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Identification of Novel Small-Molecule *Ulex Europaeus* I Mimetics for Targeted Drug Delivery

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Abstract—Lectin mimetics have been identified that may have potential application towards targeted drug delivery. Synthetic multivalent polygalloyl constructs effectively competed with *Ulex europaeus* agglutinin I (UEA1) for binding to intestinal Caco-2 cell membranes.

Introduction

Various lectins, naturally occurring proteins with affinity for sugar residues, bind specifically to oligosaccharide moieties on the surface of intestinal cells. It has therefore been proposed to use lectins as ligands for targeted oral drug delivery to enterocytes or vaccine delivery to M-cells, which are antigen presenting cells in Peyer's patch tissues.[1,2] *Ulex europaeus* agglutinin I (UEA1), an α-L-fucose specific lectin, has been of particular interest due to its M-cell specificity in the mouse model and, therefore, its applicability for proof-of-concept studies of vaccine delivery to antigen presenting cells.[3] Lectins, however, are susceptible to proteolytic degradation in the gastrointestinal tract (GIT) and during typical procedures used for preparation of oral drug formulations. Potential immunogenic and cytotoxic effects also limit the use of lectins as targeting agents to deliver drugs and vaccines to and across the human GIT.

One approach to overcome these limitations is to synthesize small organic molecules that are able to mimic the function of the lectins and, thus, would have properties applicable to targeted drug delivery. The advantages of such mimetics include their size (typically less than 1500 daltons), stability (due to non-natural composition), ease of synthesis and low cost, as well as their suitability for incorporation into delivery systems using routine chemical procedures.

Combinatorial chemistry has become increasingly useful as a means to discover new lead candidates for biological targets. Synthesis and screening of pharmaceutically relevant mixture-based compound libraries have proven to be a valid and advantageous alternative to high-throughput screening of individual compounds. The use of mixtures formatted as positional-scanning synthetic combinatorial libraries (PS-SCL) enables one to rapidly screen thousands to millions of compounds effectively and efficiently.[4,5] In this study, PS-SCLs were screened and UEA1 mimetic candidates were identified that effectively competed with the biotinylated lectin for binding to human intestinal Caco-2 cell membranes in a competition assay. The PS-SCLs included peptides, peptidomimetics and non-peptide small molecules.

Results and Discussion

The most active mixture-based PS-SCL using this bioassay was an *N*-acylamine structure containing three positions of diversity[6] (Fig. 1). Subsequent screening of individual *N*-acylamine compounds, derived from the most active PS-SCL mixtures, confirmed the level of activity observed with the mixtures. Among the top inhibitors from this series of compounds, a prevalence of substituted aromatic groups is seen in the R_3 position, which is incorporated through N-terminal acylation with

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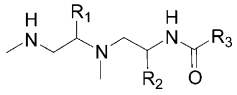


Figure 1. Generic structure of *N*-acylamine library.

a carboxylic acid. Specifically, gallic acid (3,4,5-trihydroxybenzoic acid) and other hydroxy- and methoxy-substituted benzoic acids were most prevalent at this position of the pharmacophore. Structural specificities of the R_1 and R_2 positions could not be deduced, however, as there was no apparent SAR evident within the activity data. Therefore, the role of the R_3 group was further explored in order to determine the importance of the library scaffold versus the carboxylic acid functionality.

When designing the initial constructs for carboxylic acid presentation, the nature of UEA1-carbohydrate binding was considered. The structure of this lectin contains multiple carbohydrate binding sites, therefore multivalent ligands could potentially achieve stronger receptor–ligand interactions than monovalent molecules.[7,8] For that reason, both monovalent and multivalent constructs were chosen to substantiate binding activity as well as to test the multivalency theory. D-Lysine was chosen as a versatile scaffold component and gallic acid was selected as a representative active R_3 group. Individual lysine scaffolds acylated with gallic acid at one or both amino positions were synthesized, producing 'multiple copy' constructs of the acid via an amide linkage (Table 1). Following testing, it became clear from the IC_{50} data (see Biology section in the Experimental) that compounds having multiple galloyl moieties resulted in greater inhibition of UEA1 binding. However, the level of inhibition did not increase significantly when more than four polyphenolic groups were present (data not shown).

Isomeric derivatives of gallic acid were incorporated into a series of compounds for the purpose of evaluating the arrangement of the aromatic ring functionalities. Two-copy and four-copy constructs of 2,3,4- and 2,4,6-trihydroxybenzoic acids were synthesized on lysine in the same manner as those presented in Table 1. Additionally, the resin-bound lysine amino groups were reductively alkylated at both the alpha and epsilon positions with 3,4,5-trihydroxybenzaldehyde, producing the N^{α},N^{ε}-bis-alkylated compound **2d** (Table 2) following cleavage from the solid support. The data presented in Table 2 clearly demonstrate the requirement of the 3,4,5- hydroxyl substitution pattern for high inhibitory activity since the constructs derived from 2,4,6- and 2,3,4-trihydroxybenzoic acid (**2b**, **2c**, **4b**, **4c**) were significantly less active than those derived from gallic acid. Furthermore, the N^{α},N^{ε}-bis-3,4,5-trihydroxybenzylated compound failed to inhibit UEA1 at a reasonable concentration, thus confirming the need for the carboxyl functionality adjacent to the polyphenolic ring.

Additional multimeric lysinyl scaffolds of various hydroxy- and methoxy-substituted cyclic carboxylic acids were synthesized. The selection of carboxylic acids included the active R_3 groups from the libraries as well as other structurally similar derivatives in order to determine the optimal number of functionalities on the aromatic ring. Two- and four-copy constructs of shikimic and quinic acids **2i**, **2j**, **4i**, **4j** (Table 3) were included in the synthesis, due to the role of these carboxylic acids in gallic acid biosynthesis and a recent publication detailing their use as carbohydrate mimics.[9] The greater flexibility of these structures is of value in elucidating the effects of ring planarity and aromaticity on activity. IC_{50} values for this series of compounds are shown in Table 3. Results from this series confirm the need for planar aromatic groups for successful inhibition of UEA1. Most interesting was the moderate activity demonstrated by the multimeric structures of citrazinic acid (**2f**, **4f**), where the carbinol group at position 4 has been replaced by nitrogen.

Table 1. Four D-lysine scaffolds with one, two, three or four galloyl moieties

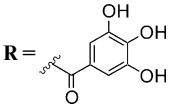
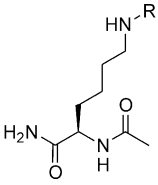
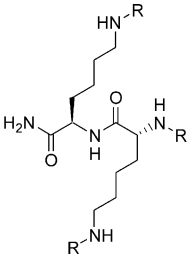
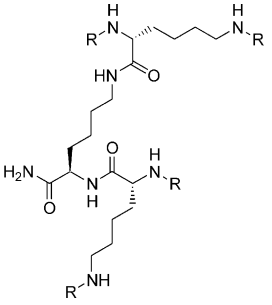
R =	Compound	IC_{50} (μM)
	**1a**	211
	**2a**	96
	**3a**	56
	**4a**	3

Table 2. Trihydroxybenzoyl derivatives on two- and four-copy scaffolds

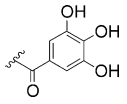
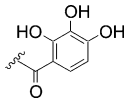
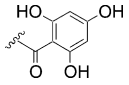
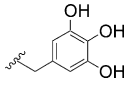
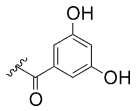
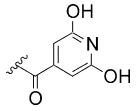
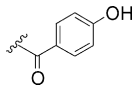
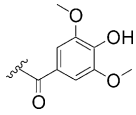
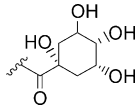
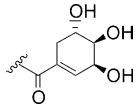
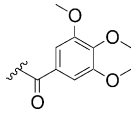
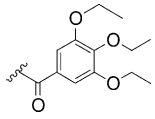
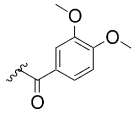
[R]	Two copy	IC_{50} (μM)	Four copy	IC_{50} (μM)
 galloyl	**2a**	96	**4a**	3
 2,3,4-trihydroxy-benzoyl	**2b**	> 200	**4b**	94
 2,4,6-trihydroxy-benzoyl	**2c**	> 200	**4c**	> 200
 3,4,5-trihydroxy-benzyl (reductive alkylation)	**2d**	> 200	**4d**	N/A

Table 3. Trihydroxybenzoyl derivatives on two- and four-copy scaffolds

[R]	Two copy	IC_{50} (μM)	Four copy	IC_{50} (μM)
	**2e**	> 200	**4e**	> 200
	**2f**	88	**4f**	41
	**2g**	> 200	**4g**	> 200
	**2h**	57	**4h**	96
	**2i**	> 200	**4i**	> 200
	**2j**	> 200	**4j**	129
	**2k**	> 200	**4k**	> 200
	**2l**	> 200	**4l**	92
	**2m**	200	**4m**	93

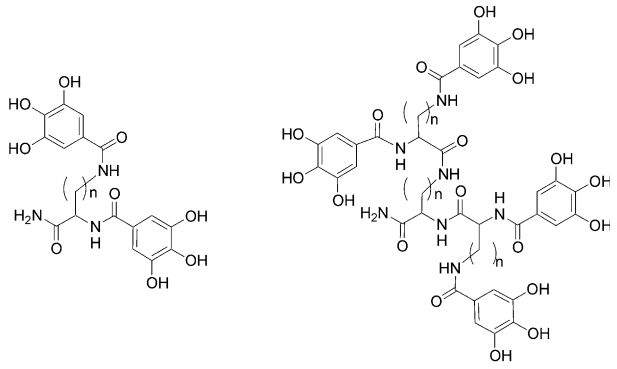
In order to investigate the effect of spatial arrangements of the polyphenolic functionalities, the amino acid side-chain lengths of the two-copy and four-copy gallic acid constructs were varied (Table 4). These scaffolds, synthesized from commercially available protected derivatives of diaminopropionic acid, diaminobutyric acid and ornithine, ranged from one to three carbons in the spacer length of the amino acid side chain. The data for the two-copy constructs showed variation in activity. The ornithine-derived compound **7a** was 5-fold more active (IC_{50} 13 μM) than the construct derived from diaminopropionic acid **5a** (IC_{50} 59 μM). Most notable from this series was the increase in activity as the scaffold chain length was decreased on compounds bearing four galloyl moieties. In this group, the IC_{50} values were as low as 100 nM. Attaining this level of activity with multimeric galloyl constructs prompted further investigation into the source and biological activity of other polyphenolic compounds.

Gallic acid is ubiquitous in the plant kingdom and readily available from natural sources. Numerous polyphenolic molecules, or tannins, have been isolated and identified as active components in medicinal plant preparations.[10] Their antioxidant activity has been documented in numerous studies. Both hydrolyzable and condensed tannins have been isolated and evaluated for their biological activity in various applications. Hydrolyzable tannins, in particular, are a class of macromolecules composed of multiple galloyl esters arranged around a sugar core. Due to the correlation between gallic acid and tannins, commercially available polyphenolic compounds were tested for inhibitory activity. Tannic acid, for example, showed a high level of inhibition (IC_{50} 1.3 μg/mL). However, this commercial product was a mixture of polyphenolic components and therefore difficult to characterize. In contrast, free gallic acid, several alkyl gallates and 3,4,5-trihydroxybenzamide were all inactive in the binding assay. Likewise, condensed tannins such as epicatechin, epigallocatechin and myricetin were marginal in activity. Ellagic acid also failed to demonstrate binding inhibition using the described assay protocol.

Conclusion

In conclusion, a series of novel small molecule mimics of UEA1 has been identified. Employing mixture-based PS-SCLs as a source of chemical diversity, SAR information was quickly obtained identifying the polyphenolic group as an active component in the

Table 4. Two and four copy scaffolds with varying amino acid chain lengths



2 Copy 4 Copy

Compound	Scaffold amino acid	*n*	R =	#R groups	IC_{50} (μM)
5a	L-Diaminopropionic acid	1	Galloyl	2	59
6a	L-Diaminobutyric acid	2	Galloyl	2	34
7a	L-Ornithine	3	Galloyl	2	13
2a	D-Lysine	4	Galloyl	2	96
8a	L-Diaminopropionic acid	1	Galloyl	4	0.1
9a	L-Diaminobutyric acid	2	Galloyl	4	0.1
10a	L-Ornithine	3	Galloyl	4	0.1
4a	D-Lysine	4	Galloyl	4	3

competition binding assay. The synthetic galloyl constructs, designed from the PS-SCL activity data, have been evaluated as UEA1 mimics by competition assay in which they exhibited IC_{50} values less than 1 μM. While UEA1 is known as an α-L-fucose specific lectin, these synthetic constructs demonstrate significantly higher binding inhibition than α-L-fucose in the UEA1 binding assay (IC_{50} 15,000 μM). Scaffolds with four galloyl groups situated in close proximity to each other show the highest activities **8a**, **9a**, and **10a** (Table 4). Clearly, multivalency plays a role in UEA1 binding since multiple galloyl moieties are required for sufficient inhibition of the lectin binding to Caco-2 cell membranes. Synthetic constructs such as these are expected to have greater stability than their naturally derived counterparts due to their small size, amide linkages (instead of esters) and unnatural scaffold building blocks. These advantages enhance the significance of such UEA1 mimetics and their potential use as drug delivery vehicles for cellular targeting of therapeutically relevant molecules. While recent publications have described artificial lectins for other applications,[11,12] there is no publication to our knowledge that describes novel synthetic small molecules that mimic the binding of this lectin. Further biological studies addressing the in vitro and in vivo characterization of these compounds will be published elsewhere.[13]

Experimental

Chemistry

Amino acids were purchased from Novabiochem (San Diego, CA, USA). All other synthesis solvents and reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA). Methylbenzhydrylamine resin (mBHA) was purchased from Advanced ChemTech (Louisville, KY, USA). All compounds were analyzed by a Finnigan LCQ Duo Liquid Chromatography/Mass Spectrometry (LC-MS) system (ThermoQuest Corporations, CA, USA) using electrospray ionization (ESI) and UV detection at 214 nm on a Thermo Hypersil-Keystone Betasil (C_{18}, 5 μm, 100 Å, 3×50 mm) column (Thermo Hypersil-Keystone, PA); solvent system A: 0.1% trifluoroacetic acid (TFA) in water, B: 0.1% TFA in acetonitrile with a gradient from 5 to 95% B over 6 min. Exact mass was determined at the University of California Riverside Mass Spectroscopy Facility (Riverside, CA, USA) using a Micromass Ultima Global QTOF high resolution spectrometer. ^{1}H NMR analysis was performed at Numega (San Diego, CA, USA).

A combination of standard *t*Boc and Fmoc peptide chemistry were used with mBHA resin for the synthesis of all compounds generated. Couplings were performed in the following manner.

Resin was sealed within a polypropylene mesh packet.[14] Fmoc-D-Lys(Boc)-OH was coupled to the resin amine(s) (6 equiv protected amino acid, 6 equiv 1-hydroxybenzotriazole (HOBt), 6 equiv diisopropylcarbodiimide (DIC), 0.2 M in dimethylformamide (DMF), 2 h). The tBoc group was removed with a 55% trifluoroacetic acid (TFA)/dichloromethane (DCM) solution. The resin was washed twice with isopropanol and twice in DCM. The resin was then treated with 20% piperidine/DMF (30 min) to remove the Fmoc group. The resin was then washed 3×DMF and 3×DCM. These steps were repeated to produce the desired scaffold precursor. Acylation of the scaffold by the appropriate carboxylic acid was performed in the same manner as for coupling

amino acids. Cleavage from the solid support was achieved using anhydrous HF with anisole (5%) as a scavenger (0 °C, 90 min). Subsequent extraction of the material with an acetic acid and water solution (10 or 95% AcOH, depending on the hydrophobicity of the construct), followed by lyophilization furnished the crude material. Theoretical yields were calculated based upon the loading of the resin. Crude purities were determined from the relative peak areas (%) from the UV component of the LC–MS chromatograms and are reported below. Small samples of active compounds were purified for NMR and HRMS analysis.

Compounds from initial syntheses which contained any trihydroxybenzoic acid (gallic acid, 2,3,4-trihydroxybenzoic acid and 2,4,6-trihydroxybenzoic acid) were of low purity due to phenolic esters that formed during the final acylation step. These esters are hydrolyzed during the cleavage with HF and subsequently form an insoluble by-product with anisole. Residual gallic acid also remained in the crude material obtained. Therefore, the following method was implemented to hydrolyze and remove the galloyl esters prior to HF cleavage. Following acylation with gallic acid, the resin-bound construct was treated twice with a solution of 2% hydrazine in 10% methanol/90% dioxane for 3 h and then overnight. The resin was then washed twice in dioxane and twice in DCM. Compounds synthesized with other carboxylic acids were not subject to this treatment.

N^2,N^6-Bis(3,4,5-trihydroxybenzoyl)-D-lysyl-N^6-[N^2,N^6-bis(3,4,5-trihydroxybenzoyl)-D-lysyl]-D-lysinamide (4a). Yield: 78%; purity: 75%; ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.67 (s, 1H), 8.65 (s, 1H), 8.61 (s, 1H), 8.60 (s, 1H), 8.05–8.06 (m, 3H), 7.89–7.91 (d, 1H, $J = 10$ Hz), 7.86 (m, 1H), 7.75–7.77 (d, 1H, $J = 10$ Hz), 7.33 (s, 1H), 7.00 (s, 1H), 6.86 (s, 4H), 6.80 (s, 4H), 4.27–4.30 (m, 2H), 4.16–4.17 (m, 1H), 3.13–3.16 (m, 6H), 1.67, 1.70 (m, 6H), 1.46 (m, 6H), 1.35–1.38 (m, 6H). *m/z* (HRMS) calcd for $C_{46}H_{55}N_7O_{19}$ $(M+H)^+$ 1010.3632, found 1010.3687.

N^2-Acetyl-N^6-(3,4,5-trihydroxybenzoyl)-D-lysinamide (1a). Yield: 112%; purity: 83%; *m/z* (HRMS) calcd for $C_{15}H_{21}N_3O_6$ $(M+Na)^+$ 362.1328, found 362.1369.

N^2,N^6-Bis(3,4,5-trihydroxybenzoyl)-D-lysinamide (2a). Yield: 101%; purity: 91%; *m/z* (HRMS) calcd for $C_{20}H_{23}N_3O_9$ $(M+H)^+$ 450.1513, found 450.1540.

N^2,N^6-Bis(3,4,5-trihydroxybenzoyl)-D-lysyl-N^6-(3,4,5-trihydroxybenzoyl)-D-lysinamide (3a). Yield: 140%; purity: 48%; *m/z* (HRMS) calcd for $C_{33}H_{39}N_5O_{14}$ $(M+H)^+$ 730.2573, found 730.2571.

***N*-{(1*S*)-(Aminocarbonyl)-3-[(3,4,5-trihydroxyl)amino]-ethyl}-3,4,5-trihydroxybenzamide (5a).** Yield: 157%; purity: 44%; *m/z* (HRMS) calcd for $C_{17}H_{17}N_3O_9$ $(M+H)^+$ 408.1044, found 408.1047.

***N*-{(1*S*)-(Aminocarbonyl)-3-[(3,4,5-trihydroxyl)amino]-propyl}-3,4,5-trihydroxybenzamide (6a).** Yield: 102%; purity: 49%; *m/z* (HRMS) calcd for $C_{19}H_{21}N_3O_9$ $(M+H)^+$ 422.3662, found 422.1227.

N^2,N^5-Bis(3,4,5-trihydroxybenzoyl)-L-ornithinamide (7a). Yield: 97%; purity: 59%; *m/z* (HRMS) calcd for $C_{19}H_{21}N_3O_9$ $(M+H)^+$ 436.1357, found 436.1355.

***N*-(3,4,5-trihydroxylbenzoyl)-3-[(3,4,5-trihydroxylbenzoyl)amino]-L-alanyl-3-({*N*-(3,4,5-trihydroxybenzoyl-3-[(3,4,5-trihydroxybenzoyl)amino]-L-alanyl}amino)-L-alaninamide (8a).** Yield: 81%; purity: 47%; *m/z* (HRMS) calcd for $C_{37}H_{37}N_7O_{19}$ $(M+H)^+$ 884.2223, found 884.2198.

***N*-{(1*S*)-1-({[(3*S*)-4-Amino-3-({(2*S*)-2,4-bis[(3,4,5-trihydroxybenzoyl)amino]butanoyl}amino) - 4 - oxobutyl]amino}carbonyl)-3-[(3,4,5-trihydroxylbenzoyl)amino]propyl}-3,4,5-trihydroxybenzamide (9a).** Yield: 82%; purity: 71%; *m/z* (HRMS) calcd for $C_{40}H_{43}N_7O_{19}$ $(M+H)^+$ 926.2693, found 926.2650.

N^2,N^5-Bis(3,4,5-trihydroxybenzoyl)-L-ornithyl-N^5-[N^2,N^5-bis (3,4,5-trihydroxybenzoyl)-L-ornithyl]-L-ornithinamide (10a). Yield: 71%; purity: 67%; *m/z* (HRMS) calcd for $C_{43}H_{49}N_7O_{19}$ $(M+H)^+$ 968.3162, found 968.3163.

N^2,N^6-Bis(2,3,4-trihydroxybenzoyl)-D-lysinamide (2b). Yield: 82%; purity: 54%; *m/z* (ESI-MS) 449.5 $(M+H)^+$.

N^2,N^6-Bis(2,4,6-trihydroxybenzoyl)-D-lysinamide (2c). Yield: 83%; purity: 42%; *m/z* (ESI-MS) 449.5 $(M+H)^+$.

N^2,N^6-Bis(3,4,5-trihydroxybenzyl)-D-lysinamide (2d). Yield: 113%; purity: 79%; *m/z* (ESI-MS) 422.1 $(M+H)^+$.

N^2,N^6-Bis(3,5-trihydroxybenzoyl)-D-lysinamide (2e). Yield: 101%; purity: 87%; *m/z* (ESI-MS) 418.0 $(M+H)^+$.

N^2,N^6-Bis(2,6-dihydroxyisonicotinoyl)-D-lysinamide (2f). Yield: 91%; purity: 55%; *m/z* (ESI-MS) 420.1 $(M+H)^+$.

N^2,N^6-Bis(4-hydroxybenzoyl)-D-lysinamide (2g). Yield: 107%; purity: 92%; *m/z* (ESI-MS) 385.7 $(M+H)^+$.

N^2,N^6-Bis(4-hydroxy-3,5-dimethoxybenzoyl)-D-lysinamide (2h). Yield: 144%; purity: 85%; *m/z* (ESI-MS) 507.4 $(M+H)^+$.

N^2,N^6-bis{[(3*R*,5*S*)-1,3,4,5-tetrahydroxycyclohexyl]carbonyl}-D-lysinamide (2i). Yield: 79%; purity: 82%; *m/z* (ESI-MS) 493.6 $(M+H)^+$.

N^2,N^6-bis{[(3*S*,4*R*,5*S*)-3,4,5-trihydroxycyclohex-1-en-1yl]-carbonyl}-D-lysinamide (2j). Yield: 63%; purity: 86%; *m/z* (ESI-MS) 457.5 $(M+H)^+$.

N^2,N^6-Bis(3,4,5-trimethoxybenzoyl)-D-lysinamide (2k). Yield: 84%; purity: 81%; *m/z* (ESI-MS) 534.7 $(M+H)^+$.

N^2,N^6-Bis(3,4,5-triethoxybenzoyl)-D-lysinamide (2l). Yield: 94%; purity: 80%; *m/z* (ESI-MS) 640.7 $(M+Na)^+$.

N^2,N^6-Bis(3,4-Dimethoxybenzoyl)-D-lysinamide (2m). Yield: 98%; purity: 75%; *m*/*z* (ESI-MS) 473.9 $(M+H)^+$.

N^2,N^6-Bis(2,3,4-trihydroxybenzoyl)-D-lysyl-N^6-[N^2,N^6-bis(2,3,4-trihydroxybenzoyl)-D-lysyl]-D-lysinamide (4b). Yield: 83%; purity: 13%; *m*/*z* (ESI-MS) 1010.2 $(M+H)^+$.

N^2,N^6-Bis(2,4,6-trihydroxybenzoyl)-D-lysyl-N^6-[N^2,N^6-Bis(2,4,6-trihydroxybenzoyl)-D-lysyl]-D-lysinamide (4c). Yield: 89%; purity: 28%; *m*/*z* (ESI-MS) 1010.3 $(M+H)^+$.

N^2,N^6-Bis(3,5-dihydroxybenzoyl)-D-lysyl-N^6-[N^2,N^6-bis(3,5-dihydroxybenzoyl)-D-lysyl]-D-lysinamide (4e). Yield: 103%; purity: 84%; *m*/*z* (ESI-MS) 946.3 $(M+H)^+$.

N^2,N^6-Bis(2,6-dihydroxyisonicotinoyl)-D-lysyl-N^6-[N^2,N^6-bis(2,6-dihydroxyisonicotinoyl)-D-lysyl]-D-lysinamide (4f). Yield: 70%; purity: 36%; *m*/*z* (ESI-MS) 949.6 $(M+H)^+$.

N^2,N^6-Bis(4-hydroxybenzoyl)-D-lysyl-N^6-[N^2,N^6-Bis(4-hydroxybenzoyl)-D-lysyl]-D-lysinamide (4g). Yield: 89%; purity: 81%; *m*/*z* (ESI-MS) 881.9 $(M+H)^+$.

N^2,N^6-Bis(4-hydroxy-3,5-dimethoxybenzoyl)-D-lysyl-N^6-[N^2,N^6-Bis(4-hydroxy-3,5-dimethoxybenzoyl)-D-lysyl]-D-lysinamide (4h). Yield: 95%; purity: 58%; *m*/*z* (ESI-MS) 1123.0 $(M+H)^+$.

N^2,N^6-bis{[(3*R*,5*S*)-1,3,4,5-tetrahydroxycyclohexyl]carbonyl}-D-lysyl-N^6-(N^2,N^6-bis{[(3*R*,5*S*)-1,3,4,5-tetrahydroxycyclohexyl]carbonyl}-D-lysyl)-D-lysinamide (4i). Yield: 58%; purity: 36%; *m*/*z* (ESI-MS) 1098.7 $(M+H)^+$.

N^2,N^6-bis{[(3*R*,4*R*,5*S*)-3,4,5-trihydroxycyclohex-1-en-1yl]-carbonyl}-D-lysyl-N^6-(N^2,N^6-bis{[(3*R*,4*R*,5*S*)-3,4,5-trihydroxycyclohex-1-en-1-yl]carbonyl}-D-lysyl)-D-lysinamide (4j). Yield: 62%; purity: 36%; *m*/*z* (ESI-MS) 1026.7 $(M+H)^+$.

N^2,N^6-Bis(3,4,5-trimethoxybenzoyl)-D-lysyl-N^6-[N^2,N^6-bis(3,4,5-trimethoxybenzoyl)-D-lysyl]-D-lysinamide (4k). Yield: 82%; purity: 89%; *m*/*z* (ESI-MS) 1177.7 $(M+H)^+$.

N^2,N^6-Bis(3,4,5-triethoxybenzoyl)-D-lysyl-N^6-[N^2,N^6-bis(3,4,5-triethoxybenzoyl)-D-lysyl]-D-lysinamide (4l). Yield: 79%; purity: 79%; *m*/*z* (ESI-MS) 673.9 $(M+2H)^{2+}$.

N^2,N^6-Bis(3,4-dimethoxybenzoyl)-D-lysyl-N^6-[N^2,N^6-bis(3,4-dimethoxybenzoyl)-D-lysyl]-D-lysinamide (4m). Yield: 88%; purity: 37%; *m*/*z* (ESI-MS) 1080.9 $(M+Na)^+$.

Biology

Caco-2 cells were purchased from ATCC (Rockville, MD, USA). Sodium bicarbonate, orthophenyl diamine substrate, and bovine serum albumin were purchased from Sigma (St. Louis, MO, USA). Dulbecco's phosphate buffered saline (DPBS) was purchased from Gibco (NY, USA). Biotin labeled UEA1 was purchased from Vector Laboratories (Peterborough, UK). Horseradish peroxidase was purchased from Calbiochem (San Diego, CA, USA).

For this competition assay, Caco-2 cell membrane fractions were prepared from confluent cell monolayers grown in 150-cm^2 flasks for up to 1 week at 37 °C and 5% CO_2 as previously described.[15,16] Membrane fractions were then coated onto 96-well microtiter plates in 0.05 M carbonate buffer overnight at 4 °C, after which the plates were blocked with bovine serum albumin in Dulbecco's phosphate buffered saline for 1 to 4 h at room temperature. After washing in distilled water, samples were added in an equal volume of 1.5% BSA-DPBS, and biotin-labeled UEA1 was added at a final concentration of 1 μg/mL. Following an overnight incubation at 4 °C, the plates were again washed and the biotin-labeled UEA1 bound was detected using streptavidin-horseradish peroxidase and an orthophenyl diamine substrate, measured at 490 nm using a conventional 96-well plate spectrophotometer. Results are reported as the percentage of inhibitory activity or by an inhibitory constant (IC_{50}) value (i.e., the concentration of a compound at which 50% inhibition of UEA1 binding occurred).

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