From natural products to biological tools*

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Abstract: The development of a total synthetic approach for the antimitotic disorazole C_1 and the design of a peptide isostere linked to the reactive oxygen scavenger 2,2,6,6-tetra-methylpiperidine-*N*-oxyl (TEMPO) demonstrate established as well as novel strategies for mining the therapeutic potential of natural products.

Keywords: natural products; disorazole; gramicidin S; antimitotic; mitochondria.

INTRODUCTION

Traditionally, synthetic chemists have dedicated major efforts toward the fundamental study of natural products. After the initial isolation and structure determination, which usually spreads out over several years, it is often an interesting phenotype description, an unusual structure, the desire to showcase a particular methodology or strategy, or, more mundanely, the race toward a "hot" target molecule that triggers the synthetic efforts (Fig. 1). A disadvantage of this structure- and chemistry-driven approach for the development of natural products is that the molecular target or mechanism of action remains often unclear, and thus the clinical relevance of the lead structure from nature is speculative. While the synthetic work can provide valuable insights into structure–activity relationships (SARs), it is a rare occasion when any derivative reaches the practical application stage, and if it does against all odds, this generally occurs decades after the initial isolation of the parent compound.
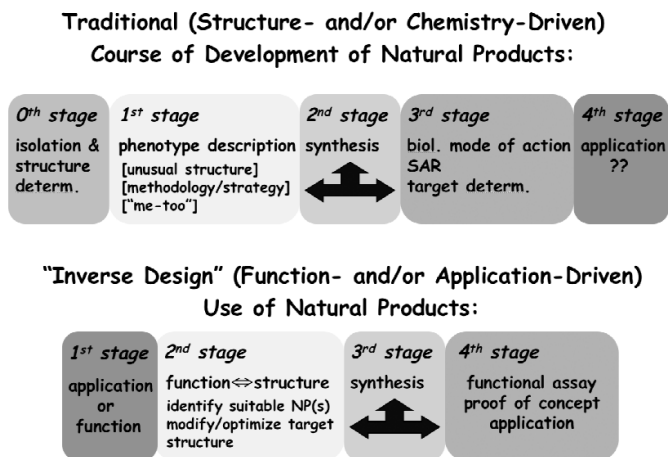

Fig. 1 Strategies in natural product chemistry.

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In recent years, we have become increasingly interested in exploring an alternative, "inverse design" strategy, wherein it is the formulation of a desirable application or function that lays the foundation of a natural product-based synthetic project. The key intellectual exercise is then the correlation of defined function to a lead structure, which depends on the identification of a suitable natural product, or a family of secondary metabolites, that can rationally be expected to provide the desired function in an efficacious and innovative fashion. Obviously, this task is greatly facilitated by the current availability of hundreds of thousands of natural product structures, and the sophistication of databases and modern data mining tools that link structures, properties, and bioassays. Chemical synthesis still fulfills a central role in the inverse design process, since it is very likely that several iterative steps are necessary to modify and optimize the natural product-based lead structure. However, since the application or utility has been formulated, functional assays are immediately available, and therefore the proof of concept for the validity of the design process should be rapid and unambiguous. Accordingly, we envision the inverse design concept to allow many more natural product-inspired practical applications to be realized, and, while by no means replacing the traditional investigations, to greatly enhance the utility of the vast pool of natural products that we now have at our disposal.

In this short assay, we present our recent studies with disorazoles and gramicidins, two examples for a traditional and an inverse design project, respectively.

TOTAL SYNTHESIS AND STRUCTURE–ACTIVITY STUDIES OF DISORAZOLE C_1
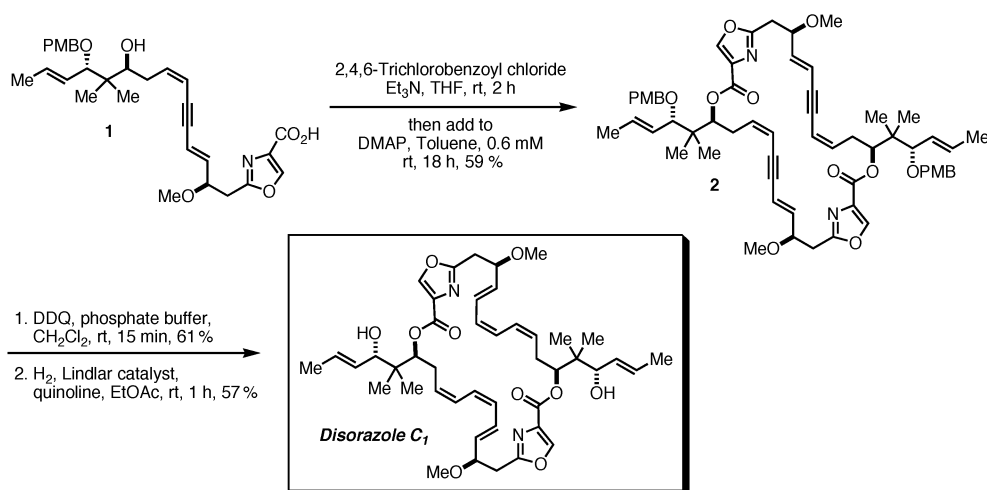
The disorazoles represent a family of 29 macrodiolides that were isolated in 1994 by Jansen et al. from a strain of sorangicin-producing myxobacteria [1]. Initially, no relative or absolute configurations were available, until a report in 2000 clarified some of the structural ambiguities for disorazole A_1 [2]. The latter compound was also biochemically characterized in 2004 as an extremely cytotoxic tubulin polymerization inhibitor, with a likely binding site in the vinca domain [3]. Since then, the biosynthetic gene cluster of the disorazoles has been identified [4], and a methanolysis product was found [5]. Prior to our total synthesis of (–)-disorazole C_1 [6], several fragment syntheses were reported [7]. Our selection of disorazole C_1, a minor metabolite of *Sorangium cellulosum*, was based on its presumably increased chemical stability compared to disorazole A_1, as well as its simplified dimeric structure, representing the southern half of disorazole A_1, thus allowing a more direct access to synthetic analogs (Scheme 1).

The sensitivity of the conjugated *Z,Z,E*-triene segment toward isomerization and polymerization as well as the sterically congested *anti*-1,3-hydroxyester moiety posed strategic and protective group challenges. Retrosynthetically, we took advantage of the symmetry of the target molecule by ester disconnections as well as Sonogashira couplings, which led to two readily available vinyl iodide and alkyne segments. While this implied a late-stage selective hydrogen addition across the alkyne portion of a dienyne, we hypothesized that the presence of the alkyne in between the two alkenes would stabilize the polyunsaturated system and facilitate ring closure. Similarly, the allylic alcohol protective group had to be carefully selected, since base-mediated conditions might lead to undesired acyl transfer reactions or elimination of the homoallylic ester function, and under acidic conditions, alkene *cis/trans*-isomerization or elimination processes would lead to rapid decomposition. We selected the *p*-methoxybenzyl (PMB) ether group since oxidative conditions, while not entirely innocuous to the dienyne and allylic alcohol moieties, appeared to be the least incompatible with the dense array of functionalities in the late-stage synthetic intermediates.
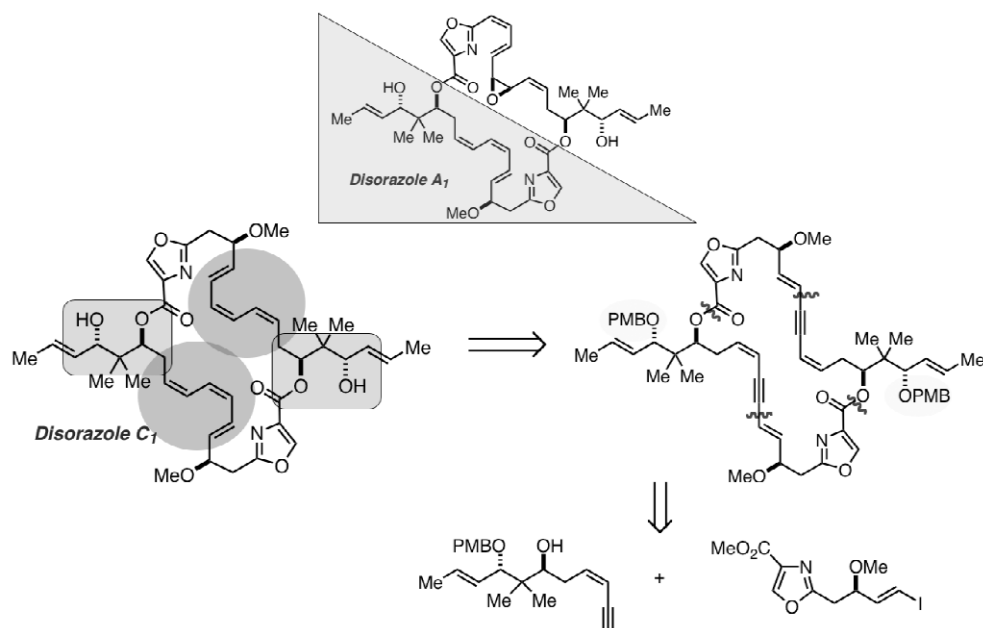
Scheme 1 Target structures and retrosynthetic plan.

Cyclodimerization of hydroxy acid **1** [6] was accomplished under Yamaguchi conditions, and the macrodiolide **2** was isolated in 59 % yield (Scheme 2). Not surprisingly, removal of the PMB ether with dichlorodicyano-*p*-benzoquinone (DDQ), and Lindlar hydrogenation of the dienyne required extensive optimization of reaction conditions and were batch- and scale-dependent. We ultimately identified reproducible protocols for both that delivered the natural product in 30–40 % yield over the last two steps, and in 2 % yield over 17 steps from commercially available starting materials [8].

Scheme 2 Cyclodimerization and final deprotection.

Immediately after completion of this total synthesis, we tried to resolve the lingering question of the biological activity of disorazole C_1. Only assay results for disorazole A_1 were available in the literature, and it was not clear if the potent biological properties in this class of natural products were contingent on the presence of the reactive vinyl epoxide function, which was absent in the C_1 metabolite. Fortunately, in collaboration with the Lazo group in Pittsburgh, we were able to demonstrate that disorazole C_1 continued to be a very potent antimitotic agent, with IC_{50}'s below 4 nM in five different cell lines (Fig. 2) [9]. In comparison, vincristine and vinblastine had IC_{50}'s of 5–22 and 1–2 nM, respectively, in the same assays. However, the alkyne precursor showed a very substantial decrease in activity to single-digit μM. In fact, any analog of disorazole C_1 synthesized in our lab to date experienced this order of magnitude drop off in activity. This observation points toward a tight fit to the biological target, requiring both a correct backbone orientation as well as the presence of the side-chain functionalities (Fig. 3). Truncated and acyclic analogs were also inactive, and a demethoxy analog was too unstable to be isolated and tested. Furthermore, since the side-chain cyclopropyl alcohol analog was inactive, it is feasible that the allylic alcohol is oxidized intracellularly to an enone, which functions as a Michael acceptor for covalent attachment to nucleophilic groups on the target protein(s). However, the definitive mechanism of action and the relevant molecular target remain to be established.

Cell Line	GI$_{50}$ [nM] ± SEM			
	(-)-disorazole C$_1$	alkyne precursor	vincristine	vinblastine
A549	2.21 ± 0.23 (8)	3485 (8)	21.62 ± 2.68 (8)	1.52 ± 0.09 (7)
PC3	1.57 ± 0.10 (8)	2640 (8)	4.68 ± 0.29 (8)	0.86 ± 0.08 (8)
MDA-MB-231	3.53 ± 0.19 (8)	6568 (8)	7.16 ± 0.37 (8)	1.34 ± 0.21 (8)
2008	1.91 ± 0.23 (8)	6726 (8)	21.81 ± 2.92 (8)	2.24 ± 0.16 (8)
WI-38	1.34 ± 0.04 (4)	6947 (4)	8.85 ± 1.15 (4)	1.83 ± 0.12 (4)

Fig. 2 Cell viability assays (72 h MTT) [9].

Fig. 3 Summary of SAR studies [8].

Our studies with disorazole C_1, which were performed exclusively with synthetic material, illustrate the ability of organic synthesis to provide unique structure–activity information efficiently and precisely. However, our inability to identify improved analogs after several iterations illustrates the challenge to improve upon the biological activities, even beyond the effort that would be necessary to identify the biological target, validate its clinical relevance, and engineer appropriate absorption, distribution, metabolism, excretion, and toxicity (ADMET) properties for the natural product or its congeners.

"INVERSE DESIGN"-BASED DEVELOPMENT OF NATURAL PRODUCTS: CONTROL OF REACTIVE OXYGEN SPECIES ESCAPE FROM MITOCHONDRIA

The first stage in the inverse design process is the definition of a practical application that could be controlled at the molecular level through the use of a natural product-based probe molecule. In discussions with the Fink, Kagan, and Greenberger groups at Pittsburgh we became interested in the control of reactive oxygen species (ROS) and reactive nitrogen species (RNS) by-products of cellular oxidative phosphorylation. ROS and RNS, in particular $O_2^{-\bullet}$, but also H_2O_2, $HO^\bullet$, $^1O_2$, O_3, ROOR, $NO^\bullet$, and $ONOO^-$, are formed in the conversion of O_2 into H_2O in complexes I, II, and III in the inner mitochondrial membrane. Efficient enzymatic scavenging systems such as superoxide dismutase usually reduce ROS, but in certain disease states, ROS and nitric oxide synthase (NOS) escape, activate cellular stress pathways, and damage many intracellular targets [10]. In 1956, Harman proposed the "free radical theory" of aging and associated degenerative diseases, and there is now overwhelming evidence that atherosclerosis, Alzheimer's disease, Parkinson's disease, neuronal death including ischemic stroke, acute and chronic degenerative cardiac myocyte death, and cancer may be among the consequences of ROS leakage [11]. Accordingly, regulation of ROS/NOS species at the source of generation in the mitochondrial membrane would be highly beneficial and therapeutically significant. By accumulating pro-drugs at the inner membrane, the active drugs are in close proximity to mitochondrial NO and $O_2^{-\bullet}$ species upon their release into the matrix.

While oxygen radical-scavenging substances such as the stable nitroxyl radical 2,2,6,6-tetramethylpiperidine-*N*-oxyl (TEMPO) and antioxidants such as hydroxylamines and vitamins C, E, and Q are well known to reduce the intracellular ROS/RNS levels [12], the goal to use these agents at therapeutically useful low concentrations and guide them to the mitochondrial membrane to prevent diffusion of ROS and RNS has been elusive [10,13]. We decided that it should be possible to engineer bacterial membrane targeting antibiotics to deliver catalytically active ROS/RNS scavengers selectively to the mitochondrial membrane. As a first scaffold for the development of these subcellular-targeting probes, we selected the cyclic decapeptide gramicidin S (GS), a topical antibiotic isolated from *Bacillus brevis* (Fig. 4) [14]. While a large number of membrane active natural antibiotics are potential candidates for this type of delivery, GS is particularly suitable since is has a strong interaction with microbial membrane lipids [15], including the anionic phospholipid cardiolipin (CL, diphosphatidyl glycerol) [16]. CL is closely associated to cytochrome C in the inner mitochondrial membrane and is converted to cardiolipin hydroperoxide (CL-OOH) by escaping ROS and RNS. Furthermore, we chose 4-amino-TEMPO (4-AT) as the scavenging moiety, since the nitroxide radical and the corresponding *N*-nitroso and hydroxylamine functionalities are readily interconverted, and the 4-amino substituent offers a suitable linkage point to GS.
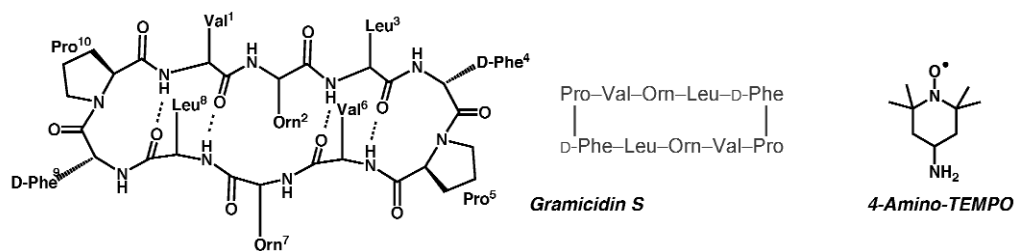
Fig. 4 GS and 4-AT.

Due to the strong toxicity of GS to human red blood cells (hemolysis), we embarked on identifying a suitable fragment that would both maintain the antiparallel β-sheet secondary structure of the natural product as well as offer a carboxyl function for attachment to 4-AT. We also aimed at improving bioavailability by replacing critical amide bonds with (*E*)-alkene peptide isosteres [17]. A circular dichroism (CD)-guided fragment analysis of GS isosteres revealed that ester **3**, which contains the β-turn inducing D-Phe-Pro subunit, assumed a solution conformation resembling that of the parent cyclodecapeptide (Fig. 5), and therefore this sequence was selected for conjugation to 4-AT. The preparation of the (*E*)-alkene peptide isostere **5** was based on our Zr/Zn methodology (Scheme 3) [18]. Hydrozirconation [19] of alkyne **4** with Cp_2ZrHCl followed by transmetalation to Me_2Zn and addition of *N*-Boc-isovaleraldimine afforded the corresponding allylic amide as a mixture of diastereomers, which were separated after desilylation and acetylation [20]. After oxidation of **5**, segment condensation of acid **6** and tripeptide amine H-Pro-Val-Orn(Cbz)-OMe was accomplished using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) as a coupling agent. Saponification of **7** followed by coupling with 4-AT afforded the desired conjugate **8**, in which the Leu-D-Phe peptide bond had been replaced with an (*E*)-alkene.
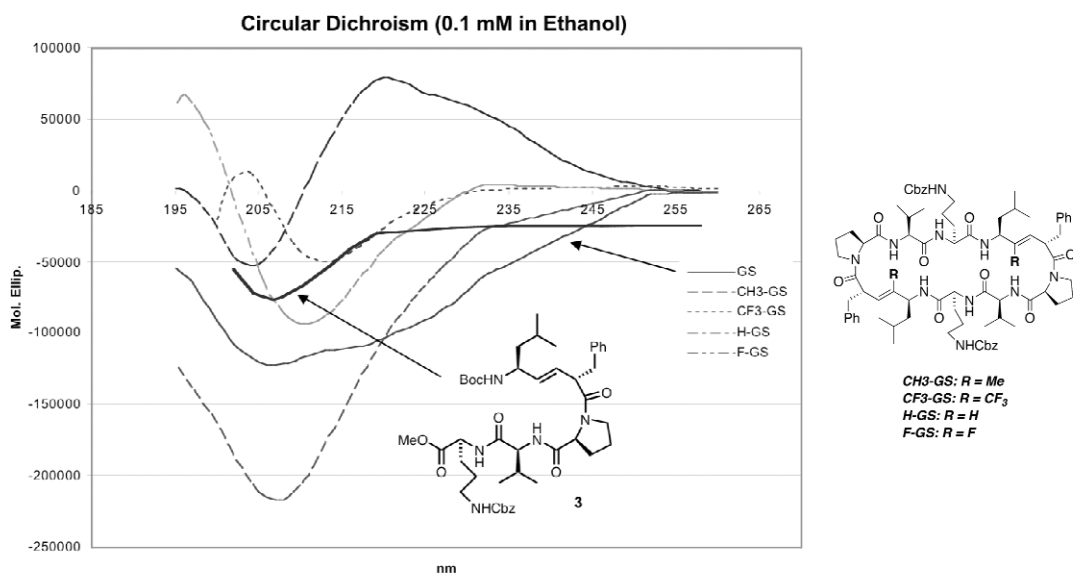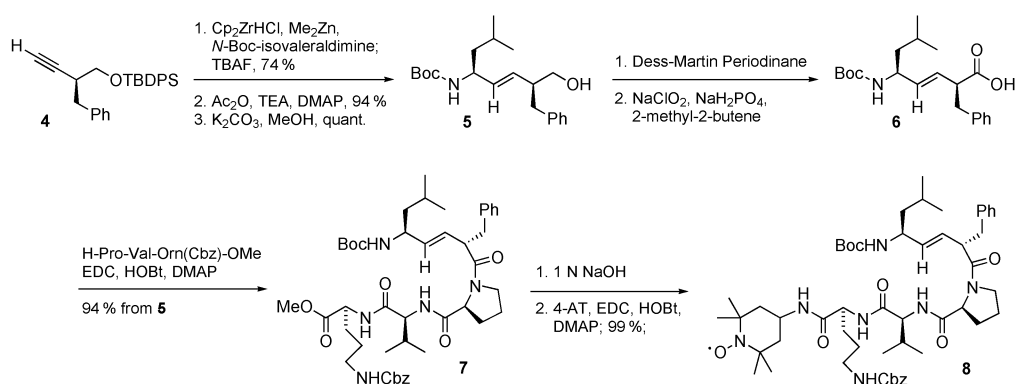

Fig. 5 CD analysis of GS analogs with substituted *(E)*-alkene isosteric replacements of the Leu-D-Phe peptide bond, and fragment ester **3** [17].

Scheme 3 Synthesis of 4-AT/GS conjugate **8**.

Our biological collaborators used electron paramagnetic resonance (EPR) spectroscopy to monitor cellular delivery and metabolic fate of **8** [20]. Distinctive triplet signals of nitroxide radicals were detected in mouse embryonic cells (MECs) incubated with 10 µM **8** as well as in mitochondria isolated from these cells. We further tested the ability of **8** to protect cells against apoptosis triggered by actinomycin D (ActD), and found that the AT-conjugate offered protection at relatively low 10 µM concentrations. These and additional biological studies confirmed that GS-derived **8** is a potent ROS/NOS scavenger.

Subsequent to establishing proof of principle, we further evaluated the therapeutic utility of **8** in an animal model for hemorrhagic shock in collaboration with the Fink group. Hemorrhagic shock results from inadequate oxygen delivery and effects the central nervous, cardiac, and renal systems, frequently resulting in death (Fig. 6). As a consequence of oxygen deprivation, adenosine 5'-triphosphate (ATP) levels are reduced, and reduced nicotinamide adenine dinucleotide (NADH) is increased in mitochondria. Upon reoxygenation, a burst of ROS escapes the enzymatic scavenging system and results in massive mitochondrial damage, the release of cytochrome C, programmed cell death, and organ dysfunction [21].
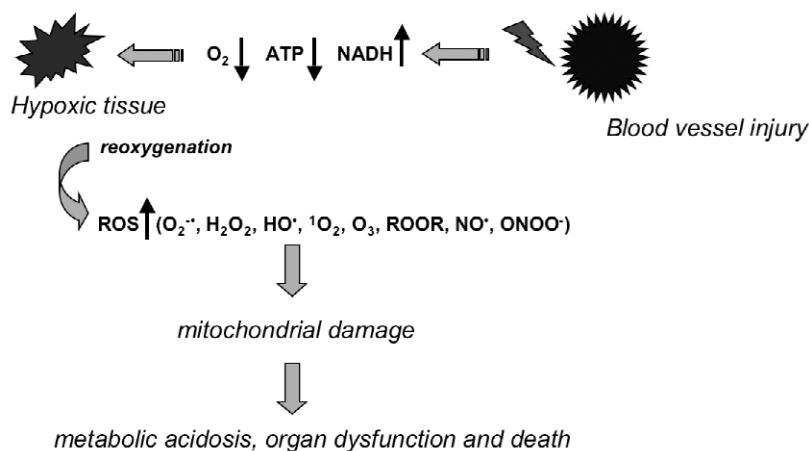

Fig. 6 ROS are generated during recovery from trauma.

We were able to demonstrate that delayed treatment with the superoxide dismutase mimic **8** significantly prolonged the survival of rats subjected to lethal hemorrhagic shock, even in the absence of resuscitation with asanguinous fluid or blood [22]. Presently, we are continuing these studies, with the goal to identify clinically viable derivatives of **8**.

In summary, we completed a total synthesis of disorazole C_1, which allowed us to investigate the natural product and demonstrate that this minor *Sorangium* metabolite is also a potent and irreversible inhibitor of cell proliferation with profound effects on microtubule structure and cell cycle. The synthetic route is convergent and amenable to SAR studies. Future directions of this research include the elucidation of the mode of action of disorazoles as well as the cellular target. However, this traditional line of investigation of natural products is time-intensive, and practical applications are speculative. In contrast, the use of natural products as the starting point for an "inverse design" approach has the potential to move much more quickly toward and beyond the proof of principle stage. We showcased the use of hemigramicidin S peptide isosteres to target the mitochondrial membrane and augment the effect of the ROS scavenger TEMPO. An in vivo model demonstrated the utility of the targeted conjugate to prevent hemorrhagic shock. Due to the vast pool of biologically annotated natural products, and the ever-expanding database mining toolsets that allow a reasonably rational function–structure correlation, the inverse design strategy bears great promise for the future of natural products chemistry.

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