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Iodine scanning of a phenazine inhibitor of vacuolar sorting

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ABSTRACT

Affinity reagents are often used to address the target identification problem in chemical genetics. The design of such reagents so that the linker does not occlude interactions with protein targets is an ongoing challenge. This work describes a systematic approach to synthesize derivatives of a bioactive that should avoid interference with binding to targets and be readily converted to affinity reagents.

Forward genetics based on mutagenesis and phenotypic screening for modified function is a powerful method for the study of biological pathways. Forward chemical genetics offers a complementary approach in which a small molecule plays a role similar to that of a modified (and readily identified) gene in a classical forward genetics experiment. One aim of forward chemical genetics is to use the small molecule to identify its protein target(s) and its role in the pathway under study. Powerful methods for target identification based on small molecules are therefore needed.[1]

Past work from one of our laboratories described the methyl scanning approach to mapping the interactions between a bioactive compound and its receptor protein.[2] The activities of a family of systematically synthesized derivatives were used to design and prepare an affinity reagent for use in identification of unknown targets.[3] While effective, this approach had the drawback that once positions that can be substituted are identified, a de novo synthesis of the affinity reagent is required. A streamlined approach would use a group that could not only be scanned to identify sites that can be modified without compromising activity, but would also be convertible to the affinity reagent. We propose the use of iodine for this purpose based on its van der Waals radius (2.15 Å), which is comparable to methyl groups (1.7 Å) that proved able to perturb small molecule–protein interactions in our initial studies. C–I bonds are also synthetically versatile, participating in radical-based bond formation, organometallic cross-coupling, and nucleophilic substitution. The method of iodine scanning developed here was applied to a compound discovered via chemical genetics to play a role in gravitropism in plants.

Past work from the other of our laboratories focused on the interplay between gravitropic signal transduction and the plant endomembrane system, which is functionally and genetically complex. Plants utilize many of the same components for vesicular trafficking through the endoplasmic reticulum as animals and fungi, but also carry out unique functions, such as storage of proteins and the biosynthesis of cell wall precursors.[4] Furthermore, many of the gene families that encode trafficking proteins in plants have many more members than their animal counterparts. Such a multiplicity of trafficking genes has confounded our studies of the plant endomembrane system. Many genes that encode its components are essential for viability, limiting the utility of knockout mutants.[5] Conversely, many point mutants have no phenotypes.[6] The latter could be due to gene redundancy, a situation to which chemical genetics may be particularly applicable.

Classical genetics screens had already demonstrated that the plant endomembrane system is intimately involved in gravitropic signal transduction. This pathway is not well understood, but mutations in a number of genes that encode endomembrane system components result in agravitropic phenotypes. Screening of a commercial chemical library for compounds that interfere with gravitropic responses in the model plant *Arabidopsis* identified four compounds.[7] They not only inhibit gravitropism via the endomembrane system, but also cause changes in vacuole morphology. One such compound is 5271050 (**1**; *N*-phenylphenazin-2-amine; Fig. 1). To discern structure–function relationships, we examined analogs of **1** that were also available commercially. These included the dioxide **2** and the parent ring system, phenazine itself. Neither of these compounds was active in either phenotypic readout, gravitropism or formation of aggregates of a prototypical vacuolar cargo protein, tonoplast intrinsic protein (δTIP), which has been labeled with green fluorescent protein (GFP) (vide infra).

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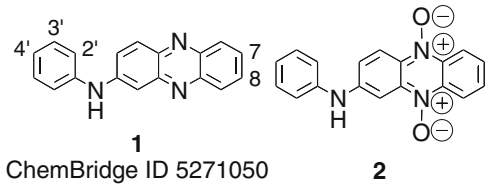
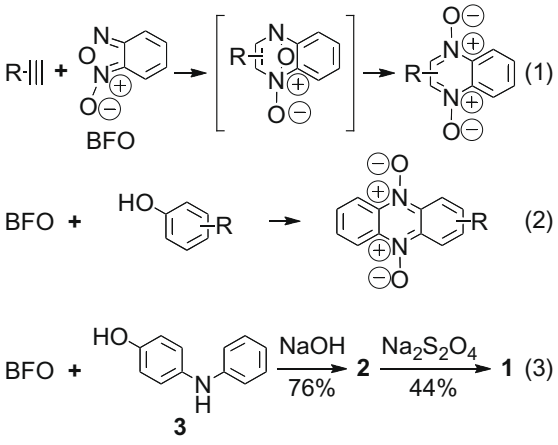


Figure 1. Structures of the vacuolar sorting inhibitor 5271050 (**1**) and its dioxide analog **2**.

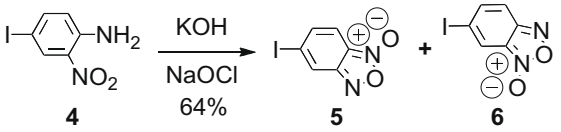
We next became interested in applying the concept of iodine scanning enumerated above to compound **1** so as to permit the generation of **1**-based affinity reagents for identification of its target(s). The only method reported for the preparation of **1**[8] was unlikely to have been used in a high-throughput synthesis for the generation of a screening library. Both the presence of **2** in the ChemBridge library and a review of phenazine natural products[9] suggested to us a versatile synthetic route to phenazines based on the Beirut reaction[10] of benzofurazan-*N*-oxide (BFO; Scheme 1). The most common version of the Beirut reaction is a hetero-Diels–Alder cycloaddition between BFO and acetylenic dienophiles, after which bond reorganization and aromatization of the cycloadduct gives quinazoline-*N,N'*-dioxides (Eq. 1). When the Beirut reaction of BFO is instead conducted with a phenol in the presence of base, phenazine-*N,N'*-dioxides result (Eq. 2).

This approach toward **1** was readily tested, as both BFO and 4-hydroxydiphenylamine (**3**) are commercial. Their reaction under standard conditions provides **2**, which could be reduced to **1** under mild reaction conditions (Eq. 3). The wide availability of phenols and the simplicity of this route suggest that this is very likely the synthesis ChemBridge used to prepare 5271050.

This re-discovered route provided the convergent and modular synthesis of **1** that is essential to efficient application of the iodine scanning concept. The synthesis of iodinated derivatives of **1** simply required the preparation of the two building blocks of this synthesis, BFO and 4-hydroxydiphenylamine, in iodine-substituted forms. Several synthetic routes to BFOs are available; we adopted the oxidation of *o*-nitroanilines, in part owing to the commercial availability of 4-iodo-2-nitroaniline (**4**; Scheme 2). This compound is oxidized with NaOCl under basic conditions to give a mixture of the known[11] 5- and 6-iodinated BFOs **5** and **6**.[12] This mixture is a result of the facile equilibration of the two positional isomers of any substituted benzofurazan-*N*-oxide via *o*-dinitroso intermediates. This process is invisible when the BFO is symmetrical, but if it is not, both isomers are routinely observed. Even if only one of these two isomers were present, it is unlikely that a substituent distant from the site of the Beirut reaction would have a significant effect on the cycloaddition, with the result that a mixture of regiochemical outcomes is expected. Therefore, both 7- and 8-iodo-substituted derivatives of **1** should be formed. Though a mixture of compounds is not ideal, syntheses that would give one compound exclusively were much more involved.

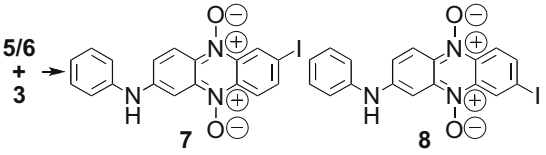


Scheme 1. Methods for phenazine synthesis based on BFO.



Scheme 2. Synthesis of 5/6-iodo-BFO.

With the **5**/**6** mixture in hand, the Beirut reaction with **3** was performed as before (Scheme 3), delivering a 3:2 mixture of **7** and **8** (50% yield), which proved to be inseparable. Upon dithionite



Scheme 3. Synthesis of 7/8-iodo-**1**.

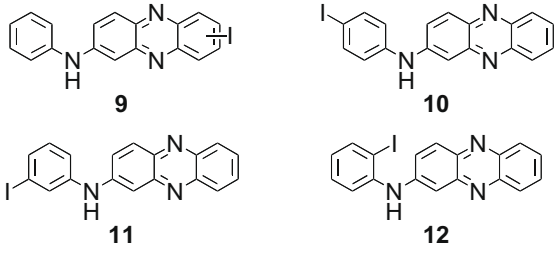
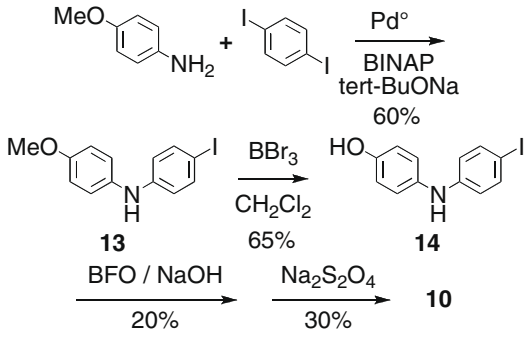
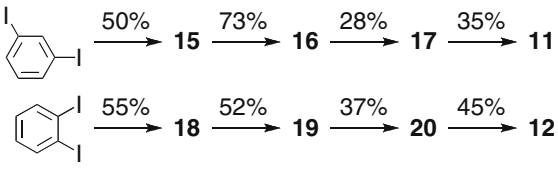


Figure 2. Iodinated analogs of **1** that were prepared.



Scheme 4. Synthesis of analog **10**.



Scheme 5. Synthesis of analogs **11** and **12**.

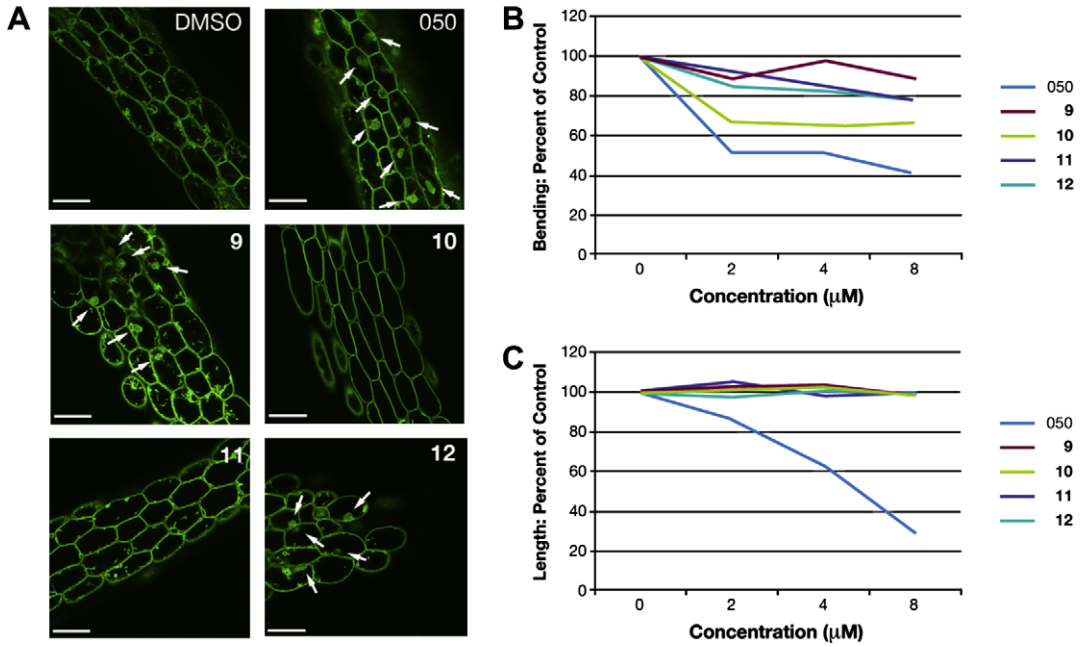


Figure 3. The effects of iodinated derivatives of 5271050 on aggregate formation in GFP:δTIP seedlings. (**A**) Hypocotyls were examined by laser scanning confocal microscopy following germination and growth for seven days in the light and on the designated chemicals: DMSO control, 2 μM 050, or 16 μM compounds **9**–**12**. Bars = 50 μm. (**B**) The effects of iodinated compounds on gravitropic bending. Four days old seedlings grown in the presence of 050 and its iodinated derivatives were gravistimulated for 24 h, and the angle of curvature of the hypocotyl from a vertical position was measured. The values, in degrees ± standard error of the mean, for the DMSO control of each treatment were as follows: 050, 55.4 ± 3.2; **9**, 48.7 ± 3.7; **10**, 58.2 ± 3.3; **11**, 53.9 ± 5.2; and **12**, 46.6 ± 3.2. (**C**) The effects of iodinated compounds on hypocotyl growth. Values for the DMSO control of each treatment, reported as mm ± standard error of the mean, were as follows: 050, 15.4 ± 0.5; **9**, 15.1 ± 0.4; **10**, 14.7 ± 0.3; **11**, 15.3 ± 0.5; and **12**, 14.6 ± 0.4. The results in panels (**B**) and (**C**) are reported as percent of DMSO controls, where the DMSO control was 100%.

reduction, the **7**/**8** mixture gave our initial target, **9**, as a 4:1 mixture (40% yield; Fig. 2). We cannot explain the variance of the isomer ratios in these products and have not assigned the structure of the major isomer. While in principle it should be possible to use this route to prepare 6- and 9-iodo-substituted derivatives of **1**, substituents adjacent to the heterocycle severely compromise reactivity of BFOs.[10]

The remainder of the targeted analogs should be accessible via iodinated versions of phenol **3**. This route was successfully applied to the preparation of **10**–**12**.

After initial investigation of some other strategies that proved unsuccessful, a general route to iodinated 4-hydroxydiphenylamines was adopted that involved coupling of *p*-methoxyaniline to three diiodobenzene isomers using Buchwald–Hartwig conditions. With *p*-diiodobenzene (Scheme 4), compound **13** results, from which the methyl ether is cleaved with boron tribromide. The known[13] phenol **14** is then taken through the already proved synthesis to give target **10**.

By a similar sequence of reactions starting with *o*- and *m*-diiodobenzene, the isomers **11** and **12** were accessed (Scheme 5).

Versions of **1** iodinated in the central benzene ring would in principal be available from iodination of the phenol ring of **3**, but in practice it proved impossible to obtain such compounds cleanly, owing largely to the strong tendency of **3** to be oxidized by electrophilic halogenating reagents rather than being halogenated. We were forced to be satisfied with the four analogs **9**–**12**. While they do not constitute a comprehensive set of iodo-substituted versions of **1**, they do at least represent functionalization at the extremities of the molecule.

Treatment of *Arabidopsis* seedlings with compounds **9**–**12** revealed varying degrees of efficacy (Fig. 3). Bioactivity testing of **9**–**12** using the same approaches and treatment with **1** as a positive control and DMSO as a negative control showed diverse effects of the four compounds on three phenotypic responses: hypocotyl length, gravitropic bending, and aggregate formation. First, none of the iodinated compounds reduced the hypocotyl length of treated seedlings (Fig. 3a), indicating that these four positions must be unsubstituted for growth inhibition. The parent molecule, **1**, decreased gravitropic bending by approximately 50% at the lowest treatment concentration, 2 μM. Derivative **10**, which features the iodine atom at the 3′ position, had only a slight effect on inhibition of gravitropic bending; treatment with 2 μM compound reduced the bending angle by approximately 35%. The remaining three derivatives had a greater impact on inhibition of gravitropism. In their effects on inhibition of gravitropic bending, compound **9** deviated only slightly from the negative DMSO control, and **10** and **11** were intermediate. Taken together, these results show that the 7/8 positions on the molecule must be unsubstituted for inhibition of gravitropic bending, while the 2′ and 3′ positions also contribute, albeit to a lesser extent. Compound **12** (iodine in the 2′ position) induced aggregate formation at concentrations of 16 μM (compared with 2 μM for the parent **1**). No effects were observed at concentrations of 2, 4, or 8 μM. Compound **11** showed sporadic aggregate formation at 16 μM, whereas no aggregates were observed for compound **10** at this or higher concentrations. Compound **9** (iodine in the 7/8 positions) induced aggregate formation $\geqslant$4 μM.

Our conclusion from these results is twofold. First, one of the bioactivity determinants of 5271050 for modifying the endomembrane system morphology surely resides in its aniline moiety. Second, the growth/gravitropic and endomembrane morphology phenotypes are clearly separable, implying that 5271050 has multiple targets. The observation that all of the iodinated derivatives abrogate the growth effects and three out of the four derivatives affect gravitropism inhibition of 5271050 suggests that both ends of the molecule are important for these processes.

We are currently cloning and characterizing two *Arabidopsis* EMS mutants that do not form aggregates when treated with 5271050. Identification of these targets will further our under-

standing of early trafficking events in the plant endomembrane system. A more complete understanding of aggregate induction by 5271050 will provide a tool to dissect trafficking through the endoplasmic reticulum in much the same way brefeldin A is used to dissect trafficking through the Golgi and endosome.[14]

With respect to iodine scanning methodology and target identification for 5271050, the agent that is obviously of the most interest for future studies is **9**. This material should be readily biotinylated using known reagents,[15] and the resulting compound should be useful to identify endoplasmic reticulum protein targets relevant to the activity of 5271050 regarding vacuole morphology.

Acknowledgment

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12. *^{1}H NMR data*: *Compound* **5**/**6**: δ 7.42 (br, 1H), 7.64 (br, 1H). 8.18 (br, 1H). **7**/**8**: δ 9.47 (s, 1H), 8.78–8.82 (m, 1H), 8.39–8.43 (m, 1H), 7.99–8.23 (m, 2H), 7.81 (d, J = 2.4, 1H), 7.59–7.63 (m, 1H), 7.33–7.46 (m, 4H), 7.12–7.17 (m, 1H). **9**: δ 9.31 (s, 1H), 8.49–8.57 (m, 1H), 7.74–8.09 (m, 4H), 7.43–7.51 (m, 5H), 7.14–7.19 (m, 1H). **10**: δ 8.02–8.11 (m, 3H), 7.56–7.74 (m, 5H), 7.42 (dd, J = 9.1, 2.4, 1H), 7.05 (d, J = 8.7, 2H), 6.22 (br, 1H). **11**: δ 8.10–8.20 (m, 3H), 7.70–7.82 (m, 2H), 7.61–7.68 (m, 2H), 7.45–7.54 (m, 2H), 7.32–7.38 (m, 1H), 7.08–7.14 (m, 1H), 6.30 (br, 1H). **12**: δ 8.12–8.20 (m, 3H), 7.70–7.82 (m, 2H), 7.61–7.68 (m, 2H), 7.45–7.54 (m, 2H), 7.32–7.38 (m, 1H), 7.08–7.14 (m, 1H), 6.30 (br, 1H). **13**: δ 7.45 (d, J = 6.6, 2H), 7.05 (d, J = 6.6, 2H), 6.87 (d, J = 7.8, 2H), 6.66 (d, J = 6.3, 2H), 5.50 (br, 1H), 3.81 (s, 3H). **14**: δ 7.48 (d, J = 6.6, 2H), 7.01 (d, J = 6.3, 2H), 6.80 (d, J = 6.5, 2H), 6.64 (d, J = 6.6, 2H), 5.45 (br, 1H), 4.67 (br, 1H). **15**: δ 7.21–7.22 (m, 1H), 7.06–7.15 (m, 3H), 6.79–6.83 (m, 4H), 5.47 (br, 1H), 3.80 (s, 3H). **16**: δ 7.21 (s, 1H), 7.15 (d, J = 7.5, 1H), 6.80–7.02 (m, 6H), 6.06 (br, 1H), 5.50 (br, 1H). **17**: δ 9.49 (s, 1H), 8.46–8.55 (m, 3H), 7.81–7.92 (m, 3H), 7.60–7.69 (m, 2H), 7.47 (d, J = 7.5, 1H), 7.40 (d, J = 7.5, 1H), 7.40 (t, J = 8.1, 1H).
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