Novel Phospholipase-Resistant Lipid/Peptide Synthetic Lung Surfactants

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Abstract: Animal-derived drugs are currently widely-used to treat clinical lung surfactant deficiency, but synthetic surfactants have significant advantages as pharmaceutical agents. This article examines exogenous surfactants containing novel synthetic phospholipase-resistant lipids of extremely high surface activity. Mixtures of these lipid analogs with purified native surfactant apoproteins are detailed as a proof of concept for related fully-synthetic surfactants containing laboratory-produced peptides. The chemistry and biophysics of relevant lipid analogs and peptides are reviewed in the context of developing new synthetic drugs of utility for patients with surfactant deficiency or lung injury-related surfactant dysfunction.

Key Words: Synthetic lung surfactants, exogenous surfactants, phosphonolipids, lipid analogs, amphipathic peptides, surfactant peptides, acute lung injury.

I. INTRODUCTION

Pulmonary surfactant is a complex mixture of lipids and apoproteins that is synthesized, stored, secreted, and recycled in type II pneumocytes in the alveolar epithelial lining of air-breathing animals. Since its discovery in the 1950's, pulmonary surfactant has been the subject of extensive basic and clinical research (see [1] for detailed review). Much of this research was stimulated by the early recognition that a deficiency of lung surfactant in premature infants led to lethal Hyaline Membrane Disease (now known as the neonatal respiratory distress syndrome, RDS or NRDS) [2]. Prior to the advent of effective exogenous surfactant replacement therapy, this condition was the largest cause of neonatal mortality in the developed countries of the world [1]. Surfactant replacement therapy has greatly decreased the mortality and morbidity of premature infants, but many still develop NRDS and associated lung injury in the perinatal period. Moreover, surfactant therapy has not yet been effectively extended to the lethal syndromes of clinical acute lung injury (ALI) and the acute respiratory distress syndrome (ARDS) in patients of all ages [1,3,4].

ALI/ARDS results from multiple direct and indirect pulmonary causes in adult and pediatric patients. Direct pulmonary causes of ALI/ARDS include gastric or meconium aspiration, pulmonary infection, hyperoxia, lung contusion, near-drowning, smoke inhalation, and thoracic radiation. Indirect causes of these conditions include sepsis, hypovolemic shock, generalized trauma, burn injury, pancreatitis, fat emboli, and a number of others [1,5-9]. The incidence of ALI has been estimated to be 20-65 cases per 100,000 persons per year in the United States, with approximately 50-150,000 adults developing ARDS (by definition all patients with ARDS also have ALI) [10,11]. The pathophysiology of ALI/ARDS is complex, and includes inflammation, vascular dysfunction, and cell/tissue injury in addition to surfactant dysfunction. However, surfactant dysfunction is an important contributor to respiratory failure in many patients [5-8,12], providing a clear rationale for exogenous surfactant therapy. Although surfactant therapy cannot be expected to completely reverse the complex multifaceted inflammatory lung injury pathology of ALI/ARDS, it has the potential to provide crucial respiratory benefits for affected patients.

Exogenous surfactants used to treat ALI/ARDS and severe NRDS must have the highest possible activity and inhibition resistance. Basic science understanding indicates that exogenous surfactants containing biophysically-optimal lipids and proteins would have significant efficacy in ALI/ARDS if delivered in adequate amounts to the pulmonary alveoli [3]. The current review focuses on novel lipids and peptides as constituents of new synthetic surfactant drugs for treating ALI/ARDS and NRDS. Synthetic surfactants have substantial advantages over animal-derived surfactants as pharmacologic agents, as detailed below. In addition, the synthetic phospholipase-resistant lipids described have structural, biophysical, and inhibition resistance properties designed specifically to increase their utility in exogenous surfactants used in inflammatory lung injury.

II. SYNTHETIC VERSUS ANIMAL-DERIVED EXOGENOUS SURFACTANTS FOR DISEASE THERAPY

Current animal-derived exogenous lung surfactant drugs are known to be more active than available first generation synthetic preparations [1,13-17]. However, synthetic lung surfactants manufactured under controlled conditions in the laboratory have significant advantages in purity, reproducibility, and quality control compared to animal-derived preparations. As biological products, FDA-approved animal surfactants have inherent batch-to-batch variability that necessitates complex compositional testing and bioactivity analysis.

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Synthetic surfactants in principle require less elaborate quality control, and become increasingly cost-effective over time. Synthetic surfactants are also completely free from concerns about prion-caused animal disease (e.g., bovine spongioform encephalitis) that can constrain animal lung supplies and raise costs, and they are not subject to cultural and religious issues associated with bovine- or porcine-derived preparations.

In addition to the general advantages of synthetic lung surfactants, dosage considerations show the practical importance of such preparations for treating ALI/ARDS. On a bodyweight basis, total surfactant doses 50-100 times larger than those used in premature infants are required for an equivalent administered quantity of drug in adults. A surfactant dose of 100 mg/kg bodyweight translates to 5-10 gm of tracheally-instilled surfactant in typical older adolescents and adults weighing 50-100 kg, as opposed to 100 mg in a 1,000 gm premature infant. In addition, an increased number of surfactant doses per patient may be necessary to mitigate severe respiratory failure in patients with ALI/ARDS, further increasing total required drug amounts relative to NRDS. Costs for animal-derived drugs are strongly dependent on animal usage, which is directly proportional to required drug dosage. The cost of multiple 5-10 gm doses of exogenous surfactant per patient would be prohibitive at a level even closely proportional to the expense of current 100-200 mg infant vials of animal-derived surfactant drugs. In contrast, the production of synthetic surfactants *in vitro* can be scaled up to larger amounts with substantially reduced expense. If ALI/ARDS is to be cost-effectively treated with surfactant therapy in the future, fully-synthetic exogenous surfactants are much more likely to achieve this goal.

III. NOVEL SYNTHETIC PHOSPHOLIPASE RESISTANT PHOSPHOLIPID (PHOSPHONOLIPID) ANALOGS

III.A. Native Lung Surfactant Glycerophospholipids

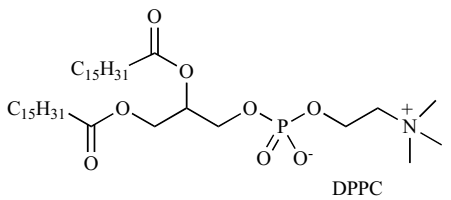
Biophysically-functional endogenous lung surfactant contains about 90-95% lipid on a weight basis, with the remainder being surfactant proteins (SP) [1,4,18,19]. Glycerophospholipids account for the great majority of lung surfactant lipid, with zwitterionic phosphatidylcholine (PC) comprising about 80% of the total phospholipid fraction. Dipalmitoyl phosphatidylcholine (DPPC; C16:0, C16:0) is the most prevalent single phospholipid (40-50% of total PC), although multiple other saturated and unsaturated PC species are also present. Endogenous surfactant also contains a mix of saturated and unsaturated phospholipids with anionic headgroups: phosphatidylglycerol (PG), phosphatidylinositol (PI), and phosphatidylserine (PS), which make up 10-15% of total phospholipid. Three biophysically-active surfactant apoproteins SP-A, -B, and -C are also present in functional lung surfactant. The various isoforms of SP-A have monomeric molecular weights of about 28-36 kilo-Daltons (kD) (an average monomer weight of 35 kD is typically used). SP-B and SP-C are much smaller and more hydrophobic than SP-A, and have monomer molecular weights of about 8.7 kD and 4.2 kD, respectively [1,4,18,19]. These apoproteins interact with glycerophospholipids at the molecular level to generate the set of surface properties required for lung surfactant to achieve its physiologically-essential roles of reducing the work of breathing, stabilizing alveoli against collapse and over-distension, and lowering the pressure driving force for pulmonary edema [1].

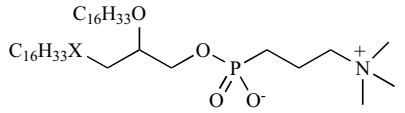
All current exogenous lung surfactants contain a substantial content of glycerophospholipids including DPPC. As a result, all of these surfactants are subject to degradation by phospholipases A_1, A_2, C, and D. Lytic phospholipase enzymes are active in normal intracellular metabolism, but can be elaborated in high concentrations in the interstitium and alveoli during inflammatory lung injuries associated with ALI/ARDS (e.g., [20-27]). Phospholipase-induced degradation of surfactant glycerophospholipids not only reduces the concentration of these active components, but also generates reaction products such as lysophosphatidylcholine (LPC) and free fatty acids that are known biophysical inhibitors of lung surfactant activity [1,28-30]. Moreover, LPC and free fatty acids can directly damage the integrity of the alveolocapillary membrane [31,32]. Thus, when surfactant is exposed to phospholipases in injured lungs, the result is a loss of functional phospholipids, increased biophysical inhibition, and potentially increased alveolar and capillary permeability. Similar effects can occur if phospholipases act on exogenous surfactants administered to treat a deficiency or dysfunction of exogenous surfactant. The synthetic lung surfactants described in this article incorporate novel phospholipid analogs designed to have specific structural resistance to phospholipases along with beneficial surface-active properties.

III.B. Synthesis of Phospholipase-Resistant Phospholipid (Phosphonolipid) Analogs

The synthesis, purification, and biophysical behavior of several novel phospholipase-resistant phosphonolipids having potential utility as constituents in synthetic lung surfactants has been reported [33-46]. Two extremely surface-active C16:0 phosphonolipid analogs identified in these studies are DEPN-8 [34,36-42,44] and SO_2-lipid [43-45] (Fig. **1**). Ether linkages between the fatty chains and glycerol backbone in DEPN-8 increase its molecular flexibility and hydrophobicity compared to ester-linked DPPC, with only a minimal steric penalty in packing ability [34,36]. Thus, DEPN-8 has the ability to reduce surface tension to <1 mN/m in compressed surface films, while having superior adsorption and film respreading behavior compared to DPPC [34,36-38]. SO_2-lipid also reaches very low surface tensions in compressed interfacial films while having improved adsorption and film respreading over DPPC [43-45]. Structural resistance to phospholipases A_1, A_2, and D in phosphonolipid analogs does not prevent these compounds from being taken up and utilized in lung surfactant recycling pathways in alveolar type II epithelial cells [1,40,47], and exogenous surfactants containing DEPN-8 do not have short-term pulmonary toxicity in animals [40].

Synthesis methods for producing gram quantities of DEPN-8 have been defined [42,44], and pathways for the preparative synthesis of SO_2-lipid and related sulfur-containing lipid analogs are similarly available [44,45]. The protocol for construction of the glycerol framework in these compounds requires strategic functional group protection enabling the selective placement of the long chains and polar





1a, X = O, DEPN-8
2, X = S, S-lipid
3, X = SO_2, SO_2-lipid

Fig. (1). Chemical structures of natural dipalmitoyl phosphatidylcholine (DPPC) and synthetic phosphonocholines (compounds 1a, 2, 3). The compounds shown were synthesized by Notter, Schwan, and co-workers [42-44]. Molecular substitutions relative to dipalmitoyl phosphatidylcholine (DPPC) confer resistance to phospholipase A_1, A_2, and D, as well as partial resistance to phospholipase C in DEPN-8 through steric hindrance [41]. See text for details on the synthesis and biophysics of highly-active DEPN-8 and SO_2-lipid.

head groups. The conceptual approach to accomplish this was established some time ago [48], and led to the initial preparation of DEPN-8 [35]. Commercially available solketal **4** was alkylated with an *n*-hexadecyl electrophile, and the acetal protecting unit was removed to afford 1-hexadecyloxy-2,3-propanediol **5** (Fig. **2**). The terminal of the two liberated hydroxyl groups of **5** was then protected selectively with a large trityl (Tr, triphenylmethyl) group, permitting subsequent selective hexadecylation of the remaining secondary hydroxyl to yield **6a**. Detritylation provided diether functionalized glycerol **7a** for head group installation. In initial synthesis work, the alkylation of solketal was performed using harsh KOH in refluxing xylenes [35,48], whereas more recent syntheses [42,44] have adopted the reagent conditions of powdered KOH in dimethyl sulfoxide (DMSO) [49] at or slightly above room temperature for the Williamson etherification steps, which have proved amenable for this chemistry [50]. Moreover, it has proved possible to place the C16:1 group at the sn-2 position to form **7b** by way of the KOH in DMSO conditions, which did not perturb the position or geometry of the double bond [44]. In this latter reaction, the commercially available methanesulfonate ester of palmitoleyl alcohol ($C_{16}H_{31}$OMs) successfully functions as the applicable electrophile [44].

After obtaining compound **7a**, the synthesis of DEPN-8 is completed by first reacting this glycerol with 3-bromopropylphosphonic dichloride **8**, and then hydrolyzing the coupled intermediate (Fig. **3**). Although intermediate **9** has been isolated and purified in some related syntheses [35], the final reaction step can be performed on the crude material [44]. Hence treatment of the unpurified bromopropyl phosphonate **9** with aqueous trimethylamine provides DEPN-8 (compound **1a**). DEPN-8 is typically chromatographed on silica gel, and recrystallized to homogeneity prior to its evaluation of surface properties [44]. The lipid analog possessing the C16:1 group at the *sn*-2 position (compound **1b**, UnDEPN-8) is prepared in the same manner.

To secure SO_2-lipid (compound **3**), the starting point requires commercially available 1-thioglycerol **10** (Fig. **4**). There is no need to introduce any protecting group strategies at this point, as the high nucleophilicity of thiol groups over hydroxyl groups permits selective alkylation of the sulfur [45]. Following the protocol outlined above for access to glycerols **7**, sulfur analog **11** is readily obtained. Conversion of **11** to the phosphonolipid creates the thioether analog **2** (S-lipid) [43,44]. Oxidation of the sulfur of **11** to the sulfone oxidation state with *meta*-chloroperoxybenzoic acid afforded **12,** which was readily converted to compound **3** (SO_2-lipid) [43,44]. As is the case with DEPN-8, this sulfur-containing lipid has significant surface activity both as a pure compound and when combined with bovine hydrophobic surfac-

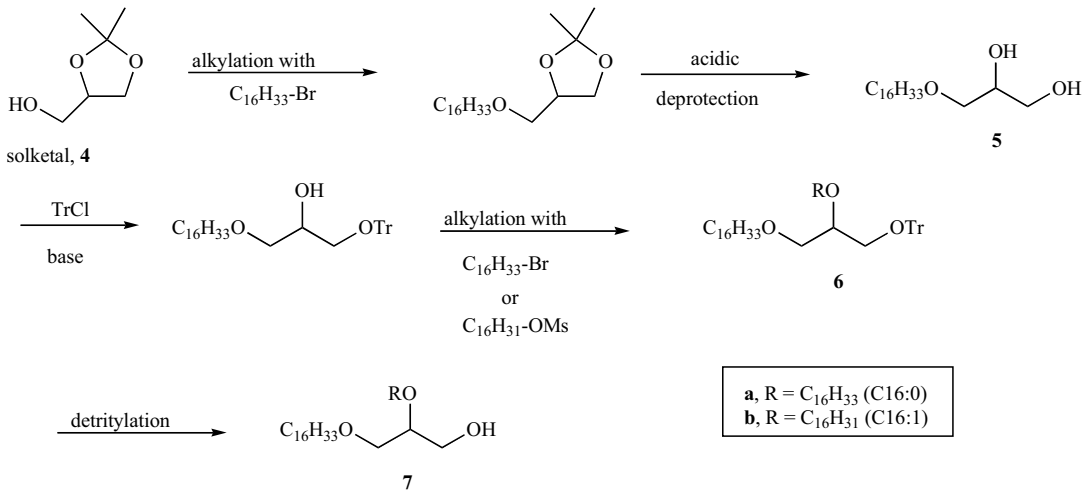


Fig. (2). General synthetic sequence for the formation of 1,2-dialkoxyglycerols utilized in the synthesis of DEPN-8. The figure diagrams reaction schemes for the production of dialkoxyglycerols **7a,b** from solketal **4**, as detailed by Notter, Schwan, Turcotte and co-workers [34,35,42,44]. See text for details.

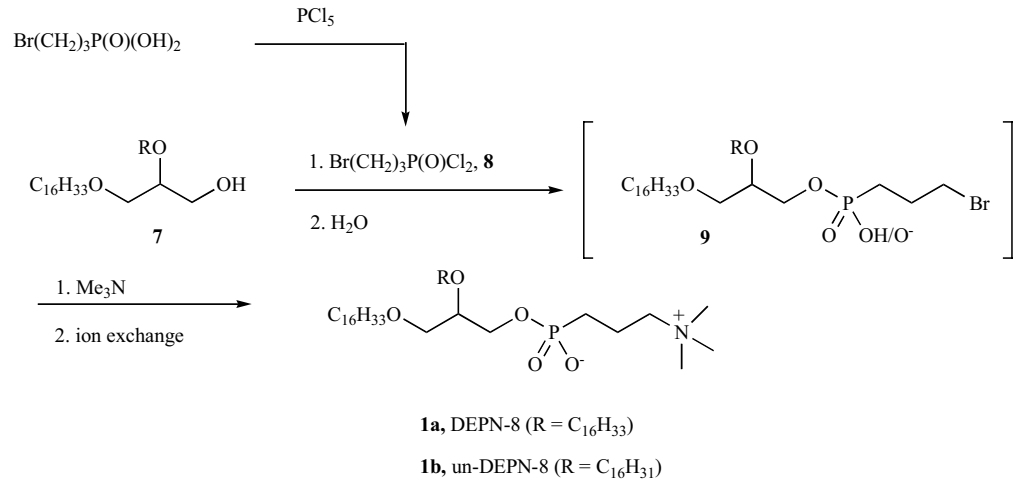


Fig. (3). Synthetic protocol for the placement of the phosphonocholine polar headgroup in DEPN-8 and related compounds. DEPN-8 (compound **1a**) and related diether phosphonolipids (e.g., UnDEPN-8, compound **1b**) are synthesized from dialkoxyglycerols **7** as shown schematically based on the work of Notter, Schwan, and co-workers [42,44]. See text for details.

tant proteins SP-B/C in model lung surfactants (see following section III.B.).

Recent research has also defined synthetic methods for producing three new diether PG analogs for combination with DEPN-8 or SO_2-lipid in synthetic exogenous lung surfactants [46] (Fig. **5**). PG analogs **13a**, **13b**, and **14** all have structural resistance to PLA_1 and PLA_2, and compound **14** is also resistant to phospholipase D. Anionic PG-analogs are important for inclusion with DEPN-8 or SO_2-lipid in synthetic lung surfactants because of their potential for interactions with charged amino residues in surfactant proteins and related synthetic peptides. The synthesis of phosphoglycerol derivatives **13a** and **13b** was realized by standard phosphoglycerol chemistry [51-53] (Fig. **6**). With either of the C16:0 and C16:1 units, at the sn-2 position, glycerols **4-7** were treated with phosphorus oxychloride for the eventual placement of the phosphate functionality [46]. The unpurified dichloride was then treated with solketal to introduce the last three carbons of the glycerol and to complete phosphate formation. The final step involving deacetalization proceeded smoothly to provide phosphoglycerols **13a** and **13b** after purification by flash chromatography and recrystallization [46].

The structural differences between phosphoglycerols and phosphonoglycerols demanded a conceptually different synthetic approach for the production of PG phosphonate analog **14**. Due to the P-C bond of the phosphonate, it was felt that the polar head group fragment should be presented as a near-complete unit bearing appropriately placed protecting groups [46]. Specifically, alcohol **7a** needed to be treated with a phosphonic dichloride bearing a 4-carbon unit with the hydroxyl protected. Dihydroxybutane derivative **15** represents the requisite compound, protected as an acetal by analogy to the phosphoglycerol chemistry (Fig. **7**). Usually the phos-

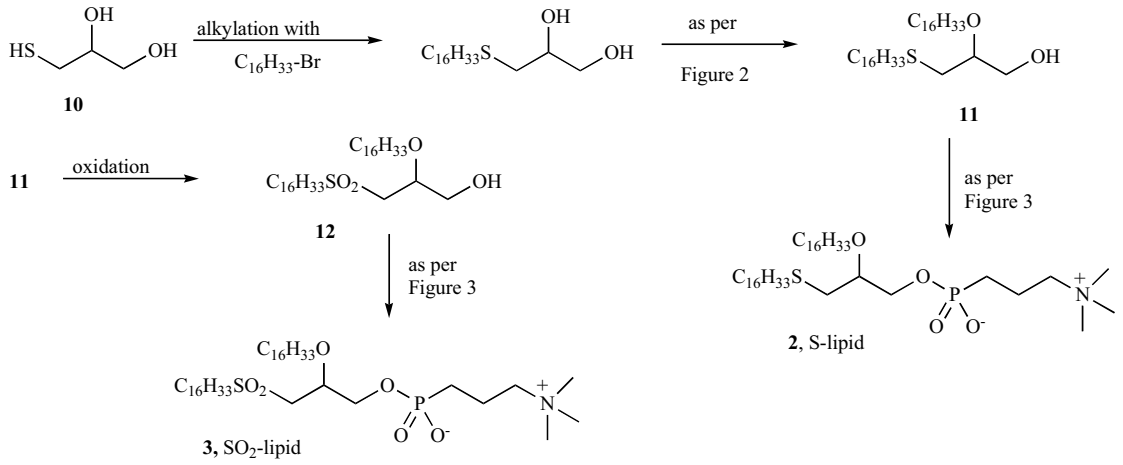


Fig. (4). General synthetic scheme for the preparation of phosphonocholines bearing sulfur and oxygen substitution at the *sn*-1 and *sn*-2 position, respectively. The synthetic pathway for producing sulfur-containing lipids such as S-lipid (compound 2) and SO_2-lipid (compound 3) is diagrammed based on the work of Notter, Schwan, and co-workers [43,44]. See text for details.

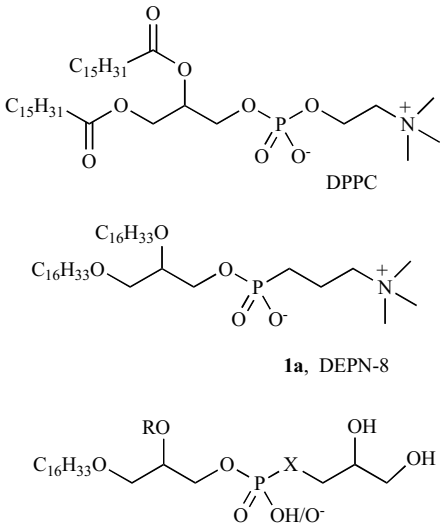


Fig. (5). Schematic diagram of three novel synthetic anionic phosphatidylglycerol (PG) analogs (compounds 13a, 13b, 14). Structures of PG analogs **13-14** [46] are illustrated in relation to the C16:0 diether phosphonolipid DEPN-8 (compound **1a**) and DPPC. The R group in **13a** and **14** is C16:0, while it is C16:1 in **13b** to increase molecular fluidity. See text for details.

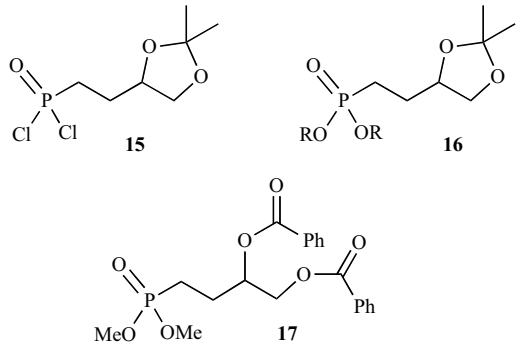


Fig. (7). Structures of potential components for the synthesis of the phosphonoglycerol polar headgroup. Shown are the structures of compounds considered to achieve the synthesis of the novel PG phosphonate **14** (Fig. **5**) as described by Notter *et al.* [46]. See text for details.

phonic dichloride **15** is formed through adaptation of the phosphonodiester functionality of compound **16**. However, the acetal is not amenable to the reagents required to convert diester to dichloride [54,55]. Hence a new protocol was developed to bypass this limitation and incorporate the phosphorus atom with its 4-carbon unit using compound **17**, with carboxylic ester protecting groups on the hydroxyl units [46]. The preparation of **17** was adapted from the work of Bittman *et al.* [56].

In terms of the specific synthesis of PG phosphonate **14**, commercially available dimethyl methylphosphonate **18** was treated with strong base to make lithiated intermediate **19**, which was reacted with glycidol derivative **20** in the presence of $BF_3 \bullet EtO_2$ (Fig. **8**) [46]. The resulting alcohol **21** was then readily benzoylated to position the second carboxylate ester protecting group of **22**. Treatment of **22** with trimethylsilyl bromide, followed immediately with oxalyl chloride [57], gave phosphonic dichloride **23** with negligible disruption of the ester protecting groups. Dichloride **23** was then reacted as a crude material with glycerol **7a** to produce **24**. The overall yield of coupled product **24** from alcohol **21** was found to be 65%. The final step in the preparation of phosphonoglycerol **14** involved alkaline methanolysis of **24** to remove the benzoyl protecting groups, which provided the lipid after acidification and purification [46]. The four-carbon phosphonoglycerol unit represents a new functional group in lipid chemistry. The preparative protocol outlined is expected to be generally applicable for the preparation of multiple phosphonoglycerol analogs in addition to **14**. Further, the procedure is also expected to permit the incorporation of chirality onto both the polar headgroup and the dietherglycerol module of the lipid.

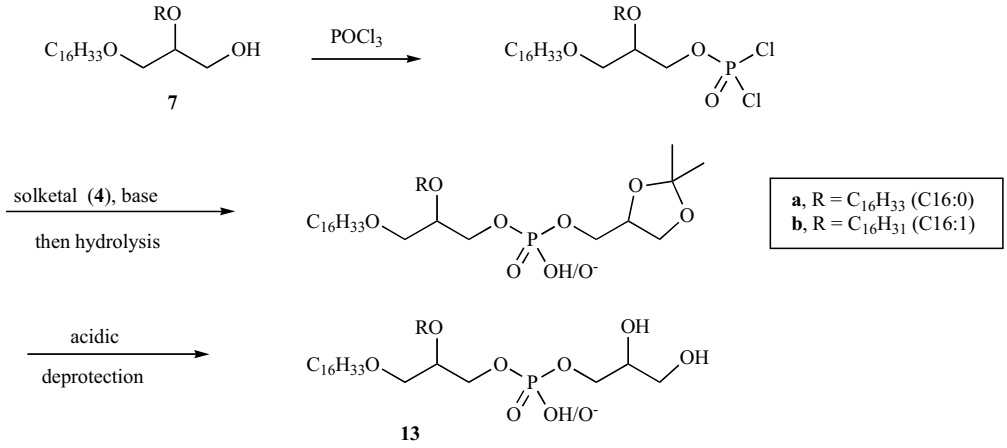


Fig. (6). General synthetic scheme for the installation of the phosphoglycerol polar headgroup. The synthetic pathway for the production of PG analogs **13a,b** from dialkoxyglycerols **7** (Fig. **3**) is shown based on the paper of Notter *et al.* [46]. See text for details.

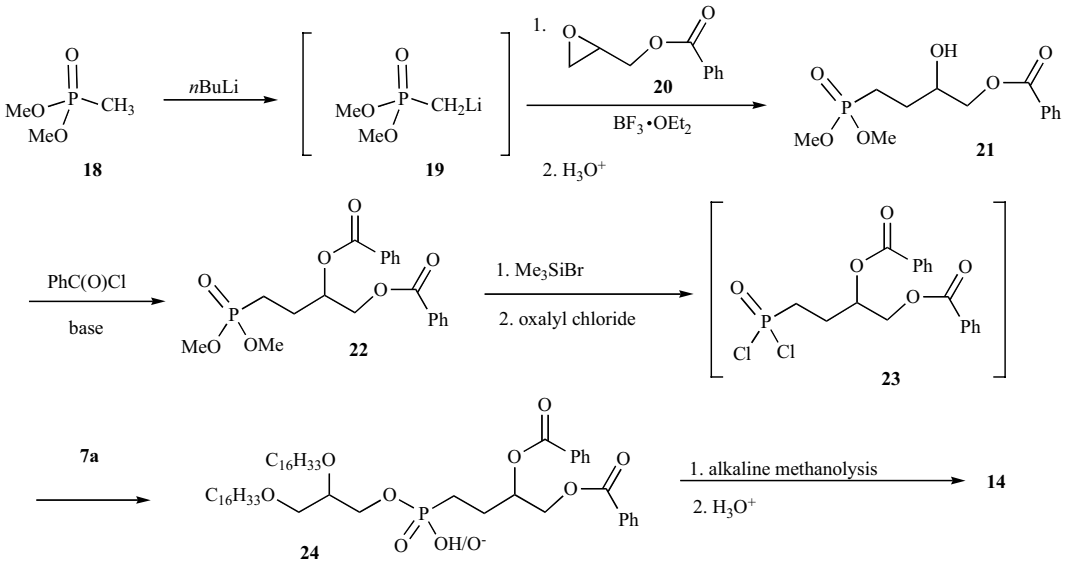


Fig. (8). Synthetic scheme for the synthesis of the novel phosphonoglycerol polar headgroup in PG analog 14. The diagram shows the specific synthetic scheme for the phosphonoglycerol headgroup present in PG phosphonate **14** (Fig. **5**) [46]. See text for details.

III.C. Surface Activity of Lipid Analogs Combined with Native SP-B/C

Studies showing the high surface activity and inhibition resistance of model mixtures of phosphonolipid analogs plus purified animal-derived SP-B/C provide a proof of concept for the ultimate development of fully-synthetic exogenous surfactants containing laboratory-prepared peptides (see Section IV). Several studies have now documented that mixtures of DEPN-8 or SO_2-lipid + 1.5% (by wt) of purified bovine SP-B/C have extremely high surface activity in addition to the ability to resist inhibition by phospholipase A_2 (PLA_2) [42-44]. When examined on a pulsating bubble surfactometer in the presence or absence of inhibitor substances, these model surfactant mixtures rapidly reduce surface tension to values <1 mN/m [42-44]. An example of this behavior is shown in Fig. **9**, where DEPN-8 + 1.5% bovine SP-B/C has dynamic surface tension lowering ability equal to calf lung

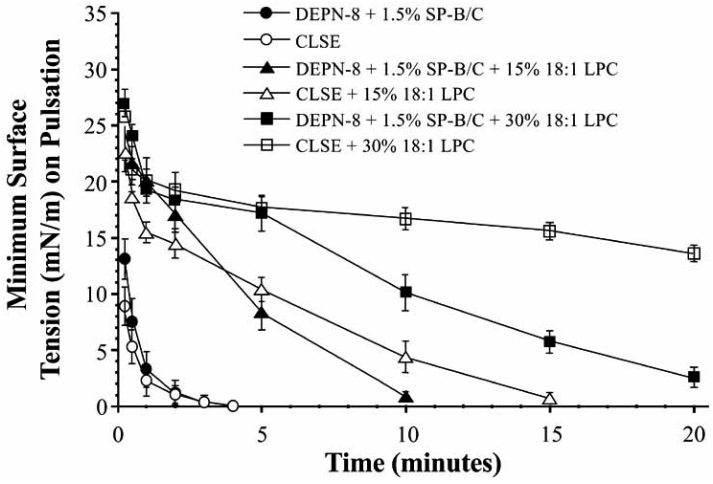


Fig. (9). Dynamic surface activity of DEPN-8 + 1.5% bovine SP-B/C compared to calf lung surfactant extract (CLSE) in the presence and absence of lysophosphatidylcholine (LPC) on a pulsating bubble surfactometer. Minimum surface tension (surface tension at minimum bubble radius) is plotted as a function of time for dispersions of DEPN-8 + 1.5% bovine SP-B/C and CLSE alone or mixed with C18:1 LPC at a content of 15% or 30% by weight relative to surfactant lipid. The pulsating bubble surfactometer in these experiments is widely-used to assess lung surfactant biophysics, because it gives a physiologically-relevant measure of overall activity that combines dynamic surface tension lowering and adsorption at physical conditions similar to those in the lungs *in vivo* (37°C, 20 cycles/min, 50% surface area compression) [1,143]. An air bubble is formed in the surfactant dispersion, which is held in a sample chamber mounted on the pulsator unit of the surfactometer. The bubble is then pulsated between maximum and minimum radii (R) of 0.4 and 0.55 mm, respectively, while the pressure across the air-water interface (ΔP) is measured with a precision transducer. Surface tension (σ) is calculated from Laplace's Law ($\Delta P = 2\sigma/R$). Experiments were done at a uniform surfactant phospholipid (phosphonolipid) concentration of 1 mg/ml in 0.15 M NaCl + 1.5 mM $CaCl_2$ + 10 mM HEPES buffer (pH 7). See text for details. Data are Mean ± SEM (n = 4-8). Redrawn from Ref [42].

Table 1. Phospholipase A_2 (PLA_2) Chemically-Degrades CLSE but not Model Surfactants Containing DEPN-8 or SO_2-lipid + 1.5% Bovine SP-B/C

Phospholipid Class	CLSE	CLSE + PLA_2	DEPN-8 or SO_2-lipid + SP-B/C	DEPN-8 or SO_2-lipid + SP-B/C + PLA_2
Lysophosphatidylcholine	0.4 ± 0.2	29.5 ± 2.4		
Sphingomyelin	1.0 ± 0.2	1.2 ± 0.5		
Phosphatidylcholine	84.4 ± 0.4	55.1 ± 3.2	100 ± 0	100 ± 0
Phosphatidylinositol	4.0 ± 0.6	3.8 ± 0.7		
Phosphatidylethanolamine	3.7 ± 0.7	3.8 ± 1.0		
Phosphatidylglycerol	4.7 ± 0.3	4.1 ± 0.6		
Residue	1.8 ± 0.2	2.5 ± 0.2		

Phospholipid (phosphonolipid) classes are given in weight percent relative to total phospholipid based on phosphate analysis [138] of thin layer chromatographic bands. Surfactants were exposed to 0.1 Units/ml of PLA_2 for 30 min, followed by extraction with chloroform:methanol [139] and analysis by thin layer chromatography using solvent system C of Touchstone et al [140]. Calf lung surfactant extract (CLSE) was prepared as described by Notter and co-workers (e.g., [1,13,141]). Bovine SP-B/C were purified from CLSE by column chromatography [42,142]. Data are Mean ± SEM (n = 3). Compiled from Refs [42,44].

surfactant extract (CLSE) in the absence of inhibitors, and activity superior to CLSE in the presence of C18:1 LPC. DEPN-8 + 1.5% SP-B/C has also been shown to have an ability to resist inhibition by serum albumin that is equivalent to CLSE [42]. Plasma proteins like albumin, and lysophospholipids such as LPC, can both be present in the alveoli and pulmonary interstitium in animals and humans with inflammatory pulmonary injury. The ability of model mixtures of DEPN-8 + 1.5% bovine SP-B/C to resist inhibition by such substances to an equal or greater extent than CLSE is promising, since CLSE is the substance of the clinical exogenous surfactant drug Infasurf® that is known to be highly efficacious in treating surfactant deficiency and dysfunction in humans ([1,3,58] for review).

The ability of model surfactants containing DEPN-8 or SO_2-lipid to resist chemical degradation and maintain high surface activity in the presence of phospholipases has also been explicitly documented ([42,44]. Exposure of CLSE to 0.1 Units/ml of phospholipase A_2 (PLA_2) results in severe chemical degradation of lung surfactant glycerophospholipids, with a loss of normal two-chain PC compounds and a great increase in LPC (Table **1**). In contrast, mixtures of DEPN-8 or SO_2-lipid + 1.5% bovine SP-B/C are fully resistant to chemical degradation by PLA_2 (Table **1**). These findings on chemical degradation are directly consistent with the surface activity of these surfactants on the pulsating bubble surfactometer in the presence of PLA_2 (Fig. **10**). The surface activity of CLSE is significantly reduced by exposure to PLA_2 (0.1 U/ml), while mixtures of DEPN-8 or SO_2-lipid + 1.5% bovine SP-B/C (0.75% SP-B + 0.75% SP-C) are unaffected by this lytic enzyme (Fig. **10**). These findings of high surface activity for model mixtures of DEPN-8 or SO_2-lipid + 1.5% bovine SP-B/C in the presence of PLA_2 provide further support for the potential utility of related synthetic lipid/peptide exogenous surfactants in ALI/ARDS. Recent biophysical data also indicate that phospholipase-resistant PG analogs (compounds **13a**, **13b**, and **14**) can further increase the already high surface activity of DEPN-8 both in binary lipid mixtures and in the presence of added bovine SP-B/C [46].

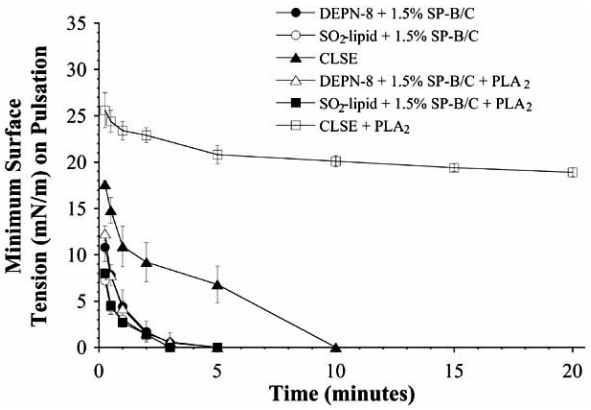


Fig. (10). Effects of PLA_2 on the dynamic surface activity of model surfactants containing SO_2-lipid or DEPN-8 plus purified bovine SP-B and SP-C. Surface tension at minimum bubble radius was measured as a function of time of pulsation on a bubble surfactometer (37ºC, 20 cycles/min, 50% area compression, 0.5 mg phosphonolipid/ml in 0.15 M NaCl + 1.5 mM $CaCl_2$) for DEPN-8 or SO_2-lipid + 1.5% by weight of purified bovine hydrophobic apoproteins (0.75% SP-B + 0.75% SP-C) in the presence and absence of PLA_2 (0.1 Units/ml). Also shown for comparison is the activity of calf lung surfactant extract (CLSE) with and without PLA_2. Data are Mean ± SEM for n = 6-8. Redrawn from Ref [44].

IV. SYNTHETIC PEPTIDES FOR COMBINATION WITH LIPID ANALOGS IN FULLY-SYNTHETIC EXOGENOUS LUNG SURFACTANTS

The model exogenous surfactants in Section III above contain apoproteins purified from animal lung surfactant, which are studied as a proof of concept. In order to develop fully-synthetic exogenous lung surfactants, active laboratory-produced peptide constituents as well as lipid constituents

are required. Synthetic peptides of almost any primary amino acid sequence of reasonable length can be prepared by solid phase synthesis and related techniques. For applications involving lung surfactants, attention has focused largely on hydrophobic or amphipathic peptides having varying degrees of homology to native SP-B and SP-C [59-104]. Because native SP-B has been shown in multiple studies to have greater biophysical activity than native SP-C in enhancing the surface activity of glycerophospholipids in lung surfactants [1,69,89,105-111], particular emphasis has been given to synthetic peptides related to SP-B (e.g., [64,68,69,71,72, 80,86,91,93-104]). Synthetic peptides related to SP-C, however, have also been examined (e.g., [59,67,78,85-87,92]), as well as regional SP-A peptides [77]. Studies on less specific synthetic amphipathic or hydrophobic peptides have also been reported (e.g., [63,65,72,76,81,82,84,112-115], with KL4 being one prominent example [63,65,72,81,113-115].

Finding optimal synthetic substitutes for the highly-active full-length apoproteins in endogenous lung surfactant is challenging. Basic research has documented that synthetic surfactants containing glycerophospholipids combined with a number of laboratory-produced SP-B/C peptides have significant surface activity and in selected cases can improve surfactant-deficient lung mechanics or function (e.g., [1,89, 93,96,116,117] for review). However, the great majority of synthetic lung surfactants containing laboratory-produced peptides plus normal diester glycerophospholipids have not been found to have activity fully equal to analogous preparations containing purified native surfactant proteins. Lipid analogs like DEPN-8 and SO_2-lipid have several enhanced molecular behaviors compared to DPPC and other typical glycerophospholipids, and have the potential to produce more active exogenous lung surfactants when combined with synthetic peptides. Moreover, recent advances that improve the molecular design and structural stability of laboratory-produced surface-active peptides further increase the potential for developing fully-synthetic peptide/lipid analog surfactants of this kind. One novel surfactant peptide (Mini-B) is discussed below as a representative example of such peptides, along with synthetic perspectives applicable for producing additional new surfactant peptide compounds.

IV.A. Native SP-B and a Modified Regional Mini-B Construct

As noted earlier, native SP-B is the most active apoprotein in interacting with glycerophospholipids in alveolar surfactant, making it an important focus of synthetic peptide research. SP-B is a member of the saposin or SAPLIP family of proteins based on its disulfide connectivity, amphipathic helical segment sequence alignments, and disulfide connectivities [118,119]. This combination of structural motifs results in a tertiary ensemble that is an amphipathic helix hairpin stabilized by disulfide linkages and turn/bend regions that join five helical segments, and is termed the "saposin fold". Such a disposition of structural elements is thought to allow the maintenance of relatively stable helical regions together with specific types of protein flexibility for optimal interaction with target lipid substrates [120,121]. Over the past decade, there have been a large number of structures determined for the saposin protein family using both solution NMR and high-resolution X-ray crystallography [119-125]. Although the complete three dimensional structure of full length human SP-B is yet to be determined, many important domains have been solved by isotope enhanced FTIR and solution NMR based on the modern template homology modeling of the ubiquitous saposin fold protein backbone (Fig. **11**) [96,126-128]. Moreover, *in vitro* and *in vivo* activity correlations have been determined for a number of regional SP-B peptides in mixtures with normal ester-linked glycerophospholipids [59,62,73,87,94-96,99-104]. This work has helped to identify several molecular characteristics of putative functional importance in the design of new peptides.

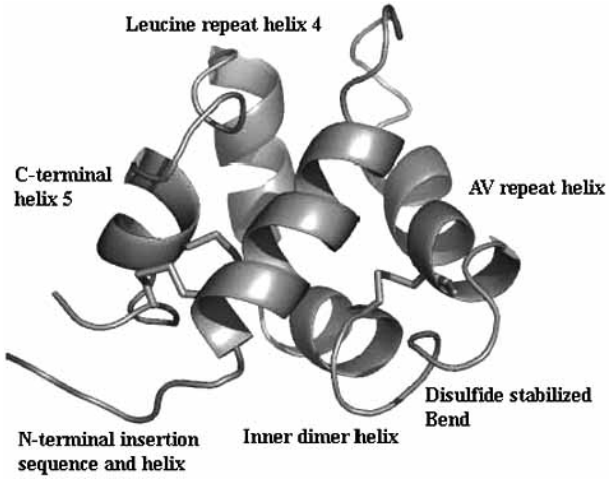


Fig. (11). A molecular illustration of human surfactant protein B (SP-B) based on homology modeling of sequence onto the NK-lysin backbone conformation. The backbone structure of the lowest energy conformer of NK-lysin [119] was used as a template for SP-B. The two proteins were aligned by sequence similarities using T-COFFEE [144], and SP-B was modeled using the server-based SWISS-MODEL program [145]. Five helical regions of SP-B are present based on this approach.

One important example of a modified regional SP-B peptide of potential utility in synthetic surfactants with lipid analogs is the N-terminal/C-terminal SP-B construct Mini-B [96,126]. Although full-length native SP-B has five amphipathic helixes, the clustering of cationic charged residues (lysine, K; arginine, R) that interact with anionic surfactant lipids is primarily associated with the N-terminal and C-terminal helix domains (Fig. **12**). This clustering of cationic charged lipid-active surfaces with disulfide-stabilized side-by-side helical geometry is a crucial feature to maintain in synthetic peptide analogs to generate activity approaching that of native SP-B. Mini-B was specifically designed using modern homology templating algorithms to maintain related disulfide-stabilized helical geometry [96,129]. A loop was also engineered between the N-terminal and C-terminal amphipathic helixes of Mini-B, facilitating solvent-mediated folding of the overall construct [130]. This allows the cysteine residues of Mini-B to come into close proximity and pair correctly to form disulfide linkages, providing similar intramolecular connectivity as in the native full-length protein (Fig. **13**) [96]. Details on the chemical synthesis of Mini-B are given below.

Fig. (12). Amino acid sequence of human SP-B with the approximate assignments for secondary structure derived from homology modeling. Homology modeling as illustrated in Fig. **11** was used to assign specific aspects of secondary structure to the primary 79 amino acid sequence of human SP-B. The five amphipathic helical regions are noted, along with four bend regions and one turn region. Multiple cysteine (C) residues that can participate in disulfide connectivity are present in the primary sequence of SP-B, which contains multiple lysine (K) and arginine (R) residues that are positively charged at neutral pH. The sequence of SP-B additionally contains two negatively charged residues (glutamic acid, E; aspartic acid, D). Charged residues, coupled with the large number of hydrophobic residues also present, give SP-B an overall amphipathic structure that interacts strongly with both the fatty chains and headgroups of lipids. See text for details.

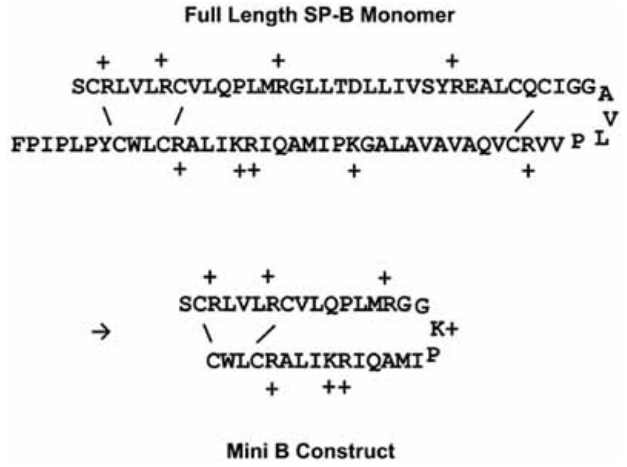


Fig. (13). Amino acid sequence and disulfide connectivity of full-length human SP-B and the N-terminal/C-terminal 34 amino acid mini-B construct. The diagrams indicate the cluster of cationic residues in the N-terminal and C-terminal amphipathic alpha helixes of both native SP-B and synthetic Mini-B [96]. A bend region is also illustrated on the right hand side of each molecule. These molecular features, along with the disulfide connectivities also shown, are thought to be important for the molecular biophysical activity of these proteins in interacting with lipids. See text for details.

IV.B. Chemical Synthesis, Folding, Purification, and Concentration of Mini-B

The chemical synthesis of Mini-B is performed as a stepwise process that starts with the assembly of the peptide as linear sequence using an Applied Biosystems ABI 431A solid phase peptide synthesizer configured for FastMoc™ chemistry [131]. A low substitution (0.3 mmole/gm) pre-derivatized Fmoc-serine (tBu) resin is generally used to help prevent cross-talk of the peptide chain during synthesis that can result in truncated sequences. To allow pairing of disulfide residues to obtain the potential for molecular connectivities similar to the native SP-B sequence, cysteine residues at positions 1 and 33 in the linear Mini-B molecule are coupled by using acid-labile Fmoc-Cys trityl [Fmoc-Cys(Trt)], while acid-resistant Fmoc-Cys acetamidomethyl (ACM) side chain-protecting groups are employed for cysteine insertion at positions 4 and 27 [96]**.** In addition, Fmoc Gln(DMCP)-OH that has greater solubility in coupling solvent [132] is used for the Glutamine residues in the Mini-B sequence, as opposed to more conventional Fmoc-Gln(Trt)-OH that has limited solvent solubility and lower coupling efficiency. All residues are also double-coupled to the resin during Mini-B synthesis to insure optimal yield [96].

After synthesis of the linear Mini-B sequence, the peptide is cleaved from the resin and deprotected using a mixture of 0.75 gm phenol, 0.25 ml ethanedithiol, 0.5 ml of thioanisole, 0.5 ml of deionized water and 10ml trifluoroacetic acid per gram of resin [96,133]. The cleavage-deprotection mixture is first chilled to 5 ^{o}C and added to the resin while stirring, and then allowed to come to 25 ^{o}C with continuous stirring over a period of 2 hrs to insure complete deprotection of the peptide [96]. After cleavage and deprotection, the crude peptide is separated from the resin by filtration. This process is accomplished first by vacuum-assisted filtration in the cleavage-deprotection solution, followed by washing the resin on a medium porosity sintered glass filter with first trifluoroacetic acid and then dichloromethane to remove any peptide remaining on the resin. The filtrate containing the peptide is precipitated with ice cold tertiary butyl ether and separated from the ether deprotection solution by centrifugation at 2000 x g for 10 min. The precipitate is then subjected to several ether-peptide-precipitation centrifugation cycles to remove excess amounts of cleavage-deprotection byproducts. The crude peptide, which is in the reduced state, is then dissolved in solution of trifluoroethanol (TFE):10 mM HCl (1:1, v:v) and freeze-dried so that the material has a uniform powder consistency that can easily be dissolved in organic solvents for further purification [96]. Crude peptide is purified using preparative scale HPLC and the mass of the peptide is confirmed by MALDI TOF mass spectrometry [96].

Folding of HPLC-purified, reduced Mini-B peptide into a disulfide-linked helical structure closely resembling the saposin fold of native SP-B is facilitated by specific solvents [91]. Incubation of purified peptide (0.1 mg/ml) for at least 24 hr at 25 ^{o}C in a solution of TFE and 10 mM ammonium bicarbonate buffer (4:6, v:v) at pH 8.0 results in almost full air-oxidation of the disulfide linkages between cysteines at positions 1 and 33. TFE is an ideal solvent for structures like Mini-B, since it interacts with the peptide backbone in a manner that optimizes both the helix elements in the sequence as well as the turn of the engineered bend domain. The buffered TFE solvent system enhances the folding of Mini-B to facilitate the formation of a helix hairpin structure with the N-terminal and C-terminal helical domains in close proximity to one another, which is a preferred geometry for the formation of properly indexed disulfide connectivities (Fig. **14**) [96]. The final disulfide linkage between cysteine residues 4 and 27 is then accomplished by iodine oxidation of the ACM protected side chains.

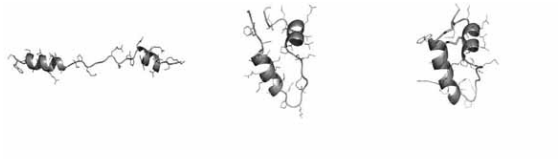


Fig. (14). Molecular illustration of solvent-mediated backbone folding and formation of disulfide linkages of Mini-B based on the Protein Data Bank (PDB) coordinate set. The PDB coordinate set for Mini-B (PDB 1DFW) was used as a template to derive the molecular models shown. The models illustrate that unfolded Mini-B (left image) becomes folded into a helix hairpin structure (middle image) in the presence of buffered Trifluoroethanol (TFE). This solvent-mediated folding brings the cysteine residues in the N- and C-terminal domains in alignment so that oxidation of the disulfide linkages can take place in a period of 24 hr to form a stable saposin-like fold (right image) similar to that of native full-length SP-B. See text for details.

Following oxidation, the final Mini-B peptide is re-purified by reverse phase HPLC using the same boundary conditions as employed for the crude material [96]. This procedure allows separation of any residual reduced peptide away from the desired final oxidized Mini-B product. The molecular mass of oxidized Mini-B is re-checked by mass spectroscopy, and the disulfide connectivity confirmed by mass spectroscopy of enzyme-digested fragments (trypsin and chymotrypsin digestion). The oxidized Mini-B peptide is then dissolved in ethanol:10 mM HCl (4:6, v:v), and dialyzed against ethanol:0.1 mM HCl (4:6, v:v) to remove any trichloroacetic acid counter ions from the HPLC purification, as well as excess chloride salt that would have adverse effects on *in vivo* and *in vitro* applications. After dialysis, the purified desalted peptide is freeze-dried to give a uniform powder that can be combined with lipids for synthetic surfactant applications.

Even freeze-dried powders of dialyzed purified peptides by necessity contain some anionic counter ions associated with cationic peptide residues, and they typically contain variable amounts of residual salt. This means that simple weighing of purified peptide powders can be an inaccurate method for estimating their active concentration for applications involving synthetic lung surfactants. A more precise quantitation of peptide concentration can be obtained spectrophotometrically. Mini-B has a tryptophan residue (position 2) plus two disulfide linkages that give it strong UV absorbance at 280 nm, and a calculated molar extinction coefficient of 5930 M^{-1} cm^{-1} [134]. Since TFE is basically transparent at 280 nm, it is a convenient solvent for quantitation of Mini-B. Once dissolved in TFE, the concentration of peptide can be determined from the measured absorbance and known extinction coefficient using the Beer-Lambert law: $A = \varepsilon lC$, where A is absorbance, ε is the molar extinction coefficient, l is the sample cuvette pathlength in centimeters, and C is the molar concentration of peptide.

IV.C. Combination of Peptides with Lipids in Synthetic Exogenous Surfactants

One important step in the preparation of synthetic lipid/peptide lung surfactants is the physicochemical incorporation of peptide constituents together with lipid constituents *in vitro*. Compared to native SP-B or other endogenous surfactant apoproteins, synthetic peptides can be less easy to insert into lipid multilayer vesicles (MLV's) *in vitro*. This may in part reflect secondary or tertiary structural differences between native surfactant apoproteins and laboratory-synthesized peptides. The ability of native surfactant apoproteins to combine with phospholipids *in vitro* may also be facilitated by the presence of anionic "core" lipid that has become closely-associated with these materials in the type II cell or in the alveolar spaces. The presence of anionic lipid associated with surfactant apoproteins isolated from animal lung lavage has been demonstrated, for example, by Notter *et al.* [135]. The combination of synthetic lipids and peptides *in vitro* is also influenced by the methods and conditions utilized to co-disperse these constituents in the aqueous phase. One important variable for dispersion and formulation is temperature, which can be adjusted to exceed or remain below the gel to liquid crystal transition temperature(s) of specific lipid components. The physical dispersion method used can also influence the degree of mixing and association of lipid and peptide components in the aqueous phase, e.g., sonication, mechanical vortexing, extrusion, or other methods of generating liposomal or vesicular dispersions.

In addition to the influence of peptide structure, core lipid, and physical variables on lipid-peptide combination *in vitro*, studies with several amphipathic peptides have shown that their insertion into lipid multilayers depends on the organic solvent systems used during formulation [136,137]. For example, TFE is a weak Bronsted acid, and hence is a good hydrogen bond donor that has a protonating effect on peptides and proteins (36) that may affect their combination with lipids. Peptides initially dissolved in TFE are often co-solvated with desired lipid mixtures in chloroform [132,137]. Co-solvated lipid-peptide mixtures are then dried under nitrogen to form a multilayer film in a glass vial (vacuum drying for 12 hrs or more can be used to help remove organic solvent). The dried lipid/peptide multilayer is then hydrated and physically suspended in the aqueous phase as above, and repetitive cycles of freeze-drying and re-suspension can be used to further remove residual organic solvent if necessary.

V. SUMMARY

In summary, current medicinal chemistry research demonstrates that highly-active exogenous lung surfactants can be prepared by combining novel synthetic phospholipase-resistant lipid analogs such as DEPN-8 or SO_2-lipid with apoproteins purified from native lung surfactant. This provides a proof of concept for the development of fully-synthetic lung surfactant drugs containing lipid analogs plus laboratory-produced amphipathic peptides with homology to human surfactant proteins. In addition to designed structural resistance to phospholipases, DEPN-8 and SO_2-lipid have significantly improved surface-active properties compared to DPPC, the primary glycerophospholipid constituent of endogenous lung surfactant. Studies combining DEPN-8 or SO_2-lipid with 1.5% purified bovine SP-B/C have demonstrated that these model mixtures have surface activity in the absence of inhibitors that is equivalent to CLSE, the substance of the highly-active animal-derived clinical surfactant drug Infasurf®. In addition, model surfactants containing

DEPN-8 or SO_2-lipid + 1.5% bovine SP-B/C maintain full activity in the presence of PLA_2, while CLSE is degraded chemically and severely reduced in activity in the presence of this enzyme. In addition, DEPN-8 + 1.5% bovine SP-B/C surpasses CLSE in its inhibition resistance to LPC, and has equal inhibition resistance to serum albumin. All of these substances (phospholipases, LPC, and albumin) are known to be present in the lungs during states of inflammatory injury (ALI/ARDS).

Organic chemical research has identified methods for the efficient synthesis and purification of DEPN-8 and SO_2-lipid, as well as for the production of additional synthetic lipids such as active PG analog compounds. Moreover, progress in protein synthetic chemistry and molecular design is now at a point where peptides having biophysical activities approaching those of native surfactant apoproteins can be produced for use with lipid analogs in fully-synthetic surfactants. One representative synthetic peptide described here for such applications is Mini-B, an N-terminal/C-terminal construct related to human SP-B. In addition, a variety of new lung surfactant peptides are currently under development. Fully-synthetic lung surfactants composed of lipid analogs and peptides have significant advantages as pharmaceutical products compared to current animal-derived exogenous surfactant drugs used in treating NRDS or ALI/ARDS. These practical advantages, together with their potential for extremely high surface activity and resistance to inhibition or degradation in the lungs during inflammatory injury, makes synthetic lipid/peptide lung surfactants highly promising for future therapeutic applications.

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