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Spiropyran-based photoisomerizable α-amino acid for membrane-active peptide modification

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Abstract: Photoisomerizable peptides are promising drug candidates in photopharmacology. While azobenzene- and diarylethene-containing photoisomerizable peptides have already demonstrated their potential in this regard, reports on the use of spiropyrans to photoregulate bioactive peptides are still scarce. This work focuses on the design and synthesis of a spiropyran-derived amino acid, (*S*)-2-amino-3-(6´-methoxy-1´,3´,3´-trimethylspiro-[2H-1-benzopyran-2,2´-indolin-6-yl])propanoic acid, which is suitable for the preparation of photoisomerizable peptides. The utility of this amino acid is demonstrated by incorporating it into the backbone of BP100, a known membrane-active peptide, and by examining the photoregulation of the membrane perturbation by the spiropyran-containing peptides. The toxicity of the peptides (against the plant cell line BY-2), their bacteriotoxicity (*E. coli*), and actin–auxin oscillator modulation ability were shown to be significantly dependent on the photoisomeric state of the spiropyran unit.

Introduction

Peptides exhibit highly diverse biological activities and are promising therapeutics and theranostic tools.[1] In particular, reversibly photoisomerizable (photoswitchable) bioactive peptides have attracted intense research attention for many decades.[2] The photoregulation of peptides is achieved by incorporating light-responsive fragments, the so-called photoswitches, in their molecules, offering exciting prospects for drug development and *in vivo* studies of molecular processes using precisely focused optical stimuli as modulators.[3] Hence, photoswitch-modified bioactive peptides are promising drug candidates in a rapidly evolving medicinal chemistry area that is called “photopharmacology”[4] and as research tools.

Most of the currently described photoswitchable peptides have been designed using azobenzene as the light-responsive unit.[3b,c,g,i] In contrast, other known photoswitches, such as diarylethenes, stilbenes, hemithioindigos, fulgides, and spiropyrans, are underexplored in this structural context,[3i] which is surprising given the vast knowledge accumulated on these photoswitches. For example, the photoinduced reversible transformation of spiropyrans (Figure 1) has been intensively studied since 1952.[5] Spiropyrans belong to a unique type of photoswitches exhibiting diverse responsiveness to different stimuli.[5f] However, spiropyran-modified bioactive compounds are still rare,[6] even though conformational changes upon

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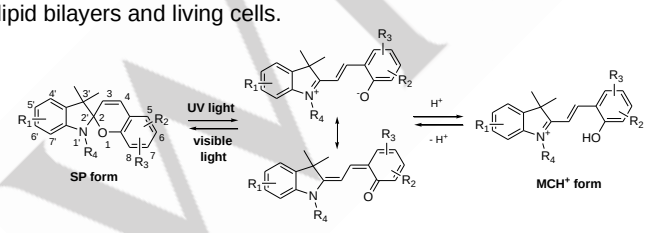
photoswitching were demonstrated decades ago for several spiropyran-modified proteins and polypeptides.[3b,7] The influence of the environment on spiropyran-containing peptide photoisomerization equilibria and kinetics has been ingeniously used in analytical[8] and imaging applications[9] and for the development of photoresponsive hydrogels.[10] However, the impact of spiropyran photoisomerization on the biological activity of peptides has been scarcely explored.[11] One of the reasons for this *status quo* is the limited availability of photoswitch-containing building blocks suitable for the chemical synthesis of spiropyran-modified peptides. Almost none of the abovementioned works used specifically designed spiropyran-based amino acids, which can be considered as the most appropriate building blocks.[12]

A distinct advantage of spiropyran photoswitches is the dramatic change in structure and polarity induced by photoisomerization.[5f] Spiropyran photoisomers (SP form, Figure 1) usually possess low polarity similar to that of hydrophobic proteinogenic amino acids, whereas merocyanine photoisomers (MC/MCH$^+$ form, Figure 1) are highly polar/charged. Notably, both photoforms differ substantially in their structure and steric size. Therefore, we envisaged the development of a spiropyran-based α-amino acid whose major photoisomeric forms would mimic proteinogenic amino acids while differing in their steric size and polarity. The difference in the polarity of the SP and MC forms in the side chain should enable the modulation of the hydrophobic–hydrophilic balance (amphipathicity) of a peptide carrying such an amino acid. For many natural and synthetic membrane-active peptides, e.g., antimicrobial,[13] fusogenic,[14] and cell-penetrating[15] peptides, this balance is important for their activity because it determines critical peptide–membrane interactions.[16] We also hypothesized that photoisomerization of the spiropyran residue in an amphiphilic peptide could be an efficient tool to control its biological activity with light, at least concerning its membrane interactions, as was previously demonstrated for azobenzene-derivatized peptides.[16b,c]

Herein, we describe the design, synthesis, and characterization of a spiropyran-based α-amino acid. We incorporated a novel amino acid into the membrane-active peptide BP100 and studied the effects of this modification on various BP100 interactions with lipid bilayers and living cells.



1a, $R_1 = R_2 = R_3$ =H, R_4 = Me; **1b**, $R_1 = R_2$ = H, R_3 = 7-NEt_2, R_4 = Me; **1c**, $R_1 = R_2$ = H, R_3 = 6-OMe, R_4 = Me; **1d**, $R_1 = R_2$ = H, R_3 = 8-OMe, R_4 = Me; **1e**, R_1 = H, $R_2 + R_3$ = 6,7-OCH_2O-, R_4 = Me; **1f**, $R_1 = R_2$ = H, R_3 = 6-Ph, R_4 = Me; **1g**, $R_1 = R_2$ = H, R_3 = 6-Br, R_4 = Me; **1h**, $R_1 = R_2$ = H, R_3 = 6-Cl, R_4 = Me; **1i**, R_1 = H, R_2 = 6-Br, R_3 = 8-Br, R_4 = Me; **1j**, R_1 = H, R_2 = 6-Cl, R_3 = 8-Cl, R_4 = Me;
2a, R_1 = 5'-OMe, $R_2 = R_3$ = H, $R_4 = (CH_2)_3{}^+NMe_3$; **2b**, $R_1 = R_2 = R_3$ = H, R4 = $(CH_2)_3{}^+NMe_3$; **2c**, R_1 = 5'-OMe, $R_2 = R_3$ = H, $R_4 = (CH_2)_3SO_3H$;
3, R_1 = 5'-OMe, R_2 = 6-$CH_2CH(NH_2)CO_2H$, R_3 = H, R_4 = Me;

Figure 1. Overview of the photoisomerization and acidochromism of spiropyrans, the model spiropyran-derived compounds studied in this work.

Results and Discussion

Design of spiropyran-derived α-amino acid

It is known that spiropyran photoswitches are not perfectly bistable.[5f] Usually, only one photoform (either SP or MC) is stable in the absence of light, whereas the other form converts to the stable photoisomer in the dark. This process is known as thermally induced dark adaptation. Under irradiation of the stable photoform, a photostationary state is usually established due to a competition between light- and thermally induced isomerization. Considering these facts, we set the following criteria for the photophysical and chemical properties of the target amino acid toward the design of functional light-controllable peptides to be used in living organisms: (i) the thermally stable photoform of the modified peptides should be less biologically active than the thermally unstable photoform. In this case, the less active form of the peptides could be safely applied to a living system, and its activity could then be switched “on” by light and turned “off” thermally in the absence of light, thereby minimizing side effects. (ii) The more active form of the peptides should be generated upon irradiation with visible light, preferably red light (~630–650 nm wavelength), because this light is less damaging to living organisms and penetrates deeper into the tissue of multicellular organisms than UV light. Combining both criteria, the thermally stable and less active spiropyran-based peptides should exist in their MC photoform and be capable of converting to the SP form under visible (preferably red) light irradiation. Spiropyran derivatives exhibiting such behavior were named “inverse (reverse, negative) photochromic systems”.[17] (iii) As a third criterion, the MC-to-SP photoconversion should be as fast and complete as possible. (iv) In addition, the photoswitching molecular fragment must be sufficiently chemically stable, in particular, resistant to chemical degradation in aqueous media under physiological conditions and toward photodegradation upon irradiation.

Fulfilling simultaneously the above criteria is highly challenging. Although many known spiropyran photoswitches are thermally stable in the MC photoform in polar solvents, thus demonstrating the desired inverse photochromism [criterion (ii)],[5f] the compounds reported as suitable for biological applications were poorly photoswitchable or photodegradable in most cases.[18] In addition, most spiropyran fragments incorporated into polypeptides contain metabolically unstable nitro groups in the chromophore.[3b,7a,b,10b] Nitro groups can cause toxicity issues *in vivo* and are avoided in drug candidates.[19] Therefore, we first decided to synthesize model spiropyran-based compounds bearing substituents other than nitro groups at the spirocyclic core to determine the substitution pattern that favors inverse photochromism, efficient photoswitching, and sufficient chemical stability [criteria (ii)–(iv)].

Although the influence of substituents on the photoswitching properties of spiropyrans has been intensively investigated,[20] the literature data are fragmentary and the measurements described in different publications were performed under different conditions. Thus, we compared the behavior of our model compounds under identical conditions in polar solvents.

In the first series of model compounds, we varied the R_2 and R_3 substituents in the benzopyran part of the core chromophore and synthesized compounds **1a–j** (Figure 1) via the condensation reaction of various *o*-hydroxybenzaldehydes with 1,3,3-trimethyl-

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2-methylene-indoline (Fischer's base) as a key synthetic step, which is the most widely used synthetic approach to spiropyrans.[5d]

Since the compounds turned out to be insoluble in water, we selected methanol as another polar solvent to identify the substitution pattern that promotes inverse photochromism in a polar environment. The absorption spectra of compounds **1a–j** in methanol revealed that the MC form was the prevailing stable photoisomer only in one case. Compound **1b** bearing an electron-donating substituent NEt_2 in the benzopyran fragment exhibited a typical MC spectrum in the dark-adapted form (Figure 2; supplementary (SI) Figure S1).

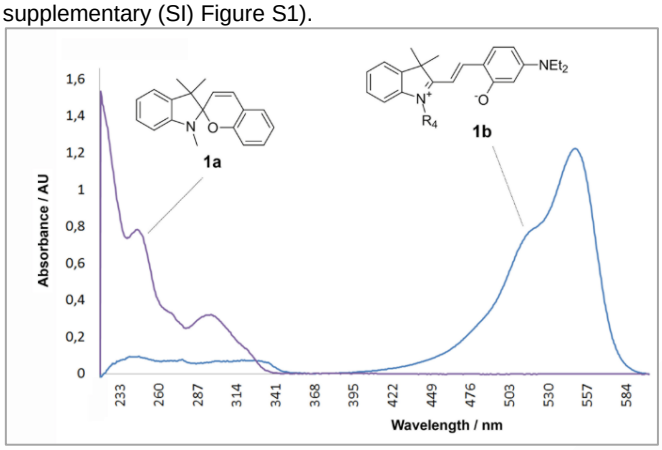
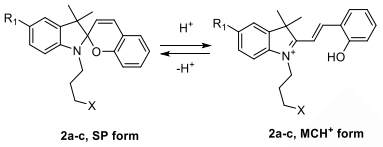


Figure 2. Representative absorption spectra of **1a** and **1b** recorded at a concentration of 10 µM in methanol in a 10 mm light path cuvette at ambient temperature after 30 min of dark adaptation.

MC stabilization by electron-donating substituents in the indoline fragment was previously reported for spiropyran photoswitches.[29b,c] Therefore, we reconsidered the substitution pattern and installed an electron-donating substituent into the indoline fragment. We also attached a positively charged $(CH_2)_3{}^+NMe_3$ group to the nitrogen atom of the indoline fragment to increase the water solubility. Dark-adapted aqueous solutions of the resulting compound **2b** contained a much higher amount of the MC photoisomer than those of nonsubstituted **2a** (Table 1). Moreover, the MC form of **2b** could be converted almost completely into the SP form within minutes upon irradiation with visible light (420–550 nm).

Another known strategy for promoting the desired inverse photochromism is to introduce acidic groups at appropriate positions in the spiropyran chromophore.[17a, 21] Such groups can donate protons, which may further shift the equilibrium toward the fully protonated MCH^+ form in polar solvents (Figure 1).[22] Thus, we synthesized model compound **2c** with an acidic group (SO_3H) to compare its behavior in water with that of **2a** and **2b**. As shown in Table 1 (see SI, Fig. S2 for the ^1H NMR spectra), compound **2c** exhibited the most pronounced inverse photochromism. Interestingly, the MCH^+ content increased in solutions at lower pH (compounds **2a** and **2b**), obviously due to protonation. Such proton-donating effects could stabilize the MC form in peptides bearing neighboring carboxylic or protonated amino groups in neutral and especially in acidic solutions.

Table 1. Dark-adapted equilibria for compounds **2a–c**, measured via ^1H NMR in aqueous ~10 mM solutions at physiologically relevant pH values at 25°C.

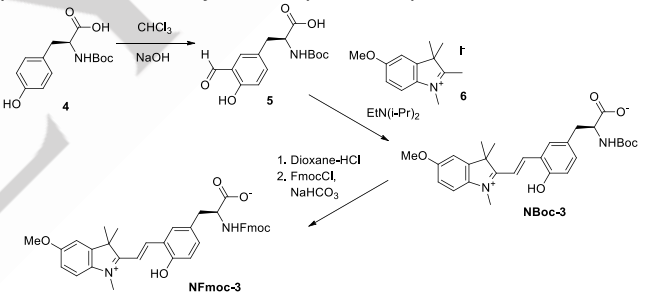


Compound	R_1	X	MCH^+ content, %	
			pH 7.4	pH 5.5
2a	H	$^+NMe_3$	9	17
2b	OMe	$^+NMe_3$	11	63
2c	OMe	SO_3^-	59	ND[a]

[a] ND – not determined

Taken together, the model studies described above suggested that the inverse photochromism of the spiropyran moiety is maintained in peptides when the residue bears a 5'-OMe-substituted spiropyran core. The presence of abundant ionizable functional groups in peptides could further stabilize the MC form in aqueous solutions. Accordingly, we designed the target amino acid derivative bearing the fluorenylmethoxycarbonyl (=Fmoc) protecting group (N-Fmoc-**3**, Figure 1, Scheme 1).

The synthesis of N-Fmoc-**3** was performed starting from N-Boc-protected natural L-tyrosine **4** (Scheme 1).



Scheme 1. Synthesis of the target α-amino acid **3** in N-Fmoc-protected form.

Design and synthesis of the photoswitchable analogs of BP100

The next step was to incorporate **3** into a peptide sequence using solid phase peptide synthesis (SPPS), Fmoc chemistry. We selected the representative membrane-active α-helical peptide BP100 (KKLFKKILKYL-NH_2),[23] whose conformational and functional features are well known. To satisfy criteria (i) and (ii), a careful choice of the position of **3** in place of a natural amino acid residue in the BP-100 sequence was required.

The sequence of BP100 was designed as a chimera from two natural antimicrobial peptides, i.e., cecropin A (from the moth *Hyalophora cecropia*) and melittin (the main component of the *Apis mellifera* venom), representing a peptide that was specifically developed to treat plant infections.[23] This peptide is amphiphilic because it folds into an α-helix and can interact with various cell membranes.[24] We aimed to synthesize a BP100 analog that was less active in the dark due to a perturbed amphiphilic profile but whose membrane interactions could be boosted by switching the spiropyran fragment under visible light.

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We reasoned that the polar MC photoform (dark adapted) should perturb the amphiphilic character of the peptide when situated on the hydrophobic face of the BP100 α-helix. Therefore, we considered BP100 analogs in which **3** substituted the natural hydrophobic Ile7 or Phe4 residues. We may anticipate that the amphiphilicity of BP100 can be restored upon MC-to-SP photoswitching because the SP photoisomer possesses low polarity. Figure 3 illustrates the peptide design and sequences of the BP100 analogs. We synthesized two spiropyran-containing analogs: monosubstituted BP100-7/MC [KKLFKK(**3**)LKYL-NH_2] and disubstituted BP100-4,7/MC [KKL(**3**)KK(**3**)LKYL-NH_2]. N-Fmoc-**3** was fully compatible with standard SPPS protocols; the spiropyran-modified peptides were obtained in moderate yields with high purity (>95%) after HPLC purification (SI, Figure S3).

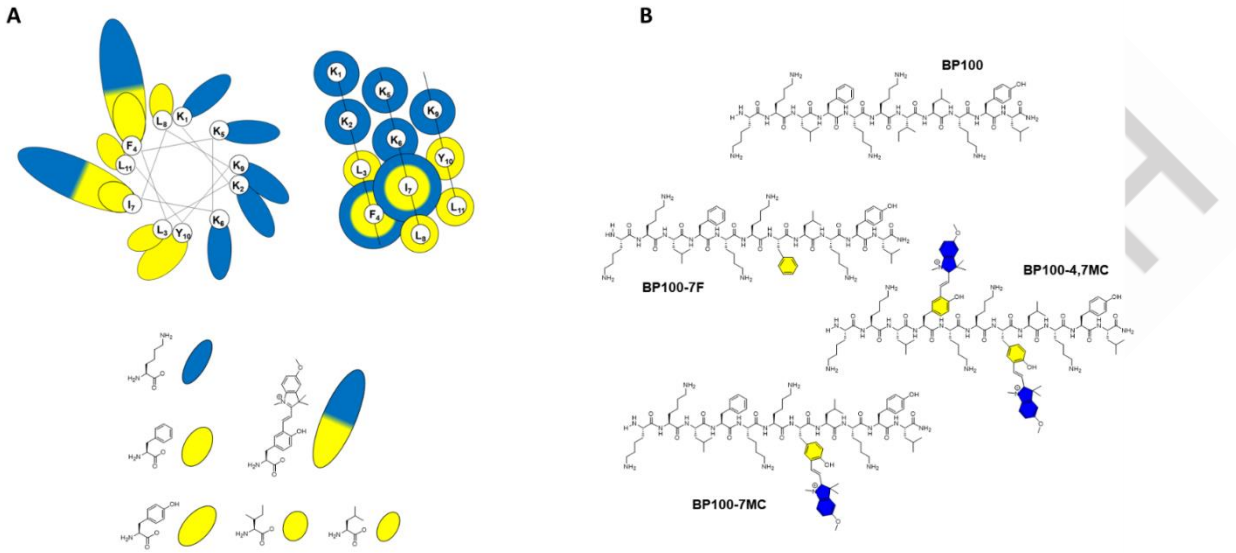

Figure 3. Peptides used in this study. (**A**) Amphiphilicity of the α-helically folded BP100. Helical wheel (top left) and helical mesh (top right) representations of amino acids in one-letter code. The polar amino acids are displayed in blue, the nonpolar ones in yellow, and the photoswitchable spiropyran residue in yellow/blue. (**B**) Structures and nomenclature of the studied peptides. The residue **3** is shown in the MC photoform.

Characterization of spiropyran-containing peptides

Both BP100-7MC and BP100-4,7MC were soluble in phosphate buffer (PB) at pH values spanning the entire physiological range (5.5–8).[25] As predicted in the design of **3** based on the data for our model compounds, the absorption spectra of the peptides in these solutions were characteristic of the MC photoforms (SI, Figure S4).[26] The absorption of the MC forms was pH dependent, as illustrated in Figure 4A for BP100-7MC. Upon increasing pH, an increase in absorbance at λ_{max} = 550 nm and a gradual disappearance of the band at λ_{max} = 425 nm were observed. This pH dependence is a known phenomenon for merocyanines[27] and reflects the equilibrium between protonated MCH^+ and nonprotonated MC forms.

Next, we studied the light-induced photoisomerization and thermally induced dark adaptation of the two peptides at different pH values. Irradiation of acidic, neutral, and alkaline solutions with blue light (λ = 460 nm, power density 1000 mW/cm^2) resulted in a fast (~10 s) decoloration. The UV spectra of the decolorized solutions corresponded to the SP forms of the photoswitch chromophore,[28] demonstrating the required inverse photochromism of both peptides.

A quantitative comparison of the irradiated spectra with the spectra of pure MC forms revealed that the MC-to-SP photoisomerization of peptides proceeded almost completely. Notably, a fast MC-to-SP photoisomerization of the two peptides was observed at pH 7 and 8 even upon irradiation with a redshifted light of 570 nm wavelength (yellow), which is important for *in vivo* applications.

The SP-to-MC adaptation of peptides in the dark proceeded with complete restoration of the initial MC photoforms within minutes in most cases, as illustrated in Figure 4B, except for the solutions of BP100-4,7/MC at pH 7.0 and 8.0. Under these conditions, the dark-adapted spectra were not identical to the initial spectra (pure MC forms), indicating that certain amount of SP form remained in equilibrium with the MC form even after prolonged relaxation.

The MC forms of the peptides exhibited weak fluorescence with maxima at ~550 nm upon excitation at 440 nm. The fluorescence of the spiropyran side chain can be used as a sensor to probe the local environment in living cells.[9,11] The fluorescence intensity was pH dependent, reaching the highest value at the lowest pH measured (5.0). In the presence of excess sodium dodecylsulfate (SDS) micelles, the fluorescence intensity increased sharply and almost lost its pH dependence (Figure 4C, 4D and Figure S5).

Our peptides underwent no remarkable photodegradation; the degradation of BP100-7/MC was less than 7% after 15 photoswitching cycles in pH 6.0 PB (Figure S4).

Structure of photoswitchable BP100 in membrane-mimicking environments

The conformational behavior of the peptides was studied for the MC form using circular dichroism (CD) spectropolarimetry, and the results were compared with those of the nonphotoswitchable original peptide BP100 and the phenylalanine mutant BP100-7F. CD spectra were collected in aqueous buffers and in media mimicking cell membranes, i.e., 50% 2,2,2-trifluoroethanol (TFE) in PB, micellar solutions of anionic sodium dodecyl sulfate (SDS) and zwitterionic dodecylphosphorylcholine (DPC), and large unilamellar vesicles (LUV, *ca.* 100 nm diameter) prepared from

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1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) and 1,2-dimyristoyl-*sn*-glycero-3-(phospho-*rac*-(1-glycerol)) (DMPG) (1:1 molar ratio). Representative CD spectra are shown in Figure 5.

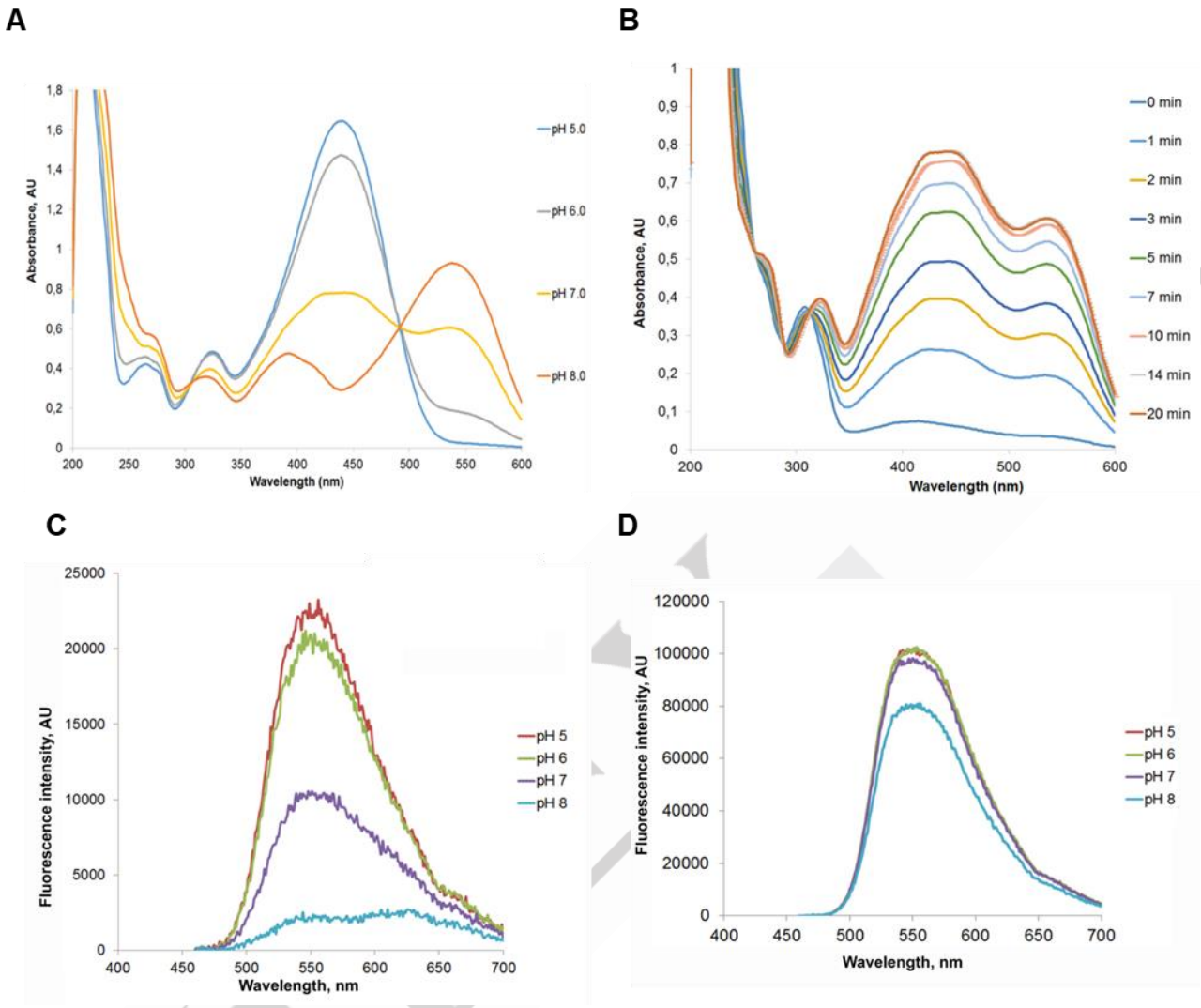

Figure 4. Spectroscopic characterization of photoswitchable peptides. (**A**) Absorption spectra of aqueous BP100-7MC solutions at different pH values (measured in 10 mM buffers, 100 mM NaCl, r. t.) and a peptide concentration of 70.4 μM. (**B**) Kinetics of the dark adaptation of the spiropyran moiety of BP100-7MC in PB (pH 7). Fluorescence spectra of BP100-7MC recorded (**C**) at different pH values and (**D**) in the presence of SDS micelles. See SI for the specific conditions.

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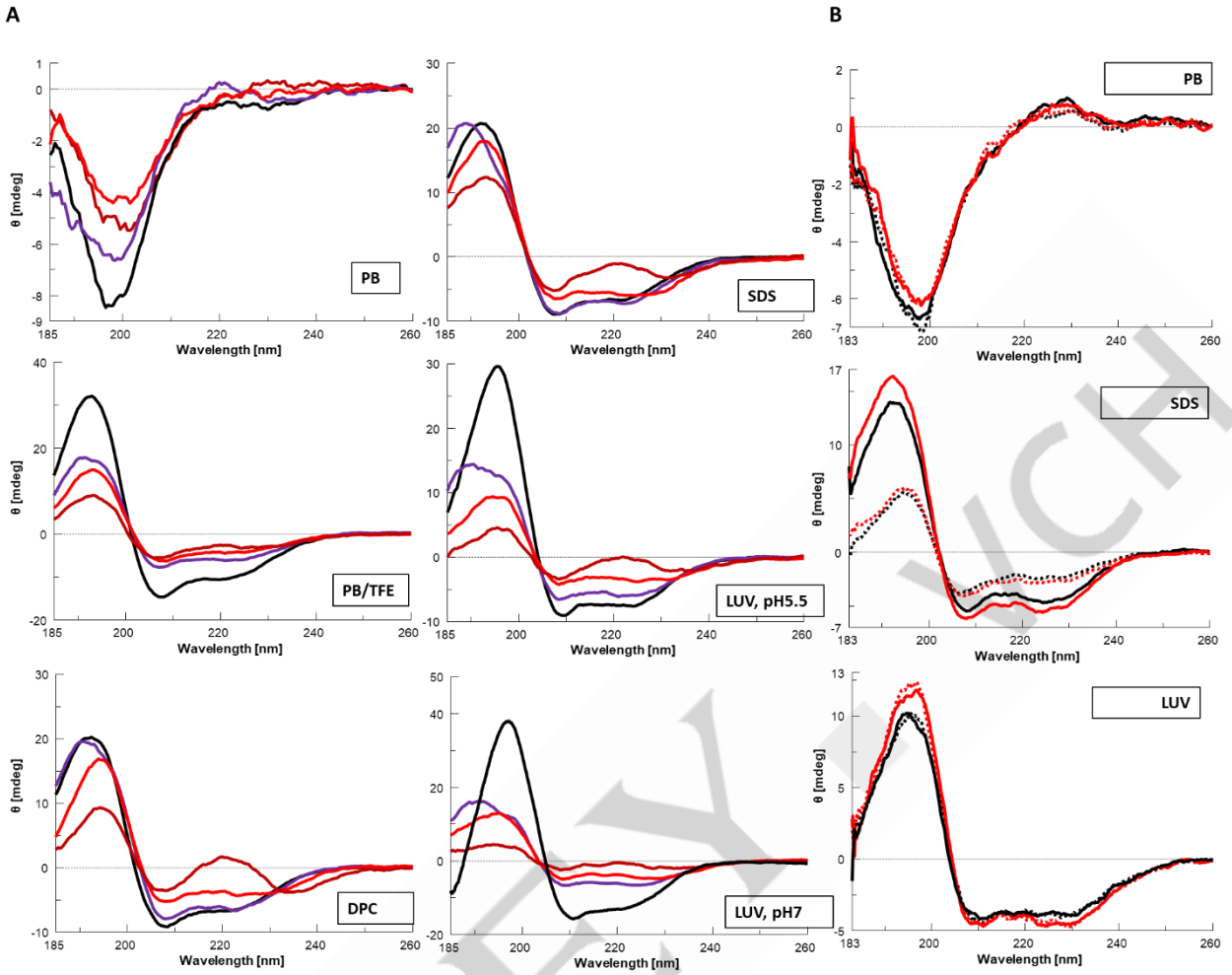

Figure 5. Structural studies of BP100 analogs in membrane-mimicking environments. (**A**) CD spectra of the peptides in 10 mM PB, PB/TFE, isotropic micelles (DPC and SDS, 7 mM) at pH 5.5, 140 mM NaF, 25°C: BP100, black; BP100-7F, violet; BP100-7MC, light red; BP100-4,7MC, dark red. Peptides reconstituted in DMPC/DMPG LUVs (peptide/lipid = 1/20, mol/mol) are shown at pH 5.5 and 7.0. The peptide concentration in all samples was 70 or 100 μM for LUVs. (**B**) Photoswitching (MC-to-SP photoisomerization) of the spiropyran-containing BP100 analogs in PB (top), in the presence of membrane mimics (5 mM SDS solution, middle), and in lipid bilayers (DMPC/DMPG LUVs, bottom). BP100-7MC, red; BP100-4,7MC, black; MC photoform of the side chains, dotted lines; SP photoform, solid lines.

All peptides were largely unstructured in aqueous environment irrespective of pH. In the hydrophobic helix-promoting PB/TFE mixture, the peptides showed characteristic helix CD signals, with the intensity and degree of helicity decreasing in the order: BP100 > BP100-7F > BP100-7MC > BP100-4,7MC. This behavior strongly suggests a considerable helix-perturbing potential of the spiropyran residue in the MC form, as anticipated. When reconstituted in both types of SDS and DPC micelles, the spectra of the peptides demonstrated the same trend, but also revealed additional features. Thus, BP100 and BP100-7F displayed the typical α-helical CD spectra with characteristic negative bands at 208 and 222 nm of similar intensity, whereas the **3**-derived peptides showed a different n -> π* transition signal at around 230 nm, containing a positive ellipticity feature around 220 nm for BP100-4,7MC. The spectrum of BP100-7MC reflects a reduced helicity, while the positive signal at 220 nm in the spectrum of BP100-4,7MC suggests the presence of a kinked or partially unfolded structure probably associated with the residue of **3** at the fourth position. Finally, in the presence of LUVs, only BP100 was helical and well folded.

In a separate set of experiments, we photoisomerized the spiropyran-modified peptides *in situ* and compared the CD spectra in PB, SDS, and LUV environments at pH 7.0. As seen in Figure 5B, substantial photoinduced structural changes occurred in both spiropyran-modified peptides in SDS and, to a lesser extent, in DPC (data not shown). We note, that the obvious differences of the MC spectra in SDS may be attributed to the below cmc conditions of the latter experiment (cmc = 7-8 mM).

Interactions between photoswitchable BP100 analogs and lipid membranes

Next, we compared the peptides containing **3** with BP100 and BP100-7F in lipid bilayers mechanically oriented between thin glass slides (se the samples preparation in the SI) using solid-state NMR spectroscopy (ssNMR). First, we characterized peptide-induced bilayer perturbations via ^{2}H ssNMR in zwitterionic DMPC-d_{54} and anionic DMPG-d_{54}. Changes in the quadrupolar splitting of deuterated glycerophospholipids in the fluid lamellar phase in the presence of BP100 compared with pure bilayers are indicative of a strong bilayer thinning.[29] We observed thinning with all our peptides in the fluid-state DMPG-d_{54} and DMPC-d_{54} bilayers (T > 23°C) (Figure 6A). In contrast, membrane thickening due to interaction with the peptides was observed in gel-state bilayers (T < 23°C) in both DMPG-d_{54} and DMPC-d_{54}.

We further performed ^{2}H ssNMR measurements of the same samples upon *in situ* MC-to-SP photoswitching (Figure 6B). Irradiation of the samples containing BP100-7MC and BP100-4,7MC caused decolorization and changes in the ^{2}H ssNMR spectra. Above the phase-transition temperature, the splittings increased by approximately 3 kHz during irradiation, indicating that switching from the MC to the SP forms resulted in lesser bilayer thinning.

Using ^{31}P ssNMR in 1,2-di-(9Z-octadecenoyl)-*sn*-glycero-3-phosphoethanolamine (DOPE) bilayers, we evaluated the impact of BP100 and its analogs on the bilayer curvature. Since the lipid headgroup–associated surface of phospholipid assemblies is negatively curved in the inverted hexagonal phase state (H_{II} phase, inverted cylindrical micelles), and the lamellar phase state (L_{α} phase) can be assumed to have a close to zero curvature, the transition between two states can be associated with the induction of a positive or negative curvature by membrane-active compounds.[30] Temperature-dependent ^{31}P ssNMR spectra were collected using samples of peptide/DOPE bilayers aligned on the glass slides (as above). As shown in Figure 6C, BP100 and its analogs increased (*ca*. 11°C) and broadened the DOPE phase transition, unambiguously demonstrating a positive curvature induction even at low peptide concentrations (peptide/lipid = 1/100 mol/mol). The phase-transition temperature further increased by an additional 3°C–6°C upon *in situ* irradiation of the BP100 analogs inducing the SP forms.

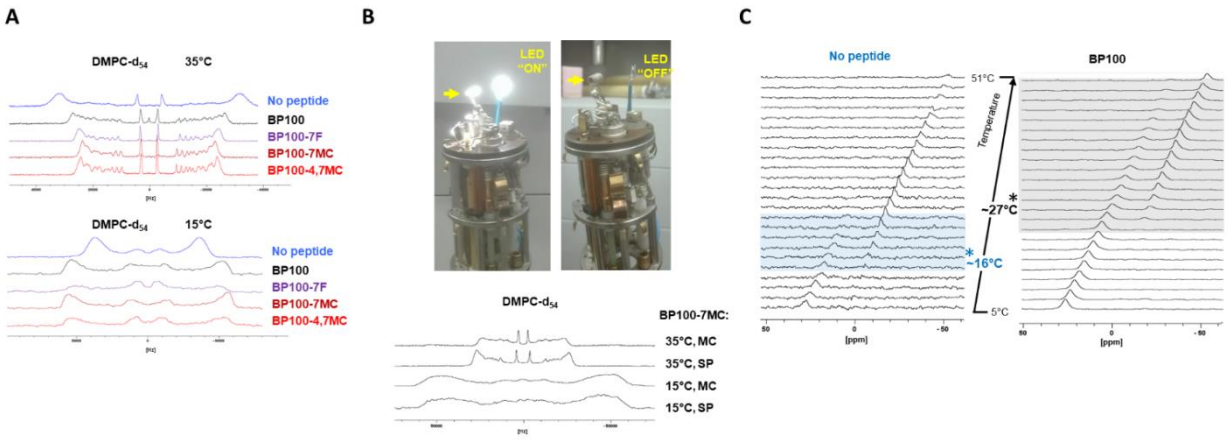

Figure 6. Solid-state NMR (ssNMR) studies of peptide/lipid interactions. (**A**) Representative ^{2}H ssNMR spectra of BP100 analogs in proteobilayers of DMPC-d_{54}. The spectra were measured above (35°C, top stacks) and below (15°C, bottom stacks) the main thermotropic phase transition (Tm, *ca*. 23°C). Fully hydrated proteobilayers with peptide/lipid = 1/10, mol/mol; spiropyran-modified peptides are in the dark-adapted form (MC). (**B**) Photoswitching (MC-to-SP photoisomerization) of the spiropyran-containing BP100 analogs. Top: installment of a LED into a static ssNMR probe. The arrow points to the sample-containing coil. Bottom: representative spectra in darkness ("MC") compared with the spectra under continuous LED illumination ("SP"). The traces above (35°C) and below (15°C) Tm were measured for B100-7MC in DMPC-d_{54}. (**C**) Representative ^{31}P ssNMR spectra of supported DOPE membranes undergoing thermotropic L_{α}-to-H_{II} phase transition. Pure lipid (left column) compared with the BP100/lipid ratio of 1/100, mol/mol, mixture (right column). $T_{L->H}$ (maximum) of the transition is indicated with an asterisk. The temperature range in which both phases coexist is highlighted with a background color.

Bioactivity of photoswitchable BP100 analogs

To determine whether the differential behavior of the spiropyran-derived BP-100 photoforms in lipid membranes described in the previous section manifests itself in living cells, we checked the toxicity of the novel peptides against a tobacco cell line, *Nicotiana tabacum L.* cv. Bright Yellow No. 2 (BY-2)[31] because BP100 was developed to treat plant pathogens.

BY-2 cell death induced by BP100 and its analogs at different concentrations was assessed via microscopy (Figure 7A). We qualitatively demonstrated *in situ* cytotoxicity photoswitching for samples containing 32 µg/mL of the spiropyran-modified peptides after incubation with the cells for 2 h. A photoinduced cytotoxicity enhancement was observed for both spiropyran-containing peptides (Figure 7B). A moderate increase in cell toxicity was induced by irradiating the cultures containing the peptides bearing **3**, causing the MC-to-SP photoisomerization.

We were surprised to observe overall high cytotoxicities against eukaryotic (plant) cells because BP100 is considered an antibacterial peptide with a relatively high therapeutic index.[32] The standard culturing pH for plant cells is somewhat acidic (pH 5.8), whereas antibacterial and hemolytic activities are generally

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measured at neutral values. Considering that the pH lowering may affect the MCH^+/MC equilibrium (see Table 1 and Figure 1 and 4), resulting in a more pronounced difference in amphiphilicity for our photoswitchable BP100 analogs (SP photoforms being apolar, MC polar, and MCH^+ charged), we studied the pH dependence of the antibacterial cytotoxicity of our peptides. As can be seen in Figure 7C, the SP photoforms of both **3**-substituted peptides were bacteriotoxic at pH 5 (16 μM concentration). In contrast, the photoisomerized peptides with destroyed amphiphilicity exerted virtually no effect on the growth of overnight cultures at this concentration, at any pH. The pH dependence of the BP100 bioactivity is intriguing but was not the focus of our study. We were delighted to find conditions affording dramatic differences in the amphiphilicity-dependent bioactivities.

Finally, the photoswitchable BP100 analogs were evaluated for their ability to interfere with the actin–auxin oscillator of plant cells, for which BP100 is active when applied at nontoxic concentrations and with short exposure times.[33] In these experiments, after the addition of the peptides photoisomerized to the SP forms (continuously illuminated with a white-light LED), the cells were immediately transferred to the microscopy stage to ensure that the photoconversion of the SP-to-MC photoforms occurred in real time.

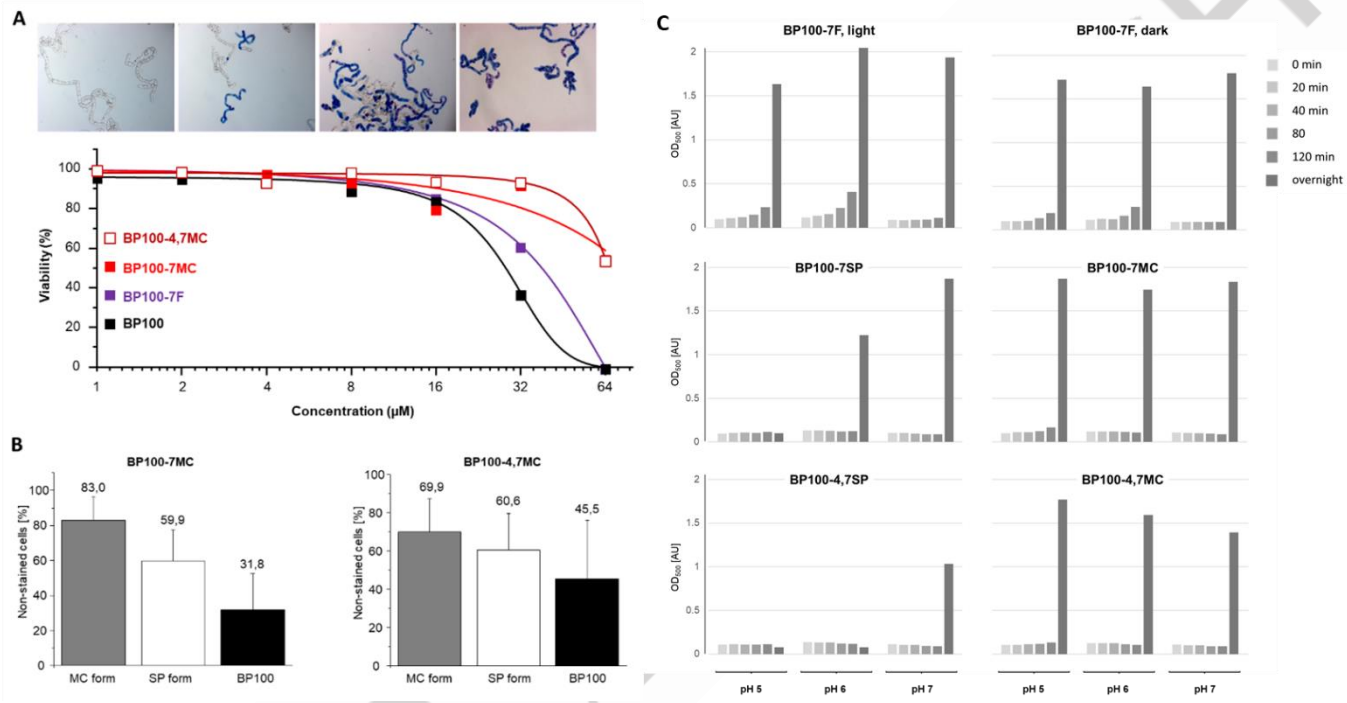

Figure 7. Cytotoxicity of the spiropyran-modified BP100 analogs. (**A**) Photographs of the cytotoxicity assessment of BP100 against BY-2 cells (left to right): 100%, 66%, 33%, and 0% nonstained (alive) cells. Results of cell counting (% alive) for the four peptides at different concentrations after 24 h coincubation. Photoswitchable peptides in the MC photostate. (**B**) Comparison of the cytotoxicity of BP100 and its analogs against BY-2 cells (peptide concentration, 32 μg/mL; incubation with cells, 2 h). (**C**) pH dependence of the antibacterial activity (*E. coli*) of BP100 analogs. Nonphotoswitchable, BP100-7F, and spiropyran-modified BP100 analogs were added to bacterial suspensions at 16 μM and incubated under continuous white LED light illumination (left column, indicated as “light” or “SP”) or in darkness (right column, indicated as “dark” or “MC”)

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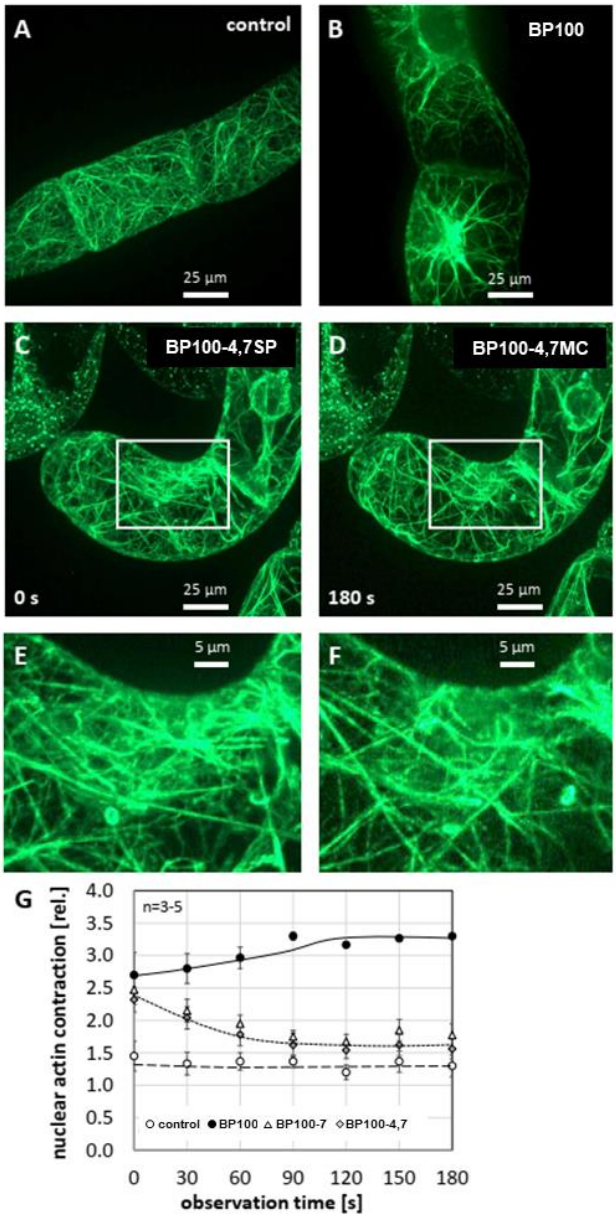

Figure 8. Response of plant actin filaments to BP100 and its photoswitchable analogs. Representative tobacco BY-2 cells expressing the actin-binding domain 2 of fimbrin 1 from *Arabidopsis thaliana* in fusion with GFP were imaged via spinning-disc confocal microscopy without treatment (control, **A**) and in the presence of 5 µM of BP100 (BP100, **B**) and BP100-4,7, respectively, during SP-to-MC isomerization (BP100-4,7SP/BP100-4,7MC, **C–F**). (**C**) Representative first frame (0 s) of a cell treated with BP100-4,7 in the SP photostate and (**D**) image of the same cell 180 s later. To evaluate the changes in nuclear actin contraction, the regions within the white rectangles in **C** and **D** were magnified (**E** and **F**, respectively). (**G**) Time-dependent changes in nuclear actin contraction in the presence of different peptides, quantified from the time-lapse series according to ref. [34]. Data represent mean and standard error (three to five individual cells were examined).

This observation revealed dark adaptation–associated changes in actin remodeling (Figure 8). Remarkably, the SP photoisomers of both photoswitchable peptides caused filament contraction at almost the same level as the wild-type peptide. After approximately 90 s, when most of the spiropyran residues should have been converted to the MC photostates, the actin dynamics were restored to levels compatible with the control (nontreated) cells (Figure 8G). Overall, this result suggests that for the BP100-induced actin–auxin oscillator modulation, the amphiphilicity of the peptide is important, and that a spiropyran photoisomeric state of the residue **3** can modify the biological activity of membrane-active peptides.

Conclusion

In summary, we demonstrated the utility of a spiropyran photoswitch for modulating the structure and function of a membrane-active peptide using the known peptide BP100 as a representative example. Amino acid **3** bearing a spiropyran core in its side chain was designed to fulfill strict criteria for the practical application of the corresponding peptides in living systems. Photoswitchable analogs of BP100 in SP and MC photostates were found to be differentially active in the induction of membrane thinning and in membrane curvature modulation. Furthermore, their helicity substantially differed in detergent solutions, and their cytotoxicity correlated with the degree of structuring, being lower in the MC photostates where amphiphilicity was destroyed. In the SP photostates, BP100 analogs demonstrated a degree of actin dynamics modulation similar to that of the wild-type peptide in live plant cells. In contrast, the MC isomers did not interfere with cell cytoskeleton dynamics. This work has laid out the fundamental design principles for spiropyran-derived photoswitchable amino acids, paving the way for future applications of spiropyran-modified peptides in photopharmacology and beyond.

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Keywords: membrane-active peptides • BP100 • molecular photoswitches • photopharmacology • spiropyran

[1] M. Muttenthaler, G.F. King, D.J. Adams, P.F. Alewood, *Nat. Rev. Drug Discov*. **2021**, *20*, 309.

[2] a) M. Goodman, A. Kossoy, *J. Am. Chem. Soc.* **1966**, *88*, 5010; b) M. Goodman, , M.L. Falxa, *J. Am. Chem. Soc.* **1967**, *89*, 3863.

[3] a) O. Pieroni, , A. Fissi, G. Popova, *Progr. Polym. Sci.* **1998**,*23*, 81; b) O. Pieroni, A. Fissi, N. Angelini, F. Lenci, *Acc. Chem. Res*. **2001**, *34*, 9; c) C. Renner, L. Moroder, *ChemBioChem* **2006**, *7*, 868; d) J.E. Bock, J. Gavenonis, J.A. Kritzer, *ACS Chem. Biol*. **2013**, *8*, 488; e) R.J. Mart, R.J. Errington, C.L. Watkins, S.C. Chappell, M. Wiltshire, A.T. Jones, P.J. Smith, R.K. Allemann, *Mol. BioSyst*. **2013**, *9*, 2597; f) S. Wiedbrauk, H. Dube, *Tetrahedron Lett*. **2015**, *56*, 4266; g) R.J. Mart, R.K. Allemann, *Chem. Commun*. **2016**, *52*, 12262; h) S. Kitzig, M. Thilemann, T. Cordes, K. Rück-Braun, *ChemPhysChem* **2016**, *17*, 1252; i) L. Albert, O. Vázquez, *Chem. Commun*. **2019**, *55*, 10192; j) T.C. de Souza‐Guerreiro, G. Bondelli, I. Grobas, S. Donini, V. Sesti, C. Bertarelli, G. Lanzani, M. Asally, G.M. Paternò, *Adv. Sci.* **2023**, *10*, 2205007; k) F. Höglsperger,

B.E. Vos, A.D. Hofemeier, M.D. Seyfried, B. Stövesand, A. Alavizargar, L. Topp; A. Heuer, T. Betz, B.J. Ravoo, *Nat. Commun*. **2023**, *14*, 3760.

[4] a) W.A. Velema, W. Szymanski, B.L.Feringa, *J. Am. Chem. Soc*. **2014**,*136*, 2178; b) J. Broichhagen, J.A. Frank, D. Trauner, *Acc. Chem. Res*. **2015**, *48*, 1947; c) M.M. Lerch, M.J. Hansen, G.M. van Dam, W. Szymanski, B.L. Feringa, *Angew. Chem. Int. Ed*. **2016**, *55*, 10978; d) K. Hüll, J. Morstein, D. Trauner, *Chem. Rev*. **2018**, *118*, 10710.

[5] a) E. Fischer, Y. Hirshberg, *J. Chem. Soc.* **1952**, 4522; b) Y. Hirshberg, E. Fischer, *J. Chem. Soc*. **1953**, 629; c) G. Berkovic, V. Krongauz, V. Weiss, *Chem. Rev*. **2000**, 100, 1741; d) V.I. Minkin, *Chem. Rev*. **2004**,104, 2751; e) R. Klajn, *Chem. Soc. Rev*. **2014**, *43*, 148; f) L. Kortekaas, W.R. Browne, *Chem. Soc. Rev*. **2019**, *48*, 3406.

[6] a) J. Andersson, S. Li, P. Lincoln, J. Andréasson, *J. Am. Chem. Soc.* **2008**, *130*, 11836;b) W.A.Velema, M.J.Hansen, M.M. Lerch, A.J. Driessen, W. Szymanski, B.L. Feringa, *Bioconjugate Chem*. **2015**, *26*, 2592.

[7] a) E. Zahavy, S. Rubin, I. Willner, *Mol. Cryst. Liq. Cryst. A*. **1994**, *246*, 195; b) A. Fissi, O. Pieroni, N. Angelini, F. Lenci, *Macromolecules* **1999**, *32*, 7116; c) T.M. Cooper, L.V. Natarajan, C.G. Miller, *Photochem. Photobiol*. **1999**, *69*, 173; d) K. Fujimoto, M. Amano, Y. Horibe, M. Inouye, *Org. Lett*. **2006**, *8*, 285.

[8] a) K.Y. Tomizaki, H. Mihara, *J. Mater. Chem*. **2005**, *15*, 2732; b) K.Y. Tomizaki, H. Mihara, *Mol. BioSyst.* **2006**, *2*, 580.

[9] L. Chen, J. Wu, C. Schmuck, H. Tian, *Chem. Comm*. **2014**, *50*, 6443.

[10] a) Z. Qiu, H. Yu, J. Li, Y. Wang, Y. Zhang, *Chem. Comm.* **2009**, 3342; b) W. Wang, J. Hu, M. Zheng, L. Zheng, H. Wang, Y. Zhang, *Org. Biomol. Chem.* **2015**, *13*, 11492.

[11] L. Chen, Y. Zhu, D. Yang, R. Zou, J. Wu, H. Tian, *Sci Rep*. **2014**, *4*, 1.

[12] The paper of Tomizaki, et al.[8a] described the use of lysine-derived spiropyran derivatives in peptide synthesis. However, this work was not focused on the amino acid design and synthesis.

[13] Y. Huan, Q. Kong, H. Mo, H. Yi, *Front. Microbiol*. **2020**, *11*, 2559.

[14] B. Apellániz, N. Huarte, E. Largo, J.L. Nieva, *Chem. Phys. Lipids* **2014**, *181*, 40.

[15] K. Desale, K. Kuche, S. Jain, *Biomaterials Sci* **2021**, *9*, 1153.

[16] F.G. Avci, A.B. Sariyar, E. Ozkirimli, *Biomolecules* **2018**, *8*, 77.

[17] a) I. Shimizu, H. Kokado, E. Inoue, *Bull. Chem. Soc. Jpn*. **1969**, *42*, 1730; b) J. Zhou, Y. Li, Y. Tang, F. Zhao, X. Song, E. Li, *J. Photochem. Photobiol. A* **1995**, *90*, 117.

[18] a) T. Stafforst, D. Hilvert, *Chem Commun*. **2009**, 287; b) C. Özçoban, T. Halbritter, S. Steinwand, L.M. Herzig, J. Kohl-Landgraf, N. Askari, F, Groher, B. Fürtig, C. Richter, H. Schwalbe, B. Suess, J. Wachtveitl, A. Heckel, *Org. Lett*. **2015**, *17*, 1517.

[19] K. Nepali, H.Y. Lee, J.P. Liou, *J. Med. Chem*. **2018**, *62*, 2851.

[20] a) C.J. Roxburgh, P.G. Sammes, A. Abdullah, *Dyes Pigm*. **2009**, *83*, 31; b) E.I. Balmond, B.K.Tautges, A.L. Faulkner, V.W. Or, B.M. Hodur, J.T. Shaw, A.Y. Louie, *J. Org. Chem.* **2016**, *81*, 8744; c) M. Schulz-Senft, P.J. Gates, F.D. Sönnichsen, A. Staubitz, *Dyes Pigm*. **2017**, *136*, 292.

[21] a) Z. Shi, P. Peng, D. Strohecker, Y. Liao, *J. Am. Chem. Soc*. **2011**, *133*, 14699; b) V.K. Johns, Z. Wang, X. Li, Y. Liao, *J. Phys. Chem. A* **2013**, *117*, 13101; c) C. Fu, J. Xu, C. Boyer, *Chem. Commun*. **2016**, *52*, 7126; d) H. Gao, T. Guo, Y. Chen, Y. Kong, Z. Peng, *J. Mol. Struct*. **2016**, *1123*, 426; e) D. Moldenhauer, F. Gröhn, *Chem. Eur. J.* **2017**, *23*, 3966.

[22] L. Kortekaas, J. Chen, D. Jacquemin, W.R. Browne, *J. Phys. Chem. B* **2018**, *122*, 6423.

[23] E. Badosa, R. Ferre, M. Planas, L. Feliu, E. Besalú, J. Cabrefiga, E. Bardají, E. Montesinos, *Peptides* **2007**, *28*, 2276.

[24] P. Wadhwani, E. Strandberg, J. van den Berg, C. Mink, J. Bürck, R.A.M. Ciriello, A.S. Ulrich, *Biochim. Biophys. Acta* **2014**, *1838*, 940.

[25] A. Roos, W.F. Boron, *Physiol. Rev*. **1981**, *61*, 296.

[26] a) S.R. Keum, M.S. Hur, P.M. Kazmaier, E. Buncel, *Can. J. Chem*. **1991**, *69*, 1940; b) X. Song, J. Zhou, Y. Li, Y. Tang, *J. Photochem. Photobiol. A* **1995**, *92*, 99.

[27] L. Cui, H. Zhang, G. Zhang, Y. Zhou, L. Fan, L. Shi, C. Zhang, S. Shuang, C. Dong, *Spectrochim. Acta A* **2018**, *202*, 13.

[28] N.W. Tyer Jr, R.S. Becker, *J. Am. Chem. Soc*. **1970**, *92*, 1289.

[29] a) S.L. Grage, S. Afonin, S. Kara, G. Buth, A.S. Ulrich, *Front. Cell Dev Biol.* **2016**, *4*, 65; b) J. Misiewicz, S. Afonin, A.S. Ulrich, *Biochim. Biophys. Acta* **2015**, *1848*, 833.

[30] a) S. Pujals, H. Miyamae, S. Afonin, T. Murayama, H. Hirose, I. Nakase, K. Taniuchi, M. Umeda, K. Sakamoto, A.S. Ulrich, S. Futaki, *ACS Chem. Biol.* **2013**, *8*, 1894; b) W.Y. Hsu, T. Masuda, S. Afonin, T. Sakai, J. V. V.Arafiles, K. Kawano, H. Hirose, M. Imanishi, A.S. Ulrich, S. Futaki, *Bioorg. Med. Chem. Lett.* **2020**, *30*, 127190.

[31] T. Nagata, Y. Nemoto, S. Hasezawa, *Int. Rev. Cytol*. **1992**, *132*, 1

[32] I.M.Torcato, Y.H.Huang, H.G. Franquelim, D. Gaspar, D.J. Craik, M.A. Castanho, S.T. Henriques, *Biochim. Biophys. Acta Biomembr.* **2013**, *1828*, 944.

[33] K. Eggenberger, P. Sanyal, S. Hundt, P. Wadhwani, A.S. Ulrich, P. Nick, *Plant Cell Physiol.* **2017**, *58*1,71.

[34] H. Wang, M. Riemann, Q. Liu, J. Siegrist, P. Nick, *Plant Sci*. **2021**, *302*, 110712

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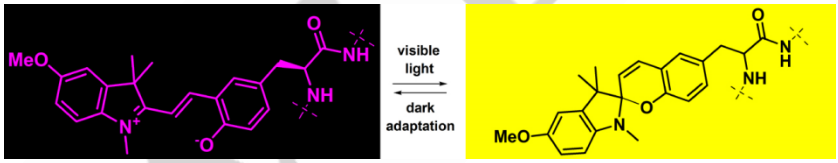

A spiropyran-derived α-amino acid was carefully designed for the synthesis of photocontrollable bioactive peptides. Dramatic changes in structure and polarity occurring upon light exposure or dark adaptation of the spiropyran side chain can cause changes in the behavior of peptides in lipid membranes and living cells, as demonstrated on spiropyran-containing analogs of the membrane-active peptide BP100 (KKLFKKILKYL-NH_2).

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