Sensing living bacteria in vivo using D-alanine derived $^{11}$C radiotracers

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A. Synthetic procedures:

A.1. General:

All chemical reagents were purchased from commercial sources (Acros Organics, Alfa Aesar, & Sigma-Aldrich) and used without further purification unless otherwise stated. All separatory cartridges were purchased from Waters. All reactions were performed under inert atmosphere of dry nitrogen and monitored by thin layer chromatography (TLC) on precoated (250 µm) silica gel 60 F254 aluminum sheets and visualized under a UV-254 lamp followed by staining with cerium ammonium molybdate or potassium permanganate. Flash
chromatography was performed on silica gel (60Å pore size). $^1$H and $^{13}$C NMR spectra were obtained on a Bruker Avance III HD 400 mHz instrument at the UCSF Nuclear Magnetic Resonance Laboratory and data were processed using the accompanying Topspin software package. Chemical shifts (§) were reported in ppm relative to known solvent (7.28 ppm for CDCl$_3$, 3.33 ppm for MeOD, & 2.5 ppm for DMSO-d6). Abbreviations are as follows: s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet). High resolution mass spectra (HRMS) services were provided by Notre Dame Mass Spectrometry & Proteomics Facility. Analytical HPLC was performed using a Waters pump equipped with a manual Rheodyne injector (1 mL loop) and a UV detector and a RAD detector. Reversed-phase chromatography used a Phenomenex Luna C18 column stationary phase, a mobile phase of 70:30 acetonitrile/4 mM sodium formate (aqueous) at a flowrate of 1 mL/min, and a 254 nm observation wavelength. Chiral chromatography used a Phenomenex Chirex 3126 column stationary phase, a mobile phase of 1 mM copper sulfate (aqueous) at a flowrate of 1 mL/min, and a 254 nm observation wavelength.

A.2. Synthesis of previously reported molecules:

$O$-allyl-$N$-(9-anthracenylmethyl)cinchoninium bromide (1) was synthesized according to published procedure. Identification and purity were assessed by NMR and matched the reported literature$^1$.

$N$-(diphenyl)glycine tert-butyl ester (2) was synthesized according to published procedure. Identification and purity were assessed by NMR and matched the reported literature$^1$.

**Scheme S1.** a) Benzophenone imine, DCM, rt, o/n; b) KOH, THF, MeOH, 0°C, 10 min; c) benzophenone, NaH$_3$CN, MeOH, water, 90°C, 18hr; d) EDCI, HOAT, NMP, rt, o/n; e) Boc$_2$O, DMAP, THF, t-BuOH, rt, o/n.
N-(diphenylmethylene)glycinate potassium (S2). Glycine methyl ester (S1) (300 mg, 2.07 mmol) was suspended in dichloromethane (4 mL) and treated with benzophenone imine (381 mL, 1.82 mmol). The reaction was stirred at room temperature overnight. The precipitate was filtered and the filtrate evaporated to dryness to yield N-(diphenyl)glycine methyl ester as a white solid (Yield: 472 mg, 77%). To a solution of N-(diphenyl)glycine methyl ester (200 mg, 0.748 mmol, 1 eq.) in THF (6 mL) and methanol (1.5 mL) at 0°C, was added potassium hydroxide (41.9 mg, 0.748 mmol, 1 eq.) and water (150 µL). The reaction medium was stirred at 0°C for 10 minutes. The solvents were evaporated, and the sample resuspended in water (15 mL). The aqueous layer was washed ether x2, then evaporated and dried to yield compound S2 (146 mg, 70%): NMR (400 MHz, D2O): 4.05 (s, 2H), 6.88-6.93 (m, 2H), 7.08-7.19 (m, 6H), 7.39 (d, J=7.7 Hz, 2H). 13C-NMR (100 MHz, D2O): 57.8, 127.7, 128.1, 128.5, 128.6, 128.9, 130.5, 135.7, 139.3, 171.8, 177.8. These NMR values matched the reported literature.

(R)-2-(N-(Diphenylmethyl)amino)propanoic Acid (S4). A solution of D-alanine (S3) (4.45 g, 50 mmol) in water (10 mL) was added to a solution of benzophenone (9.1 g, 50 mmol) in MeOH (100 mL). NaBH3CN (4.7 g, 75 mmol) was added slowly to this mixture which was heated to 90 °C. After being refluxed for 18 h, the mixture was concentrated, and water (100 mL) and 10% NaOH (20 mL) were added. The resulting solution was washed with Et2O and adjusted to pH 5 with 10% HCl aqueous solution. The precipitated solid was collected and dried to yield compound S4 (2.6 g, 20%): NMR (400 MHz, CDCl3) (broad s, 10), 5.21 (s, 1H), 3.80 (s), 3.38 (q, J = 7.3 Hz, 1H), 1.40 (d, J = 7.2 Hz, 3H). 13C-NMR (100 MHz, CDCl3): 171.5, 169.8, 139.4, 136.2, 130.3, 130.1, 128.6, 128.3, 128.0, 127.7, 81.0, 56.3. These NMR values matched the reported literature.

A.3. Synthesis of novel compounds

D-Ala-D-Ala Precursor (3). To a solution of (S2) (146 mg, 0.524 mmol, 1 eq.) and HOAT (428 mg, 3.14 mmol, 6 eq.) in NMP (3 mL) was added EDCI (111 mg, 0.576 mmol, 1.1 eq.). The reaction was stirred for 90 mins at room temperature. This solution was added in one portion to a solution of (S4) (134 mg, 0.524 mmol, 1 eq.) in NMP (2 mL). The reaction was stirred overnight and then quenched with saturated bicarbonate solution. The mixture was extracted with EtOAc and the organic portions were washed with saturated ammonium chloride solution followed by brine. The organic portion was dried over sodium sulfate, filtered and concentrated. To the
residue in 1:1 THF/t-BuOH (10 mL) was added Boc₂O (685 mg, 3.14 mmol, 6 eq.) followed by DMAP (19 mg, 0.157 mmol, .3 eq). The reaction was stirred overnight and concentrated. The residue was purified by column chromatography using hexanes/EtOAc (100mg, 36%).

NMR (400 MHz, DMSO-D₆): 1.49 (d, 3H), 1.52 (s, 9H), 3.99 (d, 2H), 4.58-4.65 (m, 1H), 5.29 (s, 1H), 7.15-7.7 (m, 20H). 13C-NMR (100 MHz, DMSO-D₆): 172.0, 170.2, 170.1, 142.2, 138.7, 136.1, 132.4, 130.7, 130.0, 128.9, 128.5, 128.2, 127.9, 127.2, 127.0, 126.5, 85.0, 81.8, 56.5, 48.4, 28.0, 18.9. HRMS (ESI) calculated C₃₅H₃₆N₂O₃: 533.3293 ([M+H]+), found 533.3310 ([M+H]+).

B. Radiochemistry:

Radiochemical procedure for [¹¹C]Mel generation: [¹¹C]CO₂ was produced in target by the ¹⁴N(p,a)¹¹C nuclear reaction of 17 MeV protons on N₂ in the UCSF radiopharmaceutical facility. [¹¹C]CO₂ was converted to [¹¹C]CH₃I using the gas-phase method on a GE FX/C Pro automated synthesis module. [¹¹C]CH₃I (g) was transferred from a Porapak N column into a glass reactor vial, previously charged with precursor in 500 µL of toluene, with the reactor vial temperature maintained at 0°C during the transfer. The contents of the reaction vial were then transferred to a leaded hot cell for further synthetic manipulation.

**Radiosynthesis of D-[¹¹C]alanine:** In a reaction vessel, cinchonidinium catalyst 1 (1.5 mg, 2.4 µmol) and CsOH-H₂O (90 mg, 600 µmol) were suspended in a mixture of toluene/dichloromethane (400 µL, 9:1, v/v). The reaction vessel was cooled in an ice bath while stirring rapidly, and [¹¹C]CH₃I and precursor 2 (5.67 mg, 21 µmol) in 500 µL of toluene was added dropwise. Following the addition, the mixture was stirred for 15 min. A sample was taken for analysis on a Phenomonex Luna RP18 column (acetonitrile/sodium formate 4 mM 70/30, v/v) with a retention time (tᵣ) of 29 min for D-[¹¹C]alanine. The reaction mixture was passed through a silica light sep pak and collected as Fraction 1. 1 mL of MeCN was used to rinse the reaction vessel and passed through the silica light sep pak and collected as Fraction 2. 1 mL of TFA was added to Fraction 2 and the mixture was heated to 100°C for 15 min. A sample was taken for analysis on a Phenomonex Luna RP18 column (acetonitrile/sodium...
formate 4 mM 70/30, v/v) with a retention time ($t_R$) of 9.1 min of compound 3. The reaction mixture was concentrated and diluted with 1mL of MeCN x 3 to remove all the TFA. The residue was dissolved in pH 7.4 PBS and a sample was taken for analysis on a Phenomex Chirex 3126 chiral column (1mM CuSO$_4$) with a $t_R$ of 13 min for L-$[^{11}\text{C}]$alanine and 18 min for D-$[^{11}\text{C}]$alanine.

**HPLC Analysis of D-$[^{11}\text{C}]$alanine**

(A) Reversed-phase HPLC after first step. Solvent and catalyst were observed in the UV trace (blue) and the radiolabeled alanine intermediate elutes at 29 min (red). (B) Reversed-phase HPLC after cartridge purification and second synthetic step. Nothing is observed in the UV trace (blue) and the deprotected radiolabeled alanine elutes at 9.1 min (red). (C) Chiral HPLC of final radiolabeled D-$[^{11}\text{C}]$alanine ($R_T$ 18 min). (D) Chiral HPLC of final radiolabeled D-$[^{11}\text{C}]$alanine ($R_T$ 18 min) co-injected with 1mg/mL of racemic alanine.

![HPLC Analysis of D-$[^{11}\text{C}]$alanine](image)
Radiosynthesis of L-[^11]C]alanine

In a reaction vessel, catalyst ent-1 (1.5 mg, 2.4 µmol) and CsOHxH₂O (90 mg, 600 µmol) were suspended in a mixture of toluene/dichloromethane (400 µL, 9:1, v/v). The reaction vessel was cooled in an ice bath while stirring rapidly, and[^11]CCH₃I and precursor 2 (5.67 mg, 21 µmol) in 500 µL of toluene was added dropwise. Following the addition, the mixture was stirred for 15 min. The reaction mixture was passed through a silica light sep pak and collected as Fraction 1. MeCN (1 mL) was used to rinse the reaction vessel and passed through the silica light sep pak and collected as Fraction 2. TFA (1 mL) was added to Fraction 2 and the mixture was heated to 100°C for 15 min. The reaction mixture was concentrated and diluted with 1mL of MeCN x 3 to remove all the TFA. The residue was dissolved in pH 7.4 PBS and a sample is taken for analysis on a Phenomonex Chirex 3126 chiral column (1mM CuSO₄) with a tᵣ of 13 min for L-[^11]C]alanine and 20 min for D-[^11]C]alanine.

HPLC Analysis of L-[^11]C]alanine

(A) Chiral HPLC of final radiolabeled L-[^11]C]alanine (Rᵣ 13 min). (B) Chiral HPLC of final radiolabeled L-[^11]C]alanine (Rᵣ 13 min) co-injected with 1mg/mL of glycine and racemic alanine (the first and third peaks in blue correspond to glycine and the undesired D-alanine enantiomer respectively).
Radiosynthesis of D-[^11]C]alanyl-D-alanine

In a reaction vessel, catalyst 1 (1.5 mg, 2.4 μmol) and CsOHxH₂O (90 mg, 600 μmol) were suspended in a mixture of toluene/dichloromethane (400 μL, 9:1, v/v). The reaction vessel was cooled in an ice bath while stirring rapidly, and [^11]CCH₃I and precursor 3 (5.67 mg, 21 μmol) in 500 μL of toluene were added dropwise. Following the addition, the mixture was stirred for 15 min. The reaction mixture was passed through a silica light sep pak and collected as Fraction 1. MeCN (1 mL) was used to rinse the reaction vessel and passed through the silica light sep pak and collected as Fraction 2. TFA (1 mL) was added to Fraction 2 and the mixture was heated to 100 °C for 15 min. The reaction mixture was concentrated and diluted with 1mL of MeCN x 3 to remove all the TFA. The residue was dissolved in pH 7.4 PBS and a sample is taken for analysis on a Phenomonex Chirex 3126 chiral column (1 mM CuSO₄) with a tᵣ of 14 min for D-[^11]C]alanyl-D-alanine and 19 min for L-[^11]C]alanyl-D-alanine.

HPLC Analysis of D-[11C]alanyl-D-alanine

(A) Chiral HPLC of final radiolabeled D-[^11]C]alanyl-D-alanine (tᵣ 14 min). (B) Chiral HPLC of final radiolabeled D-[^11]C]alanyl-D-alanine (tᵣ 14 min) co-injected with 1mg/mL of 1:1 D-alanyl-D-alanine and L-alanyl-D-alanine.
Radiosynthesis of $^{68}$Ga-gallium citrate

$^{68}$Ga Citrate synthesis was performed at UCSF on an iQS® Ga-68 Fluidic Labeling Module with a pre-assembled cassette. $^{68}$Ga was eluted off an ITG GMP Ge-68/Ga-68 Generator with 4 mL of 0.05M HCl at a rate of 2 mL/min into a reactor with 2.2 mL of 0.075 M NaOH buffer. The solution was mixed by bubbling of Gallium (III) Chloride and NaOH. After one minute, the solution was transferred over an Accel Plus CM cation exchange cartridge, trapping the Gallium (III) cation with residuals sent to waste. The cartridge was then rinsed with 0.5 mL of 0.9% NaCl. The Accel Plus CM cartridge was subsequently removed from the module to be eluted in 0.5 mL incremental fractions with 1 mL of sodium citrate followed by 3 mL of 0.9% NaCl.

Radiosynthesis of $^{18}$FFluoro-2-deoxy-D-glucose

2-$^{18}$FFluoro-2-deoxy-D-glucose (FDG) production was manufactured at UCSF on a GE FASTlabTM synthesizer using an FDG citrate disposable cassette. Approximately 7 Ci of $^{18}$F was delivered to the synthesizer after the bombardment of $^{18}$O-water with hydrogen. $^{18}$F$^-$ was trapped on a quaternary methyl ammonium (QMA) anion exchange cartridge and eluted with a 0.5 mL solution of Kryptofix (K2.2.2), potassium carbonate, acetonitrile (MeCN), and water solution. Acetonitrile additions form an azeotropic mixture for evaporation of residual water. Mannose triflate precursor dissolved in MeCN was added to the reaction vessel for a 3-minute labelling reaction at 125 °C. The resulting FTAG (2-$^{18}$F-fluoro-1,3,4,6-tetra-O-acetyl-D-glucose was then diluted with water prior to transfer to the reversed-phase tC18 cartridge. FTAG was retained on the tC18 while unreacted $^{18}$F and impurities flow through the cartridge to waste. Alkaline hydrolysis of the trapped FTAG on the tC18 was performed with 2N NaOH. FDG was eluted with water and neutralized in 3.2 mL of citrate buffer before final purification through a tC18 plus cartridge and Alumina N cartridge1. Following this 25-minute synthesis, the final product was delivered through a 0.22µm vented filter to the final product vial. The synthesis resulted in approximately 80% decay-corrected yield.
C. $^1$H and $^{13}$C NMR spectra:
Figure S4. Comparison of D-[\textsuperscript{11}C]ala and D-[\textsuperscript{11}C]ala-D-alal uptake in bacterial pathogens. (A) \textit{In vitro} tracer studies performed in \textit{E. coli} and \textit{S. aureus}. The accumulation of D-[\textsuperscript{11}C]ala was much higher than that of D-[\textsuperscript{11}C]ala-D-alal in \textit{S. aureus}, an important organism to which this approach was targeted. Both D-[\textsuperscript{11}C]ala and D-[\textsuperscript{11}C]ala-D-alal did not show activity in heat-killed bacteria. (B) A partial screen for D-[\textsuperscript{11}C]ala-D-alal did show promising uptake in several other disease pathogens.

Figure S5. Comparison of D-[\textsuperscript{11}C]ala and D-[\textsuperscript{11}C]met uptake across a range of gram-positive and gram-negative pathogens. Similar trends of susceptibility can observed for both labeled amino acids with D-[\textsuperscript{11}C]ala showing superior accumulation in every pathogen tested.
Figure S6. Accumulation of D-[¹¹C]ala and L-[¹¹C]ala in mammalian and bacterial cells. As highlighted separately D-[¹¹C]ala showed significantly lower uptake in mammalian cells. However as depicted here there was high uptake of both tracers in *E. coli* as expected given the known role of L-alanine in bacterial metabolism and cell wall synthesis.

Figure S7. D-[¹¹C]ala accumulation in conventionally raised (CONVR) and germ-free (GF) mice. Data were obtained via *ex vivo* gamma counting of harvested tissues following radiotracer administration. (A) Typical sagittal μPET-CT image obtained for a CONVR animal highlighting background D-[¹¹C]ala signal, seen particularly in the lungs, pancreas, kidneys and liver. (B) Biodistribution of D-[¹¹C]ala in a conventionally raised (CONVR) mouse cohort versus a germ-free (GF) mouse cohort. Of note this uptake was quite similar to that seen in CONVR organisms (P > 0.05 in all cases) with exception of signals detected from the colon. (C) Comparison between colon uptake in CONVR and GF animals. Higher colonic accumulation of D-[¹¹C]ala in normal animals suggests that commensal bacteria are incorporating activity. No other significant organ-specific differences were observed.
Figure S8. µPET-CT imaging using D-[¹¹C]ala in *E. coli*. The red arrows indicate the site of inoculation with live bacteria with heat-killed organisms introduced into the opposite shoulder. *Ex vivo* data was obtained following tissue harvesting on a gamma counter. These data closely approximate those obtained for *S. aureus*.

Figure S9. µPET-CT imaging using [¹⁸F]FDG in *E. coli*. The red arrows indicate the site of inoculation with live bacteria with heat-killed organisms introduced into the opposite shoulder. *Ex vivo* data was obtained following tissue harvesting on a gamma counter.
**Figure S10.** µPET-CT imaging using [$^{68}$Ga]gallium citrate in *E. coli*. The red arrows indicate the site of inoculation with live bacteria with heat-killed organisms introduced into the opposite shoulder. *Ex vivo* data was obtained following tissue harvesting on a gamma counter.

**Figure S11.** Comparison of *ex vivo* data of pneumonia infection cohort (n = 5) and a vehicle cohort (n = 5) obtained following tissue harvesting and analysis on a gamma counter.
| Bacteria Strain   | ATCC   | Growth Temperature (°C) | Media                |
|-------------------|--------|-------------------------|----------------------|
| E. coli           | 25922  | 37                      | Luria Broth          |
| P. aeruginosa     | 10145  | 37                      | Luria Broth          |
| K. pneumoniae     | 13883  | 37                      | Luria Broth          |
| A. baumannii      | 19606  | 37                      | Luria Broth          |
| S. typhimurium    | 29630  | 37                      | Luria Broth          |
| E. cloacae        | 7256   | 37                      | Luria Broth          |
| P. mirabilis      | 29906  | 37                      | Luria Broth          |
| S. aureus         | 12600  | 37                      | Luria Broth          |
| S. epidermidis    | 14990  | 37                      | Luria Broth          |
| L. monocytogenes  |        | 37                      | Brain Heart Infusion Broth |
| E. faecalis       | 19433  | 37                      | Brain Heart Infusion Broth |
| M. marinum        | 927    | 30                      | Luria Broth          |

**Figure S12.** Bacterial strains and growth conditions used for this study.

**E. References:**

1. Filp, U., Pekošak, A., Poot, A. J. & Windhorst, A. D. Enantioselective synthesis of carbon-11 labeled L-alanine using phase transfer catalysis of Schiff bases. *Tetrahedron*. 72, 6551-6557 (2016).

2. Mai, T. T., Viswambharan, B., Gori, D., Guillot, R., Naubron, J., Kouklovsky, C. & Alezra, V. Frozen chirality of tertiary aromatic amides: access to enantioenriched tertiary alpha-amino acid or amino alcohol without chiral reagent. *Chem. Eur. J.* 2017, 23, 5787-5798 (2017).

3. Carceller, E., Merlos, M., Giral, M., Almansa, C., Bartroli, J., Garcia-Rafanell, J. & Forn, J. Synthesis and structure-activity relationship of 1-Acyl-4-((2-methyl-3-pyridyl)cyanomethyl)piperazines as PAF antagonists. *J Med. Chem.* 36, 2984-2997 (1993).