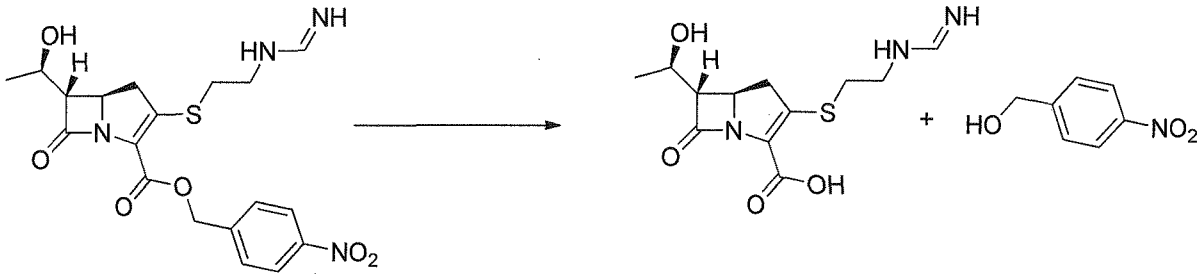
particular, the engineered pNB esterase polypeptides of the present disclosure have been engineered for efficient removal a pNB protecting group from the imipenem precursor compound of compound **(2)** thereby converting it to the product compound **(1)**, imipenem, as shown in Scheme 1.

Scheme 1



(2) **(1)**

[0010] The amino residue differences are located at residue positions affecting various enzyme properties, including among others, activity, stability, product selectivity, and product tolerance. These amino residue differences, although evolved for the removal of pNB protecting groups from the imipenem precursor of compound **(2)**, can also be used to evolve engineered pNB esterase polypeptides having activity useful for removing pNB protecting groups of structurally analogous carbapenem precursor compounds including, but not limited to, pNB-protected precursor compounds of thienamycin meropenem, doripenem, ertapenem, biopenem, and panipenem.

In one aspect, ~~the present disclosure provides~~ there is provided an engineered polypeptides having pNB esterase activity, ~~where the engineered polypeptide comprises~~ comprising an amino acid sequence having at least 80% identity to SEQ ID NO: 2 and one or more residue differences as compared to SEQ ID NO:2 at residue positions selected from: X108, X115, X116, X130, X193, X214, X219, X273, X276, X321, and X362. In some embodiments, the residue differences as compared to SEQ ID NO: 2 at the residue positions X108, X115, X116, X130, X193, X214, X219, X273, X276, X321, and X362 are selected from X108L/Y, X115Q/W, X116S, X130T, X164T, X193A/D/E/V, X214G, X219A/D/L/V, X273A/E/T/V, X276A/T/L, X321A, and X362A/D/Q/S/V.

[0011] In some embodiments, the engineered polypeptide having pNB esterase activity comprises an amino acid sequence having at least 80% identity to SEQ ID NO: 2 and one or more residue differences as compared to SEQ ID NO: 2 selected from: X108L/Y, X193A/D/E/V, X219A/D/L/V, X273A/E/T/V, and X362A/D/Q/S/V.

[0012] In some embodiments, the engineered polypeptide having pNB esterase activity comprises an amino acid sequence having at least 80% identity to SEQ ID NO: 2 and a residue difference as compared to SEQ ID NO: 2 at position X193 selected from: X193A/D/E/V. In some embodiments, the amino acid residue difference as compared to SEQ ID NO: 2 at position X193 is X193V.

polypeptides of **Table 2**), but in which the amino acid sequence does not comprise a residue difference as compared to SEQ ID NO: 2 at positions X60, X144, X317, X322, X334, X358, and X370.

[0018] In some embodiments, the engineered polypeptides having pNB esterase activity of the present disclosure having at least 80% identity to SEQ ID NO: 2 and any of the amino acid residue differences disclosed herein, further comprises at least 1.2 fold, 2 fold, 5 fold, 10 fold, 20 fold, 25 fold, 50 fold, 75 fold, 100 fold, or greater increased activity as compared to the polypeptide of SEQ ID NO: 4 in converting compound **(2)** to compound **(1)** under suitable reaction conditions.

[0019] In some embodiments, the engineered polypeptides having pNB esterase activity comprises an amino acid sequence having at least 80%, 85%, 87%, 88%, 89%, 90% 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identity to a sequence selected from SEQ ID NO: 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38,40, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, 64, 66, 68, 70, 72, 74, 76, 78, 80, 82, 84, 86, 88, 90, 92, 94, 96, 98, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, and 120.

[0020] In some embodiments, that disclosure further provides any of the engineered polypeptides having pNB esterase activity as disclosed herein, wherein the polypeptide is immobilized on a solid support. In some embodiments, the solid support is a bead or resin comprising polymethacrylate with epoxide functional groups, polymethacrylate with amino epoxide functional groups, styrene/DVB copolymer or polymethacrylate with octadecyl functional groups

[0021] Exemplary engineered polypeptides having pNB esterase activity and amino acid sequences incorporating the residue differences disclosed herein, including various combinations thereof, and having improved properties (*e.g.,* capable of converting compound **(2)** to compound **(1)** under suitable reaction conditions) are disclosed in **Table 2**, and the Examples. The amino acid sequences are provided in the Sequence Listing and include SEQ ID NO: 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38,40, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, 64, 66, 68, 70, 72, 74, 76, 78, 80, 82, 84, 86, 88, 90, 92, 94, 96, 98, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, and 120.

[0022] ~~In another aspect, t~~The present disclosure also provides polynucleotides encoding the engineered polypeptides having pNB esterase activity, as well as expression vectors comprising the polynucleotides, and host cells capable of expressing the polynucleotides encoding the engineered polypeptides.

In one aspect, there is provided a polynucleotide encoding the engineered pNB esterase polypeptide as defined herein.

In one aspect, there is provided a polynucleotide encoding the engineered pNB esterase polypeptide as defined herein comprising a nucleotide sequence selected from SEQ ID NO: 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 63, 65, 67, 69, 71, 73, 75, 77, 79, 81, 83, 85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109, 111, 113, 115, 117, and 119. Exemplary polynucleotide sequences are provided in the Sequence Listing incorporated

by reference herein and include SEQ ID NO: 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 63, 65, 67, 69, 71, 73, 75, 77, 79, 81, 83, 85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109, 111, 113, 115, 117, and 119.

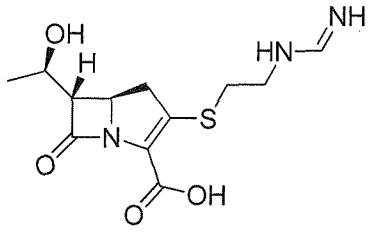
In one aspect, there is provided an expression vector comprising the polynucleotide as defined herein.

In one aspect, there is provided a host cell comprising the polynucleotide as defined herein or the expression vector as defined herein.

[0023] In ~~some embodiments~~one aspect, ~~the present disclosure also provides~~there is provided a methods ~~of manufacturing~~ preparing the engineered polypeptide~~s having pNB esterase activity~~as defined herein, ~~where the method can comprise~~ comprising culturing a

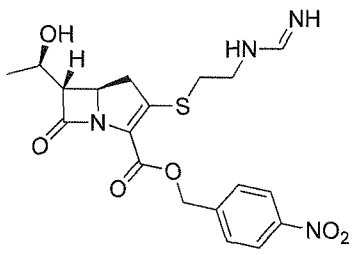
the host cell ~~capable of expressing a polynucleotide encoding the engineered polypeptide~~ as defined herein under conditions suitable for expression of the polypeptide. In some embodiments, the method for manufacturing the engineered pNB esterase polypeptide can also include: (a) synthesizing a polynucleotide encoding a polypeptide comprising an amino acid sequence selected from SEQ ID NO: 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38,40, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, 64, 66, 68, 70, 72, 74, 76, 78, 80, 82, 84, 86, 88, 90, 92, 94, 96, 98, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, and 120, and having one or more residue differences as compared to SEQ ID NO:2 at residue positions X108, X115, X116, X130, X193, X214, X219, X273, X276, X321, and X362 selected from X108L/Y, X115Q/W, X116S, X130T, X164T, X193A/D/E/V, X214G, X219A/D/L/V, X273A/E/T/V, X276A/T/L, X321A, and X362A/D/Q/S/V; and (b) expressing the pNB esterase polypeptide encoded by the polynucleotide. As further provided in the detailed description, additional variations can be incorporated during the synthesis of the polynucleotide to prepare engineered polypeptides with corresponding differences in the expressed amino acid sequences.

[0024] The structural features of the engineered pNB esterase polypeptides allow for the conversion of the pNB-protected substrate of compound **(2)** to their corresponding un-protected product of compound **(1)**, imipenem. Thus, in another aspect, ~~the present disclosure provides~~ there is provided a process for preparing ~~carbapenem antibiotic of~~ compound **(1)**, ~~imipenem,~~ or a salt or hydrate of compound **(1)**,



(1)

wherein the method comprises contacting a substrate compound **(2)**, or a salt of hydrate of compound **(2)**,



(2)

with an engineered pNB esterase polypeptide ~~of the present disclosure~~as defined herein, under suitable reaction conditions. As provided herein, the processes using the engineered pNB esterases can be carried out under a range of suitable reaction conditions, including, among others, pH, temperature, buffer, solvent system, substrate loading, polypeptide loading, cofactor loading, pressure, and reaction time. In some embodiments, the suitable reaction conditions for a biocatalytic process using the engineered pNB esterases of the present disclosure can comprise: (a) substrate loading at about 2 g/L to 200 g/L; (b) about 0.1 to 10 g/L of engineered pNB esterase polypeptide; (c) about 0.05 to 0.5 M MES buffer; (d) about 5% to about 20% (v/v) DMF co-solvent; (e) pH of about 6 to 8; and/or (f) temperature of about 10 to 35°C. Further, guidance on the choice of engineered pNB esterases, preparation of the biocatalysts, and parameters and reaction conditions for carrying out the processes are described in the detailed description that follow.

5. DETAILED DESCRIPTION

[0025] As used in this specification and the appended claims, the singular forms "a", "an" and "the" include plural referents unless the context clearly indicates otherwise. Thus, for example, reference to "a polypeptide" includes more than one polypeptide. Similarly, "comprise," "comprises," "comprising" "include," "includes," and "including" are interchangeable and not intended to be limiting. It is to be further understood that where descriptions of various embodiments use the term "comprising," those skilled in the art would understand that in some instances, the embodiment can be alternatively described using the terms "consisting essentially of" or "consisting of."

[0026] It is to be understood that both the foregoing general description, and the following detailed description are exemplary and explanatory only and are not restrictive of this disclosure. The section headings used herein are for organizational purposes only and not to be construed as limiting the subject matter described.

5.1 Abbreviations

[0027] The abbreviations used for the genetically encoded amino acids are conventional and are as follows:

Amino Acid	Three-Letter Abbreviation	One-Letter Abbreviation
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartate	Asp	D
Cysteine	Cys	C
Glutamate	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	HIS	H