Controlling Antibacterial Activity Exclusively with Visible Light: Introducing a Tetra-*ortho*-Chloro-Azobenzene Amino Acid

Xavier Just-Baringo,*[a, b] Alejandro Yeste-Vázquez,[a] Javier Moreno-Morales,[c] Clara Ballesté-Delpierre,[c] Jordi Vila,[c, d] and Ernest Giralt[a]

Dedicated to Prof. Mercedes Álvarez on her retirement

Abstract: The introduction of a novel tetra-*ortho*-chloroazobenzene amino acid (CEBA) has enabled photoswitching of the antimicrobial activity of tyrocidine A analogues by using exclusively visible light, granting spatiotemporal control under benign conditions. Compounds bearing this photoswitchable amino acid become active upon irradiation with red light, but quickly turn-off upon exposure to other visible light wavelengths. Critically, sunlight quickly triggers isomerisation of the red light-activated compounds into their original *trans* form, offering an ideal platform for self-deactivation upon release into the environment. Linear analogues of tyrocidine A were found to provide the best photocontrol of their antimicrobial activity, leading to compounds active against *Acinetobacter baumannii* upon isomerisation. Exploration of their *N*- and *C*-termini has provided insights into key elements of their structure and has allowed obtaining new antimicrobials displaying excellent strain selectivity and photocontrol.

[a] *Dr. X. Just-Baringo, A. Yeste-Vázquez, Prof. E. Giralt*
Institute for Research in Biomedicine (IRB Barcelona)
Barcelona Institute of Science and Technology (BIST)
Baldiri Reixac 10, 08028 Barcelona (Spain)

[b] *Dr. X. Just-Baringo*
Laboratori de Química Orgànica
Facultat de Farmàcia
IBUB, Universitat de Barcelona
08028 Barcelona (Spain)
E-mail: xavier.just@ub.edu

[c] *J. Moreno-Morales, Dr. C. Ballesté-Delpierre, Prof. J. Vila*
Institute for Global Health (ISGlobal)
Hospital Clínic - Universitat de Barcelona
Barcelona (Spain)

[d] *Prof. J. Vila*
Department of Clinical Microbiology – CDB
Hospital Clínic - University of Barcelona
Barcelona (Spain)

Supporting information for this article is available on the WWW under https://doi.org/10.1002/chem.202102370

© 2021 The Authors. Chemistry - A European Journal published by Wiley-VCH GmbH. This is an open access article under the terms of the Creative Commons Attribution Non-Commercial NoDerivs License, which permits use and distribution in any medium, provided the original work is properly cited, the use is non-commercial and no modifications or adaptations are made.

Antibiotic resistance is an increasing concern worldwide. Accumulation of antibiotics in the environment increases the evolutionary pressure on microorganisms, which evolve and adapt at a great pace. Moreover, the number of new antibiotic classes entering the pipeline has been alarmingly low during the last decades.[1] Although new antimicrobials are still needed, a change of paradigm on how we fight bacterial infections is on high demand as new drugs are bound to suffer from the emergence of resistances as well.[2] On this regard, gaining the ability to switch drugs on and off offers a unique opportunity to avoid the release of active drugs into the environment, thus reducing the chances of resistance arising and disseminating. In particular, visible light can be used as a clean and harmless stimulus to modulate the structure of a molecule and thus, its biological profile.[3]

Natural antimicrobial peptides play a crucial role as first line of defence against pathogenic bacteria.[4] These are produced by a plethora of microorganisms, but also by complex animals, including humans.[5]

Previous works have shown how the introduction of photoswitches into small molecules can allow control over the activity of antimicrobial compounds, but only recently has the control of antibacterial activity with visible light been reported.[6] In this context, a photoswitchable amino acid fully operated under visible light is a coveted goal in order to gain control over the structure of peptides under harmless conditions.[7,8] Tetra-*ortho*-chloro-azobenzenes can be isomerised efficiently under red or green light into their *cis* form and will quickly return to their *trans* isomer under other visible light sources unless kept in the dark. Importantly, the use of harmful UV light is avoided. This feature offers a unique platform for safe in situ activation of drugs, also through living tissue, and subsequent deactivation upon release into the environment after their therapeutic use, once exposed to sunlight.

The pioneering work of Feringa and co-workers showed that photocontrol of antibacterial activity to fight the appearance of resistances is possible.[6a] Short after, Ulrich and Komarov reported the use of visible light to trigger the antimicrobial activity of a gramicidin S photoswitchable peptidomimetic, which could be deactivated upon irradiation with UV light thanks to a diaryl-ethane photoswitch (Figure 1A).[6b,f,i] Other works have also used gramicidin S as a platform to study the photocontrol of antibacterial activity in

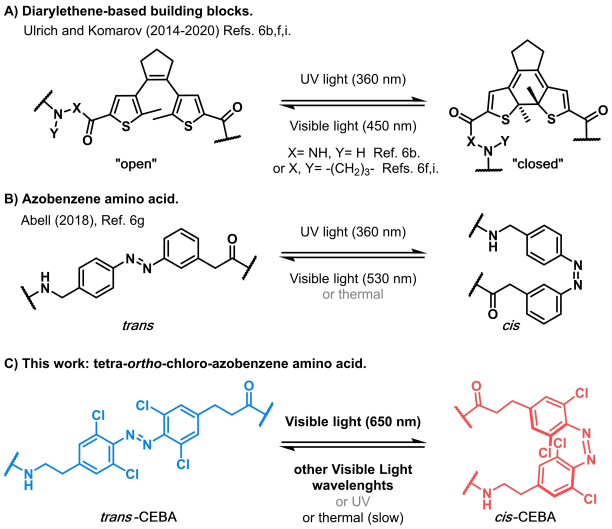


Figure 1. Photoswitchable building blocks used to photocontrol the antibacterial activity of tyrocidines' and gramicidins' analogues.

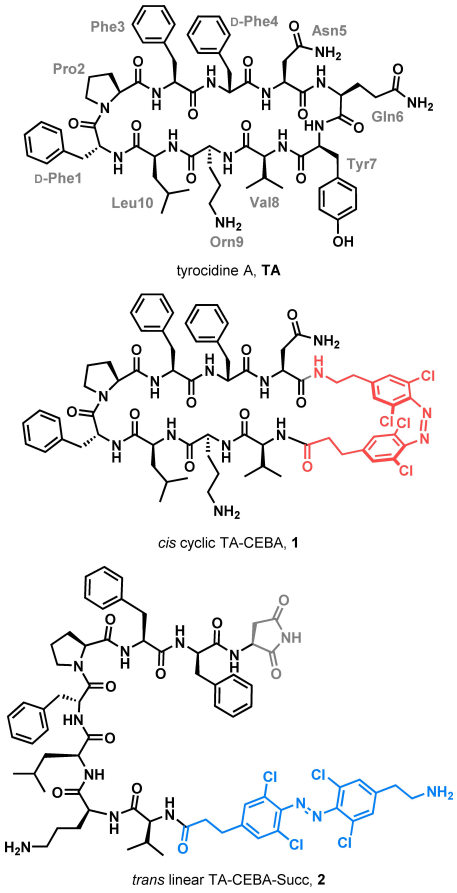
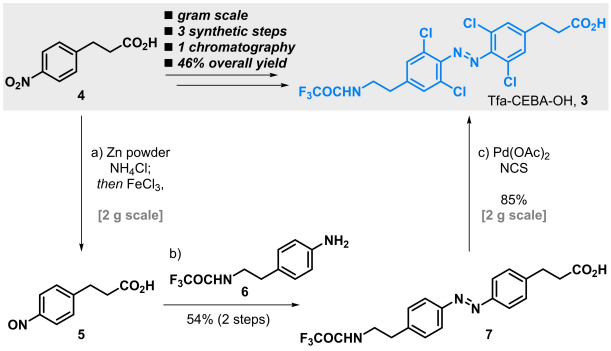


Figure 2. Tyrocidine A and examples of cyclic and linear visible light active photoswitchable analogues described in this work.



Scheme 1. Synthesis of tetra-*ortho*-chloro-azobenzene amino acid **3** (Tfa-CEBA-OH). Reaction conditions: a) Zn powder (2.5 equiv), NH_4Cl (1.5 equiv), H_2O, 2-methoxyethanol, 0 °C to rt, 17 h; *then* $FeCl_3$ (3.0 equiv), EtOH, H_2O, 0 °C, 1.5 h. b) **6** (1 equiv), AcOH, rt, 18 h, 54% (2 steps). c) $Pd(OAc)_2$, NCS (6 equiv), AcOH, 140 °C, 19 h, 85%. Tfa = trifluoroacetyl; NCS = *N*-chlorosuccinimide.

cyclic peptidomimetics (Figure 1B).[6g] However, all reports on the photocontrol of peptidomimetic's antibacterial activity require the use of UV light for one of the isomerisations. In this context, we embarked on the design of a photoswitchable amino acid building block that could be operated with visible light for both *trans*→*cis* and *cis*→*trans* isomerisations, resulting in CEBA (tetra-*ortho*-chloro-diethylelene-azobenzene Amino acid) (Figure 1C). Importantly, it was crucial to achieve fast *cis*→*trans* upon exposure to sunlight and other white light sources in order to achieve self-deactivation of the compounds upon release into the environment.

Tyrocidine A is a cyclic decapeptide with antimicrobial activity originally found along gramicidin in tyrothricin, a mixture of peptide antibiotics isolated from *Brevibacillus brevis*. Like gramicidin, tyrocidine is primarily active against Gram-positive bacteria. Modification of its structure has granted access to analogues with improved biological profiles such as an expanded panel of susceptible bacterial strains and higher therapeutic indexes.[9] However, its precise mechanism of action has remained elusive.[10]

We hypothesized that modification of tyrocidine A's structure by introducing our tetra-*ortho*-chloro-azobenzene amino acid (CEBA) in substitution of . Gln6 and Tyr7 would distort the natural peptide's β-hairpin secondary structure while in its *trans* configuration. However, upon isomerisation under red light, the *cis* form would become the major isomer and re-establish the biologically active secondary structure of the natural product (Figure 2).

Azobenzene amino acid **3** possesses the required features and its synthesis proved to be reliable and easily scalable, offering a useful route to this building block (Scheme 1). First, 3-(4-nitrophenyl)propanoic acid **4** was turned into the corresponding nitroso derivative **5** and upon condensation with aniline **6**, the azobenzene scaffold was set, yielding amino acid **7**. Subsequent palladium-catalysed chlorination gave the desired amino acid photoswitch **3** in very good yield.[11–13] Overall, Tfa-CEBA-OH (**3**) was obtained in 46% yield in just three steps, needing only one chromatographic purification at the end of the synthesis.

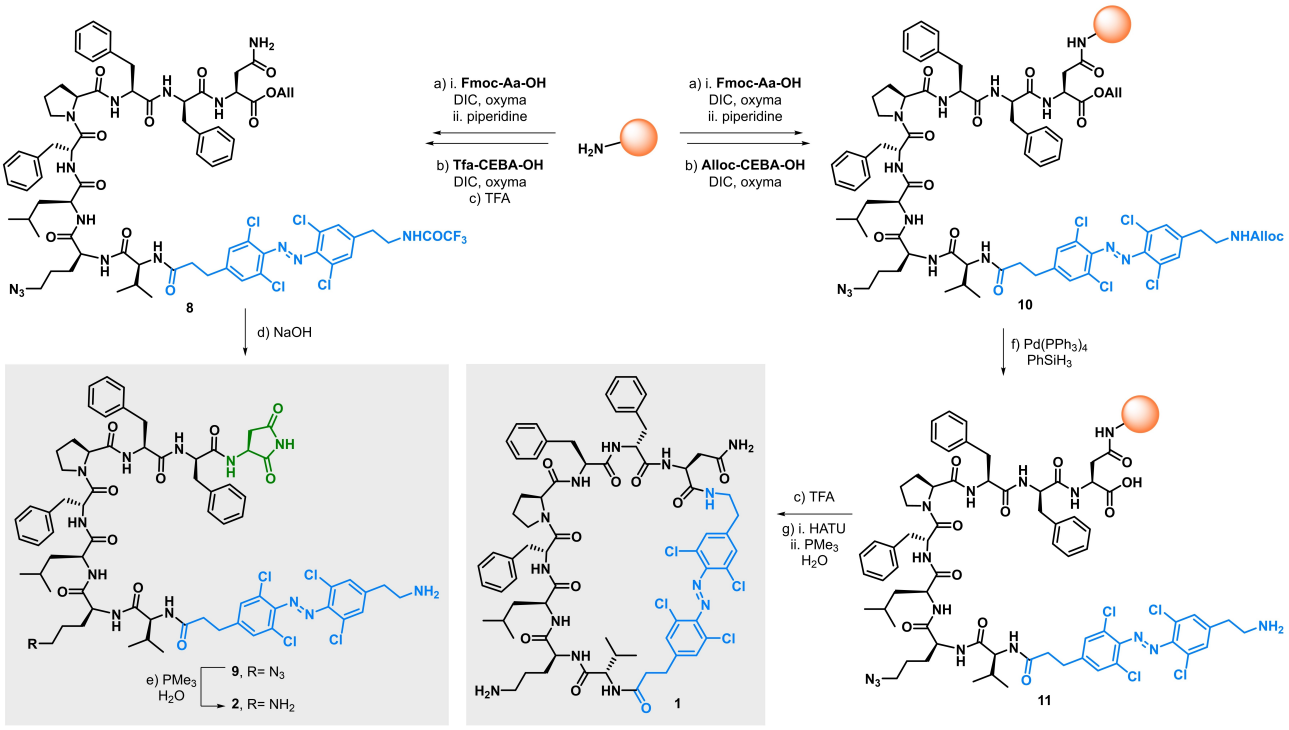
At this stage, we were ready to synthesize a photoswitchable analogue of tyrocidine A using solid phase peptide

© 2021 The Authors. Chemistry - A European Journal published by Wiley-VCH GmbH

synthesis (SPPS) starting from Gln5 anchored from its amide side chain to the resin (Scheme 2). Elongation of the peptide was performed uneventfully and TFA-CEBA-OH (**3**) was introduced last, prior to acidic cleavage to produce **8**. Subsequent basic hydrolysis in solution did not yield the expected *N*- and *C*-termini unprotected peptide, but a product with the same *m/z* as the target photoswitchable cyclic tyrocidine A analogue. Further analysis and mechanistic studies revealed that basic conditions had triggered cyclisation of the Asn residue side chain onto the allyl ester to form succinimide **9**, whose unanticipated structure prompted us to study it further and carry on with the remaining deprotection step.[14] Thus, the azide group in **9** was reduced to yield the corresponding amine **2** via a mild Staudinger reduction.[15] Alternatively, and in order to obtain the desired cyclic analogue **1**, The photoswitch was introduced as its allyl carbamate analogue Alloc-CEBA-OH, which was obtained quantitatively from **3** in a two-step, one-pot operation.[14] Once peptide **10** was ready, both termini were deprotected under Pd catalysis to yield **11**. At this stage, conditions were screened to optimise cyclisation of the peptide, which proved to be very reagent and solvent dependent.[14] In the end, the cyclised product was most efficiently obtained in solution, and a one-pot Staudinger reduction was performed last to reveal the free amine in **1**.

Photoisomerisation of the compounds was studied using **1** and **2** as models for cyclic and linear compounds, respectively. A kinetic profile of *trans* to *cis* isomerisation under red light (650 nm) was recorded and in both cases a virtually identical behaviour was observed.[14] Upon exposure to red light, the amount of *cis* isomer reaches a plateau after ca. 40 min, indicating the minimum time required to achieve the highest ratio of *cis* isomer under these conditions. The reverse process was also studied using daylight to irradiate the samples, as it would be the ideal source of light to trigger deactivation of the compounds.[14] To our delight, less than three minutes were required for the opposite isomerisation, highlighting the viability of the proposed idea of self-deactivation upon release into the environment (Figure 3). Importantly, the same behaviour was observed for all compounds described in this work.

With **1** and **2** in our hands, we carried out some preliminary antibacterial activity assays, which showed a more interesting biological profile for **2** (see below). This finding prompted us to investigate further the role of the *N*- and *C*-termini moieties present in this linear analogue. Thus, using the same synthetic approach, we prepared a small library of linear peptides with modifications on both termini. These included substitution of the photoswitchable amino acid CEBA for a photoswitchable carboxylic acid analogue without the 2-aminoethyl tail (**12**) and substitution of the Gln-derived succinimide moiety for the corresponding glutamine carboxylic acid or glutamine amide (Figure 4).



Scheme 2. Synthesis of linear and cyclic Visible Light photoswitchable tyrocidine A analogues **1** and **2**. Reaction conditions: a) see Supporting Information for details on manual and microwave-assisted SPPS methods. b) Alloc-CEBA-OH or Tfa-CEBA-OH (2.3 equiv), DIC (2.5 equiv), oxyma (2.5 equiv), DMF, rt, 16 h. c) TFA/CH_2Cl_2 (95:5), rt, 5×45 min. d) NaOH (3.5 equiv), THF, MeOH, H_2O, rt. e) PMe_3 (3 equiv), H_2O, THF, 0 °C to rt. f) $Pd(PPh_3)_4$ (10 mol%), $PhSiH_3$ (20 equiv), CH_2Cl_2, rt, 15 min. g) HATU (2 equiv), DIPEA (6 equiv), DMF, rt, 16 h; *then* PMe_3 (5 equiv), H_2O, 0 °C to rt. Alloc = Allyloxycarbonyl; DIC = *N,N'*-diisopropylcarbodiimide; oxyma = ethyl cyanohydroxyiminoacetate; DMF = dimethylformamide; TFA = trifluoroacetic acid; THF = tetrahydrofuran HATU = 1-[bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxide hexafluorophosphate; DIPEA = diisopropylethylamine.

© 2021 The Authors. Chemistry - A European Journal published by Wiley-VCH GmbH

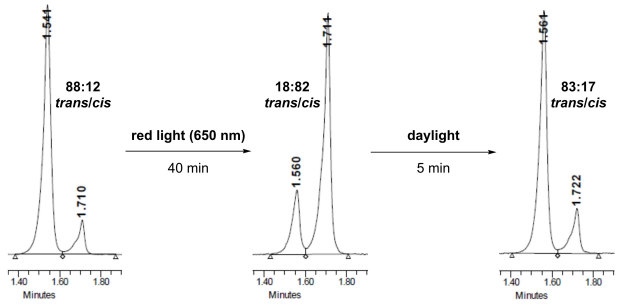


Figure 3. Photoisomerisation of 2. UPLC analysis of sequential *trans*→*cis* and *cis*→*trans* isomerisation of 2.

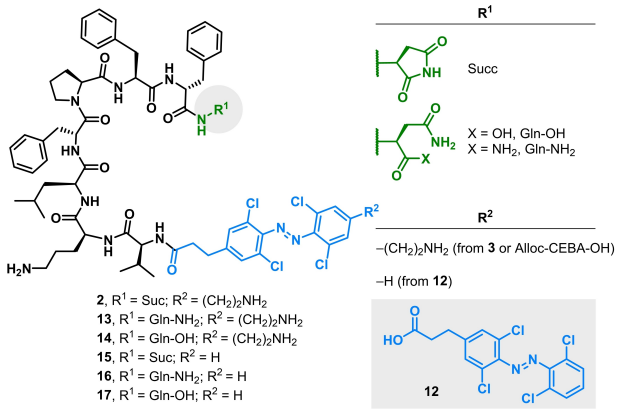


Figure 4. Library of linear photoswitchable analogues of tyrocidine A.

With all the analogues in hand, we next tested them against a panel of Gram-positive and Gram-negative bacterial strains (see the complete table in the Supporting Information). To our surprise, cyclic analogue **1** displayed a lack of photocontrol against all strains tested and its biological profile was very similar to **TA**, with the exception of *A. baumannii* (Table 1). Very interestingly, linear analogue **2** also had a similar profile, although photoswitchability against *A. baumannii* (both a susceptible -ATCC 19606- and a colistin-resistance strain -CR17-) was now observed and at least a 3-fold decrease in the MIC of the compound was observed upon activation with red light. Substitution of the Gln-derived succinimide for the corresponding glutamine amide (**13**) or carboxylic acid (**14**) had completely opposite effects on the compounds' activity against *A. baumannii*, showing lower MICs for analogue **13** (8–16 μg mL^{-1}) than analogue **14** (>64 μg mL^{-1}) in either photostationary state, whereas they behaved very similarly against the rest of the panel. Removal of the 2-aminoethyl tail from CEBA (analogues **15**–**17**) drastically changed the biological profile of the peptides, as they lost activity against most strains, including Gram-positive bacteria. However, upon irradiation at 650 nm, an increased susceptibility was observed for compounds **16** and **17** against *S. pyogenes*. In particular, **17** displayed great selectivity against this strain, whereas it remained inactive against all the other ones tested during this study.

Depending on the compound, differences were also observed in terms of their haemolytic effect.[14] **TA**, **2**, **13** and **15** were less haemolytic in their non-irradiated state compared to the irradiated one, whereas compounds **1**, **14**, **16** and **17** showed the opposite effect. In the case of compound **17**, which showed good antibacterial activity against *S. pyogenes* in the irradiated state, a high IC50 value (173 μg mL^{-1}) was also observed (low haemolytic effect), which can likely be attributed to its lack of a net positive charge under the assay conditions and is an optimistic starting point for therapeutic purposes.

In conclusion, we have developed an efficient synthesis of the novel tetra-*ortho*-chloro-photoswitchable azobenzene amino acid (CEBA) that can be incorporated into peptidic sequences to grant photocontrol using exclusively visible light. This has led to the development of **2**, a photoswitchable tyrocidine A linear analogue with a *C*-terminal succinimide moiety that can be activated under red light to become active against a clinically relevant Gram-negative strain such as *A. baumannii*. Importantly, the compound quickly becomes inactive against this pathogenic strain upon exposure to sunlight, enabling self-immolation upon release to the environment. Taking into consideration the available data for the linear analogue **17**, selectivity and photocontrol have been observed against *S. pyogenes*, achieving the highest IC50 value in both photostationary states out of all the analogues tested. To confirm their antimicrobial effect against this species, further studies increasing the number of *S. pyogenes* strains should be conducted. In addition, the decreased toxicity (haemolytic assay) observed in some of the non-irradiated compounds is an important added value of this strategy that may also contribute to preserving the ecosystem and should be considered in further studies. We hope that these findings will help advancing in the development of more efficient strain selective and self-

Table 1. Selected in vitro antibacterial activities of **TA** and its photoswitchable analogues.[a]

	TA		**1**		**2**		**13**		**14**		**15**		**16**		**17**	
	NoIrr	Irr[b]	NoIrr	Irr[b]	NoIrr	Irr[b]	NoIrr	Irr[b]	NoIrr	Irr[b]	NoIrr	Irr[b]	NoIrr	Irr[b]	NoIrr	Irr[b]
Bacillus subtilis[c]	4	4	4	8	2	2	1	2	4	16	>64	>64	64	64	>64	>64
Streptococcus pyogenes 016[c]	4	4	4	4	4	4	4	4	8	4	>64	64	**>64**	**16**	**>64**	**16**
Escherichia coli ATCC 25922[d]	>64	>64	>64	>64	>64	64	>64	32	>64	>64	>64	>64	>64	>64	>64	>64
Acinetobacter baumannii ATCC 19606[d]	16	16	>64	>64	**64**	**8**	8	8	>64	>64	>64	>64	>64	>64	>64	>64
Acinetobacter baumannii CR17[d]	32	16	>64	>64	>64	32	16	8	>64	>64	>64	>64	>64	>64	>64	>64

[a] Measured as minimum inhibitory concentration (MIC) in μg mL^{-1}. [b] Plates were irradiated at 650 nm for 40 min before incubation in the dark. [c] Gram-positive strain. [d] Gram-negative strain.

© 2021 The Authors. Chemistry - A European Journal published by Wiley-VCH GmbH

deactivating photoswitchable antimicrobials that help diminish the increasing threat of antibiotic resistance.

Acknowledgements

We thank AGAUR and Generalitat de Catalunya for a Beatriu de Pinós fellowship to X.J.B. We acknowledge support from the Spanish Ministry of Science and Innovation through the "Centro de Excelencia Severo Ochoa 2019–2023" Program (CEX2018-000806-S), and support from the Generalitat de Catalunya through the CERCA Program. Supported by Plan Nacional de I+D+i 2013–2016 and Instituto de Salud Carlos III, Subdirección General de Redes y Centros de Investigación Cooperativa, Ministerio de Economía, Industria y Competitividad, Spanish Network for Research in Infectious Diseases (REIPI RD16/0016/0010) - co-financed by European Development Regional Fund "A way to achieve Europe", Operative program Intelligent Growth 2014–2020. We would also like to thank Prof. Pau Gorostiza and Dr. Rossella Castagna for lending us their 650 nm LED lamp.

Conflict of Interest

The authors declare no conflict of interest.

Keywords: antibiotics · azobenes · peptidomimetics · photochemistry · tyrocidine A

[1] a) World Health Organization. Worldwide country situation analysis: Response to antimicrobial resistance, April 2015; http://apps.who.int/iris/bitstream/10665/163468/1/9789241564946_eng.pdf?ua=1&ua=1; b) E. Tacconelli, E. Carrara, A. Savoldi, S. Harbarth, M. Mendelson, et al. *Lancet Infect. Dis.* **2018**, *18*, 318–327; c) U. Theuretzbacher, K. Outterson, Al. Engel, A. Karlén, *Nat. Rev. Microbiol.* **2020**, *18*, 275–285.

[2] For reviews on alternative approaches to tackle antibacterial resistance, see: a) Y. Liu, R. Li, X. Xiao, Z. Wang, *Crit. Rev. Microbiol.* **2019**, *45*, 301–304; b) C.-H. Huang, Y.-H. Hsieh, Z. M. Powers, C.-Y. Kao, *Int. J. Mol. Sci.* **2020**, *21*, 1061.

[3] For selected reviews on photopharmacology, see: a) W. A. Velema, W. Szymanski, B. L. Feringa, *J. Am. Chem. Soc.* **2014**, *136*, 2178–2191; b) J. Broichhagen, J. A. Frank, D. Trauner, *Acc. Chem. Res.* **2015**, *48*, 1947–1960; c) M. M. Lerch, M. J. Hansen, G. M. van Dam, W. Szymanski, B. L. Feringa, *Angew. Chem. Int. Ed.* **2016**, *55*, 10978–10999; *Angew. Chem.* **2016**, *128*, 11140–11163; d) K. Hüll, J. Morstein, D. Trauner, *Chem. Rev.* **2018**, *118*, 10710–10747; e) M. J. Fuchter, *J. Med. Chem.* **2020**, *63*, 11436–11447.

[4] a) J. M. Ageitos, A. Sánchez-Pérez, P. Calo-Mata, T. G. Villa, *Biochem. Pharmacol.* **2017**, *133*, 117–138; b) Y. Huan, Q. Kong, H. Mou, H. Yi, *Front. Microbiol.* **2020**, *11*, 582779.

[5] A. Zipperer, M. C. Konnerth, C. Laux, A. Berscheid, D. Janek, C. Weidenmaier, M. Burian, N. A. Schilling, C. Slavetinsky, M. Marschal, M. Willmann, H. Kalbacher, B. Schittek, H. Brötz-Oesterhelt, S. Grond, A. Peschel, B. Krismer, *Nature* **2016**, *535*, 511–516.

[6] For reports on photoswitchable antibiotics, see: a) W. A. Velema, J. P. van der Berg, M. J. Hansen, W. Szymanski, A. J. M. Driessen, B. L. Feringa, *Nat. Chem.* **2013**, *5*, 924–928; b) O. Babii, S. Afonin, M. Berditsch, S. Reißer, P. K. Mykhailiuk, V. S. Kubyshkin, T. Steinbrecher, A. S. Ulrich, I. V. Komarov, *Angew. Chem. Int. Ed.* **2014**, *53*, 3392–3395; *Angew. Chem.* **2014**, *126*, 3460–3463; c) Y. Hu, J. B. Marlow, R. Ramanathan, W. Zou, H. G. Tiew, M. J. Pottage, V. Bansal, R. F. Tabor, B. L. Wilkinson, *Aust. J. Chem.* **2015**, *68*, 1880–1884; d) W. A. Velema, M. J. Hansen, M. M. Lerch, A. J. M. Driessen, W. Szymanski, B. L. Feringa, *Bioconjugate Chem.* **2015**, *26*, 2592–2597; e) M. Wegener, M. J. Hansen, A. J. M. Drieseen, W. Szymanski, B. L. Feringa, *J. Am. Chem. Soc.* **2017**, *139*, 17979–17986; f) O. Babii, S. Afonin, A. Y. Ishchenko, T. Schober, A. O. Negelia, G. M. Tolstanova, L. V. Garmanchuk, L. I. Ostapchenko, I. V. Komarov, A. S. Ulrich, *J. Med. Chem.* **2018**, *61*, 10793–10813; g) Y. Q. Yeoh, J. Yu, S. W. Polyak, J. R. Horsley, A. D. Abell, *ChemBioChem* **2018**, *19*, 2591–2597; h) H.-B. Cheng, X. Li, N. Kwon, Y. Fang, G. Baek, J. Yoon, *Chem. Commun.* **2019**, *55*,12316–12319; i) S. Afonin, O. Babii, A. Reuter, V. Middel, M. Takamiya, U. Strähle, I. V. Komarov, A. S. Ulrich, *Beilstein J. Org. Chem.* **2020**, *16*, 39–49; j) Y. Q. Yeoh, J. R. Horsley, J. Yu, S. W. Polyak, B. Jovcevski, A. D. Abell, *ChemMedChem* **2020**, *15*, 1505–1508.

[7] To the best of our knowledge, only one visible light operated azobenzene amino acid has been reported to date: L. Albert, A. Peñalver, N. Djokovic, L. Werel, M. Hoffarth, D. Ruzic, J. Xu, L.-O. Essen, K. Nikolic, Y. Dou, O. Vázquez, *ChemBioChem* **2019**, *20*, 1417–1429.

[8] For reviews on photoswitchable peptides, see: a) F. Ciardelli, S. Bronco, O. Pieroni, A. Pucci in *Molecular Switches Chapter 13*: Photoswitchable Polypeptides, 2nd Ed. (Edd. B. L. Feringa, W. R. Browne), Wiley-VCH Verlag GmbH, **2011**, pp. 321–360; b) A. A. Beharry, G. A. Woolley, *Chem. Soc. Rev.* **2011**, *40*, 4422–4437; c) W. Szymanski, J. M. Beierle, H. A. V. Kistemaker, W. A. Velema, B. L. Feringa, *Chem. Rev.* **2013**, *113*, 6114–6178; d) S. Kitzig, M. Thilemann, T. Cordes, K. Rück-Braun, *ChemPhysChem* **2016**, *17*,1252–1263; e) L. Alvert, O. Vázquez, *Chem. Commun.* **2019**, *55*, 10192–10213; f) V. Peddie, A. D. Abell, *J. Photochem. Photobiol. C* **2019**, *40*, 1–20.

[9] For selected reports on tyrocidine A's SAR, see: a) R. M. Kohli, C. T. Walsh, M. D. Burkart, *Nature* **2002**, *418*, 658–661; b) X. Bu, X. Wu, G. Xie, Z. Guo, *Org. Lett.* **2002**, *4*, 2893–2895; c) C. Qin, X. Zhong, X. Bu, N. L. J. Ng, Z. Guo, *J. Med. Chem.* **2003**, *46*, 4830–4833; d) M. A. Marques, D. M. Citron, C. C. Wang, *Bioorg. Med. Chem.* **2007**, *15*, 6667–6677; e) Q. Xiao, D. Pei, *J. Med. Chem.* **2007**, *50*, 3132–3137; f) G. Munyuki, G. E. Jackson, G. A. Venter, K. E. Kövér, L. Szilágyi, M. Rautenbach, B. M. Spathelf, B. Bhattacharya, D. van der Spoel *Biochemistry* **2013**, *52*, 44, 7798–7806; g) A. N.-N. Leussa, M. Rautenbach, *Chem. Biol. Drug Des.* **2014**, *84*, 543–557; h) A. J. Cameron, P. J. B. Edwards, E. Harjes, V. Sarojini, *J. Med. Chem.* **2017**, *60*, 9565–9574.

[10] For recent reports on tyrocidine A's mechanism of action, see: a) P. J. Loll, E. C. Upton, V. Nahoum, N. J. Economou, S. Cocklin, *Biochim. Biophys. Acta* **2014**, *1838*, 1199–1207; b) M. Wenzel, M. Rauntenbach, J. A. Vosloo, T. Siersma, C. H. M. Aisenbrey, E. Zaitseva, W. E. Laubscher, W. van Rensburg, J. C. Behrends, B. Bechinger, L. W. Hamoen, *mBio* **2018**, *9*, e00802.

[11] For the tetra-*ortho*-halogenation of azobenzenes, see: a) D. B. Konrad, J. A. Frank, D. Trauner, *Chem. Eur. J.* **2016**, *22*, 4364–4368; b) T. H. L. Nguyen, N. Gigant, D. Joseph, *ACS Catal.* **2018**, *8*, 1546–1579; c) V. Poonthiyil, F. Reise, G. Despras, T. K. Lindhorst, *Eur. J. Org. Chem.* **2018**, 6241–6248; d) Q. Liu, X. Luo, S. Wei, Y. Wang, J. Zhu, Y. Liu, F. Quan, M. Zhang, C. Xia, *Tetrahedron Lett.* **2019**, *60*, 1715–1719.

[12] For selected alternative methods to access tetra-*ortho*-halogenated azobenzenes, see: a) D. Bléger, J. Schwarz, A. M. Brouwer, S. Hecht, *J. Am. Chem. Soc.* **2012**, *134*, 20597–20600; b) S. Samanta, A. A. Beharry, O. Sadovsi, T. M. McCormik, A. Babalhavaeji, V. Tropepe, G. A. Woolley, *J. Am. Chem. Soc.* **2013**, *135*, 9777–9784; c) M. J. Hansen, M. M. Lerch, W. Szymanski, B. L. Feringa, *Angew. Chem. Int. Ed.* **2016**, *55*, 13514–13518; *Angew. Chem.* **2016**, *128*, 13712–13716.

[13] For recent reports on the properties of tetra-*ortho*-halogenated azobenzenes, see: a) L. N. Lameijer, S. Budzak, N. A. Simeth, M. J. Hansen, B. L. Feringa, D. Jacquemin, W. Szymanski, *Angew. Chem. Int. Ed.* **2020**, *59*, 21663–21670; *Angew. Chem.* **2020**, *132*, 21847–21854; b) D. B. Konrad, G. Savasci, L. Allmendinger, D. Trauner, C. Ochsenfeld, A. M. Ali, *J. Am. Chem. Soc.* **2020**, *142*, 6538–6547.

[14] See Supporting Information. Blood samples were purchased from Zenbio (https://www.zen-bio.com/products/serum/human-blood-products.php).

[15] A. P. A. Janssen, D. van der Vliet, A. T. Bakker, M. Jiang, S. H. Grimm, G. Campiani, S. Butini, M. van der Stelt, *ACS Chem. Biol.* **2018**, *13*, 2406–2413.

Manuscript received: July 1, 2021
Accepted manuscript online: July 6, 2021
Version of record online: July 22, 2021

 © 2021 The Authors. Chemistry - A European Journal published by Wiley-VCH GmbH