# Enzyme-Mediated Directional Transport of a Small-Molecule Walker With Chemically Identical Feet

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S Supporting Information

ABSTRACT: We describe a small-molecule "walker" that uses enzyme catalysis to discriminate between the relative positions of its "feet" on a track and thereby move with net directionality. The bipedal walker has identical carboxylic acid feet, and "steps" along an isotactic hydroxyl-group-derivatized polyether track by the formation/breakage of ester linkages. Lipase AS catalyzes the selective hydrolysis of the rear foot of macrocyclized walkers (an information ratchet mechanism), the rear foot producing an (R)-stereocenter at its point of attachment to the track. If the hydrolyzed foot reattaches to the track in front of the bound foot it forms an (S)-stereocenter, which is resistant to enzymatic hydrolysis. Only macrocyclic walker-track conjugates are efficiently hydrolyzed by the enzyme, leading to high processivity of the walker movement along the track. Conventional chemical reagents promote formation of the ester bonds between the walker and the track. Iterative macrocyclization and hydrolysis reactions lead to 68% of walkers taking two steps directionally along a three-foothold track.
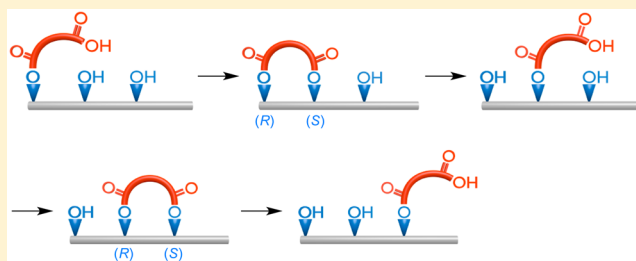
INTRODUCTION

Bipedal motor proteins perform tasks in the cell by directionally "walking" along microtubule tracks.[1] A well-studied example is kinesin I, a homodimeric protein with two chemically identical "feet" that typically takes 75−175 directional steps before fully detaching from the track.[2] Several synthetic small-molecule walkers have been developed;[3−5] however, only two[4] are able to walk along molecular tracks with net directionality. Both rely on the walker having chemically distinct feet that undergo orthogonal chemistries with the track to achieve the key property of processivity, that is, to enable each foot to remain attached to the track under conditions where the other one moves so that the walker does not fully detach from the track. Kinesin I uses mechanical strain to differentiate the reactivity of its identical feet during the walking cycle.[6] We wondered if it would be possible to achieve a similar outcome for a small-molecule walker with two identical feet, by exploiting the difference in stereochemistry of front and rear foot attachments (within a macrocycle) to the essentially[7] prochiral footholds of an isotactic oligomer track (Figure 1).

Upon macrocyclization of *1-1* (Figure 1), handedness is induced at each site of attachment to isotactic functional groups on the track (*1,2-2*). If that difference in stereochemistry can be exploited to make the rear foot more reactive to a chiral catalyst or reagent (e.g., by stereoselective hydrolysis by an enzyme; Figure 1, process II), the rear foot should be cleaved in preference to the front foot. The resulting open-chain species (*2-1*, Figure 1) is attached to the track through one, now essentially achiral,[7] attachment point. Reattachment of the dangling foot of *2-1* through macrocyclization with the track (Figure 1, process I) then forms a mixture of *1,2-2* (in which
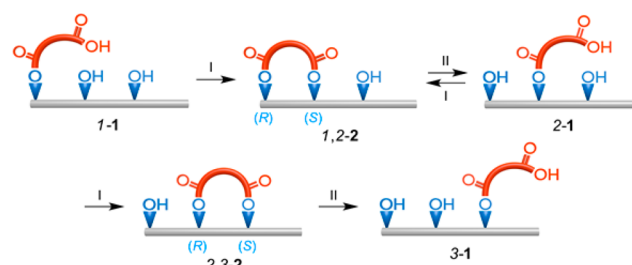

Figure 1. Directional transport of a small-molecule walker with two chemically identical feet: I, reagent-promoted macrocyclization (no selectivity in site of attachment); and II, enzymatic hydrolysis (rear foot selectively hydrolyzed: (R-) hydrolyzed much faster than (S-)).

the walker has returned to its original position on the track) and *2,3-2* (in which the walker has taken a step forward). Crucially for the directional walking mechanism, in *2,3-2* the foot that was the front (unreactive) (S)-attached foot in *1,2-2* has now become the rear (reactive) (R)-attached foot. Iterative repetition of the unselective macrocyclization and stereoselective hydrolysis steps (I, II, I, II, ...etc.) should lead to directional transport of the walker with chemically identical feet along the isotactic track (Figure 1).

RESULTS AND DISCUSSION

Walker and Track Design. We experimentally explored this concept with a molecular walker, **3**, based on a C_2-symmetric (R,R)-(+)-hydrobenzoin motif (Scheme 1a) and a

Scheme 1. Directional Transport of a Small-Molecule Biped with Chemically Identical Feet[a]

Cycle (n)	0	1	2	3	4
1-1	100	15	6	10	10
2-1	0	56	38	24	22
3-1	0	29	56	66	**68**
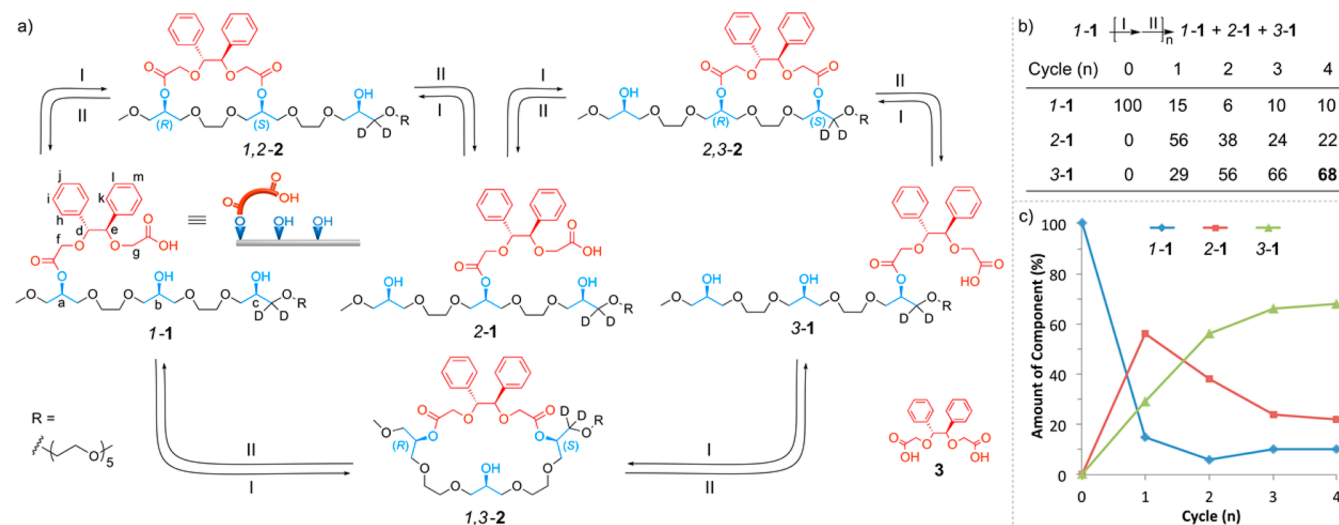
[a](a) Interconversion of walker-track conjugates under operation cycles consisting of (I) macrocyclization (2,4,6-TCBC, DMAP, Et$_3$N, CHCl$_3$, 0.05 mM, rt, 20 h) and (II) enzyme hydrolysis (lipase AS (3.0 equiv w/w), H$_2$O, 18 mM, 40 °C, 40−64 h). (b) Table and (c) graph showing the population of walker-track positional isomers 1-1, 2-1, and 3-1 after each operation cycle (see the Supporting Information, margin of error ±3%). TCBC, trichlorobenzoyl chloride; DMAP, 4-(dimethylamino)pyridine; lipase AS, lipase AS "Amano" (lipase from *Aspergillus niger*).

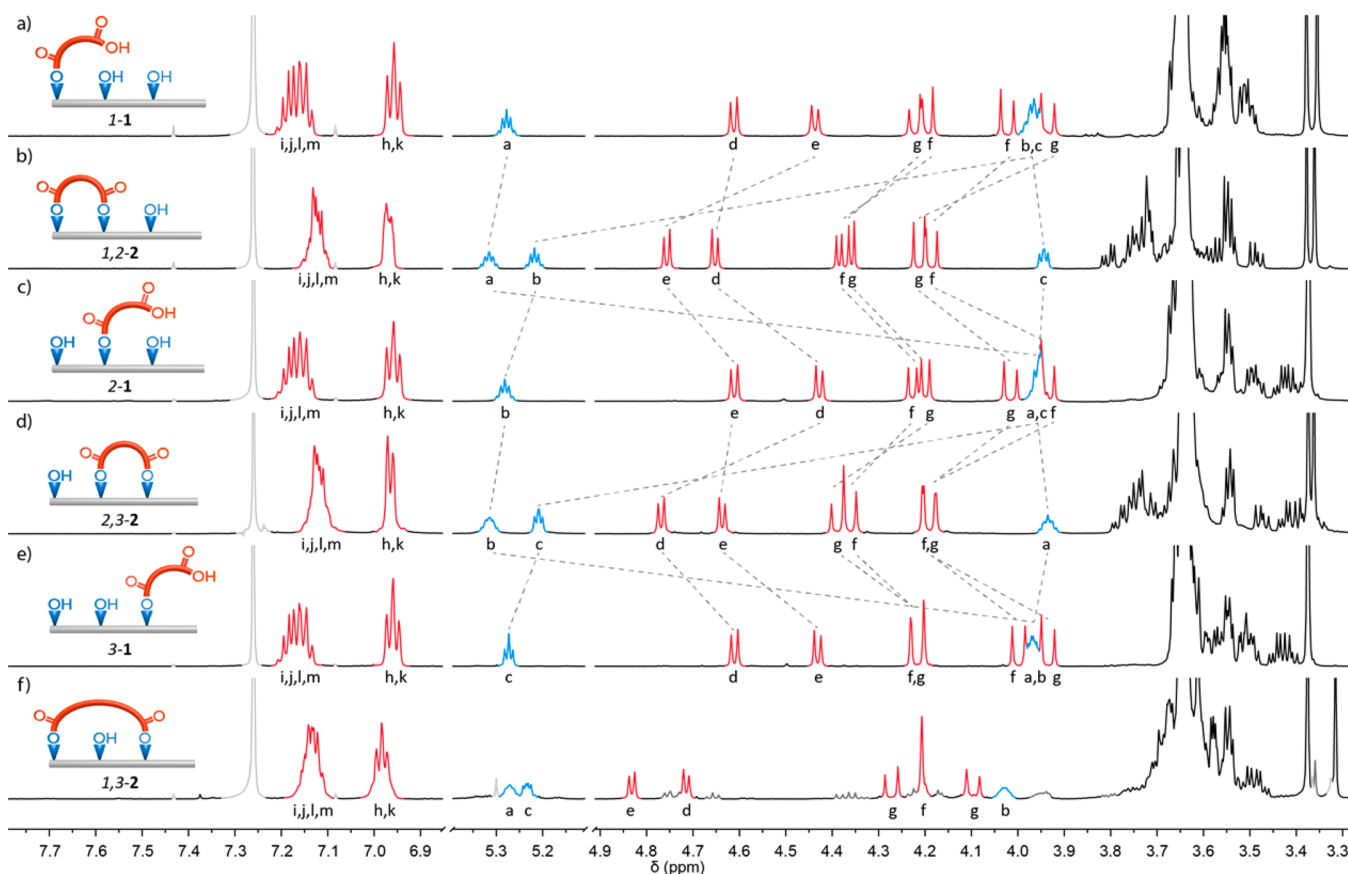**Figure 2.** Partial ^1H NMR spectra (600 MHz, CDCl$_3$, 298 K) of walker-track conjugates: (a) *1-1*, (b) *1,2-2*, (c) *2-1*, (d) *2,3-2*, (e) *3-1*, and (f) *1,3-2*. Dashed lines connect the methine protons (H$_a$, H$_b$, and H$_c$, blue) of the track footholds, the methine protons (H$_d$ and H$_e$) and the methylene protons (H$_f$ and H$_g$) of the walker (red), and are diagnostic of the walker's position on the track. Proton assignments correspond to the lettering in Scheme 1a. Signals due to residual solvents are shown in light gray.

track consisting of a poly(ethylene glycol) chain featuring an isotactic triad of three secondary alcohol footholds at one end that can attach to the carboxylate feet of the walker through the

formation of ester linkages. The phenyl groups of the walker are UV-chromophores that aid analysis during synthesis and purification. It is important that the walker "legs" have the

same chirality (or none) in the absence of the track: if a *meso*-(R,S)-walker was used, the walker-track conjugate could potentially have differing behaviors in the enzyme active site depending on whether the rear leg stemmed from the (R-) or (S-) part of the hydrobenzoin unit. Three footholds on the track is sufficient to demonstrate the directional transport principle aided by unambiguous determination of the position of the walker on the track at each stage, a feat that becomes significantly more difficult with more than three footholds.[5c,e] The polyether track confers solubility in aqueous solvents suitable for enzymatic hydrolysis and has some flexibility to adopt conformations that allow access to the active site of the enzyme. The hydrolysis of chiral and prochiral diesters by lipases generally has high substrate tolerance and often proceeds with excellent regio- and stereochemical control.[8,9]

Scheme 1a shows the walking process. The walker begins as hydroxy-acid *1-1*, which cyclizes with the track to form macrocycle *1,2-2* (Scheme 1a, top sequence, I). The ester linkages that fix the walker to the track in *1,2-2* are stereochemically distinct. Lipase AS, identified as a suitable enzyme in screening studies (see the Supporting Information), selectively hydrolyzes the ester linkage at the (R)-stereocenter (Scheme 1a, top sequence, II) leading to the formation of *2-1*; that is, the rear leg of the walker has become detached from the track. At this stage, the walker has taken one step along the track (from starting position *1-1*) via a passing-leg gait.

Lipase AS does not hydrolyze the remaining ester linkage in *2-1* quickly (the rate of reaction of *1,2-2* or *2,3-2* is >20× faster than that of *2-1*), enabling good processivity for the walking process. A second (nondirectional) intramolecular macrocyclization reaction should result in approximately 50% of the walkers forming the new positional isomer *2,3-2* (the other 50% reforms *1,2-2*). As the relative positions of the walker's feet change, so does the stereochemistry at the occupied footholds; the front leg of *1,2-2*, which was previously attached to the track through a center whose stereochemistry was (S), has become the rear leg of *2,3-2*, and the (same) center it is attached to has become (R) by virtue of the change in the macrocycle position on the track. As the enzyme selectively hydrolyzes the rear (R)-attached foot each time, the enzyme hydrolyzes *2,3-2* to form *3-1*, after which the walker has taken two steps directionally along the three-foothold track (Scheme 1a, top sequence). Because the directional walking results from the enzyme's selective hydrolysis of whichever foot is to the rear, the mechanism corresponds to an information ratchet type of Brownian ratchet mechanism.[10]

Motor-proteins take occasional double steps, and the flexibility of the polyether track should make overstepping a significant process for synthetic walker **3** too. Hydrolysis of the rear leg of *1,3-2*, still reacting in preference to the front leg as the rear leg is attached through an (R)-stereocenter, allows the walker to step directionally to the terminal position of the track through a "double-step" mechanism (Scheme 1, bottom sequence).

Characterization of Different Positions of the Walker on the Track. Acid *1-1* and macrocycles *1,2-2* and *2,3-2* were prepared unambiguously through synthesis, acids *2-1* and *3-1* were isolated from enzymatic hydrolysis of *1,2-2* and *2,3-2*, respectively, and *1,3-2* was obtained by preparative thin-layer chromatography of a mixture of *1,3-2* and *1,2-2* following macrocyclization of *1-1* (see the Supporting Information for synthetic procedures and characterization data). Each compound could be distinguished from the others by ^{1}H NMR

spectroscopy (Figure 2). The chemical shifts of the methine protons of the glycerol subunits, H_a, H_b, and H_c, are diagnostic of the position of the walker on the track. Esterification of the footholds leads to downfield shifts in the ^{1}H NMR spectrum from 3.9−4.0 to 5.2−5.3 ppm. In the macrocyclic conjugates, the methine signal at the (R)-stereocenter (H_a in Figure 2b and f and H_b in Figure 2d) is 0.5−1.5 ppm further downfield than that of the equivalent (S)-stereocenter. Additional clarity in determining the walker position on the track was provided by deuterium labeling of the methylene group adjacent to H_c. When the walker is at positions 1- or 2-, a pentet is observed at 5.2−5.3 ppm (Figure 2a−d and f), whereas when the walker reaches the final foothold the corresponding signal is a triplet (Figure 2d−f). The similarity of the ^{1}H NMR spectra of *1,2-2* (Figure 2b) and *2,3-2* (Figure 2d) suggests these macrocycles adopt very similar conformations.

Ring-Opening and Ring-Closing Experiments. The individual parts of the walking mechanism were initially studied by subjecting macrocycles *1,2-2* and *2,3-2* to enzymatic hydrolysis−macrocyclization operation conditions (Scheme 1a, and see the Supporting Information).

Walker-track conjugate *1,2-2* was treated with lipase AS (Scheme 1a, process II: lipase AS, 3.0 equiv w/w, H_2O, 18 mM, 40 °C, 40 h). After filtration and evaporation of the solvent, ^{1}H NMR spectroscopy indicated that the enzyme had hydrolyzed *1,2-2* with excellent regioselectivity, giving *1-1*:*2-1* in a 3:97 ratio (Figure S5) accompanied by 4% of diacid **3**, the product of fully detaching the walker from the track. The formation of relatively little **3** shows that lipase AS distinguishes effectively between macrocycle *1,2-2*, its preferred substrate, and the open-chain form, *2-1*; this is a key result for achieving significant processivity during the walking sequence.

Macrocyclization of the *1-1*:*2-1* (3:97) product mixture was carried out using a Yamaguchi protocol[11] (Scheme 1a, process I: 2,4,6-TCBC, DMAP, CHCl₃, 0.05 mM, rt, 20 h), leading to a 48:51:1 ratio of *1,2-2*:*2,3-2*:*1,3-2*. Following the essentially nondirectional macrocyclization of *2-1* (to form *1,2-2* and *2,3-2*), 51% of walkers had taken one step directionally along the track by a passing-leg gait after one hydrolysis−macrocyclization cycle.

Walker-track conjugate *2,3-2* was similarly subjected to lipase AS (3.0 equiv w/w, H_2O, 12 mM, 40 °C, 40 h) leading to 93% conversion to *2-1* and *3-1*. Hydrolysis occurred preferentially at the ester linkage adjacent to the (R)-stereocenter, affording *3-1* with 94% selectivity (Figure S6). Diacid **3** again constituted ≤4% of the product mixture. Macrocyclization of the product mixture led to a 2:53:45 ratio of *1,2-2*:*2,3-2*:*1,3-2*. The formation of *1,3-2* as a major component in this reaction suggests that the double-step mechanism could play a significant role if the walker was used to traverse an extended form of the track with additional footholds.

Four Walking Cycles Starting from Walker-Track Conjugate 1-1. With the outcomes of the ring-opening and ring-closing reactions established for each intermediate, the small-molecule walker *1-1* was operated through four cycles of macrocyclization/enzymatic hydrolysis (Scheme 1). The changing distribution of the complex mixture was consistent with modeling the transformations as a series of Markov chains[10d,12] using the ring-opening/closing experimental data (see the Supporting Information). Macrocyclization of walker-track conjugate *1-1* (Scheme 1, I) led to a 58:42 mixture of macrocycles *1,2-2* and *1,3-2* (Figure S8b and Table S5). This mixture was treated with the enzyme (Scheme 1, II) generating

a 15:56:29 mixture of *1*-**1**:*2*-**1**:*3*-**1** (cycle 1, Scheme 1b,c and Figure S9). Lipase AS hydrolyzes the 25-membered macrocycle *1,3*-**2** with 70% selectivity for the (*R*)-stereocenter, that is, the rear leg of the walker (Figure S9). As a result, 29% of walkers reach the terminal foothold after only one operation cycle by double-stepping. The majority of walkers (56%) take one step directionally along the track, forming *2*-**1** by a passing-leg gait (cycle 1, Scheme 1b and c).

After repeating the macrocyclization–hydrolysis operations a further three times (cycles 2–4, Scheme 1b,c), the distribution approaches a steady-state in which 68% of the walkers have taken two steps directionally along the track (forming *3*-**1**). Of the other walkers, 22% had taken one step (*2*-**1**), while 10% remained at the starting position (*1*-**1**) (cycle 4, Scheme 1b,c). The processivity remained good over four cycles of operation, with 1–4% of diacid **3** lost during each enzyme hydrolysis stage. This suggests that the small-molecule walker should be able to take an average of at least 17 steps before fully dissociating when directionally walking along a longer track (see the Supporting Information).

CONCLUSIONS

The chemically identical feet of a small-molecule walker can be discriminated on an isotactic track by exploiting the stereo-chemical differences in foot environment induced by macro-cyclization of the walker with the track. Lipase AS hydrolyzes the rear foot ester linkages of a (*R,R*)-(+)-hydrobenzoin-based walker with up to 97% regioselectivity. The process can be combined with (unselective) macrocyclization reactions to produce directional migration of the walker along the track. After four macrocyclization–hydrolysis operations on a three-foothold track, 90% of walkers had moved away from the starting position, with 68% two steps further down the track. To favor passing-leg over double-step mechanisms, it may be necessary to employ more rigid strand designs for extended tracks.

All biomolecular walkers are also enzymes (their directional movement is powered by their catalysis of ATP hydrolysis).[1] The use of an enzyme to control the directionality of an artificial small-molecule walker marries a biological machine with a synthetic one in a new form of hybrid biosynthetic walker mechanism.

ASSOCIATED CONTENT

ⓈSupporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.7b06503.

> Experimental procedures, spectroscopic data, and oper-ation details of the molecular walkers (PDF)

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Notes

The authors declare no competing financial interest.

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