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Synthesis of novel isoluminol probes and their use in rapid bacterial assays

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ABSTRACT

Rapid assays for bacteria have been developed utilizing novel LysLysLys-isoluminol (**14**) and GluGlu-isoluminol (**16**) probes that have been derived from peptides which potentially mimic bacteriophage attachment protein binding regions. Compared to two conventional methods that are widely used, namely nucleic acid probes and polymerase chain reaction (PCR) assays, these types of probes may eventually have certain advantages, such as high sensitivity, and short preparation and assay time.

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Many new rapid assays for bacteria have been developed during the last decade. Two of the most sensitive tests on the market are nucleic acid probes and polymerase chain reaction (PCR) assays. Nucleic acid probes have been developed that contain 20–50 bases and can be prepared after 30–60 min of hybridization. Detection can be as low as 10 colony formation units (cfu)/ml after 48 h.¹ PCR methods are better for identifying different types of organisms rather than enumeration, but the detection limit may be as low as one cell in purified media, even when other bacterial cells are present. However, some compounds impede the amplification process of PCR techniques and limit the sensitivity to greater than 10⁴ cfu/ml.¹ Most other tests, such as radioimmunoassay and ELISA assays, require even higher levels of specific bacteria for enumeration. Therefore, if a rapid assay could be developed that had high sensitivity and short preparation and assay time, it would find immediate commercial application.

The non-pathogenic *Lactococcus lactis* ssp. *lactis* C2 (ATCC 11454) host and ϕ c2 bacteriophage is one of the best characterized host/phage systems in the literature.² Therefore, C2 host and ϕ c2 phage constitute an ideal model system for studying C2 host interactions with ϕ c2 phage protein, because the protein in the membrane of the C2 host and its corresponding gene (pip) have been identified. This 99-kDa membrane protein contains the receptor configuration that the ϕ c2 phage binds to irreversibly.³ Also, the attachment protein (protein F) of the ϕ c2 phage has been identified and partially sequenced,³ and both the N-terminal and calcium binding sites of the ϕ c2 phage F protein have been determined.⁴

Ustunol and Hicks⁵ theorized that whey peptides that blocked immune protein receptors to reduce culture agglutination might also be used to block bacteriophage adsorption and inhibit phage proliferation. When whey peptides were added to an M17 medium, it was observed that the whey peptides caused *L. lactis* ssp. *cremoris* WWA culture to grow at a slower rate than when in normal M17 medium.⁶ However, time from phage inoculation to bacteriolysis was extended when peptides were present. Extension of growth time was thought to result from the peptides blocking the phage attachment site on the lactic bacteria. It was concluded that the whey peptides blocked receptors that phage bound to, as well as those receptors used for metabolic processes.⁷ Therefore, it was reasoned that peptides prepared from phage attachment proteins might be a more effective blocker than the whey peptides.⁸ When Hicks et al.⁸ tested phage peptides they were found to be more effective in blocking phage attachment than whey peptides. Thus, it was concluded that rapid assay probes might be developable from the above sources if the specificity of these attachment proteins could be maintained in the probe development.

As proof-of-principle, rather than using whole phage peptides, two small model peptides LysLysLys and GluGlu were selected because of their importance as receptor binding inhibitors⁹ and as a calcium binding point in the ϕ c2 phage F protein.⁴ The rationale for using the GluGlu peptide was based upon the fact that this unit is in the pocket of the F-protein that binds with calcium and forms the initial reversible attachment between the ϕ c2 phage and the membrane (pip-gene protein) of the *L. lactis* ssp. *lactis* C2 bacteria.^{3,4} GluGlu has two free carboxyl groups that can bind calcium ion, and it seemed reasonable to hypothesize that the GluGlu peptide would bind with receptors on *L. lactis* ssp. *lactis* C2. It was reasoned that the LysLysLys and GluGlu peptides might be more

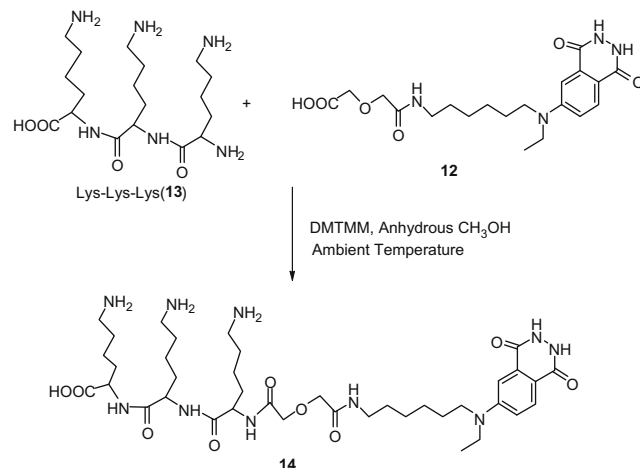
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general in their binding characteristics, would bind to any Lys receptor or calcium binding receptor, respectively, but would be adequate to establish proof-of-concept that probes derived from peptides which mimic bacteriophage attachment protein binding regions could be utilized in rapid bacterial assays. An isoluminol reporter was selected because the molecule could be patented, and could be easily monitored using generally available fluorescence equipment; background fluorescence as determined from controls could then be subtracted from fluorescence generated from the prepared probes. Although many other reporter molecules could have been chosen, an isoluminol probe was utilized to allow for broad patent coverage of many other reporter molecules.

The isoluminol probe molecule (**12**) was synthesized starting from phthalic anhydride, utilizing a nine-step procedure. The key steps in the synthesis of this probe molecule are shown in Scheme 1. Phthalic anhydride (**1**) was reacted with 6-aminohexanol (**2**) under Dean–Stark conditions in anhydrous toluene at 120 °C for 12 h to yield 2-(6-hydroxyhexyl)-isoindole-1,3-dione (**3**), which was then brominated with PBr₃ to afford **4**. In a separate series of reactions, 4-nitrophthalic acid (**5**) was converted into anhydride **6** in refluxing acetic anhydride, followed by treatment of **6** with CH₃NH₂/EtOH to afford **7**; reduction of **7** with SnCl₂/HCl yielded aminophthalimide **8**. Compound **8** was then reacted with 2-(6-bromohexyl)-isoindole-1,3-dione (**4**) in anhydrous DMF to afford the coupled product, *N*-methyl-4-*N*-(6-(*N*-phthalimido)hexyl)aminophthalimide (**9**). Compound **9** was *N*-ethylated to **10**, followed by ring expansion of the phthalimide moiety in refluxing NH₂NH₂ to afford the dihydropthalazine analogue **11**, which was then reacted with diglycolic anhydride/triethylamine to afford the isoluminol probe molecule, **12**.

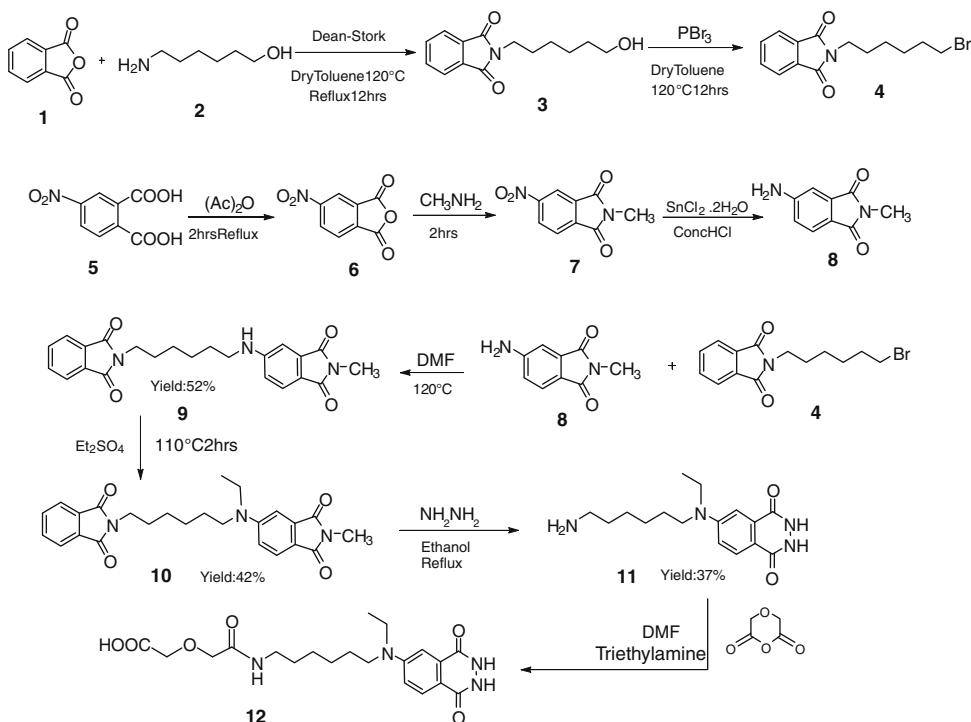
The intermediates from each synthetic step were isolated and their structure and purity verified by ¹H and ¹³C NMR spectroscopy, and mass spectrometric analysis.¹⁰ The synthesized isoluminol probe was then coupled to LysLysLys (**13**) and GluGlu (**15**) peptides in two separate reactions (see Schemes 2 and 3) using



Scheme 2. Synthesis of the LysLysLys-isoluminol probe.

the coupling reagent dimethoxytriazole methyl morphonium iodide (DMTMM) and methanol as solvent under ambient conditions to afford isoluminol conjugated peptides **14** and **16**, respectively. The coupled products were then evaluated for their ability to bind to the host surface of *L. lactis* spp. *lactis* C2 bacteria. The data obtained was used to calculate the amount of probe retained by the bacterial cell surfaces (see below for details).

Measurement of the binding of the isoluminol probe to *L. lactis* spp. *lactis* C2 was carried out as follows: *L. lactis* spp. *lactis* C2 was grown in M17 media (4% inoculation, v/v) for 18 hrs at 27 °C (cultures were grown to 10⁸ cfu/ml). 200 µl of the culture (in triplicate) was accurately transferred to 1 ml microcentrifuge tubes and centrifuged (9000g) at 2 °C for 10 min. The supernatant was separated and transferred to a micro-plate (96-well) for fluorescence readings. Supernatant background fluorescence was subtracted from all other readings. The appropriate peptide conjugated isoluminol



Scheme 1. Synthesis of the isoluminol probe molecule **12**.

probe was dissolved in two drops of DMF and diluted to 200 μ l with phosphate buffer (0.1 ionic strength, 0.01 M, pH 6.4). The culture (pellet) remaining in the microcentrifuge tubes was then dissolved in the peptide-isoluminol probe solution and vortex-mixed. The peptide-isoluminol probe was allowed to react with the culture pellet for 10 min at 27 °C and the mixture was then carefully transferred to micro-plates for fluorescence readings (excitation λ = 360 nm and emission λ = 430 nm). The mixture from each well was carefully transferred to 1 ml microcentrifuge tubes and centrifuged (9000g) at 2 °C for 10 min and the supernatant separated for fluorescence analysis. The culture pellet was again dissolved in phosphate buffer (0.1 ionic strength, 0.01 M, pH 6.4) and the above steps were repeated for four additional washings followed by fluorescence measurements.

Cell numbers were determined at 10^3 cfu/ml dilutions. Fluorescence readings revealed that approximately 34% of the LysLysLys-isoluminol probe was absorbed to the surface of the *L. lactis*, ssp. *lactis* C2 bacteria and 66% remained in solution. After the first and second washes with buffer (Fig. 1), approximately 23% and 10%, respectively, of the initial LysLysLys-isoluminol probe remained on the surface of the *L. lactis* ssp. *lactis* C2 bacteria (solution).

Salmonella typhimurium (ATCC 14028) and *Escherichia coli* (ATCC 47076) were also placed in LysLysLys-isoluminol probe solutions under the same conditions, washed, and the amount of the tritylsine probe that was absorbed onto the bacterial cell surface determined. The data from *E. coli* and *S. typhimurium* were compared to the C2 *lactis* data, as presented in Figure 2.

Initial amounts of absorbed LysLysLys-isoluminol probe were slightly higher on *L. lactis* ssp. *lactis* C2 bacteria. However, after the fourth washing, similar amounts remained on all three bacteria, suggesting that the LysLysLys-isoluminol probe was non-specific and would bind to both gram positive and gram negative bacterial membranes.

The GluGlu-isoluminol probe produced via the synthetic procedure illustrated in Scheme 3 was also evaluated for its ability to bind to *L. lactis* ssp. *lactis* C2, *S. typhimurium*, *E. coli* and *Staphylococcus aureus* (ATCC 25923) with and without calcium ion added to the medium. No differences were observed in the amount of GluGlu-isoluminol probe bound to the cells when calcium was absent or present in the medium. Figure 3 shows the amount of probe binding to four different bacterial strains. Fluorescence readings indicated that only 15–20% of the GluGlu-isoluminol probe was absorbed to the surface of the bacteria; this percentage decreased on subsequent washings. As expected, *L. lactis* ssp. *lactis* C2 bound more probe initially than either *S. aureus* or *E. coli* (Fig. 3), and

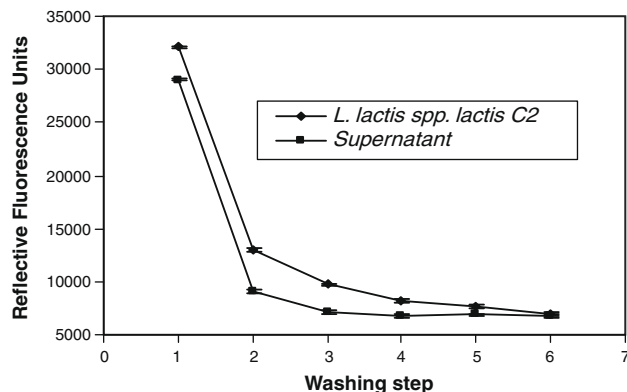


Figure 1. Binding of the LysLysLys-isoluminol probe to *L. lactis* ssp. *lactis* C2. (♦). LysLysLys in wash solution (■) after repeated washing cycles in phosphate buffer (0.1 ionic strength, 0.01 M, pH 6.4). Fluorescence excitation was at 360 nm and emission was at 430 nm.

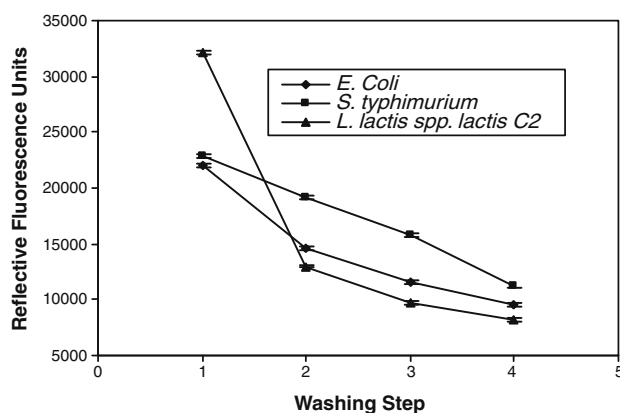
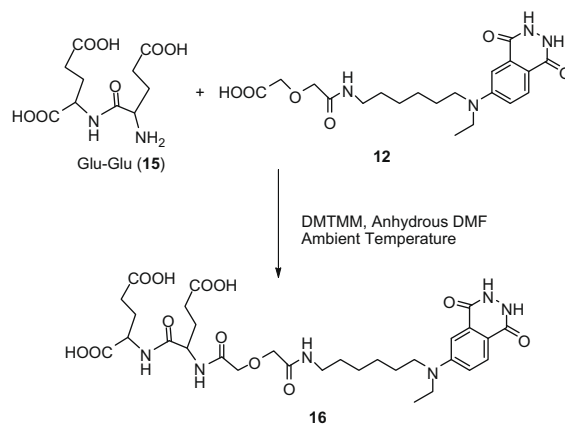


Figure 2. Comparison of concentration of the LysLysLys-isoluminol probe bound to *E. coli*, *S. typhimurium* and *L. lactis* ssp. *lactis* C2 after repeated washing cycles in phosphate buffer (0.1 ionic strength, 0.01 M, pH 6.4). Fluorescence excitation was at 360 nm and emission was at 430 nm.



Scheme 3. Synthesis of the GluGlu-isoluminol probe.

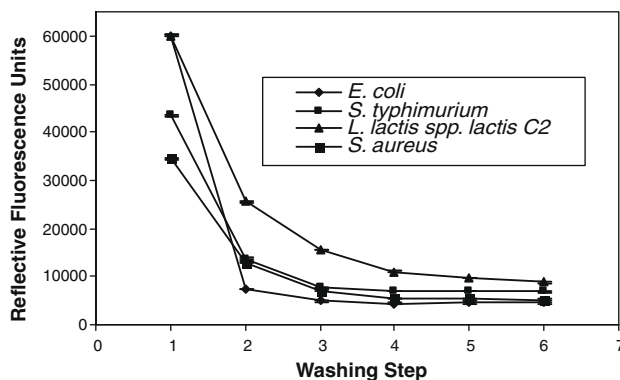


Figure 3. Binding of the GluGlu-isoluminol probe to *E. coli*, *S. typhimurium*, *L. lactis* ssp. *lactis* C2 and *S. aureus* after repeated washing cycles in phosphate buffer (0.1 ionic strength, 0.01 M, pH 6.4). Fluorescence excitation was at 360 nm and emission was at 430 nm.

bound significant amounts of probe, even after the second and third washings. No additional amounts of probe were washed off the surfaces of either *E. coli*, *S. aureus* or *S. typhimurium* after the second washing.

The data obtained indicate that the LysLysLys-isoluminol probe was absorbed onto the surface of all the bacterial strains tested. Washing of the bacteria removed a proportional amount of the

LysLysLys-isoluminol probe from all bacteria equally. However, sufficient probe remained after the second or third washings to allow bacterial estimates to be observed. A second isoluminol probe containing GluGlu as the active binding moiety appeared to be slightly more specific than the LysLysLys-isoluminol probe, in that it had a greater affinity for *L. lactis* ssp. *lactis* C2 than for either *S. typhimurium*, *S. aureus*, or *E. coli*. This specificity was most noticeable after the second and third washings, which would be the expected clean-up procedure for a commercial test. These data suggest that reporter probes derived from peptides which mimic bacteriophage attachment protein binding regions can be synthesized using the chemistry described. As more complex peptide sequences are utilized in the synthesis and design of second generation probes, the specificity is expected to improve. Thus, specific probes suitable for a rapid assay procedure might be produced using these procedures, and these could be utilized to identify a particular genus of bacteria, such as *Listeria* and *E. coli*, which are known to be problematic pathogens in foods.

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- Representative characterization data for conjugates and intermediates. 2-(6-Hydroxyhexyl)-isoindole-1,3-dione (**3**): to a mixture of phthalic anhydride (14.8 g, 100 mmol) and 6-aminoheptan-1-ol (11.7 g, 100 mmol) in a round-bottomed flask was added 120 ml of anhydrous toluene. The flask was then fitted with a Dean-Stark apparatus and the mixture refluxed for 12 h at 120 °C. The water formed during the reaction was removed as an azeotrope. On completion of the reaction, toluene was removed on a rotovac. Little unreacted amine was detected is the resulting residue when monitored by silica gel thin layer chromatography. The residue was subjected to flash column chromatographic separation over silica gel (230–400 mesh) with ethyl acetate and petroleum ether as eluant, to afford 2-(6-hydroxyhexyl)-isoindole-1,3-dione (**3**) as a white solid; yield: 22.7 g (92%), mp 49–50 °C. ¹H NMR (CDCl₃, 300 MHz): δ 7.85 (2H, m), 7.65 (2H, m), 3.70 (2H, t, J = 7.0 Hz), 3.65 (2H, t, J = 7.0 Hz), 1.70 (2H, m), 1.55 (4H, m), 1.40 (4H, m) ppm. ¹³C NMR (CDCl₃, 75 MHz): δ 168.2, 133.6, 131.8, 122.9, 62.5, 37.7, 32.4, 28.4, 26.4, 25.1 ppm. MS: m/z 247 (M⁺). 2-(6-Bromohexyl)-isoindole-1,3-dione (**4**): to 6-hydroxyhexylphthalimide (22.5 g, 0.09 mol) in 100 ml of anhydrous toluene at 110 °C was slowly added phosphorous tribromide in anhydrous toluene (17 g, 0.063 mol in 20 mL of toluene) through a pressure-equalizing funnel. The mixture was refluxed for 3 h and allowed to cool to room temperature. The orange precipitate that formed was removed by filtering the reaction mixture through Celite. The filtrate was then concentrated on a rotovac to afford an oil. The resulting oil was crystallized from absolute ethanol to afford 2-(6-bromohexyl)-isoindole-1,3-dione (**4**) of analytical purity; yield: 25.1 g (90%), mp 59–60 °C. ¹H NMR (CDCl₃, 300 MHz): δ 7.82 (2H, m), 7.65 (2H, m), 3.65 (2H, t, J = 7.0 Hz), 3.40 (2H, t, J = 7.0 Hz), 1.85 (2H, m), 1.65 (2H, m), 1.45 (2H, m), 1.34 (2H, m) ppm. ¹³C NMR (CDCl₃, 75 MHz): δ 168.1, 133.6, 131.8, 122.9, 37.7, 33.6, 32.5, 28.3, 27.6, 25.9 ppm. MS: m/z 310 (M⁺). 5-Nitro-isobenzofuran-1,3-dione (**6**): to 4-nitrophthalic acid (21.1 g, 0.1 mols) in a round-bottomed flask was added acetic anhydride (40 ml) and the mixture refluxed for 1 h. The mixture was brought to room temperature and the acetic anhydride was removed under vacuum. The concentrated mixture was allowed to stand overnight. The solid obtained was recrystallized from toluene to afford 5-nitro-isobenzofuran-1,3-dione (**6**); yield: 9.9 g (51%), mp 119 °C. ¹H NMR (CDCl₃, 300 MHz): δ 8.90 (1H, s), 8.80 (1H, m), 8.40 (1H, m) ppm. ¹³C NMR (CDCl₃, 75 MHz): δ 162.3, 153.6, 136.8, 133.8, 127.2, 121.1 ppm. MS: m/z 193 (M⁺). 2-Methyl-5-nitro-isoindole-1,3-dione (**7**): to 5-nitro-isobenzofuran-1,3-dione (**6**) (9.5 g, 49.2 mmol) in a round-bottomed flask, was added 20 ml of 33% methylamine solution in absolute ethanol and the mixture was stirred for 2 h at 0 °C. The reaction mixture was acidified to pH 2.0 with concentrated hydrochloric acid and then concentrated under vacuum. The solid obtained was refluxed with 20 ml of acetic anhydride for 15 min and allowed to stand overnight. The crystals obtained were washed with acetic acid, then water, and dried under vacuum. The mother liquor, which contained additional product, was evaporated and the slurry was subjected to silica gel (240–300 mesh) flash column chromatography with ethyl acetate and petroleum ether as eluant to afford additional amounts of **7**. The total yield of 2-methyl-5-nitro-isoindole-1,3-dione (**7**) was 4.9 g (48%), mp 177 °C. ¹H NMR (CDCl₃, 300 MHz): δ 8.74 (1H, s), 8.62 (1H, d, J = 7.0 Hz), 8.10 (1H, d, J = 7.0 Hz), 3.21 (3H, s) ppm. ¹³C NMR (CDCl₃, 75 MHz): δ 165.9, 151.4, 136.3, 133.3, 129.0, 124.1, 118.4, 24.5 ppm. MS: m/z 206 (M⁺). 5-Amino-2-methyl-isoindole-1,3-dione (**8**): to a solution of SnCl₂ dihydrate (19.2 g, 85 mmol) in 8 ml water and 24 ml of concentrated hydrochloric acid was added 2-methyl-5-nitro-isoindole-1,3-dione (**7**) (4.5 g, 21.8 mmol). The mixture was stirred vigorously for 2 h, during which time the cloudy solution turned clear. The reaction was then allowed to stand overnight, and the mixture concentrated to three fourths its volume on a rotovac. The solid obtained was filtered and washed with water several times (Note: on washing with water the solid becomes yellow in color). The resulting solid was then dried under vacuum to afford 5-amino-2-methyl-isoindole-1,3-dione (**8**); yield: 2.1 g (54.7%), mp 247–248 °C. ¹H NMR (DMSO-d₆, 300 MHz): δ 7.50 (1H, m), 6.90 (1H, m), 6.76 (1H, m), 6.44 (2H, s), 2.95 (3H, s) ppm. ¹³C NMR (DMSO-d₆, 75 MHz): δ 168.1, 154.6, 134.4, 124.5, 116.7, 106.8, 23.3 ppm. MS: m/z 176 (M⁺). N-Methyl-4-N-[6-(N-phthalimido)hexyl]aminophthalimide (**9**): to 2-(6-bromohexyl)-isoindole-1,3-dione (**4**) (7.04 g, 22.7 mmol) in 20 ml of anhydrous DMF under a nitrogen atmosphere was added 5-amino-2-methyl-isoindole-1,3-dione (**8**) (4 g, 22.7 mmol). The mixture was stirred under nitrogen at 120 °C for 30 h, and then cooled to room temperature and poured into ice-cold water with stirring. A yellow precipitate was obtained, which was filtered and recrystallized from 70% aqueous acetic acid to afford N-methyl-4-N-[6-(N-phthalimido)hexyl]amino phthalimide (**9**); yield: 4.5 g (49%), mp 169–170 °C. ¹H NMR (DMSO-d₆, 300 MHz): δ 7.82 (4H, m), 7.46 (1H, m), 6.96 (1H, m), 6.86 (1H, d, J = 7.0 Hz), 6.74 (1H, m), 3.55 (2H, t, J = 9.0 Hz), 3.20 (2H, m), 2.90 (3H, s), 1.60 (4H, m), 1.30 (4H, m) ppm. ¹³C NMR (DMSO-d₆, 75 MHz): δ 168.1, 167.6, 153.8, 134.4, 134.1, 124.4, 122.7, 116.2, 114.3, 105.6, 42.3, 37.3, 28.6, 27.9, 26.09, 26.04, 23.4 ppm. MS: m/z 405 (M⁺). 4-[N-Ethyl-N-(6-N-phthalimido)hexyl]amino-N-methylphthalimide (**10**): to N-methyl-4-N-[6-(N-phthalimido)hexyl]aminophthalimide (**9**) (3.7 g, 9.2 mmol) in a flask under nitrogen was added diethyl sulfate (11 ml, 70.84 mmol). The mixture was gradually heated with stirring at 110 °C for 2 h, and then at 160 °C for a further 20 min, during which time the reaction turned brown in color. The mixture was then cooled to room temperature and poured into ice-cold water with stirring. The yellow precipitate obtained was filtered and recrystallized from 70% aqueous acetic acid to yield 4-[N-ethyl-N-(6-N-phthalimido)hexyl]-amino-N-methylphthalimide (**10**); yield: 2.3 g (58%), mp 195–196 °C. ¹H NMR (DMSO-d₆, 300 MHz): δ 7.80 (4H, m), 7.45 (1H, m), 6.90 (1H, s), 6.80 (1H, m), 3.54 (2H, t, J = 9.0 Hz), 3.44 (2H, m), 3.34 (2H, t, J = 7.0 Hz), 2.94 (3H, s), 1.56 (4H, m), 1.32 (4H, m), 1.10 (3H, t, J = 12.0 Hz) ppm. ¹³C NMR (DMSO-d₆, 75 MHz): δ 168.2, 167.7, 134.5, 134.2, 131.4, 124.5, 122.8, 115.7, 114.0, 104.5, 49.8, 44.7, 37.3, 27.9, 26.6, 26.1, 25.9, 23.4, 11.9 ppm. MS: m/z 434 (M⁺). 6-[N-(6-Aminoethyl)-N-ethylamino]-2,3-dihydrophthalazine-1,4-dione (**11**): to 4-[N-ethyl-N-(6-N-phthalimido)hexyl]amino-N-methylphthalimide (**10**) (1.75 g, 4.04 mmol) in 20 ml absolute ethanol under nitrogen was added hydrazine (5 ml, 147.1 mmol). The reaction mixture was refluxed for 3 h under nitrogen. The solvent was removed under vacuum and the resulting solid dried at 110 °C under vacuum. The residue was then dissolved in 10% HCl and filtered to remove insoluble impurities. The filtrate was then adjusted to pH 8 with potassium hydroxide solution. The precipitate obtained was filtered and crystallized from aqueous DMF to yield 6-[N-(6-aminoethyl)-N-ethylamino]-2,3-dihydro-phthalazine-1,4-dione (**11**); yield: 220 mg (18%) mp 151–152 °C. ¹H NMR (DMSO-d₆, 300 MHz): δ 7.80 (1H, m), 7.1 (2H, m), 3.45 (2H, m), 3.34 (2H, t, J = 9.0 Hz), 2.73 (2H, t, J = 7.0 Hz), 1.54 (4H, m), 1.34 (4H, m), 1.14 (3H, t, J = 12.0 Hz) ppm. ¹³C NMR (DMSO-d₆, 75 MHz): δ 156.3, 155.5, 149.8, 129.8, 126.9, 116.0, 103.6, 49.4, 44.3, 29.9, 26.3, 25.9, 25.8, 12.0 ppm. MS: m/z 304 (M⁺). {6-[(1,4-Dioxo-1,2,3,4-tetrahydrophthalazin-6-yl)-ethylamino]-hexylcarbamoyl}-methoxy-acetic acid (**12**): to 6-[N-(6-aminoethyl)-N-ethylamino]-2,3-dihydrophthalazine-1,4-dione (**11**) (608 mg, 2 mmol) in 10 ml of anhydrous DMF under nitrogen was added diglycolic anhydride (232 mg, 2 mmol) and triethylamine (205 mg, 2 mmol). The reaction mixture was stirred under nitrogen for 18 h. The solvent was removed under vacuum and the resulting oil was dissolved in anhydrous methanol and kept at 4 °C. The product crystallized from the solution after 18 hours and was filtered and dried under vacuum; yield: 420 mg (50%) mp 170–171 °C. ¹H NMR (DMSO-d₆, 300 MHz): δ 7.82 (1H, m), 7.16 (1H, m), 7.10 (1H, s), 4.04 (2H, s), 3.94 (2H, s), 3.46 (2H, m), 3.36 (2H, t, J = 9.0 Hz), 3.14 (2H, t, J = 7.0 Hz), 1.56 (2H, m), 1.44 (2H, m), 1.32 (4H, m) ppm. ¹³C NMR (DMSO-d₆, 75 MHz): δ 176.0, 170.9, 156.3, 155.5, 149.8, 129.8, 126.9, 116.0, 103.6, 74.7, 73.6, 49.4, 44.3, 29.9, 26.3, 25.9, 25.8, 12.0 ppm. MS: m/z 420 (M⁺). 402 (loss of H₂O), 218 (base peak), 2,5,8-Tris-(4-aminobutyl)-22-(1,4-dioxo-1,2,3,4-tetrahydrophthalazin-6-yl)-4,7,10,14-tetraoxo-12-oxa-3,6,9,15,22-pentaazatetracos- an-1-oic acid (**14**): to the isoluminol probe (**12**) (0.042 g, 0.01 mmol) in 5 ml of anhydrous methanol was added the coupling agent DMTMM (0.041 g, 0.015 mmol) and the resulting mixture stirred under ambient conditions for 30 min followed by addition of LysLysLys (**13**) (0.040 g, 0.01 mmol). The reaction mixture was stirred for 5 h under ambient conditions and then solvent was removed under vacuum. Flash chromatography of the residue utilizing a Sephadex G-25 column under

nitrogen gas afforded compound **14** as cream colored solid; yield: 39.0 mg (48%) mp 162–163 °C. ^1H NMR (CD_3OD , 300 MHz): δ 8.01 (1H, m), 7.25–7.16 (2H, m), 4.36–4.32 (1H, m), 4.22 (2H, s), 4.20–4.18 (1H, m), 4.07 (2H, s), 3.60–3.24 (7H, m), 2.98–2.88 (6H, m), 1.96–1.08 (26H, m), 1.22 (3H, t, J = 12.0 Hz) ppm. ^{13}C NMR (CD_3OD , 75 MHz): δ 173.1, 172.0, 170.2, 166.3, 152.4, 128.4, 126.2, 117.2, 114.1, 108.1, 68.4, 54.1, 53.3, 52.0, 50.9, 41.8, 31.5, 30.2, 29.6, 29.0, 28.2, 27.0, 26.3, 22.4, 12.9 ppm. MS: 805 MH^+ (positive ion Maldi). 2-(2-(2-Carboxyethyl)-16-(1,4-dioxo-1,2,3,4-tetrahydrophthalazin-6-yl)-4,8-dioxo-6-oxa-3,9,16-triazaoctadecanamido)pentane dioic acid (**16**): to the isoluminol probe (**12**) (0.042 g, 0.01 mmol) in 5 ml of anhydrous DMF was added the coupling agent DMTMM (0.041 g, 0.015 mmol) and the mixture stirred under ambient conditions for 30 min followed by addition of GluGlu (**15**) (0.027 g,

0.01 mmol; note: a few drops of DMF was added to solubilize the GluGlu). The reaction mixture was stirred for 5 h under ambient conditions and then solvent was removed under vacuum. Flash chromatography of the residue obtained utilizing a Sephadex G-25 column under nitrogen afforded **16** as a sticky mass; yield: 18.1 mg (27%). Compound **16** was found to undergo some decomposition when allowed to stand overnight in solvents such as methanol and dimethyl sulfoxide. ^1H NMR ($\text{DMSO}-d_6$, 300 MHz): δ 8.70 (2H, broad), 8.22 (1H, broad), 8.15 (1H, s), 7.81 (1H, m), 7.18 (1H, m), 7.02 (1H, s), 4.38–3.05 (17H, m), 2.43–1.20 (12H, m), 1.12 (3H, t, J = 12.0 Hz). ^{13}C NMR (CD_3OD , 75 MHz): δ 176.4, 172.3, 166.2, 164.6, 128.1, 117.1, 115.3, 108.4, 126.2, 67.6, 67.2, 53.8, 53.4, 49.1, 43.7, 37.2, 29.6, 29.0, 28.4, 26.2, 25.8, 11.9 ppm. MS 679 M^2 (positive ion Maldi).