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Reviews

Synthetic strategies in construction of organic low molecular-weight carrier-drug conjugates

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A B S T R A C T

Inefficient transportation of polar metabolic inhibitors through cell membranes of eukaryotic and prokaryotic cells precludes their direct use as drug candidates in chemotherapy. One of the possible solutions to this problem is application of the 'Trojan horse' strategy, i.e. conjugation of an active substance with a molecular carrier of organic or inorganic nature, facilitating membrane penetration. In this work, the synthetic strategies used in ra-tional design and preparation of conjugates of bioactive agents with three types of organic low molecular-weight carriers have been reviewed. These include iron-chelating agents, siderophores and cell-penetrating peptides. Moreover, a less known but very promising "molecular umbrella" conjugation strategy has been presented. Spe-cial attention has been paid on appropriate linking strategies, especially these allowing intracellular drug release after internalisation of a conjugate.

1. Introduction

Many organic compounds, known as effective inhibitors of enzymes of crucial importance for human pathogenic microorganisms or cancer cells, do not exhibit any chemotherapeutic activity. This is often due to the poor diffusion of an active substance through biological membranes. The 'Trojan horse' strategy is based on the idea of the conjugation of membrane-impermeable substance with any molecular carrier. As a result of this conjugation, unfavourable properties of drug molecules become masked in a way that makes them able to cross the cell membrane. In an ideal case, once internalized, the active component is released from the conjugate and reaches the intracellular target. Several types of drug carriers are known. One of them is inorganic nanoparticles, like quantum dots, silver and gold or iron oxide nanoparticles [1–5]. Another group comprises macromolecular organic carriers, carbon nanotubes [6–10] and dendrimers [11–14]. Both are supramolecular structures, able to penetrate biological membranes in a rather cell-unspecific manner (direct translocation or endocytosis), due to the overall shape and physico-chemical character of the macromolecular carrier.

In this review, attention is focused on another group of organic molecular carriers, low molecular-weight compounds, which due to the specific interactions of their particular functional groups with components of biological membranes (lipids or proteins), can effectively act as cell membrane penetrating agents. The most popular members of this group are cell-penetrating peptides (CPPs), composed of 5 – 30 amino acid residues of often cationic character [15–19]. Another members of this group, siderophores, are definitely cell-specific since they can effectively act as molecular carriers delivering cargo exclusively to cells containing membrane-located siderophore uptake systems [20,21]. Less extensively studied than aforementioned systems but conceptually interesting "molecular umbrellas" are rationally designed membrane translocators of potentially universal application [22,23]. Carrier-drug conjugates can be formed either through non-covalent interactions or covalent bonds. In the case of the covalent approach, functionalisation often requires the incorporation of an additional moiety (linker) to the molecule. In some cases, non-specific linkers are used - for example, di-

Abbreviations: Boc, *tert*-butoxycarbonyl group; Cbz, benzyloxycarbonyl group; DIC, *N,N'*-diisopropylcarbodiimide; DIPEA, diisopropylethylamine; DMAP, 4-(*N*-dimethylamino)pyridine; DPPA, diphenylphosphoryl azide; Fmoc, fluorenylmethyloxycarbonyl group; HATU, *N*-[(dimethylamino)-1*H*-1,2,3-triazolo-[4,5-*b*]pyridin-1-ylmethylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide; HBTU, 2-(1*H*-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; IBCF, isobutyl chloroformate; *m*-CPBA, 4-chloroperoxybenzoic acid; NaAsc, sodium ascorbate; NHS, *N*-hydroxysuccinimide; PEG, poly(ethylene glycol); PMB, *para*-methoxybenzyl group; SAR, structure-activity relationship; SPPS, solid-phase synthesis; TBTU, 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium tetrafluoroborate; TBDMS, *tert*-butyldimethylsilyl group; TDBTU, *N,N,N',N'*-tetramethyl-*O*-(benzotriazol-1-yl)-uronium tetrafluoroborate; TBTA, tris[1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine; TFA, trifluoroacetic acid; THF, tetrahydrofuran.

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carboxylic acid esters and amides, ω-amino acids or diamines, alkylated ureas, click chemistry derived triazoles or squaric acid diamides (Fig. 1A). The more popular approach takes advantages of stimuli-responsive linkers, like glutathione-sensitive activated disulfides, pH-labile linkers (3-thiosuccinimide, (acyloxy) alkyl esters, hydrazones), esterase-labile 'trimethyl-lock' linkers or cathepsin-sensitive Val-Cit dipeptide (Fig. 1B).

2. Siderophores – cell-specific carriers of natural origin

Siderophores are low molecular-weight organic molecules, produced by micro-organisms, especially bacteria, able to chelate metal ions, particularly Fe(III). In nature, Fe(III)-chelating siderophores are produced by microbial cells under conditions of low iron content (below 10^{-18} M) [24,25], in order to ensure iron acquisition at the level necessary for optimal growth and metabolism. Until now, more than 500 siderophores of natural origin have been identified. Most of them belong to one of the four groups, depending on the structure of the iron-chelating fragment of a siderophore molecule: (i) catecholates, (ii) hydroxamic acids, (iii) α-hydroxycarboxylic acids, mainly citric acid derivatives and (iv) mixed-type. Less abundant are siderophores containing α-aminocarboxylate, hydroxyphenyloxazolone or α-hydroxyimidazole functionalities (Fig. 2) [26–29]. The readers interested in siderophores' biological functions and mechanisms of siderophore-mediated iron acquisition by micro-organisms may refer to several review articles [30–34,20].

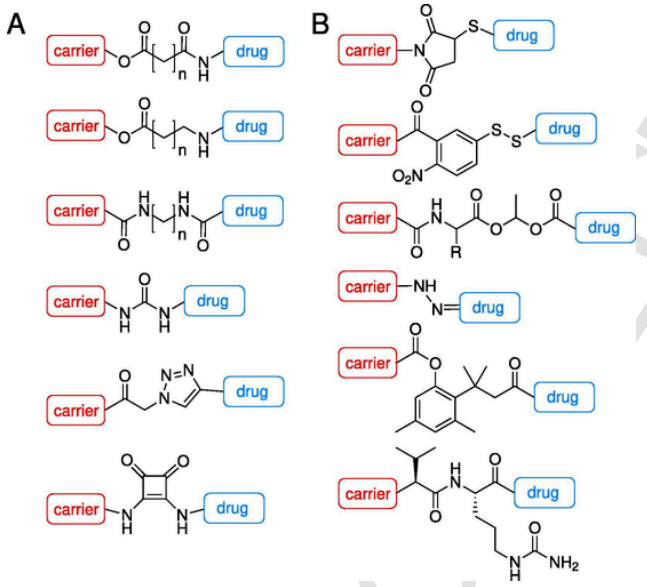

Fig. 1. Examples of linkers used in carrier-drug conjugates: (A) non-specific linkers, from top to bottom: amidoester of a dicarboxylic acid, ω-amino acid ester, diamine, disubstituted urea, triazole, squaric acid diamide; (B) stimuli-responsive linkers, from top to bottom: thiol-maleimide linker, activated disulfide linker, (acyloxy) alkyl ester, hydrazone linker, esterase-labile 'trimethyl lock' linker, valine-citrulline linker.

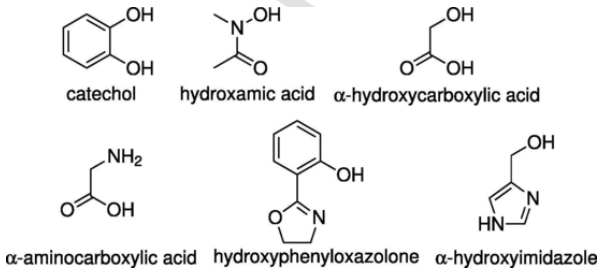

Fig. 2. Iron-chelating motifs present in siderophores of natural origin.

2.1. Siderophore-antimicrobial agents conjugates – sideromycins

Siderophores are molecules of an exceptional potential for very efficient and specific translocation through the microbial cell membrane, due to the presence of siderophore-specific transport proteins. An idea of using siderophores as carriers for antimicrobials in a 'Trojan horse-like' fashion arose from the discovery of several groups of antibiotics called sideromycins. These are composed of structural analogs of bacterial siderophores linked to low molecular weight antimicrobials. Known sideromycins include hydroxamate-containing albomycin [35–37], ferrimycins, and salmycin [38], as well as catecholate-type microcins [39,40]. Following an inspiration coming from the mechanism of action of sideromycins, a number of conjugates composed of an iron-chelating part and an antimicrobial substance have been designed and synthesized. The conjugates are composed of three main components: (i) siderophore-like fragment, effectively chelating Fe(III) ions (a derivative of siderophore of natural origin or its synthetic analogue), (ii) a metabolic inhibitor, and (iii) a linker part, joining (i) with (ii), usually containing a fragment that can be cleaved in microbial cytosol to release and active inhibitor [29]. Following this general scheme, a number of sideromycin-like conjugates containing β-lactam antibiotics, sulfonamides, fluoroquinolones or other antimicrobials have been constructed (Table 1).

Table 1
Siderophore-antimicrobial agent conjugates.

Type of siderophore	Antimicrobial agent	Type of linker	Ref.
Citrate-based	fluoroquinolone	no linker	[41,42]
		5-aminopentanoyl	[43]
		2-aminoethanoyl	[43]
		2-succinoyl diamide	[43]
Hydroxamate	β-lactam	no linker	[44,45]
		succinoyl diamide	[44–46]
		thiol-maleimide ester	[47]
	fluoroquinolone	succinate (amidoester)	[46,48]
		succinoyl diamide	[45,46,48]
		thiol-maleimide ester	[47]
		trimethyl lock	[48,49]
	5-fluorouracil	amino acid ester	[50]
	aminoglycoside antibiotic	succinate (amidoester)	[46]
	macrolide antibiotic	succinoyl diamide	[50]
	triclosan	succinate (amidoester)	[45]
	sulfonamide	nicotinamide	[51]
Catecholate	β-lactam	succinoyl diamide	[44,52]
		no linker	[53] [54]
	oxazolidinone	(acyloxy) alkyl amide	[55]
	fluoroquinolone	(acyloxy) alkyl ester	[56]
		disulfide	[57]
Mixed hydroxamate-catecholate	vancomycin	succinoyl diamide	[58]
	β-lactam	succinoyl diamide	[59]
	fluoroquinolone	trimethyl lock	[49]
		succinoyl diamide	[59]

2.2. Synthesis of siderophores and their analogs

Although siderophores are compounds of natural origin, the iron-chelating components of siderophore-drug conjugates are always products of chemical syntheses. These molecular carriers are either identical with original microbial siderophores, or more often are their analogs, containing the iron-chelating functionalities in a more simplified structural scaffold. Therefore, in this paragraph, the major synthetic methods of siderophore-like molecules preparation are presented, since these are important components of the overall strategy of siderophore carrier-drug congeners synthesis. In the synthesis of siderophore molecules, the main challenge is formation of iron-chelating functionalities. In the case of hydroxamic siderophores, the hydroxamic group usually derive from amines or amino acids, prepared upon *N*-acylation of 1-amino-ω-(*N*-hydroxyamino)-alkanes or ω-(*N*-hydroxyamino)-α-amino acids, respectively (Fig. 3A) [60]. The hydroxamic acids can be obtained by direct oxidation of an aliphatic amino group to *N*-hydroxylamine and subsequent selective acylation of a nucleophilic nitrogen atom. In general, hydroxylamines can be prepared by several different methods, both by amine oxidation or by reduction of nitro or nitroso functionalities [61]. However, in siderophore chemistry, it is important that generation of the hydroxamic group should be performed upon mild conditions because of other labile functionalities present in the siderophore structure. One of the promising oxidative agents for this purpose is dimethyldioxirane (DMD), which oxidises the appropriate amine to the corresponding hydroxylamine in a one-step reaction (Fig. 3B) [60,62,63].

Another amine oxidizing agent used for the formation of *N*-hydroxylamines from amines is benzoyl peroxide, originally applied by Milewska and co-workes for preparation of ω-*N*-hydroxy-L-lysine and γ-*N*-hydroxy-L-ornithine from respective amino acids, as components of citric acid siderophores [64,65]. Some other siderophores have also been synthesized using this approach. Bergeron et al. [66] obtained a mixed-type siderophore nannochelin, based on the structure of citric acid and cinnamoyl hydroxamic acid. The starting material was Boc-protected L-lysine which was selectively oxidized with benzoyl peroxide. Subsequent *N*-acylation and condensation with a citric acid derivative led to the target structure (Fig. 4A) [66]. The usage of benzoyl peroxide as an oxidation agent was also accomplished by Wang et al. [67] who synthesized another naturally occurring siderophore, acinetoferrin which was previously discovered by Okujo et al. [68]. This is the mixed type siderophore, consisting of citric acid and hydroxamate functionalities. Authors have used the Boc-protected diamine as a starting material that was oxidised by benzoyl peroxide. Subsequent acylation with (*E*)-oct-2-enoic acid and condensation with a citric acid derivative resulted in the ultimate formation of acinetoferrin (Fig. 4B) [67].

It seems, that preparation of ω-*N*-hydroxy-diaminoalkanes by reaction of a Boc-protected diamine with benzoyl peroxide is not optimal, since such compounds can be much more efficiently obtained using an appropriate dibromoalkane as a starting substrate and the procedure of Kolasa and Chimiak [69,70]. On the other hand, in the case of ω-*N*-hydroxy-α-amino acids, their preparation by oxidation of easily obtainable α-*N*-protected esters of lysine or ornithine is a method of choice, preferably by the DMD methodology. In the alternative oxidation with benzoyl peroxide, formation of *N*-benzoyl derivatives results in lower yields of desirable *O*-benzoyl products.

In the synthesis of catecholate-type siderophores, a 2,3-dihydroxybenzoic acid derivative is used for *N*-acylation of an amino component (Fig. 6A). In the total synthesis of enterobactin, a starting substrate was *N*-trityl-L-serine, converted into β-lactone. Three molecules of

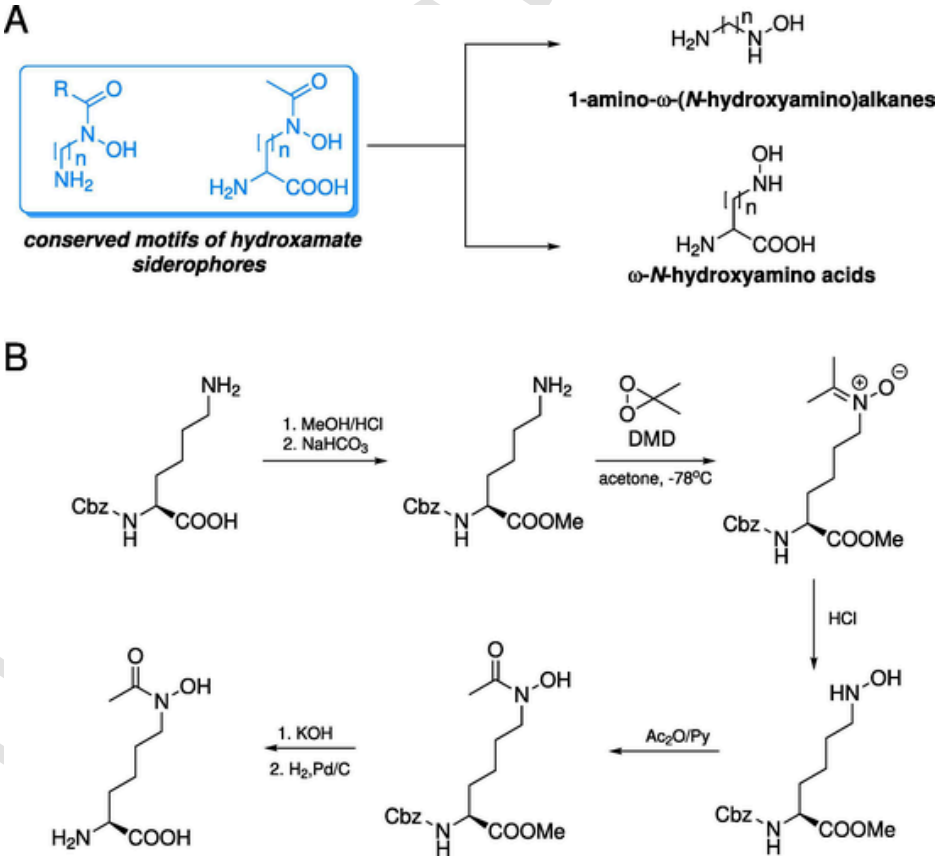

Fig. 3. (A) The common hydroxamate motifs of naturally occurring siderophores; (B) synthesis of hydroxamate functionality by direct oxidation of amine with dimethyldioxirane (DMD).

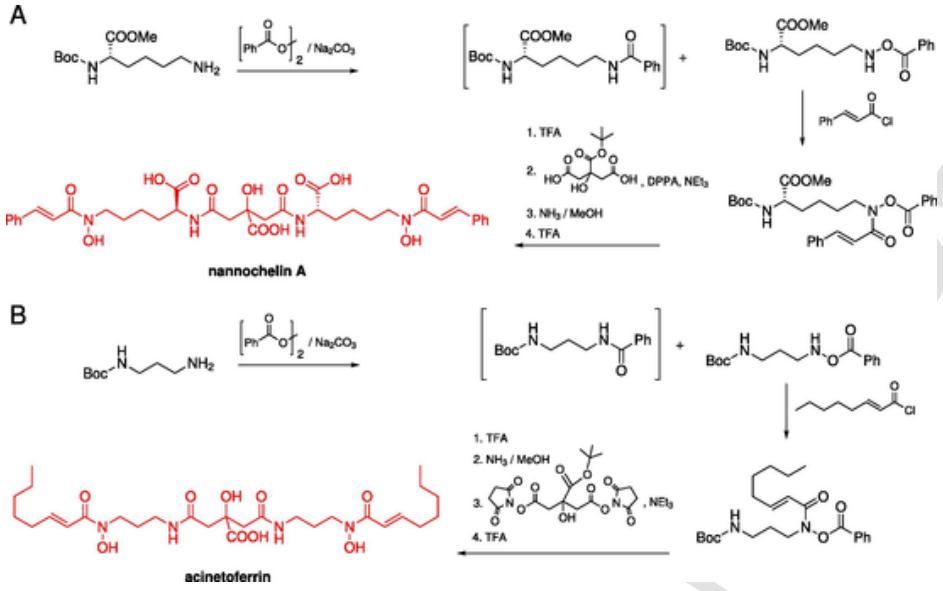

Fig. 4. Simplified schemes of hydroxamate siderophore synthesis with the use of benzoyl peroxide as an oxidising agent: (A) nanochelin A; (B) acinetoferrin.

N-trityl-L-serine β-lactone were condensed in the presence of stannoxane to give the enterobactin skeleton. After removal of the trityl protection, the amino groups were *N*-acylated with 2,3-dihydroxybenzoic acid active ester to the final enterobactin (Fig. 5) [71].

This strategy of preparation of enterobactin and enterobactin-like systems is undoubtedly elegant, straightforward and thus superior to other approaches described in literature [72].

Studies on the enterobactin-mediated Fe(III) ions transport to bacteria revealed that the trilactone skeleton is not necessary and can be substituted by other molecular scaffolds [25]. In an artificial triscatecholate enterobactin analogue [52], the trilactone ring was substituted with three aminotrimethylene groups (Fig. 6B). The tris[3-(*N-tert*-butoxycarbonylamino-propyl]methylamine intermediate, well known in dendrimer chemistry [73], was prepared from nitrotrinitrile by nitrile hydroboration, protection of amines formed and the final reduction of nitro functionality (Fig. 6C) [74]. Simplification of a siderophore structure, not affecting its carrier efficiency, is of primary importance for the construction of carrier-drug conjugates. Such approach has been in the recent years successfully taken in the construction of drug-artificial siderophore conjugates.

2.3. Preparation of siderophore – drug conjugates

Synthesis of siderophore-drug conjugates can be accomplished following one of the general strategies: (i) synthesis of the siderophore part, followed by addition of a metabolic inhibitor – either directly or through a linker, (ii) total synthesis. Siderophores of natural or synthetic origin can be directly linked with drugs by carboxyl or amino functionalities. In this way, conjugates of vancomycin and a siderophore analog containing spermidine-based catechol ligands [58], and mixed catechol and hydroxamate ligands [58], were obtained. Analogously, conjugates of carbacephalosporin with albomycin and agrobactin [44], cefiderocol (catechol-cephalosporin conjugate) [75], and ampicillin and amoxicillin congener with a mixed type siderophore [53] were synthesised (Fig. 7). In the BAL 30072 compound, the sul-

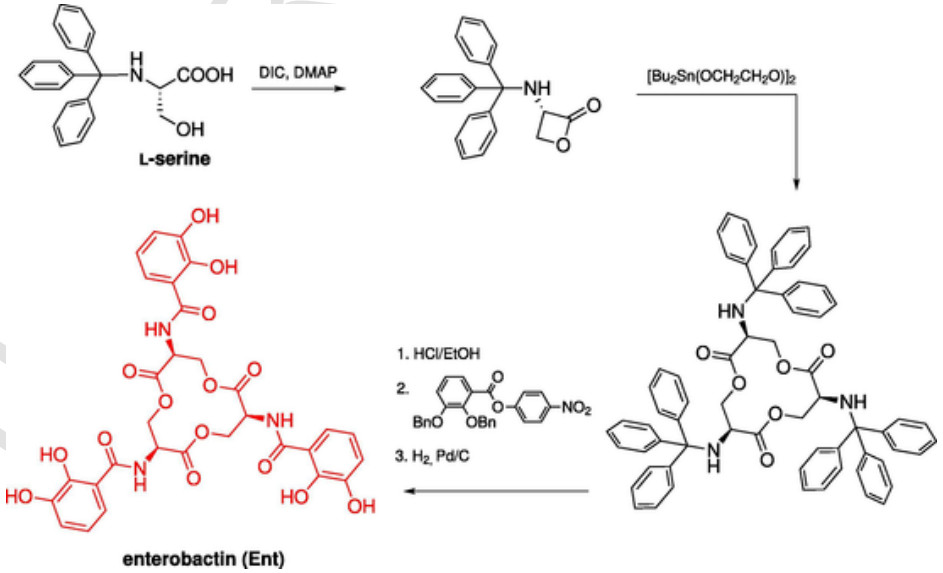

Fig. 5. A schematic representation of the total synthesis of enterobactin using an organotin reagent.

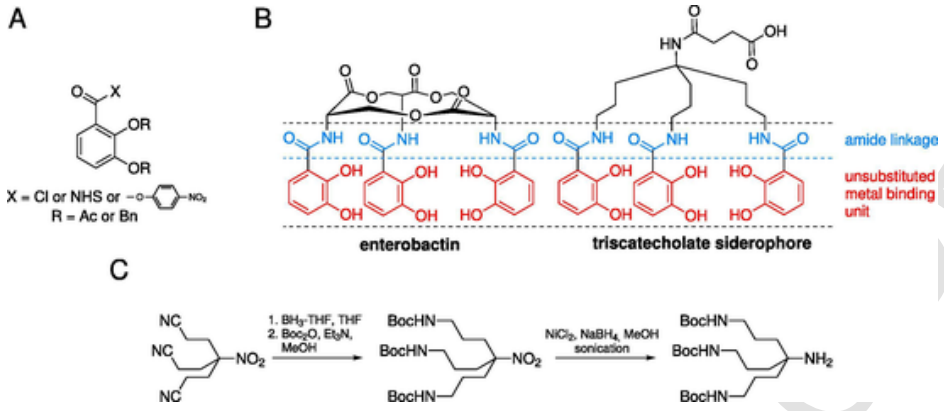

Fig. 6. Structures of (A) derivatives of 2,3-dihydroxybenzoic acid; (B) enterobactin and tris-catecholate siderophore [52]; (C) scheme of synthesis of the tris-catechola.te siderophores.

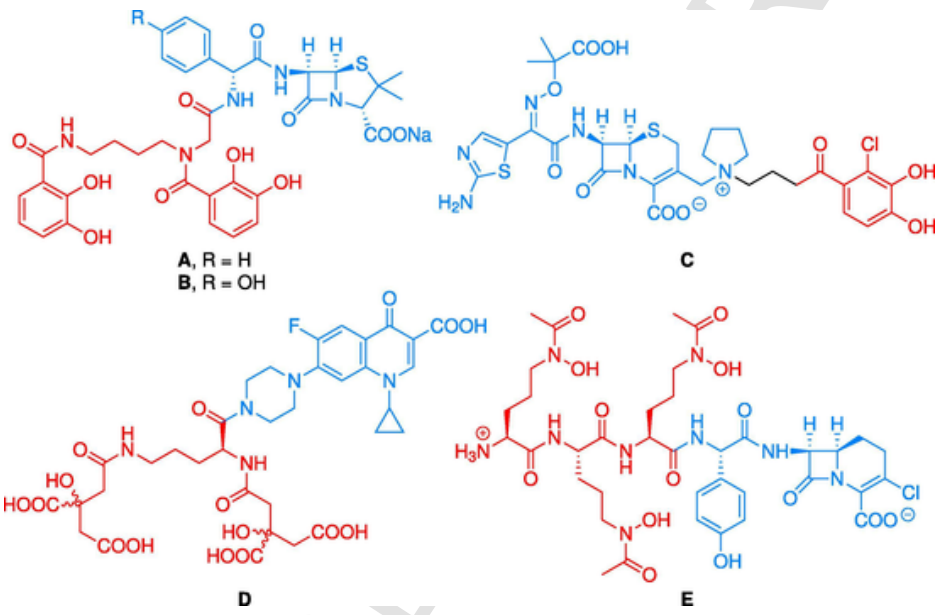

Fig. 7. Examples of direct connection of siderophore and antibacterial agents: (A) catecholate siderophore-ampicilin conjugate; (B) catecholate siderophore-amoxicilin conjugate; (C) catecholate siderophore-cephalosporin conjugate (cefiderocol); (D) staphyloferrin-ciprofloxacin conjugate; (E) hydroxamate siderophore-carbacephalosporin conjugate.

factam molecule is linked with *N*-hydroxy-dihydropyridone iron-chelating group through the = N-O-linkage (Fig. 12A) [76].

The more advanced approach in siderophore-drug conjugation is the application of linkers being independent molecules incorporated between cargo and siderophore structures. The linkers can be of a cleavable or non-cleavable type. In the latter, the most often used precursors are bifunctional molecules, such as diamines, dicarboxylic acids or ω-amino acids. Cleavable linkers usually contain the reducible disulfide bond, (acyloxy) alkyl group or esterase-triggered 'trimethyl lock' component. In Fig. 8, examples of hydroxamate siderophore-antibacterial agent drug conjugates containing different kinds of 'trimethyl lock' linkers are shown [48,49].

An entirely novel approach was presented by Liu et al. [77] who synthesized a dual drug conjugate of siderophore linked to a cephalosporin, with oxazolidinone attached. Cephalosporin component of the conjugate was prepared in four steps from readily available 7-aminocephalosporanic acid. Treatment of cephalosporin carbonate with aminooxazolidinone, followed by reaction with TFA to remove the Boc group and *tert*-butyl ester, afforded cephalosporin-oxazolidinone conjugate. This was subsequently subjected to reaction with bis-catechol active ester (NHS), to give the dual drug-artificial siderophore conjugate (Fig. 9) [77].

A novel, promising approach in construction of entero/-bactin-/-containing siderophore-/-drug conjugates is based on the use of glucosylated enterobactin derivatives as a siderophore carrier. *C*-glucosylated derivatives of enterobactin, known as salmochelins (Fig. 10) are produced by virulent strains of gram negative bacteria, like *E. coli* and *S. enterica.* The biosynthesis and utilisation of salmochelins are important for virulence because these siderophores allow these bacteria to evade the enterobactin-scavenging host-defense protein lipocalin-2 [78]. Therefore, conjugates of salmochelins with antibiotics may demonstrate enhanced selectivity towards pathogenic bacterial strains. Unfortunately, chemical synthesis of such derivatives is multistep and difficult [79].

An access to salmochelin-containing siderophore-drug is facilitated by the use of bacterial enzymes catalyzing enterobactin glucosylation. One of them, IroB, transfers glucosyl groups from uridine-5'-diphosphoglucose (UDP-Glc) to C5 of one, two, or three of the 2,3-dihydroxybenzoyl units of enterobactin to yield monoglucosyl-*C*-Ent (MGE), diglucosyl-*C*-Ent (DGE) and triglucosyl-*C*-Ent. On the other hand, another enzyme, MceC, catalyses only formation of MGE and DGE. Both enzymes were used by Chairatana *et al.* in their chemoenzymatic synthesis of MGE and DGE (known also as salmochelin S4, Fig. 10) conjugates with β-lactam antibiotics [80]. In this synthesis, Ent was functionalized with PEG_3-N_3 prior to the enzymatic transglycosylation, to enable conjuga-

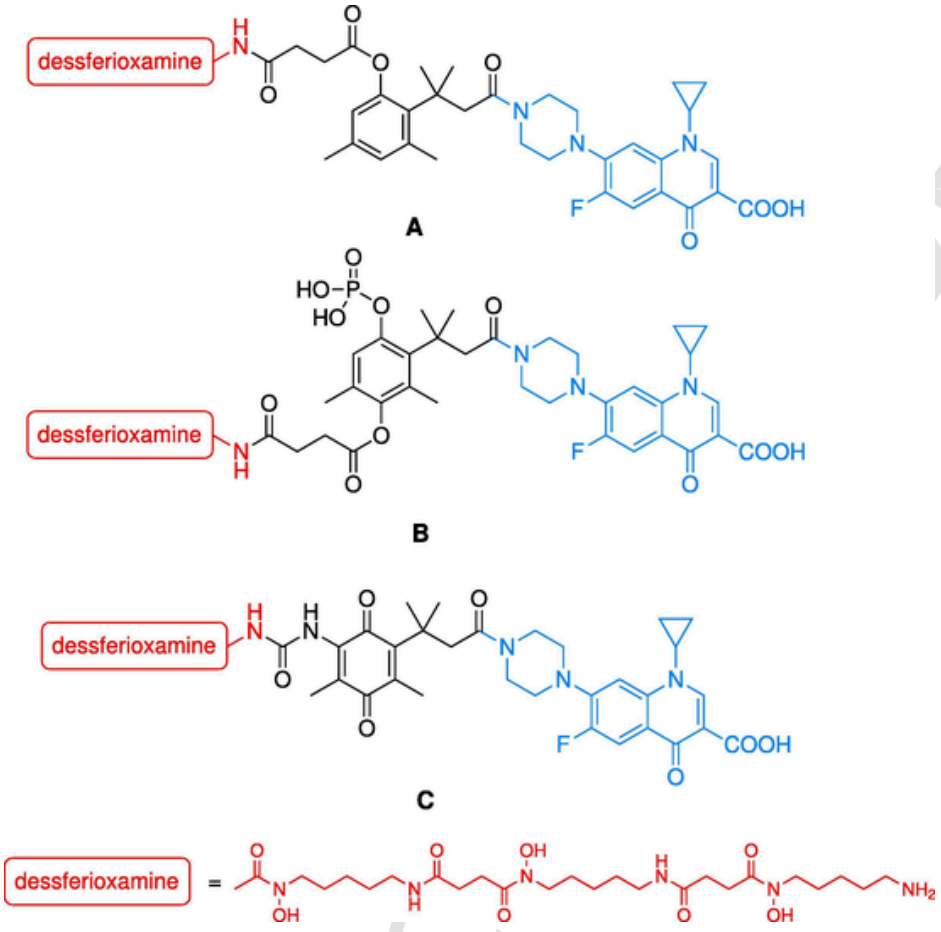

Fig. 8. Desferrioxamine B-ciprofloxacin conjugates containing different types of 'trimethyl lock' linkers: (A) conjugate with an esterase-labile 'trimethyl lock' linker [48]; (B) conjugate with a phosphatase-labile 'trimethyl lock' linker [48]; (C) conjugate with the oxidoreductase-labile 'trimethyl lock' linker [49].

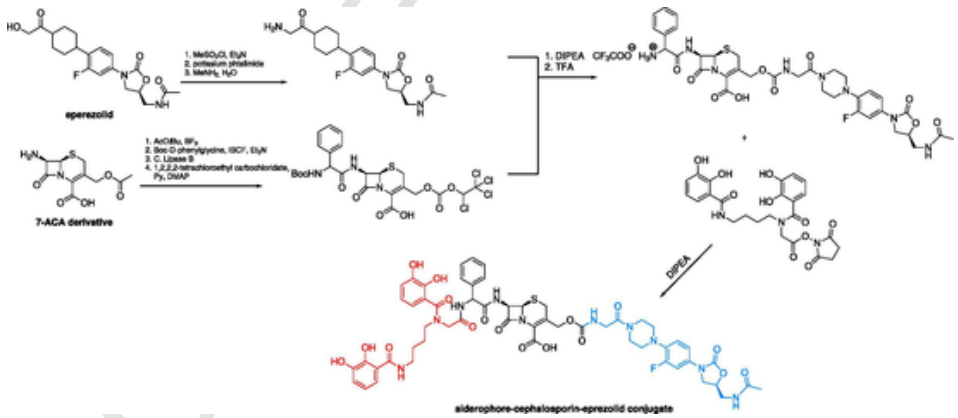

Fig. 9. Scheme of synthesis of conjugate consisting of catecholate siderophore, cephalosporin linker and oxazolidinone antibiotic.

tion of glucosylated Ent with ampicillin or amoxicillin by click chemistry in the last step. (Fig. 11, path A and B) [80]. On the other hand, Lee and co-workers [81] proposed enzymatic transglycosylation of enterobactin with functionalised glucose derivatives derived from UDP-sugar. Presence of bromine, azide or thiol functionality in such derivatives offers a broad range of possibilities of further attachment of a drug molecule. Recently, the chemoenzymatic approach was applied in construction of salmochelin S4-inspired ciprofloxacin conjugate, where not the entire salmochelin S4 moiety but only two C-glucosylated catechol units are present (Fig. 12B) [82].

Construction of glycosylated siderophore-drug conjugates involving chemoenzymatic approach seems a very promising way to novel antibacterials selectively targeting virulent versus non-virulent strains, provided this chemoenzymatic methodology could be effectively scaled up. It is worth mentioning, that Neumann et al. proposed an alternative approach to selective targeting of virulent bacteria by the siderophore-drug conjugate. They prepared the Ent-ciprofloxacin conjugate (Fig. 12A) which is effectively internalized by the virulent and non-virulent *E. coli* cells, however only in the former, the conjugate is activated due to the action of the IroB cytoplasmic esterase [83].

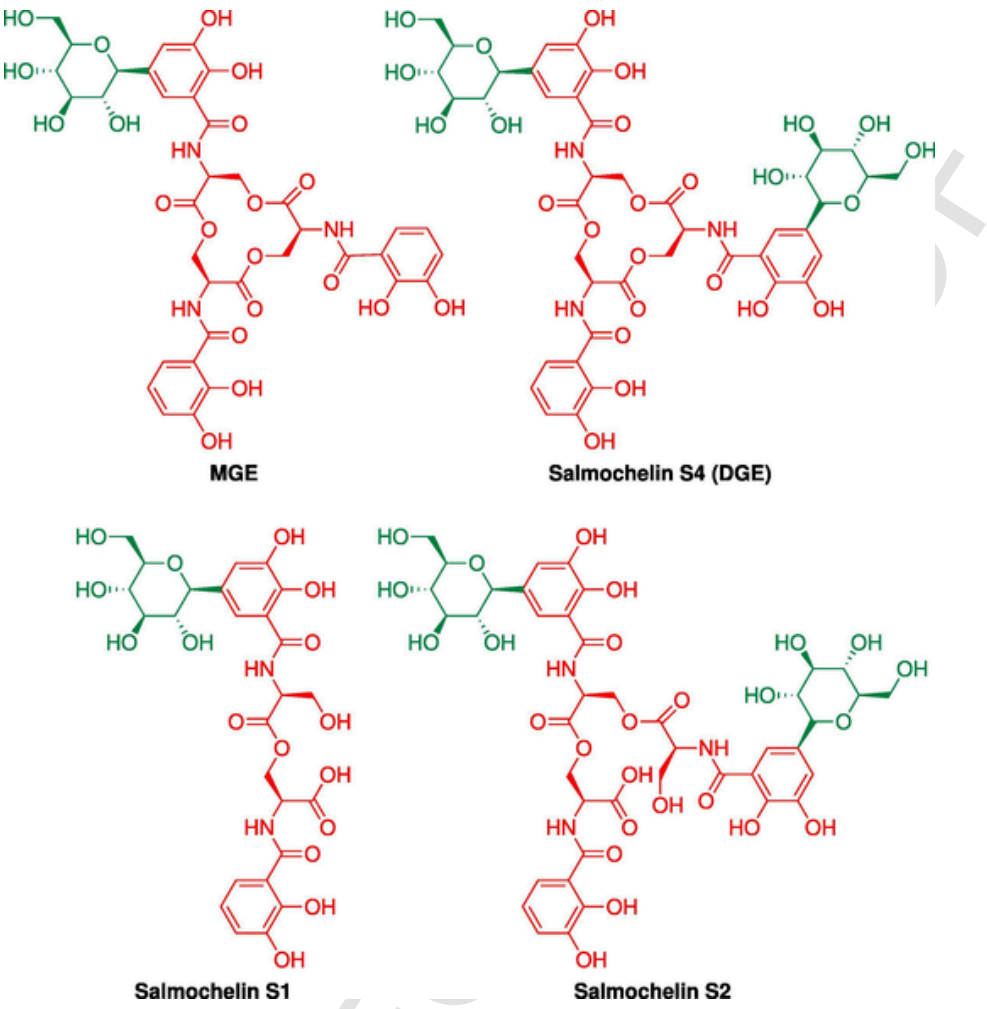

Fig. 10. Chemical structures of glucosylated Enterobactin derivatives.

2.4. Synthesis of drug-artificial siderophore conjugate

A novel, especially promising approach in construction of drug conjugates with iron-chelating compounds is application of synthetic siderophore mimics (artificial siderophores) as carriers. It was found that an appropriately modified catechol moiety or heterocycles containing *N*-hydroxy functionalities can successfully substitute the entire siderophore molecule as a carrier components in conjugates. Linking of unnatural siderophores with antimicrobials through linkers usually calls for the total synthesis. Starting from the simple precursors, like acrylonitrile, acetaldehyde and cyclohexylamine, derivatives of tri(3-aminopropyl)methylamine [52] or tri(3-aminopropyl)methanol [84] can be obtained. Tris[3-(*N*-*tert*-butoxycarbonylamino)propyl]methylamine is treated with methyl succinyl chloride and subsequently, after prior removal of protecting group, with 2,3-diacyloxybenzoyl chloride. Obtained artificial siderophore was coupled *via* mixed anhydride (isobutyl chloroformate) with ampicillin and amoxicillin. After the final deprotection, artificial siderophore-β-lactam antibiotic conjugates were generated (Fig. 10, path A) [85]. In another approach, appropriately protected triaminomethanol was acylated with 2,3-dibenzyloxybenzoyl fluoride and then, after removal of the sillyl protection, *O*-acylated with chloroacyl chloride. The resulting chloroester was treated with ciprofloxacin, thus leading to the ultimate formation of the artificial siderophore-ciprofloxacin conjugate (Fig. 10, path B) [84]. The synthesis of aminochelin- and vanchrobactin-norfloxacin conjugates [86] started with the coupling of 2,3-diisopropyloxybenzoic acid with *tert*-butyl(4-aminobutyl)carbamate or *tert*-butyl N^5-Cbz-D-ornithine, using TBTU as the coupling agent. After deprotection, the free amines were condensed with chloroacetyl chloride and then treated with norfloxacin, giving aminochelin-norfloxacin and vanchrobactin-norfloxacin conjugates (Fig. 10, path C) [86].

Cefiderocol is a conjugate of a synthetic cephalosporin with artificial siderophore, namely 2-chloro-3,4-dihydroxybenzoate. This compound was approved for clinical use as an antibacterial drug in the United States in November 2019, and in the European Union in April 2020. It is sold under the trade name of Fetroja. Synthesis of such congener can be accomplished in two ways. The appropriately protected cephalosporin chloride is either first conjugated with (2-aminothiazolyl)iminoacetic acid derivative and this intermediate is subsequently treated with a tertiary amine catechol derivative (Fig. 11) or formation of cephalosporin-catechol conjugate is followed by reaction with (2-aminothiazolyl)iminoacetic acid derivative. Substrates for conjugation are prepared as follows: 2-chloro-3,4-dihydroxybenzaldehyde is oxidized under Pinnick conditions to the respective acid and subsequently conjugated with diamine, while (2-aminothiazolyl) iminoacetic acid is prepared from respective 2-oxoacetic acid and *O*-alkylhydroxylamine. In sequence presented in Fig. 11, product of cephalosporin chloride conjugation with 2-aminothiazolyl) iminoacetic acid derivative is oxidized with *m*-CPBA to give the intermediate sulfoxide and then chloride is exchanged for iodide. Reaction between the resulting iodide and the tertiary amine catechol derivative affords the intermediate conjugate

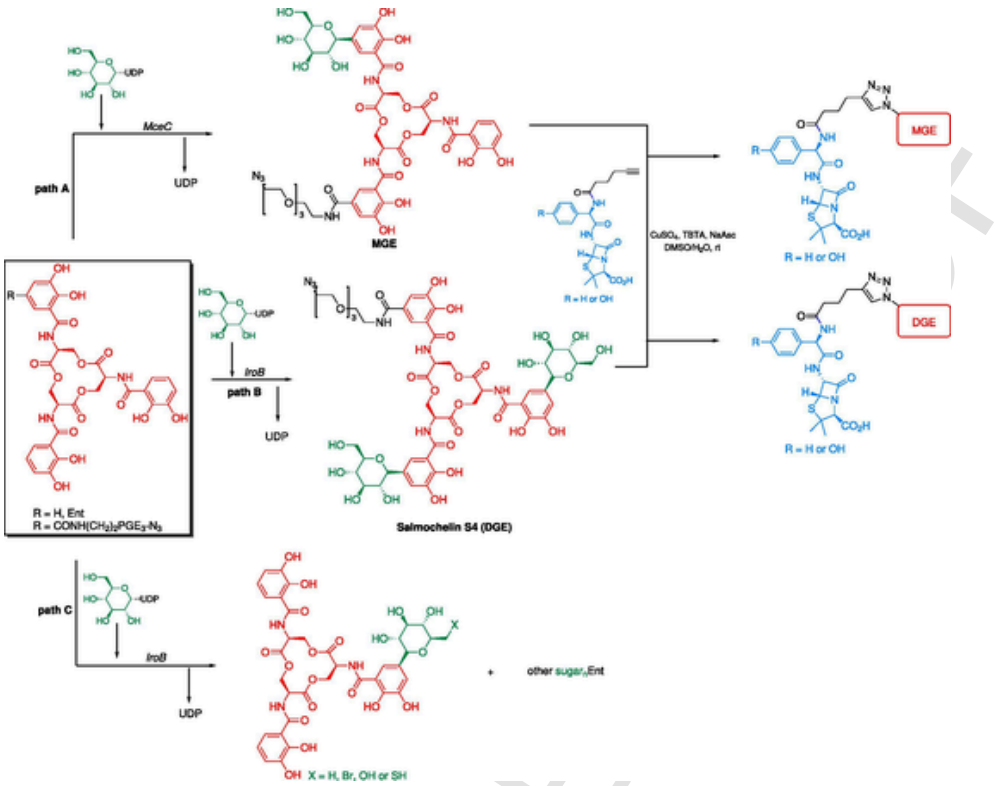

Fig. 11. Chemoenzymatic syntheses of GlcEnt and its conjugates with β-lactam antibiotics. The synthetic routes consist of MceC- or IroB-catalysed glycosylation of Ent-PGE_3-N_3 (path A and B) or enterobactin (path C).

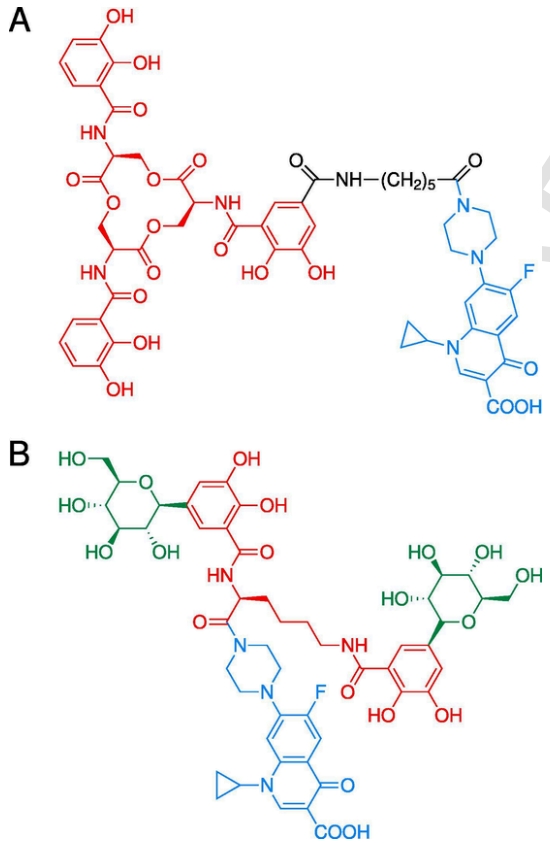

Fig. 12. Chemical structures of the enterobactin-ciprofloxacin conjugate (A) and salmochelin S4-inspired Trojan Horse antimicrobial agent (B).

containing the quaternary amine. Reduction of the 1-sulfoxide gives the quaternary ammonium salt, which is treated with Lewis acid such as aluminium trichloride in the presence of anisole to produce the final cephalosporin-siderophore conjugate (Fig. 11) [87,88].

The conjugate known as BAL 30072, containing *N*-hydroxydihydropyridone (Fig. 12A), developed by Basilea Pharmaceutica, has ever been investigated in phase I clinical studies, however was abandoned for severe hepatotoxicity [89]. Nevertheless, its perfect activity against*Pseudomonas aeruginosa* and *Acinetobacter baumanni* is worth mentioning [90,91]. A series of SAR modifications of BAL 30072 were proposed, especially at C4 in β-lactam ring (Fig. 12B), introducing spiro-, heterocyclic or alkoxy substituents [76].

The starting substrates were amino acids of natural or synthetic origin, appropriately protected at amino or carboxyl functionalities. In the A synthetic path, the amino acid methyl ester was oxidized with SeO_2/*t*BuOOH and subsequently converted into *N*-acetylated hydroxamic acid. The Mitsunobu reaction was performed to transform this intermediate into corresponding α-amino-β-lactam (Fig. 13, path A). On the other hand, starting from benzyl *N*-*tert*-butoxycarbonyl glycinate, β-lactams with a spiro substituent at C3 were obtained. The aldol reaction was carried out between various cyclic ketones and a glycine derivative to get a substituted serine derivative. The hydrogenolytic removal of the benzyl group yielded intermediate acids, which were coupled with $BnONH_2$ to give *O*-benzyl hydroxamic acids. The Mitsunobu reaction was carried out to transform these derivatives of hydroxamic acids to the corresponding *rac*-α-amino-β-lactams (Fig. 13, path B).

An optically active α-amino-β-lactam containing the cyclopentyl substituent was prepared from the *N*-Boc-L-serine methyl ester, to which two allyl moieties were attached. Ring-closure metathesis reaction of the diallyl derivative was performed using the 2nd generation Grubbs catalyst to afford cyclopentenylserine. Reduction of the double bond in the five-membered ring compounds with Wilkinson's catalyst gave a saturated product. This intermediate was converted into *O*-ben-

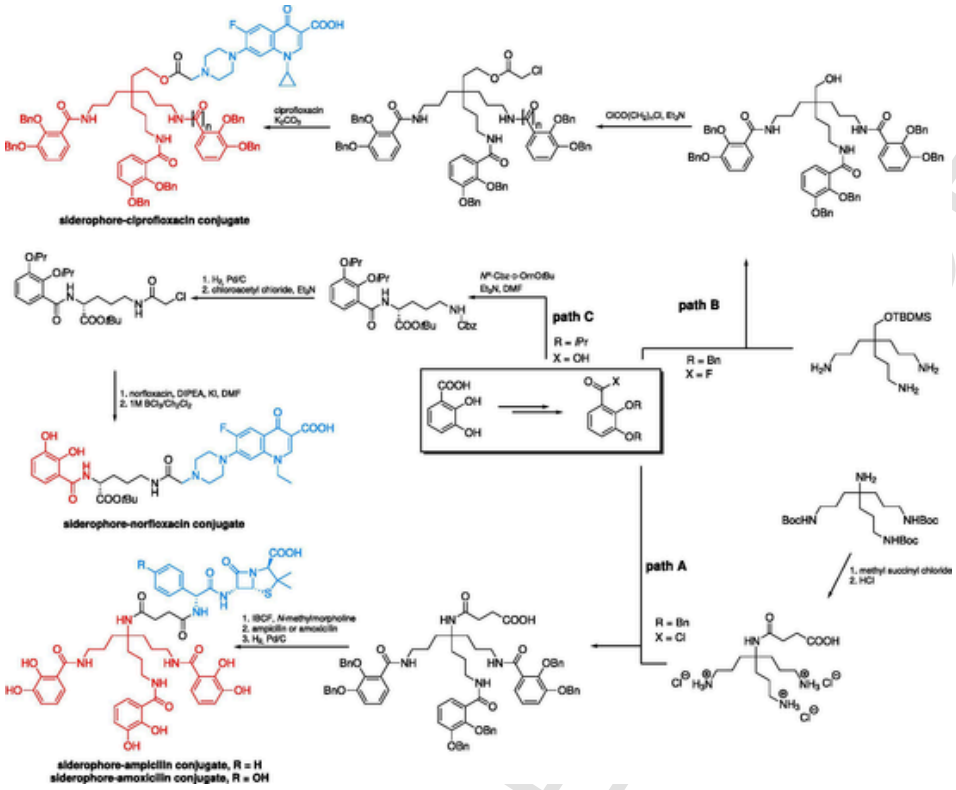

Fig. 13. Scheme of synthesis of artificial siderophores conjugates with antibiotics.

zyl hydroxamic acid, and subsequently under Mitsunobu conditions in α-amino-β-lactam (Fig. 13, path C). Sulfonation of α-amino-β-lactams with an excess of SO_3 x DMF or pyridine complex gave tetra-*tert*-butylammonium salts, followed by deprotection of the Boc group under acid conditions, to give the corresponding α-amino-β-lactam. A condensation reaction was carried out between α-iminocarboxylic acid and α-amino-β-lactam to afford intermediate compound, which was converted into final compounds by removal of the protecting groups (Fig. 14) [90].

3. Cell-penetrating peptides – universal carriers

Structurally the most known CPPs are short (up to 30 amino acid residues), linear peptides made up of L-amino acids [15]. In terms of origin about half of existing CPPs are of the natural origin and the other half can be described either as chimeric (combining both natural and artificial features) or de novo designed structures [15]. Known CPPs can be classified by amino acid composition and physicochemical properties into three major groups: (i) cationic, (ii) amphipathic and

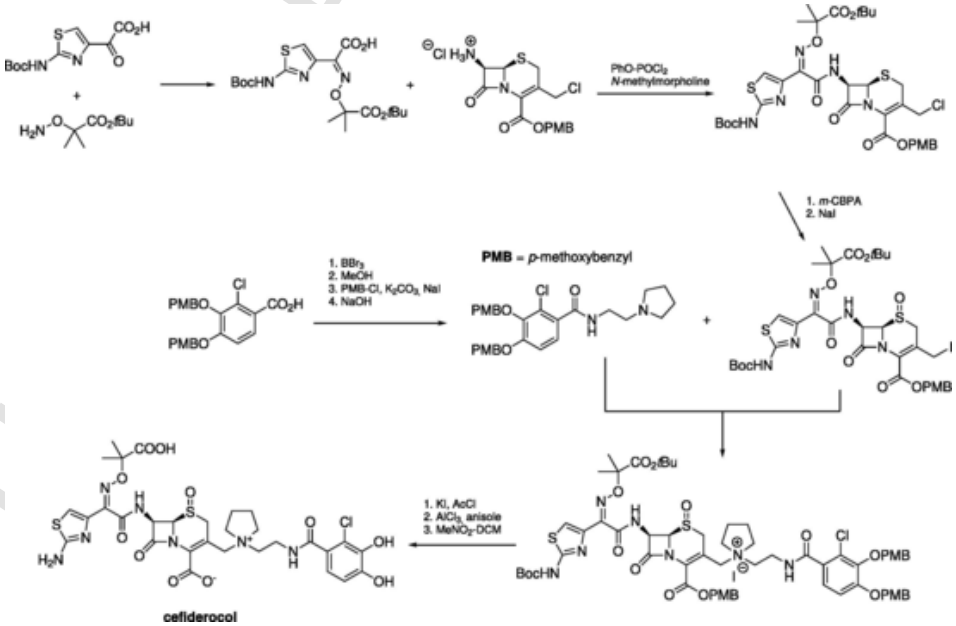

Fig. 14. Synthesis of cefiderocol.

(iii) hydrophobic. Cationic peptides are in most cases derived or inspired from Tat-peptide. Those CPPs are mostly composed of arginine, lysine, and histidine. Due to this composition, they exhibit salt-like behaviour which is crucial in biological delivery system development. Amphipathic peptides (often chimeric or synthetic in origin) have lipophilic and hydrophilic tails that are responsible for their cell-penetrating properties. This 'two-in-one' property unlocks many ways in drug delivery synthetic strategies [16]. Moreover, KLA (lysine, leucine, alanine) sequence is often present [92]. Class of hydrophobic CPPs has the fewest number of representatives. They contain either only nonpolar amino acids or incorporate only a few charged amino acids [16]. Notable examples of known cell-penetrating peptides are summarized in Table 2.

It is worth to mention that CPPs affect the plasma membrane at very low concentrations, without causing any significant damage to it [101]. When compared to other cytoplasmic delivery systems CPPs display low toxicity [102]. Mechanism of CPPs translocation through a membrane is still debated. Although, it is generally accepted that it can be described as either direct membrane translocation without energy input and endocytosis [103].

3.1. Functionalization of CPPs – designing CPP and cargo conjugation

Generally, cell-penetrating peptides are synthesized using solid-phase peptide synthesis. The whole peptide sequence can be achieved in this step-wise manner and is usually performed from*C*- to *N*-terminus utilizing Fmoc or Boc strategy. After obtaining the desired peptide sequence, the product can be either cleaved and used in further syntheses or modified on-resin. Cell-penetrating peptide-cargo attachment can be performed by utilizing non-covalent (usually electrostatic) interactions or by direct covalent bonding often utilizing some kind of linker moiety.

3.2. Non-covalent approach

In many cases, non-covalent complex formation is the simplest and most straightforward way of utilizing CPPs as nanocarrier systems. This approach is commonly applied for macromolecular cargos with charged moieties like oligopeptides or oligonucleotides. The simple salt complex of fosmidomycin and octaarginine (Fig. 15A) prepared by Sparr and co-workers [104] is one of the simplest examples of the non-covalent approach. This seemingly trivial approach has dramatically increased

Table 2
Examples of known CPPs classified by their chemical structure.

Class	Name of CPP	Amino acid sequence	Ref.
Cationic	oligoarginines	R_n, n = 8–12	[93]
	penetratin	RQIKIWFQNRRMKWKK	[94]
	HIV Tat	RKKRRQRRR	[95]
	FHV coat	RRRRNRTRRNRRRVR	[93]
Amphipathic	MAP	KLALKLALKALKAALKLA	[92]
	CADY	Ac-GLWRALWRLLRSLWRLLWRAC-NH_2	[96]
	Pep-1	KETWWETWWTEWSQPKKKRKV	[97]
	transportan	AGYLLGKINLKALAALAKKIL	[98]
Hydrophobic	integrin β3 fragment	VTVLAGALAGVGVG	[99]
	Kaposis sarcoma FGF	AAVALLPAVLLALLAP	[100]
	fusion sequence HIV-1 gp41	GALFLGFLGAAGSTMGA	[97]

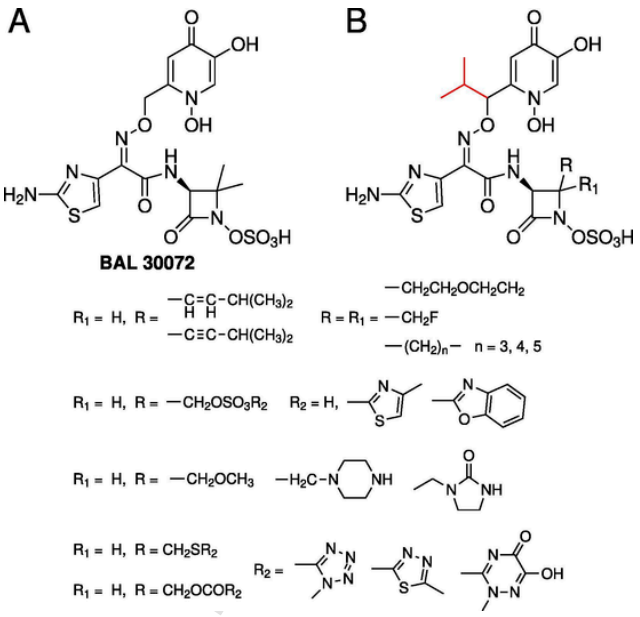

Fig. 15. Structure of BAL 30072 and its modifications.

cargo's permeability through several pathogens' membranes leading to increased inhibitory activity of fosmidomycin against those cells. In recent literature, there have been several cases of non-covalent CPP-based insulin delivery systems. Kamei and co-workers [105] presented their study with simple mixed solutions of known CPPs and insulin. The resulting complex provided increased insulin uptake by brain cell-lines, suggesting that this strategy might be an efficient solution to overcome issues with the blood-brain barrier in drug delivery. Fukuoka et al. [106] presented pH-dependent insulin controlled release system based on hydrogels loaded with insulin and hexaarginine. Studies with those complexes and rat ileal fragments showed that such complexes could be further developed into promising strategy for the oral delivery of insulin. Non-covalent complexation using hydrophobic interactions has also been used. Niu et al. [107] prepared a nanocomplex between insulin and hydrophobically-modified octaarginine enveloped by protecting PEG-PGA polymer (Fig. 15B). In their work, octaarginine has been covalently coupled with stearic acid to enable hydrophobic interactions in the system. In such complex, insulin molecules were efficiently protected from degradation in simulated intestinal fluids and proved to be able to diffuse through intestinal mucus. Hayashi et al. [108] developed polyhistidine-modified liposomes that turned out to be able to deliver fluorescent cargo into human fibrosarcoma cells *via* endocytosis (Fig. 15C).

CPP-based liposomes in which there is a covalent bonding between peptide and nanoparticle-forming elements have been reported [109–111]. Deshpande et al. [110] prepared doxorubicin-loaded liposomes covalently surface-modified with octaarginine. Authors have reported good tumor-cell penetrating level of the complex and high doxorubicin concentration in target cells.

Veiman et al. [111] have formulated PEG-CPP based nanoparticles for delivery of plasmid DNA. Strategy of selective pDNA delivery to tumor cells have been proven to be effective.

3.3. Covalent approach

In many reported studies CPPs are covalently bonded directly to cargo or connected covalently through some kind of linker. Fig. 16 presents schematically the most common ways of covalent CPP-cargo conjugations.

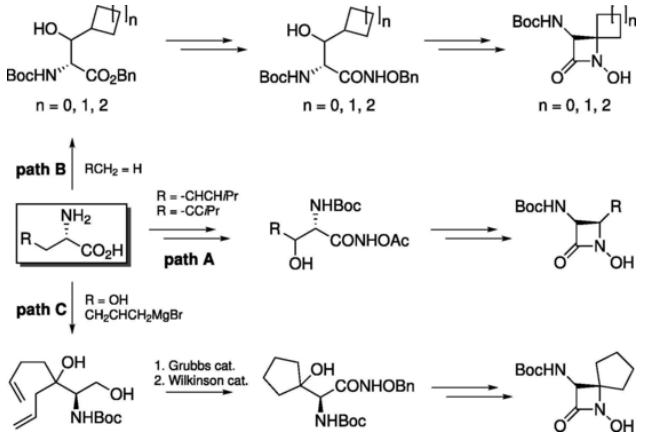

Fig. 16. General route of synthesis α-amino-β-lactams, intermediates in obtaining of drug-artificial siderophore conjugates.

Cargo molecules of peptidic nature can be attached directly to a CPP through a peptide or amide bond [112–121]. This often enables to synthesize the whole molecule using solid-phase peptide synthesis (SPPS) if the cargo molecule has a free carboxylic function available. Soudah et al. [112] synthesized peptide nucleic acid (PNA)-CPP conjugate using that advantage. This conjugate managed to target and modulate Mnk2 gene - a therapeutic target in cancer. Astriab-Fisher et al. [115] synthesized conjugates of antisense nucleotides with several CPPs. Those compounds effectively entered targeted cells and thus internalized nucleotides were detected in nuclei. Samuel et al. [114] presented a series of polyarginine-triclosan conjugates accompanying various linkers to achieve amide-type conjugation to a cell-penetrating peptide. Triclosan moiety was bound to conjugates through labile phenolic ester bond. Conjugates were proved to be able to enter *Toxoplasmosa Gondi* tachyzoites and inhibit specific enzymes (Fig. 17). Purkayashta et al. [119] used this strategy to synthesize simple β-polyarginine-fluoroquinolone conjugates (Fig. 17). Limited anti-bacterial activity of conjugates was attributed to lack of cleavage that would lead to "free" enzymatic inhibitor. Natamycin-tat proteins using simple amide bonds have been prepared by Jain et al. leading to significantly more potent antifungal agent than the pure natamycin (Fig. 17) [116]. Sparr and co-workers prepared covalent octaarginine-fosmidomycin conjugate using butanoic acid-based linker (Fig. 17), but received worse results when comparing to noncovalent analogue discussed in previous subsection [104].

Another widely used strategy in CPP-cargo conjugations is disulfide bridge formation. However, this approach requires the presence of a thiol group in a CPP molecule. This complication is easily avoided by the addition of cysteine linker in the structure of CPP [122–127]. Jones et al. [123] synthesized octaarginine-luciferin cleavable conjugate, using carbonate-modified luciferin derivative and introduced thiol group to it using 2,2'-dithiopyridin allowing easy disulfide bridge-type conjugation with cysteine-modified octaarginine (Fig. 18A). This conjugate proved to be able to both penetrate prostate cancer cells and be cleaved under intracellular conditions. A thiol group was similarly introduced to gemcitabine by Vale and co-workers in their penetratin-based conjugate (Fig. 18B) [124]. In this case, an additional ω-thioacyl linker was used to introduce the -SH functionality. This conjugate presented remarkable increase of anti-proliferative activity against human cancer-cell lines [128,125,127,129,109,130,56,131]. Kim et al. [129] synthesized conjugates of resveratrol (RSV) with leucine lysine-rich (LK) and Tat CPPs. In their approach, they modified RSV molecule by acylation of phenolic oxygen with 4-maleimidobutyric acid which allowed them to conjugate it to a peptide through a cysteine residual chain. (Fig. 18C). The obtained conjugate turned out to be able to penetrate into the nasal epihelium in the mouse model and efficiently inhibited development of specific nasal diseases in low doses.

An interesting case of pH labile CPP-drug conjugate has been shown by Rothbard et al. [132]. Heptaarginine-cyclosporin A obtained by them undergoes intramolecular amide (lactam) formation which releases neat cyclosporin molecules (Fig. 19A). This conjugate was efficiently transported into mice and human skin cells while simultanously inhibiting cutaneous inflammation. Cleavable thiazolidine and imine linkages in CPP-drug conjugates have been reported by Maity et al. [133]. Vale and co-workers [134] have synthesized amphipathic CPP-rasagiline conjugate utilizing copper(I) catalyzed 1,3-dipolar cycloaddition of azide and alkyne. Azide functionality was introduced to a peptide by SPPS by conjugating *N*-terminus with FmocLys(N_3) (Fig. 19B). This example presents an alternative way of designing CPP-cargo conjugates. For the "cost" of synthesizing (usually less desired) non-cleavable connections it enables the use of "click chemistry", which is often very convenient for synthesis.

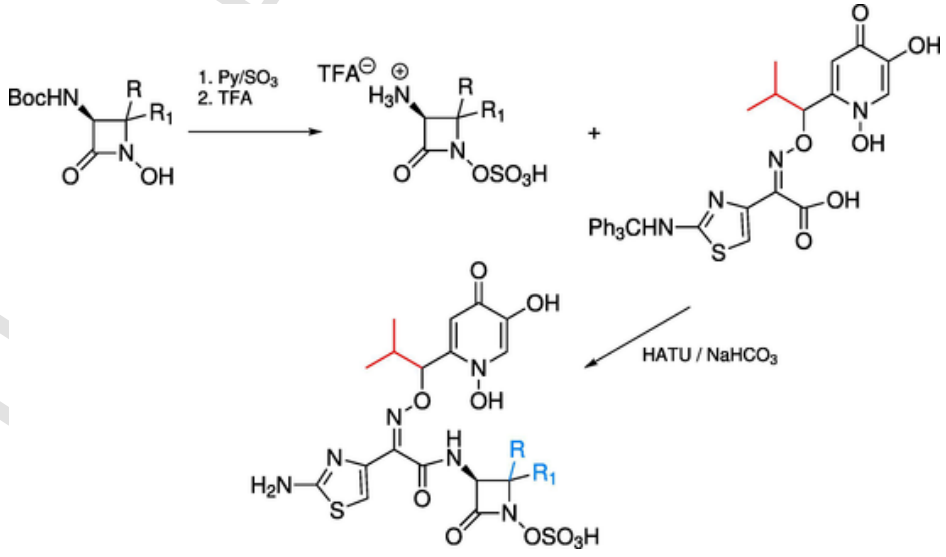

Fig. 17. Synthesis of BAL30072 derivatives on C4 carbon atom.

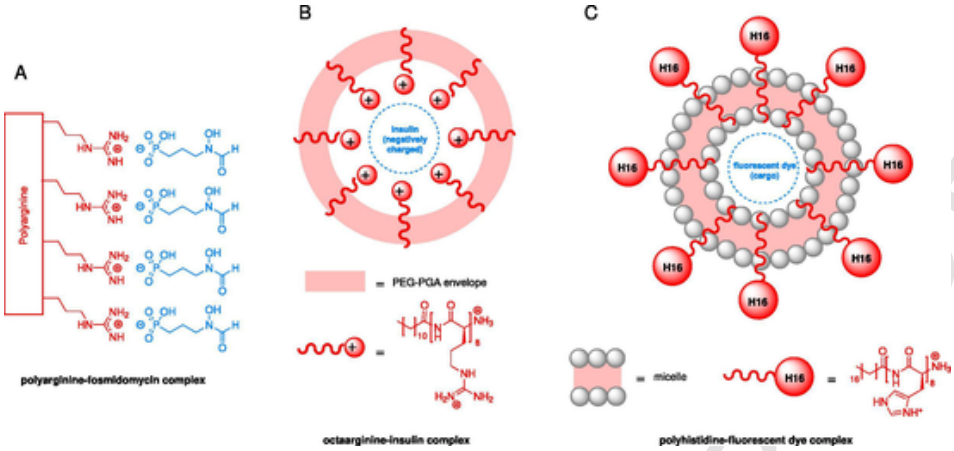

Fig. 18. Examples of arginine- and histidine-based CPP-cargo non-covalent complexes: (A) polyarginine-fosmidomycin complex; (B) octaarginine-insulin complex; (C) polyhistidine-fluorescent agent complex.

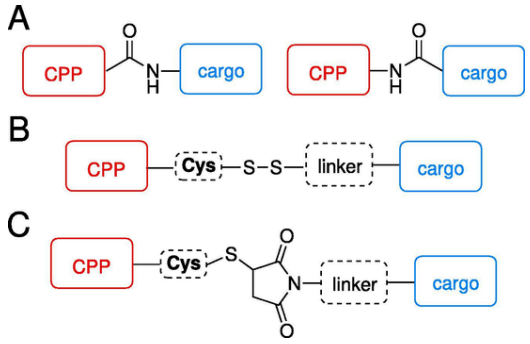

Fig. 19. Most common ways of achieving covalent CPP-cargo conjugations: (A) amide linkage; (B) disulfide linkage; (C) maleimide linkage.

4. Molecular umbrellas – promising novel carriers?

In 1996, Janout et al. [135] proposed a new class of potential molecular carriers, so-called molecular umbrellas. Most commonly, mentioned structures consist of steroids residues, particularly cholic and deoxycholic acids, connected together *via* the polyamine (spermidine) chain (Fig. 20A). Due to the presence of amphiphilic bile acid moieties, the whole structure of molecular umbrella is also amphiphilic and capable of specific conformational changes related to the polarity of the surrounding environment. Moreover, the conformational changes lead to shielding of polar functionalities of bile acid moieties. This property was applied in the construction of molecular umbrella-polar drug conjugates. Molecular umbrellas are able to mask the polar character of cargo molecules and allow for its translocation across lipid bilayers (Fig. 20B) [135–137,22].

In contrast to the most widely described low molecular-weight carriers i.e. siderophores and cell-penetrating peptides, as well as macromolecular structures of carbon nanotubes, fullerenes, dendrimers, quantum dots, silver, gold and iron oxide nanoparticles. The relatively "young" molecular umbrellas are rarely mentioned in the literature. This could be a result from a low number of research groups undertaking this subject. Nevertheless, the recent paper of Palermo's research group [138] takes up the subject of cationic molecular umbrellas. Simplified synthesis of those compounds are presented on Fig. 24. In con-

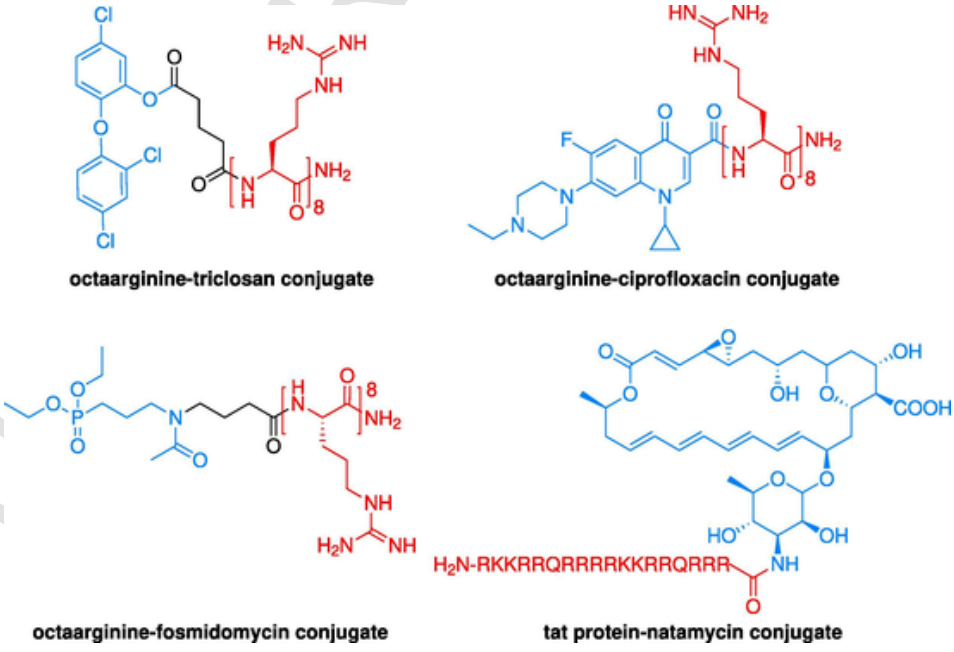

Fig. 20. Examples of octaarginine and Tat conjugates with antimicrobial agents.

trast to structures proposed by Regen and co-workers [135], Palermo's dendron-like constructs do not consist of steroids residues but are based on 2,2-bis(hydroxymethyl) propinic acid residues.

4.1. Synthesis of molecular umbrellas-cargo conjugates

The synthetic pathway leading to particular molecular umbrella conjugates is highly related with the overall structure of molecular umbrella itself and type of the linker mediated between cargo and nanocarrier. To date, several types of connection have been reported. These include mainly linkers based on disulfide bonds a nd ω-aminoacyl structure.

4.1.1. Disulfide-based linkers

The very first a pplication o f m olecular u mbrellas i n t he transportation of cargo molecule across the lipid bilayer was accomplished with nanocarriers loaded with glutathione. Janout et al. [139] attached glutathione molecule to the diwalled molecular umbrella *via* a cleavable 5-disulfanyl-2-nitrobenzoyl linker, undergoing reduction reaction with release of cargo molecule as a free thiol [139]. In this case, the synthetic strategy depended on the formation of molecular umbrella structure, the subsequent generation of the linker and eventually conjugation with cargo molecule. Nanocarrier was formed on the way of amide bond formation between two molecules of cholic acid and one molecule of spermidine. The reaction was accomplished using NHS-DCC method of amide bond generation. Subsequent condensation of molecular umbrella *via* secondary amine group with active ester of 5,5'-dithiobis(2-nitrobenzoic acid), containing disulfide bond, led to the symmetric disulfide of the molecular umbrella. The final thiol exchange reaction between obtained disulfide and thiol functionality of cargo (glutathione) molecule resulted in final conjugate (Fig. 21) [139,140].

The disulfide linker based on 5-disulfanyl-2-nitrobenzoyl structure was also used by Janout et al. [141] for the formation of labile connection of molecular umbrellas with nucleotides and oligonucleotides. Using the same methodology as described for glutathione derivatives, ATP and oligonucleotide conjugates have been obtained. Noteworthy is the fact that oligonucleotide and ATP molecules were enriched with thiol functionalities before conjugation process (Fig. 22) [141].

Another disulfide-based labile linker used in molecular umbrella conjugation, described by Janout et al. [142,143] is based on 3-disulfanylpropionic acid structure. Acylation of the secondary amine group of the molecular umbrella with TDBTU-derived active ester of 3-(pyridine-2-yldisulfanyl) propanoic acid results in asymmetric disulfide, which undergoes subsequent reaction of thiol exchange with cargo molecule. This synthetic approach was successfully applied in the generation of octawalled molecular umbrella conjugates with thiolated siRNA

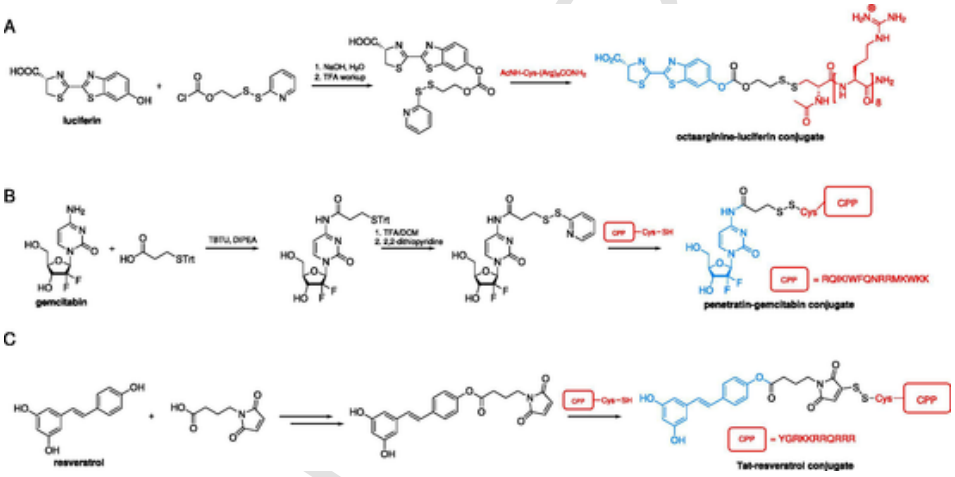

Fig. 21. Simplified representation of syntheses of CPPs conjugates with bioactive molecules attached *via* disulfide bond: (A) synthesis of octaarginine-luciferin conjugate; (B) synthesis of penetratin-gemcitabine conjugate; (C) synthesis of tat-resveratrol conjugate.

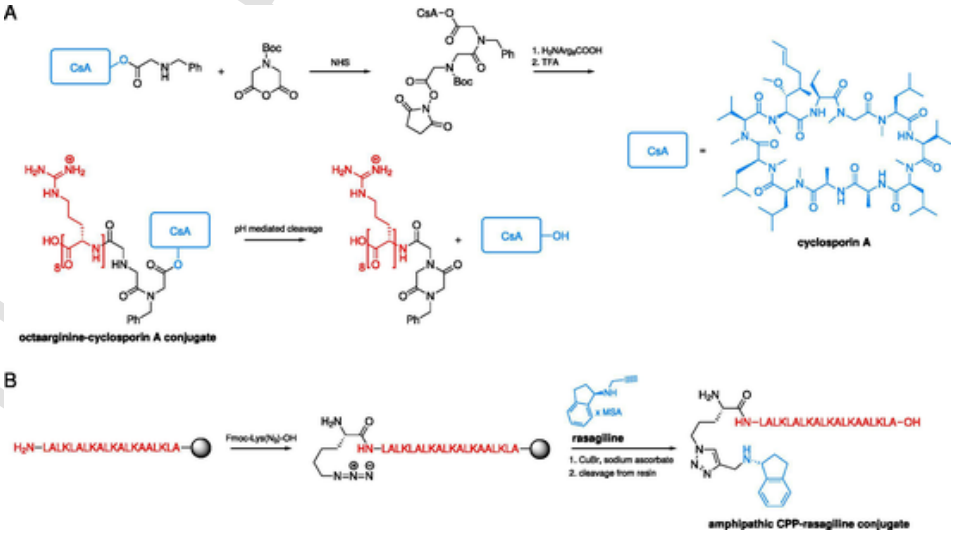

Fig. 22. Simplified representation of the synthesis of: (A) octaarginine-cyclosporin A conjugate; (B) amphipathic CPP-rasagiline conjugate.

cargo (Fig. 23) [142] and amphotericin B (Fig. 24) [143]. The octawalled structure of nanocarrier was obtained from cholic acid, spermidine and lysine. The lysine ramifies the umbrella scaffold allowing to attach eight steroids moieties (Fig. 23) [142–144].

Formation of disulfide 5 -disulfanyl-2-nitrobezoyl a nd 3-disulfanylpropanoyl linkers described so far calls for the presence of the thiol group in cargo molecule. That is usually related to extra modifications of cargo molecules that do not possess that kind of functionality.

That problem solves another disulfide-based l inker, w hich w as applied in molecular umbrella chemistry – *o*-dithiobenzoyl carbamate. Decomposition of this linker depends on thiol exchange reaction with reduced glutathione molecule what leads to degradation of the whole linker structure with the release of gaseous carbon dioxide and cargo molecule. A cargo molecule is attached to linker structure by urethane bond, which means that cargo bearing amine functionalities can be easily incorporated into the conjugate structure. The synthesis of *o*-dithiobenzoyl carbamate linker is based on commercially available 2-mercaptobenzyl alcohol. The hydroxyl group serves as a nucleophile for the generation of urethane bond whereas the thiol functionality is transformed into unsymmetrical activated disulfide t hat s erves as an electrophile for conjugation with molecular umbrella structure. *o*-Dithiobenzoyl carbamate linker was successfully applied in the synthesis of molecular umbrella-oligopeptide H-Tyr-D-Ala-Gly-D-Phe-D-Leu-OH conjugate, where the cargo molecule was attached to the nanocarrier *via* urethane bond formed from primary *N*-terminal amine group (Fig. 25). [145,146]

The synthetic approach leading to molecular umbrella-pentapeptide conjugate is related to the incorporation of thiol functionality to molecular umbrella structure. This was accomplished by condensation of two molecules of nanocarrier with an active ester of 3,3'-dithiopropanoic acid, which results in symmetrical disulfide dimer. Reduction of dimer structure to thiol derivative and subsequent thiol exchange reaction with activated disulfide lead to molecular umbrella bearing benzyl alcohol residue. This alcohol serves a nucleophile for condensation reaction with carbonic acid derivatives (e.g. carbonic acid di-*N*-hydroxysuccinimidyl ester) and the resulting structure is ready for condensation with nucleophilic primary *N*-terminal amine functionality of pentapeptide (Fig. 25) [145].

4.1.2. ω-Aminoacyl linkers

Research work of Regen and co-workers focused on increasing of selective toxicity of amphotericin B, resulted in the formation of diwalled molecular umbrellas with macrolide structure connected *via* a chemically sTable 8-aminooctanoyl linker. The synthesis of mentioned conjugates was accomplished with the usage of properly protected spermidine molecule that was acylated with TDBTU-derived active ester of Boc-protected 8-aminooctanoic acid. Subsequent condensation with cholic acid gave molecular umbrella carrying 8-carbon nucleophilic

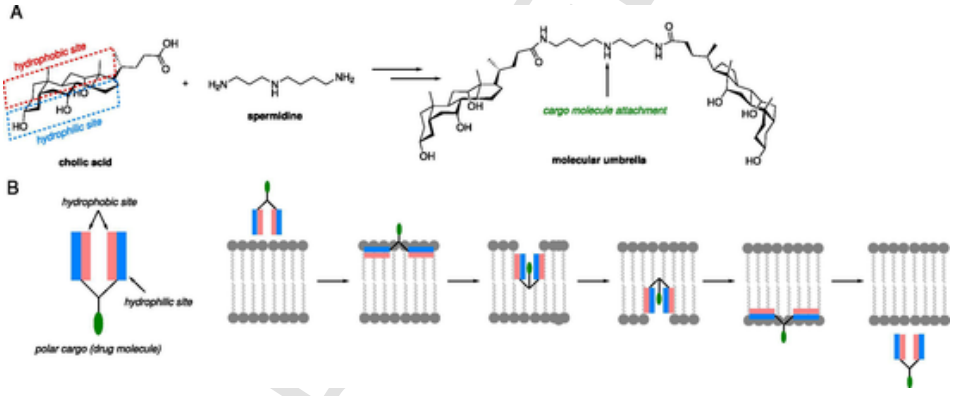

Fig. 23. (A) Chemical components of molecular umbrellas; (B) schematic representation of molecular umbrella and proposed mechanism of translocation of polar cargo across lipid bilayer in assistance of molecular umbrella.

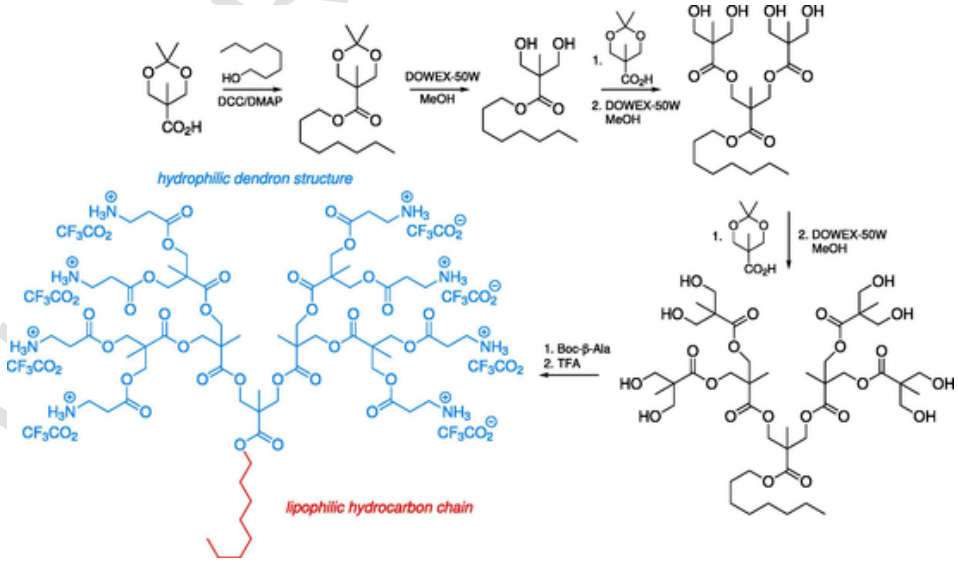

Fig. 24. Simplified synthesis of Palermo's molecular umbrellas.

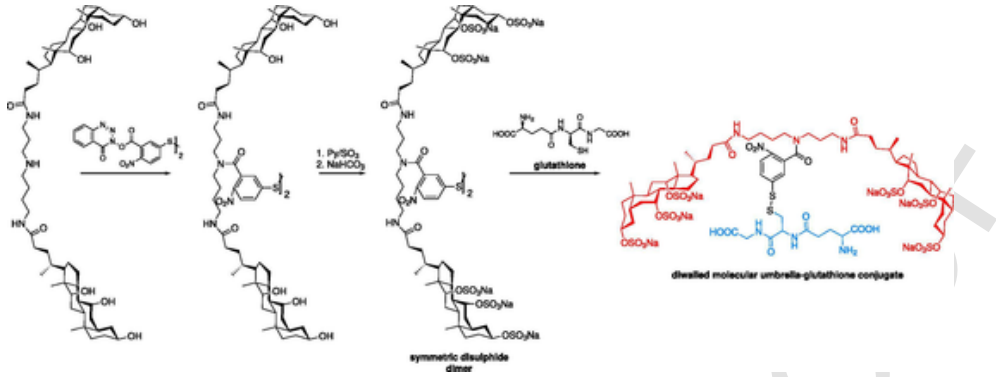

Fig. 25. Applications of 5-thiol(2-nitrobenzoyl) linker in molecular umbrella conjugates; glutathione conjugate.

linker. Coupling with TDBTU activated amphotericin B resulted in needed conjugate (Fig. 26, path A) [147].

In our previous research work, aimed at the selective toxicity of molecular umbrella-amphotericin B and nystatin conjugates, we presented an alternative synthetic procedure, based on direct acylation of diwalled molecular umbrella structure with NHS active ester of appropriate ω -amino acid (Fig. 26, path B) [23].

4.1.3. Maleimide linker

Cline et al [148] elaborated synthetic methods for obtaining of molecular umbrella containing octaarginine electrostatic anchors for nucleotide and nucleic acids conjugation. In their work, diwalled and tetrawalled molecular umbrellas were provided with *N*-(2-carboxyethyl)maleimide linker, which precursor was *N*-(2-carboxyethyl)maleimide TDBTU-derived active ester. The maleimide linker possesses α,β-unsaturated functionality, which serves an electrophile for Michael addition of thiolated octaarginine (Fig. 27) [148].

4.1.4. Molecular umbrellas are still unexploited as molecular carriers

The original concept of molecular umbrellas was focused on their possible application as carriers facilitating drug transport through biological membranes. Indeed, it was shown that conjugates of molecular umbrellas with ATP, glutathione derivatives and nucleotides were effectively internalized to liposomes [139–141] and those with siRNA to HeLa cells [148]. Nevertheless, no examples of molecular umbrella-drug conjugates demonstrating antimicrobial activity due to their Trojan horse-like action have been presented so far. On the other hand, conjugation of molecular umbrellas with an antifungal antibiotic Amphotericin B resulted in congeners showing much better selective toxicity than the mother antibiotic. This effect was achieved not due to the carrier-like action but thanks to the "taming" of antibiotic molecules in the outer layer of a cytoplasmic membrane [147]. An analogous effect was found for the conjugates of amphotericin B with "semi-umbrellas" (only one cholic acid residue) [149]. However, in the case of molecular umbrella-nystatin conjugates, their selective toxicity appeared much worse than that of the intact nystatin [23], thus showing that the taming effect of conjugation with molecular umbrella is not universal for all antifungal polyene macrolide antibiotics. (see Figs. 28–31).

5. Conclusions and perspectives

In this review, we presented the current state of knowledge about strategies of syntheses of low molecular weight organic carrier-drug conjugates as novel potential drugs, especially antimicrobials. In our opinion, three types of such carriers presented in this work, namely siderophores, cell penetrating peptides and molecular umbrellas are most promising and their further development should lead to elaboration of successful drug candidates, as it already has happened in the case of the artificial siderophore-synthetic cephalosporin conjugate cefiderocol. Obviously, conjugates containing siderophores, siderophore derivatives or artificial siderophores as carriers cannot be broad spectrum antimicrobials. Microbial systems of iron acquisition use species-specific siderophores and rarely accept other iron-complexing com-

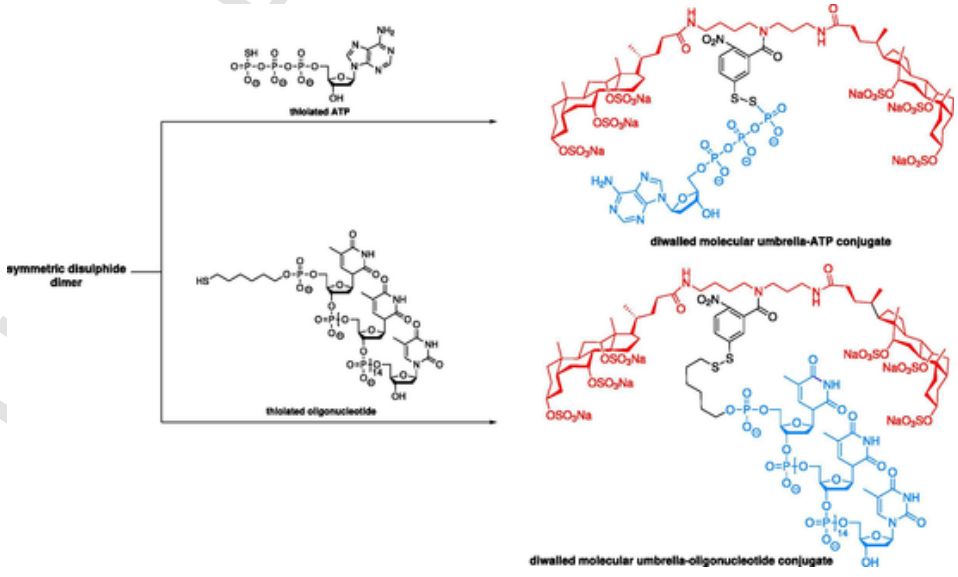

Fig. 26. Applications of 5-thiol(2-nitrobenzoyl) linker in molecular umbrella-gluthathione conjugate. Conjugation with ATP and oligonucleotide.

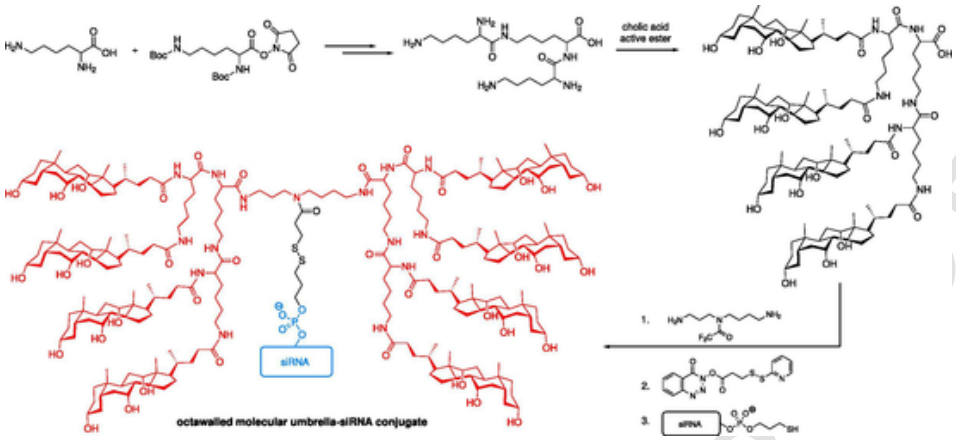

Fig. 27. Application of 3-disulfanylpropanoyl linker in synthesis of molecular umbrella conjugates with thiolated siRNA.

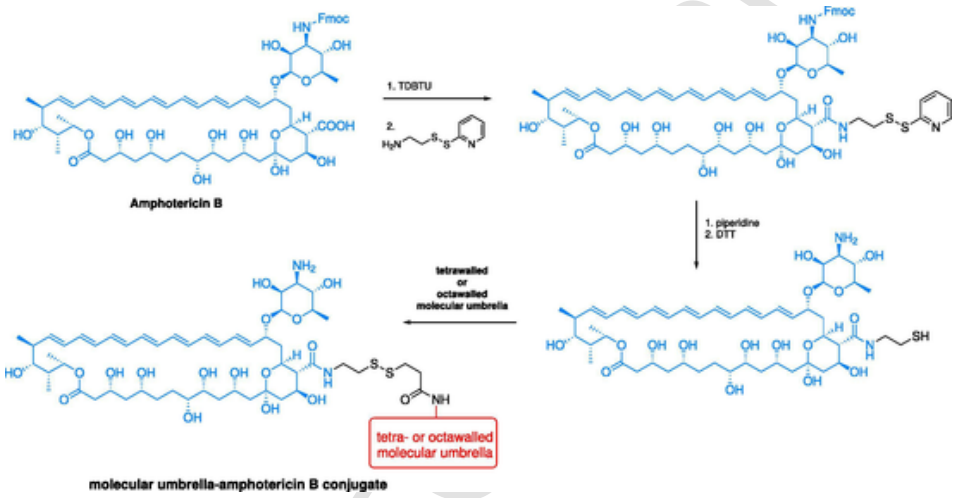

Fig. 28. Application of 3-disulfanylpropanoyl linker in synthesis of molecular umbrella conjugates with amphotericin B.

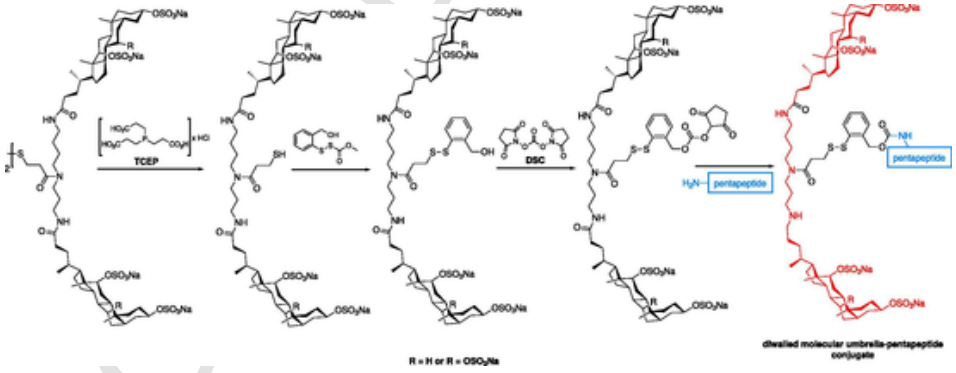

Fig. 29. Synthesis of molecular umbrella-pentapeptide conjugate bearing *o*-dithiobenzoyl carbamate linker.

pounds. Paradoxically, this feature is a chance. Several siderophore-drug conjugates with enhanced selectivity towards pathogenic *vs.* non-pathogenic bacterial strains have been reported (for example, uropathogenic *E. coli vs.* strains of this bacterium constituting human microflora). It is worth mentioning therefore, that development of narrow-spectrum antibiotics is a strategy recommended by WHO. Examples of successful application of this approach are presented in this review. These include conjugates contaning enterobactin *C*-glycosylated analogues, obtained by chemoenzymatic synthesis. Undoubtedly, it is worth pursuing further, possibly also with artificial catecholate siderophores, instead of enterobactin itself. This suggestion is in line with another direction of siderophore-drug conjugate design and synthesis observed in the recent years, i.e. simplification of structures of the siderophore component. Examples of cefiderocol and BAL 30072 indicate that it is possible and can be successful. Another advantage of this approach is facilitation of synthesis and possible reduction of a number of synthetic steps. While catecholate siderophores and their analogs have been extensively studied as drug carriers, much less attention has been paid to their hydroxamate counterparts. By analogy with artificial catecholate siderophores used as carriers for antibacterial agents targeting specific bacteria, simplified hydroxamate siderophores could be possibly used for construction of novel antifungals, active

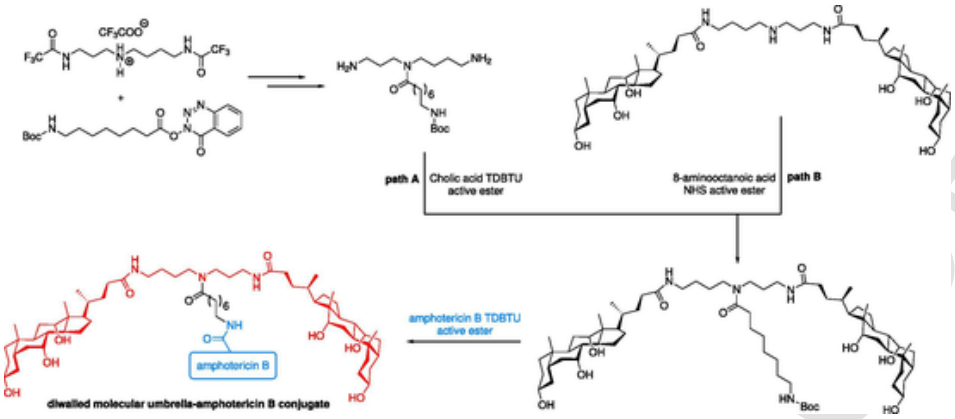

Fig. 30. Alternative synthetic pathways for synthesis of molecular umbrella-amphotericin B conjugates bearing 8-aminooctanoyl linker.

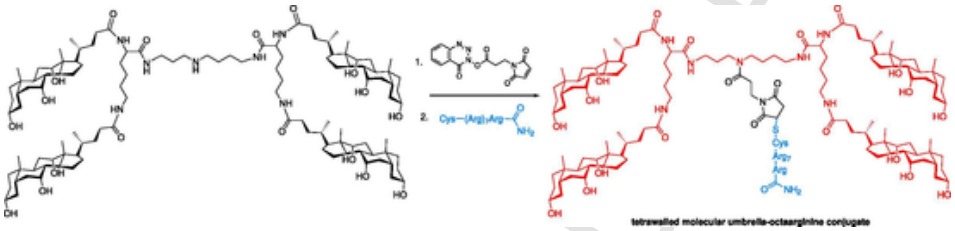

Fig. 31. Simplified synthetic pathway for tetrawalled molecular umbrella carrying octaarginine residues attached *via* maleimide linker.

against selected human pathogenic fungi. Chemistry of these systems has been well elaborated, so it seems that not too many further efforts are needed to develop it in a required direction. Application of CPPs as drug carriers offers a chance for a broad spectrum of activity of CPP-containing conjugates. On the other hand, a huge number of CPPs of natural or synthetic origin reported in literature is a challenge in terms of selection of the optimal carrier. Although, there are examples of CPPs that target specific types of cells, in most cases these carriers are not cell-specific. Most of the CPPs of natural origin are somewhat large (20–30 aa) and their amino acid sequence is complicated. For that reason, the most popular CPP applied for construction of carrier-drug conjugates is relatively simple and small synthetic octaarginine, used as a carrier for construction of many antimicrobial and anticancer conjugates. The undoubted advantage of CPP-based conjugates is relative simplicity of their preparation involving manual or automated solid-phase peptide synthesis. A chemical variety of functional groups provided by side chains of amino acids leads to virtually unrestricted possibilities of conjugation to cargo molecules and the design of cleavable linkers. If conjugate's cleavability is not a necessity, synthetically convenient "click chemistry" reaction can be used, utilizing amino groups in side chains of lysine or ornithine, converted into azide functionalities. On the other hand, some CPPs, due to their can be used in construction of noncovalent complexes like liposomes or PEG-based nanoparticles. Molecular umbrellas constitute the "youngest" group of low molecular weight carriers. So far, their use for facilitated transport of bioactive substances has not been very extensive. Molecular umbrellas give rise to their coupling with active molecules via various cleavable or non-cleavable linkers. Moreover, the ability to transport large molecules as siRNA deserves a special attention. The chemistry of obtaining molecular umbrella-cargo conjugates described so far is based primarily on the formation of non-cleavable C-N bonds, both amide and amine type or glutathione-cleavable S-S bonds, however, the application of trimethyl lock or other easily cleavable linkers is possible. The recently reported cationic molecular umbrellas have not been recognized as drug carriers, but exhibit antibacterial activity themselves. Nevertheless, the proved ability to permeabilization of prokaryotic membranes with simultaneous low hemotoxicity give a chance for those construct to be used as drug carriers in the future. One feature which is common for all three types of low molecular weight carriers presented in this review is presence or absence of cleavable linkers between a carrier and a drug. The cleavable linker is required if the drug component of a conjugate has its intracellular target and can interact with it only after release from a conjugate). This is not the case of conjugates containing β-lactams (the target is extracellular) or fluoroquionolones (the target is intracellular but interaction with it is possible also for a drug molecule not released from a conjugate. For conjugates containing drugs that must be released intracellularly, several types of cleavable linkers have been proposed, including the sulfur-base ones, active esters, hydrazones and trimethyl lock. Sulfur-based linkers, especially the disulfide bonds, afford a possibility of creation of conjugates undergoing non-enzymatic cleavage, based on the reductive activity of intracellular glutathione. That is particularly important because it overcomes the problem of narrow substrate specificity of intracellular enzymes. From the synthetic point of view, creation of disulfide bonds by thiol exchange reaction with activated disulfides is favorable because of mild conditions and high yields, what is an important factor in the synthesis of such complex compounds. Moreover, the syntheses of conjugates are based on readily available sulfur-containing reagents, such as the Ellman's reagent, 3-(pyridine-2-yldisulfanyl) propionic acid and 3,3-dithiopropionic acid. It must be mentioned however, that there have been reports indicating that intracellular reduction of disulfide bonds in microbial cells is species- and even strain-specific, so that this linker is not of general applicability. Other stimuli-responsive linkers, like acidic pH-triggered hydrazone or active asters have not been used extensively. In our opinion, esterase- or phosphatase-triggered trimethyl locks, originally introduced in siderophore-drug conjugates, deserve broader application but efforts could be continued towards designing linkers cleavable exclusively in microbial, not mammalian cells. Majority of reviewed conjugates were only studied under *in vitro* conditions, thus there is still need for further studies in order to gain data on *in vivo* activity and mammalian toxicity. As there are hundreds of siderophore- and CPP-based conjugates being investigated for the delivery of therapeutic small molecules, a successful transition to the clinical applications is very likely. 'Molecular umbrellas' are relatively "young"

carriers and there are not many reported conjugates that could be used in the medicinal applications, nevertheless, the rational design behind the idea of this transporter is very promising. We believe that gathering synthetic strategies from seemingly distinct fields of study in a concise review may cause researchers from different fields to draw inspiration from each other for the future design of novel conjugates as drug candidates.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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