Cation-Binding Cyclic Peptides with Lipophilic Tails

C. M. DEBER* and P. D. ADAWADKAR, *Research Institute, Hospital for Sick Children, Toronto, M5G 1X8, and Department of Biochemistry, University of Toronto, Toronto, M5S 1A8, Ontario, Canada*

Synopsis

We have synthesized and characterized a series of cation-binding cyclic octapeptides which may function as potential ionophoric substances. The materials contain varying degrees of hydrophobic character, which was controlled systematically through the incorporation of *N*-alkylglycine residues where *N*-alkyl = methyl, *n*-hexyl, cyclohexyl, or *n*-decyl. The peptides reported include cyclo(Phe-Sar-Gly-Sar)$_2$, cyclo(Glu(OBzl)-Sar-Gly-Sar-Glu(OBzl)-Sar-Gly-(*N*-decyl)Gly), cyclo(Glu(OBzl)-Sar-Gly-(*N*-decyl)Gly)$_2$, cyclo(Glu-(OBzl)-Sar-Gly-(*N*-hexyl)Gly)$_2$, cyclo(Glu(OBzl)-Sar-Gly-(*N*-cyclohexyl)Gly)$_2$, and the corresponding free diacid forms of the Glu-containing compounds. Using ^{13}C- and ^{1}H-nmr spectra, we demonstrated that the mixture of *cis/trans* peptide bond-isomer conformers, characteristic of the free-peptide benzyl esters in solution, was converted to unique C_2-symmetric, presumably all-*trans* conformers on complexation with calcium ions. Cation-transport experiments, using the thick-liquid model of transport in a Pressman cell, established that these compounds transport a variety of cations and that one peptide examined in detail, cyclo(Glu(OBzl)-Sar-Gly-(*N*-decyl)Gly)$_2$ (selectivity $Ca^{2+} > Na^{+} > K^{+} > Mn^{2+} > Cu^{2+} > Mg^{2+} > Co^{2+} > Zn^{2+}$), transports calcium about an order of magnitude more efficiently than magnesium.

Physiological and biochemical investigations of transmembrane ion transport of cations, anions, and small organic molecules (e.g., amino acids) have been aided in recent years by a new class of carrier molecules—termed *ionophores*—which selectively increase the ion permeability of biological membranes.[1-3] Many ionophores are of microbial origin (having been recognized through their antibiotic activity[4]) and have diverse chemical structures, often cyclic or pseudocyclic chains containing amino and hydroxyl groups.[5] However, few have the structural features of materials normally found in animal tissues. Gates in nerve or pores and channels in membranes which may regulate ion transport *in vivo* are fairly certain to consist of proteinlike materials. This circumstance suggested that suitably chosen peptides should have ion-binding and perhaps ion-transporting capability. With respect to ease of synthesis, systematic modification of sequence, structural resemblance of functional sites to natural ionophores, and amenability of studying by biophysical techniques, the cyclic peptides are ideal candidates for a new series of ionophoric substances.

* To whom correspondence should be addressed.

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Attempts to date to prepare synthetic ionophores have been limited largely to those which are analogs of known natural ionophores, particularly the cyclodepsipeptide valinomycin.[6,7] A survey of the structural features, solubility properties, etc., of these and other naturally occurring ionophores suggested that among several features which must be present concomitantly to generate cation-transporting activity, lipophilic character is essential. Since the potentially ionophoric peptides must contain polar (carbonyl) or perhaps ionizable groups (carboxylate) for direct interaction with charged cations, the problem of solubilization (for partitioning) into the membranous phase must be overcome through incorporation of hydrophobic residues. In naturally occurring substances, this is usually accomplished with Val, Leu, Ileu, Phe residues, etc.; in the synthetic peptides described below, we have used *N*-alkylglycine residues, where the size, shape, and lipophilicity of the alkyl group can be varied systematically. Further advantages of this approach are that *N*-alkylglycines are optically inactive, thus avoiding any racemization problems during synthesis, and the residues themselves are *imino* acids whose presence (*vide infra*) influences conformation favorably and produces potential liganding sites.

Because of the universal importance of calcium ions in "cell communication"[8]—for example, as the coupling factor between excitation and contraction in all forms of muscle—we have focused on the development of calcium-transporting ionophores. The best-known naturally occurring ionophore largely specific for transporting divalent cations versus monovalents, A23187,[9] does not select between calcium and magnesium transport in many systems.[10] Reports to date of the design of synthetic calcium-selective ionophores have emphasized the chelating properties of certain diglycolamic acids and diamides and their hydrophobic analogs.[11-13] It is, in part, with the goals of producing peptides which will transport divalent cations such as calcium or magnesium selectively, as well as gaining information concerning the structural requirements for binding sites of transport proteins, that the following investigation was undertaken.

EXPERIMENTAL

Materials

t-Butyloxycarbonyl (Boc) amino acids were purchased from Peninsula Laboratories, San Carlos, California. *N*-methyl-morpholine, *iso*-butyl-chloroformate, *N*-decyl aldehyde, *N*-decylamine, and sodium cyanoborohydride were bought from Aldrich Chemical Co. Chloroform-*d* was ordered from Merck Sharp and Dohme Canada, Limited. Cyclohexylamine and iodoacetic acid were purchased from British Drug House (BDH), Toronto. Pyridine "Baker Instra-analyzed" was obtained from Can-Lab, Toronto.

General Synthetic Methods

t-Butyloxycarbonyl (Boc) groups were used for *N*-protection, while benzyl esters (-OBzl) provided *C*-protection. Coupling between peptide fragments was accomplished by mixed anhydride reactions using *N*-methylmorpholine and *iso*-butylchloroformate. Removal of Boc groups was effected with HCl–ethyl acetate. Benzyl esters were converted to free acids by hydrogenation with 10% palladium/charcoal in *t*-butyl alcohol. Precursor peptides for cyclization were prepared by activation of *C*-terminal acids with *p*-nitrophenylesters followed by removal of *N*-terminal Boc groups using anhydrous HCl. The resulting peptide-active ester hydrochlorides were dissolved in dimethylformamide and added to pyridine under conditions of high dilution. Throughout, intermediate peptides were crystallized where possible, and generally characterized by a combination of ir, nmr, and TLC (thin-layer chromatography) procedures.

Details of Synthesis

Boc-Gly-Sar-OBzl (1)

Boc-Gly-OH (43.75 g, 0.25 mol) was dissolved in 250 ml chloroform, and the solution was cooled to −20°C using CCl_4/dry ice. *N*-methylmorpholine (26.9 ml, 0.25 mol) and isobutylchloroformate (33.4 ml, 0.25 mol) were added, and stirring was continued for 20 min at −20°C. H-Sar-OBzl·tosylate (87.75 g, 0.25 mol) was then added, followed by an additional equivalent of *N*-methylmorpholine (26.9 ml). The reaction temperature was maintained at −20°C for 1 hr, then allowed to warm to room temperature slowly as the dry ice evaporated, and stirring was continued at room temperature overnight. The reaction mixture was transferred into a separatory funnel and washed twice with cold water, twice with 5% $NaHCO_3$ solution, once with saturated NaCl solution, then dried and concentrated, to give a pale yellow oil, 59.5 g, 71% yield. Crystallization from diethyl ether/hexane gave 31.5 g (53%) of dipeptide, mp 62–63°C.

HCl-H-Gly-Sar-OBzl (2)

Boc-dipeptide (1) was dissolved in ethyl acetate, cooled in an ice bath, and anhydrous HCl was bubbled in for 5 min with stirring. After 1 hr at 0°C, and an additional 15 min without the ice bath, solvent was removed, and the crude product was triturated with ether to yield a white powder in quantitative yield. This material displayed appropriate TLC and was used directly in the preparation of tripeptide (3).

Z-Sar-Gly-Sar-OBzl (3)

The preparation of tripeptide 3 was accomplished by the mixed anhydride procedure as described for Boc-Gly-Sar-OBzl. After similar work-up, the tripeptide was obtained as a syrup, which was crystallized from ethyl acetate/ether in 80% yield, mp 82–84°C.

H-Sar-Gly-Sar-OH (4)

The protected tripeptide (3, 19.1 g) was dissolved in 150 ml of methanol, and 10% Pd/C (500 mg, suspended in 3 ml water) was added, along with 2.5 ml of glacial acetic acid. Then hydrogen gas was bubbled into the reaction mixture overnight with stirring at room temperature. As the reaction proceeded, the product precipitated as a white solid and was redissolved by adding more water. The catalyst was then removed by filtration through Celite, and the solvents were evaporated to obtain the tripeptide as a white solid (6.7 g, 71% yield).

Boc-Glu(γOBzl)-Sar-Gly-Sar-OH (5)

A solution of Boc-Glu-(γOBzl)-OH (8.1 g, 0.024 mol) in 100 ml dimethoxyethane was cooled to −20°C with dry ice–CCl_4. *N*-methylmorpholine (2.68 ml, 0.024 mol) and isobutylchloroformate (3.14 ml, 0.024 mol) were added in succession, with stirring. After 20 min at −20°C, a solution of H-Sar-Gly-Sar-OH (5.8 g, 0.0264 mol) and NaOH (0.07 g, 0.0264 mol) in 50 ml water was added. The reaction mixture was allowed to warm to room temperature as the dry ice evaporated while stirring was continued at room temperature overnight. Solvent was then removed by rotary evaporation, water was added to the oily residue, the mixture was adjusted to pH 3 using 0.1*N* citric acid, and extracted three times with 50 ml ethyl acetate. The ethyl acetate layers were combined, washed once with water, twice with saturated NaCl, and dried over sodium sulfate, and the solvent was removed to yield the tetrapeptide as a white fluffy solid. Traces of unreacted Boc-Glu-(γOBzl)OH were removed by trituration with ether. After drying over high vacuum, tetrapeptide 5 (11.4 g) was obtained in 89% yield. TLC: single spot ($MeOH/CHCl_3$, 1:3), R_f = 0.14.

HCl-H-Glu-(γOBzl)-Sar-Gly-Sar-OH (6)

This hydrochloride was prepared by removal of the Boc group of tetrapeptide 5, as described above for tripeptide 2.

Boc-(Glu(γOBzl)-Sar-Gly-Sar)$_2$-OH (7)

Octapeptide 7 was prepared from peptides 5 and 6 by the mixed anhydride method using the same procedure as described above for the preparation of tetrapeptide 5 with the following modifications: (1) the amine

component (HCl-Glu(γOBzl)-Sar-Gly-Sar-OH, 6) was dissolved in dimethylformamide rather than in water, and (2) 2 eq of *N*-methylmorpholine were added after addition of 6. After evaporation of solvents, and work-up as described for 5 and trituration with ether, the Boc-octapeptide 7 was obtained as a white powder (7.49 g, 84% yield).

HCl[Glu(γOBzl)-Sar-Gly-Sar]$_2$-ONp (8)

Boc-octapeptide (7) (3.12 g, 0.003 mol) was treated with *p*-nitrophenol (417 mg, 0.003 mol) and dicyclohexylcarbodiimide (DCC) (618 mg, 0.003 mol) in 50 ml chloroform at 4°C and stirred overnight. After removal of solvent, 20 ml acetone was added to the gummy product, and the insoluble urea was removed by filtration. Then five drops of glacial acetic acid were added to the filtrate, and the reaction was stirred at 4°C for 1 hr. Additional urea formed was removed by filtration. Removal of solvent and trituration with ether gave Boc-octapeptide-*p*-nitrophenyl ester as an off-white powder (2.29 g, 99% yield).

This *p*-nitrophenyl ester was dissolved directly in ethyl acetate and treated with anhydrous HCl as described above for tripeptide 2. After trituration with ether, the product (8) was obtained as a white powder (2.1 g, 97% yield), which was used directly in the ensuing cyclization procedure.

Cyclo[Glu-(γOBzl)-Sar-Gly-Sar]$_2$ (9)

The *p*-nitrophenyl ester hydrochloride octapeptide 8 (700 mg) was dissolved in dimethylformamide (5 ml, dried over anhydrous sodium sulfate and containing 2–4 drops of glacial acetic acid) and added dropwise over 3-hr period with efficient stirring to 300 ml of spectral-grade pyridine at room temperature. Stirring was continued for 48 hr at room temperature, during which the reaction mixture took on a bright yellow color. Solvents were then removed completely by a combined rotary evaporator–high vacuum pump system at 45°C. The residue was treated with a few ml of acetone, and the acetone-insoluble powdery material (156 mg) was isolated. This product displayed appropriate ir, nmr, and TLC behavior and was confirmed to be cyclic octapeptide by mass spectrum (molecular ion peak 836, Table I).

[The acetone-soluble fraction on removal of solvent gave a gummy product, which was treated with Rexyn (Fisher I-300) mixed-bed ion-exchange resin (10 g) in 1:1 ethanol/H_2O (300 ml) with stirring for 1 hr. The resin was removed by filtration, and after removal of solvent, 220 mg of residue was obtained. This material showed one major spot on TLC (MeOH/$CHCl_3$, 1:3, R_f = 0.5). When crystallized from chloroform/ethyl acetate, 150 mg of white solid was obtained, for which available characterization data—particularly the observation of a molecular ion peak in the mass spectrum at 1672—suggest the structure of a cyclic(hexadecyl)-peptide, cyclo(Glu(OBzl)-Sar-Gly-Sar)$_4$.]

TABLE I
Physical Characteristics of Cyclic Octapeptides[a]

	(Phe-Sar-Gly-Sar)$_2$	OR (Glu-Sar-Gly-Sar)$_2$	OR (Glu-Sar-Gly-Sar- OR Glu-Sar-Gly-*N*-(decyl)Gly)	OR (Glu-Sar-Gly-*N*-(decyl)Gly)$_2$	OR (Glu-Sar-Gly-*N*-(hexyl)Gly)$_2$	OR (Glu-Sar-Gly-*N*-(cyclohexyl)Gly)$_2$
Method of isolation as benzyl esters[b] (% yield)	Resin treatment; acetone-insoluble fraction (41%)	Crystallized from MeOH (25%)	Resin treatment; crystallized from ethyl acetate/diethyl ether (22%)	Resin treatment; crystallized from MeOH (12%)	Crystallized from MeOH/ diethyl ether (13%)	Resin treatment; crystallized from H_2O/MeOH[c] (9%)
		Ester Acid	Ester Acid	Ester Acid	Ester Acid	Ester Acid
Mol. wt.	692	836 656	962 782	1088 908	976 796	972 792
Mass spectrum (m/e)	692 (m^+)	836 (m^+)	962 (m^+) 963 (m + 1); 964 (m + 2)	1089 (m + 1); 1090 (m + 2)	976 (m^+) 977 (m + 1); 978 (m + 2)	973 (m + 1); 974 (m + 2); 975 (m + 3)

mp (°C)	284–285	264–265	200–202	232–233	142–147	212–213	131–133	240–242	226	244–245	256–257
Soluble[d] in	TFE, $CHCl_3$(Sl)	TFE	TFE, H_2O	TFE, $CHCl_3$, EtOH	TFE, H_2O, $CHCl_3$, EtOH	TFE, $CHCl_3$	TFE, $CHCl_3$, EtOH	TFE, $CHCl_3$, EtOH	TFE, H_2O, $CHCl_3$, EtOH	TFE, $CHCl_3$, EtOH	TFE, $CHCl_3$, H_2O, EtOH
Insoluble in	H_2O, EtOH	H_2O $CHCl_3$ EtOH	$CHCl_3$ EtOH	H_2O	—	H_2O EtOH	H_2O	H_2O	—	H_2O	—
TLC[e] (R_f)	0.09[f]	0.78[g]	0.38[g]	0.24[g]	0.24[h]	0.88[f]	0.48[h]	0.34[f]	0.37[h]	0.33[f]	0.30[h]

[a] R = Bzl, benzyl ester; R = H, free acid.

[b] Except for Phe compound. Acids were obtained by hydrogenation of benzyl esters in t-BuOH or MeOH, using 10% Pd/C, and isolated as ether-insoluble fraction.

[c] Cyclic octapeptide obtained via cyclodimerization of linear tetrapeptide.

[d] Given as "soluble" if 1 mg/ml dissolved.

[e] Quanta-Gram Q6F silica gel plate.

[f] TLC solvent system, ethyl acetate/acetone/methanol/acetic (40:40:15:5).

[g] TLC solvent system, n-BuOH/acetone/water/acetic acid (4:3:2:1).

[h] TLC solvent system, ethyl acetate/acetone/methanol/acetic acid (30:30:30:10).

N-*Decyl-Glycine Benzyl Ester* (*10*): *Method A*

To a 100-ml round-bottom flask were added 4.04 g (0.012 mol) glycine benzyl ester *p*-tosylate, 1.56 g (0.01 mol) decyl-aldehyde, 0.377 g (0.006 mol) of sodium cyanoborohydride, and 50 ml methanol. The mixture was stirred for 1 hr at room temperature. Solvent was removed, water was added to the semisolid product, the pH was adjusted to 2 using aqueous hydrochloric acid, and the mixture extracted three times with 50-ml portions of ether. Ether extracts were combined, washed once with pH 2 water, once with saturated NaCl, and finally dried over anhydrous Na_2SO_4. Solvent was removed to obtain 3.0 g (52.5%) of oily product which crystallized partially on standing overnight. Ether was added and the mixture was filtered to obtain a first crop of white solid 10, as the *p*-toluenesulfonate (0.73 g, 12.8%) mp 84–86°C.

TLC: silica gel (3% MeOH in $CHCl_3$), R_f = 0.54. NMR (220 MHz ^1H, $CDCl_3$): triplet, 0.89 ppm (3 H, tail CH_3); multiplet, 1.18 (14 H, $+CH_2+_n$); multiplet, 1.70 (2 H, —N—CH_2—CH_2); singlet, 2.34 (3 H, tosylate CH_3); multiplet, 3.05 (2 H, N—CH_2—CH_2···); multiplet, 4.00 (2 H, α-CH_2); singlet, 5.14 (2 H, —O$CH_2\phi$); singlet, 7.32 (5 H, ϕ protons); doublets, 7.75, 7.16 (4 H, tosylate ring protons); broad singlet, 8.90 (1 H, N-*H*).

N-*Decyl-Glycine* (*11*)

N-Decylamine (70 ml, 360 mmol) was dissolved in a mixture of 24 ml water and 50 ml ethanol and cooled in an ice bath with stirring. Iodoacetic acid (15 g, 81 mmol) was then added slowly. After standing at room temperature for 20 hr, the contents of the reaction were poured into acetone. A white precipitate of *N*-decyl-glycine formed, which, after filtering and drying, weighed 13.7 g (79%); mp after recrystallization from methanol, 229–230°C.

ANAL.: *Calcd. for* $C_{12}H_{25}NO_2$: C, 66.98; H, 11.63; N, 6.51. *Found:* C, 66.21; H, 11.65; N, 6.58.

N-*Decyl-Glycine Benzyl Ester* (*10*): *Method B*

N-Decyl-glycine (12.9 g, 60 mmol), *p*-toluene sulfonic acid (12.54 g, 66 mmol), benzyl alcohol (30 ml), and benzene (80 ml) were combined in a 250-ml round-bottom flask. The water generated during the reaction was removed by azeotropic distillation. Upon overnight standing at room temperature, the ester crystallized. Ether was added, the mixture was filtered, washed several times with ether, and dried to obtain a white solid (23.7 g, 83%) identical to 10 obtained by Method A.

TABLE II
Characterization of *N*-Alkyl-glycines

N-Alkyl-glycine	Elemental Analysis		NMR (220 MHz ^1H, D_2O)
N-Cyclohexyl-glycine ($C_8H_{15}NO_2$)	*Calcd:*	C, 61.15; H, 9.55; N, 8.92	Multiplet(s), 1.07–2.20 ppm (10 H, ring CH_2's); multiplet, 3.09 (1 H, N—CH—); singlet, 3.64
	Found:	C, 61.38; H, 9.63; N, 8.92	(2 H, α-CH_2)
N-Hexyl-glycine ($C_8H_{17}NO_2$)	*Calcd:*	C, 60.38; H, 10.69; N, 8.81	Triplet, 0.90 ppm (3 H, —CH_3); multiplet(s), 1.35 (6 H, $+CH_2+_n$); multiplet(s), 1.71 (2 H, N—CH_2—CH_2···); triplet, 3.08 (2 H, N—CH_2—CH_2···); singlet, 3.63 (2 H, α-CH_2)
	Found:	C, 60.25; H, 10.58; N, 8.68	

N-*Hexyl-Glycine and* N-*Cyclohexyl-Glycine*

These *N*-alkyl-glycines and their corresponding benzyl esters were synthesized in an identical manner to that reported above for *N*-decyl-glycine and its benzyl ester (method B). Yields and melting points of these products are as follows: *N*-hexyl-glycine (see also Ref. 14) (52%, 162–163°C); *N*-hexyl-glycine benzyl ester (89%, 102–104°C); *N*-cyclohexyl-glycine (84%, 228–229°C); and *N*-cyclohexyl-glycine benzyl ester (96%, 137°C). For further characterization of the acids, see Table II.

Boc-Sar-Gly-OBzl (12)

Boc-Sar-OH (8.5 g, 45 mmol) was coupled with Gly-OBzl *p*-tosylate (15.17 g, 45 mmol) using the mixed anhydride procedure as described for the preparation of Boc-Gly-Sar-OBzl (1). After work-up, the syrupy product (12) was isolated (14.0 g, 93% yield) which resisted attempts at crystallization. TLC: silica gel (5% MeOH in $CHCl_3$), R_f = 0.82.

Boc-Sar-Gly-OH (13)

Boc-Sar-Gly-OBzl (14.0 g, 41.8 mmol) was dissolved in 50 ml *t*-butanol, 50 mg 10% palladium/charcoal catalyst was added, and the mixture shaken on a Paar hydrogenation apparatus under 30 psi of H_2 pressure. The oily product (13) was isolated after filtration and removal of solvent (8.7 g, 85% yield). TLC: silica gel (BuOH/acetone/H_2O/HOAc, 4:3:2:1), R_f = 0.79.

*Boc-Sar-Gly-*N*-Decyl-Gly-OBzl (14)*

Boc-Sar-Gly-OH (8.0 g, 32.5 mmol) was coupled with *N*-decyl-Gly-OBzl-tosylate (15.5 g, 32.5 mmol) using the mixed anhydride procedure as in the preparation of Boc-Gly-Sar-OBzl (1). After work-up, the syrupy product (16.0 g) was isolated in 95% yield and used directly in the ensuing procedure.

*Boc-Sar-Gly-*N*-Decyl-Gly-OH (15)*

Boc-Sar-Gly-*N*-decyl-Gly-OBzl (16.0 g, 31 mmol) was de-esterified using *t*-BuOH and 10% Pd/C, using a Paar hydrogenator as for the preparation of dipeptide 13. The syrupy product crystallized on overnight standing to yield tripeptide 15 (6.2 g, 46.6%), mp 116–117°C. TLC: (BuOH/acetone/H_2O/HOAc, 4:3:2:1), R_f = 0.9.

*HCl-Sar-Gly-*N*-Decyl-Gly-OH (16)*

Boc-Sar-Gly-*N*-decyl-Gly-OH (6.17 g) was dissolved in 100 ml ethyl acetate and cooled in an ice bath, and HCl gas was bubbled into the reaction mixture for 5 min. After stirring at ice-bath temperature for 1 hr, the solvent was removed on the rotary evaporator, and the crude product was washed several times with petroleum ether, then with diethyl ether, and dried under vacuum to provide a white solid (4.9 g, 89% yield).

*Boc-Glu(γ-OBzl)-Sar-Gly-*N*-Decyl-Gly-OH (17)*

Boc-Glu(γ-OBzl)-OH (9.1 g, 27 mmol) was dissolved in 300 ml $CHCl_3$, and the resulting solution was cooled to −20°C using a dry ice–CCl_4 bath. Then 3.1 ml (27 mmol) *N*-methylmorpholine and 3.5 ml (27 mmol) of *iso*-butyl chloroformate were added, and stirring was continued for 10 min at −20°C. To this 11.27 g (29.7 mmol) HCl·H-Sar-Gly-*N*-decyl-Gly-OH (16) and 6.6 ml (59.4 mmol) *N*-methylmorpholine were added, and stirring at low temperature was continued for 2 hr, after which the mixture was allowed to warm to room temperature and stirred overnight. Upon removal of solvent, an oily product was obtained, to which water was added; the mixture was cooled in an ice bath, the pH of the solution adjusted to 3 using 0.1*N* citric acid, and the aqueous mixture was extracted three times with 150 ml ethyl acetate. Extracts were combined and washed once with water, twice with saturated NaCl, and dried over Na_2SO_4. The crude product obtained after removal of solvent was triturated with petroleum ether and then with ether. Tetrapeptide 17 was isolated as a syrup (14.7 g, 82% yield). TLC: silica gel ($CHCl_3$/MeOH/AcOH, 85:15:5), R_f = 0.75.

*HCl·H-Glu(γ-OBzl)-Sar-Gly-*N*-Decyl-Gly-OH (18)*

Boc-Glu-(γ-OBzl)-Sar-Gly-*N*-decyl-Gly-OH (7.8 g, 11.8 mmol) was dissolved in 100 ml ethyl acetate, cooled in an ice bath, anhydrous HCl was bubbled into the flask, and stirring was continued for an hour at ice-bath temperature. Solvent was removed, and the residue was washed several times with ether and dried to yield a solid product (6.9 g, 97%).

*Boc[Glu-(γ-OBzl)-Sar-Gly-*N*-Decyl-Gly]$_2$-OH (19)*

Boc-Glu-(γ-OBzl)-Sar-Gly-*N*-decyl-Gly-OH (6.88 g, 10.4 mmol) was coupled with HCl·H-Glu-(γ-OBzl)-Sar-Gly-*N*-decyl-Gly-OH (6.85 g, 11.44 mmol) using mixed anhydride procedures as described for the preparation of tetrapeptide 17. Yield, 12.8 g (95%).

*Cyclo[Glu-(γ-OBzl)-Sar-Gly-*N*-Decyl-Gly]$_2$ (20)*

Boc-[Glu-(γ-OBzl)-Sar-Gly-*N*-decyl-Gly]$_2$-OH (12.06 g, 10 mmol) was treated with 10 mmol *p*-nitrophenol and 10 mmol DCC in $CHCl_3$ to obtain the octapeptide *p*-nitrophenyl ester. The Boc group was removed by treatment with anhydrous HCl in the usual manner, and the resulting hydrochloride was dissolved in DMF (25 ml) and added dropwise to 3900 ml pyridine over a 5-hr period. Stirring at room temperature was continued for 68 hr. After thorough removal of solvents, the residue was treated with Rexyn I-300 mixed-bed ion-exchange resin to give 7.18 g of crude product. When this product was dissolved in MeOH and the solution cooled, a white solid soon separated, which was filtered, dried, and isolated (1.3 g, 12% overall yield). Characterization is given in Table I.

*Cyclo(Glu-Sar-Gly-*N*-Decyl-Gly)$_2$ ("DECYL-2") (21)*

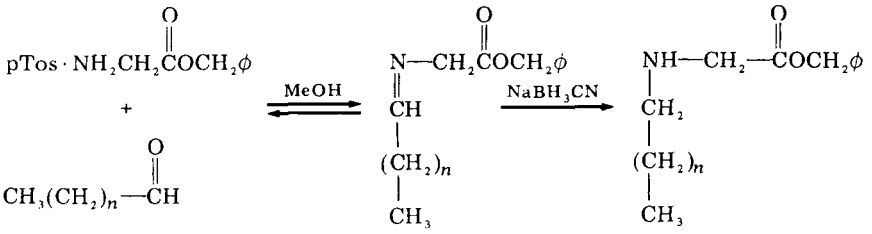
When 420 mg cyclic octapeptide 20 was dissolved in *t*-BuOH, hydrogenated using 10% Pd/C, in a Paar hydrogenator by the usual procedure, the free diacid 21 was obtained as a white solid (318 mg, 91%) (characterized as reported in Table I).

RESULTS AND DISCUSSION

Chemical Synthesis of Peptide Ionophores

One approach to achieving the requisite balance of hydrophobic/hydrophilic forces is to incorporate covalently bound lipid "tails" into potentially ionophoric peptides. These resemble fatty acid side-chain groups of natural membranes with respect to solubility and structure, and as such, can be expected to interact favorably with membranes. The crucial intermediates required for this phase of the work are the *N*-alkylated glycines, which contain hydrophobic groups (linked to glycine through the backbone

nitrogen atom), whose length and shape can be varied systematically. To synthesize the desired materials, we initially employed a modification of the method of Borch et al.[15] to prepare a Schiff's base of glycine benzyl ester (I). This species was then subjected to mild reduction with sodium cyanoborohydride to "fix" the covalent attachment of the tail, giving the *N*-alkylglycine derivative (II) as shown:



(I) (II)

Subsequently, we adopted a more general method which led to higher isolated yields. Alkylamine (III) and iodoacetic acid (IV) are mixed in water/ethanol, leading to direct formation of *N*-alkylglycine (V) (modification of procedure of Rowley et al.[16]):

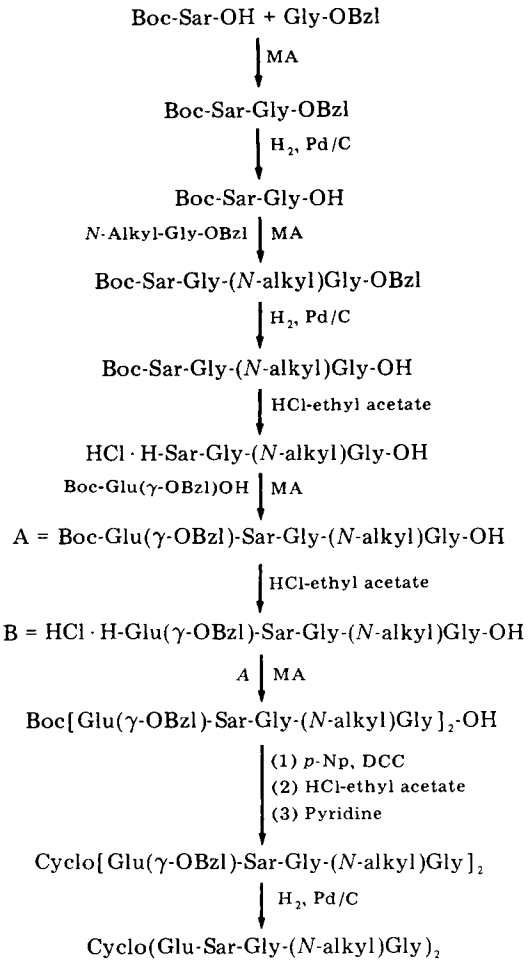
$$\underset{\text{(III)}}{CH_3(CH_2)_n—NH_2} + \underset{\text{(IV)}}{ICH_2COOH} \rightarrow \underset{\text{(V)}}{CH_3(CH_2)_n—NHCH_2COOH} + HI$$

N-alkylated glycines which we have now prepared, characterized, and used in further syntheses include *N*-decyl, *N*-hexyl, and *N*-cyclohexyl. *N*-alkylated glycines prepared by this latter method were converted to benzyl esters by treating the *N*-alkyl-glycine with benzyl alcohol in the presence of *p*-toluenesulfonic acid and removing water by azeotropic distillation with benzene.

Evidence from the limited existing data on synthesis of homodetic cyclic octapeptides suggests that much as cyclic hexapeptides arise from linear tripeptide precursors[17] and cyclic decapeptides arise from linear pentapeptide precursors,[18] cyclic octamers can also readily be synthesized from linear tetrapeptides.[19-21] This route would seem particularly attractive in the present instances because all peptides (except one series, see Table I) have C_2-symmetry in their primary sequences, and it is obviously simpler to proceed directly to cyclization from a linear tetrapeptide than carry out further steps to first prepare a linear octapeptide. To determine the best synthetic tactics, cyclization reactions were performed both ways in a few instances; for example, cyclo(Phe-Sar-Gly-Sar)$_2$ prepared either from linear tetra- or octapeptide active esters gave identical major products, which were shown to be the octapeptide. Similarly, cyclo(Glu(OBzl)-Sar-Gly-Sar)$_2$ could be prepared by both routes. However, as the investigation progressed, we became convinced that the method of choice was cyclization from the linear octapeptide, as the tetrapeptide cyclizations tended to give

rise to mixtures of products, including (most likely) the cyclic tetrapeptide, with relative yields a function of primary sequence and reaction conditions.

Scheme I shows the synthesis of cyclo(Glu(OR)-Sar-Gly-(*N*-alkyl)Gly)$_2$ as the benzyl ester and, after removal of benzyl groups by hydrogenation, as the free acid. Key intermediates are the tripeptides of the type Sar-Gly-*N*(*R*)-Gly, where *R* is the hydrophobic tail; we have discussed the



Scheme 1. Outline of synthesis scheme for synthetic ionophoric peptides. Complete details of representative syntheses of cyclo(Glu(OBzl)-Sar-Gly-Sar)$_2$, cyclo(Glu(OBzl)-Sar-Gly-(*N*-decyl)Gly)$_2$, and the corresponding free acids are given in the text (Experimental). Other peptides were similarly prepared. In the case of cyclo(Glu(OBzl)-Sar-Gly-Sar-Glu(OBzl)-Sar-Gly-(*N*-decyl)Gly), peptide A contained the *N*-methyl, peptide B contained the *N*-decyl group. (Symbols and abbreviations: Boc, *t*-butyloxycarbonyl (*N*-protecting group); Sar, *N*-methyl-glycine; -OBzl, benzyl ester; HCl, anhydrous hydrochloric acid; MA, mixed anhydride, peptide coupling reaction; 10% Pd/C, palladium-on-charcoal, hydrogenation catalyst; *p*-Np = *p*-nitrophenol, active ester; DCC, dicyclohexylcarbodiimide, coupling reagent.)

conformational properties of some of these.[22] The syntheses of cyclo-(Glu-Sar-Gly-Sar)$_2$ and of a peptide widely studied in our laboratory, cyclo(Glu-Sar-Gly-(*N*-decyl)-Gly)$_2$ (DECYL-2),[23,24] are described in detail in the Experimental section. The molecular structure of a representative peptide, cyclo(Glu(OR)-Sar-Gly-Sar-Glu(OR)-Sar-Gly(*N*-decyl)Gly) is given schematically in Fig. 1.

Using analogous methods to assemble and cyclize linear octapeptides, we have completed the total syntheses of several sets of peptides in neutral (ester) and acid forms. Results of these syntheses are outlined in Table I. Characterization of the cyclic products (in the ester forms) rests on criteria such as (1) homogeneity on TLC; (2) lack of end groups (i.e., these materials survived mixed-bed ion-exchange resin treatment); (3) observation of molecular ion peaks by mass spectrometry; (4) spectroscopic characterization principally by ir methods (all peptides displayed appropriate ester/acid carbonyl, amide I, and amide II bands) and nmr methods (peptides displayed ^{13}C- and ^{1}H-resonances consistent with constituent residues); and (5) subsequent study of conformational properties, principally by nmr and CD, which confirm peptide-bond isomerizations and metal-binding properties anticipated for cyclic octapeptides (but not for corresponding cyclic tetrapeptides).

Cyclic peptides in Table I which are similar except for the degree of alkyl substitution display an array of solubility properties as a function of these hydrophobic "tails." All benzyl esters with tails larger than two methyls

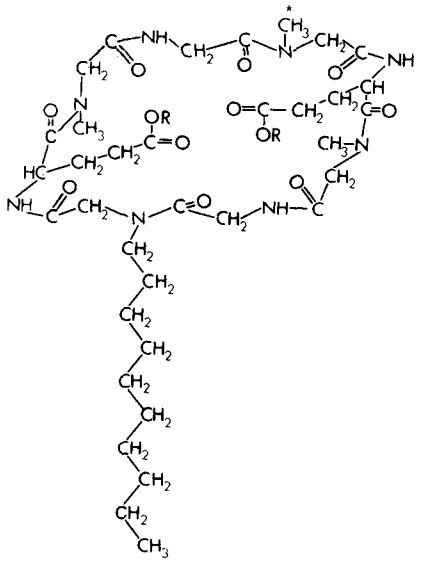


Fig. 1. Molecular structure typical of synthetic cyclic octapeptides reported herein. Shown is cyclo(Glu(OR)-Sar-Gly-Sar-Glu(OR)-Sar-Gly-(*N*-decyl)Gly). R = CH_2Ph in benzyl ester forms, and R = H in free acid forms of Glu side chains. In the compounds containing two hydrophobic tails, the second tail would replace the Sar methyl group marked (*) to give a C_2-symmetric sequence.

were chloroform soluble. It was also our intention to produce peptide *di-acids,* which were organic-soluble despite the presence of two ionizable Glu side chains; this was achieved in the four such diacids prepared, although it was subsequently determined that among these, only DECYL-2 remained completely in the chloroform phase when extracted against an aqueous phase.

NMR Studies of Cyclic Octapeptides and Their Metal Complexes

We have used ^{13}C- and ^{1}H-nmr as primary methods to characterize newly synthesized materials by noting numbers and chemical shifts of carbon resonances of both intermediates and products and correlating them with expectation based on primary sequence. Beyond this valuable use, we employed these techniques to study conformational aspects of these peptides both free in solution and on interaction with metal cations.

In Fig. 2 the ^{13}C spectrum of cyclo(Glu(OBzl)-Sar-Gly-Sar)$_2$ in trifluoroethanol (TFE) solvent is presented. This set of spectra of what might

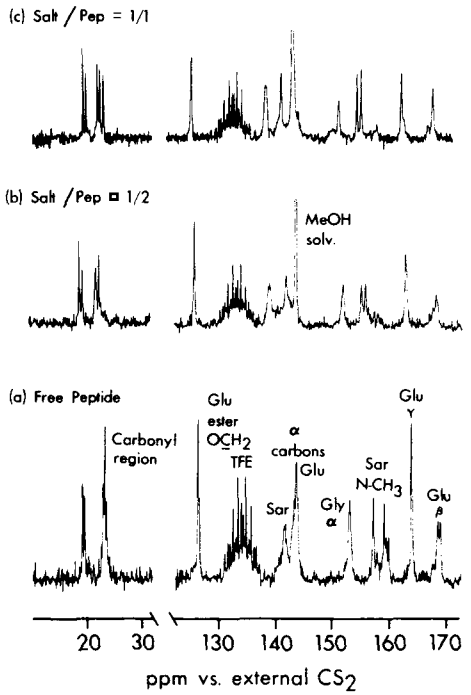


Fig. 2. ^{13}C-nmr spectra (25 MHz) of the cyclic octapeptide cyclo(Glu(OBzl)-Sar-Gly-Sar)$_2$ titrated with calcium perchlorate. Peptide concentration: 36 mg/ml. Solvent: trifluoroethanol-d_3 (TFE). The calcium salt was added as aliquots in methanol solution for solubility reasons. Tentative resonance assignments are indicated. Chemical shifts versus external tetramethylsilane (TMS) may be obtained by subtracting the values given from 193.8 ppm.

be termed a "tailless" cyclic octapeptide affords the clearest upfield spectral region (Glu side chain C^{β} and C^{γ} carbons, Sar *N*-methyl carbons) among the present peptides. Figure 2(a) demonstrates that the free cyclic octapeptide exists as a mixture of conformers whose interconversion is slow on the nmr time scale. This spectral process is readily attributable to *cis/trans* peptide bond isomerism about the Gly-Sar and/or Glu-Sar ("*X*-imino acid") peptide bonds.[25-27] Several conformers may arise, including an all-*trans*, a four-*cis*, and a few types of one-*cis* and two-*cis*. Those conformers actually present are not identified or resolved individually, but the Glu C^{β} provides a monitor of the conformational transitions which take place when calcium ions (as $Ca(ClO_4)_2$) are added to the peptide. This resonance, near 170 ppm in Fig. 2(a), appears as a doublet in the free peptide but sharpens to a single peak (with a residual downfield shoulder) at a 1:1 calcium/peptide ratio [Fig. 2(c)]. The Sar *N*-CH_3 region also changes significantly from a group of several resonances in Fig. 2(a) to two sharp singlets near 155 ppm in Fig. 2(c). It is also observed that the five possible C=O resonances are resolved in Fig. 2(c) between 20 and 25 ppm. The spectral effects are consistent with the formation of a single complexed conformer—presumably a 1:1 calcium/peptide complex—with the highest symmetry allowed by the peptide sequence (C_2) and, by analogy to similar complexes with other imino acid-containing cyclic peptides,[21,28] with all peptide bonds *trans*. The affinity of this peptide for calcium is apparent from the observation that despite the relatively high polarity of the solvent (TFE), only 1 eq of calcium is needed to complex the available peptide. (This affinity persisted even at the orders-of-magnitude lower concentration employed for corresponding binding studies in TFE using CD spectra,[24] thus precluding direct determination of binding constants.)

When hydrophobic tails are introduced, ^{13}C spectra take on the characteristics shown in Fig. 3, where spectra of cyclo(Glu(OBzl)-Sar-Gly-(*N*-hexyl)Gly)$_2$ titrated with calcium perchlorate are presented. Calcium perchlorate itself is insoluble in chloroform solution, so that only the calcium *complexed* by the peptide will be solubilized. Indeed, cyclo-(Glu(OBzl)-Sar-Gly-(*N*-hexyl)Gly)$_2$ was observed to dissolve $Ca(ClO_4)_2$ into $CDCl_3$ up to, but not further than, a 1:1 stoichiometry. This result provides substantive evidence for the formation of a 1:1 calcium/peptide complex. The spectra in Fig. 3 are broadly analogous to those in Fig. 2, except that six hexyl carbon resonances replace one Sar methyl carbon resonance. Four of these hexyl tail carbon resonances, superimposed over the region of the Glu beta/gamma carbons, could be tentatively identified by the fact that their chemical shifts remain virtually unchanged during the calcium titration, in accord with the general observation that conformational transitions are reflected maximally in resonances due to backbone and adjacent atoms more than in side-chain resonances. Note, for example, that the side chain benzyl ester OCH_2 carbon does not change its position from Fig. 3(a) to (c).

Assignments given in Fig. 3(c) for hexyl tail carbons are based on the

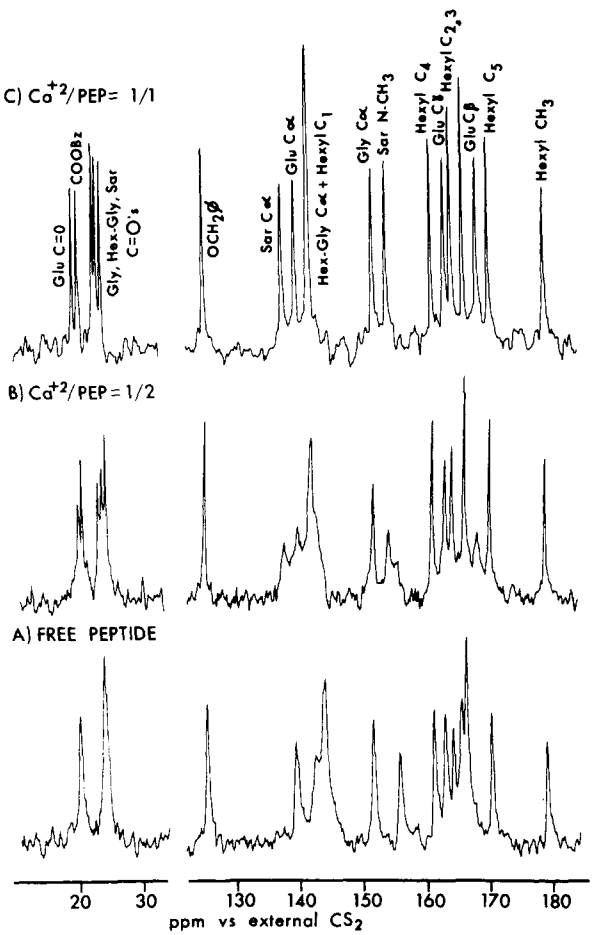


Fig. 3. ^{13}C-nmr spectra (25 MHz) of the cyclic octapeptide cyclo(Glu(OBzl)-Sar-Gly-(*N*-hexyl)Gly)$_2$ titrated with calcium perchlorate. Solvent: chloroform-*d*. Peptide concentration: 30 mg/ml. The calcium salt was added as solid portions which were solubilized by the peptide. Tentative resonance assignments indicated.

expectation that hexyl-C_1 (bound to a nitrogen atom) will be furthest downfield and hexyl-C_6 (a terminal CH_3 rather than CH_2) will be at highest field. Carbons 2–5 were tentatively assigned using T_1 spin lattice relaxation values obtained from studies on the model compound *N*-hexylglycine. In this experiment (spectra not shown), the six hexyl carbons displayed similar chemical shifts to those in Fig. 3 and had NT_1 values (proceeding from downfield to upfield) of 1.36, 1.82, 1.74, 1.74, 2.30, and 3.54 sec. Since molecular motion (and hence NT_1) is expected to increase on moving from N-CH_2 to CH_3 out along the hexyl chain, these results permitted the assignments shown in Fig. 3(C). However, the similarity between some of these NT_1 values indicates that assignments of carbons 2–4 could be interchanged.

In order to obtain further details concerning the structure of the cyclo-(Glu(OBzl)-Sar-Gly-(*N*-hexyl)Gly)$_2$ octapeptide–calcium complex, we recorded 220-MHz ^{1}H-nmr spectra on a series of samples similar to those in Figs. 3(A)–(C). The results of these ^{1}H-nmr studies are shown in Fig. 4. Again, the C_2-symmetry of the final calcium/peptide complex [Fig. 4(C)] is apparent from several aspects of the spectra, such as the appearance of only one Gly and one Glu NH resonance for each of the two Gly and Glu residues, a single AB quartet for the two benzyl ester methylene groups, and a nearly first-order α-proton region where most resonances could be delineated. Spin-decoupling experiments (not shown) performed on this and on the related *N*-decyl system confirmed the assignments of Glu and Gly NH and α-protons. Pairs of AB quartets for Sar and *N*-hexyl Gly α-methylene protons were identified, but the data do not allow a choice between these. Peptide NH resonances appeared to move generally upfield over the course of the experiment, suggesting that added metal is competing successfully for peptide carbonyl groups previously occupied in (intramolecular?) hydrogen bonds to peptide NH groups in (some) free peptide conformers.

Values of vicinal coupling constants obtained from expanded versions of related spectra (Glu NH-C$^\alpha$-H, J = 9.2 Hz; Gly NH-C$^\alpha$-H's, J = 8.8 Hz and 3.6 Hz) may be used to limit the range of backbone conformations

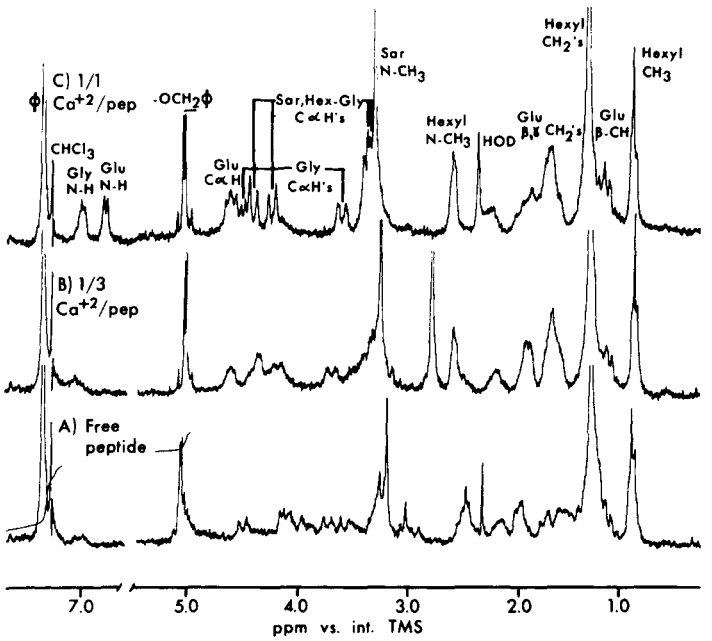


Fig. 4. ^{1}H-nmr spectra (220 MHz) of the cyclic octapeptide cyclo(Glu-(OBzl)-Sar-Gly-(*N*-hexyl)Gly)$_2$ titrated with calcium perchlorate. Solvent: chloroform-*d*. Peptide concentration: 20 mg/ml. The calcium salt was added as solid portions which were solubilized by the peptide. Tentative resonance assignments indicated.

possible for the calcium/peptide complex in chloroform solution. These coupling constant data, along with CPK model-building studies, eliminate all but two general categories of conformers, which differ by the group of four carbonyl groups (amino- or imino-acid bound) which are oriented toward the peptide central cavity for participation in metal binding. While a definitive determination will await appropriate experiments (e.g., using manganese/peptide complexes as shift reagents), molecular models (and inference from structures previously deduced for related peptide–metal complexes)[20,21] suggest that the most likely site of calcium interaction is the group of imino acid-bound carbonyls (two Glu and two Gly C=O's) oriented in a coplanar configuration in the best plane of the cyclic peptide disk. Perchlorate ions above or below this plane could fill remaining (octahedral) coordination sites of the calcium ion. Although it is difficult to separate concomitant spectral effects of *cis/trans* mixed conformers converting to all-*trans* and chemical shift changes due specifically to metal binding, the combination of ^{13}C and ^{1}H spectra (Figs. 3 and 4) of cyclo-(Glu(OBzl)-Sar-Gly-(*N*-hexyl)Gly)$_2$ does not suggest direct involvement of side-chain benzyl ester carbonyls in calcium binding.

Cation Transport Across "Thick-Liquid" Membranes

A major goal of this work is the preparation of peptides which will not only bind or extract metals into organic phases, but ultimately transport them across membranous phases. If cation-selective transport can be demonstrated, these peptides may be of considerable practical utility as reagents in a variety of biochemical and physiological studies.

Cation transport may be evaluated using the so-called "thick-liquid membrane" model. The experimental setup consists of a U-shaped glass vessel (also called a "Pressman cell") in which two aqueous phases are separated by an organic phase (in our experiments, chloroform).[29] In a typical procedure, the ionophore is dissolved in the organic phase. The metal(s) to be studied is (are) dissolved, as the chloride, in aqueous phase 1. The aqueous solution is buffered with HEPES and adjusted to pH 7 with lithium hydroxide. If the peptide contains no ionizable group (e.g., the benzyl ester series), picric acid (2,4,6-trinitrophenol) must be added to aqueous phase 1 in order to provide the peptide with a lipophilic anion; it is unlikely that a metal chloride complex will have sufficient solubility in the organic phase. An advantage of using picric acid is its characteristic yellow color; this enables the experimentalist to observe transport in the cell visually by noting the color intensity of various phases and to measure transport quantitatively by absorption spectroscopy.

Typical results of thick-liquid membrane transport experiments are presented in Fig. 5. After it was found that the benzyl esters in Table I transport calcium with approximately equal effectiveness in Pressman cells, one compound, cyclo(Glu(OBzl)-Sar-Gly-(*N*-decyl)Gly)$_2$ (DECYL-2E), was chosen and its selectivity profile studied in detail. As shown in Fig.

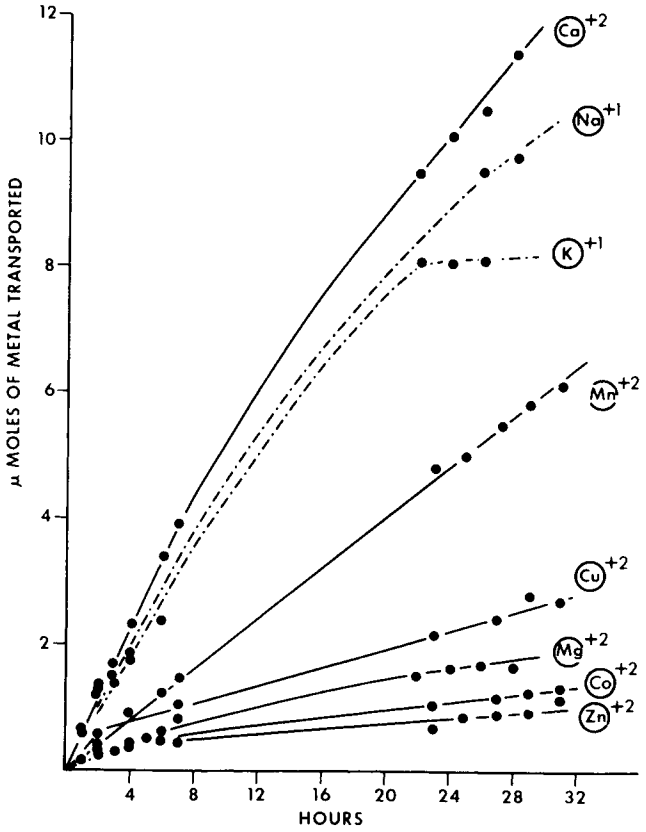


Fig. 5. Cation transport across a thick-liquid membrane (Pressman cell) mediated by synthetic ionophoric cyclic octapeptide, cyclo(Glu-(OBzl)-Sar-Gly-(*N*-decyl)Gly)$_2$. Organic phase: chloroform. Aqueous phase 1 initially contained 10 m*M* HEPES buffer at pH 7, 20 m*M* metal chloride, and 50 m*M* picric acid. Concentration of peptide in organic phase: 200 μM. Transport was monitored by absorption spectroscopy, measuring picrate concentration in aqueous phase 2. A consistency check with radioactive calcium ion ($^{45}Ca^{2+}$) demonstrated that picrate accompanied cation in a stoichiometric manner.

5, this peptide transports calcium from one aqueous phase to another with a capacity greater than all other cations surveyed (sodium, potassium, manganese, copper, magnesium, cobalt, and zinc). The capacity of DECYL-2E to deliver calcium to the second aqueous phase is approximately equal—and somewhat superior—to calcium transport exhibited by the naturally occurring ionophore A23187[9,10] (e.g. under experimental conditions identical to those in Fig. 5, where DECYL-2E had transported ca. 12 μmol of calcium, ca. 8 μmol were delivered by A23187).

With respect to cation selectivity, it was noted that the micromoles of calcium delivered by DECYL-2E to aqueous phase 2 were nearly an order of magnitude greater than the magnesium delivered. Among divalent ions studied, only manganese had substantial activity besides calcium. However, the peptide did not discriminate between mono- and divalent cations,

as evidenced by its capacity to transport sodium and potassium; this may be attributed to the fact that cation selectivity by the present peptides (in their benzyl ester forms) should be greatest for cations complementary in ionic radius to the cross-sectional radius of the central cavity formed by peptide carbonyl groups. For cations of comparable radii, net charge and preferred coordination geometry will additionally influence binding and/or transport. In the model for the calcium complex tentatively deduced from nmr data, the oxygen–oxygen contact distance across the cavity is ca. 2.5 Å, thus providing a better fit for cations such as Ca^{2+} (0.99 Å ionic *radius*), Na^+ (0.97 Å), and K^+ (1.33 Å) than others of ionic radius of 0.6–0.7 Å (Co^{2+}, Cu^{2+}, Mg^{2+}, and Zn^{2+}). The cation Mn^{2+} (radius 0.80 Å) is intermediate between the two groups in size and degree of transport.

Our laboratory is presently investigating the detailed cation selectivity profiles of the peptides described, with particular interest in the diacid forms. In such peptides, transport may be mediated by a proton(s)-for-metal exchange mechanism, whereby a net neutral cation–peptide complex becomes the transporting species. Such a mechanism was shown to operate for the extraction of calcium into organic phases by cyclo(Glu-Sar-Gly-(*N*-decyl)Gly)$_2$ (DECYL-2).[23,24] It is anticipated that the presence of two negative carboxylate groups per single molecule, participating along with peptide carbonyls in a binding cavity of appropriate dimensions and ligand geometry, should create a favorable situation for possible calcium-selective transport. Concomitantly, preliminary results suggest that systematic incorporation of lipophilic tails will prove valuable for establishing and regulating the membrane-partitioning properties of these peptide–metal complexes.

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