REMARKS

The specification has been amended to delete the word "Novel" from the title of the application as requested by the Examiner.

Claims 1-8, 10-12, 14-17, 20-23, 25-33, 36, 38, 40, 43, 45, 46, 48, 50, and 52 were pending in the application. All claims have been cancelled, without prejudice and new claims 54-93 have been added. Accordingly, following entry of these amendments, claims 54-93 will remain pending in the application.

Support for the new claims may be found throughout the specification and originally filed claims. Specifically, claims 54 and 60 find support in original claim 12 and the specification at page 39, lines 9-10. Claims 55, 56, 61 and 62 find support in the paragraph bridging pages 39 and 40 and the paragraph bridging pages 41-42 of the specification as filed. Claims 57 and 63 find support in the specification at page 40, lines 19-20 and page 42, lines 18-20. Claims 58 and 64 find support in the specification at page 44, lines 4-5. Claims 59 and 65 find support in the specification at page 46, line 27. Claims 66 and 80 find support in original claims 21 and 22, respectively. Claims 67-72 and 81-86 find support in original claims 27-32 and the specification at page 36, line 1-2 and 17-20. Claims 73 and 87 find support in the specification at page 43, lines 27-28. Claims 74 and 88 find support in the specification at page 43, lines 28-30. Claims 75 and 89 find support in the specification at page 43, line 30 through page 44, line 1. Claims 76 and 90 find support in the specification at page 44, lines 23-26. Claims 77 and 91 find support in the specification at page 44, lines 8-9. Claims 78 and 92 find support in the specification at page 44, lines 10-12. Claims 79 and 93 find support in the specification at page 10, line 17. No new matter has been added.

Objection to Specification

The Examiner has objected to the title of the application for including the word "novel." To expedite prosecution, the title has been amended to remove the word "novel," thereby rendering this objection moot. Accordingly, reconsideration and withdrawal of this objection is respectfully requested.

Rejection of Claims 1-8, 10-12, 14-17, 20-23, 25-33, 36, 38, 40, 43, 46, 48, 50 and 52 Under 35 U.S.C § 101

The Examiner has rejected claims 1-8, 10-12, 14-17, 20-23, 25-33, 36, 38, 40, 43, 46, 48, 50 and 52 under 35 U.S.C. § 101 as allegedly being drawn to patent ineligible subject matter.

In the interest of expediting prosecution and in no way acquiescing to the validity of the Examiner's rejection, Applicants have cancelled the composition of matter claims and have presented herein pharmaceutical composition claims and method of treatment claims. Applicants respectfully submit that these claims are patent eligible for the following reasons.

I. Eligibility of Pharmaceutical Composition Claims

New claims 54 and 60, and claims dependent therefrom, are directed to pharmaceutical compositions comprising the compound of Formula (II) and Formula (III), respectively, and a pharmaceutically-acceptable carrier that efficiently solubilizes the compound of Formula (II) or (III), respectively.

Claims 54 and 60 are analogous to claim 6 of Example 3 of the Nature-Based Product Examples found in *2014 Procedure For Subject Matter Eligibility Analysis Of Claims Reciting Or Involving Laws Of Nature / Natural Principles, Natural Phenomena, And / Or Natural Products* (hereinafter "the Guidelines"):

> 6. A stable aqueous composition comprising: amazonic acid; and a solubilizing agent.

The Guidelines provide that this claim is patent eligible based on the following analysis:

> Claim 6: Eligible. In nature, amazonic acid is insoluble in water. As explained in the specification, however, when amazonic acid is combined with a solubilizing agent, it becomes soluble in water and forms a stable solution. ***This changed property (solubility) between amazonic acid as a part of the claimed stable aqueous composition and amazonic acid in nature is a marked difference. Accordingly, the claimed composition has markedly different characteristics, and is not a "product of nature" exception.*** Thus, the claim is not directed to an exception (Step 2A: NO), and

> qualifies as eligible subject matter. (See Example 3 of the Guidelines, emphasis added).

Similar to claim 6 from Example 3 of the Guidelines, claims 54 and 60 presented herein are directed to a pharmaceutical composition comprising a compound of Formula (II) or (III), respectively, and a pharmaceutically acceptable carrier that efficiently solubilizes the compound. Just as amazonic acid in Example 3 is insoluble in water, the compound of Formula (II) also exhibits very low solubility in water (see the Declaration under 37 CFR § 1.132 of Aaron Peoples ("the Declaration") submitted herewith, paragraph 3). Furthermore, similar to the use of a solubilizing agent with amazonic acid to produce a stable aqueous solution in Example 3 of the Guidelines, Applicants have determined that when the compound of Formula (II) is combined with a pharmaceutically acceptable carrier that efficiently solubilizes the compound, the solubility of the compound of Formula (II) increases and a stable solution is formed (see the Declaration, paragraph 4).

Accordingly, similar to claim 6 in Example 3 of the Guidelines, ***"[t]his changed property (solubility) between . . . [the compound of Formula (II)] as part of the claimed stable aqueous composition and . . . [the compound of Formula (II)] in nature is a marked difference. Accordingly, the claimed composition has markedly different characteristics, and is not a 'product of nature' exception."*** Therefore, just as claim 6 from Example 3 of the Guidelines qualifies as patent eligible subject matter, claims 54 and 60, and claims dependent therefrom, also qualify as patent eligible subject matter under U.S.C. § 101.

II. Eligibility of Method of Treatment Claims

New claims 66 and 80, and claims dependent therefrom are directed to methods for treating a bacterial infection in a subject by administering to the subject an effective amount of a compound of Formula (II) or (III), respectively.

The Guidelines provide that method claims for nature-based products are patent-eligible subject matter under 35 U.S.C. § 101. Specifically, the Guidelines indicate that

> [a] ***process claim is not subject to the markedly different analysis for nature-based products used in the process***, except in the

> limited situation where a process claim is drafted in such a way that there is no difference in substance from a product claim (e.g., "a method of providing an apple."). (The Guidelines, I.3.a., *emphasis added; internal citations omitted).*

Moreover, claims 66 and 80 are analogous to claim 8 of Example 3 of the Nature-Based Product Examples of the Guidelines:

> 8. A method of treating breast or colon cancer, comprising: administering an effective amount of purified amazonic acid to a patient suffering from breast or colon cancer.

The Guidelines provide that this claim is patent eligible because:

> [a]lthough claims 7-8 recite nature-based products (amazonic acid), ***a full eligibility analysis of these claims is not needed because the claims clearly do not seek to tie up all practical uses of the nature-based products***. (Emphasis added).

Example 3 of the Guidelines continues to provide a more detailed explanation of why such claims are patent eligible:

> Claim 8: Eligible. Although the claim recites a nature-based product (amazonic acid), ***analysis of the claim as a whole indicates that the claim is focused on a process of practically applying the product to treat a particular disease (breast or colon cancer), and not on the product per se. Thus, it is not necessary to apply the markedly different characteristics analysis in order to conclude that the claim is not directed to an exception (Step 2A: NO). The claim qualifies as eligible subject matter.*** (Emphasis Added).

Similar to claim 8 from Example 3 of the Guidelines, claims 66 and 80, and claims dependent therefrom, are patent eligible. Following the analysis set forth in Example 3, although claims 66 and 80 recite a nature-based product (a compound of Formula (II)), "***analysis of the claim as a whole indicates that the claim is focused on a process of practically applying the product to treat a particular disease . . . [a bacterial infection] and not on the product per se.***" Thus, it is not necessary to apply the markedly different characteristics analysis in order to conclude that the claim is not directed to an exception. Therefore, just as claim 8 from Example

3 of the Nature-based Product examples in the Guidelines qualifies as patent eligible subject matter, claims 66 and 80, and claims dependent therefrom, also qualify as patent eligible subject matter under 35 U.S.C. § 101.

In view of all of the foregoing, Applicants respectfully request reconsideration of this rejection under 35 U.S.C. § 101 and an indication that new claims 54-93, presented herein, are patent eligible and in condition for allowance.

Finally, and notwithstanding the cancellation of the composition of matter claims, and the obviation of this rejection as it relates to those claims, Applicants would like to present the following arguments for the record.

III. Eligibility of Composition of Matter Claims

Prior to entry of the claim amendments presented herein, claims 1-3, and 5-8 were directed to an isolated compound of Formula (I); claim 4 was drawn to an isolated compound of Formula (II); claim 10 was drawn to an isolated compound of Formula (III) and claim 11 was drawn to an isolated compound of Formula (IV). The Examiner is of the opinion that these claims are directed to a product and that this "product is an isolated compound from nature which is not sufficient to be patent eligible." (Office Action, page 3).

Applicants submit that claims 1-8, 10-12, 14-17, 20-23, 25-33, 36, 38, 40, 43, 46, 48, 50 and 52 are patent-eligible under 35 U.S.C. § 101, which provides that

> [w]hoever invents or discovers any new and useful process, machine, manufacture, or composition of matter, or any new and useful improvement thereof, may obtain a patent therefor, subject to the conditions and requirements of this title.

Furthermore, Applicants submit that claims 1-8, 10-12, 14-17, 20-23, 25-33, 36, 38, 40, 43, 46, 48, 50 and 52 are in compliance with the Guidelines.

Purified or isolated forms of natural products have long been held to be patentable, and the recent decisions cited by the Examiner, *Ass'n for Molecular Pathology v. Myriad Genetics,*

Inc., 133 S. Ct. 2107, 2116, 106 USPQ2d 1972 (2013); and *Mayo Collaborative Svcs. v. Prometheus Laboratories, Inc.*, 132 S. Ct. 1289, 101 USPQ2d 1961 (2012), did not overturn the precedent acknowledging the eligibility of purified or isolated forms of a natural product. To the contrary, the Supreme Court sought clarification during oral arguments of the *Myriad* proceedings that the purified or concentrated compositions of small molecule natural products were not before the Court. In particular, Justice Alito engaged in the following exchange with counsel for the Association for Molecular Pathology ("AMP") at the beginning of oral arguments, posing a hypothetical similar to the amazonic acid example (Example 3) in the Guidelines:

> JUSTICE ALITO: Can I take you back to -- to Justice Ginsburg's question, because I'm -- I don't -- I'm not sure you got at what troubles me about that. Suppose there is a substance, a -- a chemical, a molecule in the -- the leaf -- the leaves of a plant that grows in the Amazon, and it's discovered that this has tremendous medicinal purposes. Let's say it -- it treats breast cancer. A new discovery, a new way -- a way is found, previously unknown, to extract that. You make a drug out of that. Your answer is that cannot be patent -- patented; it's not eligible for patenting, because the chemical composition of the -- of the drug is the same as the chemical that exists in the leaves of the plant.
>
> MR. HANSEN: If there is no alteration, if we simply pick the leaf off of the tree and swallow it and it has some additional value, then I think it is not patentable. You might be able to get a method patent on it, you might be able to get a use patent on it, but you can't get a composition patent. But as –
>
> JUSTICE ALITO: But you're making -- you keep making the hypotheticals easier than they're intended to be. It's not just the case of taking the leaf off the tree and chewing it. ***Let's say if you do that, you'd have to eat a whole forest to get the – the value of this. But it's extracted and – and reduced to a concentrated form. That's not patent --that's not eligible?***
>
> MR. HANSEN: ***No, that may well be eligible, because you have now taken what was in nature and you've transformed it in two ways. First of all, you've made it substantially more concentrated than it was in nature; and second, you've given it a function.*** If it doesn't work in the diluted form but does work in a concentrated

> form, you've given it a new function. And the -- by both changing its nature and by giving it a new function, you may well have [a] patent.[1]

Thus, the Supreme Court in *Myriad* did not seek to set forth a new precedent for the eligibility of claims drawn to "products of nature" generally and instead took time to distinguish from DNA the natural products present in the natural environment. Furthermore, the Supreme Court rendered a narrow holding applicable to DNA.[2] Similar to Justice Alito's hypothetical chemical, which would constitute patent eligible subject matter upon isolation because it does not exist in nature in a useable form, the compound of Formula (II) is not present in nature in useable form because it is present in extremely low concentrations in the soil, and in order to get the value of it a subject would need to consume a vast volume of dirt that would not only be impossible to achieve, but likely very dangerous as the soil may contain other chemicals or pathogenic organisms that would sicken the subject consuming the dirt. Similar to the hypothetical discussed by Justice Alito in the situation where a natural product does not exist in nature in a form that is useable for a therapeutic purpose, isolation and concentration of the natural product changes the nature of the natural product and transforms it into a patent eligible chemical with a new function. Thus, Applicants' isolation of the compound of Formula (II) transformed the compound as it existed in nature into a "new and useful . . . composition of matter," or at the very least a "new and useful improvement thereof," as required under U.S.C. § 101.

Thus, based upon the concession of the AMP attorney during oral arguments that an isolated natural product would be patentable if a new property results from the isolation, coupled with the narrow holding of the Court limited to DNA, it does not follow that the precedents of decisions such as *Parke-Davis* and *Merck*, which acknowledge the patentability of purified or isolated forms of natural product were overturned by *Myriad*. Instead, the USPTO is still bound

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[1] Transcript of Oral Argument at 6:24-8:12, *Ass'n for Molecular Pathology v. Myriad Genetics, Inc.*, 133 S. Ct. 2107 (2013) (No. 12-398). (*Emphasis Added*).

[2] *Ass'n for Molecular Pathology v. Myriad Genetics, Inc.*, 133 S. Ct. 2107, 2111, 106 USPQ2d 1972, 1975 (2013) (holding that a naturally occurring DNA segment is a product of nature and not patent eligible merely because it has been isolated, but that cDNA is patent eligible because it is not naturally occurring.)

to follow such holdings. In particular, the *Parke-Davis* court found that adrenaline, purified from gland tissue, was patentable[3]:

> [the inventor] was the first to make it available for any use by removing it from the other gland-tissue in which it was found, and, while it is of course possible logically to call this a purification of the principle, ***it became for every practical purpose a new thing commercially and therapeutically***. That was a good ground for a patent.

Analogous to the purifying of adrenaline from the gland tissue, which "ma[d]e it available for any use" and transformed it "for every practical purpose [to] a new thing commercially and therapeutically," the Applicants of the present application identified and isolated the compound of Formula (II) from a previously unknown bacterial species, and in doing so, "ma[d]e it available for any use" and transformed it "for every practical purpose [to] a new thing commercially and therapeutically," a feat not achieved prior to Applicants' invention.

Similarly, a purified composition of vitamin B_{12} as isolated from a fermentation broth of certain fungal species was also found to be patentable in *Merck & Co. v. Olin Mathieson Chem. Corp.*,[4]:

> The fact, however, that a new and useful product is the result of processes of extraction, concentration and purification of natural materials does not defeat its patentability.

The *Merck* court found that the purified composition of vitamin B_{12} was new in part because such a purified composition did not exist prior to the patentees' invention and that the resulting composition had advantages over the products known to those in the art.

> ***Until the patentees produced them, there were no such B_{12} active compositions.*** No one had produced even a comparable product. ***The active substance was unidentified and unknown.*** The new

[3] *Parke-Davis & Co. v. H.K. Mulford Co.*, 189 F. 95, 103 (C.C.S.D.N.Y. 1911) (Hand, J.) (emphasis added) (sustaining product claims of a patent to purified adrenalin).
[4] *Merck & Co. v. Olin Mathieson Chem. Corp.*, 253 F.2d 156, 163-64 (4th Cir. 1958) (finding claim to purified vitamin B_{12} to be within the scope of patentable subject matter because "the natural fermentates are quite useless, while the patented compositions are of great medicinal and commercial value").

> product, not just the method, had such advantageous characteristics as to replace the liver products. What was produced was, in no sense, an old product.[5]

Analogous to the purified composition of vitamin B_{12}, which the court held did not exist before the patentee's invention, prior to Applicant's invention no one had "produced even a comparable product" that included the isolated compound of Formula (II). Indeed, similar to *Merck*, "the active substance [*i.e.,* the compound of Formula (II)] was unidentified and unknown" prior to Applicants' invention. Finally, Applicants' invention, namely, the isolated compound of Formula (II) now has well-documented advantages as it is a new class of antibiotic, and its discovery generated much interest and excitement from the scientific community as evident from the widespread publication and commentary on this invention. See for example, "A New Antibiotic Kills Pathogens Without Detectable Resistance," Ling *et al.*, (2015) Nature: Vol 517, 455–459, submitted herewith as Appendix A; and "Antibiotics: US discovery labelled 'game-changer' for medicine," James Gallagher, BBC News, 7 January 2015; accessed at http://www.bbc.com/news/health-30657486, submitted herewith as Appendix B.)

Accordingly, purification or isolation of a substance (*e.g.*, adrenaline in *Parke-Davis* and vitamin B_{12} in *Merck*) leads to patent-eligible subject matter, because the purification/isolation step transforms the material from an unusable substance to a therapeutically effective composition. The present invention is similar to both the inventions deemed patentable in *Parke-Davis* and *Merck* in that the isolated compound of Formula (II) ***"became for every practical purpose a new thing commercially and therapeutically"*** after Applicants identified and isolated it, and accordingly, the isolated compound of Formula (II) is patent-eligible subject matter under U.S.C. § 101.

Furthermore, and in line with the *Parke-Davis* and the *Merck* holdings above, the Guidelines state that purified or isolated products are patent eligible if a marked difference can be shown between the purified or isolated product and its naturally occurring counterpart:

> In accordance with this analysis, ***a product that is purified or isolated, for example, will be eligible when there is a resultant***

[5] *Id.*

> ***change in characteristics sufficient to show a marked difference from the product's naturally occurring counterpart.*** (Section I.A.3.b., emphasis added, internal citations omitted).

This standard from the Guidelines affirms the position that a purified or isolated product is patent eligible if purification or isolation of the product transforms the product of nature in some way to result in a marked difference between the two. In particular, a "product of nature" is patent eligible if the product has markedly different characteristics as compared to "its naturally occurring counterpart in its natural state" (the Guidelines, I.A.3.b.):

> The markedly different characteristics analysis compares the ***nature-based product limitation to its naturally occurring counterpart in its natural state.*** When there is no naturally occurring counterpart to the nature-based product, the comparison should be made to the closest naturally occurring counterpart. (Emphasis added).

The nature-based product limitation is an isolated compound of Formula (II). Isolated is defined in the specification as meaning "being substantially free from other materials associated with it in its natural environment" (see specification, at page 15, lines 25-26). Therefore, the "nature-based product limitation" as described above in the Guidelines is a compound of Formula (II), substantially free from other materials associated with it in its natural environment. However, the "naturally occurring counterpart in its natural state" of the isolated compound of Formula (II) is difficult to determine. While Applicants have demonstrated that the bacterial isolate ISO18629 can be cultured to produce a compound of Formula (II) under laboratory conditions, it is unknown whether this bacterial isolate produces the compound of Formula (II) in nature, and if produced, it is unknown what the function of this compound is in nature. Applicants note that ISO18629 belongs to a new genus of bacteria related to *Aquabacteria,* and that this group of Gram-negative organisms is not known to produce antibiotics (see, "A New Antibiotic Kills Pathogens Without Detectable Resistance," Ling *et al.*, (2015) Nature: Vol 517, 455–459, submitted herewith as Appendix A). Indeed, if the compound of Formula (II) was produced in nature, it would be present in the dirt in such miniscule amounts and mixed with

contaminants (including other compounds, minerals, and other organisms) that it would be almost impossible to determine its function.

Further, the Guidelines provide that no bright-line rule exists for establishing the "markedly different characteristics" and that these characteristics can include both functional as well as structural differences:

> ***Markedly different characteristics can be expressed as the product's structure, function, and/or other properties, and will be evaluated based on what is recited in the claim on a case-by-case basis.*** As seen by the examples that are being released in conjunction with this Interim Eligibility Guidance, even a small change can result in markedly different characteristics from the product's naturally occurring counterpart. (Section I.A.3.b., emphasis added, internal citations omitted).

Thus, markedly different properties include a new function of the nature-based product limitation. The Office's rejection under U.S.C. § 101 must be based upon the assumption that the compound of Formula (II) as produced in nature not only has the same structure, but also the same function as the naturally occurring counterpart in its natural state. However, no evidence to support the same function has been presented.

To distinguish between the different functions (rather than structural differences) between the natural product limitation and the product's naturally occurring counterpart, Example 3 of the Nature-Based Examples in the Guidelines is illustrative. In Example 3, claim 1 is directed to "purified amazonic acid," which was deemed to be patent ineligible because the "purified amazonic acid is structurally and functionally identical to the amazonic acid in the leaves." According to the fact pattern provided in Example 3, the "leaves of the Amazonian cherry tree contain a chemical that is useful in treating breast and colon cancers. Many have tried and failed to isolate the cancer-fighting chemical from the leaves." As the function of the amazonic acid was known prior to isolation from the leaves, the amount of amazonic acid in the leaves must have been sufficient to identify amazonic acid as anti-cancer agent even when contained in the leaves. Thus, the comparison of "nature-based product limitation" (*i.e.*, purified amazonic acid) to its "naturally occurring counterpart in its natural state" (*i.e.*, amazonic acid in the leaves of the

trees) does not yield any markedly different properties because the two have the same structure and function.

In contrast to the amazonic acid in Example 3, it is not known whether the isolated compound of Formula (II) is functionally equivalent to its naturally occurring counterpart. As discussed above, the "naturally occurring counterpart *in its natural state*" of the isolated compound of Formula (II) exists in dirt in miniscule amounts mixed with other potentially harmful contaminants. Where the comparison in Example 3 is between purified amazonic acid and amazonic acid in the leaf, here the comparison is an isolated compound of Formula (II) and a compound of Formula (II) in dirt. Even if the compound of Formula (II) is present in nature, its "naturally occurring counterpart in its natural state" would be an undetectable amount of compound mixed in dirt, a variety of organisms, and other compounds and minerals. Dirt containing the compound of Formula (II) does not have the same antibacterial function as the isolated compound of Formula (II). Indeed, the compound of Formula (II) gains its antibiotic functionality when it is isolated from its natural environment. Such a gain in function is a markedly different property, and accordingly, the isolated compound of Formula (II) is patent-eligible subject matter. Moreover, the isolated compound of Formula (II) is markedly different from its naturally occurring counterpart in its natural state because the isolated compound of Formula (II) is suitable for a pharmaceutical use, whereas the naturally occurring counterpart in its natural state does not have therapeutic value in its natural form.

Just as adrenaline was purified from its natural form from gland tissue and vitamin B_{12} was purified from the fermentation of certain fungi, the compound of Formula (II) is a purified product that has markedly different properties than its naturally occurring counterpart. Accordingly, the present invention is similar to both the inventions deemed patentable in *Parke-Davis* and *Merck* in that the isolated compound of Formula (II) "became for every practical purpose a new thing commercially and therapeutically" after Applicants identified and isolated it.

Accordingly, the isolated compound of Formula (II) constitutes patent eligible subject matter under U.S.C. § 101.

Rejection of Claims* 1-3 and 5-8 *Under 35 U.S.C. § 112 First Paragraph

The Examiner has rejected claims 1-3 and 5-8 under 35 U.S.C. § 112(a) or 35 U.S.C. § 112 (pre-AlA), first paragraph, as allegedly failing to comply with the enablement requirement. Specifically, the Examiner is of the opinion that "the specification, while being enabling for the compound of Formula (II), for example (Claim 4), is not enabled for the compound of Formula (I)"

Without acquiescing to the validity of the Examiner's rejection and solely in the interest of expediting prosecution of the application, Applicants have cancelled claims 1-3 and 5-8, without prejudice, thereby rendering this rejection moot.

Rejection of Claims* 21-23, 25-33, 36, 38, 40, 43, 46, 48, 50 and 52 *Under 35 U.S.C. § 112 First Paragraph

The Examiner has rejected claims 21-23, 25-33, 36, 38, 40, 43, 46, 48, 50 and 52 under 35 U.S.C. § 112(a) or 35 U.S.C. § 112 (pre-AlA), first paragraph, as allegedly failing to comply with the written description requirement. Specifically, the Examiner is of the opinion that

> [w]hile having written description for a few bacterium [sic], the specification is simply void of describing the various disorders one can treat. A disorder from an undisclosed virus reads on Ebola, HCV, yellow fever, etc. . . the specificity of the compound being nearly a magic bullet for any disorder from any microbial infection. Treating a bacterial infection is described, but the disorders are not.

Without acquiescing to the validity of the Examiner's rejection and solely in the interest of expediting prosecution of the application, Applicants have cancelled claims 21-23, 25-33, 36, 38, 40, 43, 46, 48, 50 and 52, thereby rendering this rejection moot.

New claims 66 and 80, and claims dependent therefrom, are directed to methods for treating a bacterial infection in a subject by administering to the subject an effective amount of a compound of Formula (II) or Formula (III), respectively. Based on the teachings in Applicants' specification, one of skill in the art would conclude that Applicants were in possession of the

claimed invention at the time of filing. Specifically, methods of using and testing the compounds of Formula (II) and (III) to treat Gram-negative and Gram positive bacterial infections are described at page 33, line 4 to page 34, line 3; and page 34, line 27 through page 36, line 22 of the specification. The antibiotic spectrum of the compounds of Formula (II) and (III) is described in Example 2 at page 54, line 16 through page 60, line 20 of the specification. Example 6 at page 68, lines 8-24, describes the effectiveness of the compound of Formula (II) in a mouse septicemia protection assay. Example 7 at page 69, line 1 through page 70, line 7, describes the effectiveness of a compound of Formula (II) against MRSA in the neutropenic mouse thigh infection model. Example 8 at page 69, line 8 through page 71, line 5, describes the effectiveness of the compound of Formula (II) against Streptococcus pneumoniae in an immunocompetent mouse pneumonia model.

Based on the foregoing teachings in Applicants' specification, one of skill in the art would conclude that Applicants were in possession of the claimed invention at the time of filing. Accordingly, Applicants respectfully request reconsideration and withdrawal of this rejection.

SUMMARY

In view of the above amendments, Applicants believe that the pending application is in condition for allowance. If a telephone conversation with Applicants' Attorney would expedite the prosecution of the above-identified application, the Examiner is urged to call the undersigned at (617) 449-6512.

If a fee is due, please charge our Deposit Account No. 50-4876, under Order No. 122733-00103, from which the undersigned is authorized to draw.

Dated: March 2, 2015

Respectfully submitted,

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ARTICLE

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A new antibiotic kills pathogens without detectable resistance

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Antibiotic resistance is spreading faster than the introduction of new compounds into clinical practice, causing a public health crisis. Most antibiotics were produced by screening soil microorganisms, but this limited resource of cultivable bacteria was overmined by the 1960s. Synthetic approaches to produce antibiotics have been unable to replace this platform. Uncultured bacteria make up approximately 99% of all species in external environments, and are an untapped source of new antibiotics. We developed several methods to grow uncultured organisms by cultivation *in situ* or by using specific growth factors. Here we report a new antibiotic that we term teixobactin, discovered in a screen of uncultured bacteria. Teixobactin inhibits cell wall synthesis by binding to a highly conserved motif of lipid II (precursor of peptidoglycan) and lipid III (precursor of cell wall teichoic acid). We did not obtain any mutants of *Staphylococcus aureus* or *Mycobacterium tuberculosis* resistant to teixobactin. The properties of this compound suggest a path towards developing antibiotics that are likely to avoid development of resistance.

Widespread introduction of antibiotics in the 1940s, beginning with penicillin[1,2] and streptomycin[3], transformed medicine, providing effective cures for the most prevalent diseases of the time. Resistance development limits the useful lifespan of antibiotics and results in the requirement for a constant introduction of new compounds[4,5]. However, antimicrobial drug discovery is uniquely difficult[6], primarily due to poor penetration of compounds into bacterial cells. Natural products evolved to breach the penetration barriers of target bacteria, and most antibiotics introduced into the clinic were discovered by screening cultivable soil microorganisms. Overmining of this limited resource by the 1960s brought an end to the initial era of antibiotic discovery[7]. Synthetic approaches were unable to replace natural products[6].

Approximately 99% of all species in external environments are uncultured (do not grow under laboratory conditions), and are a promising source of new antibiotics[8]. We developed several methods to grow uncultured organisms by cultivation in their natural environment[9,10], or by using specific growth factors such as iron-chelating siderophores[11]. Uncultured organisms have recently been reported to produce interesting compounds with new structures/modes of action—lassomycin, an inhibitor of the essential mycobacterial protease ClpP1P2C1 (ref. 12); and diverse secondary metabolites present in a marine sponge *Theonella swinhoei* which are actually made by an uncultured symbiotic *Entotheonella* sp.[13].

Here we report the discovery of a new cell wall inhibitor, teixobactin, from a screen of uncultured bacteria grown in diffusion chambers *in situ*.

Identification of teixobactin

A multichannel device, the iChip[10], was used to simultaneously isolate and grow uncultured bacteria. A sample of soil is diluted so that approximately one bacterial cell is delivered to a given channel, after which the device is covered with two semi-permeable membranes and placed back in the soil (Extended Data Fig. 1). Diffusion of nutrients and growth factors through the chambers enables growth of uncultured bacteria in their natural environment. The growth recovery by this method approaches 50%, as compared to 1% of cells from soil that will grow on a nutrient Petri dish[10]. Once a colony is produced, a substantial number of uncultured isolates are able to grow *in vitro*[14]. Extracts from 10,000 isolates obtained by growth in iChips were screened for antimicrobial activity on plates overlaid with *S. aureus*. An extract from a new species of β-proteobacteria provisionally named *Eleftheria terrae* showed good activity. The genome of *E. terrae* was sequenced (Supplementary Discussion). Based on 16S rDNA and *in silico* DNA/DNA hybridization, this organism belongs to a new genus related to *Aquabacteria* (Extended Data Fig. 2, Supplementary Discussion). This group of Gram-negative organisms is not known to produce antibiotics. A partially purified active fraction contained a compound with a molecular mass of 1,242 Da determined by mass spectrometry, which was not reported in available databases. The compound was isolated and a complete stereochemical assignment has been made based on NMR and advanced Marfey's analysis (Fig. 1, Extended Data Figs 3 and 4 and Supplementary Discussion). This molecule, which we named teixobactin, is an unusual depsipeptide which contains enduracididine, methylphenylalanine, and four D-amino acids. The biosynthetic gene cluster (GenBank accession number KP006601) was identified using a homology search (Supplementary Discussion). It consists of two large non-ribosomal peptide synthetase (NRPS)-coding genes, which we named *txo1* and *txo2*, respectively (Fig. 1). In accordance with the co-linearity rule, 11 modules are encoded. The *in silico* predicted adenylation domain specificity perfectly matches the amino acid order of teixobactin (Fig. 1), and allowed us to predict the biosynthetic pathway (Extended Data Fig. 5).

Resistance and mechanism of action

Teixobactin had excellent activity against Gram-positive pathogens, including drug-resistant strains (Table 1 and Extended Data Table 1).

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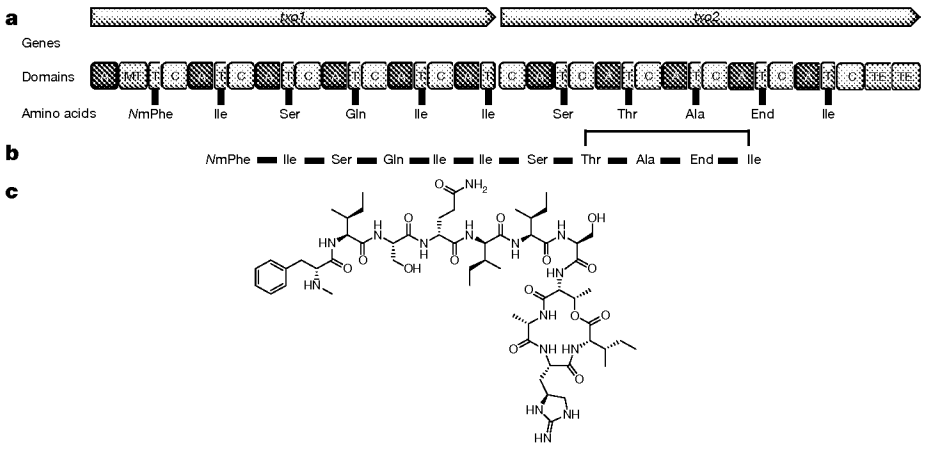

Figure 1 | The structure of teixobactin and the predicted biosynthetic gene cluster. a, The two NRPS genes, the catalytic domains they encode, and the amino acids incorporated by the respective modules. Domains: A, adenylation; C, condensation; MT, methylation (of phenylalanine); T, thiolation (carrier); and TE, thioesterase (Ile-Thr ring closure). *N*mPhe, *N*-methylated phenylalanine. **b**, Schematic structure of teixobactin. The *N*-methylation of the first phenylalanine is catalysed by the methyltransferase domain in module 1. The ring closure between the last isoleucine and threonine is catalysed by the thioesterase domains during molecule off-loading, resulting in teixobactin. **c**, Teixobactin structure.

Potency against most species, including difficult-to-treat enterococci and *M. tuberculosis* was below 1 μg ml^{-1}. Teixobactin was exceptionally active against *Clostridium difficile* and *Bacillus anthracis* (minimal inhibitory concentration (MIC) of 5 and 20 ng ml^{-1}, respectively). Teixobactin had excellent bactericidal activity against *S. aureus* (Fig. 2a), was superior to vancomycin in killing late exponential phase populations (Fig. 2b), and retained bactericidal activity against intermediate resistance *S. aureus* (VISA) (Extended Data Fig. 6a). Note that frequent clinical failure in patients with *S. aureus* MRSA treated with vancomycin has been linked to the poor bactericidal activity of this compound[15,16]. Teixobactin was ineffective against most Gram-negative bacteria, but showed good activity against a strain of *E. coli asmB1* with a defective outer membrane permeability barrier (Table 1).

We were unable to obtain mutants of *S. aureus* or *M. tuberculosis* resistant to teixobactin even when plating on media with a low dose (4 × MIC) of the compound. Serial passage of *S. aureus* in the presence of sub-MIC levels of teixobactin over a period of 27 days failed to produce resistant mutants as well (Fig. 2d, Supplementary Discussion). This usually points to a non-specific mode of action, with accompanying toxicity. However, teixobactin had no toxicity against mammalian NIH/3T3 and HepG2 cells at 100 μg ml^{-1} (the highest dose tested). The compound showed no haemolytic activity and did not bind DNA. In order to determine specificity of action of teixobactin, we examined its effect on the rate of label incorporation into the major biosynthetic pathways of *S. aureus*. Teixobactin strongly inhibited synthesis of peptidoglycan, but had virtually no effect on label incorporation into DNA, RNA and protein (Fig. 3a). This suggested that teixobactin is a new peptidoglycan synthesis inhibitor.

Resistance has not developed to this compound, suggesting that the target is not a protein. The essential lack of resistance development through mutations has been described for vancomycin which binds lipid II, the precursor of peptidoglycan. We reasoned that teixobactin could be acting against the same target. Treatment of whole cells of *S. aureus* with teixobactin (1–5 × MIC) resulted in significant accumulation of the soluble cell wall precursor undecaprenyl-*N*-acetylmuramic acid-pentapeptide (UDP-MurNAc-pentapeptide), similar to the vancomycin-treated control cells (Fig. 3b), showing that one of the membrane-associated steps of peptidoglycan biosynthesis is blocked. Teixobactin inhibited peptidoglycan biosynthesis reactions *in vitro* in a dose-dependent manner with either lipid I, lipid II or undecaprenyl-pyrophosphate (Fig. 3c) as a substrate. Quantitative analysis of the

Table 1 | Activity of teixobactin against pathogenic microorganisms

Organism and genotype	Teixobactin MIC (μg ml^{-1})
S. aureus (MSSA)	0.25
S. aureus + 10% serum	0.25
S. aureus (MRSA)	0.25
Enterococcus faecalis (VRE)	0.5
Enterococcus faecium (VRE)	0.5
Streptococcus pneumoniae (penicillin^R)	≤ 0.03
Streptococcus pyogenes	0.06
Streptococcus agalactiae	0.12
Viridans group streptococci	0.12
B. anthracis	≤ 0.06
Clostridium difficile	0.005
Propionibacterium acnes	0.08
M. tuberculosis H37Rv	0.125
Haemophilus influenzae	4
Moraxella catarrhalis	2
Escherichia coli	25
Escherichia coli (*asmB1*)	2.5
Pseudomonas aeruginosa	>32
Klebsiella pneumoniae	>32

The MIC was determined by broth microdilution. MSSA, methicillin-sensitive *S. aureus*; VRE, vancomycin-resistant enterococci.

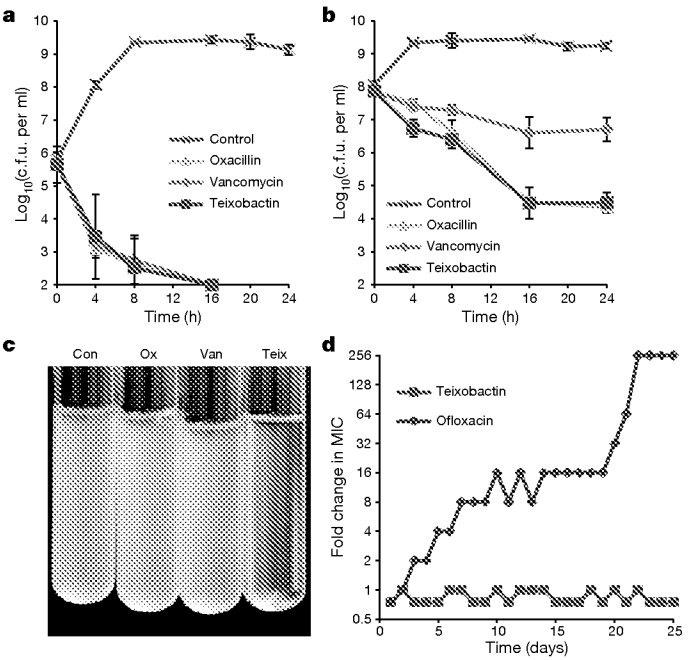

Figure 2 | Time-dependent killing of pathogens by teixobactin. a, **b**, *S. aureus* were grown to early (**a**), and late (**b**) exponential phase and challenged with antibiotics. Data are representative of 3 independent experiments ± s.d. **c**, Teixobactin treatment resulted in lysis. The figure is representative of 3 independent experiments. **d**, Resistance acquisition during serial passaging in the presence of sub-MIC levels of antimicrobials. The *y* axis is the highest concentration the cells grew in during passaging. For ofloxacin, 256 × MIC was the highest concentration tested. The figure is representative of 3 independent experiments.

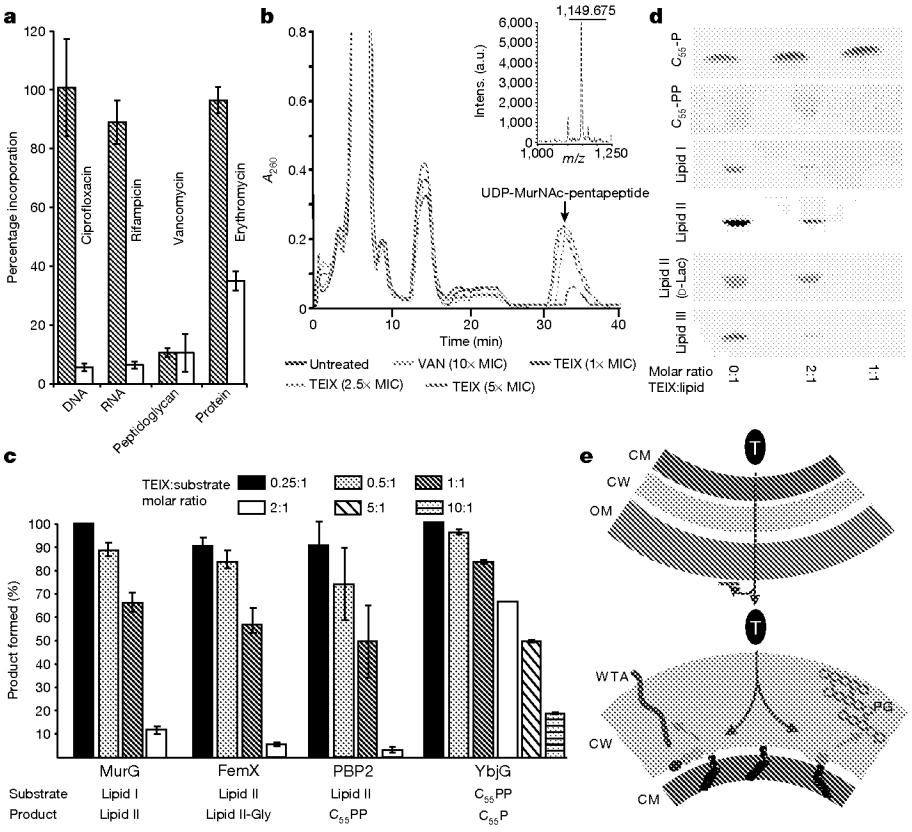

Figure 3 | Teixobactin binds to cell wall precursors. **a**, Impact of teixobactin (TEIX) on macromolecular biosyntheses in *S. aureus*. Incorporation of ^{3}H-thymidine (DNA), ^{3}H-uridine (RNA), ^{3}H-leucine (protein), and ^{3}H-glucosamine (peptidoglycan) was determined in cells treated with teixobactin at 1 × MIC (grey bars). Ciprofloxacin (8 × MIC), rifampicin (4 × MIC), vancomycin (2 × MIC) and erythromycin (2 × MIC) were used as controls (white bars). Data are means of 4 independent experiments ± s.d. **b**, Intracellular accumulation of the cell wall precursor UDP-MurNAc-pentapeptide after treatment of *S. aureus* with teixobactin. Untreated and vancomycin (VAN)-treated (10 × MIC) cells were used as controls. UDP-MurNAc-pentapeptide was identified by mass spectrometry as indicated by the peak at *m/z* 1,149.675. The experiment is representative of 3 independent experiments. **c**, The effect of teixobactin on precursor consuming reactions. Experiments were performed in 3 biological replicates and data are presented as mean ± s.d. **d**, Complex formation of teixobactin with purified cell wall precursors. Binding of teixobactin is indicated by a reduction of the amount of lipid intermediates (visible on the thin-layer chromatogram). The figure is representative of two independent experiments. **e**, A model of teixobactin targeting and resistance. The teixobactin producer is a Gram-negative bacterium protected from this compound by exporting it across the outer membrane permeability barrier (upper panel). In target Gram-positive organisms lacking an outer membrane, the targets are readily accessible on the outside where teixobactin binds precursors of peptidoglycan (PG) and WTA. CM, cytoplasmic membrane; CW, cell wall; OM, outer membrane; T, teixobactin.

MurG-, FemX-, and PBP2-catalysed reactions using radiolabelled substrates, showed an almost complete inhibition at a twofold molar excess of teixobactin with respect to the lipid substrate (Fig. 3c). The addition of purified lipid II prevented teixobactin from inhibiting growth of *S. aureus* (Extended Data Table 2). These experiments showed that teixobactin specifically interacts with the peptidoglycan precursor, rather than interfering with the activity of one of the enzymes. In order to evaluate the minimal motif required for high affinity binding of teixobactin, the direct interaction with several undecaprenyl-coupled cell envelope precursors was investigated. Purified precursors were incubated with teixobactin at different molar ratios, followed by extraction and subsequent thin-layer chromatography analysis (Fig. 3d). In agreement with the results obtained from the *in vitro* experiments, lipid I and lipid II were fully trapped in a stable complex that prevented extraction of the lipid from the reaction mixture in the presence of a twofold molar excess of the antibiotic, leading to the formation of a 2:1 stoichiometric complex. Teixobactin was active against vancomycin-resistant enterococci that have modified lipid II (lipid II-D-Ala-D-Lac or lipid II-D-Ala-D-Ser instead of lipid II-D-Ala-D-Ala)[17–19]. This suggested that, unlike vancomycin, teixobactin is able to bind to these modified forms of lipid II. Indeed, teixobactin bound to lipid II-D-Ala-D-Lac and lipid II-D-Ala-D-Ser (Extended Data Fig. 6b). Moreover, teixobactin efficiently bound to the wall teichoic acid (WTA) precursor undecaprenyl-PP-GlcNAc (lipid III). Although WTA is not essential *per se*, inhibition of late membrane-bound WTA biosynthesis steps is lethal due to accumulation of toxic intermediates[20]. Furthermore, teichoic acids anchor autolysins, preventing uncontrolled hydrolysis of peptidoglycan[21]. Inhibition of teichoic acid synthesis by teixobactin would help liberate autolysins, contributing to the excellent lytic and killing activity of this antibiotic. Teixobactin was also able to bind undecaprenyl-pyrophosphate, but not undecaprenyl-phosphate (Fig. 3d and Extended Data Table 2). Although teixobactin efficiently binds lipid I *in vitro*, this is probably less significant for antimicrobial activity, as this is the intracellular form of the precursor, unlike surface-exposed lipid II and the undecaprenyl-PP-GlcNAc WTA precursors (Fig. 3e and Extended Data Fig. 7). Binding to the target primarily relies on the interaction of the antibiotic with the pyrophosphate moiety, and the first sugar moiety attached to the lipid carrier, as higher concentrations of teixobactin were required to completely inhibit the YbjG-catalysed monophosphorylation of undecaprenyl-pyrophosphate, involved in the recycling process of the essential lipid carrier (Fig. 3c and Extended Data Fig. 7). Corroborating this result, a tenfold higher concentration of undecaprenyl-pyrophosphate was required to antagonize the antimicrobial activity of teixobactin compared to lipid II (Extended Data Table 2). The exact nature of this first sugar is therefore not important, explaining why teixobactin is active against *M. tuberculosis*, where it probably binds to decaprenyl-coupled lipid intermediates of peptidoglycan and arabinogalactan. Teixobactin is also likely to bind to prenyl-PP-sugar intermediates of capsular polysaccharide biosynthesis which is important for virulence in staphylococci[22] and whose inhibition of biosynthesis is lethal in streptococci[23].

In vivo efficacy

Given the attractive mode of action of this compound, we investigated its potential as a therapeutic. The compound retained its potency in the presence of serum, was stable, and had good microsomal stability and low toxicity (Supplementary Discussion). The pharmacokinetic

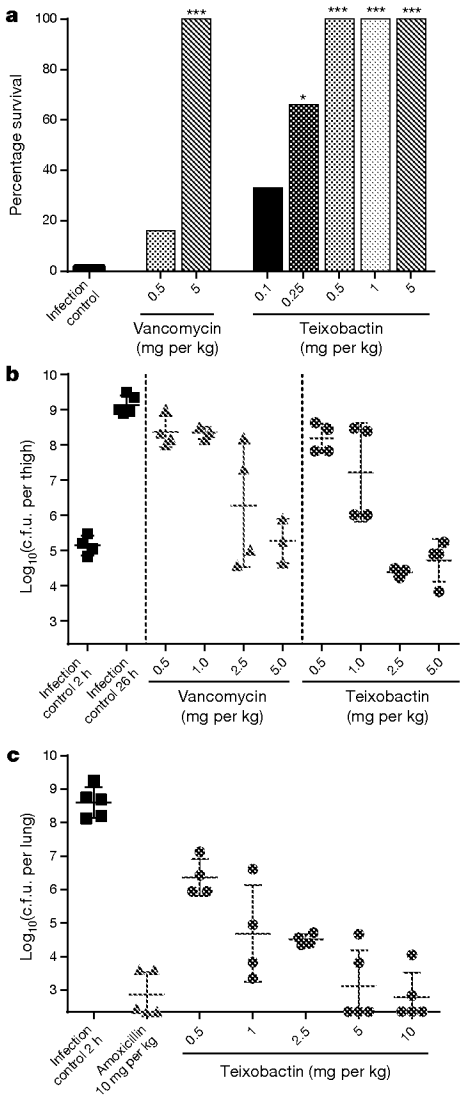

Figure 4 | Teixobactin is efficacious in three mouse models of infection. **a**, Single dose treatment (i.v., 1 h post-infection, 6 mice per group) with teixobactin and vancomycin in septicemia protection model using MRSA. Survival is depicted 48 h after infection. **b**, Single dose (i.v., 2 h post-infection, 4 mice per group) treatment with teixobactin and vancomycin in neutropenic mouse thigh infection model using MRSA. For drug-treated animals, thigh colony-forming units (c.f.u.) were determined at 26 h post-infection. For controls, c.f.u. in thighs were determined at 2 h and 26 h post-infection. **c**, Two dose treatment, 5 mice per group, with teixobactin (i.v., 24 h and 36 h post-infection) and single dose treatment with amoxicillin (subcutaneous, 24 h post-infection) in immunocompetent lung infection model using *S. pneumoniae*. Lung c.f.u. were determined at 48 h post-infection. The c.f.u. from each mouse are plotted as individual points and error bars represent the deviation within an experimental group. $*P < 0.05$, $***P < 0.001$ (determined by non-parametric log-rank test).

parameters determined after i.v. injection of a single 20 mg per kg dose in mice were favourable, as the level of compound in serum was maintained above the MIC for 4 h (Extended Data Fig. 8). An animal efficacy study was then performed in a mouse septicemia model. Mice were infected intraperitoneally with methicillin-resistant *S. aureus* (MRSA) at a dose that leads to 90% of death. One hour post-infection, teixobactin was introduced i.v. at single doses ranging from 1 to 20 mg per kg. All treated animals survived (Fig. 4a), and in a subsequent experiment the PD_{50} (protective dose at which half of the animals survive) was determined to be 0.2 mg per kg, which compares favourably to the 2.75 mg per kg PD_{50} of vancomycin, the main antibiotic used to treat MRSA. Teixobactin was then tested in a thigh model of infection with *S. aureus*, and showed good efficacy as well (Fig. 4b). Teixobactin was also highly efficacious in mice infected with *Streptococcus pneumoniae*, causing a 6 $\log_{10}$ reduction of c.f.u. in lungs (Fig. 4c).

Discussion

This study, as well as previous work[12,13,24] suggests that new organisms such as uncultured bacteria are likely to harbour new antimicrobials[25]. This is consistent with resistance mechanisms in soil bacteria being stratified by phylogeny, with horizontal transmission limited[26] (as compared to pathogens) and the pattern of antibiotic production correlating with resistance. Exploiting uncultured bacteria is likely to revive the Waxman platform of natural product drug discovery[7]. Teixobactin is a promising therapeutic candidate; it is effective against drug-resistant pathogens in a number of animal models of infection. Binding of teixobactin to WTA precursor contributes to efficient lysis and killing, due to digestion of the cell wall by liberated autolysins. This is akin to the action of another natural product with excellent killing ability, acyldepsipeptide, which converts the ClpP protease into a non-specific hydrolase that digests the cell[27]. These examples show that natural products evolved to exploit the inherent weaknesses of bacteria[28], and additional compounds that subvert important enzymes into killing devices are likely to be discovered. Teixobactin binds to multiple targets, none of which is a protein (Fig. 3e and Extended Data Fig. 7). Polyprenyl-coupled cell envelope precursors, such as lipid II, are readily accessible on the outside of Gram-positive bacteria and represent an 'Achilles heel' for antibiotic attack[28]. The target of teixobactin, the pyrophosphate-sugar moiety of these molecules, is highly conserved among eubacteria. The producer is a Gram-negative bacterium, and its outer membrane will protect it from re-entry of the compound (Fig. 3e and Extended Data Fig. 7). This suggests that the producer does not employ an alternative pathway for cell wall synthesis that would protect it from teixobactin, and which other bacteria could borrow. Resistance could eventually emerge from horizontal transmission of a resistance mechanism from some soil bacterium, and given the highly conserved teixobactin binding motif, this would likely take the form of an antibiotic modifying enzyme. However, although determinants coding for enzymes attacking frequently found antibiotics such as β-lactams or aminoglycosides are common, they are unknown for the rare vancomycin. The recently discovered teixobactin is even less common than vancomycin. After its introduction into the clinic, it took 30 years for vancomycin resistance to appear[29]. The lipid II modification pathway resulting in vancomycin resistance probably originated in the producer of vancomycin, *Amycolatopsis orientalis*[19]. It will probably take even longer for resistance to the better-protected teixobactin to emerge. Teixobactin is the first member of a new class of lipid II binding antibiotics, structurally distinct from glycopeptides, lantibiotics[30,31], and defensins[32]. The properties of teixobactin suggest that it evolved to minimize resistance development by target microorganisms. It is likely that additional natural compounds with similarly low susceptibility to resistance are present in nature and are waiting to be discovered.

Online Content Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

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Supplementary Information is available in the online version of the paper.

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Author Contributions K.L. and T.S. designed the study, analysed results, and wrote the paper. L.L.L. designed the study and analysed results. A.J.P. designed the study, performed compound isolation and structure determination and analysed data. B.P.C. designed the study, performed susceptibility experiments and wrote the paper. D.E.H. oversaw preclinical work including designing studies and analysing data. S.E. designed cultivation experiments and analysed data. M.J., L.L. and V.A.S. designed and performed experiments on structure determination and analysed data. I.E. and A.M. designed and performed experiments on mechanism of action. A.L.S., D.R.C., C.R.F., K.A.F., W.P.M., A.G.N., A.M.Z. and C.C. performed experiments on compound production, isolation, susceptibility testing and data analysis. T.F.S. identified the biosynthetic cluster.

Author Information The biosynthetic gene cluster for teixobactin has been deposited with GenBank under accession number KP006601. Reprints and permissions information is available at www.nature.com/reprints. The authors declare competing financial interests: details are available in the online version of the paper. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to K.L. (k.lewis@neu.edu).

METHODS

Isolation and cultivation of producing strains. A sample of 1 g of soil sample collected from a grassy field in Maine was agitated vigorously in 10 ml of deionized H_2O for 10 min. After letting the soil particulates settle for 10 min, the supernatant was diluted in molten SMS media (0.125 g casein, 0.1 g potato starch, 1 g casamino acids, 20 g bacto-agar in 1 litre of water) to achieve an average concentration of one cell per 20 µl of medium. Then 20 µl aliquots were then dispensed into the wells of an iChip. The iChip was placed in direct contact with the soil. After one month of incubation, the iChips were disassembled and individual colonies were streaked onto SMS agar to test for the ability to propagate outside the iChip and for colony purification.

Extract preparation and screening for activity. Isolates that grew well outside the iChip were cultured in seed broth (15 g glucose, 10 g malt extract, 10 g soluble starch, 2.5 g yeast extract, 5 g casamino acids, and 0.2 g $CaCl_2 \cdot 2H_2O$ per 1 litre of deionized H_2O, pH 7.0) to increase biomass, followed by 1:20 dilution into 4 different fermentation broths. After 11 days of agitation at 29 °C, the fermentations were dried and resuspended in an equal volume of 100% DMSO. Then 5 µl of extracts were spotted onto a lawn of growing *S. aureus* NCTC8325-4 cells in Mueller-Hinton agar (MHA) plates. After 20 h of incubation at 37 °C, visible clearing zones indicated antibacterial activity. The extract from this isolate, which was provisionally named *Eleftheria terrae* sp., produced a large clearing zone. Although *E. terrae* sp. produced antibacterial activity under several growth conditions, the best activity (that is, largest clearing zone) was seen with R4 fermentation broth (10 g glucose, 1 g yeast extract, 0.1 g casamino acids, 3 g proline, 10 g $MgCl_2$-$6H_2O$, 4 g $CaCl_2$-$2H_2O$, 0.2 g K_2SO_4, 5.6 g TES free acid (2-[[1,3-dihydroxy-2-(hydroxymethyl) propan-2-yl]amino]ethanesulfonic acid) per 1 litre of deionized H_2O, pH 7).

Sequencing of the strain. Genomic DNA of *E. terrae* was isolated. Sequencing was performed at the Tufts University Core Facility (Boston, MA). A paired-end library with an insert size of approximately 800 bases was generated and sequenced using Illumina technology. The read length was 251 bases per read.

Strain identification. A suspension of cells was disrupted by vigorous agitation with glass beads (106 nm or smaller) and the supernatant used as template to amplify the 16S rRNA gene, using GoTaq Green Master Mix (Promega M7122), and the universal primers E8F and U1510R[33]. The thermocycler parameters included 30 cycles of 95 °C for 30 s, 45 ° C for 30 s and 72 °C for 105 s. The amplified DNA fragment was sequenced by Macrogen USA (Cambridge, MA), and the sequence compared by BLAST to cultured isolates in the Ribosomal Database Project.

The assembled genome for *E. terrae* was submitted to the RAST genome annotation server at (http://rast.nmpdr.org/)[34] which produced a list of closest relatives with published genomes. These are *Alicycliphilus denitrificans, Leptothrix cholodnii, Methylibium petroleiphilum,* and *Rubrivivax gelatinosus*, and their genomes were downloaded from the NCBI ftp site (ftp://ftp.ncbi.nih.gov/genomes/ASSEMBLY_BACTERIA/). DNA–DNA hybridization (DDH) values of these genomes to *E. terrae* were then predicted by the Genome-to-Genome Distance calculator 2.0, formula 2, (http://ggdc.dsmz.de/)[35–37]. Note that *M. petroleiphilum* and *R. gelatinosus* are present on the phylogeny tree of *E. terrae* (Extended Data Fig. 2).

Biosynthetic gene cluster identification. By screening the draft genome of *E. terrae*, obtained by Illumina sequencing, many gene fragments putatively belonging to NRPS coding genes were identified. The assembly was manually edited and gap closure PCRs were performed. Sanger sequencing of the resulting fragments allowed the closure of the gene locus corresponding to the teixobactin biosynthetic gene cluster. The specificity of the adenylation domains was determined using the online tool NRPSpredictor2 (ref. 38).

Strain fermentation and purification of teixobactin. Homogenized colonies were first grown with agitation in seed broth. After 4 days at 28 °C, the culture was diluted 5% (v/v) into the R4 fermentation media, and production monitored with analytical HPLC. For scale-up isolation and purification of teixobactin, 40 litres of cells were grown in a Sartorius Biostat Cultibag STR 50/200 Bioreactor for about 7 days. The culture was centrifuged and the pellet extracted with 10 litres of 50% aqueous acetonitrile and the suspension again centrifuged for 30 min. The acetonitrile was removed from the supernatant by rotary evaporation under reduced pressure until only water remained. The mixture was then extracted twice with 5 litres of n-BuOH. The organic layer was transferred to a round bottom flask and the n-BuOH removed by rotary evaporation under reduced pressure. The resulting yellow solid was dissolved in DMSO and subjected to preparatory HPLC (SP: C18, MP: H_2O/MeCN/0.1% TFA). The fractions containing teixobactin were then pooled and the acetonitrile removed by rotary evaporation under reduced pressure. The remaining aqueous mixture was then lyophilized to leave a white powder (trifluoroacetate salt). Teixobactin was then converted to a hydrochloride salt, and endotoxin removed as follows. 100 mg of teixobactin (TFA salt) was dissolved in 100 ml of H_2O and 5 g of Dowex (1×4 Cl^- form) was added and the mixture incubated for 20 min with occasional shaking. A 10 g Dowex (1×4 Cl^- form) column was prepared and the mixture was then poured onto the prepared column and the solution was allowed to elute slowly. This solution was then poured over a fresh 10 g Dowex (1x4 Cl^- form) column and the resulting solution filtered through a Pall 3K Molecular Weight Centrifugal filter. The clear solution was then lyophilized to leave a white powder.

Minimum inhibitory concentration (MIC). MIC was determined by broth microdilution according to CLSI guidelines. The test medium for most species was cation-adjusted Mueller-Hinton broth (MHB). The same test medium was supplemented with 3% lysed horse blood (Cleveland Scientific, Bath, OH) for growing Streptococci. *Haemophilus* Test Medium was used for *H. influenzae* (Teknova, Hollister, CA), Middlebrook 7H9 broth (Difco) was used for mycobacteria, Schaedler-anaerobe broth (Oxoid) was used for *C. difficile,* and fetal bovine serum (ATCC) was added to MHB (1:10) to test the effect of serum. All test media were supplemented with 0.002% polysorbate 80 to prevent drug binding to plastic surfaces[39], and cell concentration was adjusted to approximately 5×10^5 cells per ml. After 20 h of incubation at 37 °C (2 days for *M. smegmatis*, and 7 days for *M. tuberculosis*), the MIC was defined as the lowest concentration of antibiotic with no visible growth. Expanded panel antibacterial spectrum of teixobactin was tested at Micromyx, Kalamazoo, MI, in broth assays. Experiments were performed with biological replicates.

Minimum bactericidal concentration (MBC). *S. aureus* NCTC8325-4 cells from the wells from an MIC microbroth plate that had been incubated for 20 h at 37 °C were pelleted. An aliquot of the initial inoculum for the MIC plate was similarly processed. The cells were resuspended in fresh media, plated onto MHA, and the colonies enumerated after incubating for 24 h at 37 °C. The MBC is defined as the first drug dilution which resulted in a 99.9% decrease from the initial bacterial titre of the starting inoculum, and was determined to be $2 \times$ MIC for teixobactin. Experiments were performed with biological replicates.

Time-dependent killing. An overnight culture of cells (*S. aureus* HG003; vancomycin intermediate *S. aureus* SA1287) was diluted 1:10,000 in MHB and incubated at 37 °C with aeration at 225 r.p.m. for 2 h (early exponential) or 5 h (late exponential). Bacteria were then challenged with antibiotics at $10 \times$ MIC (a desirable concentration at the site of infection), oxacillin (1.5 µg ml^{-1}), vancomycin (10 µg ml^{-1}) or teixobactin (3 µg ml^{-1}) in culture tubes at 37 °C and 225 r.p.m. At intervals, 100 µl aliquots were removed, centrifuged at 10,000*g* for 1 min and resuspended in 100 µl of sterile phosphate buffered saline (PBS). Tenfold serially diluted suspensions were plated on MHA plates and incubated at 37 °C overnight. Colonies were counted and c.f.u. per ml was calculated. For analysis of lysis, 12.5 ml of culture at $A_{600\,nm}$ (OD_{600}) of 1.0 was treated with $10 \times$ MIC of antibiotics for 24 h, after which, 2 ml of each culture was added to glass test tubes and photographed. Experiments were performed with biological replicates.

Resistance studies. For single step resistance, *S. aureus* NCTC8325-4 at 10^{10} c.f.u. were plated onto MHA containing 2×, 4×, and $10 \times$ MIC of teixobactin[40]. After 48 h of incubation at 37 °C, no resistant colonies were detected, giving the calculated frequency of resistance to teixobactin of $< 10^{-10}$. For *M. tuberculosis*, cells were cultured in 7H9 medium and plated at 10^9 cells per ml on 10 plates and incubated for 3 weeks at 37 °C for colony counts. No colonies were detected.

For resistance development by sequential passaging[40,41], *S. aureus* ATCC 29213 cells at exponential phase were diluted to an $A_{600\,nm}$ (OD_{600}) of 0.01 in 1 ml of MHB supplemented with 0.002% polysorbate 80 containing teixobactin or ofloxacin. Cells were incubated at 37 °C with agitation, and passaged at 24 h intervals in the presence of teixobactin or ofloxacin at subinhibitory concentration (see Supplementary Discussion for details). The MIC was determined by broth microdilution. Experiments were performed with biological replicates.

Mammalian cytotoxicity. The CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega) was used to determine the cytotoxicity of teixobactin. Exponentially growing NIH/3T3 mouse embryonic fibroblast (ATCC CRL-1658, in Dulbecco's Modified Eagle's medium supplemented with 10% bovine calf serum), and HepG2 cells (ATCC HB-8065, in Dulbecco's Modified Eagle's medium supplemented with 10% fetal calf serum) were seeded into a 96-well flat bottom plate, and incubated at 37 °C. After 24 h, the medium was replaced with fresh medium containing test compounds (0.5 µl of a twofold serial dilution in DMSO to 99.5 µl of media). After 48 h of incubation at 37 °C, reporter solution was added to the cells and after 2 h, the $A_{490\,nm}$ (OD_{490}) was measured using a Spectramax Plus Spectrophotometer. Experiments were performed with biological replicates.

Haemolytic activity. Fresh human red blood cells were washed with PBS until the upper phase was clear after centrifugation. The pellet was resuspended to an A_{600nm} (OD_{600}) of 24 in PBS, and added to the wells of a 96-well U-bottom plate. Teixobactin was serially diluted twofold in water and added to the wells resulting in a final concentration ranging from 0.003 to 200 µg ml^{-1}. After one hour at 37 °C, cells were centrifuged at 1,000*g*. The supernatant was diluted and A_{450nm} (OD_{450}) measured using a Spectramax Plus Spectrophotometer. Experiments were performed with biological replicates.

Macromolecular synthesis. *S. aureus* NCTC8325-4 cells were cultured in minimal medium (0.02 M HEPES, 0.002 M $MgSO_4$, 0.0001 M $CaCl_2$, 0.4% succinic acid,

0.043 M $NaCl_2$, 0.5% $(NH_4)_2SO_4$) supplemented with 5% tryptic soy broth (TSB). Cells were pelleted and resuspended into fresh minimal medium supplemented with 5% TSB containing test compounds and radioactive precursors to a density of 10^8 cells per ml. The radioactive precursors were glucosamine hydrochloride, D-[6-^{3}H(N)] (1 mCi ml^{-1}), leucine, L-[3,4,5-^{3}H(N)] (1 mCi ml^{-1}), uridine, [5-^{3}H] (1 mCi ml^{-1}), or thymidine, [methyl-^{3}H] (0.25 mCi ml^{-1}) to measure cell wall, protein, RNA, and DNA synthesis, respectively. After 20 min of incubation at 37 °C, aliquots were removed, added to ice cold 25% trichloroacetic acid (TCA), and filtered using Multiscreen Filter plates (Millipore Cat. MSDVN6B50). The filters were washed twice with ice cold 25% TCA, twice with ice-cold water, dried and counted with scintillation fluid using Perkin Elmer MicroBeta TriLux Microplate Scintillation and Luminescence counter. Experiments were performed with biological replicates.

Intracellular accumulation of UDP-*N*-acetyl-muramic acid pentapeptide. Analysis of the cytoplasmic peptidoglycan nucleotide precursor pool was examined using *S. aureus* ATCC 29213 grown in 25 ml MHB. Cells were grown to an A_{600nm} (OD_{600}) of 0.6 and incubated with 130 μg ml^{-1} of chloramphenicol for 15 min. Teixobactin was added at 1, 2.5 and 5 × MIC and incubated for another 60 min. Vancomycin (VAN; 10 × MIC), known to form a complex with lipid II, was used as positive control. Cells were collected and extracted with boiling water. The cell extract was then centrifuged and the supernatant lyophilized[42]. UDP-linked cell wall precursors were analysed by RP18-HPLC[43] and confirmed by MALDI-ToF[44] mass spectrometry. Experiments were performed with biological replicates.

Cloning, overexpression and purification of *S. aureus* UppS and YbjG as His_6-tag fusions. *S. aureus* N315 *uppS* (SA1103) and *ybjB* (SA0415) were amplified using forward and reverse primers uppS_FW-5′-TCGGAGGAAAGCATATGTTAAAAAGC-3′, uppS_RV-5′-ATACTCTCGAGCTCCTCACTC-3′, SA0415_FW-5′-GCGCGGGATCCATGATAGATAAAAAATTAACATCAC-3′ and SA0415_RV-5′-GCGCGCTCGAGAACGCGTTGTCGTCGATGAT-3′, respectively and cloned into a modified pET20 vector[44] using restriction enzymes NdeI (*uppS*) or BamHI (*ybjG*) and XhoI, to generate C-terminal His_6-fusion proteins. Recombinant UppS-His_6 enzyme was overexpressed and purified as described for MurG[32]. For overexpression and purification of YbjG-His_6 *E. coli* BL21(DE3) C43 cells transformed with the appropriate recombinant plasmid were grown in 2YT-medium (50 μg ml^{-1} ampicillin) at 25 °C. At an A_{600nm} (OD_{600}) of 0.6, IPTG was added at a concentration of 1 mM to induce expression of the recombinant proteins. After 16 h, cells were harvested and resuspended in buffer A (25 mM Tris/HCl, pH 7.5, 150 mM NaCl, 2 mM β-mercaptoethanol, 30% glycerol, and 1 mM $MgCl_2$). 2 mg ml^{-1} lysozyme, 75 μg ml^{-1} DNase and 75 μg ml^{-1} RNase were added; cells were incubated for 1 h on ice, sonicated and the resulting suspension was centrifuged (20,000*g*, 30 min, 4 °C). Pelleted bacterial membranes were washed three times to remove remaining cytoplasmic content. Membrane proteins were solubilized in two successive steps with buffer A containing 17.6 mM *n*-dodecyl-β-D-maltoside (DDM). Solubilized proteins were separated from cell debris by centrifugation (20,000*g*, 30 min, 4 °C) and the supernatant containing recombinant proteins was mixed with Talon-agarose (Clontech) and purification was performed[42]. Purity was controlled by SDS–PAGE and protein concentration was determined using Bradford protein assay (Biorad).

***In vitro* peptidoglycan synthesis reactions.** *In vitro* peptidoglycan biosynthesis reactions were performed as described using purified enzymes and substrates[32,45]. The MurG activity assay was performed in a final volume of 30 μl containing 2.5 nmol purifed lipid I, 25 nmol UDP-*N*-acetyl glucosamine (UDP-GlcNAc) in 200 mM Tris-HCl, 5.7 mM $MgCl_2$, pH 7.5, and 0.8% Triton X-100 in the presence of 0.45 μg of purified, recombinant MurG-His_6 enzyme. Reaction mixtures were incubated for 60 min at 30 °C. For quantitative analysis 0.5 nmol of [^{14}C]-UDP-GlcNAc (9.25 GBq mmol^{-1}; ARC) was added to the reaction mixtures. The assay for synthesis of lipid II–Gly_1 catalysed by FemX was performed as described previously without any modifications[32,45]. Enzymatic activity of *S. aureus* PBP2 was determined by incubating 2 nmol [^{14}C]-lipid II in 100 mM MES, 10 mM $MgCl_2$, pH 5.5 in a total volume of 50 μl. The reaction was initiated by the addition of 5 μg PBP2–His_6 and incubated for 2.5 h at 30 °C. Monophosphorylation of C_{55}-PP was carried out using purified *S. aureus* YbjG–His_6 enzyme as described previously for *E. coli* pyrophosphatase[46], with modifications. 0.5 nmol [^{14}C]-C_{55}-PP (1.017 kBq) was incubated with 0.6 μg YbjG-His^6 in 20 mM Tris/HCl, pH 7.5, 60 mM NaCl, 0.8% Triton X-100 for 10 min at 30 °C.

In all *in vitro* assays teixobactin was added in molar ratios ranging from 0.25 to 8 with respect to the amount of [^{14}C]-C_{55}-PP, lipid I or lipid II and [^{14}C]-lipid II, respectively. Synthesized lipid intermediates were extracted from the reaction mixtures with *n*-butanol/pyridine acetate, pH 4.2 (2:1; vol/vol) after supplementing the reaction mixture with 1 M NaCl and analysed by thin-layer chromatography (TLC). Quantification was carried out using phosphoimaging (Storm imaging system, GE Healthcare) as described[32,45]. Experiments were performed with biological replicates.

Synthesis and purification of lipid intermediates. Large scale synthesis and purification of the peptidoglycan precursors lipid I and II was performed[45]. Radiolabelled lipid II was synthesized using [^{14}C]-UDP-GlcNAc (9.25 GBq mmol^{-1}; ARC) as substrate. For synthesis of the lipid II variant with a terminal D-Lac residue, UDP-MurNAc-depsipeptide (Ala-Glu-Lys-Ala-Lac) was purified from *Lactobacillus casei* ATCC393 . Briefly, *L. casei* was grown in MRS broth to an A_{600nm} (OD_{600}) of 0.6 and incubated with 65 μg ml^{-1} of chloramphenicol for 15 min. Intracellular accumulation was achieved by incubation with Bacitracin (10 × MIC, 40 μg ml^{-1}) in the presence of 1.25 mM zinc for another 60 min. For synthesis of lipid II ending D-Ala-D-Ser the UDP-MurNAc-pentapeptide (Ala-Glu-Lys-Ala-Ser) was used. The wall teichoic acid precursor lipid III (undecaprenyl-PP-GlcNAc) was prepared using purified TarO enzyme[44]. In short, purified recombinant TarO protein was incubated in the presence of 250 nmol C_{55}-P, 2.5 μmol of UDP-GlcNAc in 83 mM Tris-HCl (pH 8.0), 6.7 mM $MgCl_2$, 8.3% (v/v) dimethyl sulfoxide, and 10 mM *N*-lauroylsarcosine. The reaction was initiated by the addition of 150 μg of TarO-His_6 and incubated for 3 h at 30 °C. Lipid intermediates were extracted from the reaction mixtures with *n*-butanol/ pyridine acetate (pH 4.2) (2:1; vol/vol), analysed by TLC and purified. C_{55}-P and C_{55}-PP were purchased from Larodan Fine Chemicals, Sweden. [^{14}C]-C_{55}-PP was synthesized using purified *S. aureus* UppS enzyme based on a protocol elaborated for *E. coli* undecaprenyl pyrophosphate synthase[47]. Synthesis was performed using 0.5 nmol [^{14}C]-farnesyl pyrophosphate (ARC; 2.035 GBq mmol^{-1}), 5 nmol isopentenyl pyrophosphate (Sigma–Aldrich) and 5 μg UppS enzyme in 100 mM HEPES, pH 7.6, 50 mM KCl, 5 mM $MgCl_2$, and 0.1% Triton X-100. After 3 h of incubation at 30 °C radiolabelled C_{55}-PP was extracted from the reaction mixture with BuOH and dried under vacuum. Product identity was confirmed by TLC analysis. Experiments were performed with biological replicates.

Antagonization assays. Antagonization of the antibiotic activity of teixobactin by potential target molecules was performed by an MIC-based setup in microtitre plates. Teixobactin (8 × MIC) was mixed with potential HPLC-purified antagonists (C_{55}-P, farnesyl-PP [C_{15}-PP; Sigma Aldrich], C_{55}-PP, UDP-MurNAc-pentapeptide, UDP-GlcNAc [Sigma Aldrich], lipid I, lipid II, and lipid III) at a fixed molar ratio (fivefold molar excess) or at increasing concentrations with respect to the antibiotic, and the lowest ratio leading to complete antagonization of teixobactin activity was determined. *S. aureus* ATCC 29213 (5 × 10^5 c.f.u. per ml) were added and samples were examined for visible bacterial growth after overnight incubation. Vancomycin (8 × MIC) was used as a control. Experiments were performed with biological replicates.

Complex formation of teixobactin. Binding of teixobactin to C_{55}-P, C_{55}-PP, lipid I, lipid II, lipid II-D-Ala-D-Ser, lipid II-D-Ala-D-Lac and lipid III was analysed by incubating 2 nmol of each purified precursor with 2 to 4 nmoles of teixobactin in 50 mM Tris/HCl, pH 7.5, for 30 min at room temperature. Complex formation was analysed by extracting unbound precursors from the reaction mixture with n-butanol/pyridine acetate (pH 4.2) (2:1; vol/vol) followed by TLC analysis using chloroform/methanol/water/ammonia (88:48:10:1, v/v/v/v) as the solvent and detection of lipid-containing precursors by phosphomolybdic acid staining[48]. Experiments were performed with biological replicates.

hERG inhibition testing. Teixobactin was tested for inhibition of hERG activity using an IonWorksTM HT instrument (Molecular Devices Corporation), which performs electrophysiology measurements in a 384-well plate (PatchPlate). Chinese hamster ovary (CHO) cells stably transfected with hERG (cell-line obtained from Cytomyx, UK) were prepared as a single-cell suspension in extracellular solution (Dulbecco's phosphate buffered saline with calcium and magnesium pH 7), and aliquots added to each well of the plate. The cells were positioned over a small hole at the bottom of each well by applying a vacuum beneath the plate to form an electrical seal. The resistance of each seal was measured via a common ground-electrode in the intracellular compartment and individual electrodes placed into each of the upper wells. Experiments were performed with three biological replicates.

Cytochrome P450 inhibition. Teixobactin and control compounds were incubated with human liver microsomes at 37 °C to determine their effect on five major human cytochromes P450s (CYP). The assay included probe substrates (midazolam for Cyp3A4, testosterone for Cyp3A4, tolbutamide for Cyp2C9, dextro-methorphan for Cyp2D6, S-mephenytoin for Cyp2C19, and phenacetin for Cyp1A2, 2 mM NADPH, 3 mM $MgCl_2$ in 50 mM potassium phosphate buffer, pH 7.4. The final microsomal concentration was 0.5 mg ml^{-1}. NADPH was added last to start the assay. After ten minutes of incubation, the amount of probe metabolite in the supernatant was determined by LC/MS/MS using an Agilent 6410 mass spectrometer coupled with an Agilent 1200 HPLC and a CTC PAL chilled autosampler, all controlled by MassHunter software (Agilent). Experiments were performed with three biological replicates.

***In vitro* genotoxicity.** Teixobactin was tested in an *in vitro* micronucleus test that employs fluorescent cell imaging to assess cytotoxicity and quantify micronuclei. The assay was performed with CHO-K1 cells in the presence or absence of Aroclor

(to induce CYP activity)-treated rat liver S9 fraction (contains phase I and phase II metabolizing enzymes) to determine if any genotoxic metabolites are produced. No evidence of genotoxicity was observed with teixobactin up to 125 $\mu g\,ml^{-1}$ (the highest concentration tested) under either condition. Experiments were performed with three biological replicates.

DNA binding. Compounds were serially diluted and mixed with sheared salmon sperm DNA (6.6 $mg\,ml^{-1}$ final concentration). An aliquot was spotted onto a lawn of growing *S. aureus* NCTC 8325-4 cells, and the zones of growth inhibition measured after 20 h of growth at 37 °C. A reduction in the inhibition zone size in the presence of DNA would indicate loss of antibacterial activity due to binding to the DNA. Experiments were performed with three biological replicates.

Plasma protein binding. Protein binding of teixobactin in rat plasma was determined using a Rapid Equilibrium Dialysis (RED) kit (Pierce) with LC–MS/MS analysis. Teixobactin (10 $\mu g\,ml^{-1}$) and rat plasma in 5% dextrose containing 0.005% polysorbate 80 were added to one side of the single-use RED plate dialysis chamber having an 8kD MW cutoff membrane. Following four hours of dialysis the samples from both sides were processed and analysed by LC/MS/MS. The teixobactin concentration was determined, and the percentage of compound bound to protein was calculated. Teixobactin exhibited 84% plasma protein binding. Experiments were performed with three biological replicates.

Microsomal stability. The metabolic stability of teixobactin was measured in rat liver microsomes (Invitrogen/Life Technologies, CA) using NADPH Regeneration System (Promega) by monitoring the disappearance of the compound over an incubation period of two hours. Teixobactin (60 $\mu g\,ml^{-1}$) or verapamil (5 μM) serving as positive control were added to 1 $mg\,ml^{-1}$ microsomes at 37 °C. Aliquots were removed at 0 h, 0.5 h, 1 h and 2 h, and the reactions stopped by addition of 3 volumes of ice-cold acetonitrile. Samples were analysed by LC/MS/MS. Experiments were performed with three biological replicates.

Animal studies. All animal studies were carried out at Vivisource Laboratories, (Waltham, MA), and University of North Texas Health Science Center (Houston, TX), and conformed to institutional animal care and use policies. Neither randomization nor blinding was deemed necessary for the animal infection models, and all animals were used. All animal studies were performed with female CD-1 mice, 6–8-weeks old.

Pharmacokinetic analysis. CD-1 female mice were injected intravenously with a single dose of 20 mg per kg in water and showed no adverse effects. Plasma samples were taken from 3 mice per time point (5, 15, 30 min; 1, 2, 4, 8 and 24 h post-dose). An aliquot of plasma sample or calibration sample was mixed with three volumes of methanol containing internal standard, incubated on ice for 5 min, and centrifuged. The protein-free supernatant was analysed by LC/MS/MS using an Agilent 6410 mass spectrometer coupled with an Agilent 1200 HPLC and a CTC PAL chilled autosampler, all controlled by MassHunter software (Agilent). After separation on a C18 reverse phase HPLC column (Agilent) using an acetonitrile-water gradient system, peaks were analysed by mass spectrometry using ESI ionization in MRM mode. The product *m/z* analysed was 134.1D, which provided a low limit of quantification of 1 $ng\,ml^{-1}$. The mean plasma concentration and the standard deviation from all 3 animals within each time point were calculated. PK parameters of test agent were calculated with a non-compartmental analysis model based on WinNonlin. The mean plasma concentrations from all 3 mice at each time point were used in the calculation.

Mouse sepsis protection model. Teixobactin was tested against clinical isolate *S. aureus* MRSA ATCC33591 in a mouse septicemia protection assay to assess its *in vivo* bioavailability and PD_{50} (protective dose resulting in 50% survival of infected mice after 48 h). CD-1 female mice were infected with 0.5 ml of bacterial suspension (3.28×10^7 c.f.u. per mouse) via intraperitoneal injection, a concentration that achieves at least 90% mortality within 48 h after infection. At one hour post-infection, mice (6 per group) were treated with teixobactin at single intravenous doses of 20, 10, 5, 2.5, and 1 mg per kg. Infection control mice were dosed with vehicle or vancomycin. Survival is observed 48 h after infection and the probability determined by non-parametric log-rank test. To obtain the $PD_{50,}$ the experiment was repeated at lower doses 5, 1, 0.5, 0.25, and 0.1 mg per kg.

Mouse thigh infection model. Teixobactin was tested against MRSA ATCC33591 in a neutropenic mouse thigh infection model. Female CD-1 mice were rendered neutropenic by cyclophosphamide (two consecutive doses of 150 and 100 mg per kg delivered on 4 and 1 days before infection). Bacteria were resuspended in sterile saline, adjusted to an A_{625nm} (OD_{625}) of 0.1, and a 0.1 ml inoculum (2.8×10^5 c.f.u. per mouse) injected into the right thighs of mice. At 2 h post-infection, mice received treatment with teixobactin at 1, 2.5, 5, 10 or 20 mg per kg administered in a single dose, intravenous injection (four mice per group). One group of infected mice was euthanized and thighs processed for c.f.u. to serve as the time of treatment controls. At 26 h post-infection mice were euthanized by CO_2 inhalation. The right thighs were aseptically removed, weighed, homogenized, serially diluted, and plated on trypticase soy agar for c.f.u. titres.

Mouse lung infection model. Teixobactin was tested against *Streptococcus pneumoniae* ATCC 6301 (UNT012-2) in an immunocompetent mouse pneumonia model to determine the compound's potential to treat acute respiratory infections. CD-1 mice were infected intranasally (1.5×10^6 c.f.u. per mouse). The compound was delivered intravenously at 24 and 36 h post-infection, whereas amoxicillin was delivered subcutaneously at a single concentration to serve as positive control. Teixobactin was delivered at doses ranging from 0.5 to 10 mg per kg per dose (5 mice per dose). At 48 h post-infection, treated mice were euthanized, lungs aseptically removed and processed for c.f.u. counts.

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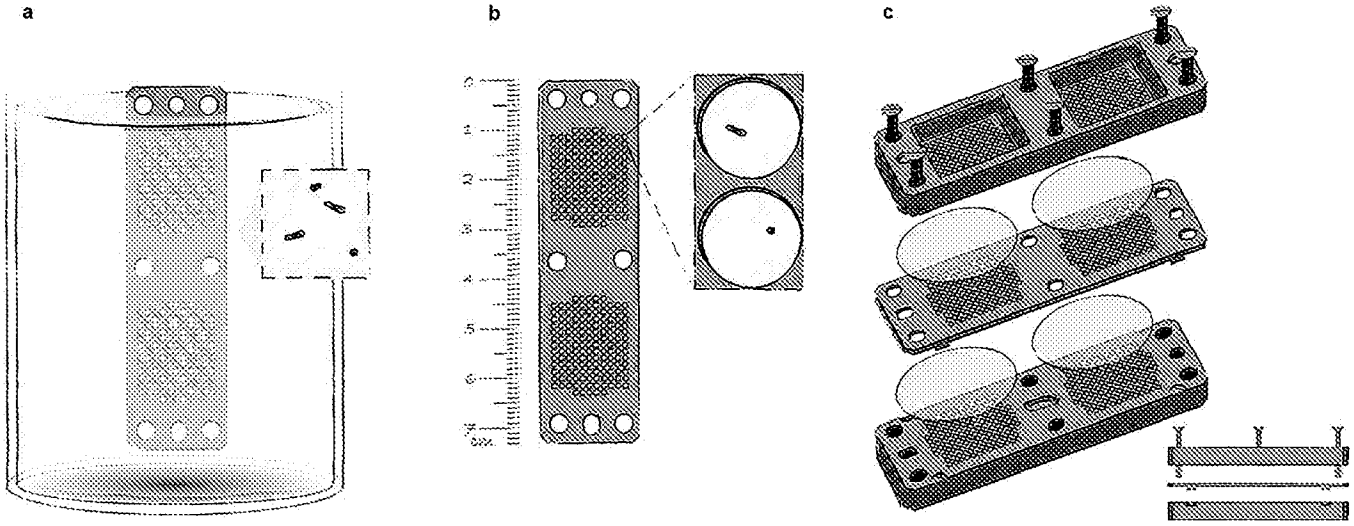
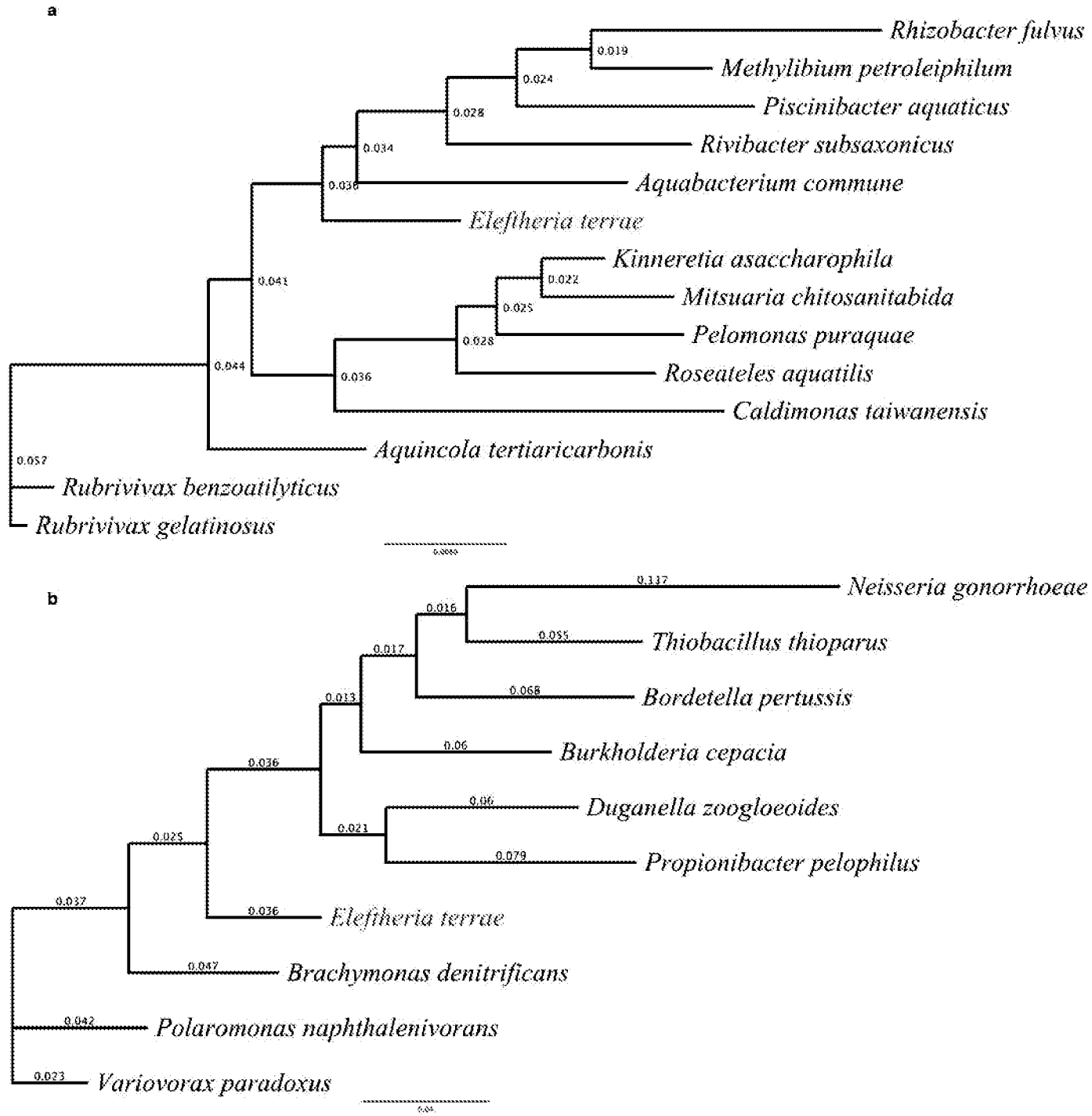


Extended Data Figure 1 | The iChip. **a**–**c**, The iChip (**a**) consists of a central plate (**b**) which houses growing microorganisms, semi-permeable membranes on each side of the plate, which separate the plate from the environment, and two supporting side panels (**c**). The central plate and side panels have multiple matching through-holes. When the central plate is dipped into suspension of cells in molten agar, the through-holes capture small volumes of this suspension, which solidify in the form of small agar plugs. Alternatively, molten agar can be dispensed into the chambers. The membranes are attached and the iChip is then placed in soil from which the sample originated.



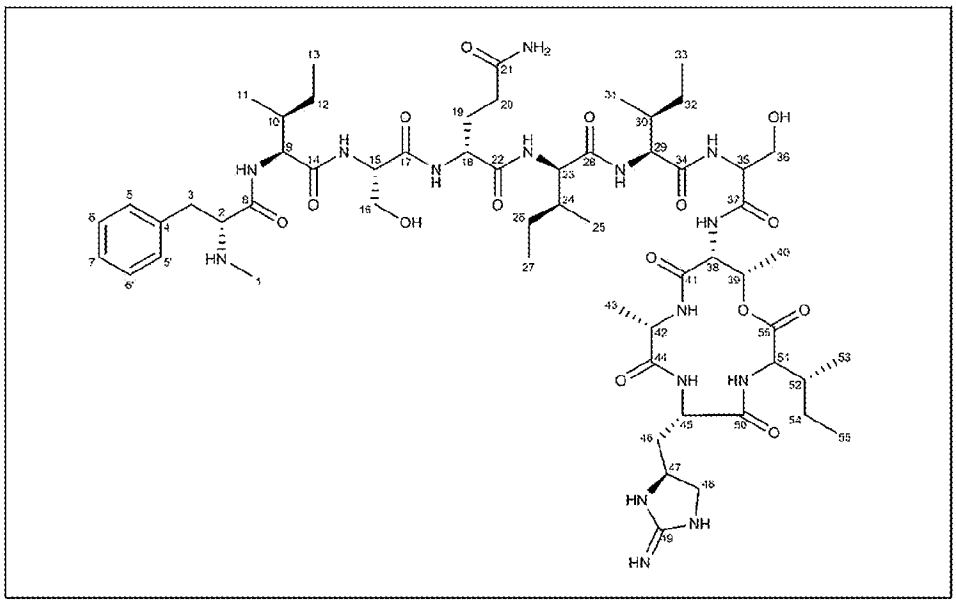
Extended Data Figure 2 | 16S rRNA gene phylogeny of *Eleftheria terrae*. **a**, The phylogenetic position of *E. terrae* within the class β-proteobacteria. The 16S rRNA gene sequences were downloaded from Entrez at NCBI using accession numbers retrieved from peer-reviewed publications. **b**, The phylogenetic position of *E. terrae* among its closest known relatives. The sequences were downloaded from NCBI using accession numbers retrieved from the RDP Classifier Database. For both trees, multiple sequence alignments (MSA) were constructed using ClustalW2, implementing a default Cost Matrix, the Neighbour-Joining (NJ) clustering algorithm, as well as optimized gap penalties. Resulting alignments were manually curated and phylogenetic trees were constructed leveraging PhyML 3.0 with a TN93 substitution model and 500 Bootstrap iterations of branch support. Topology search optimization was conducted using the Subtree–Pruning–Regrafting (SPR) algorithm with an estimated Transition–Transversion ratio and gamma distribution parameters as well as fixed proportions of invariable sites.

a

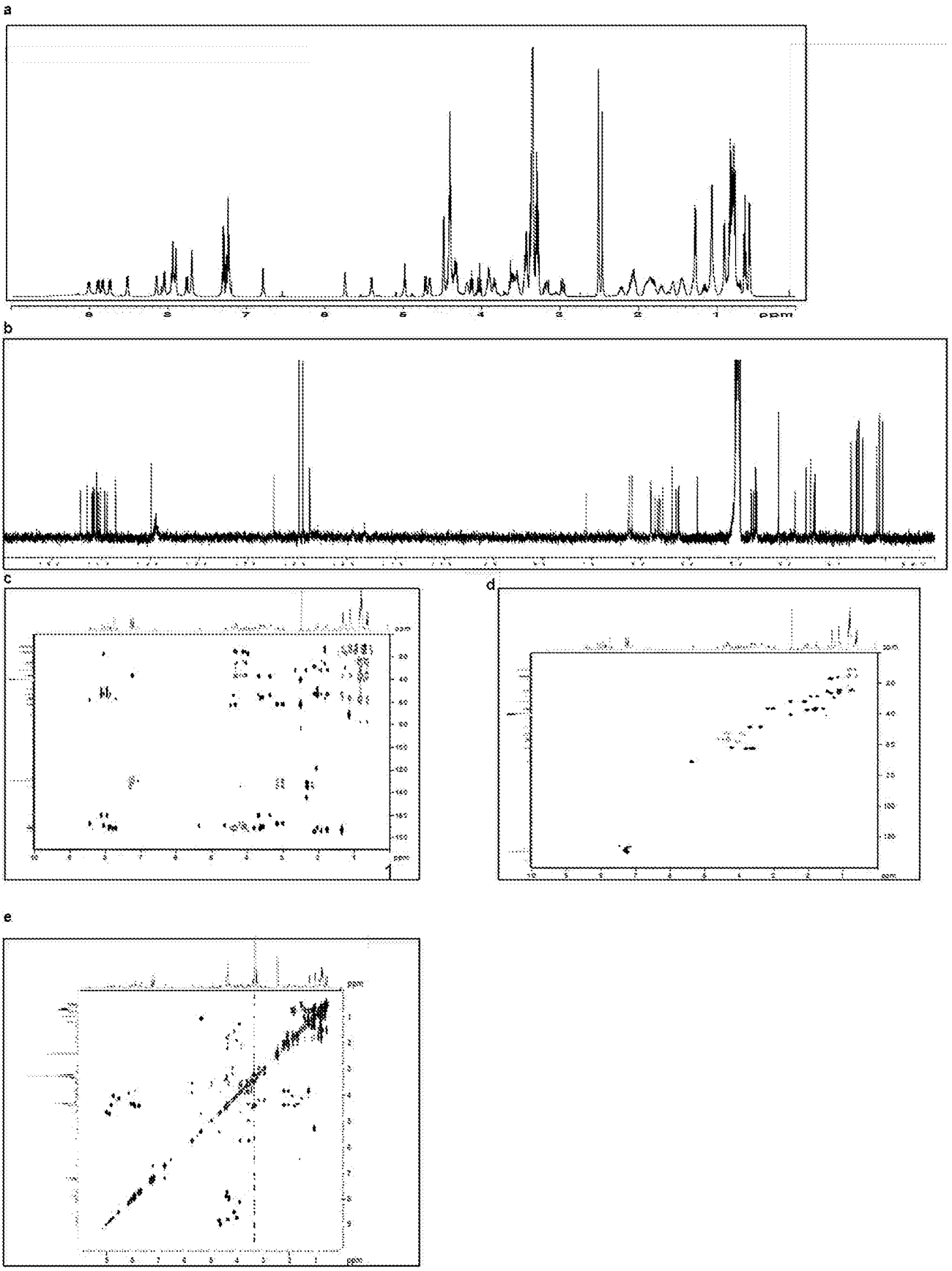
Teixobactin (DMSO-d_6)					
Position	δ_C	δ_H (mult., J in *Hz*)	Position	δ_C	δ_H (mult., J in *Hz*)
1	31.9	2.5 (3H, br s)	29	57.3	4.29 (1H, m)
2	61.9	4.21 (1H, dd, 9.4, 5.3)	29-NH		7.78 (1H, d, 8.8)
2-NH[a]		(2H, 9.3, 9.0, v br s)	30	36.9	1.83 (1H, m)
3	36.4	3.00 (1H, dd, 13.2, 9.4)	31	15.4[a]	0.84 (3H, m)
		3.15 (1H, 13.2, 5.3)	32	25.3	1.11 (1H, m)
4	135.0				1.42 (1H, m)
5,5'	129.7	7.24 (2H, m)	33	11.2[c]	0.85 (3H, m)
6,6'	128.9	7.31 (2H, m)	34	171.6[a]	
7	127.5	7.27 (1H, m)	35	56.5	4.47 (1H, dt, 5.0, 5.2)
8	167.1		35-NH		8.37 (1H, d, 5.2)
9	57.9	4.12 (1H, dd, 7.8, 7.2)	36	62.7	3.64 (1H, m)
9-NH		8.43 (1H, d, 7.2)			3.80 (1H, dd, 10.8, 5.0)
10	36.5	1.56 (1H, m)	36-OH		exchanged
11	15.5	0.62 (1H, d, 6.7)	37	171.7[a]	
12	24.4	0.76 (1H, m)	38	56.2	4.64 (1H, dd, 9.5, 2.2)
		1.07 (1H, m)	39	71.2	5.36 (1H, dq, 2.2, 6.4)
13	11.3	0.66 (3H, t, 7.1)	40	15.9	1.13 (3H, d, 6.4)
14	170.6		41	168.9	
15	55.6	4.34(1H, m)	42	52.2	3.97 (1H, dq, 5.1, 7.5)
15-NH		7.88 (1H, d, 7.9)	42-NH		8.05 (1H, d, 5.1)
16	62.4	3.57 (1H, dd, 10.8, 5.6)	43	17.1	1.34 (3H, d, 7.5)
		3.63 (1H, dd, m)	44	173.1	
16-OH		exchanged	45	52.2	4.38 (1H, m)
17	170.2		45-NH		8.32 (1H, d, 9.1)
18	52.7	4.33 (1H, m)	46	37.2	2.03 (2H, m)
18-NH		7.85 (1H, d, 7.9)	47	53.5	3.90 (1H, m)
19	31.9	2.10 (2H, m)	47-NH		7.95 (1H, br s)
20	28.4	1.74 (1H, m)	48	48.3	3.36 (1H, dd, 9.4, 7.7)
		1.92 (1H, m)			3.66 (1H, t, 9.4)
21	174.4		48-NH		8.1 (1H, br s)
21-NH_2		6.63 (1H, br s)	49	160.0	
		7.11 (1H, br s)	49-NH[c]		7.76 (2H, br s)
22	170.9[a]		50	171.8[a]	
23	56.8	4.36 (1H, m)	51	57.8	4.03 (1H, t, 9.4)
23-NH		7.70 (1H, d, 8.8)	51-NH		8.01 (1H, d 9.4)
24	37.4	1.8 (2H, m)	52	36.3	1.77 (1H, m)
25	14.7[b]	0.82 (3H, m)	53	16.0[b]	0.81 (3H, m)
26	26.2	1.09 (1H, m)	54	24.5	0.77 (1H, m)
		1.32 (1H, m)			1.07 (1H, m)
27	10.6[c]	0.82 (3H, m)	55	11.8[c]	0.82 (3H, m)
28	171.4[a]		56	169.3	

[a] Assignments may be switched due to overlap.
[b] Assignments may be switched due to overlap.
[c] Assignments may be switched due to overlap.
[d] Appears as an ammonium salt

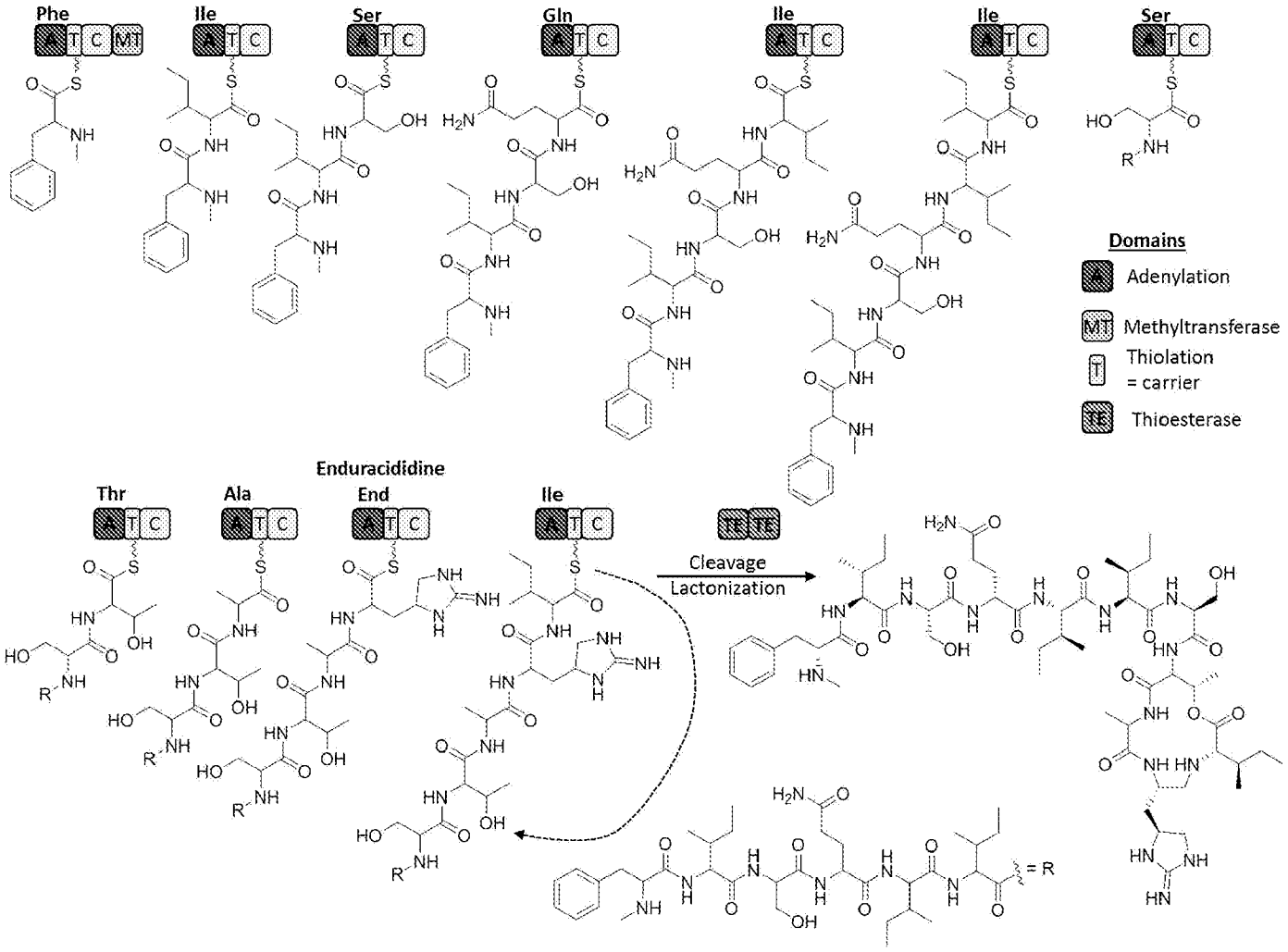
b



Extended Data Figure 3 | NMR assignment of teixobactin. a, ^{13}C-NMR of teixobactin (125 mHz, δ in p.p.m.). **b**, Structure of teixobactin with the NMR assignments.

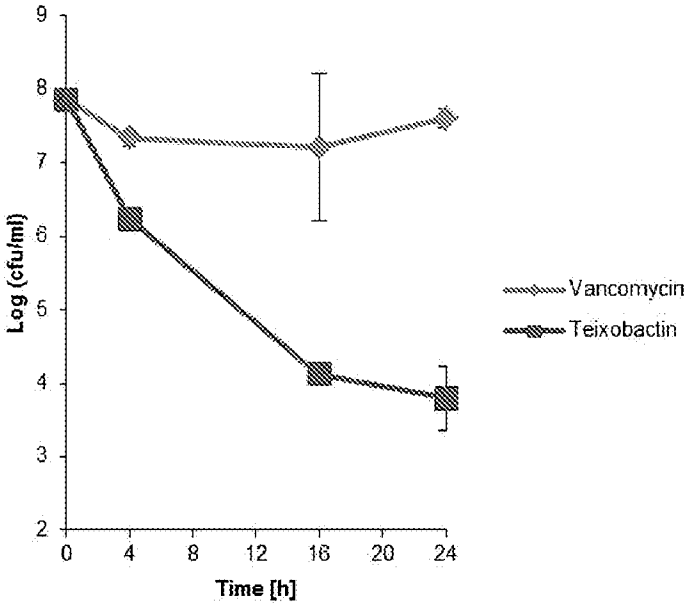


Extended Data Figure 4 | NMR spectra of teixobactin. a, ^{13}C NMR spectrum of teixobactin. **b**, ^{1}H NMR spectrum. **c**, HMBC NMR spectrum. **d**, HSQC NMR spectrum. **e**, COSY NMR spectrum.



Extended Data Figure 5 | Hypothetical biosynthesis pathway of teixobactin. The eleven modules of the non-ribosomal peptide synthetases Txo1 and Txo2 are depicted with the growing chain attached. Each module is responsible for the incorporation of one specific amino acid in the nascent peptide chain. The *N*-methylation of the first amino acid phenylalanine is catalysed by the methyltransferase domain in module 1. The ring closure (marked by a dashed arrow) between the last isoleucine and threonine is catalysed by the thioesterase domains during molecule off-loading, resulting in teixobactin.

a



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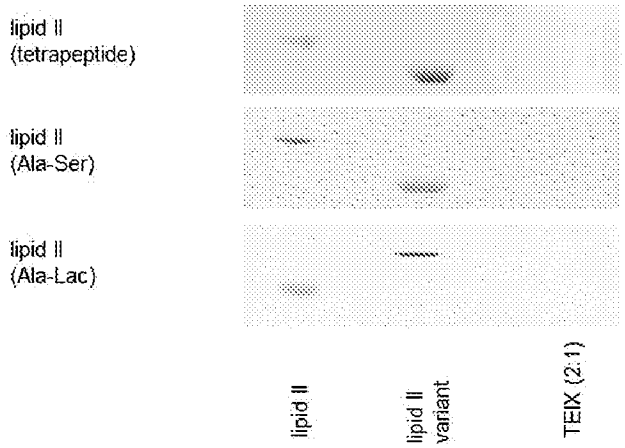
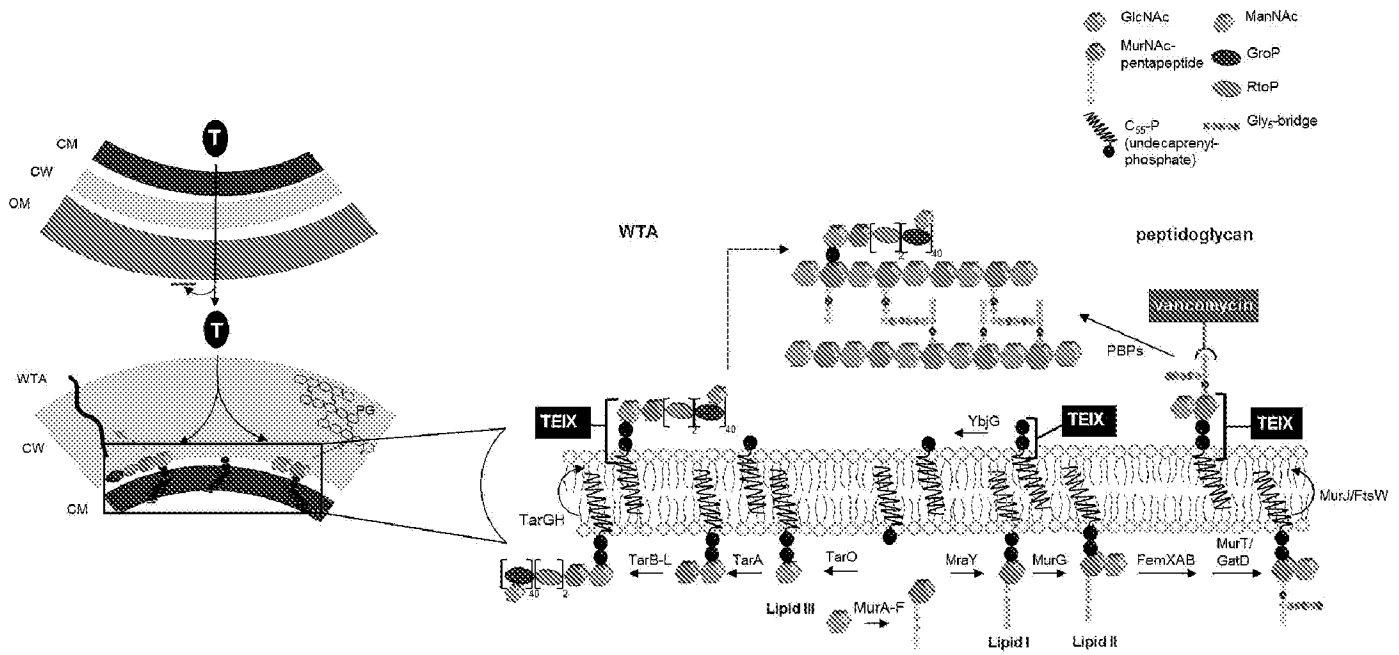
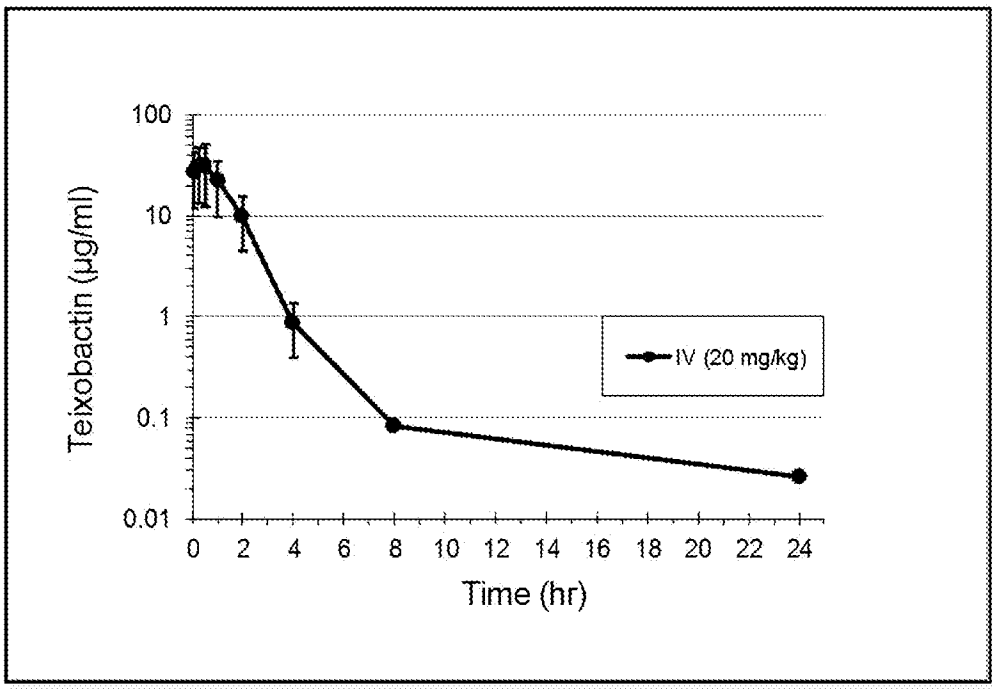


Extended Data Figure 6 | Teixobactin activity against vancomycin-resistant strains. **a**, Vancomycin intermediate *S. aureus* (VISA) were grown to late exponential phase and challenged with vancomycin or teixobactin. Cell numbers were determined by plating for colony counts. Data are representative of 3 independent experiments ± s.d. **b**, Complex formation of teixobactin with cell wall precursor variants as formed by vancomycin-resistant strains. Purified lipid intermediates with altered stem peptides were incubated with teixobactin at a molar ratio of 2:1 (TEIX:lipid II variant). Reaction mixtures were extracted with BuOH/PyrAc and binding of teixobactin to lipid II variants is indicated by its absence on the thin-layer chromatogram. Migration behaviour of unmodified lipid II is used for comparison. The figure is representative of 3 independent experiments.



Extended Data Figure 7 | Model for the mechanism of action of teixobactin. Inhibition of cell wall synthesis by teixobactin. Lipid II, precursor of peptidoglycan, is synthesized in the cytoplasm and flipped to the surface of the inner membrane by MurJ[48] or FtsW[49]. Lipid III, a precursor of wall teichoic acid (WTA), is similarly formed inside the cell and WTA lipid-bound precursors are translocated across the cytoplasmic membrane by the ABC-transporter TarGH[50]. Teixobactin (TEIX) forms a stoichiometric complex with cell wall precursors, lipid II and lipid III. Abduction of these building blocks simultaneously interrupts peptidoglycan (right), WTA (left) biosynthesis as well as precursor recycling. Binding to multiple targets within the cell wall pathways obstructs the formation of a functional cell envelope. Left panel, teixobactin targeting and resistance. The producer of teixobactin is a Gram-negative bacterium which is protected from this compound by exporting it outside of its outer membrane permeability barrier. The target Gram-positive organisms do not have an outer membrane. CM, cytoplasmic membrane; CW, cell wall; OM, outer membrane; LTA, lipoteichoic acid; WTA, wall teichoic acid.

a



b

PK parameter	Definition	Value
C0 (µg/mL)	Initial concentration	27.2
AUC to Last (µg-hr/mL)	Area Under Curve to last time point	57.8
t1/2 (hr)	Half life	4.7
Total CL (mL/hr)	Clearance	6.9
Total CL (mL/min/kg)	Clearance	5.8
V (mL)	Volume of Distribution	47
Vss (mL)	Volume of Distribution at steady state	9.7
MRTINF (hr)	Mean residence time	1.4
Last Time point (hr)	–	24

Extended Data Figure 8 | Pharmacokinetic analysis of teixobactin. **a**, The mean plasma concentrations of teixobactin after a single i.v. injection of 20 mg per kg teixobactin (3 mice per time point). Data are the mean of plasma concentration, and error bars represent the standard deviation from 3 animals in each time point. **b**, Pharmacokinetic parameters of teixobactin calculated with a non-compartmental analysis model based on WinNonlin.

Extended Data Table 1 | Antibacterial spectrum of teixobactin

a

Organism	MIC (μg/mL)	Organism	MIC (μg/mL)
Staphylococcus aureus		***Streptococcus pneumoniae***	
ATCC 29213 (MSSA)	0.16-0.31	ATCC BAA 255	0.05
NCTC 8325 (MSSA)	0.08-0.31	VL-172	0.15
ATCC 33591 (MRSA)	0.16-0.31	VL-190	0.15
NRS54 (MRSA)	0.078-0.16	ATCC 10813	0.08
NRS108 (MRSA, also synercid^{R})	0.16	ATCC 6303	0.02-0.04
NRS269 (MRSA, also tigecycline^{R})	0.16-0.31	BAA 1407	0.04
ATCC 700699 (GISA)	0.31	***Bacillus anthracis***	
S. epidermidis		Sterne	0.02
ATCC 35984=NRS101 (*mecA* positive)	0.078-0.16	***B. anthracis* BEI Resources[a] Isolates**	
NRS8 (*mecA* positive)	0.16-0.31	NR-36 NRS 1008	≤0.06
NRS34 (*mecA* positive)	0.16	NR-38 Pasteur Vaccine No.1	≤0.06
S. haemolyticus		NR-41 Graves	≤0.06
NRS9 (*mecA* positive)	0.08	NR-46 46-PY-5	≤0.06
NRS69 (*mecA* positive)	0.16	NR-411 Ames, A0462	≤0.06
Enterococcus		NR-412 Kruger B	0.06-0.125
E. faecium BM4147 (*aac(6')-Ie-aph(2")*, van^{R})	0.31	NR-413 CNEVA	0.125
E. faecium E4sol (vancomycin^{S})	0.31	NR-414 Vollum	≤0.06
E. faecalis ATCC 51575 (vancomycin^{R})	0.31-0.63	NR-415 WNA	≤0.06
E.faecalis M192 (vancomycin^{S})	0.63	NR-3838 Ames	≤0.06
Mycobacterium		**Other Gram-positive**	
Mycobacterium smegmatis mc^{2}155	0.31	*Streptococcus pyogenes* ATCC19615	0.31
M. tuberculosis H37Rv	0.125	*S. warneri* NRS138	0.02
M. tuberculosis (clinical isolate 70)	0.125	*Bacillus subtilis* 1A1	0.02
M. tuberculosis (clinical isolate 76)	0.125-0.25	*Clostridium difficile* CD196	0.005
M. tuberculosis (clinical isolate 82)	0.125-0.25	*Propionibacterium acnes* ATCC6919	0.078
M. tuberculosis (clinical isolate 102)	0.25		
Gram-negative			
Haemophilus influenzae SJ7	2.5	*E. coli* K12	25
Klebsiella pneumoniae ATCC 700603	20	*E. coli* W0153 (AB1157; *asmB1 ΔtolC::kan*)	2.5
Pseudomonas aeruginosa PA-01	>100	*E. coli* W0159 (AB1157; *asmB1 ΔrfaC::kan*)	2.5
Klebsiella pneumoniae ATCC 43816	>40	*E. coli* ATCC 25922	25
Yersinia pestis KIM 100 deletion pDC1	50-100	E. coli mutS	25
Neisseria gonorrhoeae	25	*Bacteriodes fragilis* ATCC 25825	200

b

Organism	# Isolates	Drug	MIC Range (μg/mL)	MIC_{90} (μg/mL)	Organism	# Isolates	Drug	MIC Range (μg/mL)	MIC_{90} (μg/mL)
Staphylococcus aureus MSSA	20	Teixobactin	0.06-0.25	0.25	*Enterococcus faecalis 50% VRE*	10	Teixobactin	0.5-1	0.5
		Linezolid	2-4	4			Linezolid	1-2	2
		Vancomycin	0.5-1	1			Vancomycin	0.5->32	>32
		Daptomycin	0.12-0.25	0.25			Daptomycin	0.12-8	0.5
S. aureus MRSA	20	Teixobactin	0.06-0.5	0.25	*E. faecium 50% VRE*	10	Teixobactin	0.25-1	1
		Linezolid	2-4	4			Linezolid	1-2	2
		Vancomycin	0.5-2	1			Vancomycin	0.5->32	>32
		Daptomycin	0.12-0.25	0.25			Daptomycin	0.5-1	1
S. aureus VISA	10	Teixobactin	0.12-1	0.5	*Streptococcus pneumoniae* 28.6% PSSP, 33.3% PISP, 38.1% PRSP	20	Teixobactin	≤0.03-0.06	≤0.03
		Linezolid	1-4	2			Linezolid	0.25-1	1
		Vancomycin	1-8	8			Vancomycin	0.12-0.5	0.25
		Daptomycin	0.25-1	1			Daptomycin	≤0.03-0.06	≤0.03
S. aureus Daptomycin^{NS}	5	Teixobactin	0.12-0.5	-	*S. pyogenes*	10	Teixobactin	≤0.03-0.06	0.06
		Linezolid	1-32	-			Linezolid	0.5-1	1
		Vancomycin	0.5-8	-			Vancomycin	0.25	0.25
		Daptomycin	2-8	-			Daptomycin	≤0.03-0.06	0.06
S. aureus Linezolid^{R}	5	Teixobactin	0.12-0.5	-	*S. agalactiae*	10	Teixobactin	0.06-0.12	0.12
		Linezolid	16->32	-			Linezolid	0.5-2	1
		Vancomycin	1	-			Vancomycin	0.25-0.5	0.5
		Daptomycin	0.25-0.5	-			Daptomycin	0.06-0.25	0.12
S. epidermidis	20	Teixobactin	≤0.03-0.25	0.12	Viridans Group Streptococci[1]	5	Teixobactin	≤0.03-0.12	-
		Linezolid	1-8	2			Linezolid	0.5-1	-
		Vancomycin	1-2	2			Vancomycin	0.25-0.5	-
		Daptomycin	0.06-0.25	0.25			Daptomycin	0.06-0.25	-

a, Antibacterial spectrum of teixobactin. MIC was determined by broth microdilution. [a]*B. anthracis* BB resources isolates are from NIH Biodefense and Emerging Infections Research Resources repository.
b, Antibacterial activity of teixobactin and known drugs against contemporary clinical isolates. [1]In the Viridans Group Streptococci, one isolate of each of the following was tested *S. sanguis*, *S. mitis*, *S. anginosus*, *S. intermedius* and *S. salivarius*. PISP, penicillin-intermediate *S. pneumoniae*; PRSP, penicillin-resistant *S. pneumoniae*; PSSP, penicillin-sensitive *S. pneumoniae*.

Extended Data Table 2 | Antagonization of the antimicrobial activity of teixobactin by cell wall precursors

a

antagonist	C_{55}-P	C_{55}-PP	C_{15}-PP	lipid I	lipid II	lipid III	UDP-MurNAc-pentapeptide	UDP-GlcNAc
teixobactin	-	+	+	+	+	+	-	-
vancomycin	-	-	nd	+	+	-	nd	nd

(+) antibiotic activity antagonized, (-) antibiotic activity unaffected, (nd) not determined

b

lipid intermediate	molar ratio of precursor to teixobactin						
	0 x	0.5 x	1 x	2.5 x	5 x	7.5 x	10 x
lipid II	-	+	+	+	+	+	+
C_{55}-PP	-	-	-	-	+	+	+

a, *S. aureus* ATCC 29213 was incubated with teixobactin and vancomycin at 8 × MIC in nutrient broth in a microtitre plate, and growth was measured after a 24 h incubation at 37 °C. Putative HPLC-purified antagonists (undecaprenyl-phosphate [C_{55}-P], farnesyl-pyrophosphate [C_{15}-PP], undecaprenyl-pyrophosphate [C_{55}-PP], UDP-MurNAc-pentapeptide, UDP-GlcNAc, lipid I, lipid II, and lipid III) were added in a fivefold molar excess with respect to the antibiotic. **b,** Teixobactin at 8 × MIC was exposed to increasing concentrations of putative antagonistic lipid intermediates. Experiments were performed with biological replicates.

Appendix B

BBC NEWS

HEALTH

7 January 2015 Last updated at 13:01 ET

Antibiotics: US discovery labelled 'game-changer' for medicine

By James Gallagher
Health editor, BBC News website

The decades-long drought in antibiotic discovery could be over after a breakthrough by US scientists.

Their novel method for growing bacteria has yielded 25 new antibiotics, with one deemed "very promising".

The last new class of antibiotics to make it to clinic was discovered nearly three decades ago.

The study, **in the journal Nature**, has been described as a "game-changer" and experts believe the antibiotic haul is just the "tip of the iceberg".

The heyday of antibiotic discovery was in the 1950s and 1960s, but nothing found since 1987 has made it into doctors' hands.

Since then microbes have become incredibly resistant. **Extensively drug-resistant tuberculosis** ignores nearly everything medicine can throw at it.

Back to soil

The researchers, at the Northeastern University in Boston, Massachusetts, turned to the source of nearly all antibiotics - soil.

This is teeming with microbes, but only 1% can be grown in the laboratory.

The team created a "subterranean hotel" for bacteria. One bacterium was placed in each "room" and the whole device was buried in soil.

It allowed the unique chemistry of soil to permeate the room, but kept the bacteria in place for study.

The scientists involved believe they can grow nearly half of all soil bacteria.

Chemicals produced by the microbes, dug up from one researcher's back yard, were then tested for antimicrobial properties.

The lead scientist, Prof Kim Lewis, said: "So far 25 new antibiotics have been discovered using this method and teixobactin is the latest and most promising one.

"[The study shows] uncultured bacteria do harbour novel chemistry that we have not seen before. That is a promising source of new antimicrobials and will hopefully help revive the field of antibiotic discovery."

Bacteria

Tests on teixobactin showed it was toxic to bacteria, but not mammalian tissues, and could clear a deadly dose of MRSA in tests on mice.

Human tests are now needed.

The researchers also believe that bacteria are unlikely to develop resistance to teixobactin.

It targets fats which are essential for building the bacterial cell wall, and the scientists argue it would be difficult to evolve resistance.

"Here is an antibiotic that essentially evolved to be free of resistance," said Prof Lewis. "We haven't seen that before.

"It has several independent different tricks that minimise resistance development."

Analysis
By James Gallagher, health editor, BBC News website

There are limits to the discovery of the antibiotic teixobactin, which has yet to be tested in people.

It works on only Gram-positive bacteria; this includes MRSA and mycobacterium tuberculosis.

It cannot penetrate the extra layer of protection in Gram-negative bacteria such as E. coli.

But even if their method does mark a new era of antibiotic discovery there are big questions.

Sir Alexander Fleming, who discovered penicillin, warned of the dangers of resistance back in his Nobel prize speech in 1945.

Yet even now prescriptions in England are rising, with half deemed "inappropriate" and contributing to the problem.

But can we be trusted with new antibiotics? Or will we make the same mistakes again?

BBC News: Antibiotic resistance and prescribing rise continues

Analysis: Antibiotic apocalypse

Prof Laura Piddock from the University of Birmingham said it was an "amazing" and "exciting" study and that the tool "could be a game-changer".

"The discovery of this new antibiotic, from a new class with a novel mode of action, is very exciting.

"I hope that teixobactin will now enter clinical development as the basis for a new drug to treat infections."

There is wide concern that the world is cruising into a "post-antibiotic" era.

It could leave many common infections untreatable and make many staples of modern medicine - including surgery, chemotherapy and organ transplants - impossible.

Prof Mark Woolhouse from the University of Edinburgh added: "What most excites me is the tantalising prospect that this discovery is just the tip of the iceberg."

He said it was vital the antibiotic pipeline was reopened "if we are to avert a public-health disaster".

'Unfinished business'
Dr James Mason from King's College London said the antibiotic pipeline had "all but dried up".

"It's impressive what they've done. From one soil sample they've found one new antibiotic, and their approach opens up a new route to a huge number of potential products.

"They have shown that screening soil microorganisms for antibiotics is unfinished business."

However, he cautioned that although the new antibiotic had exciting properties, it was too early to conclude it would entirely avoid the development of resistance.

The researchers said their discovery was similar to vancomycin. It was discovered in the 1950s, but it took until the late 1980s for resistance to develop.

"They argue the delay in resistance is an inherent property, but vancomycin was kept back and not used that extensively," Dr Mason said.