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SYNTHESIS AND CHARACTERIZATION OF A NEW FLUORESCENT PHOSPHOLIPID

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Summary

A novel fluorescent phospholipid, whose structure was tentatively assigned as 1-(2′-thio-1′-hydroxyethyl)-2-(ethylphosphatidyl)isoindole), was synthesized by reacting *O*-phthalaldehyde and β-mercaptoethanol with phosphatidylethanolamine. The fluorescent lipid product was purified by silicic acid chromatography. The purity was demonstrated by thin-layer chromatography. This fluorescent phospholipid could not form stable lipid vesicles. However, a mixture of phosphatidylcholine and this fluorescent phospholipid did form stable vesicles after sonication, as demonstrated by Sepharose 4B column chromatography and electron microscopy. The absorption and fluorescence properties of this lipid, both as aqueous micelles or incorporated into vesicles, have been determined. The potential usage of this new fluorescent phospholipid in membrane studies is discussed.

Introduction

The use of fluorescence analogues of lipids has greatly facilitated studies on the structure and function of biological and model membranes (see [1,2] for review). Advantage has been taken of the relatively high reactivity of the primary amino group of phosphatidylethanolamine to conjugate various fluorescent moieties to this natural phospholipid [3—6]. *O*-Phthalaldehyde, non-fluorescent by itself, reacts with primary amines, e.g. amino acids and proteins, and thiols to form some isoindole derivatives which are highly fluorescent [7—10]. This compound has been used to label proteins and facilitated the

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Abbreviation: THEPI, 1-(2′-thio-1′-hydroxyethyl)-2-(ethylphosphatidyl)isoindole.

studies of protein conformation [11]. In this communication, we report the synthesis, purification and characterization of a new fluorescent phospholipid, i.e. a conjugation product of *O*-phthalaldehyde, β-mercaptoethanol and phosphatidylethanolamine. Furthermore, the incorporation of this new fluorescent phospholipid into lipid vesicle is demonstrated.

Materials and Methods

Extraction and purification of phosphatidylcholine and phosphatidylethanolamine. Total lipids were extracted from hen egg yolks with $CHCl_3$/methanol (2 : 1, v/v) by the method of Folch et al. [12]. The phospholipids were fractionated by silicic acid column chromatography [13]. Fractions containing phosphatidylcholine or phosphatidylethanolamine were pooled, flash evaporated and redissolved in $CHCl_3$. The purity of these phospholipids was demonstrated by thin-layer chromatography (TLC) using commercial standards. Purified lipids were stored in sealed ampules under N_2 at −70°C. Lipids are quantitated by phosphorus determination [14].

Synthesis of 1-(2'-thio-1'-hydroxyethyl)-2-(ethylphosphatidyl)isoindole). The following stock solutions were prepared and stored at −20°C: *O*-phthalaldehyde (50 μmol/ml in methanol, β-mercaptoethanol (20 μmol/ml in methanol) and phosphatidylethanolamine (24 μmol/ml in $CHCl_3$). Solutions of *O*-phthalaldehyde (2.4 ml) and β-mercaptoethanol (20 ml) were mixed and incubated 30 min at 0°C. Phosphatidylethanolamine (5 ml) was then added and incubated for 10 more min. 133 μl of 100% triethylamine was then added, followed immediately by 35 μl of ice-cold HCl/methanol (1 : 1, v/v), and incubation continued at 0°C for additional 10 min. This was followed by seven additions of triethylamine (133 μl each) every 5 min, and incubation was then allowed to continue for 6 h at 0°C. The reaction mixture was flash evaporated to dryness and redissolved in 10—15 ml of petroleum ether. This solution was extracted four times with 14 ml H_2O. The aqueous layer was discarded each time. The organic phase was finally flash evaporated and residual solvent was removed in vacuum. The resulting lipid mixture was then dissolved in $CHCl_3$.

Purification of THEPI. Since the bulk of the unreacted *O*-phthalaldehyde and excess of triethylamine were already removed by H_2O extraction, and excess β-mercaptoethanol removed by evaporation, the purpose of the following chromatographic purification is mainly to separate the fluorescent THEPI from the unreacted phosphatidylethanolamine and some lyso by-product of the reaction. A column (26 × 1 cm) of activated silicic acid (Mallinckrodt cc-4 Silic AR) was first washed with about 100 ml of $CHCl_3$. The reaction products dissolved in about 2 ml $CHCl_3$ were then applied to the column. Stepwise elution was done in the following order: 400 ml $CHCl_3$, then 300 ml each of 5, 7.5, 10 and 20% methanol in $CHCl_3$. Fractions of about 4.5 ml were collected. Free *O*-phthalaldehyde was assayed by adding 0.2 ml β-mercaptoethanol to 2 ml of eluent, followed by 2 μl ethanolamine, and the relative fluorescence was then measured. Phosphatidylethanolamine was assayed by the relative intensity of the purple color after 100 μl of eluent was spotted on a chromatographic paper and sprayed with a ninhydrin reagent [15]. THEPI and its lyso-derivative were assayed by mixing 0.2 ml of eluent

with 2 ml potassium phosphate, 0.2 M, pH 7.8, and measuring the fluorescence. *O*-Phthalaldehyde was also identified by the relative intensity of orange color after spraying with a dinitrophenylhydrazine reagent [15]. The pale-yellowish fractions containing THEPI were pooled, flash evaporated to dryness and redissolved in methanol. It was stored in sealed ampules under N_2 at −70°C in the dark. The overall yield of THEPI after column purification was about 60% as determined by lipid phosphorus assay [14].

Preparation of O-phthalaldehyde-bovine serum albumin conjugates. The protein (50 μM) in 0.1 M potassium phosphate (pH 7.5) containing 0.1 mM β-mercaptoethanol was mixed with *O*-phthalaldehyde (70 μM) at 25°C and the course of the reaction monitored at 335 nm. The reaction reached completion within 7 min and the degree of labelling of the protein (1 mol of ligand/mol of protein) was determined from the absorption spectrum using a molar extinction coefficient of $1.2 \cdot 10^4\ M^{-1} \cdot cm^{-1}$ at 335 nm.

Measurements of absorption and fluorescence. Absorption spectra were measured in a Cary-15 spectrophotometer. Fluorescence spectra were obtained by using either an Aminco-Bowman or a Perkin-Elmer (model 401) spectrofluorometer. When scanning of wavelength was not necessary, the fluorescence of THEPI was measured at an excitation wavelength of 359 nm and emission wavelength of 465 nm. The quantum yield of THEPI was determined by a published method [16], using quinine sulfate ($0.89 \cdot 10^{-6}$ M in 0.2 M potassium phosphate, pH 7.8, quantum yield 0.5) as standard. The fluorescence polarization was measured in an apparatus similar to that described by Weber [17]. Illumination was provided by a xenon lamp with wavelength selected by a quartz prism monochromator. Fluorescence-polarized light was passed through a Corning-glass filter (C-S-3-72). The detector system consisted of an EMI 9502B photomultiplier and a digital voltmeter. The degree of polarization of fluorescence was measured at 25°C with a precision of ±0.02. Fluorescence lifetime measurements were made using the monophoton technique on an Ortec model 9200 nanosecond fluorimeter. A free-running flash lamp operating in air at 1 atm pressure was used as exciting light source. The excitation wavelength (maximum transmission at 365 nm) was selected by a Corning glass filter (C-S-7-83) and the emission was filtered through a C-S-3-72 Corning glass filter. Time calibration of the multichannel analyzer was performed using a 1 μM solution of quinine sulfate in 5 mM H_2SO_4 whose lifetime is 10.5 ns.

Preparation of unilamellar lipid vesicles. A thin film of dry phospholipid or mixture of phospholipids (66 μmol total) containing [^{14}C]phosphatidylcholine (Applied Science, final specific activity 0.15 Ci/mol) was suspended in 6 ml 0.2 M potassium phosphate, pH 7.8, containing 10 μCi [^{3}H]sucrose (15.8 Ci/mmol). The suspension was sonicated as described previously [18]. The sonicate was fractionated by ultracentrifugation in a Beckman 50Ti rotor, spun at 160 000 × *g* for 2 h. The upper 2/3 of the clear supernatant (60—70% of total lipid phosphorus) contained homogeneous small unilamellar vesicles [19] and was collected for optical measurements. A small aliquot (0.1 ml) of the uncentrifuged sonicate was also analysed in a Sepharose 4B column (0.8 × 20 cm). Column was eluted with 0.2 M potassium phosphate, pH 7.8, and fractions of about 0.5 ml were collected. A portion (0.2 ml) of each fraction was diluted to 2 ml with 1% Triton X-100 in 0.2 M potassium phosphate, pH 7.8, and the

fluorescence measured. Both ^{3}H and ^{14}C in each fraction were counted in a liquid scintillation counter.

Electron microscopy. Lipid vesicles were negatively stained with 1% potassium phosphotungstate, pH 7.2, and viewed in a JEM 6c electron microscope, operating at 80 kV. Pictures were taken at 12 000× magnification and further enlarged photographically.

Results and Discussion

Synthesis and purification of THEPI

The order of addition of the three reactants, i.e. *O*-phthalaldehyde, β-mercaptoethanol, and phosphatidylethanolamine, seemed to be important for the optimal yield of the final product, THEPI. Various combinations of addition order, as well as simultaneous addition of all three reactants, have been tried. The best and the most reproducible yields were obtained by mixing *O*-phthalaldehyde and β-mercaptoethanol first and then adding phosphatidylethanolamine after a short incubation. Reaction at 0°C reduced the production of lyso-derivatives. Separation of THEPI and other reactants and byproducts on silicic acid chromatography was very effective. Fig. 1 shows a typical elution profile for such a separation. THEPI was completely eluted with 5% methanol in $CHCl_3$ and well separated from phosphatidylethanolamine and the lyso-derivatives of THEPI. The purified THEPI gave a single fluorescent spot on TLC of silica gel G with a $R_F = 0.82$, when the developing solvent system was $CHCl_3$/ethyl acetate/methanol/acetic acid/H_2O (3 : 8 : 2 : 1 : 1, by vol.). In the same solvent system, phosphatidylethanolamine gave a $R_F = 0.64$. The fluores-

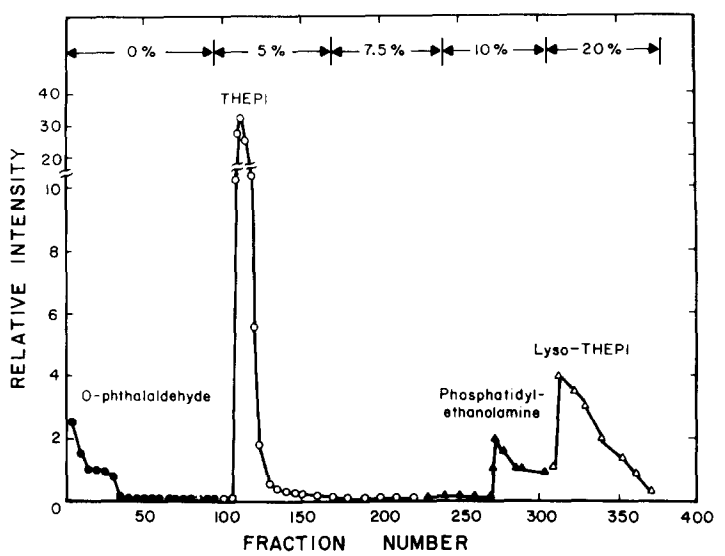
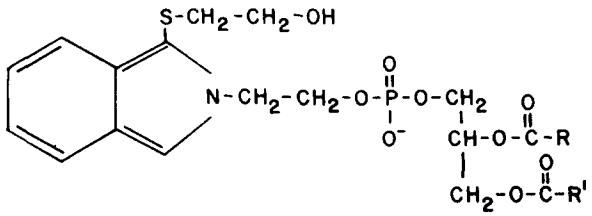


Fig. 1. Purification of THEPI by silicic acid chromatography. The reaction mixture was eluted by increasing concentrations of methanol in $CHCl_3$ (indicated at top of the graph). *O*-Phthalaldehyde (●), THEPI (○) and lyso-THEPI (△) were assayed fluorometrically; phosphatidylethanolamine (▲) was assayed by its ninhydrin reactivity (see text).



R,R' = alkyl chain

1-(2'-thio-1'-hydroxyethyl)-2-(ethylphosphatidyl)-isoindole

THEPI

Fig. 2. Tentatively assigned structure of the synthesized fluorescent phospholipid.

cence of the purified THEPI, stored at 0°C under N_2 in the dark, decayed exponentially with a half-life of 134 h.

Structure of the fluorescent phospholipid

Although the exact structure of the fluorescent phospholipid product was not determined, ample literature evidence suggests the structure shown in Fig. 2 is the most likely one. When propylamine, instead of phosphatidylethanolamine, was used in a reaction similar to the one described here, the structure of the major product was identified as 1-(2'-thio-1'-hydroxyethyl)-2-propylisoindole by infrared and proton NMR spectroscopies [20]. The structure shown in Fig. 2 is also consistent with the expected elution property in silicic acid column chromatography and the expected R_F value in TLC.

Generation of small unilamellar vesicles containing THEPI

When pure THEPI was sonicated in an attempt to generate small unilamellar vesicles, the sonicate did not turn clear. After ultracentrifugation, most of the phospholipid was recovered in the pellet. There was also a thick layer of fluffy, turbid material floating on the top of the supernatant. We concluded that THEPI alone does not form stable bilayer vesicles under this condition. When a mixture of THEPI and phosphatidylcholine (mole ratio up to 1 : 3.4) was used, however, stable unilamellar vesicles were generated. Fig. 3 shows an elution profile of the sonicate chromatographed on a Sepharose 4B column. [^{14}C]-Phosphatidylcholine was eluted as a single peak after the void volume. THEPI, assayed by fluorescence, coeluted with this peak, along with some trapped [^{3}H]sucrose as a marker for the vesicle internal aqueous volume. The upper 2/3 supernatant of the sonicate after ultracentrifugation was also examined in electron microscopy after negative staining. The average diameter of the vesicle seen (Fig. 4) was 325 ± 65 Å, a value that agrees favorably with the one for pure phosphatidylcholine vesicles [21].

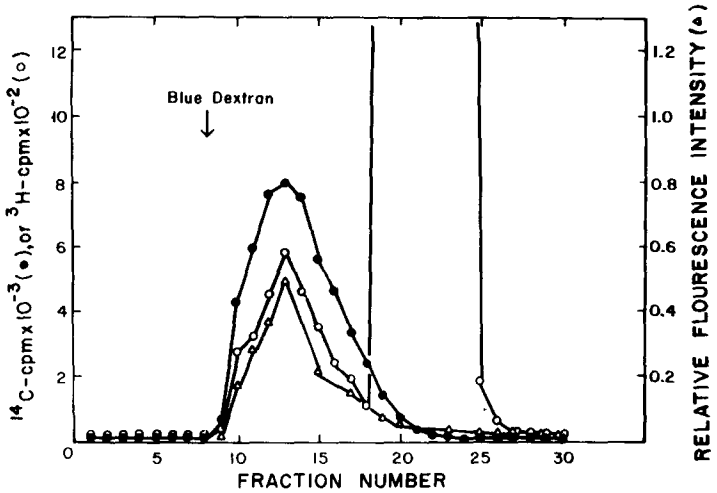


Fig. 3. Sepharose 4B elution profile of the sonicated THEPI and phosphatidylcholine (mol ratio 1 : 3.4) in aqueous buffer. [^{14}C]Phosphatidylcholine (●) and [^{3}H]sucrose (○) were assayed by liquid scintillation counting; THEPI (△) was assayed fluorometrically (see text). Blue dextran was used as a void volume marker.

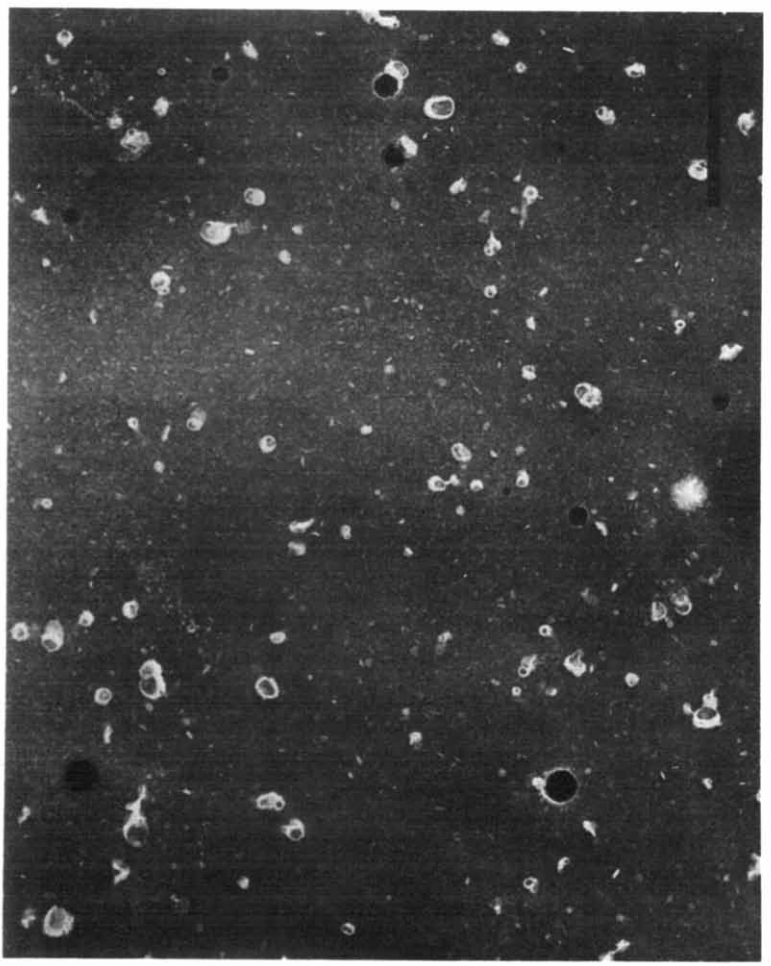


Fig. 4. Electron micrograph of the sonicated THEPI-phosphatidylcholine (mol ratio 1 : 3.4) vesicles after fractionation by ultracentrifugation. Sample was negatively stained. Bar is 1 μm.

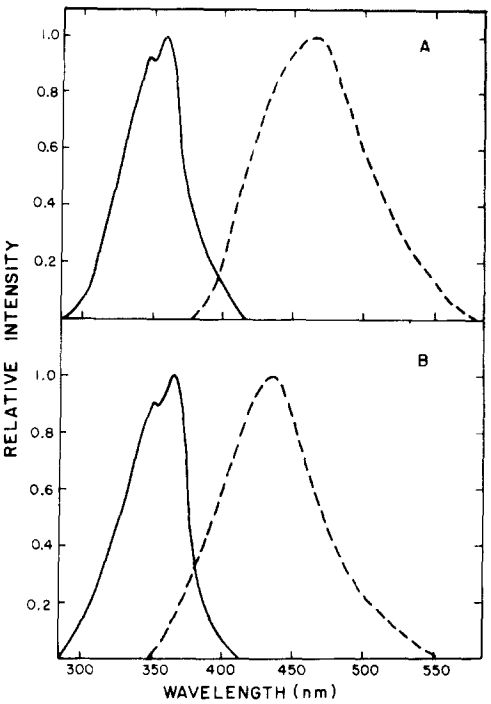


Fig. 5. Excitation (———) and emission (- - - - -) spectra of THEPI (2 mM in 0.2 M potassium phosphate, pH 7.8, panel A) and THEPI-phosphatidylcholine (2.1 mM THEPI, panel B) after fractionation by ultracentrifugation.

Optical properties of THEPI

The absorption spectra of THEPI, either in aqueous solution or incorporated in lipid vesicles, were very similar to the fluorescence excitation spectra in the range of 350—380 nm after correction for the light scattering. The fluorescence excitation and emission spectra of THEPI are shown in Fig. 5. There was a small red shift (about 5 nm) in the excitation maximum when the fluorescent phospholipid was incorporated into lipid bilayer, accompanied with a reduction in the molar extinction coefficient (ϵ_{359nm} = 400 $M^{-1} \cdot cm^{-1}$ for free THEPI in 0.2 M potassium phosphate, pH 7.8, ϵ_{365nm} = 145 $M^{-1} \cdot cm^{-1}$ in vesicles). There was a large blue shift in emission maximum (466 nm for free THEPI, 436 nm in vesicles), with a slight reduction in quantum yield (0.02 for free THEPI, and 0.018 in vesicles). Since the critical micellar concentrations of naturally occurring phospholipids are generally in the range of 10^{-10}—10^{-8} M [22], a millimolar solution of 'free' THEPI used in these measurements probably contained mostly micelles.

Polarization and life time of THEPI fluorescence

The fluorescence polarization of THEPI-phosphatidylcholine vesicles was measured to be 0.146 at 25°C. In comparison, *O*-phthalaldehyde-bovine serum albumin conjugate gave a fluorescence polarization of 0.24. Since the Stoke's

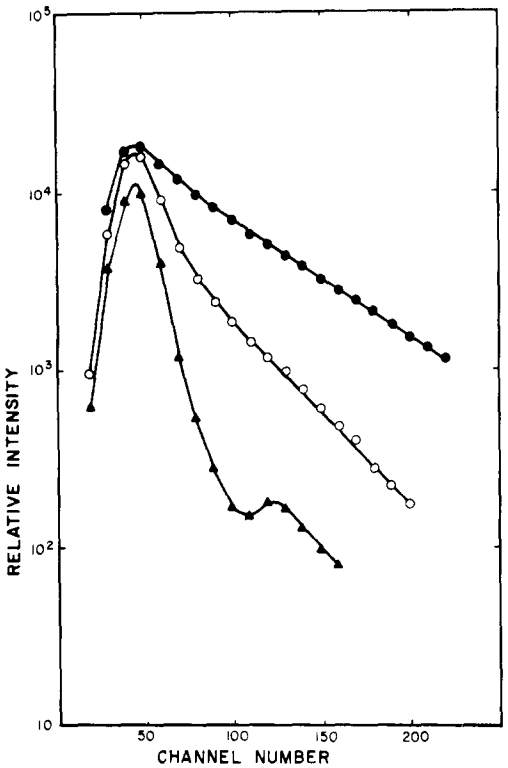


Fig. 6. Time-dependent fluorescence decay of THEPI-phosphatidylcholine vesicles (2.1 mM THEPI, ○), and *O*-phthalaldehyde-bovine serum albumin conjugate (50 μM in 0.2 M potassium phosphate, pH 7.8, ●). Decay of the lamp intensity (▲) is also shown. Sixty channels equal to 16 ns.

radius of small unilamellar vesicles is much greater than that of the bovine serum albumin molecule, the lower polarization value for THEPI-phosphatidylcholine vesicles must indicate a rapid rotational motion of the isoindole moiety of THEPI on the vesicle surface, instead of rapid tumbling of the entire vesicles. Since it is reasonable to assume the isoindole moiety of THEPI locates at the interface of bilayer and bulk aqueous solution, its interaction with H_2O and neighboring charged quarternary amino groups of phosphatidylcholine would result in a relatively short fluorescence life time. Indeed, direct measurement with a nanosecond photon-counting device showed THEPI fluorescence decayed in a multiexponential manner (Fig. 6). Although it is difficult to resolve the fast decay component from that of the lamp, the slow component gave a half-life of 11.1 ns. In comparison, *O*-phthalaldehyde-bovine serum albumin conjugate gave a single decay with a half-life of 16.6 ns. *O*-Phthalaldehyde-labelled proteins generally have a longer half-life and higher quantum yield [11] as compared with *O*-phthalaldehyde-labelled amino acid, e.g. lysine and polylysine (Churchich, J.E., personal communication). It was interpreted that the isoindole moiety locates in a relatively non-polar region in the protein [11].

Potential usage of THEPI in membrane studies

Although THEPI alone cannot form stable lipid vesicles, it does so together with phosphatidylcholine. Most of the fluorescence probes used in membrane studies are hydrophobic and hence only monitor the non-polar interior of the membrane [1,2]. In contrast, the fluorescence moiety of THEPI locates in the hydrophilic region of the membrane. Since the life time, quantum yield and the emission maximum of the isoindole moiety are sensitive to the polarity of the surrounding solvent [23], THEPI may prove valuable in such studies as lipid-protein interactions and the interaction of peripheral proteins with membranes. The emission spectrum of THEPI in lipid vesicles also gives substantial overlap with the excitation spectra of other commonly used fluorescent molecules, e.g. eosin and fluorescein, it may be useful for the energy transfer experiments similar to the dansyl-labelled phospholipid recently described by Fung and Styer [4].

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