4-[4-[(2-Hydroxybenzoyl)amino]phenyl]butyric Acid as a Novel Oral Delivery Agent for Recombinant Human Growth Hormone

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A series of *N*-acetylated, non-α, aromatic amino acids was prepared and shown to promote the absorption of recombinant human growth hormone (rhGH) from the gastrointestinal tract. Seventy compounds in this family were tested *in vivo* in rats. Of the compounds tested, 4-[4-[(2-hydroxybenzoyl)amino]phenyl]butyric acid was identified as a preclinical candidate and was used to demonstrate the oral delivery of rhGH in primates. A significant positive correlation was found between the relative log *k′* of the delivery agents, as determined by HPLC on an immobilized artificial membrane (IAM) column, and serum rhGH concentrations following oral or colonic dosing in rats. Structure−activity relationships have also been developed on the basis of electronic effects and hydrogen-bonding characteristics of the aromatic amide substituents.

Introduction

Dramatic progress in the field of biotechnology during the past decade has resulted in a large increase in the number of commercially available protein drugs.[1] While these new drugs have enormous therapeutic potential, they represent a major clinical challenge for oral delivery[2] because the function of the gastrointestinal tract, by design, is to degrade proteins[3] and prevent their absorption.[4] Acid-induced hydrolysis in the stomach, enzymatic degradation throughout the gastrointestinal tract, and bacterial fermentation in the colon are among the many barriers that can prevent the oral delivery of proteins and large peptides. Physical barriers to delivery include poor solubility in the intestinal environment and lack of permeation through the epithelial cells. The latter can exclude the passage of compounds across the tissue on the basis of size, charge, and/or lipophilicity. Given these barriers, it is not surprising that the oral delivery of protein and peptide drugs has been considered impossible or, at best, difficult. Taken together, these characteristics result in minimal oral bioavailibility of protein drugs[5] and necessitate administration by injection in order to achieve therapeutic concentrations of the drug in the blood.

Human growth hormone is a protein drug that has been used to treat growth hormone deficiency in children since 1958.[6] Endogenously, it is a heterogeneous mixture of polypeptides whose main component is a single-chain, 191 amino acid protein (MW 22 124) incorporating two intramolecular disulfide bonds and a free amino terminus.[7] The drug was obtained exclusively from human, cadaveric, pituitary extracts until 1985 when recombinant human growth hormone (rhGH) became one of the first products of the biotechnology industry. Ample supplies of rhGH are currently obtained from various bacterial strains and mammalian cells that have been modified by the addition of the gene for human growth hormone.[6]

Currently, all of the FDA-approved growth hormone preparations are parenteral formulations. The drug can be administered by subcutaneous or intramuscular injection; however, subcutaneous injection is preferable because it is less painful.[8] rhGH therapy has been approved for a number of medical indications, but the clearest clinical indication for rhGH is replacement therapy in pediatric isolated growth hormone deficiency.[9] The clinical manifestation of this condition is a lack of growth as evidenced by a height that is significantly below that expected on the basis of age and sex. Growth hormone therapy increases growth and improves bone density in this population of patients with relatively few adverse side effects.[10] Daily subcutaneous administration is recommended because it has proven to be more effective than the three times/week or once weekly dosing schedules used previously.[11] The daily dosing schedule also more closely mimics the natural, circadian cycle of normal, endogenous growth hormone release.

Because rhGH is most commonly used in pediatric patients, a noninjectable dosage form would greatly facilitate patient compliance. To date, only two studies in this area have been reported, and both have met with limited success. Ron *et al.*[12] have developed a controlled-release system for long-term rhGH therapy that uses a biodegradable polymer implant. Implantation of this polyanhydride polymer impregnated with bovine growth hormone (bGH) into the lower abdominal cavity of rats caused the release of biologically active bGH into the circulation for up to 3 weeks. In the other study, serum levels of rhGH were reported following intranasal administration in humans.[13] These studies notwithstanding, oral delivery of rhGH would still be preferred to parenteral, but, to our knowledge, has not yet been achieved.

As part of continuing efforts to design and develop oral drug delivery agents, a family of novel compounds has been prepared that promotes the gastrointestinal absorption of rhGH in rats and primates. These compounds were selected from the third generation of delivery agents designed and synthesized in our laboratories. In a previous report,[14] the first generation of drug delivery agents has been presented. These mate-

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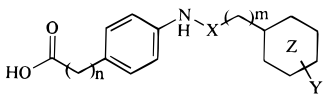


Figure 1. Generic structural representation of *N*-acylated non-α-amino acid oral drug delivery agents.

rials, which formed microspheres, were highly complex, structurally uncharacterized mixtures of thermally condensed α-amino acids called proteinoids.[14] Subsequently, microsphere formation and oral delivery of salmon calcitonin in rats and primates was demonstrated by the use of structurally defined *N*-acylated α-amino acids.[15] These latter compounds were also shown to promote the oral delivery of both salmon calcitonin and interferon-α in rats and primates when dosed as an aqueous solution instead of a microsphere suspension.[16] Herein is reported the successful oral delivery of rhGH in rats and primates from aqueous solutions using a series of *N*-acylated, non-α-amino acids as novel drug delivery agents.

Results and Discussion

Synthetic Design. A family of oral drug delivery agents has been prepared comprising 70 compounds generically represented by the chemical formula shown in Figure 1. All of the compounds described herein are derivatives of either 4-aminobenzoic acid ($n = 0$), 2-(4-aminophenyl)acetic acid ($n = 1$), 3-(4-aminophenyl)-propionic acid ($n = 2$), or 4-(4-aminophenyl)butyric acid ($n = 3$). These compounds were designed as part of an effort to expand upon our earlier leads, which were *N*-acylated, α-amino acids.[16] Of the current compounds, **2** has been identified as a preclinical candidate (Table 1).

Synthetic Procedures. The compounds listed in Table 1 were prepared by standard techniques[17−19] in either aqueous or organic solvents. In general, aqueous reaction conditions included the dissolution of a non-α-amino acid in aqueous sodium hydroxide followed by the addition of the appropriate acid chloride. 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. A representative process in an organic solvent was the dissolution of equimolar amounts of a non-α-amino acid and triethylamine in tetrahydrofuran, followed by the addition of a selected acid chloride. After about 4 h of stirring at room temperature, the reaction mixture was quenched, and the product was purified by recrystallization. All of the compounds reported herein were prepared by either of these two methods with the exception of compounds **15**, **16**, **21**, **22**, **31**, **34**, **39**, **40**, **51**, and **70** whose syntheses are described in the Experimental Section.

Lead Identification and Structure−Activity Relationships. Each of the compounds in Table 1 was evaluated in a rat model for its ability to promote the absorption of rhGH. During the course of our screening studies, it was found that rhGH absorption following intracolonic dosing was comparable to oral gavage, but at lower doses of both delivery agent and drug. The bioavailability of rhGH relative to subcutaneous injection is 3−5% orally and 8−10% colonically. Subsequently, colonic administration replaced dosing by oral gavage. Figure 2 is a comparison of the results from an orally dosed solution of **2** (300 mg/kg) and rhGH (6 mg/kg) and a colonically dosed solution of **2** (25 mg/kg) and rhGH (1 mg/kg). The serum rhGH concentrations are similar for both routes of administration in spite of the significantly lower doses of both delivery agent and drug used in the colonic study.

The data compiled from the rat *in vivo* studies, reported in Table 2, suggest a number of interesting relationships between structure and activity. For example, consider the homologous series of compounds **1**, **29**, **35**, and **44**. An increase in the activities of these delivery agents appears to correlate with an increase (from 0 to 3) in the length of the amide carbon chain. Figure 3 is a plot of the peak rhGH serum concentrations versus the log relative *k′*, as measured by chromatography on an immobilized artificial membrane (IAM) column.[20] The ability of this group of compounds to promote the absorption of rhGH increases with increasing carbon chain length and, by implication, increasing lipophilicity in the amide portion of the molecule.

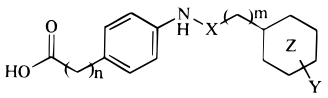
We have also identified a correlation between the electronic properties of diverse substituents at the ortho position of the amide aromatic ring and the efficiency of drug delivery. The data indicate a general trend toward improved activity for delivery agents bearing an electron-withdrawing ortho substituent. For example, **22** (Y = 2-OCF_3) is significantly more efficient at promoting the absorption of rhGH than is **20** (Y = 2-OCH_3). For the various ortho-substituted compounds, **1** (2-H), **8** (2-Cl), **9** (2-F), **13** (2-CH_3), **20** (2-OCH_3), and **22** (2-OCF_3), activity increases with increasing σ^0 values[21] (Figure 4). Two additional compounds in this series that do not follow this general trend are **2** (2-OH) and **19** (2-NO_2). Delivery agents **2** and **19** show greater and lesser activities, respectively, than predicted by the σ^0 values. This finding is not unexpected because the greatest incidence of failures in the Hammett equation are encountered in correlations involving molecules bearing substituents capable of either accepting or donating an electron pair.[21] In this case, both **2** and **19** have ortho substituents that are capable of hydrogen bonding. Hydrogen bonding between the hydroxyl hydrogen and the amide carbonyl in **2** results in the formation of a 6-membered ring. The hydrogen bonding between the nitro oxygens and the NH of the amide bond in **19** also appears to be a favorable interaction.

In an effort to use an electronic parameter that accounts for the effects of hydrogen bonding, σ^- values were substituted for the σ^0 values of **2** and **19**. This manipulation did not improve the correlation between the electronic activity and the *in vivo* data of these two compounds.

The effects of meta and para substituents on the amide aromatic ring were also investigated. In general, any substituent other than hydrogen at the para position resulted in substantially reduced (**6**, **7**, **33**, **54**, **61**, and **63**) or highly variable (**4**) protein delivery. Electron-withdrawing substituents at the meta position decreased activity (**11**, **12**, and **21**); however, the highly electron-donating meta, *N*,*N*-dimethyl substituent (**15**) increased activity.

The trend of increased lipophilicity that results in the increased delivery of drug previously mentioned is also

Table 1. Protein Oral Delivery Agents



compd	Y	X	*n*	*m*	Z	compd	Y	X	*n*	*m*	Z
1	H	C=O	3	0	phenyl	**36**	H	C=O	3	vinyl	phenyl
2	2-OH	C=O	3	0	phenyl	**37**	F	C=O	3	vinyl	phenyl
3	2,3-Ph	C=O	3	0	phenyl	**38**	2-CH_3	C=O	3	vinyl	phenyl
4	4-Ph	C=O	3	0	phenyl	**39**	2-OCH_3	C=O	3	vinyl	phenyl
5	3,4-Ph	C=O	3	0	phenyl	**40**	2-F	C=O	3	vinyl	phenyl
6	4-OCH_3	C=O	3	0	phenyl	**41**	H	C=O	3	CHMe	phenyl
7	4-F	C=O	3	0	phenyl	**42**	H	C=O	3	CHEt	phenyl
8	2-Cl	C=O	3	0	phenyl	**43**	H	C=O	3	CHEt	cyclohexyl
9	2-F	C=O	3	0	phenyl	**44**	H	C=O	3	3	phenyl
10	2,4-$(OH)_2$	C=O	3	0	phenyl	**45**	H	C=O	3	$(CH_2)_2O$	phenyl
11	3-CF_3	C=O	3	0	phenyl	**46**	H	C=O	3	$(CH_2)_2C$=O	phenyl
12	3-Cl	C=O	3	0	phenyl	**47**	H	C=O	3	$(CH_2OH)_2$	phenyl
13	2-CH_3	C=O	3	0	phenyl	**48**	H	C=O	3	3	cyclohexyl
14	2,6-$(OH)_2$	C=O	3	0	phenyl	**49**	H	C=O	3	4	phenyl
15	3-N(CH_3)	C=O	3	0	phenyl	**50**	2-OH	C=O	2	0	phenyl
16	3,4-OCH_2O	C=O	3	0	phenyl	**51**	2-NO_2	C=O	2	0	phenyl
17	2,6-diCH_3	C=O	3	0	phenyl	**52**	2,6-$(OH)_2$	C=O	2	0	phenyl
18	2-COOH	C=O	3	0	phenyl	**53**	2-OCH_3	C=O	2	vinyl	phenyl
19	2-NO_2	C=O	3	0	phenyl	**54**	4-OCH_3	C=O	2	vinyl	phenyl
20	2-OCH_3	C=O	3	0	phenyl	**55**	H	C=O	vinyl	0	phenyl
21	3-NO_2	C=O	3	0	phenyl	**56**	2-OH	C=O	1	0	phenyl
22	2-OCF_3	C=O	3	0	phenyl	**57**	H	SO_2	1	0	phenyl
23	H	SO_2	3	0	phenyl	**58**	H	C=O	1	1	phenyl
24	H	C=O	3	0	cyclohexyl	**59**	H	C=O	1	1	cyclohexyl
25	4-CH_3	C=O	3	0	cyclohexyl	**60**	H	C=O	1	2	phenyl
26	H	C=O	3	0	cycloheptyl	**61**	4-CH_3	C=O	0	0	cyclohexyl
27	2-COOH	C=O	3	0	cyclohexyl	**62**	2-OH	C=O	0	0	phenyl
28	H	C=O	3	1	cyclohexyl	**63**	4-F	C=O	0	0	phenyl
29	H	C=O	3	1	phenyl	**64**	H	SO_2	0	0	phenyl
30	2-OH	C=O	3	1	phenyl	**65**	H	C=O	0	1	cyclohexyl
31	2-OH	CH_2	3	0	phenyl	**66**	H	C=O	0	1	phenyl
32	2-F	C=O	3	1	phenyl	**67**	H	C=O	0	2	phenyl
33	4-*i*-Bu	C=O	3	1	phenyl	**68**	2-OCH_3	C=O	0	vinyl	phenyl
34	H	CH_2	3	0	phenyl	**69**	H	C=O	0	3	phenyl
35	H	C=O	3	2	phenyl	**70**	H	C=O	0	3	cyclohexyl

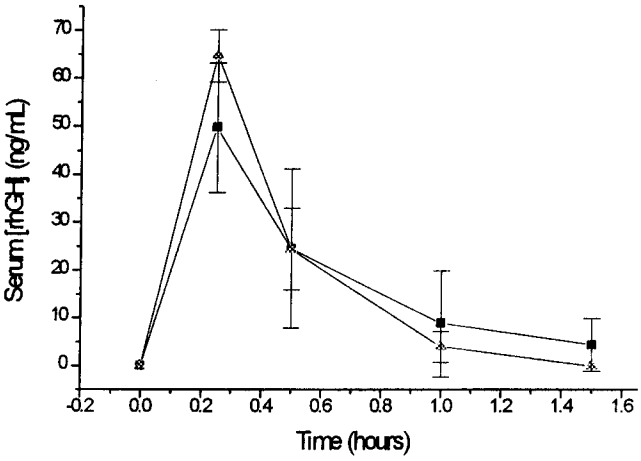


Figure 2. Comparison of oral and colonic administration of **2** and rhGH in rats. The triangles represent the response following intracolonic instillation of an aqueous solution of 2 (25 mg/kg) and rhGH (1 mg/kg). The squares represent the response following oral administration of an aqueous solution of **2** (300 mg/kg) and rhGH (6 mg/kg).

evident when the efficacy of amide delivery agents and their vinylogs is evaluated. This contention is supported by the *in vivo* activities of two pairs of compounds, namely, **1**/**36** and **20**/**39**. Compound **36** is the vinylogous amide of **1**, and **39** is the vinylogous amide of **20**. Oral dosing (Table 2) of aqueous solutions of rhGH (6 mg/kg) and either **1** (500 mg/kg) or **36** (400 mg/kg) in rats showed **36** to be at least 2 times more effective in promoting the gastrointestinal absorption of rhGH, even at a lower dose. This same trend is observed with intracolonic dosing of rats (Table 2) with aqueous solutions of rhGH (1 mg/kg) and either **20** or **39** (25 mg/kg) where **39** showed twice the activity of **20**.

On the basis of the results of the rodent studies, delivery agent **2** was chosen as a preclinical candidate and used to demonstrate oral rhGH delivery in a primate model. The pharmacokinetic profile after oral dosing of four cynomolgus monkeys with aqueous solutions of **2** (800 mg/kg) and rhGH (6 mg/kg) via nasogastric gavage is presented in Figure 5.

Once **2** was identified as the most active delivery agent in this series of compounds, the specific intramolecular hydrogen-bonding properties of this molecule and its relationship to drug delivery was more closely studied. Compounds **14**, **30**, and **31** were prepared and tested. Although structurally similar, none of these compounds has the ability to participate in the particular intramolecular hydrogen-bonding interactions that are available to **2**. Compound **14**, although it bears the necessary *o*-hydroxy group, is sterically encumbered by an additional hydroxy substituent at position 6 on the aromatic ring. Therefore, only **2** can assume a planar conformation as a result of the through-space interaction between the phenolic hydroxyl hydrogen and the amide carbonyl forming a 6-membered ring (Figure 6). The substitution of an amine function in **31** for the

Table 2. Delivery of Recombinant Human Growth Hormone (rhGH) in Rats

compd	compound dose (mg/kg)	rhGH dose (mg/kg)	route of administration	peak [rhGH] (ng/mL)	compd	compound dose (mg/kg)	rhGH dose (mg/kg)	route of administration	peak [rhGH] (ng/mL)
1	500	6	OG[a]	21.7 ± 19.5[a]	**36**	400	6	OG	49.7 ± 25.0
	300	6	OG	5.4 ± 3.0	**37**	200	6	OG	31.1 ± 14.4
	25	1	IC[b]	17.5 ± 3.5	**38**	25	1	IC	16.4 ± 17.8
2	300	6	OG	49.7 ± 13.5	**39**	25	1	OG	32.9 ± 8.2
	25	1	IC	64.7 ± 5.5	**40**	25	1	IC	7.7 ± 4.2
3	300	6	OG	5.9 ± 7.8	**41**	300	6	OG	2.3 ± 2.6
4	300	6	OG	39.9 ± 34.4	**42**	300	6	OG	5.9 ± 6.6
5	400	6	OG	28.2 ± 4.5	**43**	400	6	OG	0.5 ± 0.7
6	300	6	OG	0	**44**	300	6	OG	17.9 ± 6.9
7	300	6	OG	2.8 ± 3.2	**45**	25	1	IC	44.1 ± 8.2
8	25	1	IC	52.2 ± 11.4	**46**	25	1	IC	0.6 ± 0.7
9	300	6	OG	43.4 ± 16.6	**47**	25	1	IC	2.5 ± 2.6
	25	1	IC	20.8 ± 5.8	**48**	25	1	IC	26.0 ± 5.7
10	200	6	OG	16.9 ± 14.4	**49**	25	1	IC	8.4 ± 0.5
11	25	1	IC	17.6 ± 14.5	**50**	25	1	IC	37.2 ± 17.5
12	25	1	IC	0	**51**	25	1	IC	17.6 ± 14.7
13	25	1	IC	9.08 ± 2.9	**52**	25	1	IC	9.3 ± 3.3
14	25	1	IC	20.9 ± 11.5	**53**	25	1	IC	61.7 ± 17.8
15	25	1	IC	50.7 ± 14.5	**54**	25	1	IC	5.0 ± 5.6
16	25	1	IC	0	**55**	400	6	OG	6.5 ± 4.6
17	25	1	IC	7.7 ± 4.2	**56**	25	1	IC	5.6 ± 6.3
18	25	1	IC	22.3 ± 5.5	**57**	500	6	OG	0
19	25	1	IC	14.9 ± 6.8	**58**	300	6	OG	9.0 ± 7.6
20	25	0.8	IC	17.8 ± 10.7	**59**	300	6	OG	15.7 ± 8.0
21	25	1	IC	33.5 ± 12.7	**60**	300	6	OG	12.6 ± 10.3
22	25	1	IC	56.8 ± 15.7	**61**	400	6	OG	0
23	500	6	OG	28.0 ± 20.3	**62**	25	1	IC	0
	250	6	OG	2.6 ± 3.1	**63**	300	6	OG	1.0 ± 1.2
24	500	6	OG	63.8 ± 7.4		25	1	IC	2.2 ± 2.5
25	400	6	OG	41.6 ± 18.5	**64**	500	6	OG	13.7 ± 14.1
26	500	6	OG	92.8 ± 27.5		25	1	IC	0
27	200	3	OG	0	**65**	500	6	OG	0
28	500	6	OG	24.2 ± 14.5	**66**	500	6	OG	0
29	300	6	OG	8.1 ± 2.6	**67**	300	6	OG	3.3 ± 4.1
30	300	6	OG	0	**68**	25	1	IC	30.4 ± 12.9
31	25	1	IC	32.5 ± 7.7	**69**	300	6	OG	11.9 ± 13.3
32	25	1	IC	7.3 ± 3.0	**70**	300	6	OG	29.3 ± 19.3
33	200	6	OG	7.8 ± 5.0	none	0	6	OG	0
34	200	6	OG	20.4 ± 17.0		0	1	IC	0
35	300	6	OG	12.8 ± 3.9					

[a] Oral gavage. [b] Intracolonic instillation. [c] Mean ± SD.

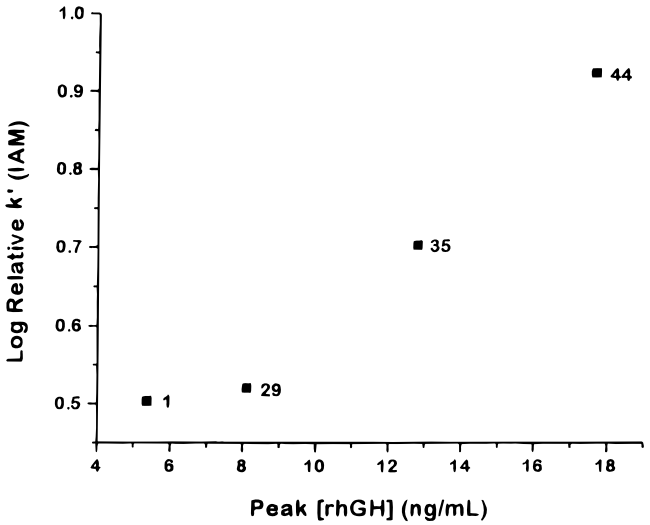


Figure 3. Correlation of log relative k' from the IAM column to the peak serum rhGH concentrations after orally dosing rats with an aqueous solution of delivery agent at 300 mg/kg and rhGH at 6 mg/kg. Compounds **1**, **29**, **35**, and **44** comprise an homologous series in which the amide carbon chain length increases from 0 to 3. A correlation coefficient of 0.98 was obtained from linear regression analysis.

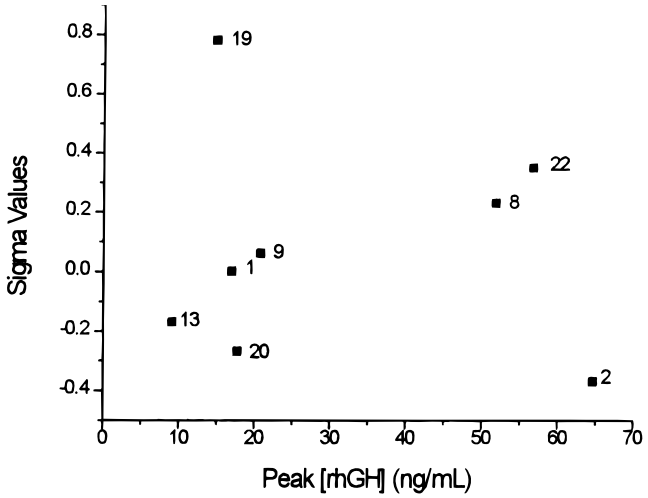


Figure 4. Correlation of σ to peak serum concentrations of rhGH after intracolonic dosing of rats with an aqueous solution of delivery agent at 25 mg/kg and rhGH at 1 mg/kg. Compounds **2**, **8**, **9**, **13**, **19**, **20**, and **22** comprise an homologous series in which the ortho substituent varies. A correlation coefficient of 0.90 was obtained from linear regression analysis when **2** and **19** were excluded.

amide in **2** prevents intramolecular hydrogen bonding completely, and intramolecular hydrogen bonding in **30** must occur through a less favorable 7-membered ring.

Consequently, neither **30** nor **31** is likely to be planar structures. Perhaps the most interesting compound in this group is **14**: the additional aromatic hydroxyl function in the 6 position presents the possibility of

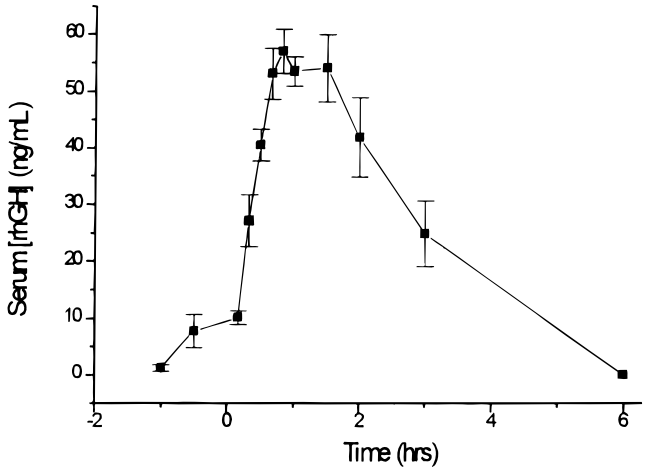
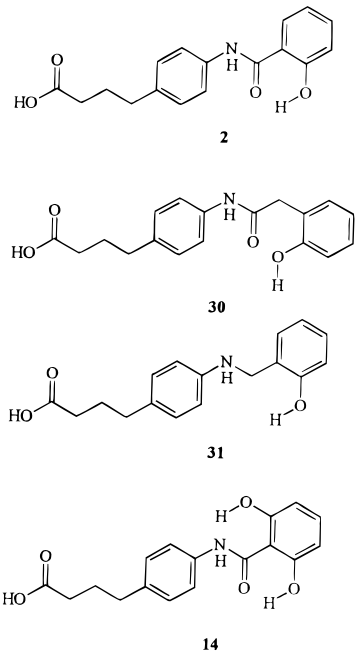


Figure 5. Pharmacokinetic profile following oral administration of an aqueous solution of **2** (800 mg/kg) and rhGH (6 mg/kg) in conscious cynomolgus monkeys.



2 **30** **31** **14**

Figure 6. Comparison of the intramolecular hydrogen-bonding capabilities of **2**, **30**, **31**, and **14**. Hydrogen bonding between the phenolic hydrogen and the amide carbonyl occurs through a 6-membered ring in **2**. The same interaction in **30** produces a 7-membered ring. The substitution of an amine for the amide in **31** prevents the hydrogen-bonding interaction completely. The additional hydroxyl in **14** allows for two intramolecular hydrogen-bonding interactions.

forming two 6-membered rings via intramolecular hydrogen bonding by the interaction of one hydroxyl hydrogen with the amide carbonyl and the other hydroxyl hydrogen with the amide nitrogen. Coincidental formation of these two hydrogen bonds would force the aromatic rings of **14** into an orthogonal orientation. Examination of the *in vivo* data for **2**, **14**, **30**, and **31** indicates that **2** is the most effective in promoting the oral absorption of rhGH. This suggests that hydrogen-bonding substituents that impose an overall planar structure may be a requirement for efficient oral protein delivery.

To gain additional information on the lead compound, homologs of **2** that varied only in the length of the acid carbon chain were examined. The compounds chosen

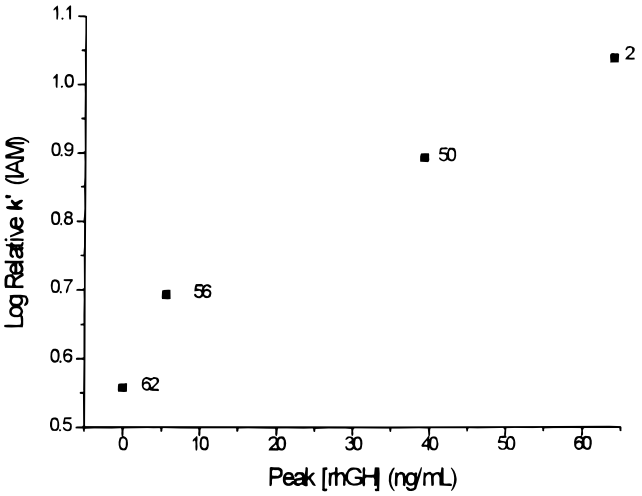


Figure 7. Correlation of log relative *k′* from IAM to peak serum concentrations of rhGH after intracolonic dosing of rats with an aqueous solution of delivery agent at 25 mg/kg and rhGH at 1 mg/kg. Compounds **2**, **50**, **56**, and **62** comprise a homologous series in which the acid carbon chain length increases from 1 to 4. A correlation coefficient of 0.98 was obtained from linear regression analysis.

for study were **50**, **56**, and **62**. The observation that **2** was significantly more active than **30** appeared to rule out the effectiveness of higher amide homologues. As expected, based on the previous observation that increased delivery agent lipophilicity within a structural series leads to improved drug delivery (Figure 3), a similar relationship exists in the series **62**, **56**, **50**, and **2** in which the rhGH serum concentrations increase as the length of the acid carbon chain increases from 1−4 (Figure 7).

Mechanistic Studies. The promising *in vivo* data generated significant speculation in our laboratories with regard to the mechanism of action of these novel protein delivery agents, including classical penetration enhancement and/or enzyme inhibition. The latter was deemed unlikely based on our previous report on similar compounds in which the delivery agents were shown to be extremely inefficient inhibitors of proteolytic enzymes.[15]

In order to address the issue of the delivery agents acting as penetration enhancers and causing drug absorption as a result of alteration in membrane structure (i.e., damage), histopathological studies were conducted on rats dosed orally or intracolonically with **2**. Groups of five or six rats were given aqueous solutions of **2** at a dose of 300 mg/kg (oral) or 25 mg/kg (colonic) and sacrificed at 30, 60, and 120 min postdosing. Necropsy of the gastrointestinal tract that included the stomach, duodenum, jejunum, and ileum of each animal, followed by histological examination, indicated no untoward pathology. These studies suggest that drug transit across the intestinal membrane is not the result of mucosal damage.

Encouraged by these findings, additional studies aimed at examining the mechanism(s) of drug delivery were started. For example, the efficiency of compound **2** in mediating rhGH delivery in rats was studied as a function of both dose and dose volume. Figure 8 shows the effects of varying the dose of **2** from 25 to 10 mg/kg. This data suggests that the minimum effective delivery agent dose is 20 mg/kg in the rat model under these

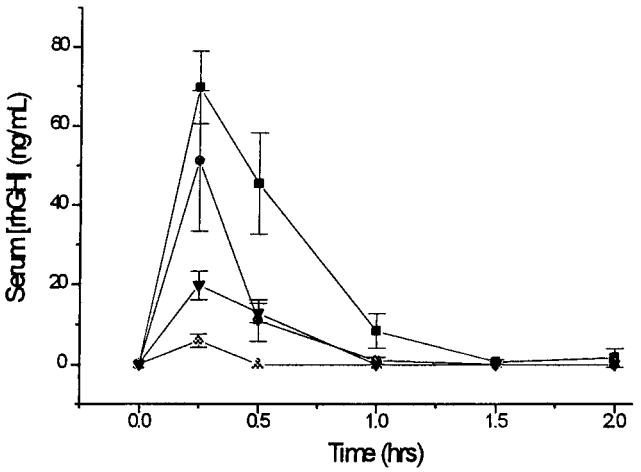


Figure 8. Effect of varying the dose of delivery agent **2** on the delivery of rhGH (1 mg/kg) in rats following intracolonic administration of 1 mL/kg of dosing solution. The squares represent the response at a delivery agent dose of 50 mg/kg. The circles represent the response at a delivery agent dose of 25 mg/kg. The inverted triangles represent the response at a delivery agent dose of 20 mg/kg. The upright triangles represent the response at a delivery agent dose of 10 mg/kg.

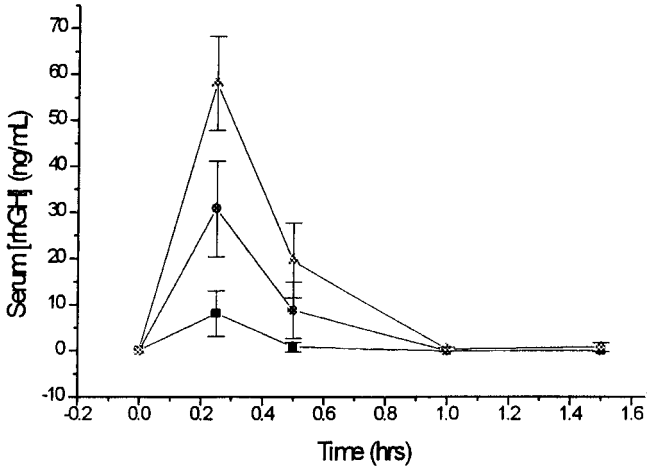


Figure 9. Effect of varying dose volume on the delivery of rhGH (1 mg/kg) in rats using delivery agent **2** (25 mg/kg). The triangles represent the response following intracolonic instillation of 0.5 mL/kg of dosing solution. The circles represent the response following intracolonic instillation of 1.0 mL/kg of dosing solution. The squares represent the response following intracolonic instillation of 1.5 mL/kg of dosing solution.

unoptimized dosing conditions. Similar studies were carried out in which the dose of compound **2** was held constant at 25 mg/kg and the dose of rhGH was varied over the range 6, 3, 1, 0.5, and 0.25 mg/kg. Under these dosing conditions, the minimum effective dose of rhGH was determined to be 0.5 mg/kg.

To determine the effect of dose volume on rhGH delivery in the presence of **2**, the doses of **2** and rhGH were held constant at 25 and 1 mg/kg, respectively, while the dose volume was varied from 0.5 to 1.5 mL/kg. It is important to note that the concentration of the delivery agent and the rhGH in the dosing solutions will increase as the dosing volume decreases because the total dose is held constant. The data presented in Figure 9 show that the efficiency of drug delivery increased with decreasing dose volume. One possible explanation for this observation is that intermolecular associations between molecules in solution increase with increasing solute concentration at concentrations below saturation. In this case, the more concentrated dosing solutions provide more favorable conditions for the intermolecular association of rhGH with **2**. Therefore, one might postulate that some type of "complex" between the protein and the delivery agent could be responsible for efficient drug absorption.

On the basis of this hypothesis, studies designed to identify techniques that would facilitate characterization of the interaction between **2** and rhGH and its role, if any, in drug delivery were initiated. Many of the conventional spectroscopic techniques commonly used to study intermolecular interactions were found not to be applicable here because of interference from the delivery compounds at the wavelengths of interest or interaction with the specific probes designed to evaluate intermolecular phenomena. However, preliminary data from differential scanning calorimetry (DSC), capillary zone electrophoresis (CZE), and molecular modeling experiments may prove useful in characterizing the nature of the interaction between the delivery agent and rhGH. Comprehensive studies using these techniques are underway.

Conclusion

We have prepared a series of 70 *N*-acylated, non-α-amino acids and demonstrated their use in two species as oral delivery agents for rhGH. Structure−activity analyses showed that increasing lipophilicity within structurally related groups of compounds enhanced their ability to promote protein absorption from the gastrointestinal tract in a rat model. The effects of substituents in the aromatic amide portion of these compounds with varying electronic properties were also examined, and electron withdrawing substituents at the ortho position were found to be the most efficacious. Preliminary mechanistic studies suggest that the formation of a drug/delivery agent complex is necessary to achieve oral drug delivery. Future studies will be focused on characterizing the mechanism by which these materials promote protein absorption following oral administration.

Experimental Section

Chemistry. NMR spectra were recorded at 300 MHz in either D_2O or DMSO-d_6. Combustion analyses were performed by Microlit Laboratories, Madison, NJ, and are within acceptable limits (C, H, N ± 0.4%). Thin layer chromatography was performed using E. Merck Kieselgel 60 F-254 plates. Reactions were monitored by high-pressure liquid chromatography on a Vydac 25 × 4.6 mm Protein and Peptide column using a gradient of 0−50% acetonitrile in water with 0.1% trifluoroacetic acid. Melting points were performed using a Mel-Temp II from Laboratory Devices.

General Procedures for the Preparations of Delivery Agents. With the exception of **18**, **27**, **31**, and **34**, one of the following three procedures was used to prepare the compounds described herein. The preparation of 4-(4-(salicyloylamino)-phenyl)butyric acid (**2**) is given as a representative example.

Method A. Preparation of 2. A 1 L round bottom flask fitted with a magnetic stirrer was charged with 4-(4-aminophenyl)butyric acid (50.0 g, 0.28 mol, 1.17 equiv) and 2 M aqueous sodium hydroxide (300 mL). Finely ground *O*-acetylsalicyloyl chloride (47.7 g, 0.24 mol, 1.00 equiv) was added portionwise over 1 h to the stirred solution. After the addition, the reaction mixture was stirred for 2.5 h at ambient temperature, and the pH of the solution was kept at ca. 10 by the addition of 10 M sodium hydroxide. The solution was then acidified with 1 M hydrochloric acid to pH 4.5 to obtain a pale

pink solid. The solid was filtered, washed with 1 M hydrochloric acid (3 × 100 mL) and water (100 mL), and air-dried. It was redissolved in boiling acetone (ca. 500 mL), decolorized with activated charcoal (3 g), and filtered. Water (1.5 L) was added to the filtrate to induce the formation of a brown oil. The brown oil solidified upon stirring at room temperature for 10 min. The crude solid was collected by filtration and recrystallized from 70% methanol-water (v/v) to afford **2** as a tan solid (35.1 g, 49%): mp 159−163 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.74 (1H, dd), 7.38 (2H, d), 7.21 (3H, m), 6.67 (1H, m), 6.57 (1H, m), 2.48 (2H, t), 2.07 (2H, t), 1.71 (2H, m). Anal. Calcd for $C_{17}H_{17}NO_4$: C, 68.22; H, 5.72; N, 4.68. Found: C, 68.29; H, 5.60; N, 4.60.

Compounds **1**, **3**−**6**, **10**, **14**, **23**−**26**, **28**−**30**, **33**, **41**−**43**, **47**−**48**, **50**, **55**, **57**−**61**, and **63**−**67** were prepared by this process.

Method B. Preparation of 2. A 2 L three-neck round bottom flask was fitted with a magnetic stirrer and two addition funnels under an argon atmosphere. A suspension of 4-(4-aminophenyl)butyric acid (50.0 g, 0.28 mol, 1.17 equiv) in ethyl acetate (700 mL) was added to the flask. A solution of *O*-acetylsalicyloyl chloride (55.60 g, 0.28 mol, 1.00 equiv) in ethyl acetate (250 mL) was charged to one of the addition funnels and added dropwise over 1 h. Triethylamine (28.20 g, 0.28 mol, 1.00 equiv) was subsequently charged to the second funnel and added dropwise over 15 min. The reaction mixture was stirred at ambient temperature for 3 h, and the solvent was evaporated *in vacuo*, giving a residual brown oil. Water (600 mL) was added to the residue followed by sodium hydroxide (2 M, 500 mL), and the mixture was stirred at ambient temperature for 3 h. The resultant brown solution was acidified with 2 M hydrochloric acid (ca. 1 L). After the mixture was cooled in an ice bath for 1 h, a yellow solid formed and was collected by filtration. The solid was washed with water (3 × 1.5 L) and recrystallized from 50% ethanol−water (v/v) to give **2** as a tan solid (56.60 g, 68%): mp 167−170 °C; ^{1}H NMR (see method A). Anal. Calcd for $C_{17}H_{17}NO_4$: C, 68.22; H, 5.72; N, 4.68. Found: C, 67.87; H, 5.85; N, 4.69.

Compounds **7**−**9**, **11**−**13**, **17**, **20**, **28**, **32**, **35**−**38**, **44**−**46**, **49**, **52**−**54**, **56**, **62**, and **68**−**69** were prepared by this process.

Method C. Preparation of 2. A 2 L round bottom flask equipped with a magnetic stirrer and a reflux condenser was charged with a suspension of 4-(4-aminophenyl)butyric acid (50.0 g, 0.28 mol, 1.17 equiv) in dichloromethane (560 mL). Chlorotrimethylsilane (62.36 g, 0.57 mol, 2.05 equiv) was added in one portion, and the mixture was heated to reflux for 1 h under argon. The reaction mixture was allowed to cool to room temperature and was placed in an ice bath (internal temperature < 10 °C). The reflux condenser was replaced with an additional funnel containing triethylamine (42.50 g, 0.42 mol, 1.50 equiv). The triethylamine was added dropwise over 15 min and a yellow solid formed during the addition. The funnel was replaced by another addition funnel containing a solution of *O*-acetylsalicyloyl chloride (55.60 g, 0.28 mol, 1.00 equiv) in dichloromethane (100 mL). The solution was added dropwise over 30 min. The reaction was stirred in the ice bath for another 30 min and at ambient temperature for 1 h. The dichloromethane was evaporated *in vacuo* to give a brown oil. The brown oil was cooled in an ice bath, and an ice-cold solution of 2 M sodium hydroxide (700 mL) was added. The ice bath was removed, and the reaction mixture was stirred for 2 h to afford a clear brown solution. The solution was acidified with 2 M sulfuric acid (400 mL) and stored at ca. 5 °C for 1 h. A yellow solid formed and was collected by filtration. The solid was washed with water (3 × 100 mL) and recrystallized from 50% ethanol−water (v/v) to afford **2** as tan needles (65.7 g, 78%): mp 174−176 °C; ^{1}H NMR (see method A). Anal. Calcd for $C_{17}H_{17}NO_4$: C, 68.22; H, 5.72; N, 4.68. Found: C, 68.27; H, 5.70; N, 4.62.

Compounds **15**−**16**, **19**, **21**−**22**, **39**−**40**, and **51** were prepared by this process. Compounds **18** and **27** were also prepared by this process using the appropriate anhydride in place of an acid chloride.

Preparation of 34. A 100 mL round bottom flask equipped with a magnetic stirrer and a reflux condenser was charged with a suspension of 4-(4-aminophenyl)butyric acid (5.0 g, 0.028 mol) in absolute methanol (60 mL). Benzaldehyde (2.96 g, 0.028 mol) was added followed by concentrated hydrochloric acid (0.5 mL). The reaction mixture was heated to reflux under nitrogen for 12 h and then cooled to ambient temperature. Sodium cyanoborohydride (2.1 g, 0.33 mol) was added and the mixture stirred for 4 h. The reaction was quenched by the addition of concentrated hydrochloric acid (10 mL), and the methanol was evaporated *in vacuo*. The residue was purified by column chromatography (40% ethyl acetate/hexanes) on silica gel to afford **34** as a colorless solid (1.4 g, 19%): mp 55−58 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.3−7.4 (m, 3H), 7.2 (m, 1H), 6.8 (d, 2H), 6.5 (d, 2H), 6.1 (s, 1H), 4.2 (s, 2H), 2.4 (t, 2H), 2.2 (t, 2H), 1.7 (q, 2H). Anal. Calcd for $C_{17}H_{19}NO_2$: C, 75.79; H, 7.12; N, 5.18. Found: C, 75.55; H, 7.49; N, 4.70.

Compound **31** was prepared by this process.

Preparation of 70. A 500 mL round bottom flask equipped with a magnetic stirrer and a reflux condenser was charged with cyclohexanebutyric acid (17.0 g, 0.10 mol) and chloroform (200 mL). *N*-Hydroxysuccinamide (12.7 g, 0.11 mol) and *N*,*N*-dicyclohexylcarbodiimide (22.7 g, 0.11 mol) were added, and the mixture was stirred at ambient temperature for 12 h. A white precipitate formed and was removed by filtration. Glacial acetic acid (5 mL) was added to the filtrate and stirred for 3 h. The solution was washed with saturated sodium bicarbonate (2 × 100 mL) and water (2 × 100 mL). The combined aqueous extracts were back-extracted with chloroform (50 mL). The combined chloroform extracts were dried over anhydrous magnesium sulfate, filtered, and evaporated. The residue was dissolved in a solution of methyl 4-aminobenzoate (15.2 g, 0.10 mol), 2 M sodium hydroxide (30 mL), and methanol (60 mL). The resulting mixture was heated to reflux for 4 h, cooled in an ice bath, and acidified to pH 1 with 1 M hydrochloric acid. A white solid formed, was collected by filtration, and was washed with petroleum ether. Recrystallization from 50% methanol−water (v/v) gave **70** as colorless crystals (22.9 g, 79%): mp 240−242 °C; ^{1}H NMR (300 MHz, alkaline D_2O) δ 7.7 (d, 2H), 7.3 (d, 2H), 2.1 (M, 2H), 1.4 (m, 7H), 1.0 (m, 6H), 0.7 (m, 2H). Anal. Calcd for $C_{17}H_{23}NO_3 \cdot 1.5H_2O$: C, 60.34; H, 7.38; N, 4.13. Found: C, 60.37; H, 6.81; N, 4.00.

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 225−250 g were used to demonstrate the delivery of rhGH. Each experiment was performed on a group of six or seven rats. The rats were housed under standard conditions with free access to water for 24 h prior to the study. All of the rats in these studies were anesthetized with 44 mg/kg ketamine and 1.5 mg/kg thorazine immediately prior to dosing. The rats were administered the dosing solutions by oral gavage or intracolonic instillation. rhGH serum concentrations were measured by an ELISA assay for recombinant human growth hormone from Medix Biotech, Inc., Foster City, CA. The data is reported as mean ± standard error. Histology studies were performed by Pharmaco LSR, East Millstone, NJ. Recombinant human growth hormone (rhGH) was a gift from Eli Lilly, Indianapolis, IN.

General Protocol for Rat Experiments. The following protocol is a general description of the rat experiments performed as part of these studies. The oral dosing of rhGH with **2** is given as a representative example. An rhGH dosing solution was prepared by dissolving compound **2** to a concentration of 300 mg/mL in water and adjusting the pH of the solution to 7.2−7.8 with aqueous sodium hydroxide (1.0 N). rhGH was added to obtain a final concentration of 3 mg/mL. Six rats were given 2 mL/kg of this rhGH dosing solution. The total dose of rhGH was 6 mg/kg and the total dose of delivery agent was 600 mg/kg for oral gavage studies, and the total dose of rhGH was 1 mg/kg and the total dose of delivery agent was 25 mg/kg for intracolonic studies. Control groups, each containing six rats, were administered a solution of rhGH or a solution of compound **2**. All of the groups were dosed at the same time. Blood samples were collected serially from the tail artery immediately prior to dosing and at 0.25, 0.5, 1.0, and

1.5 h after dosing, allowed to clot for 30−60 min, and centrifuged, and the serum was harvested and frozen until assay.

General Protocol for Primate Experiments. The following protocol is a general description of the primate experiments performed as part of these studies. An rhGH dosing solution was prepared by dissolving compound **2** to a concentration of 300 mg/mL in water and adjusting the pH of the solution to 7.2−7.8 with aqueous sodium hydroxide (1.0 N). rhGH was added to obtain a final concentration of 2.2 mg/mL. Monkeys were given 2.7 mL/kg of this rhGH dosing solution. The total dose of rhGH was 6 mg/kg and the total dose of **2** was 800 mg/kg. Three cynomolgus monkeys weighing 3−4 kg were used. The monkeys were fasted overnight, anesthesized with ketamine, placed in chairs, and allowed to regain consciousness before dosing. Conscious monkeys received a solution of rhGH and **2** by nasogastric gavage, and blood samples were collected from the saphenous vein. Blood samples were collected at 1 and 0.5 h before dosing and 0.17, 0.33, 0.5, 0.67, 0.83, 1, 1.5, 2, 3, and 6 h after dosing. The samples were allowed to clot at 25 °C for 30−60 min and centrifuged, and the serum was harvested and frozen until assay. All the samples were analyzed by ELISA for the determination of rhGH serum concentrations.

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