Microsphere Formation in a Series of Derivatized α-Amino Acids: Properties, Molecular Modeling, and Oral Delivery of Salmon Calcitonin

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A series of benzoylated and phenylsulfonylated amino acids are novel, low molecular weight, self-assembling molecules. At low pH, these compounds form microspheres that dissolve readily under neutral conditions. In a given synthetic series, those molecules with low aqueous solubility formed microspheres more readily than did the molecules possessing high water solubility, suggesting that the hydrophobicity of these compounds contributes to the ability to form microspheres. In addition, molecular modeling studies on selected compounds have shown that microsphere formation may depend also on various aromatic ring and dipole–dipole interactions, which could effect the extent and types of favorable stacking conformations between molecules. The microspheres prepared from these compounds have been used to effect the oral delivery of salmon calcitonin, a model protein drug, in both rodents and primates.

Introduction

The phenomenon of intermolecular association between similar structures has been an area of interest for many decades.[1–4] Indeed, micelles and vesicles are well known and routinely used in consumer products such as soaps and detergents. These micelles and vesicles are held together by the weak hydrophobic–hydrophilic interactions between the head and tail groups of the molecules. As such, these structures exist only in solution and collapse under dry conditions. Self-assembled molecular superstructures which maintain their integrity on drying have been reported infrequently in the literature. One of the earliest examples of such structures can be found in the work of Fox.[5] He observed that thermally-condensed amino acid mixtures had the ability to self-aggregate into spherelike microparticles. The formation of these microspheres was found to be a function of pH such that microspheres were present at low pH and dissolved under conditions of high pH.[6–10] More recently, a variety of peptide molecules have been observed to form self-assembled nanotubes with the ability to function as artificial ion channels in lipid bilayer membranes.[11–13] Similar rods and tubules have also been prepared by molecular interactions among lysines acylated with fatty acids.[14] In general, all of these structures are either peptide polymers or long chain fatty-acid-like molecules with enormous structural flexibility. However, we have documented microsphere formation in a group of conformationally-restricted, small peptides having molecular weights as low as ~600 Da. We have shown, for instance, that tetrapeptide diketopiperazines prepared from phenylalanine and glutamic or aspartic acids will self-assemble into microspheres.[15] Herein, we report on a series of even simpler, sphere-forming molecules that are derivatized single amino acids (MW < 300 Da) and that form stable, isolable microspheres either individually or as mixtures.

Previously we demonstrated that microspheres could be used to effect the oral delivery of drugs traditionally administered only by injection. Thus, microspheres prepared from thermally-condensed amino acid mixtures allowed oral delivery of heparin in both rodents and humans.[16–18] In this report, microspheres prepared from a series of benzoylated and phenylsulfonylated α-amino acids are shown to facilitate the oral delivery of a model protein drug, salmon calcitonin. Calcitonin is a 32-amino acid peptide approved for use in the treatment of Paget's disease and osteoporosis.[19] It is routinely administered by subcutaneous or intramuscular injection. Orally-dosed calcitonin is ineffective, presumably due to its instability in the gastrointestinal tract. The microspheres described herein can deliver therapeutically significant amounts of calcitonin to rats and monkeys when administered orally.

Results and Discussion

Synthesis. A series of acylated, benzoylated, and sulfonylated amino acids was prepared using standard Schotten–Baumann chemistry.[20] In general, an amino acid was dissolved in aqueous base and a selected acid chloride was added. After the mixture was stirred at room temperature for about 2 h, the product was isolated by precipitation from the acidified reaction mixture and purified by recrystallization. The compounds listed in Tables 1–3 were prepared by this procedure or purchased. All of the compounds that were prepared in our laboratory have been reported previously.[21–32]

Microsphere Formation. In order to investigate the role of structure in molecular self-assembly, we examined the microsphere-forming abilities of each derivatized amino acid individually using a light microscope at both 10- and 100-fold magnification as described in the Experimental Section of this paper. In general, no microspheres formed with either the monoderivatized basic or monoderivatized acidic amino acids under the conditions described herein. Thus, neither *N*-benzoylaspartic acid (**1**) nor *N*-benzoylglutamic acid (**2**) formed microspheres, nor did the corresponding

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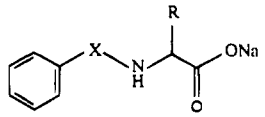
Table 1. *N*-Benzoylated and *N*-Phenylsulfonylated Amino Acids



compd	X	R	microsphere formation[a]	log *P*[b]	mp (°C)
1	C=O	CH_2CO_2H	no	−2.79	nd[c]
2	C=O	$(CH_2)_2CO_2H$	no	−2.29	nd
3	C=O	$(CH_2)_4NH_2$	no	−2.02	nd
4	C=O	$(CH_2)_4NHCOPh$	yes	−1.22	53−55
5	SO_2	$(CH_2)_4NH_2$	no	−2.80	nd
6	C=O	$(CH_2)_3NH_2$	no	−2.09	nd
7	C=O	$(CH_2)_3NHCOPh$	yes	−1.29	189−192
8	SO_2	$(CH_2)_3NHC(NH)NH_2$	no		nd
9	C=O	CH_2Ph	yes	−0.63	nd
10	SO_2	CH_2Ph	yes	−1.41	131−132
11	C=O	$CH_2CH(CH_3)_2$	yes	−0.91	nd
12	SO_2	$CH_2CH(CH_3)_2$	yes	−1.69	112−114
13	C=O	$CH(CH_3)_2$	yes	−1.30	nd
14	SO_2	$CH(CH_3)_2$	yes	−1.80	149−150
15	C=O	CH_3	no	−2.14	nd
16	C=O	H	no	−2.68	nd
17	SO_2	CH_2OH	no	−3.98	216 dec
18	C=O	$CH_2(4\text{-}C_6H_4OH)$	no	−2.24	81−82

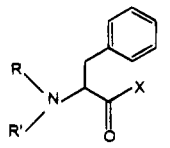
[a] Microspheres were examined using a light microscope at 10- and 100-fold magnification. Ratings of 0−2 were given a "no" designation, and ratings of 3−5 were given a "yes" designation. [b] Log P values were calculated from the reported experimental log *P* values of the amino acids using fragment constants.[33] [c] These compounds were purchased, and melting points were not measured.

lysine (**3**) or ornithine (**6**) analogs. Likewise, *N*-(phenylsulfonyl)lysine (**5**) and *N*-benzoylarginine (**8**) did not form microspheres. In contrast, *N,N'*-dibenzoylated lysine (**4**) and ornithine (**7**) derivatives formed stable microspheres. *N*-Benzoylphenylalanine (**9**) and *N*-(phenylsulfonyl)phenylalanine (**10**) also formed microspheres, as did the leucine analogs **11** and **12**. The derivatized valines **13** and **14** produced very poor microspheres, and the alanine **15** did not form microspheres at all under these conditions. *N*-Benzoylglycine (**16**), *N*-(phenylsulfonyl)serine (**17**), and *N*-benzoyltyrosine (**18**) did not form microspheres under these conditions.

One possible explanation for these results is that hydrophobicity may play an important role in the ability of these compounds to undergo molecular self-assembly into microspheres: as hydrophobicity increases, these materials are more likely to aggregate and form microspheres. Indeed, a comparison of the calculated log *P*[33] and microsphere-forming ability (Table 1) indicates that a log $P > -2.0$ is required to obtain microspheres. Within this structural series, compounds with lower log *P* values may be so hydrophilic that thermodynamic factors favor solutions over microsphere suspensions.

In order to investigate further the potential role of lipophilicity in the molecular self-assembly of derivatized amino acids, we studied microsphere formation within a group of variously *N*-substituted phenylalanines (Table 2). The results reveal, in contrast to the previously described series of compounds, that acylated phenylalanines having a calculated log $P > -2.0$ do not necessarily form spheres. Thus, *N*-methylphenylalanine (**19**), *N,N*-dimethylphenylalanine (**20**), *N*-acetylphenylalanine amide (**21**), *N*-formylphenylalanine (**22**), and *N*-acetylphenylalanine (**23**) did not form microspheres in spite of the fact that the calculated log *P* of these compounds was in the −0.43 to −1.76 range. Analogs of these compounds with similar lipophilicity (log *P* −0.27 to −1.41), however, did form microspheres as shown by *N*-cyclohexanoylphenylalanine (**29**), *N*-cyclopentanoylphenylalanine (**31**), *N*-(phenylsulfonyl)-phenylalanine (**10**), *N*-benzoylphenylalanine (**9**), and *N*-salicyloylphenylalanine (**32**).

Table 2. Derivatized Phenylalanines



compd (mp, °C)	R	R'	X	microsphere formation[a]	log *P*[b]
19	CH_3	H	OH	no	−0.93
20	CH_3	CH_3	OH	no	−0.43
21	$CH_3C(O)$	H	NH_2	no	−1.76
22	HC(O)	H	OH	no	−1.21
23	$CH_3C(O)$	H	OH	no	−0.70
24	$CH_3C(O)$	H	OCH_3	no	−0.20
25	PhC(O)	H	OCH_3	no	−0.13
26	PhC(O)	H	NH_2	no	−1.89
27	$PhC(O)CH_2C(O)$	H	OH	no	−1.34
28	$PhCH_2C(O)$	H	OH	no	−0.13
29 (169−170)	$cyclo C_6H_{11}C(O)$	H	OH	yes	−0.27
30	$PhCH_2OC(O)$	H	OH	no	−1.11
31 (110−112)	$cyclo C_5H_9C(O)$	H	OH	yes	−0.77
32 (54−55)	salicyloyl	H	OH	yes	−0.53

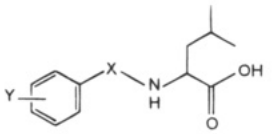
[a] Microspheres were examined using a light microscope at 10- and 100-fold magnification. Ratings of 0−2 were given a "no" designation, and ratings of 3−5 were given a "yes" designation. [b] Log *P* values were calculated from the reported experimental log *P* values of the amino acids using fragment constants.[33]

The results described above demonstrate that lipophilicity alone is not the only requirement for the formation of microspheres. Examination of the structures of these compounds indicates that an amide function having an aromatic or alicyclic component is also necessary. Substitution of a sulfonyl moiety for the carbonyl moiety in *N*-benzoylphenylalanine (**9**) had no effect on the ability of the material to form microspheres. These observations suggest that an additional structural constraint necessary for microsphere formation is juxtaposition of an amide or sulfonamide function with an aromatic or nonaromatic ring. This atomic arrangement might facilitate spatial orientations that are favorable for molecular self-assembly. The data in Table 2 clearly show that a carboxyl function is present in all of the compounds that form microspheres. Substitution of an ester (**25**) or an amide (**26**) for the carboxylic acid group results in compounds that no longer self-associate to form microspheres.

Because *N*-benzoyl and *N*-phenylsulfonyl derivatives of hydrophobic amino acids (phenylalanine, leucine) readily form microspheres, the effect of varying the substitution on the aromatic rings of these materials in a series of *N*-substituted leucines was subsequently examined (Table 3). Among benzoyl derivatives, neither electron-donating substituents (**33**, **36**) nor electron-withdrawing substituents (**34**, **35**) on the aromatic ring enhanced or attenuated the self-association of these molecules.

Molecular Modeling Studies. To understand better the molecular self-assembly of these series of de-

Table 3. Aromatic Substituents and Microsphere Formation in *N*-Benzoylated Leucines



compd	X	Y	microsphere formation[a]	mp (°C)
11	C=O	H	yes	nd[b]
33	C=O	4-OCH_3	yes	172–174
34	C=O	4-NO_2	yes	173–176
35	C=O	3-NO_2	yes	nd
36	C=O	2-OH	yes	47–50
12	SO_2	H	yes	112–114

[a] Microspheres were examined using a light microscope at 10- and 100-fold magnification. Ratings of 0–2 were given a "no" designation, and ratings of 3–5 were given a "yes" designation. [b] These compounds were purchased and melting points were not measured.

rivatized amino acids, molecular modeling studies were conducted on three representative compounds in order to examine the relationship between the conformational flexibility and structural aggregation characteristics of these molecules and their abilities to form microspheres. The molecules chosen for study were *N*-(phenylsulfonyl)-serine (**17**), *N*-(phenylsulfonyl)phenylalanine (**10**), and *N*-salicyloylphenylalanine (**32**). These compounds were chosen to exemplify the requirements for molecular self-assembly because they represent examples of poor (**17**), moderate (**10**), and excellent (**32**) microsphere formers. The modeling techniques employed used the CHARMm 22 force field within the Quanta 4.0 interface. The Boltzman Jump conformational search algorithm was used to produce ensembles of probable conformations, usually about 300 conformers, for both monomers and dimers. The dimer conformations were obtained from pairs of monomer conformations after energy minimization. The subsequent conformations generated for each compound were then clustered so as to facilitate analysis of each subset. The results of the search and cluster procedures indicated the presence of 21 *N*-(phenylsulfonyl)serine, 17 *N*-(phenylsulfonyl)phenylalanine, and 11 *N*-salicyloylphenylalanine clusters. While these results suggest differences in the dynamic flexibility of each set of dimers, they do not necessarily indicate the relative lifetimes of each conformer. For instance, even though *N*-(phenylsulfonyl)serine was shown to have 21 different clusters, the actual number might be less if the relative lifetime of each conformer is considered. However, we have assumed throughout that the barrier to interconversion is not significant because of the small energy differences between clusters. The different conformers would therefore have similar lifetimes.

N-(Phenylsulfonyl)serine exhibits the greatest flexibility and diversity among the three derivatives with 21 different low-energy conformational clusters. Most of these conformers have intermolecular phenylsulfonyl-stacking interactions and an intermolecular hydrogen bond between the molecules (Figure 1) when generated at a medium dielectric constant (~40). The presence of an intermolecular hydrogen bond is interesting because it does not seem to restrict the conformational space significantly when compared to the other derivatives.

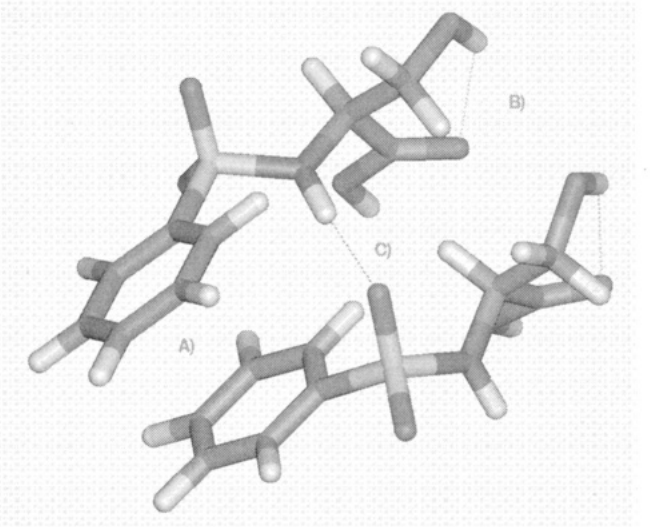


Figure 1. Representative dimer conformation of *N*-(phenylsulfonyl)serine. The dimer conformation shows an intermolecular stacking interaction between the two phenyl groups (A), an intramolecular hydrogen bond between the carbonyl oxygen and side-chain hydroxyl group of each serine moiety (B), and an intermolecular hydrogen bond between a sulfonyl oxygen and a sulfonamide nitrogen (C).

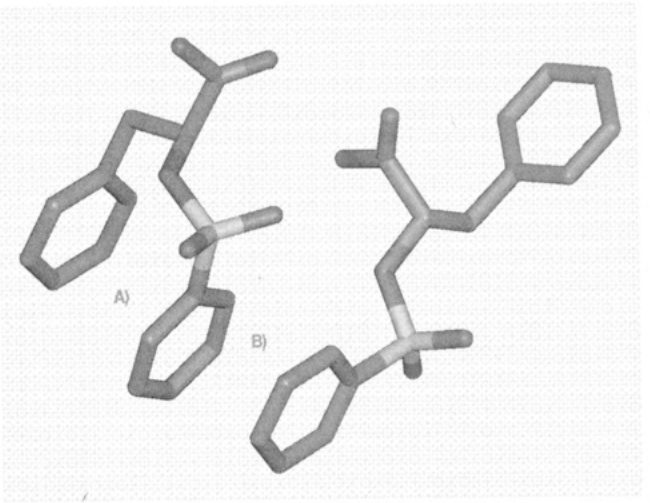


Figure 2. Representative dimer conformation of *N*-(phenylsulfonyl)phenylalanine. The dimer conformation shows an internal stacking interaction between the phenyl groups of the phenylsulfonyl and phenylalanine moieties (A) and an external stacking interaction between the two phenylsulfonyl groups (B).

The next most diverse group of conformations consists of 17 clusters generated for *N*-(phenylsulfonyl)phenylalanine. This ensemble shows that both internal and external stacking of the phenylsulfonyl group are favored. Antiparallel phenylsulfonyl/phenylalanine stacking was unstable to energy minimization and therefore considered to be unimportant. Intermolecular hydrogen bonding was observed in most clusters and predominated depending on the dielectric constant. Triple stacking of rings in the dimer conformations was also seen (Figure 2) and was especially favored at high dielectric strength (~80).

N-Salicyloylphenylalanine appeared to be the most conformationally-restricted of the three molecules studied. This was true for both the monomeric and dimeric conformations. Indeed, the small number of dimer conformational clusters reflect the rigidity of the mono-

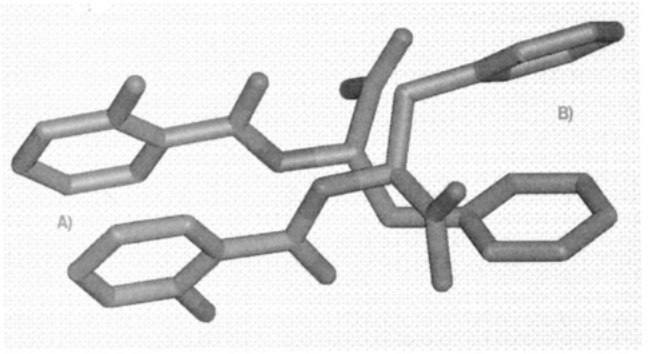


Figure 3. Representative dimer conformation of *N*-salicyloylphenylalanine. The dimer conformation shows a parallel, intermolecular stacking interaction between the aromatic rings with the salicyloyl groups on the left (A) and the phenyl groups of phenylalanine on the right (B).

mer. This rigidity arises from the internal hydrogen bonding between the carbonyl and hydroxyl of the salicyloyl moiety in combination with the planar amide bond. This lack of internal flexibility would disfavor any internally-stacked conformations. Salicyloyl−salicyloyl stacking is favored in several of the clusters (Figure 3), and three to four hydrogen bonds per dimer often developed. Interestingly, in spite of the rigidity of the *N*-salicyloylphenylalanine dimeric system compared to the aggregate structures of the other derivatives, intermolecular hydrogen bonds and ring−ring stacking were observed less frequently. This suggests that rigidity of the monomer is more important for restriction of the conformational space in the dimer than constraints between the molecules in a dimer.

Taken together, these results indicate a correlation between the conformational flexibility and the microsphere-forming ability of the molecules modeled. The most flexible molecule of this series, *N*-(phenylsulfonyl)-serine (Table 1; **17**), does not form microspheres, whereas the least flexible, *N*-salicyloylphenylalanine (Table 2; **32**), does. Thus, conformational rigidity may also be a requirement for self-association of these molecules into microspheres. This study also suggests that intramolecular hydrogen bonding, as shown by *N*-salicyloylphenylalanine, restricts the monomer conformational space resulting in dimer rigidity and the ability to form microspheres.

Microsphere Stability. The microspheres formed by *N*-benzoylphenylalanine (**9**) were transient. Within minutes of their generation, these microspheres succumbed to a higher thermodynamic order, namely, crystal formation. A dramatically-different result occurred when microsphere formation experiments were conducted on mixtures of derivatized amino acids. For example, an equimolar combination of *N*-benzoylglutamic acid (**2**), *N*-benzoylornithine (**6**), and *N*-benzoylphenylalanine (**9**) yielded excellent microspheres upon acidification. Likewise, an equimolar solution of these three derivatized amino acids plus *N*-benzoylglutamine also produced microspheres. These microspheres were isolated and analyzed for composition by reverse phase HPLC. In the three component system, the compositions of the microspheres were dependent upon the acid used to initiate their formation. When acetic acid was used to lower the pH, the resulting microspheres were formed from *N*-benzoylglutamic acid (**2**) and *N*-benzoylphenylalanine (**9**) in a ratio of 1:2. *N*-benzoylornithine (**6**) was not found in these microspheres. Acidification using citric acid gave microspheres containing all three of the compounds in a molar ratio of 1.4:2.0:1.0 *N*-benzoylglutamic acid (**2**):*N*-benzoylphenylalanine (**9**):*N*-benzoylornithine (**6**). A similar result was observed with the four-component mixture. The acetic acid-precipitated microspheres contained *N*-benzoylglutamic acid (**2**), *N*-benzoylphenylalanine (**9**), and *N*-benzoylglutamine but did not contain any of the derivatized ornithine. The microspheres formed from this four-component mixture in the presence of citric acid did contain the derivatized ornithine. In all of these cases, however, the major component in the microspheres was *N*-benzoylphenylalanine (**9**). Once again, hydrophobicity appears to be an important aspect of molecular self-assembly.

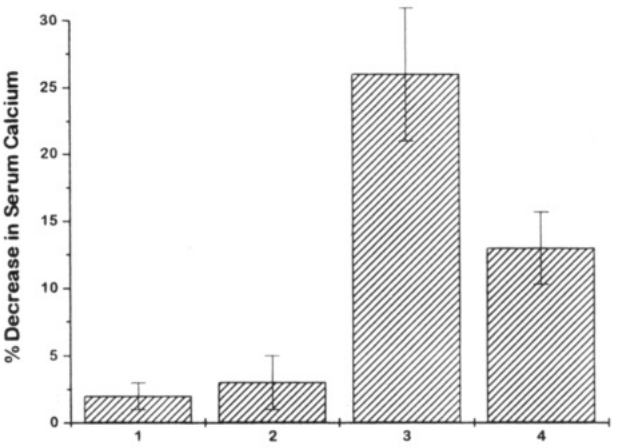


Figure 4. Oral delivery of calcitonin using microspheres prepared from *N*-phenylsulfonylated amino acids. Bar 1, calcitonin control in rats at 10 μg/kg; a similar response was obtained in primates. Bar 2, microsphere alone control in rats at 80 mg/kg; a similar response was obtained in primates. Bar 3, calcitonin-containing microspheres at 10 μg/kg calcitonin and 80 mg/kg carrier in rats 1 h after dosing. Bar 4, calcitonin-containing microspheres at 60 μg/kg calcitonin and 80 mg/kg carrier in primates 6 h after dosing.

Oral Drug Delivery Model System. Microspheres prepared from some of the derivatives were used to evaluate the feasibility of oral calcitonin delivery in rats and primates. A microsphere suspension made from a mixture of compounds **5**, **8**, **10**, **12,** and **14** and containing calcitonin was dosed by oral gavage to groups of six rats. The delivery of the drug was demonstrated by a reduction in serum calcium after dosing of the test animals and sampling of the blood. Serum calcium levels in the rats dosed with calcitonin-containing microspheres decreased by 25% compared to control experiments where calcitonin alone and empty microspheres alone were dosed (Figure 4). Similar dosing of primates caused a 15% decrease in serum calcium.

Conclusions

We have demonstrated microsphere formation in a series of *N*-benzoylated and *N*-phenylsulfonylated amino acids. To our knowledge, this is the first reported documentation of such a self-assembly process in compounds of molecular weight as low as ~300 Da. From these initial studies, certain structural features within this group of compounds are required for microsphere formation. One free carboxyl group is necessary, but no free amino groups are tolerated. Minimum calcu-

lated log P values are needed, as are substituents that facilitate hydrogen bonding between themselves and the amide carbonyl or sulfonamide oxygen. Additionally, molecular modeling studies indicate that the probability of microsphere formation increases with molecular rigidity. In future reports we will discuss structural requirements and their relationship to molecular self-assembly in more detail. Finally, microspheres prepared from derivatized amino acids can deliver therapeutically-significant amounts of salmon calcitonin as evidenced by data from rodent *in vivo* experiments providing further support for the therapeutic possibility for oral delivery of to-date orally-inactive peptides.

Experimental Section

Chemistry. Compounds **1–3**, **5**, **6**, **8**, **9**, **15**, **16**, **19–28**, and **30** were purchased from Bachem Biosciences, Inc., Philadelphia, PA. NMR spectra were recorded at 300 MHz in either D_2O or DMSO. Combustion analyses were performed by Microlit Laboratories. Thin layer chromatography was performed using E. Merck Kieselgel 60 F-254 plates. Reactions were monitored by high-pressure liquid chromatography on a Vydac 250 × 4.6 mm protein and peptide column using a gradient of 0–50% acetonitrile in water with 0.1% trifluoroacetic acid. Melting point analyses were performed using a Mel-Temp II instrument from Laboratory Devices and are in agreement with the literature values.

General Procedure for the Preparation of Derivatized Amino Acids. The following procedure was used to prepare the phenylsulfonylated and benzoylated amino acids described herein. The preparation of *N*-(phenylsulfonyl)leucine (**12**) is given as a representative example. Benzenesulfonyl chloride (70 mL, 282 mmol) was added dropwise to a solution of L-leucine (37 g, 282 mmol) in aqueous sodium hydroxide (500 mL, 2 N). During the course of this addition, the reaction temperature was maintained below 45 °C using an ice/water bath as necessary, while the pH was maintained at ca. 10 by the addition of aliquots of 14 N NaOH as necessary. After the addition was complete, the reaction mixture was stirred an additional 2 h at room temperature. The resulting clear solution was acidified to pH 2.5 by the dropwise addition of concentrated hydrochloric acid with stirring. The precipitate was collected by filtration, redissolved in a minimum amount of 12 N sodium hydroxide, reprecipitated, and filtered. This white solid was washed with dilute aqueous hydrochloric acid (750 mL, 0.1 N) to give *N*-(phenylsulfonyl)leucine as a white crystalline solid (58.6 g, 77%): ^{1}H NMR (300 MHz, DMSO-d_6) δ 12.6 (br s, 1H, OH), 8.2 (br s, 1H, NH), 7.8 (d, 2H, Ph), 7.6 (m, 3H, Ph), 3.6 (m, 1H, *CH* α to NH), 1.5 (m, 1H, *CH*CH_2), 1.3 (dd, 2H, CH_2), 0.8 (d, 3H, CH_3), 0.7 (d, 3H, CH_3). Anal. ($C_{12}H_{17}NO_4S$) C, H, N.

General Procedure for Microsphere Formation. The sodium salt of the test compound was dissolved in water at a concentration of 160 mg/mL. The pH of the resulting solution was adjusted to between 7 and 8 by use of either 2 N sodium hydroxide or 1.7 M citric acid. This essentially-neutral solution was combined with an equal volume of either 5% aqueous acetic acid or 1.7 M aqueous citric acid. The precipitate that formed was visualized under a light microscope at both 10- and 100-fold magnification. Evidence of microsphere formation was rated on a scale from 0 to 5. A value of zero was assigned to a clear solution containing no microspheres, and a value of 5 was assigned to a suspension of densely-packed microspheres. Microspheres containing salmon calcitonin were prepared similarly by dissolving the drug in the aqueous citric acid solution prior to mixing.

General Procedure for Salmon Calcitonin Microsphere Formation. The sodium salt of the test compound was dissolved in water at a concentration of 300 mg/mL. The pH of the resulting solution was adjusted to between 7 and 8 by use of either 2 N sodium hydroxide or 1.7 M citric acid. Salmon calcitonin was dissolved in an aqueous solution of citric acid (1.7 N) and gelatin (5%) at a concentration of 30 μg/mL. Equal volumes of these two solutions were mixed to give a microsphere suspension.

Molecular Modeling. Molecular modeling experiments were performed using Quanta/CHARMm software (Molecular Simulations Inc., Burlington, MA). Molecular structure files for each amino acid derivative were created using the Chemnote 2-D builder. The atom types and parameters were checked by CHARMm within Quanta. *Ab initio* HF 6-31 Gd calculations were performed on the C–SO_2–N–C dihedral angle to ensure optimal sampling of its torsional space. The parameters obtained were added to the CHARMm force field.

A conformational analysis was then performed on each of the three monomer derivatives. Energy-minimized structures of each derivatized amino acid were subjected to the Boltzmann Jump (BJ) search at a temperature of 5000 K with 40 steps of steepest descent minimization after each search step. A total of 300 structures were generated and then clustered using all non-hydrogen atoms after postprocessing with 100 steps of Powell minimization at varying dielectric constants.

For the dimer conformational analysis, initial structures were generated from the monomer BJ calculations by relative positioning and rotation of probable interacting groups. These were then optimized by energy minimization prior to BJ simulation. As before, 300 structures were generated at 5000 K with each conformer being processed with 800 steps of adopted basis Newton Raphson minimization to a gradient of 0.01 at varying dielectric strengths. Resulting structures were subsequently clustered by non-hydrogen atoms and ring-stacking distance.

Animal Experiments. All animal experimental procedures and protocols were approved in advance by the Emisphere Institutional Animal Care and Use Committee. Male Sprague–Dawley rats weighing 125–150 g were used. The rats were housed in the animal unit at Emisphere Technologies under standard conditions with free access to water. All of the rats in these studies were anesthetized with 44 mg/kg ketamine and 0.5 mg/kg thorazine immediately prior to dosing. The rats were administered 2 mL/kg calcitonin–microsphere suspension by oral gavage. The total dose of calcitonin was 30 μg/kg, and the total dose of spheres was 300 mg/kg. Blood samples were collected serially from the tail artery at 0.5, 1, 1.5, 2, and 3 h after dosing. Peak hypocalcemia was observed at 1 h. Serum calcium was determined by quantitation with a calcium kit available from Sigma Chemical Co.[34]

Four male Rhesus monkeys weighing 4–5 kg were used. The monkeys were fasted overnight and placed in primate restraint chairs for dosing and blood sampling. Conscious monkeys received microspheres containing calcitonin by nasogastric gavage, and blood samples were collected from saphenous vein catheters. Blood samples were collected at 1 and 0.5 h before dosing and at 0.25, 0.5, 1, 2, 3, 4, 5, 6, 7, and 8 h after dosing for serum calcium determination. Peak hypocalcemia was observed at 6 h.

References

(1) Ghadiri, M. R.; Granja, J. R.; Milligan, R. A.; McRee, D. E.; Khazanovich, N. Self-assembling organic nanotubules based on a cyclic peptide architecture. *Nature* **1993**, *366*, 324–327.
(2) Borman, S. Enzymes immobilized in polymeric microcapsule arrays. *Chem. Eng. News* **1993**, May 3, 45–46.
(3) Perlstein, J. Molecular Self-Assemblies. 2. A computational method for the prediction of the structure of one-dimensional screw, glide, and inversion molecular aggregates and implications for the packing of molecules in monolayers and crystals. *J. Am. Chem. Soc.* **1994**, *116*, 455–470.
(4) Brumlik, C. J.; Martin, C. R. Template Synthesis of Metal Microtubules. *J. Am. Chem. Soc.* **1991**, *113*, 3174–3175.
(5) Hsu, L. L.; Fox, S. W. Interactions between diverse proteinoids and microspheres in simulation of primordial evolution. *Biosystems* **1976**, *8*, 89–101 and references cited therein.
(6) Fox, S. W.; Harada, K.; Vegotsky, A. Thermal polymerization of amino acids and a theory of biochemical origins. *Experentia* **1959**, *15*, 81–84.
(7) Fox, S. W. A new view of the "synthesis of life". *Q. J. Fla. Acad. Sci.* **1968**, *31*, 1–15.
(8) Fox, S. W. A theory of macromolecular and cellular origins. *Nature* **1965**, *205*, 328–340.
(9) Fox, S. W. How did life begin? *Sci. Technol.* **1968**, No. 74, 51–61.

(10) Fox, S. W.; Nakashima, T. Fractionation and characterization of an amidated thermal I:I:I:-proteinoid. *Biochim. Biophys. Acta* **1967**, *140*, 155–167.
(11) Fuhrhop, J. H.; Blumtritt, P.; Lehmann, C.; Luger, C. Supramolecular assemblies, a crystal structure, and a polymer of *N*-diacetylenic gluconamides. *J. Am. Chem. Soc.* **1991**, *113*, 7437–7439.
(12) Frankel, D. A.; O'Brien, D. F. Supramolecular assemblies of diacetylenic aldonamides. *J. Am. Chem. Soc.* **1991**, *113*, 7436–7437.
(13) Dagani, R. Artificial ion channels. *Chem. Eng. News* **1994**, May 30, 4–5.
(14) Fuhrhop, J. H.; Spiroski, D.; Boettcher, C. Molecular monolayer rods and tubules made of ∝-(L-lysine),ω-(amino) bolaamphiphiles. *J. Am. Chem. Soc.* **1993**, *115*, 1600–1601.
(15) Bergeron, R. J.; Phanstiel, O.; Yao, G.; Milstein, S.; Weimar, W. R. Macromolecular self-assembly of diketopiperazine tetrapeptides. *J. Am. Chem. Soc.* **1994**, *116*, 8479–8484.
(16) Santiago, N.; Rivera, T.; Mayer, E.; Milstein, S. Proteinoid microspheres for the oral delivery of heparin. *Proc. Int. Symp. Controlled Release Bioact. Mater., 20th.* **1992**, *19*, 514–515.
(17) Milstein, S.; Baughman, R. A.; Santiago, N.; Rivera, T.; Falzarano, L.; Dewlane, P.; Welch, S. *Symp. Abstr. Am. Assoc. Pharm. Sci. Annu. Mtg.* **1992**, 35.
(18) Steiner, S.; Rosen, R. U.S. Patent 4,925,673, 1990.
(19) Azria, M. *The Calcitonins*; Karger: Basel, Switzerland, 1989; pp 133–144.
(20) Greenstein, J. P.; Winitz, M. *Chemistry of the Amino Acids*; John Wiley & Sons: New York., 1962; Vol. 2, p 1266.
(21) Cabre Castellevi, J.; Palomo Coll, A. *N*-acyl transfer. A new and easy procedure for the formation of N-acyl-α-amino acids. *Afinidad* **1986**, *43*, 401–403.
(22) Chen, M. F. F.; Benoiton, N. L. Diisopropylethylamine eliminates dipeptide formation during the acylation of amino acids using benzoyl chloride and some alkyl chloroformates. *Can. J. Chem.* **1987**, *65*, 1224–1227.
(23) Banerjee, P. K.; Amidon, G. L. Physicochemical property modification strategies based on enzyme substrate specificities. *J. Pharm. Sci.* **1981**, *70*, 1299–1303.
(24) Nishitani, T.; Iwasake, T.; Mushika, Y.; Inoue, I.; Miyoshi, M. Synthesis of 3-amino-3-(5-fluorouracil-1-yl)propionic acid and 4-amino-4,5-fluoro-1-yl)butyric acid derivatives. *Chem. Pharm. Bull.* **1980**, *28*, 1137–41.
(25) Shinkai, H.; Toi, K.; Kumashiro, I.; Seto, Y.; Fukuma, M.; Dan, K.; Toyoshima, S. N-acylphenylalanines and related compounds. A new class of oral hypoglycemic agents. *J. Med. Chem.* **1988**, *31*, 2092–2097.
(26) Piper, J. R.; Rose, L. M.; Johnston, T. P.; Grenan, M. M. S-2,ω-Diaminoalkyl dihydrogen phosphorothioates as antiradiation agents. *J. Med. Chem.* **1979**, *22*, 631–639.
(27) Scully, F. E.; Bowdring, K. Chemistry of organic chloramines. Formation of arenesulfonamides by derivatization of organic chloramines with sodium arenesulfinates. *J. Org. Chem.* **1981**, *46*, 5077–5081.
(28) Nagao, Y.; Miyasaka, T.; Seno, K.; Fujita, E.; Shibata, D.; Doi, E. Peptide bond formation, chemoselective acylation of amino acids, and crosslinking reaction between amino acids utilizing a functional five-membered heterocycle, 1,3-thiazolidine-2-thione. *J. Chem. Soc., Perkin Trans. 1* **1984**, 2439–2446.
(29) Roemmele, R. C.; Rapoport, H. Chirospecific synthesis of β-hydroxy α-amino acids. *J. Org. Chem.* **1989**, *54*, 1866–1875.
(30) Nagai, U.; Kurumi, M. Optical rotatory dispersion of nitrobenzene derivatives. *Chem. Pharm. Bull.* **1970**, *18*, 831–834.
(31) Mazzotta, D.; Giori, P.; Guarneri, M.; Benassi, C. A. Esters and amides of salicycloylamino acids. *Farmaco, Ed. Sci.* **1975**, *30*, 399–407.
(32) Shinkai, H.; Nishikawa, M.; Sato, Y.; Toi, K.; Kumashiro, I.; Seto, Y.; Fukuma, M.; Katsuaki, D.; Toyoshima, S. *N*-(Cyclohexylcarbonyl)-D-phenylalanines and related compounds. A new class of oral hypoglycemic agents. *J. Med. Chem.* **1988**, *32*, 1436–1441.
(33) Leo, A.; Hansch, C.; Elkins, D. Partition coefficients and their uses. *Chem. Rev.* **1971**, *6*, 525–616.
(34) Kessler, G.; Wolfman, M. An automated procedure for the simultaneous determination of calcium and phosphorus. *Clin. Chem.* **1964**, *10*, 686.

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