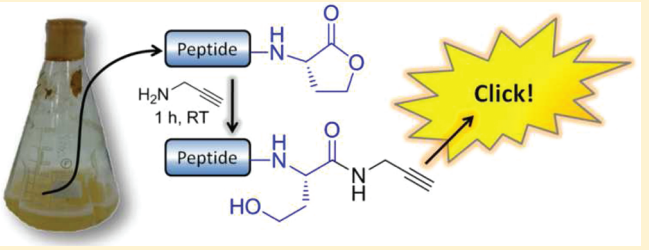
Supporting Information

ABSTRACT: *In vivo* synthesis of peptides by bacterial expression has developed into a reliable alternative to solid-phase peptide synthesis. A significant drawback of *in vivo* methods is the difficulty with which gene products can be modified post-translationally. Here, we present a method for the facile modification of peptides generated in bacterial hosts after cyanogen bromide cleavage at C-terminal methionines. Reaction of the resulting homoserine lactones with propargylamine allows efficient and selective modification with a wide variety of chemicals such as fluorescent dyes, biotin derivatives, polyprenyls, lipids, polysaccharides, or peptides. Attachment of the cell penetrating peptide octa-arginine (R_8) to peptides derived from the proapoptotic tumor suppressor Bak BH3 led to efficient cellular uptake and subsequent cytochrome *c* release from mitochondria, culminating in induction of apoptosis similar to that observed with peptides linked to R_8 via the peptide backbone. These results highlight the significant potential for use of such tools in live cells.



Protein−protein interactions are central to cellular processes and therefore provide opportunities for innovative research tools and medicines.[1−5] However, the interacting surfaces are generally hydrophobic, large, and shallow, and only a few examples exist of small molecules that target protein interfaces.[6−8] Mimicking Nature's strategy for protein recognition by using peptides is an elegant alternative and interest in therapeutic peptides is increasing;[2,9−12] the number of peptides in clinical studies has nearly doubled over the last 10 years.[13] The principal advantage of such peptides is the relative ease by which molecules with highly specific binding affinities can be synthesized through a series of simple amide bond formations. Concerns about short peptide lifetimes in the cellular environment and the difficulty with which most peptides pass across cell membranes have hitherto limited the use of peptides as therapeutics and as tools for biomedical research. However, the metabolic stability of peptides can be increased through additional chemical modification, while the use of cell penetrating peptides facilitates transport across cellular membranes. Examples of such transporter sequences include the HIV Tat peptide derived from the human immunodeficiency virus 1 trans-activating transcriptional activator,[14,15] penetratin from the *Drosophila* antennapedia homeodomain[16] and synthetic variants such as octa-arginine.[17] These peptides possess well-known abilities to enhance uptake of cargos such as peptides, proteins, DNA, and RNA into mammalian cells.[18−22] The additional length of such transporter sequences and any intervening spacer regions can, however make the chemical synthesis difficult and costly.

In recent years, new methods for high yielding syntheses of peptides in bacterial cells have been developed[23−25] and the production of peptides as fusions with larger carrier proteins has established a reliable alternative to standard solid-phase peptide synthesis. A significant drawback of the expression approach is the difficulty to efficiently and selectively modify the resulting peptides. Opportunities to attach molecules such as biotin, lipids, fluorescent dyes, glycans, or peptides in a selective way are limited by the availability of reactions that are selective for particular amino acid side chains. Here, we present a method to modify *in vivo* generated peptides to create a C-terminal alkyne group that can be transformed with a wide variety of azide reagents. Cleavage of methionine containing peptides with cyanogen bromide results in the formation of a C-terminal homoserine lactone,[26] which is amenable to nucleophilic attack. Previous reports have found homoserine lactones to be poor electrophiles that require the use of large excesses of expensive nucleophiles.[23,27] Here, the lactones were reacted with an excess of the relatively inexpensive propargylamine, resulting in the formation of a stable amide linkage between the peptide and an

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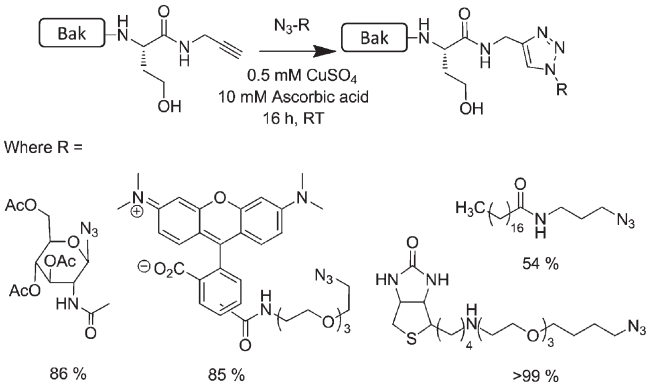
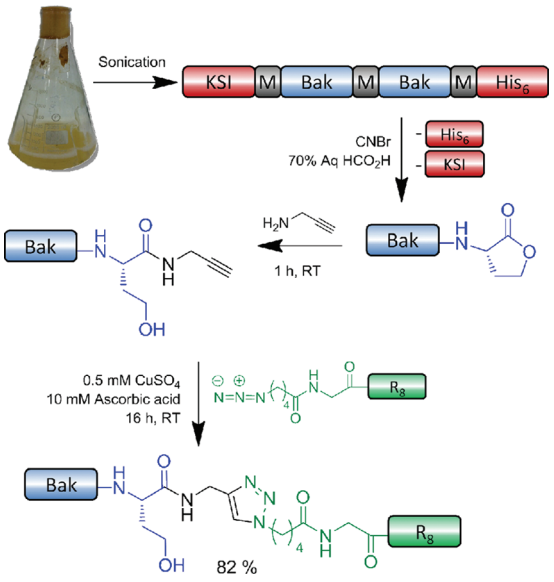


Figure 1. Structures of azides conjugated to alkyne modified peptides used in this study.

Scheme 1. Outline of the Facile Synthesis of Bioconjugates from Ketosteroid Isomerase Fusion Proteins[a]



[a] Copper catalyzed [3 + 2] cycloaddition for the production of C-terminally modified Bak peptides from *in vivo* generated fusions with ketosteroid isomerase (KSI) that were cleaved with CNBr at methionines (M).

alkyne group. The alkyne could then be reacted with a variety of different azide containing molecules and commercially available dyes *via* high-yielding copper accelerated [3 + 2] cycloadditions (Figure 1).[28,29]

In addition to the conjugation of peptides with small molecules, this method was applied to the generation of peptides derived from the protein Bak, a member of the B-cell leukemia-2 (Bcl-2) family of proteins, which play key roles in the regulation of programmed cell death.[30] Bcl-2 family proteins are characterized by the presence of up to four conserved Bcl-2 homology domains that contain α-helical segments. The pro-apoptotic tumor suppressors Bax and Bak are antagonized by antiapoptotic Bcl-2 members (eg Bcl-2 and Bcl-x_L) via protein−protein interactions. Bak is often down regulated in cancer cells[31] with evidence that diverse apoptotic stimuli can preferentially engage the Bak pathway.[32] Peptides derived from the BH3 helix of Bak

Table 1. Amino Acid Sequences of Peptides Used in This Study and Binding Affinities to Bcl-x_L

name	sequence	K_D/nM [b]
Bak wt	CGGQVGRQLAIIGDDINR-Hsl[a]	90.9 ± 9.0
Bak GR_8-click	CGGQVGRQLAIIGDDINR-triazole-GR_8	28.3 ± 4.8
Bak-$SGGR_8$	CGGQVGRQLAIIGDDINRSGGR$_8$-Hsl[a]	25.9 ± 3.7
Bak L78A	CGGQVGRQAAIIGDDINR-Hsl[a]	No Binding
Bak L78A-GR_8-click	CGGQVGRQAAIIGDDINR-triazole-GR_8	No Binding
CGR_8	CGR_8	No Binding

[a] Homoserine lactone. [b] Measured by fluorescence anisotropy using Alexa 488 labeled peptides and Bcl-x_L.

have been shown to disrupt the interaction between native Bak and its partner Bcl-x_L.[33,34] Subsequent homo-oligomerization of activated Bak leads to the formation of channels that allow the release of cytochrome *c* from mitochondria, an early commitment step of apoptosis.[35]

The synthesis of a peptide modeled on the BH3 region of Bak with an additional N-terminal cysteine residue for selective functionalization was accomplished by introducing multiple repeats of a synthetic DNA sequence of the desired peptide into the expression vector pET-31b.[23] The peptides were produced in BL21(DE3) *E. coli* cells in high yields as protein fusions with ketosteroid isomerase (KSI). KSI is known to form poorly soluble inclusion bodies in *E. coli*, thereby protecting proteins from cellular proteases and allowing facile purification. The expressed fusion proteins were treated with cyanogen bromide in formic acid and the resulting peptides purified by standard reverse-phase HPL-chromatography (Scheme 1). The product peptides were isolated as a mixture of C-terminal homoserine lactone and homoserine forms; the latter could be converted quantitatively to the lactone with trifluoroacetic acid. Typical yields were 5 to 10 mg of purified lactone peptide per liter of bacterial cell culture (for details, see Supporting Information).

Treatment of the purified homoserine lactone peptides with propargylamine furnished peptidylpropargylamides in 75−85% yields. The peptide alkynes were treated with azides derived from Fluor 546, biotin, N-acetyl glucosamine, palmitic acid, and GR_8 (82% yield) to produce the corresponding triazoles in good yields (Figure 1, SI Figures S1−S7); these were readily purified by reversed-phase HPLC and identified from their mass spectra. The presence of an N-terminal cysteine allowed the synthesis of doubly labeled peptides through reaction of Alexa Fluor 488 maleimide with the sulfydryl of the *N*-terminal cysteine of the Fluor 546-triazole peptide (Supporting Information).

While the method described here allowed the efficient extension of the sequence of *in vivo* produced peptides, the presence of the triazole linkage could be envisaged to affect the physiological properties of the peptides. Literature reports indicate that Bak-R_8 peptides can induce cytochrome *c* release from mitochondria.[36] To demonstrate how our conjugation method can alter the properties of a peptide and assess the effect of the presence of the nonpeptidic linkage, we conjugated the Bak peptide and a mutant known to remove Bcl-x_L binding and apoptotic function (Bak L78A)[34] to an azido-GR_8 peptide. For comparison, an extended peptide, Bak-$SGGR_8$, was synthesized in *E. coli* and purified (Table 1). The N-terminal cysteine residue present in both peptides allowed for the conjugation of the Alexa Fluor 488 maleimide to monitor and measure the heterogeneity of cellular uptake and delivery.

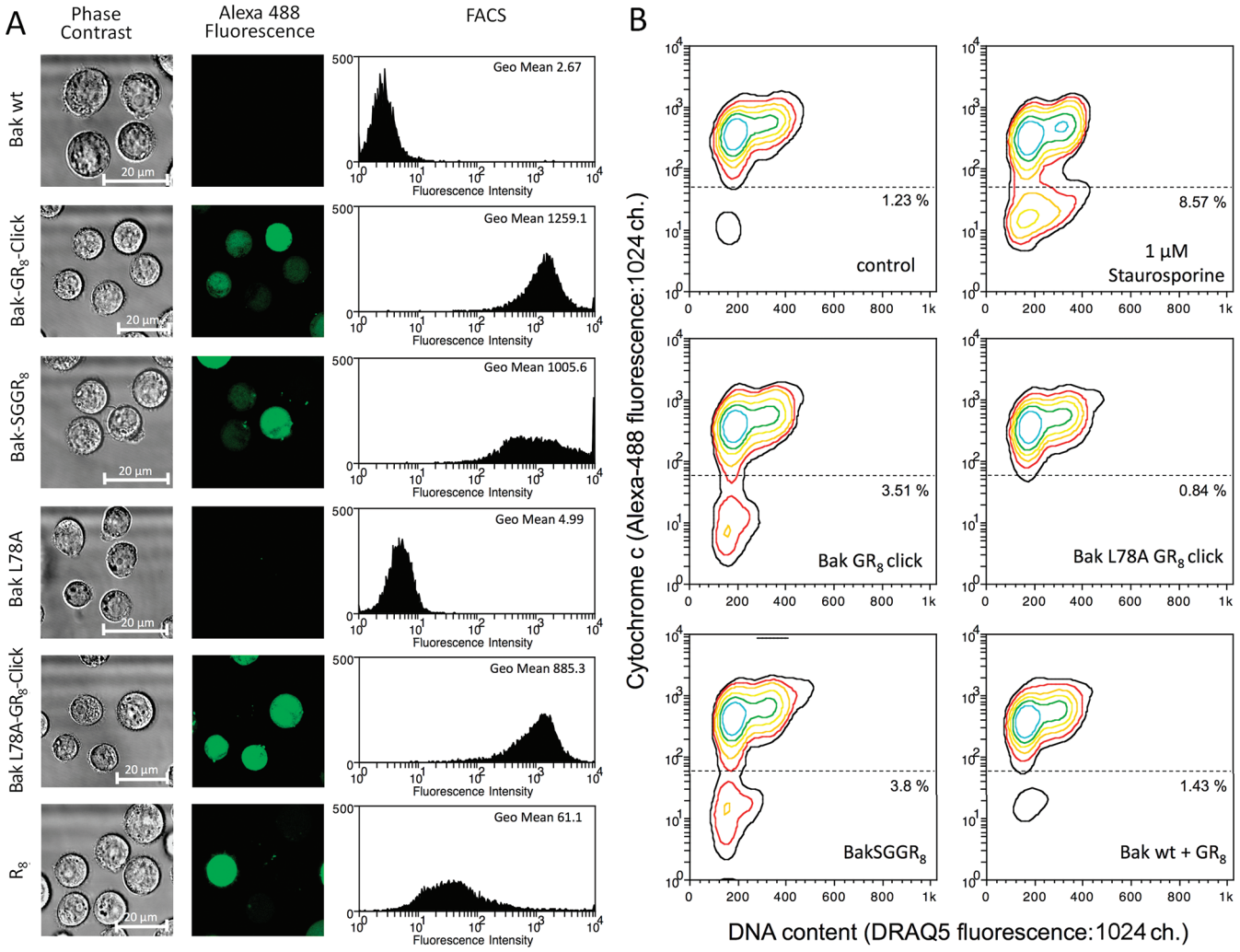


Figure 2. (a) Relative uptake of 5 μM Alexa Fluor 488 labeled peptides (Table 1) by KG1a cells for 1 h incubations at 37 °C analyzed by fluorescence microscopy and FACS (note the logarithmic scale; within the same cell population there was some degree of heterogeneity with respects to amount of internalized peptide and its subcellular distribution). (b) Contour plots from FACS (logarithmic contour spacing) showing cytochrome *c* release from mitochondria of KG1a cells after incubation with 20 μM peptides at 37 °C for 4 h.

Alexa Fluor 488 labeled $CGR_8{-}NH_2$ was synthesized by solid-phase chemistry to serve as a transporting, nonapoptotic control peptide. Fluorescence anisotropy measurements were used to determine the binding affinities of the labeled peptides to Bcl-x_L *in vitro* (Table 1).[33] As expected, no binding could be observed under the experimental conditions with Bak L78A, Bak L78A-GR_8-click, or CGR_8. Bak-$SGGR_8$ and Bak GR_8-click bound approximately 3 times more tightly to Bcl-x_L than Bak wt (Table 1 and Supporting Information). The K_D of 90.9 ± 9.0 nM for Bak wt was approximately 3 times lower than the value previously reported,[33] which is most likely attributable to the presence of Alexa Fluor 488.

Human leukemia myeloid KG1a cells were used to examine cellular uptake of the peptides. A cell population was incubated with peptide at 0.5 and 5 μM concentrations of peptides for 1 h at 37 °C and then analyzed by confocal microscopy and flow cytometry. As expected from literature precedents,[37,38] no Bak wt ingress was detectable at either concentration, whereas cells exposed to Bak GR_8-click, Bak-$SGGR_8$, and Bak L78A-GR_8-click showed fluorescent signals well above background, indicating peptide uptake. Furthermore, at 0.5 μM the fluorescence pattern was punctate indicating a possible endosomal mechanism for localization (Supporting Information). At the higher peptide concentration of 5 μM, the fluorescence signal per cell was higher and the cytoplasmic labeling diffuse, implying direct uptake (Figure 2a). Flow cytometry analysis of cells (FACS) indicated a ~350-fold increase of the mean fluorescence signal present in cells incubated with 5 μM Bak-$SGGR_8$, Bak GR_8-click, and Bak L78A-GR_8-click over control cells or those incubated with Bak wt and Bak L78A. A significant increase in fluorescence was even observed for cells incubated with 0.5 μM Bak-$SGGR_8$, Bak GR_8-click, and Bak L78A-GR8-click peptides (Supporting Information).

A further FACS experiment allowed the determination of apoptotic commitment as determined by the fraction (%) of cells showing release of cytochrome *c*. After incubation with peptides, cells are permeabilized and treated with an anti-cytochrome *c* antibody;[39] loss of signal (<60% compared to a control sample) indicated the cells had released cytochrome *c* from mitochondria into the cytoplasm and then into the buffer. As a consequence of high levels of the antiapoptotic Bcl-2 protein,[41] KG1a cells are known to be relatively resistant to the induction of apoptosis.[40] However, since effective delivery of R_8 peptide cargo into cells

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was observed here, a medium term duration incubation (4 h) of KG1a cells was chosen as sufficient for determining the activity of the peptides in the cells in terms of cytochrome *c* release. As a positive control for cytochrome *c* release, cells were exposed to 1 μM staurosporine, an alkaloid known for its pro-apoptotic effect.[39,42] This treatment showed a 6-fold increase in cells undergoing cytochrome *c* release compared to no treatment. The DNA binding fluorochrome DRAQ5 was used to co-define cells with normal range DNA content and to assess any cell cycle specific apoptotic triggering and cytochrome *c* release was found to be cell cycle independent.[43] When exposed to our peptides, low levels of cytochrome *c* release were observed after coincubations with Bak wt (no uptake) and GR_8 peptide (no bioactive peptide) (Figure 2b). However, exposure to Bak GR_8-click and Bak $SGGR_8$ led to significant increases in cytochrome *c* release, three times higher relative to Bak L78A-GR_8-click, Bak wt, GR8, and the control sample. To validate the data, a Student's one-tailed *t* test was performed, showing $P > 0.05$ for samples with 1 μM staurosporine, Bak GR_8-click, and Bak $SGGR_8$ and for the coincubations with Bak wt and GR_8 peptide (Supporting Information).

In conclusion, a reliable method for the derivation of expressed peptides with a C-terminal propargyl amide through [3 + 2] cycloaddition with various azides has been developed. The resulting triazole linkage does not appear to affect the *in vitro* and *in vivo* properties of the resulting peptides, with Bak GR_8-click and Bak $SGGR_8$ showing very similar uptake and apoptosis induction. The octa-arginine sequence enhanced the cellular uptake of Bak peptides to a level sufficient to elicit an early apoptosis response in the acute myeloid leukemia cell line KG1a. The corresponding mutant Bak peptide Bak L78A-GR_8-click showed equivalent uptake (cargo delivery remained intact) but no apoptotic induction (bioactivity compromised), confirming the expected mechanism of apoptosis induction. Our methodology provides a unique combination of high-level *in vivo* synthesis of encoded peptides with the versatile options for derivatization, normally only associated with chemical peptide synthesis. The ability to tailor peptides in a flexible and robust manner offers many possibilities for the development of next-generation biomedical research tools. The results highlight the potential for the *in vivo* use of such tools to explore and manipulate biological process in live cells, for instance, to determine the hierarchical restrictions on the induction of apoptosis in cancer cell populations.

■ ASSOCIATED CONTENT

Supporting Information. Synthetic methods, spectroscopic data, micrographs, FACS histograms, binding data, mass spectra, HPLC traces. This material is available free of charge via the Internet at http://pubs.acs.org.

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