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Development of a platform for the discovery and practical synthesis of new tetracycline antibiotics

Fan Liu and Andrew G Myers

Tetracyclines have proven to be safe and effective antibiotics over decades but to date all approved members of the class have been discovered and manufactured by chemical modification of fermentation products, which greatly limits the number of new structures that can be explored as future medicines. This review summarizes research leading to the development of a platform synthetic technology that enabled the discovery of the clinical candidate eravacycline, as well as other promising new tetracycline antibiotics, and provides the basis for a practical route for their manufacture. The approach argues for a reassessment of other antibiotic classes based on natural products for which practical, fully synthetic routes have not yet been developed, suggesting that these may represent underdeveloped resources with great potential to offer safer and more effective anti-infective agents.

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Introduction

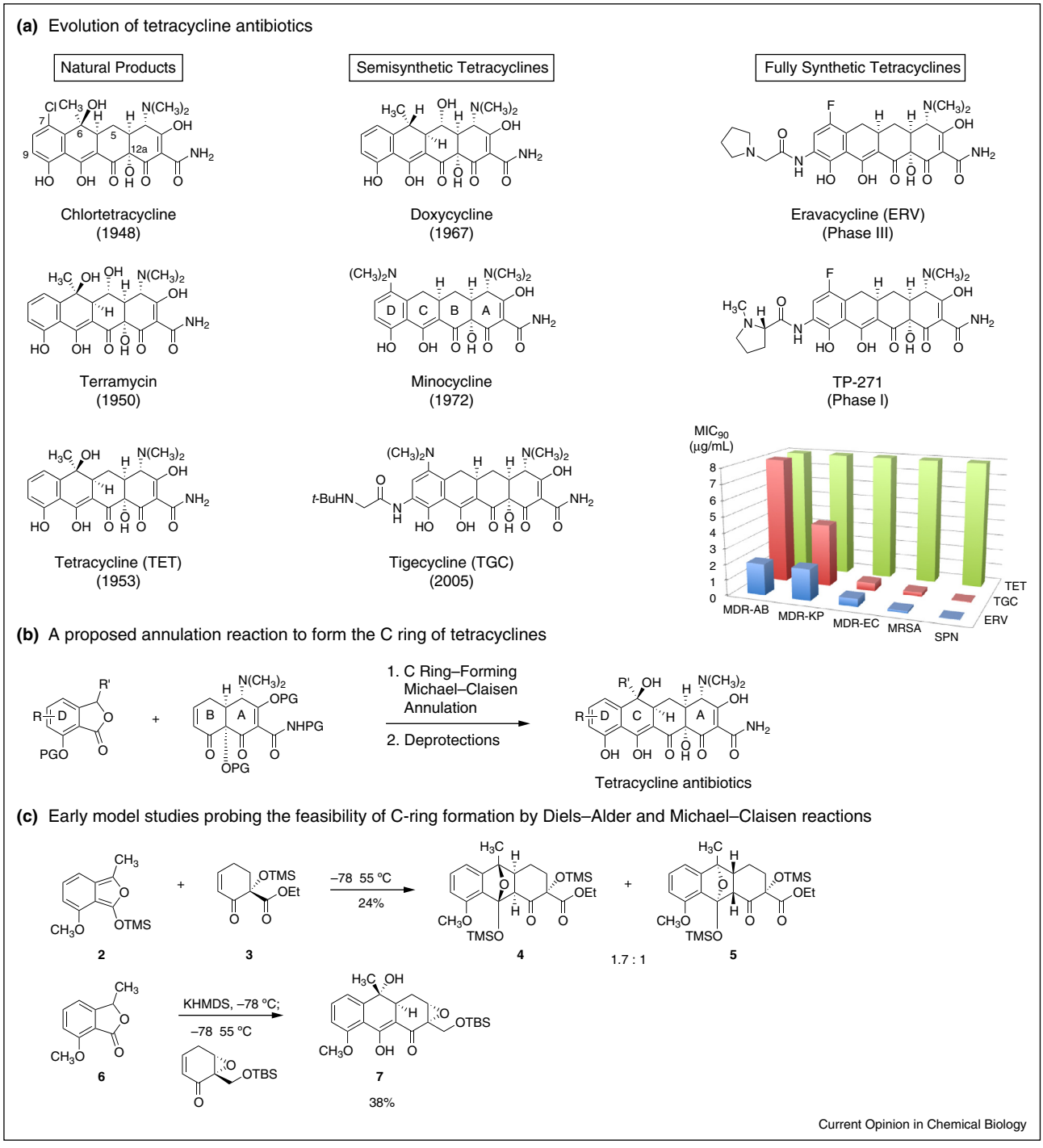
Tetracycline antibiotics have been in continuous clinical use for more than six decades and are generally regarded to be safe and effective medicines [1]. The class was discovered in 1948 when Benjamin Duggar isolated the natural product aureomycin, or 6-chlorotetracycline (chlortetracycline), from a bacterial culture (Figure 1a) [2]. Chlortetracycline was approved for human use in 1950, followed two years later with the launch of terramycin, or 5-oxytetracycline, a natural product isolated by Pfizer scientists [3]. Both natural products are unstable at the extremes of pH, but especially consequential in terms of use in humans is their propensity to undergo acid-catalyzed dehydration to form reportedly toxic anhydro-tetracycline derivatives (nephrotoxicity in particular is evidenced) [4].

Chlortetracycline was observed to undergo smooth hydrodechlorination with palladium and hydrogen, and the resulting semisynthetic molecule, tetracycline, was found to have superior stability, a better safety profile with respect to gastrointestinal toxicity, and a similar spectrum of activity relative to terramycin and aureomycin [5,6]. It was later found that tetracycline is also naturally occurring [7]. But a paradigm had been established with the chemical semisynthesis of tetracycline: that safer, more effective, and proprietary medicines can be obtained by chemical modification of naturally occurring antibiotics. This approach soon dominated discovery efforts and subsequently all approved tetracycline antibiotics were prepared by chemical modification of fermented tetracyclines. However, the chemical instabilities of tetracyclines and the dense array of polar functional groups that encircle their scaffold of four linearly fused six-membered rings make efficient and specific chemical transformations very challenging and rare. This is likely a primary reason that there have been fewer than 10 approved tetracycline antibiotics in 60 years, whereas >40 quinolone and >50 beta-lactam antibiotics (which are much more easily synthesized and modified) have been launched within the same period of time [8•].

The chemical advances leading to new semisynthetic tetracyclines are few and easily categorized. In the late 1950s scientists at Pfizer established efficient 3–4-step chemical protocols to reductively remove the labile 6-hydroxy substituents of terramycin, tetracycline, and 6-demethyltetracycline [9–11]. The resulting des-oxy products were far more stable compounds and retained good antibiotic activities [12]. In 1967, Pfizer launched the important medicine doxycycline, which they prepared in 4 steps from terramycin [13,14]. This antibiotic remains one of the most prescribed generic antibiotics in use today.

The greater acid stability of 6-deoxytetracyclines permitted for the first time limited electrophilic aromatic substitution reactions at position C7, leading to the discovery of minocycline (Lederle laboratories), which was launched in 1972 [15–17]. Like doxycycline, minocycline remains one of the most prescribed generic antibiotics, but it should be noted that resistance to both drugs, as well as all older tetracyclines, is now very widespread among both Gram-positive and Gram-negative pathogens [18]. In spite of growing resistance to doxycycline and minocycline, they remain drugs of choice for the treatment of atypical pneumonia caused by *Legionella* [19] and

Figure 1



(a) The evolutionary pathway of human development of tetracycline antibiotics, from fermentation products to semisynthetic derivatives, to fully synthetic tetracyclines. Inset, lower right: a comparison of MIC_{90} values (minimum inhibitory concentration to inhibit the growth of 90% of organisms) for natural (TET), semisynthetic (TGC), and fully synthetic (ERV) tetracyclines in multidrug-resistant (MDR) isolates of *Acinetobacter baumannii* (AB), *Klebsiella pneumonia* (KP), *Escherichia coli* (EC), methicillin-resistant *Staphylococcus aureus* (MRSA), and *Streptococcus pneumonia* (SPN) [33••]. **(b)** A proposed late-stage annulation reaction to form the C ring in fully synthetic tetracyclines, as originally conceived. PG = protective group. **(c)** Early model experiments to examine the viability of the proposed annulation reactions to construct the C ring of tetracyclines. Stereochemical assignments are tentative.

infections caused by Lyme borreliosis [20,21]. More than 30 years passed before the approval of the next and only recent tetracycline antibiotic, the important drug tigecycline [22].

Tigecycline was discovered by a team led by Dr. Frank Tally [23], then at American Cyanamid Company, who in addition to discovering tigecycline is also credited with championing the antibiotic daptomycin. The key chemical enablement leading to the discovery of tigecycline was the introduction of a second nitrogen atom at position C9 of the D ring, and to this nitrogen atom was appended a novel *N-t*-butyl glycine side chain, which is thought to contribute to the greater affinity of tigecycline for the bacterial ribosome [24] and, as a consequence, its increased efficacy against many pathogenic bacteria that had acquired resistance to all other tetracycline antibiotics [23,25,26]. Tigecycline was approved in 2005 for the treatment of complicated intra-abdominal infections (cIAIs), complicated skin and soft-tissue infections (cSSTIs), and community-acquired pneumonia (CAP). Because it is efficacious against Gram-negative bacteria that are resistant to virtually all other antibiotics, tigecycline has become a treatment of last resort for patients with infections caused by multidrug-resistant Gram-negative pathogens. Additionally, it has been prescribed alone or in combination with other antibiotic agents for the treatment of endocarditis, brucellosis, and severe *Clostridium difficile* infections [27]. Dosing of tigecycline is limited by its toxicity. To minimize the specific adverse events of nausea, vomiting, and diarrhea, initial dosing is limited to 100 mg, where approximately 25% of patients will experience incidents of nausea and vomiting [27]. In spite of this, higher dosing of 200 mg per day has been studied in critically ill patients, with the result that mortality was reduced relative to standard dosing [28].

In the mid-1990s we initiated a program that over a period of 12 years of continuous research led to the development of a practical, scalable route to tetracyclines, broadly defined [29,30]. To date, this chemistry has enabled the synthesis of more than 3000 fully synthetic tetracycline analogs, among them the clinical candidates eravacycline and TP-271 (Figure 1a) and a preclinical candidate, TP-6076.

Eravacycline, the most advanced clinical candidate, is the first fully synthetic tetracycline to enter clinical development [31]. Structurally, it differs from all previous tetracyclines by virtue of its 7-fluoro substituent, a modification that had not been possible to introduce by semisynthetic methods. Eravacycline also features a pyrrolidinoacetamido sidechain at C9 [32] and is distinct from tigecycline in that it is the first glycylcycline with oral activity in humans (Horn PT, Sutcliffe JA, Walpole SM, Leighton A, abstract 603, 49th Annual Meeting of the Infectious Diseases Society of America, Boston, MA, October 2011). Like all tetracyclines, eravacycline targets the bacterial ribosome and inhibits protein synthesis. Eravacycline exhibits greatly improved broad-spectrum antibiotic activity relative to other tetracyclines (Figure 1a), particularly against multidrug-resistant bacteria, with MIC_{90} values ranging from $\leq$0.008 to 2 μg/mL [33••]. Eravacycline has been shown to evade major tetracycline-specific resistance mechanisms such as ribosomal protection and efflux [34]. Among the Gram-positive strains tested, eravacycline shows potent *in vitro* activity against MRSA (MIC_{90} = 0.13 μg/mL), including a subset of MRSA isolates resistant to macrolides, fluoroquinolones, and linezolid. Eravacycline is highly active (MIC_{90} = 0.06 μg/mL) against strains of *E. faecium* and *E. faecalis* that are resistant to vancomycin, as well as all streptococci, including *S. pneumonia* isolates resistant to penicillin, macrolides, or both ($MIC_{90} \leq$ 0.13 μg/mL) [33••].

Eravacycline potently inhibits growth of a collection of clinically important Gram-negative bacterial species, a number of which are resistant to many or all third-generation cephalosporins, aminoglycosides, and fluoroquinolones. In one noteworthy case, eravacycline exhibited an MIC_{90} of 2 μg/mL against a panel of *A. baumannii* isolates resistant to three current antibiotic therapies (carbapenems, fluoroquinolones, and aminoglycosides) [33••]. In a Phase II trial for the treatment of cIAI, eravacycline was shown to cure 100% of patients when administered intravenously at 1.0 mg/kg of body weight every 12 h [35•]. Importantly, the incidence of adverse events, such as nausea and vomiting, in patients treated with eravacycline was low and comparable to that of the comparator drug, the carbapenem ertapenem [35•]. Subsequently, eravacycline met a primary endpoint of non-inferiority in a Phase III trial for cIAI relative to ertapenem as control. Eravacycline did not meet the primary endpoint of non-inferiority relative to the fluoroquinolone levofloxacin in a Phase III trial for complicated urinary tract infections (cUTI) involving IV to oral stepdown treatment and remains under further clinical evaluation (Tetraphase Announces Top-Line Results From IGNITE2 Phase 3 Clinical Trial of Eravacycline in cUTI, URL: http://ir.tphase.com/releasedetail.cfm?ReleaseID=930613).

TP-271 is a novel fluorocycline with potent activity against infectious agents causing anthrax, community-acquired bacterial pneumonia (CABP), tularemia, and bubonic plague, and recently entered a Phase I clinical trial (Tetraphase Pharmaceuticals, January 2016; URL: http://ir.tphase.com/releasedetail.cfm?ReleaseID=950670). A third novel tetracycline, TP-6076, is currently under preclinical evaluation for the treatment of multidrug-resistant Gram-negative bacterial infections (Tetraphase Pharmaceuticals; URL: https://www.tphase.com/our-science/pipeline/).

In this review we summarize the path that led to the development of a practical route to fully synthetic

tetracyclines, enabling the discovery of eravacycline and other clinical candidates and providing the basis for their eventual manufacture.

Background

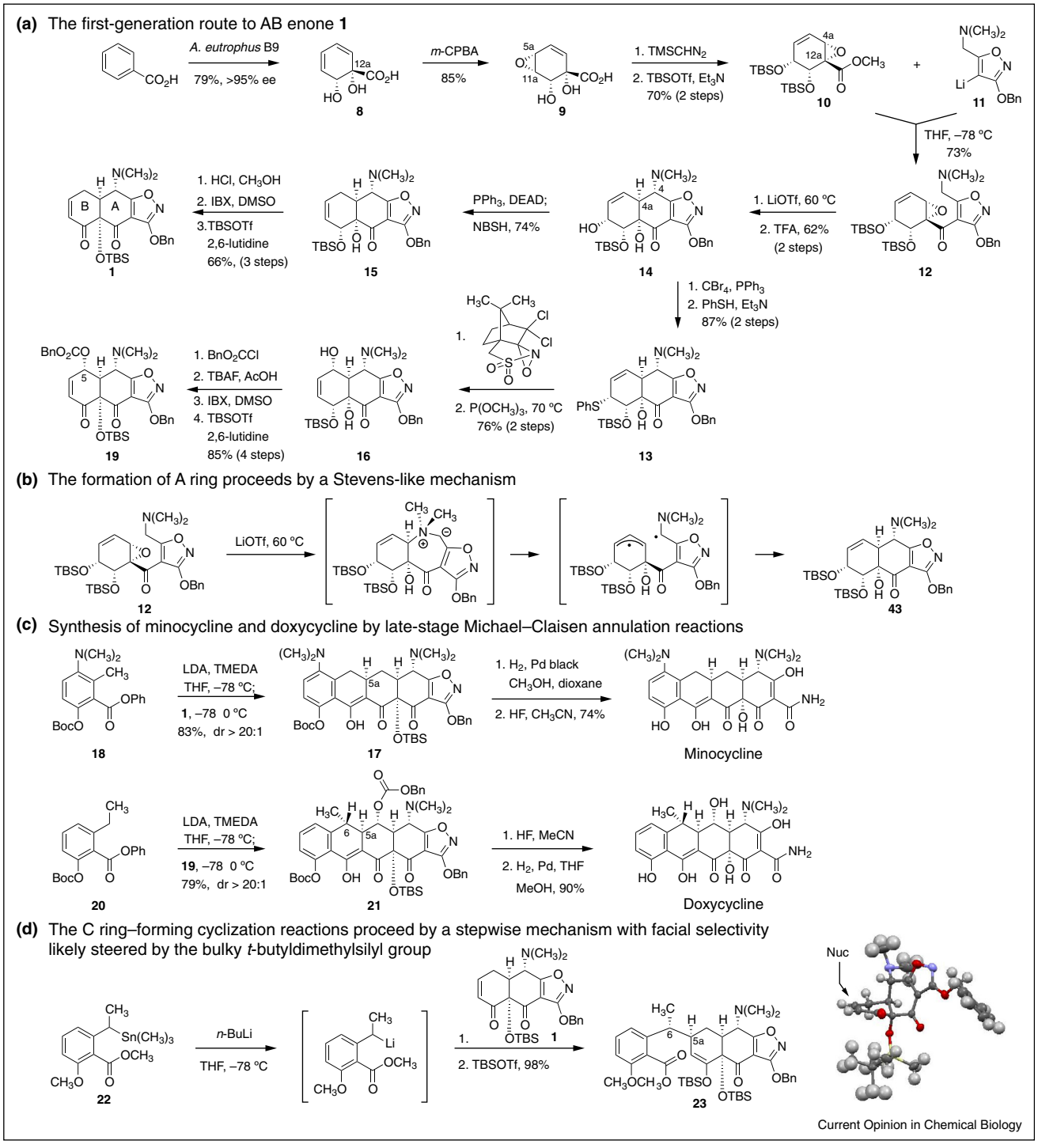
Prior synthetic routes to tetracyclines were remarkable scientific achievements in their time, but did not provide ready access to novel antibiotic candidates for evaluation, nor a basis for practical manufacturing [36–39]. During the period 1990–1995, we had developed a fully synthetic route to dynemicins that featured the convergent assembly of a central ring by using either a Michael–Claisen condensation or a Diels–Alder cycloaddition reaction (the latter using an isobenzofuran as dienophile) [40,41]. In the latter stages of this research we began to consider employing a related approach to construct the C ring of tetracyclines from AB-ring and D-ring precursors (Figure 1b). Dr. Cynthia Parrish performed initial model studies (CA Parrish, PhD thesis, California Institute of Technology, 1999) to determine whether simple cyclohexenone substrates might undergo condensations similar to those employed in our dynemicin synthetic work (which involved a much more reactive quinone imine as substrate). As shown in Figure 1c, Dr. Parrish's work revealed that the proposed condensations were feasible, but efficiencies and stereoselectivities were low; and in one case, the stereochemistry of the product was believed not to correspond to that of the tetracyclines. We determined to overlook these deficiencies, choosing instead to defer further study of the key condensation until a relevant AB-enone precursor was in hand — a goal which, remarkably, required a further nine years of study to attain. During this period, it became clear to us that 6-hydroxytetracyclines were no longer important targets (see introduction), and our attention turned from the proposal of Figure 1b to the condensation reactions of Figure 2, which afford the important 6-deoxytetracycline targets. The first synthesis of the key AB enone **1** and its 3-step transformation into fully synthetic 6-deoxytetracyclines were reported in 2005 [29]. Subsequently, two entirely different syntheses of the key AB enone were developed at Harvard [42,43], and process chemists at Tetraphase Pharmaceuticals, a company founded in 2006 to commercialize the tetracycline synthetic platform, made many further innovations to our second-generation route that permitted scaling of the AB enone to >100-kg amounts [44••,45••]. Below we summarize the evolution of the path to the AB enone and describe its successful coupling with diverse D-ring precursors through highly efficient and stereoselective Michael–Claisen condensation reactions.

The first-generation synthesis of AB enone 1 and its incorporation into fully synthetic tetracyclines

The first successful synthetic route to the AB enone **1** proceeded in 11 steps and 10% yield (Figure 2a) [29]. The sequence began with whole-cell microbial dihydroxylation of benzoic acid using a mutant strain of *A. eutrophus* to afford the diol **8** in 79% yield and >95% ee (90-g scale). Notably, this transformation established the C12a tertiary alcohol in the first step of the route (tetracycline numbering). Historically, attempts to introduce the C12a hydroxyl group at a late stage in tetracycline synthetic efforts have been problematic [36–39,46]. The diol **8** was readily transformed into the epoxide **10** in 3 steps and 60% yield. Interestingly, in the last step of this sequence (a silyl protection reaction), we observed an unexpected rearrangement of the epoxy group from positions 11a and 5a to 12a and 4a [47]. This was the first of two serendipitous and highly useful transformations discovered in this research effort. The second arose after coupling of **10** and **11** to form ketone **12** (73% yield). We note that component **11** is a 5-benzyloxyisoxazole derivative and comprises a masked form of the vinylogous carbamic acid function within the A ring of tetracyclines, a protection strategy that was developed by Stork and Hagedorn in the course of their tetracycline synthetic studies [48]. We had imagined that enolization of **12** might provide bicyclic alcohol **14** (more properly, its *t*-butyldimethylsilyl ether); it did not. Strikingly, however, when **12** was warmed in the presence of lithium trifluoromethanesulfonate, the desired product **14** was formed in 62% yield (after selective silyl deprotection with trifluoroacetic acid). Subsequent mechanistic investigations revealed that the formation of **14** likely proceeded via an ammonium ylide intermediate, which rearranged by a Stevens-like mechanism — a much more complex process than we had originally envisioned (Figure 2b, JD Brubaker, PhD thesis, Harvard University, 2007). Reductive transposition of **14** to form **15** (74% yield) was accomplished using methodology developed in another context [49]. Three simple steps then transformed **15** into the crystalline AB enone **1** in 66% yield (see Figure 2d for the solid-state structure of **1**). Separately, a different sequence transformed **14** into the C5-oxygenated AB enone **19** in 8 steps and 56% yield. AB enone **19** can also be prepared from AB enone **1** in 5 steps and 31% yield (PM Wright, PhD thesis, Harvard University, 2012).

With the fully functionalized AB-ring precursor **1** in hand, the C ring-forming Michael–Claisen cyclization reaction was shown to proceed with remarkable chemical efficiency and stereoselectivity (Figure 2c) [29,30]. For example, treatment of D-ring precursor **18** with lithium diisopropylamide (LDA) in the presence of *N,N,N′,N′*-tetramethylethylenediamine (TMEDA) at −78 °C led to deprotonation of the benzylic position. Addition of enone **1** to this bright red anion and warming of the reaction mixture to −10 °C provided the cyclization product **17** in 83% yield as a single diastereomer. A two-step deprotection sequence then afforded minocycline in 74% overall yield. Doxycycline was similarly prepared using the D-ring precursor **20** and AB enone **19** in 71% yield over three

Figure 2



(a) The first-generation synthesis of AB-ring precursors **1** and **19**. **(b)** Mechanistic investigations suggest that formation of the A ring likely proceeds by the formation of an ammonium ylide intermediate, which subsequently rearranges by a Stevens-like mechanism, a serendipitous but highly useful transformation. **(c)** Synthesis of minocycline and doxycycline through late-stage Michael–Claisen annulation reactions. **(d)** Mechanistic and stereochemical features of the C-ring annulation reaction.

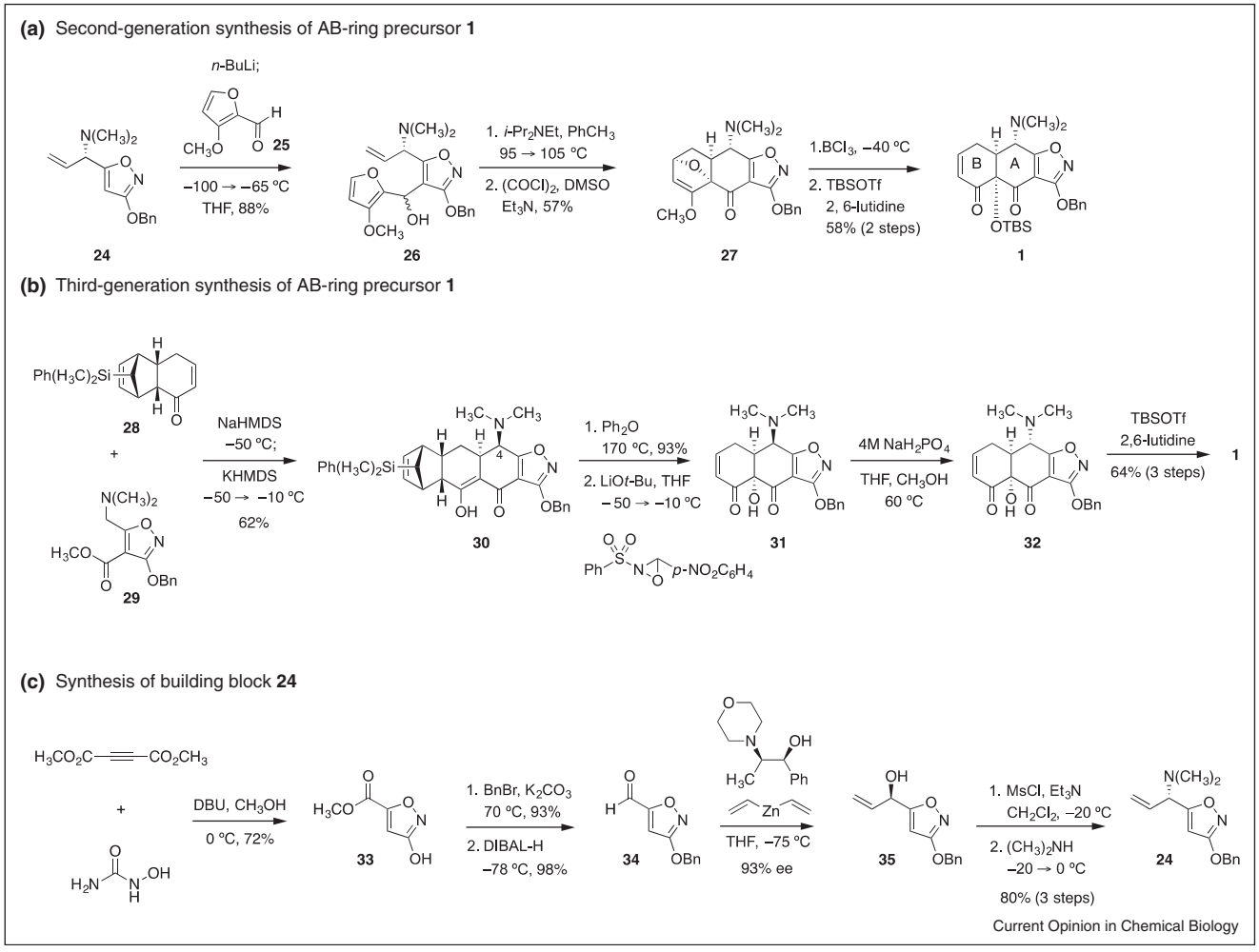
steps. The use of a phenyl ester within the D-ring toluate nucleophiles was critical, as anions derived from D-ring precursors with simple alkyl esters failed to undergo Claisen cyclization. By trapping the intermediate Michael adduct with *t*-butyldimethylsilyl trifluoromethanesulfonate at −78 °C, we demonstrated that annulation proceeds in a stepwise fashion, with the Claisen condensation as the rate-limiting step (Figure 2d). X-ray crystallographic analysis of the AB enone **1** reveals that one π-face is sterically blocked by the *t*-butyldimethylsilyl group. The relative stereochemistry at C5a and C6 is congruent with that of doxycycline and 6-deoxytetracycline [30], an exceptional outcome given that as many as four diastereomers could have been formed.

A number of different D-ring precursors were found to be suitable coupling partners in the Michael–Claisen annulation reaction, including substrates that were prone to self-coupling [30]. The new methodology also permitted the syntheses of pentacyclines and hexacyclines. These structurally unique tetracycline analogs were found to be active against both Gram-positive and Gram-negative bacteria resistant to existing tetracycline antibiotics [30,50,51].

Second-generation and third-generation syntheses of AB enone 1

While the first-generation route was sufficiently scalable to deliver quantities of the AB-ring precursor (≤10-g amounts) necessary to synthesize several structurally novel tetracycline antibiotic candidates, it was not practical beyond its application towards initial discovery chemistry. There were concerns about the viability of the microbial dihydroxylation reaction on scale, for occurrence of revertant strains (which consumed the desired product **8**) had been observed and, further, the product **8** was known to decompose when stored improperly. In addition, in the key transformation of **12** to **14**, a regioisomeric product was formed that complicated isolation of **14** and reduced its yield. For these and other reasons, we

Figure 3

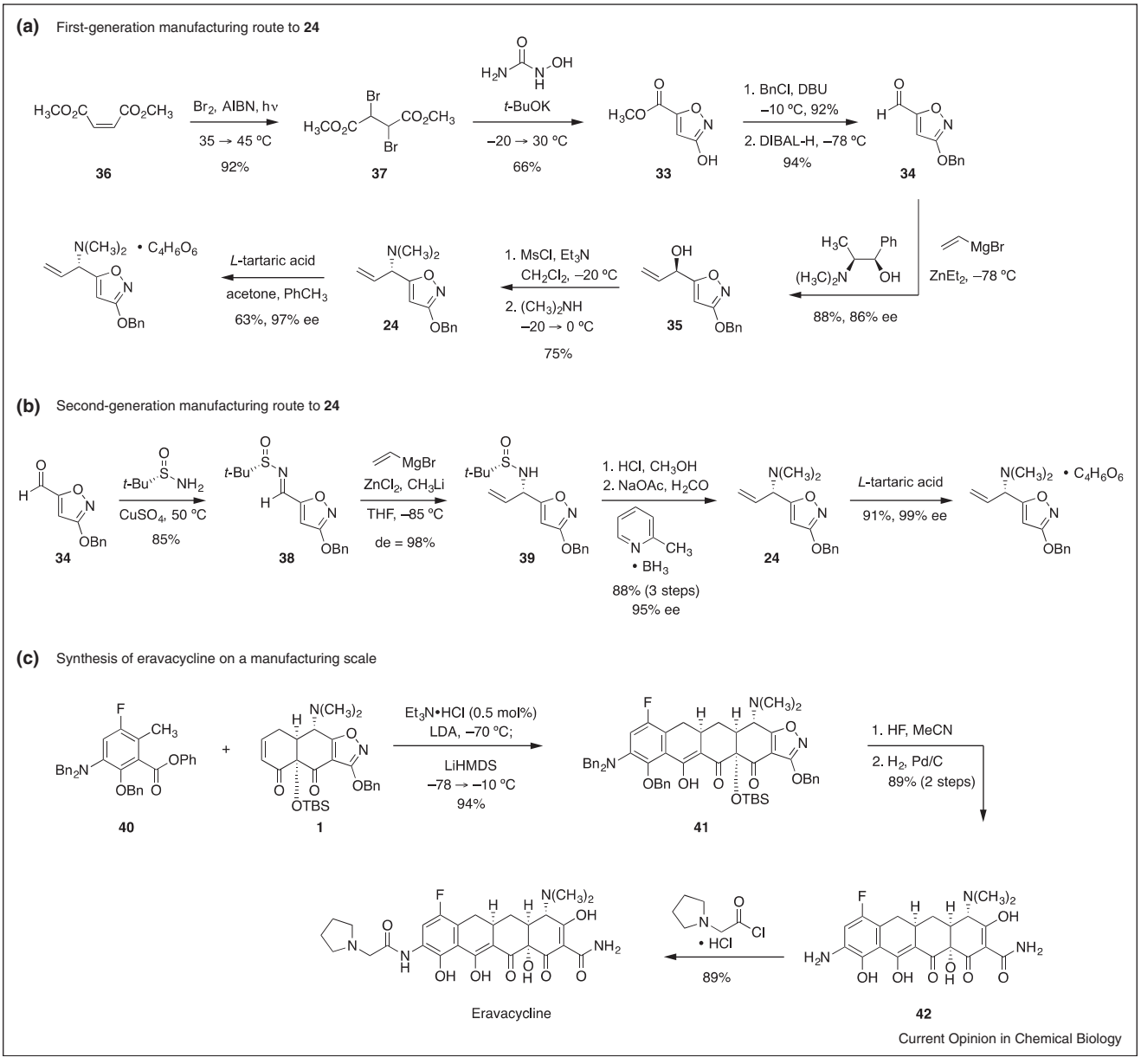


(a) The second-generation and **(b)** third-generation synthesis of the AB-ring precursor **1**. **(c)** Laboratory-scale synthesis of building block **24**.

were led to develop a second-generation route and, later, a third-generation route that provided more efficient, scalable sequences to **1**. In our second-generation approach (Figure 3a) [42], two components of similar synthetic complexity, 3-methoxyfurfural (**25**) and (*S*)-isoxazole amine **24** were coupled by a metalation–carbonyl addition sequence to afford a diastereomeric mixture of allylic alcohols **26**. Both diastereomers underwent smooth Diels–Alder cycloaddition favoring the *endo* cycloadducts; after Swern oxidation, the tetracyclic product **27** was obtained as a single pure substance. Cleavage of the allylic C–O bond of the bicyclic ether (BCl_3, −40 °C) and silyl protection then afforded the crystalline AB enone **1** in 29% yield from **24** and **25** (40-g scale). This more streamlined, efficient, and scalable route to **1** was selected for further optimization and for development of a manufacturing process (*vide infra*).

In light of the success of the Michael–Claisen reaction in constructing the C ring of tetracyclines, we imagined a third-generation synthesis of the AB enone **1** wherein the A ring would be assembled by Michael–Claisen

Figure 4



(a) First-generation manufacturing route of **24**. **(b)** Second-generation manufacturing route of **24**. **(c)** The Michael–Claisen cyclization reaction can be executed on a kilogram scale to support clinical development of eravacycline.

condensation of the optically pure cyclohexenone **28** and an enolate derived from the isoxazole ester **29**, both components of similar synthetic complexity (Figure 3b) [43]. In the event, the Michael–Claisen cyclization afforded adduct **30** as a single diastereomer, which was isolated as a crystalline hydrochloride salt in 62% yield. Thermal extrusion of 5-dimethylphenylsilylcyclopentadiene, stereoselective hydroxylation, C4-epimerization, and silylation afforded crystalline **1** in 37% yield over 5 steps from **28** and **29** (40-g scale). To date, both the second-generation and third-generation routes have been performed on >100-g scales in our laboratory and have supported the syntheses of novel tetracyclines of previously inaccessible structural variability [30,50–53].

Manufacturing fully synthetic tetracyclines on a kilogram scale

To permit clinical development of eravacycline, TP-271, and TP-6076, >50 kg of the AB-ring precursor **1** were synthesized at Tetraphase Pharmaceuticals using a route adapted from our second-generation synthesis (Figure 3a). In our original approach, building block **24** was prepared in five steps from methyl 3-hydroxy-5-isoxazolecarboxylate (**33**) [42,54], a commercial product that can be prepared from the reaction of *N*-hydroxyurea with dimethyl acetylenedicarboxylate (Figure 3c) [55]. The high cost of dimethyl acetylenedicarboxylate led chemists at Tetraphase Pharmaceuticals to develop two different routes to **24** (Figure 4a and b) [44]. In the first route (Figure 4a), dimethyl 2,3-dibromosuccinate (**37**), readily obtained by bromination of the inexpensive reagent dimethyl maleate (**36**), was substituted for the expensive reagent dimethyl acetylenedicarboxylate in coupling with *N*-hydroxyurea to furnish **33** in 66% yield. *O*-benzylation and reduction then provided the isoxazole aldehyde **34** in 86% yield. Major improvements were made in the subsequent enantioselective vinyl addition reaction; specifically, a mixture of commercial vinyl magnesium bromide and diethylzinc (in the presence of the chiral amine ligand depicted) was found to obviate the need for preparation of divinylzinc, which required a cumbersome filtration sequence that was impractical on large scale. More than 48 kg of **24**, isolated in the form of its *L*-tartrate salt, were prepared by this sequence. Later, **24** was prepared using the Ellman *t*-butyl sulfinyl auxiliary, as depicted in Figure 4b. That route has led to production of >370 kg of **24** (*L*-tartrate salt) of 99% ee.

In addition to improvements in manufacture of the AB enone component, the key Michael–Claisen condensation reaction used to prepare eravacycline has also been substantially improved (Figure 4c) [45]. On a manufacturing scale, the addition of a small amount (0.1–0.5 mol%) of triethylamine hydrochloride, which provides a source of lithium chloride, was found to be crucial to reliably generate the D-ring *o*-toluate anion. Additionally, the presence of an excess of LDA was found to promote side reactions. Use of the weaker base lithium hexamethyldisilazide to deprotonate the Claisen product proved superior. In a single batch, the modified protocol provided 934 g of **41**, in 94% isolated yield, as a single diastereomer. This product was then subjected to a conventional two-step deprotection sequence (89% yield) followed by sidechain introduction (89%) to furnish fully synthetic eravacycline.

Conclusory statement

The development of a practical fully synthetic route to tetracycline antibiotics has afforded a revolutionary discovery engine for the identification of novel antibiotic candidates. In light of this, and recognizing that practical synthetic routes are lacking for many other naturally occurring antibiotics, it seems logical to speculate that, properly focused, synthetic organic chemistry can play an important role in the development of many new medicines for the treatment of infectious disease.

Conflict of interest

A.G.M. is a co-founder of both Tetraphase Pharmaceuticals and Macrolide Pharmaceuticals and serves on the board of directors and heads the scientific advisory board at Macrolide Pharmaceuticals.

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