Usefulness of the Peptide Segment Separation Method for Asparagine-Rich Protein Syntheses.[1] Synthesis of Malaria Vaccine Analogs Having the Repeated Unit of L-Asparaginyl-L-Alanyl-L-Asparaginyl-L-Prolyl

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In order to demonstrate the usefulness of "the peptide segment separation method" for syntheses of Asn-rich proteins, the central area of circumsporozoite protein of human malaria parasite plasmodium falciparum, Boc-(Asn-Ala-Asn-Pro)$_n$-OBzl (n=2, 3, 4, 6, 9, 12, and 18), were prepared by the coupling reactions of H-(Asn-Ala-Asn-Pro)$_k$-OBzl (k=1, 2, 3, 6, 9, and 12) with Boc-(Asn-Ala-Asn-Pro)$_m$-OH (m=1, 3, or 6) using DCC and HOBt as coupling reagents. The peptide chains are separated into peptide segments by the tertiary peptide bonds of Asn-Pro moieties and they are assembled by the sequence of Asn-Ala-Asn separated by a Pro residue. Regardless of the increase in the peptide chain lengths of the amino and carboxyl components, the coupling reactions in DMF or NMP were achieved in high yields. The excellent solubility of the peptides in highly polar solvents was preserved in spite of the tendency for an Asn residue to have a high potential for aggregation through hydrogen bond formed by the side-chain amide group. The purification of the peptide series by recrystallization could be completely achieved and HPLC on a gel filtration column showed all the peptides to be monodisperse.

Establishment of chemical synthetic methods is of the utmost importance for studies of proteins, especially the syntheses of proteins containing uncorded amino acid residues. For this purpose the severe insolubility of peptide intermediates, i.e. protected peptides, must be primarily overcome as it causes difficulties for their further chain elongation, of their purification, and of their homogeneity assessment. In order to improve the solubility of large peptides, we proposed "the peptide segment separation strategy" by the insertion of tertiary peptide bonds in a peptide chain at suitable intervals.[2] The insertion of tertiary peptide bonds, which are formed by Pro and N^{α}-dimethoxybenzyl amino acid residues, induces the onset of a randomly coiled structure in the solid state and in solution to improve the solubility of large peptides.[2-5] Moreover, the dimethoxybenzyl group is known to be easily removed under suitable deprotection conditions.[6,7] Thus, the peptide segment separation strategy is applicable to the syntheses of large peptides and proteins having any desired sequence.

In a previous paper,[8] we demonstrated the usefulness of the peptide segment separation method for the synthesis of macromolecular peptides by preparing a series of sequential peptides, Boc-[(Leu)$_3$-(Pro)$_2$-Gly]$_n$-OBzl (n=1, 2, 4, 6, 8, 10, and 12). Throughout the synthesis, the coupling reactions in DMF were achieved in high yields regardless of the increase in the peptide chain length of the amino components. In the conformational study of these model proteins by molar rotation, CD,[9] and NMR measurements,[10] it was further shown that N-terminal, internal, and C-terminal segments are sufficiently solvated by highly polar solvents such as MeOH, DMF, DMA, NMP, and DMSO to have a randomly coiled structure.

In the present study, we further demonstrate the usefulness of the peptide segment separation method for the synthesis of the central area of circumsporozoite (CS) protein of human malaria parasite plasmodium falciparum, having the sequence of -(Asn-Ala-Asn-Pro)$_n$- (n=41).[11] The sequence is characteristically rich in Asn residues which have a high potential for aggregation through hydrogen bonds formed by the side-chain amide groups. The sequential peptides having the sequence of (Asn-Pro-Asn-Ala)$_n$ (n=2—4) were prepared by solid-phase peptide synthesis and were examined for their malaria vaccine activity.[12]

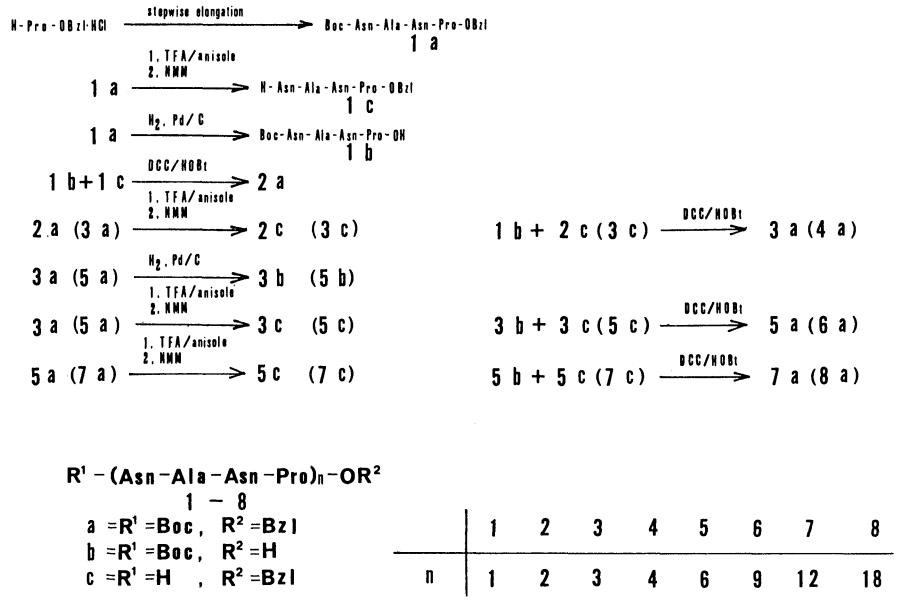
Results and Discussion

Syntheses of Boc-(Asn-Ala-Asn-Pro)$_n$-OBzl (1a—8a). The central area of CS protein has a repeated amino acid sequence of -(Asn-Ala-Asn-Pro)$_n$- (n=41), which is assembled by the sequence of -Asn-Ala-Asn- separated by a Pro residue. Namely, the peptide chain is separated into peptide segments by the tertiary peptide bonds of Asn-Pro moieties. An Asn residue having the amide group in the side chain easily forms the intermolecular hydrogen bond through the side-chain amide group, causing the decrease in the solubility of the peptide intermediates.[13] Thus, the synthesis of the monodisperse-sequential peptides **1a—8a** is a fortunate example in order to demonstrate versatility of the peptide segment separation method even for the synthesis of Asn-rich proteins.

The synthetic procedure for the sequential peptides **1a—8a** is illustrated in Scheme 1. Starting with H-Pro-OBzl as an amino component, tetrapeptide **1a** was prepared by the usual stepwise elongation. In

order to prepare **2a—4a** by fragment condensation, removal of the Bzl group from **1a** was performed in AcOH/MeOH by hydrogenolysis in the presence of Pd/C as catalyst and removal of the Boc group from **1a**, in TFA/anisole (4/1, v/v), respectively. The coupling reaction of the resulting **1b** with **1c** was carried out in DMF using DCC and HOBt[14] as coupling reagents to give **2a** in 82% yield. Peptides **3a** and **4a** were obtained by the coupling reactions of **1b** with **2c** and **3c**, which were obtained by treatment of **2a** and **3a** with TFA/anisole (4/1, v/v), respectively. The reactions were also carried out in DMF using DCC and HOBt as coupling reagents. Further elongation of the peptide chain was performed in NMP using 3.0 equiv of the carboxyl component **3b** or **5b**. The peptides **5a**, **6a**, and **7a** were obtained from **4c**, **5c**, and **6c**, respectively, in high yields regardless of the increase in the peptide chain lengths of the amino and carboxyl components. Deprotection of the carboxyl groups of **3a** and **5a** by hydrogenolysis also proceeded in high yields regardless of the peptide chain lengths, to give the corresponding acids **3b** and **5b**. The completion of the deprotection was confirmed by the UV spectra of the products which showed no absorption at 310 nm due to the Bzl group. The coupling reaction of the carboxyl component **5b** with the amino component **7c** also could be achieved in NMP to give doheptacontapeptide **8a** in 71% yield. The synthetic results of **1a—8a** are summarized in Table 1. All the peptides obtained were easily purified by repeated recrystallizations and, as a result of this purification process, each peptide gave a single peak on HPLC. The amino acid and elemental analyses of the peptides shown in Tables 1 and 2 were in good agreement with the calculated values.

Throughout the syntheses of **1a—8a**, the peptides kept excellent solubility and high reactivity. The insertion of tertiary peptide bonds into the peptide chain at suitable intervals clearly plays an important role in maintaining sufficient solvation of the peptide chains, resulting in the exposure of the reactive amino- and carboxyl-termini in solution. These results indicate that the peptide segment separation method has versatility for syntheses of proteins having



Scheme 1.

Table 1. Synthetic Results and Amino Acid Analyses of Peptides **1a—8a**

Compound	Yield/%	Recrystallization solvents	$[\alpha]_D^{25}$ (c 1.0 in DMF)	Found (Calcd)		
				Asp	Ala	Pro
1a·0.5H_2O	70	AcOEt	−59°	1.93 (2)	1.00 (1)	1.00 (1)
2a·2.5H_2O	82	i-PrOH	−68°	4.28 (4)	1.91 (2)	2.00 (2)
3a·6.0H_2O	78	MeOH/i-PrOH	−90°	6.00 (6)	2.90 (3)	3.18 (3)
4a·7.0H_2O	64	MeOH/i-PrOH	−78°	8.00 (8)	3.78 (4)	4.21 (4)
5a·14.0H_2O	92	MeOH	−86°	12.00 (12)	6.38 (6)	5.69 (6)
6a·25.5H_2O	77	H_2O/MeOH	−72°	17.46 (18)	9.74 (9)	9.00 (9)
7a·28.5H_2O	77	H_2O/MeOH	−86°	22.91 (24)	12.68 (12)	12.00 (12)
8a·35.0H_2O	71	H_2O/MeOH	−102°	34.76 (36)	18.71 (18)	18.00 (18)

Table 2. Elemental Analyses of Peptides **1a—8a**

Compound	Formula	Found (Calcd)		
		C/%	H/%	N/%
1a·0.5H_2O	$C_{28}H_{41}N_6O_{9.5}$	54.95 (54.80)	6.60 (6.73)	13.66 (13.69)
2a·2.5H_2O	$C_{44}H_{69}N_{12}O_{17.5}$	50.69 (50.51)	6.39 (6.64)	15.79 (16.06)
3a·6.0H_2O	$C_{60}H_{100}N_{18}O_{27}$	47.76 (47.86)	6.73 (6.69)	16.88 (16.74)
4a·7.0H_2O	$C_{76}H_{126}N_{24}O_{34}$	47.77 (47.54)	6.53 (6.61)	17.47 (17.50)
5a·14.0H_2O	$C_{108}H_{188}N_{36}O_{53}$	45.67 (45.69)	6.35 (6.67)	17.74 (17.76)
6a·25.5H_2O	$C_{156}H_{283}N_{54}O_{82.5}$	44.18 (44.24)	6.39 (6.73)	17.89 (17.85)
7a·28.5H_2O	$C_{204}H_{361}N_{72}O_{103.5}$	44.67 (44.72)	6.48 (6.64)	18.13 (18.41)
8a·35.0H_2O	$C_{300}H_{518}N_{108}O_{146}$	45.20 (45.19)	6.49 (6.54)	18.75 (18.97)

Table 3. Solubility Properties of Peptides **1a—8a** (c=1.0 g dl^{-1})[a,b]

Compound	Solvent												
	DMF DMA	NMP DMSO	HMPA	AcOH	MeOH	EtOH	*i*-PrOH	PrOH	AC AN AcOEt	THF Dioxane	CH_2Cl_2	TFE/CH_2Cl_2 (1/4, v/v)	TFE HFIP	H_2O
1a	A	B	A	A	B	B	B	C	B	A	A	A	B	
2a	A	A	C	A	A	B	A	C	B	C	A	A	A	
3a	A	B	C	B	C	C	B	C	C	C	A	A	A	
4a	A	B	C	C	C	C	B	C	C	C	A	A	A	
5a	A	B	C	B	C	C	B	C	C	C	A	A	A	
6a	A	C	C	C	C	C	C	C	C	C	A	A	A	
7a	A	C	C	C	C	C	C	C	C	C	A	A	A	
8a	A	C	C	C	C	C	C	C	C	C	A	A	A	

a) Solubility: A, soluble at room temperature; B, soluble at 80 °C or refluxing temperature; C, partially soluble or practically insoluble at 80 °C or refluxing temperature. b) Abbreviations: AC, acetone; AN, acetonitrile. Others, see Ref. 1.

any desired sequence.

Solubility Properties of Sequential Peptides 1a—8a. Solubility properties of sequential peptides **1a—8a** are summarized in Table 3. Compared with the solubility of Boc-[(Leu)$_3$-(Pro)$_2$-Gly]$_n$-OBzl (n=1, 2, and 4), that of peptides **1a—5a** is lower in medium-polar solvents such as AcOEt, THF, CH_2Cl_2, acetone, and dioxane, suggesting that the side-chain amide groups of the Asn residues form an intermolecular hydrogen-bonding network in the solid state. Peptides **4a—8a** are also insoluble in AcOH, MeOH, EtOH, PrOH, and *i*-PrOH, being in contrast to Boc-[(Leu)$_3$-(Pro)$_2$-Gly]$_n$-OBzl having the corresponding peptide chain lengths. The hydrogen bond-disrupting potential of hydrogen donor solvents is in good relationship with their electron acceptor numbers,[15] and those of TFE (59) and water (54.8) are slightly larger than those of AcOH (52.9) and MeOH (41.3).[15,16] Thus, it is remarkable that peptides **1a—8a** are easily soluble in TFE/CH_2Cl_2 (1/4, v/v) and water. On the other hand, the excellent solubility of all the peptides in the strong electron donor solvents DMF, NMP, DMA, and DMSO is observed regardless of the increase in the peptide chain length, indicating that the peptide segment separation method is useful to improve the solubility of Asn-rich large peptides in these solvents. Although HMPA has the highest value of electron donor number in the solvents shown in Table 3, the solubility of peptides **1a—8a** in HMPA is as poor as that of Boc-[(Leu)$_3$-(Pro)$_2$-Gly]$_n$-OBzl in HMPA.

Purification of the Sequential Peptides and Their Homogeneity Assessment by HPLC. The solubility of the peptide series in alcoholic solvents decreases gradually with the increase in the peptide chain lengths. Thus, the purification of the peptide series by recrystallization from a mixture of MeOH and *i*-PrOH or water and MeOH could be achieved by changing their ratios along with the increase in the peptide chain lengths. Each peptide purified by recrystallization gave a single peak on HPLC on a gel filtration column packed with a styrene-divinylbenzene copolymer[17] or a 2-hydroxyethyl methacrylate-ethylene dimethacrylate coplymer,[18] showing all the peptides to be monodisperse. Figure 1 shows the relationship between the molecular weight and the elution volume for peptides **1a—8a**, indicating that HPLC on a gel filtration column is valuable for assessment of the homogeneity of the peptides separated into peptide segments by tertiary peptide bonds. The linear relationship in Fig. 1a suggests that conformational preference of all the peptides in DMF is the same regardless of the peptide chain length. The structure of the peptide series will be reported elsewhere.

In conclusion, the synthetic strategy of the peptide segment separation is a remarkably promising method for synthesizing Asn-rich proteins. In addition to the excellent solubility and high reactivity of the peptides

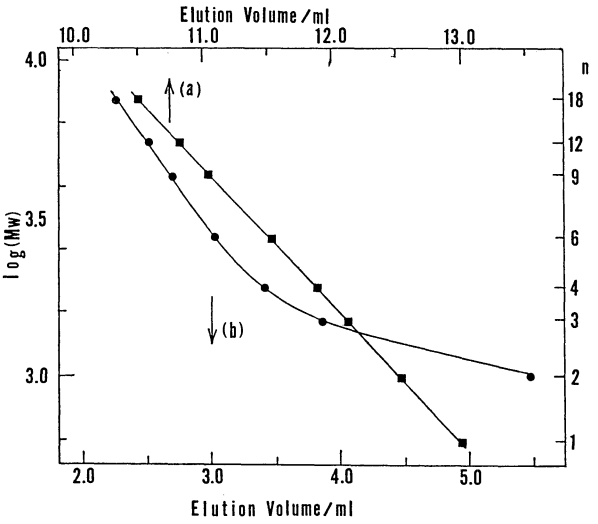


Fig. 1. Relationship between molecular weight and elution volume for **1a—8a** using gel filtration columns packed with (a) the styrene-divinylbenzene copolymer and with (b) the 2-hydroxyethyl methacrylate-ethylene dimethacrylate copolymer. The operating conditions for (a): solvent, DMF; flow rate, 1 ml min^{-1}; chart speed, 1 cm min^{-1}; temperature, at room temperature. The operating conditions for (b): solvent, water; flow rate, 0.5 ml min^{-1}; chart speed, 1 cm min^{-1}; temperature, at room temperature.

separated into peptide segments, they are readily susceptible to easy purification by recrystallization and facile assessment of homogeneity by HPLC on a gel filtration column.

Experimental

General. The uncorrected capillary melting points are reported. The optical rotations were taken in a 1 cm cell on a JASCO model ORD/UV-5 optical rotatory dispersion recorder. The amino acid compositions of acid hydrolysates were determined with a Shimazdu HPLC LC-3A all amino acid analysis system. The acid hydrolyses of the peptides were carried out with 12 M HCl (1 M=1 mol dm^{-3}) or propionic acid/12 M HCl (2/1, v/v) for 2—8 days at 115 °C in evacuated and sealed tubes. HPLC equipments for columns packed with a styrene-divinylbenzene copolymer[8] (7.5×500 mm), exclusion limit of 20000, and with a 2-hydroxyethyl methacrylate-ethylene dimethacrylate copolymer[19] (4.6×250 mm), exclusion limit of 80000, were described previously. The operating conditions of HPLC are given in the legend of Fig. 1.

Boc-Asn-Ala-Asn-Pro-OBzl. Boc-Asn-OH (30.2 g, 0.13 mol) and HOBt (17.6 g, 0.13 mol) were added to a solution of H-Pro-OBzl·HCl (24.1 g, 0.1 mol) in CH_2Cl_2 (400 ml) containing TEA (13.2 g, 0.13 mol). After a few minutes, the mixture became heterogeneous, then a solution of DCC (26.8 g, 0.13 mol) in CH_2Cl_2 (50 ml) was added. The mixture was stirred at room temperature overnight, cooled by ice-chilled bath, and filtered. The filtrate was washed with water, followed by 10% aqueous citric acid, water, 5% aqueous sodium hydrogencarbonate, and water, then dried (Na_2SO_4), and evaporated to dryness. The residue was recrystallized from AcOEt/hexane (300 ml/100 ml), yielding 36.5 g (87%) of Boc-Asn-Pro-OBzl, mp 104—106 °C (lit,[20] mp 115—116 °C). The dipeptide (36.5 g, 87 mmol) was dissolved in 3.5 M HCl-AcOEt (300 ml) and the solution was stirred for 2 h under cooling in an ice-chilled bath, then evaporated in vacuo. The residue was dissolved in CH_2Cl_2 (250 ml) containing NMM (10.6 g, 0.1 mol) and, Boc-Ala-OH (18.9 g, 0.1 mol) and HOBt (13.5 g, 0.1 mol) were added. The mixture was stirred under cooling in an ice-chilled bath, after which a solution of DCC (20.6 g, 0.1 mol) in CH_2Cl_2 (50 ml) was added. The mixture was stirred at 0 °C for 2 h, at room temperature overnight, then filtered. The filtrate was subjected to the work-up procedure mentioned above. The residue was recrystallized from AcOEt/hexane (250 ml/50 ml), yielding 35.0 g (82%) of Boc-Ala-Asn-Pro-OBzl, mp 105—108 °C. The tripeptide (34.9 g, 71 mmol) was dissolved in TFA/anisole (80 ml/20 ml), and the solution was stirred at room temperature for 1 h, then hexane/ether (1/1, v/v) was added. A residual oil was triturated under hexane and ether repeatedly. The solid was collected by filtration, dried over KOH pellets in vacuo for 3 h, and then dissolved in DMF (100 ml) together with NMM (9.3 g, 92 mmol), Boc-Asn-OH (21.3 g, 92 mmol), HOBt (12.4 g, 92 mmol), and DCC (19.9 g, 92 mmol). After stirring overnight, the solution was filtered, the filtrate was concentrated in vacuo, and the residue was washed with aqueous sodium hydrogecarbonate and water, and recrystallized from AcOEt (200 ml) to give 30.0 g (70%) of Boc-Asn-Ala-Asn-Pro-OBzl. It was recrystallized from AcOEt to give a material, mp 144—147 °C.

Boc-Asn-Ala-Asn-Pro-OH. The terapeptide (18.1 g, 30 mmol) was dissolved in MeOH containing a small amount of AcOH and hydrogenated over 10% Pd/C (1.8 g) at room temperature for 36 h. After removal of the catalyst by filtration, the filtrate was evaporated in vacuo. The residue was filtered using ether and recrystallized from AcOEt to give Boc-Asn-Ala-Asn-Pro-OH, 13.6 g (88%).

A General Procedure for the Preparation of Boc-(Asn-Ala-Asn-Pro)$_n$-OBzl (n=2, 3, and 4). Boc-(Asn-Ala-Asn-Pro)$_k$-OBzl (k=1, 2, and 3) (10 mmol) was treated with TFA/anisole (12 ml/3 ml) as usual. H-(Asn-Ala-Asn-Pro)$_k$-OBzl·TFA obtained was dissolved in DMF (30 ml) together with NMM (1.1 g, 11 mmol), Boc-Asn-Ala-Asn-Pro-OH (5.7 g, 11mmol), HOBt (1.5 g, 11 mmol), and DCC (2.2 g, 11 mmol). The mixture was stirred under cooling in an ice-chilled bath for 2 h, at room temperature for 48 h, then cooled in an ice-chilled bath, and filtered. The filtrate was poured into AcOEt and the resulting solid was collected by filtration and purified by recrystallization from suitable solvents shown in Table 1.

Boc-(Asn-Ala-Asn-Pro)$_m$-OH (m=3 and 6). Boc-(Asn-Ala-Asn-Pro)$_m$-OBzl (m=3 and 6) (2 mmol) was dissolved in water containing a small amount of AcOH and hydrogenated for 48 h. Removal of catalyst followed by evaporation gave an oily residue, which was solidified by the addition of ether. The resulting solid was collected by filtration and purified by recrystallization.

A General Procedure for the Preparation of Boc-(Asn-Ala-Asn-Pro)$_n$-OBzl (n=6, 9, 12, and 18). Boc-(Asn-Ala-Asn-Pro)$_n$-OBzl (n=6 and 9) was prepared in NMP by the coupling reactions of H-(Asn-Ala-Asn-Pro)$_k$-OBzl (k=3 and 6) with 3.0 equiv of Boc-(Asn-Ala-Asn-Pro)$_3$-OH and Boc-(Asn-Ala-Asn-Pro)$_n$-OBzl (n=12 and 18), by the cou-

pling reactions of H-(Asn-Ala-Asn-Pro)$_k$-OBzl (k=6 and 12) with 3.0 equiv of Boc-(Asn-Ala-Asn-Pro)$_6$-OH. The procedure was essentially the same as that for the preparation of Boc-(Asn-Ala-Asn-Pro)$_n$-OBzl (n=2, 3, and 4). Treatment of Boc-(Asn-Ala-Asn-Pro)$_k$-OBzl (k=3, 6, 9, and 12) with TFA/anisole (9/1, v/v) was carried out for 1.5 h instead of 1 h, the solution was then poured into ether and the resulting solid collected by filtration, washed with ether, and *i*-PrOH repeatedly, and dried over KOH pellets in vacuo.

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1) The abbreviations for amino acids are those recommended by the IUPAC-IUB Commission on Biochemical Nomenclature, *J. Biol. Chem.*, **247**, 977 (1972). Amino acid symbols except for Gly denote the L-configuration. Additional abbreviations used are the following: Boc, *t*-butoxycarbonyl; OBzl, benzyl ester; DCC, dicyclohexylcarbodiimide; HOBt, 1-hydroxy-1*H*-benzotriazole; DMF, *N*,*N*-dimethylformamide; NMP, *N*-methylpyrrolidone; HPLC, high-performance liquid chromatography; CD, circular dichroism; NMR, nuclear magnetic resonance; AcOH, acetic acid; TFA, trifluoroacetic acid; TEA, triethylamine; NMM, *N*-methylmorpholine; MeOH, methanol; DMA, *N*,*N*-dimethylacetamide; DMSO, dimethyl sulfoxide; UV, ultraviolet; AcOEt, ethyl acetate; THF, tetrahydrofuran; EtOH, ethanol; PrOH, 1-propanol; *i*-PrOH, 2-propanol; TFE, 2,2,2-trifluoroethanol; HMPA, hexamethylphosphoric triamide; HFIP, 1,1,1,3,3,3-hexafluoro-2-propanol.

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