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Epidithiodiketopiperazines: Strain-Promoted Thiol-Mediated Cellular Uptake at the Highest Tension

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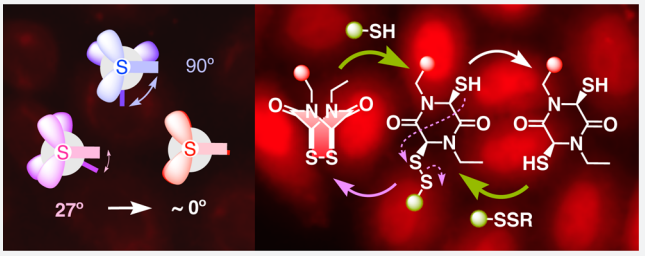
Supporting Information

ABSTRACT: The disulfide dihedral angle in epidithiodiketopiperazines (ETPs) is near 0°. Application of this highest possible ring tension to strain-promoted thiol-mediated uptake results in efficient delivery to the cytosol and nucleus. Compared to the previous best asparagusic acid (AspA), ring-opening disulfide exchange with ETPs occurs more efficiently even with nonactivated thiols, and the resulting thiols exchange rapidly with nonactivated disulfides. ETP-mediated cellular uptake is more than 20 times more efficient compared to AspA, occurs without endosomal capture, depends on temperature, and is "unstoppable" by inhibitors of endocytosis and conventional thiol-mediated uptake, including siRNA against the transferrin receptor. These results suggest that ETP-mediated uptake not only maximizes delivery to the cytosol and nucleus but also opens the door to a new multitarget hopping mode of action.



INTRODUCTION

Epidithiodiketopiperazines (ETPs) such as verticillin **1** are an intriguing family of natural products with a broad variety of biological activities (Figure 1A).[1−8] Their complex structures have attracted considerable interest in synthetic organic chemistry. The distinguishing feature of ETPs is the bicyclic disulfide with the CSSC dihedral angle $\theta \approx 0°$ (5.7° and 8.6° have been observed in crystals, Figure 1B).[5,6] This is remarkable because relaxed disulfides have $\theta \approx 90°$.[9] Despite having the highest possible strain energy, ETPs are stable, unlike 1,2-dithietanes, which occur only as reactive intermediates except for rare and remarkable exceptions such as dithiatopazine **2**.[10]

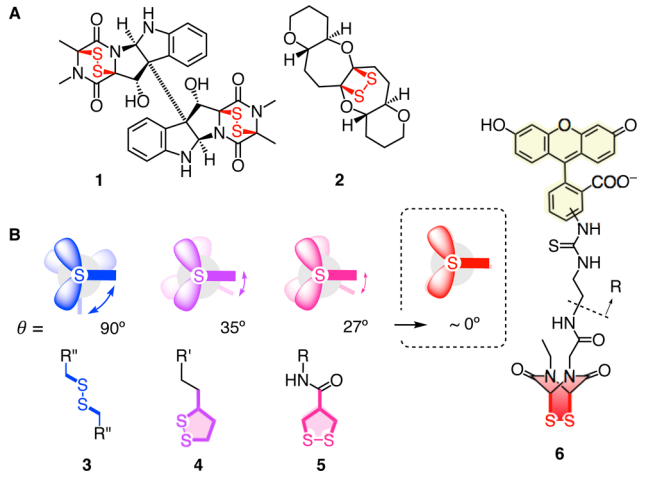


Figure 1. (A) Structure of verticillin **1**, a representative ETP natural product, and 1,2-dithietane **2**. (B) Structure of ETP transporter **6** with AspA control **5** and examples for decreasing disulfide ring tension.

We became interested in disulfide ring tension with regard to cellular uptake.[11−14] Disulfides in general are increasingly recognized to enter cells by thiol-mediated uptake, i.e., covalent attachment by disulfide exchange with exofacial thiols followed by efficient uptake via diverse, to a good part unknown mechanisms.[11−23] The emergence of thiol-mediated uptake called for the application of ring tension.[11] Uptake efficiencies were found to increase with ring tension from relaxed disulfides **3** with $\theta \approx 90°$ to lipoic acid derivatives **4** with $\theta = 35°$ and asparagusic acid derivatives **5** with $\theta = 27°$.[12,13] The most efficient "AspA tag" as in **5** allowed the delivery of functional peptides,[14] liposomes and polymersomes[13] into cells, and the transferrin receptor (TFRC) has been identified as one of the targets.[14] The power and promise of strain-promoted thiol-mediated uptake at $\theta = 27°$ provided a compelling incentive to drive disulfide ring tension to the extreme. To tackle this challenge, ETPs appeared just perfect. Their high reactivity in disulfide exchange reactions was predicted computationally and demonstrated experimentally to be crucial for the function of some natural ETPs.[1−9,23] Here, we introduce "ETP tags" for the "unstoppable" strain-promoted delivery of model probes **6** to the cytosol and nucleus, and reveal a new mechanism with distinct characteristics.

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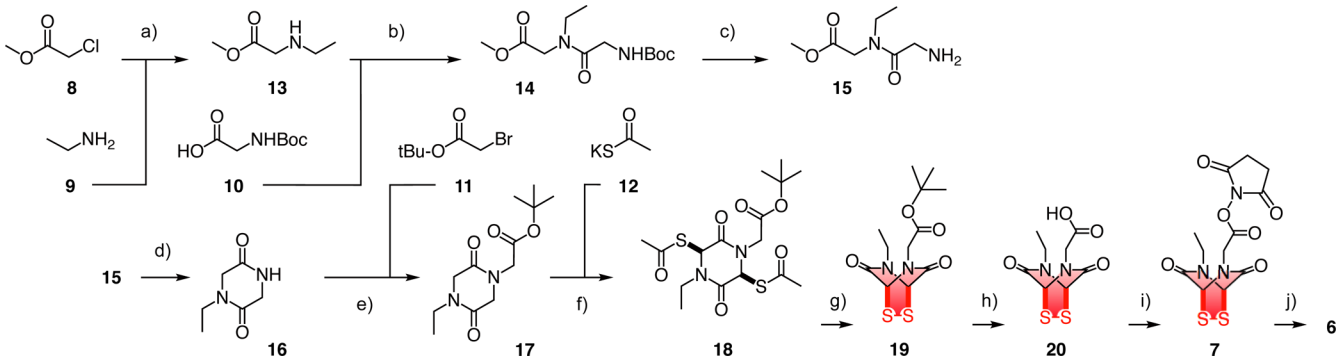
Scheme 1[a]



[a](a) K_2CO_3, CH_3CN, rt, 12 h, 54%; (b) DCC, DMAP, Et_3N, CH_2Cl_2, rt, 24 h, 74%; (c) TFA, CH_2Cl_2, 0 °C to rt, 30 min; (d) toluene, reflux, 6 h, 79% (from **14**); (e) NaH, THF, 0 °C to rt, 12 h, 87%; (f) 1. NBS, AIBN, cyclohexane, reflux, 2 h, 2. **12**, CH_2Cl_2, rt, 12 h; 34% (7.5% *trans*); (g) 1. NH_3, MeOH, rt, 30 min, 2. I_2, CH_2Cl_2, rt, 30 min, 63%; (h) TFA, CH_2Cl_2, rt, 2 h; (i) NHS, DCC, THF, rt, 24 h; (j) R-NH_2 (see Figure 1), DMF, rt, 2 h, 68% (three steps from **19**).

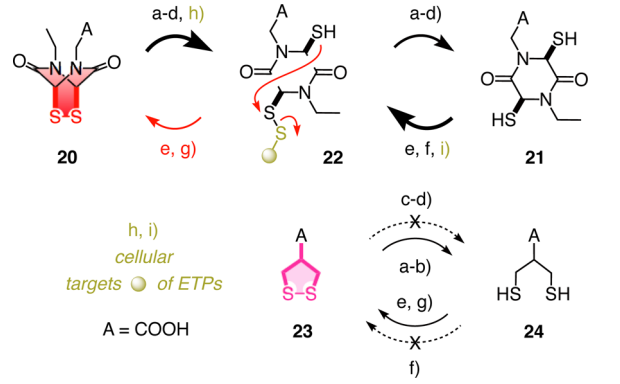
RESULTS AND DISCUSSION

The ETP tag **7** was synthesized, as in biosynthesis, using exclusively C_2 building blocks derived from acetate, i.e., **8**−**12** (Scheme 1). At the beginning, chloroacetate **8** was reacted with ethylamine **9**. The resulting secondary amine **13** was coupled with Boc-protected glycine **10**. Liberation of the amine in the obtained dipeptide **14** prepared for the cyclization of **15**. The resulting diketopiperazine heterocycle **16** was alkylated with bromoacetate **11**. With dilactam **17**, a key intermediate was reached. The sulfur atoms were introduced via radical bromination followed by substitution with thioacetate **12**.[24] The cis isomer **18** was obtained as the major product (4.5:1), easily separated from the trans isomer, and assigned by a strong NOE between the two remaining endocyclic hydrogens. Hydrolysis of the thioesters **18** with ammonia afforded the free thiols, which were immediately oxidized with molecular iodine to afford the high-tension ETP disulfide **19** in excellent 63% yield as a pale yellow solid. The bicyclic ETP scaffold remained intact during the acid-catalyzed removal of the tBu protecting group in **19**, the activation of the resulting acid **20** with *N*-hydroxysuccinimide (NHS), and reaction of the resulting ETP tag **7** with amines of free choice, here a fluorescent model substrate, under mildest conditions, to give the CF−ETP conjugate **6** in 68% yield.

In D_2O at pD 8.0, equimolar DTT reduced 5 mM ETP **20** instantaneously and completely to dithiol **21** (Table 1, entry a, Figure S10). This was also true at pD 5.5 and with 2 equiv of glutathione (GSH) at pD 8.0 (Table 1, entries b and c, Figures S9 and S14). At pD 5.5 with GSH, the consumption of the hyperstrained disulfide **20** reached 50% within the time needed to set up and record an ^{1}H NMR spectrum (Table 1, entry d, Figure S13). In sharp contrast, AspA **23** reacted slowly with DTT and failed to react with GSH under these conditions (Table 1, entries a−d, Figures S11, S12, S15, and S16).

To explore the formation of strained disulfides by disulfide exchange, dithiols **21** and **24** were prepared in situ by 1 equiv of TCEP. Subsequent addition of 1 equiv of DTNB **25** in neutral water gave rise to the strained ETP **20** and AspA **23** instantaneously (Table 1, entry e, Figures S17 and S18). With 2 equivalents of oxidized glutathione GSSG, a much less reactive disulfide, the reduced ETP **21** exchanged rapidly into the tension-free mixed disulfide **22** (Table 1, entry f, Figure S19), and, with time, ring closure into hyperstrained ETP **20** could be observed (Table 1 entry g, Figure S20). The high reactivity of reduced ETP **21** could be ascribed to the lower than usual pK_a of thiols due to the presence of lactam nitrogen and carbonyl groups on the α position. Besides high tension,

Table 1. Disulfide Exchange Cycles[a]



		ETP[a]			AspA[a]	
E[b]	S[c]	pD[d]	t[e]	η (%)[f]	t[e]	η (%)[f]
			20 → 21		**23 → 24**	
a)	DTT	8.0	<5 min	100	30 min	98
b)	DTT	5.5	<5 min	100	60 min	14
c)	GSH	8.0	<5 min	100	18 h	0
d)	GSH	5.5	<5 min	50[g]	18 h	0
			21 → 22 → 20[h]		**24 → 23**[h]	
e)	DTNB	7.2	<5 min	100 (**20**)	<5 min	100
f)	GSSG	7.2	<5 min	100 (**22**)	30 min	0
g)	GSSG	7.2	16 h	100[i]	16 h	70

[a]For ETP **20** and AspA **23** (5 mM), determined by ^{1}H NMR kinetics; for original spectra, see Figures S9−S22. [b]Entry, letters refer to reaction scheme. [c]Substrates, DTT: 1,4-dithiothreitol, 5 mM (1 equiv); GSH: glutathione, 10 mM (2 equiv), DTNB: 5,5-dithio-bis(2-nitrobenzoic acid) (**25**, Figure 3), 5 mM (1 equiv); GSSG: oxidized GSH, 5 mM (1 equiv). [d]pD in 0.1 M aqueous (D_2O) sodium phosphate buffer. [e]Reaction time at rt, in D_2O. [f]Conversion, determined from integration of NMR signals. Unless specified, only designated products are formed. [g]Unidentified product formed. [h]Fully reduced starting materials **21** and **24** were prepared in situ from **20** and **23** with 1 equiv TCEP (tris(2-carboxyethyl)phosphine, entries e−g). [i]ETP **20** (20%) could be identified from the mixture of products at least partially arisen from the decomposition of **20**.

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this increased acidity also explained the ease of ring-opening disulfide exchange (**20** → **21**/**22**) and the reluctance of ring closure (**22** → **20**). In comparison, the thiols of the reduced AspA control **24** were much less reactive toward nonactivated disulfides (Table 1, entry f, Figure S21), whereas formation of the less strained dithiolane ring was faster (Table 1, entry g, Figure S22). Control experiments without GSSG resulted in very little auto-oxidative ring closure to **20** or **23**, thus demonstrating that the rings form through the mixed disulfides, such as **22**, by disulfide exchange reactions. In summary, compared to AspAs, ETPs are (1) more reactive in ring-opening disulfide exchange with nonactivated thiols, also under acidic conditions, (2) more reactive in their reduced form with nonactivated disulfides, and (3) less efficient in ring-closing disulfide exchange to go full cycle and reproduce the hyperstrained ETPs in neutral water (Table 1).

The uptake of the green-fluorescent CF-ETP conjugate **6** into HeLa Kyoto cells was monitored by confocal laser scanning microscopy (CLSM). Incubation with 10 μM **6** in Leibovitz medium for 1 h at 37 °C resulted in intense homogeneous emission from the cytosol and particularly from the nuclei, including nuclei that were poorly stained by Hoechst 33342 (Figure 2C). This result contrasted sharply from the uptake of the AspA control **5**, which failed to reach the nucleus and produced mostly punctate emission at much lower intensity (Figure 2B). The same distinct differences between ETP **6** and AspA **5** were observed in several other cell lines (Figure 3A,B).

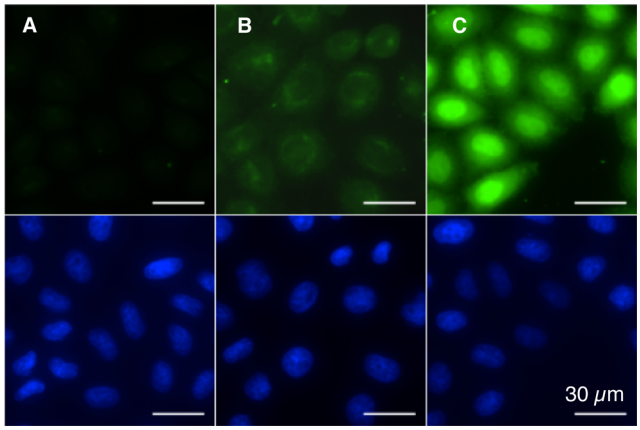

Figure 2. CLSM images of HeLa Kyoto cells after 1 h of incubation with 10 μM CF-NH_2 (A), CF-AspA **5** (B), and CF-ETP **6** (C) in Leibovitz medium at 37 °C (top), together with Hoechst 33342 to stain the nuclei (bottom).

The punctate emission obtained with AspA **5** can be assigned with confidence to receptor-mediated delivery into endosomes.[14] The absence of punctate emission suggested that contrary to AspA **5**, ETP **6** does not suffer from endosomal capture and is delivered exclusively to the cytosol and nucleus. Different from the polycationic CPDs,[11] accumulation of the overall anionic ETPs in the nuclei is not driven by ion pairing and thus not limited to the DNA-rich nucleoli. Possibly, the presence of target proteins with reactive thiols, such as histone methyl transferase,[7,8] dictates the intracellular distribution of ETPs. ETPs continued to deliver efficiently at concentrations as low as 500 nM, whereas detectable uptake of AspAs stopped below 5 μM (Figure 3C,D). Still higher intensities obtained with ETPs at 500 nM than with AspAs at 10 μM suggested that ETPs are at least 20 times more active (Figure 3C,D).

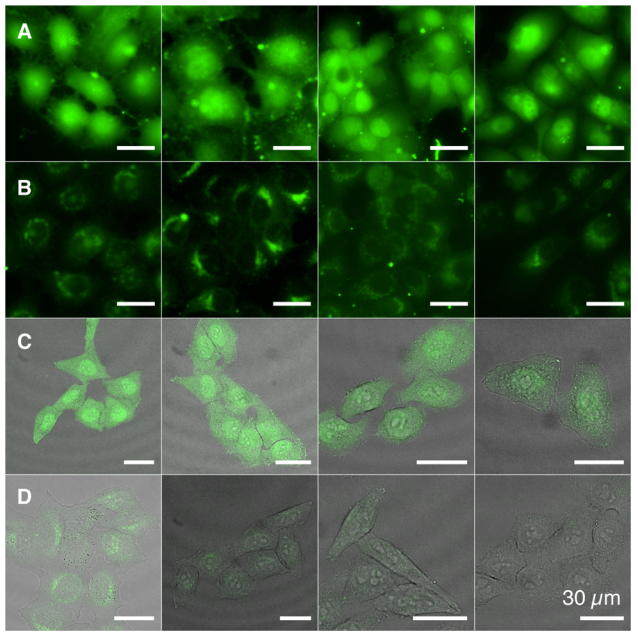

Figure 3. Microscopic images of (A, C) ETP **6** and (B, D) AspA **5** after (A, B) incubation (Leibovitz medium, 37 °C) at 10 μM with A431, Huh7, MCF7, and PC-3 cells (left to right, automated microscope images) and (C, D) incubation with HeLa Kyoto cells at 10 μM, 5 μM, 1 μM, and 500 nM (left to right; CLSM images merged with differential interference contrast (DIC)).

As many natural ETPs are toxins, the MTT assay was employed to assess the toxicity of ETP tags in HeLa Kyoto cells. This assay reports on the enzymatic conversion of the tetrazolium dye MTT into formazan, that is, the metabolic activity of the cells.[25] The positive control, polyarginine (pR), was confirmed to be cytotoxic at 10 μM (Figure 4A).[11] Under the same conditions, ETP **6** and AspA **5** were not toxic (Figure 4A).

Flow cytometry analysis confirmed the impression from CLSM images that the hyperstrained ETP **6** is much more active than the AspA control **5** (Figure 4B). The loss of essentially all activity at 4 °C is commonly interpreted as indication of uptake by endocytosis (Figure 4B). However, other possible explanations such as changes in disulfide exchange kinetics, membrane fluidity, etc., should not be forgotten, particularly since all common endocytosis inhibitors were inactive. Namely, insensitivity toward chlorpromazine (CPZ) excluded clathrin-mediated endocytosis, methyl-β-cyclodextrin (mβCD) caveolae-mediated endocytosis, and wortmannin and cytochalasin B (cytoB) ruled out macropinocytosis (Figure 4B).[11,22,26−28]

Contrary to AspA controls,[12,13] the removal of thiols on cell surfaces with maleimide **26**,[22] iodoacetamide **27**,[12] and the most powerful hypervalent iodine reagent **28**[29] failed to inactivate ETP **6** (Figure 4C). Similarly, the presence of 10% serum[22] caused only a minor ∼25% reduction of ETP uptake (Figures 4C and S5). DTNB **25** was special because this reagent converts thiols on cell surfaces into activated disulfides. After preincubation with 1.2 mM DTNB, uptake activity of ETP **6** indeed dropped to ∼65% (Figure 4C). However, unlike AspA tags,[12] ETP activity increased rather than decreased with further increasing DTNB concentration to reach saturation

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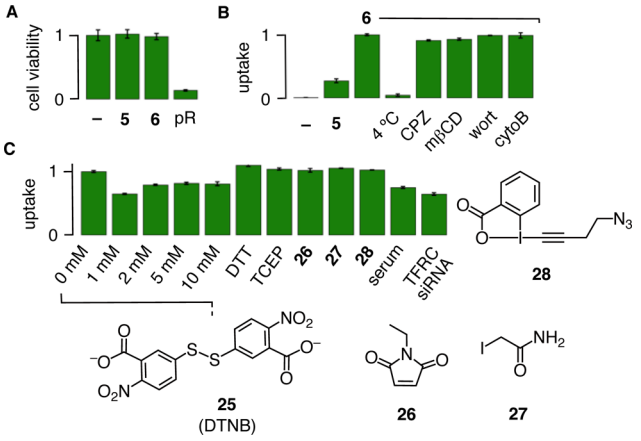

Figure 4. (A) Cell viability from MTT assay for 10 μM transporters in HeLa Kyoto cells; pR: polyarginine. (B) Flow cytometry data for HeLa Kyoto cells and **6** with endocytosis inhibitors (CPZ, mβCD, wort, cytoB), temperature dependence, and comparison to **5**, normalized to 1 for **6**. (C) Flow cytometry data for **6** and HeLa Kyoto cells that were preincubated with inhibitors (**25**–**28**, 0.02–2 mM) and activators (DTT, TCEP, 2 mM) of thiol-mediated uptake, with 10% serum, and with TFRC siRNA (quantified using automated microscope). Shown are average values ± error.

near 80%. Also unlike AspA controls,[12] preincubation of the cells with DTT or TCEP did not strongly increase the activity of ETPs (Figure 4C). Most importantly, the knockdown of the transferrin receptor (TFRC) with siRNA inhibited the uptake of AspA controls[14] but failed to inhibit ETP-mediated uptake. The observed partial inactivation by TFRC knockdown down to ~65% was most revealing (Figures 4C and S7). It supported that (1) ETPs operate by thiol-mediated uptake, that is, dynamic covalent disulfide exchange on the cell surface, (2) ETPs do not depend on single targets such as the transferrin receptor, and (3) ETPs have access to targets that are inaccessible to AspA controls.

CONCLUSIONS

In this report, we introduce ETP-mediated cellular uptake. Epidithiodiketopiperazines attracted our attention to drive ring tension in cyclic disulfides to the maximum, i.e., a CSSC dihedral angle of ~0°. However, rather than simply maximizing the efficiency of strain-promoted thiol mediated uptake,[12−14] completely new, exceptionally promising properties emerged. ETP-mediated uptake excels with the efficient, nontoxic delivery to cytosol and particularly nucleus, without any endosomal capture, sensitive to temperature but "unstoppable" by all conventional inhibitors of endocytosis and thiol-mediated uptake. This poor responsiveness to inhibitors and activators such as cytochalasin B, DTT, Ellman's reagent, TFRC siRNA, or serum indicated that the unique reactivity of ETPs is decisive for function. High reactivity of ETPs in both oxidized and reduced form allows for covalent capture by nonactivated thiols and disulfides in cellular targets[29] that are otherwise beyond reach (Table 1, entries a–d, f, h–i). Moreover, the possibility of repeated disulfide-exchange cycles in neutral water suggested that ETPs can change targets during uptake (Table 1, entries a–d, e–g). Such a multitarget hopping mechanism could explain the characteristics found for ETP-mediated uptake: namely, efficient delivery to cytosol and nucleus, without endosomal capture, without toxicity. These stunning characteristics invite the highest expectations with regard to the general, covalent, charge-free delivery of substrates of biological and medicinal relevance.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acscentsci.7b00080.

Detailed experimental procedures (PDF)

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Notes

The authors declare no competing financial interest.

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