Induced Production, Synthesis, and Immunomodulatory Action of Clostrisulfone, a Diarylsulfone from \textit{Clostridium acetobutylicum}

Toni Neuwirth,\textsuperscript{[a]} Anne-Catrin Letzel,\textsuperscript{[a]} Cedric Tank,\textsuperscript{[b]} Keishi Ishida,\textsuperscript{[a]} Michael Cyrulies,\textsuperscript{[b]} Lisa Schmölz,\textsuperscript{[c]} Stefan Lorkowski,\textsuperscript{[c]} and Christian Hertweck\textsuperscript{[a, d]}

Abstract: The anaerobe \textit{Clostridium acetobutylicum} belongs to the most important industrially used bacteria. Whereas genome mining points to a high potential for secondary metabolism in \textit{C. acetobutylicum}, the functions of most biosynthetic gene clusters are cryptic. We report that the addition of supra-physiological concentrations of cysteine triggered the formation of a novel natural product, clostrisulfone (1). Its structure was fully elucidated by NMR, MS and the chemical synthesis of a reference compound. Clostrisulfone is the first reported natural product with a diphenylsulfone scaffold. A biomimetic synthesis suggests that pentamethylchro- namol-derived radicals capture sulfur dioxide to form 1. In a cell-based assay using murine macrophages a biphasic and dose-dependent regulation of the LPS-induced release of nitric oxide was observed in the presence of 1.

Anaerobic bacteria, supposedly the oldest creatures on earth, are ubiquitously distributed in oxygen-free niches such as soils, sediments, and intestines of higher organisms. Among the best-studied anaerobic bacteria are clostridia, which play major roles in human and animal health, ecology, remediation, and industry.\textsuperscript{[1]} Because of their specialized anoxic catabolism, they are industrially used as solvent producers. An important example is \textit{Clostridium acetobutylicum}, which has played a major role in the ABE (acetone, butanol, ethanol) Weizmann fermentation process for more than a century\textsuperscript{[2]} Until recently, however, clostridia—and anaerobes in general—have been neglected as a source of non-protein natural products.\textsuperscript{[3]} With the advent of massive genome sequencing and bioinformatics, it has become apparent that certain anaerobes have a rich and diverse biosynthetic potential.\textsuperscript{[4]} However, in most cases the gene clusters are silent and need to be activated by means of particular stimuli. Several genetic and chemical approaches to triggering biosynthesis\textsuperscript{[5]} have been applied to clostridia,\textsuperscript{[3a, 6]} A range of antibiotics, such as closthioamides,\textsuperscript{[3a, 7]} clostrubins,\textsuperscript{[8]} clostrindoline,\textsuperscript{[9]} antibacterial acylloins,\textsuperscript{[10]} and a new lipopeptide\textsuperscript{[11]} have been isolated from various clostridia. Mining the genome sequence of \textit{C. acetobutylicum}\textsuperscript{[12]} pointed to a high biosynthetic potential.\textsuperscript{[4]} Apart from a small polyketide that has been implicated in the regulation of cellular differentiation or \textit{C. acetobutylicum},\textsuperscript{[13]} so far no secondary metabolites have been reported for these important industrial bacteria. Here, we report the discovery of an unusual, diarylsulfone metabolite from a \textit{C. acetobutylicum} strain disturbed in sulfur metabolism, verify its unusual structure by synthesis, and evaluate its bioactivities.

To trigger the biosynthesis of cryptic natural products in \textit{C. acetobutylicum} (DSM 792/ATCC 824), we compared the metabolic profiles of the wild type grown in standard media with those of cultures supplemented with potential elicitors. Generally, no secondary metabolites could be detected in the extracts of standard media. However, when we challenged \textit{C. acetobutylicum} with supra-physiological concentrations of L-cysteine (1 mM) in the culture broth, the metabolic profile changed markedly in the highly unpolar region (Figure 1). We detected a new compound (1) with the sum formula \textit{C}_{26}\textit{H}_{24}\textit{O}_{6}\textit{S}, deduced from its exact mass (\textit{m/z} 471.2559 [\textit{M+H}]^{+}) obtained by HR-ESI-MS measurements (Figure S1). To obtain amounts of 1 that would allow for a full structural characterization, we subjected the ethyl acetate extract of 0.5 L culture to reversed-phase HPLC to yield 0.2 mg.

The \textsuperscript{1}H and \textsuperscript{13}C NMR spectra of 1 show an unexpectedly low number of signals, which is indicative for a highly symmetric structure (Figures S2 and S3). The DEPT135 and HSQC spectral analysis indicate that 1 possesses five methyl groups (Figures S4 and S6). By the HMB correlations from 2,2-Me protons (\textit{d}_{H} 1.27) to C-2 (\textit{d} 74.0(C)/3 (\textit{d} 31.8) and from methylene protons H-3 (\textit{d} 1.78) to 2,2-Me carbons (\textit{d} 26.3(C)/3, the gem-dimethyl...
moiety is connected to the oxy-quaternary carbon C-2 next to C-3, which is adjacent to a methylene C-4 (δ 20.4) according to a 1H–1H COSY coupling signal between H3 and H4 (δ 2.55) (Figures S5 and S7). The HMBC correlations of three aromatic methyl protons and quaternary carbons, 5-Me (δH 2.21)/C-4a (δ 118.5) and C-6 (δ 134.6), 7-Me (δH 2.16)/C-6 and C-8 (δ 123.5), and 8-Me (δH 2.00)/C-7 (δ 134.17), C-8, C-8a (δ 153.5) revealed the presence of a fully substituted phenol ring. Finally, the HMBC correlations from H-4 to C-4a, C-5 (δ 134.23), and C-8a suggested that 1 harbors two 2,2,5,7,8-pentamethyl-6-chromane moieties (Figure 1). Surprisingly, this substructure is the same as the Pmc (2,2,5,7,8-pentamethylchromane-6-sulfon)yl group used for the arginine side chain. Since 1 could only be isolated in minute amounts, the presence of the sulfone bridge was initially deduced from HRMS data and the similarity of 13C NMR data of 1 and Pmc chloride (2) (Table S1).

To unequivocally confirm the unusual structure of 1 and to obtain sufficient amounts for bioactivity assays we aimed to synthesize the diarylsulfone. Therefore, we devised a short synthetic route involving a Friedel–Crafts-type aromatic sulfonation of chromane 3 with sulfonyl chloride 4, a reagent commonly used for arginine side chain protection. 2,2,5,7,8-Pentamethyl-6-chromane 3 was prepared from 2,3,5-trimethylphenol and isoprene (Figures S8 and S9). The SnCl4-mediated coupling of 3 with 4 provided the target molecule (1) in good yield (70%) (Figures S10 and S11). The proposed structure of clostrisulfone was verified by comparison of physicochemical data of the synthetic compound with the natural product. Owing to its biological origin and key structural feature we named this new compound clostrisulfone (1; Scheme 1).

The symmetrical diarylsulfone structure of clostrisulfone (1) is highly unusual. Besides sulfadixiamycins B and C from bacterial mangrove endophytes, 1 represents only the second naturally occurring diarylsulfone scaffold, and it is the first reported natural product with a diphenylsulfone substructure. Furthermore, the two chromane substructures of 1 are strikingly similar to 2,2,5,7,8-pentamethyl-6-chromanol, which plays a major role as a synthetic tocopherol (vitamin E) analogue.[16]

Chem. Eur. J. 2020, 26, 15855 – 15858 www.chemeurj.org
© 2020 The Authors. Published by Wiley-VCH GmbH
diarylsulfone was detectable in trace amounts by HRMS-HPLC. While this approach is potentially biomimetic, alternative avenues in the biosynthesis of 1 cannot be ruled out.

As diarylsulfones such as dapsone, bis-2-nitrophenylsulfone \([21]\) and diphenylsulfone \([22]\) exhibit antibacterial, antiviral, and cytotoxic activities, we tested 1 in a panel of bioassays. Clostrisulfone did not show any activity against Gram-positive or Gram-negative bacteria, mycobacteria, and HIV; it did not show any effect in antifungal, antiviral, cytotoxicity and anti-proliferative bioassays, either. Yet, the structural similarity of 1 to tocopherol analogues pointed to a possible anti-inflammatory activity.

To monitor its pro- or anti-inflammatory potential, we subjected 1 to a cell-based assay using murine macrophages. Specifically, we pre-treated the macrophages with increasing concentrations of 1 (0 to 10 \(\mu\)g mL\(^{-1}\)) prior to co-incubation with lipopolysaccharide (LPS, 100 ng mL\(^{-1}\)), which induces an inflammatory response and oxidative burst.\([23]\) At the end of the incubation time (24 h) nitric oxide release from the cells was detected by reaction with the Griess reagent (Figure 3).\([23]\) A reduced LPS-induced NO release would be indicative of an anti-inflammatory capacity of 1; for a pro-inflammatory response, contradictory results would be expected.

In the inactivated state (incubation in the absence of LPS), only the highest concentrations tested (7.5 and 10 \(\mu\)g mL\(^{-1}\)) slightly induce the release of nitric oxide (0.8 ± 0.5 \(\mu\)M nitric oxide in controls vs. 1.9 ± 0.6 \(\mu\)M nitric oxide for 10 \(\mu\)g mL\(^{-1}\) clostrisulfone). All samples treated with LPS released significantly (p < 0.05) more nitric oxide compared to LPS-free controls. While low concentrations of 1 (0.001–0.1 \(\mu\)g mL\(^{-1}\)) significantly (p < 0.05 to p < 0.01) enhance the LPS-induced NO release compared to controls treated only with LPS (24.3 ± 4.6 \(\mu\)M NO in control vs. 38.7 ± 7.6 \(\mu\)M NO for 0.05 \(\mu\)g mL\(^{-1}\) 1), higher concentrations of 1 (7.5–10 \(\mu\)g mL\(^{-1}\)) significantly (p < 0.001) reduce the LPS-induced NO release of (9.5 ± 3.4 \(\mu\)M NO for 10 \(\mu\)g mL\(^{-1}\) 1). As no cytotoxicity was observed as assessed by MTT assay, the reduction in NO release was not due to reduced cell viability. In conclusion, we observed a biphasic and dose-dependent regulation of the LPS-induced release of nitric oxide in murine macrophages in the presence of 1.

In conclusion, by disturbing the thiol landscape in the culture of \(C.\ acetobutylicum\) we have discovered an unprecedented diarylsulfone natural product. The discovery of clostrisulfone is remarkable since this anaerobe has been playing a major role in industrial processes, while the compound has remained hidden. The structure of the highly substituted diarylsulfone is unprecedented among natural products. Its symmetric architecture and a biomimetic synthesis point to a biogenetic origin involving sulfur dioxide capture. The tocopherol-like substrates may account for the dose-dependent regulation of the LPS-induced release of nitric oxide at higher concentrations of 1 and thus indicates immunomodulatory potential. These findings underscore the hidden biosynthetic potential of clostridia and may encourage similar approaches to trigger natural product formation in anaerobes for drug discovery.

**Experimental Section**

See the Supporting Information.

**Acknowledgements**

We thank A. Perner and H. Heinecke for MS and NMR measurements. Financial support by the BMBF (InfectControl) and the DFG (Leibniz Award) is gratefully acknowledged. Open access funding enabled and organized by Projekt DEAL.

**Conflict of interest**

The authors declare no conflict of interest.
Keywords: clostridia · diphenylsulfone · Griess assay · macrophages · tocopherol

[1] H. Bahl, P. Dürre, Clostridia: Biotechnology & Medical Applications, Wiley-VCH, Weinheim, 2001.

[2] D. T. Jones, D. R. Woods, Microbiol. Rev. 1986, 50, 484–524.

[3] a) T. Lincke, S. Behnken, K. Ishida, M. Roth, C. Hertweck, Angew. Chem. Int. Ed. 2010, 49, 2011–2013; Angew. Chem. 2010, 122, 2055–2057; b) S. Behnken, C. Hertweck, Appl. Microbiol. Biotechnol. 2012, 96, 61–67; c) S. J. Pidot, S. Coyne, F. Kloss, C. Hertweck, Int. J. Med. Microbiol. 2014, 304, 14–22; d) J. S. Li, C. C. Barber, W. Zhang, J. Ind. Microbiol. Biotechnol. 2019, 46, 375–383.

[4] a) A. C. Letzel, S. J. Pidot, C. Hertweck, Nat. Prod. Rep. 2013, 30, 392–428; b) A. C. Letzel, S. J. Pidot, C. Hertweck, BMC Genomics 2014, 15, 983.

[5] a) K. Scherlach, C. Hertweck, Org. Biomol. Chem. 2009, 7, 1753–1760; b) S. Behnken, C. Hertweck, PLoS ONE 2012, 7, e29609; c) B. Baral, A. Akhgari, M. Metsä-Ketelä, Synth. Syst. Biotechnol. 2018, 2, 163–178; d) X. Zhang, Hindra, M. A. Elliot, Curr. Opin. Microbiol. 2019, 51, 9–15.

[6] a) S. Behnken, T. Lincke, F. Kloss, K. Ishida, C. Hertweck, Angew. Chem. Int. Ed. 2012, 51, 2425–2428; Angew. Chem. 2012, 124, 2475–2478; b) K. L. Dunbar, H. Büttner, E. M. Molloy, M. Dell, J. Kumpfmüller, C. Hertweck, Angew. Chem. Int. Ed. 2018, 57, 14080–14084; Angew. Chem. 2018, 130, 14276–14280.

[7] a) F. Kloss, A. I. Chiriac, C. Hertweck, Chem. Eur. J. 2014, 20, 15451–15458; b) A. I. Chiriac, F. Kloss, J. Kämmerer, C. Vuong, C. Hertweck, H. G. Wahl, J. Antibiotics. Chemother. 2015, 70, 2576–2588.

[8] a) S. Pidot, K. Ishida, M. Cyrulies, C. Hertweck, Angew. Chem. Int. Ed. 2014, 53, 7856–7859; Angew. Chem. 2014, 126, 7990–7993; b) G. Schabauer, K. Ishida, S. J. Pidot, M. Roth, H. M. Dahse, C. Hertweck, Science 2015, 350, 670–674.

[9] S. SchiederDecker, G. Shabauer, U. Knuepfer, C. Hertweck, Org. Biomol. Chem. 2019, 17, 6119–6121.

[10] S. SchiederDecker, G. Shabauer, A. C. Letzel, B. Urbansky, M. Ishida-Ito, K. Ishida, M. Cyrulies, H. M. Dahse, S. Pidot, C. Hertweck, ACS Chem. Biol. 2019, 14, 1490–1497.

[11] J. S. Li, C. C. Barber, N. A. Herman, W. Cai, E. Zafir, Y. Du, X. Zhu, W. Skyrud, W. Zhang, J. Ind. Microbiol. Biotechnol. 2020, 47, 319–328.

[12] J. Nolling, G. Breton, M. V. Omelchenko, K. S. Makarova, G. Zeng, R. Gibson, H. M. Lee, J. Dubois, D. Qiu, J. Hitti, Y. I. Wolf, R. L. Tatusov, F. Sabinthe, L. Doucette-Stamm, P. Soucaille, M. J. Daly, G. N. Bennett, E. V. Koonin, D. R. Smith, J. Bacteriol. 2001, 183, 4823–4838.

[13] N. A. Herman, S. J. Kim, J. S. Li, W. Cai, H. Koshino, W. Zhang, Nat. Commun. 2017, 8, 1514.

[14] R. Ramage, J. Green, A. J. Blake, Tetrahedron 1991, 47, 6353–6370.

[15] M. Baunach, L. Ding, K. Willing, C. Hertweck, Angew. Chem. Int. Ed. 2015, 54, 13279–13283; Angew. Chem. 2015, 127, 13477–13481.

[16] E. Niki, A. Kawakami, M. Saito, Y. Yamamoto, J. Tsuchiya, Y. Kamiya, J. Biol. Chem. 1985, 260, 2191–2196.

[17] K. L. Dunbar, D. H. Scharf, A. Litomska, C. Hertweck, Chem. Rev. 2017, 117, 5521–5577.

[18] a) Q. Zhang, H. Li, L. Yu, Y. Sun, Y. Zhu, H. Zhu, L. Zhang, S. M. Li, Y. Shen, C. Tian, A. Li, H. W. Liu, C. Zhang, Chem. Sci. 2017, 8, 5067–5077; b) M. Baunach, L. Ding, T. Bruhn, G. Bringmann, C. Hertweck, Angew. Chem. Int. Ed. 2013, 52, 9040–9043; Angew. Chem. 2013, 125, 9210–9213.

[19] R. B. Tompkin, L. N. Christiansen, A. B. Spaparis, Appl. Environ. Microbiol. 1980, 39, 1096–1099.

[20] a) G. André, E. Haudecoeur, M. Monot, K. Ohtani, T. Shimizu, B. Dupuy, BMC Microbiol. 2010, 10, 234; b) T. Dubois, M. Dancer-Thibonniez, M. Monot, A. Hamiot, L. Bouillaut, O. Soutourina, I. Martin-Verstraete, B. Dupuy, Infect. Immun. 2016, 84, 2389–2405; c) H. Gu, K. Shi, Z. Liao, H. Qi, S. Chen, H. Wang, S. Li, Y. Ma, J. Wang, Microbiol. Res. 2018, 215, 114–125.

[21] J. B. Mahonon, R. J. Gulakowski, O. S. Weislow, R. J. Schultz, V. L. Narayan, D. J. Clanton, R. Pedemonte, F. W. Wassmundt, R. W. J. Bucikheit, W. D. Decker, C. H. White, J. P. Bader, M. R. Boyd, Antimicrob. Agents Chemother. 1993, 37, 754–760.

[22] J. J. Chen, C. Y. Duh, I. S. Chen, Planta Med. 2005, 71, 370–372.

[23] L. Schmolz, M. Wallert, S. Lorkowski, J. Immunol. Methods 2017, 449, 68–70.

Manuscript received: July 27, 2020
Revised manuscript received: September 29, 2020
Accepted manuscript online: September 30, 2020
Version of record online: November 3, 2020