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Diazo Reagents with Small Steric Footprints for Simultaneous Arming/SAR Studies of Alcohol-Containing Natural Products *via* O−H Insertion

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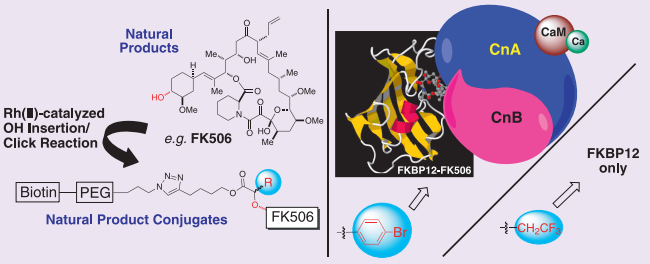
Supporting Information

ABSTRACT: Natural products are essential tools for basic cellular studies leading to the identification of medically relevant protein targets and the discovery of potential therapeutic leads. The development of methods that enable mild and selective derivatization of natural products continues to be of significant interest for mining their information-rich content. Herein, we describe novel diazo reagents for simultaneous arming and structure−activity relationship (SAR) studies of alcohol-containing natural products with a small steric footprint, namely, an α-trifluoroethyl (HTFB) substituted reagent. The Rh(II)-catalyzed O−H insertion reaction of several natural products, including the potent translation inhibitor lactimidomycin, was investigated, and useful reactivity and both chemo- and site (chemosite) selectivities were observed. Differential binding to the known protein targets of both FK506 and fumagillol was demonstrated, validating the advantage of the smaller steric footprint of α-trifluoroethyl derivatives. A *p*-azidophenyl diazo reagent is also described that will prove useful for photoaffinity labeling of low affinity small molecule protein receptors.



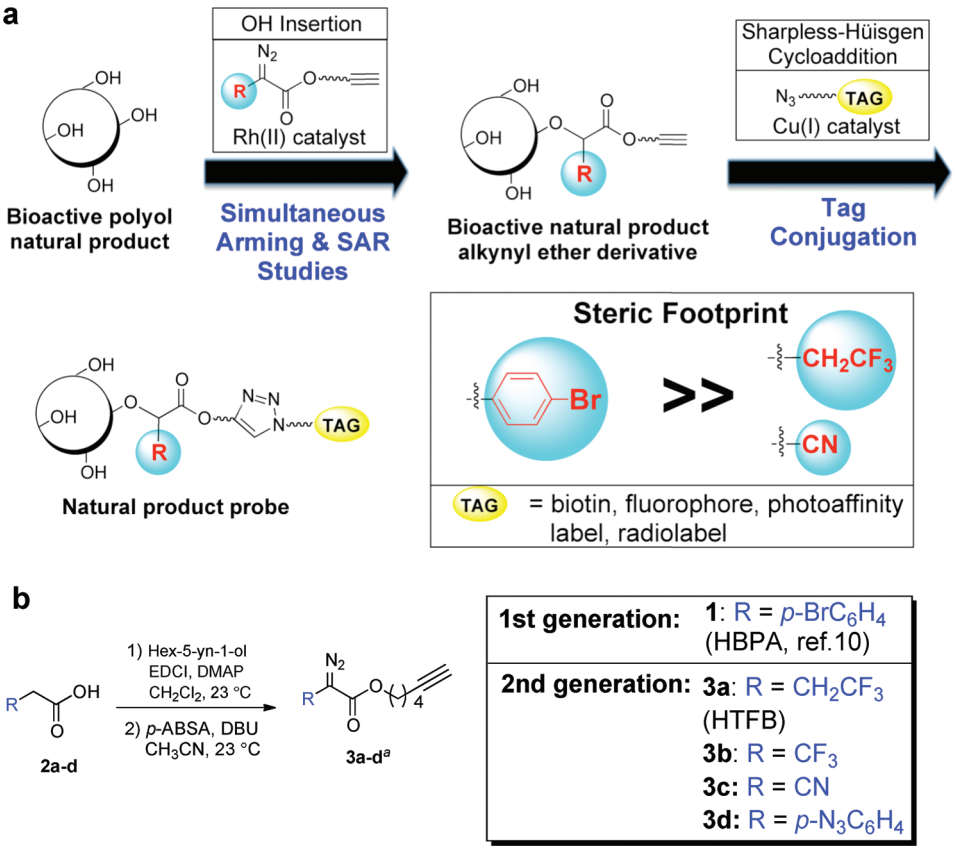
Over the past 50 years, the discovery and study of novel natural products from diverse terrestrial and marine organisms has had a profound impact on human health.[1,2] Indeed, natural products are an immense source of chemical diversity.[3] These complex molecules are capable of interacting with numerous human cellular proteins and typically have intrinsic cell permeability.[4] As a result, many natural products or natural product-inspired small molecules are currently in clinical use as antibiotics, antitumor agents, immunosuppressants, antiviral drugs, and enzyme inhibitors.[5] Furthermore, natural products have a rich history as tools for basic cellular studies leading to the discovery of potential cellular targets for intervention of human disease[6] and in this way contain enormous information content that should be exploited fully. Gaining an understanding of the detailed mode of action of biologically active natural products, including the identification of putative protein targets, continues to be a highly useful strategy for the discovery of human therapeutics.[7,8] These studies are greatly facilitated by detailed structure−activity relationship (SAR) studies and the synthesis of cellular probes derived from a particular natural product of interest. These cellular probes, which typically contain a functional group for *in vitro* or *in vivo* attachment of reporter tags such as biotin or a fluorophore, continue to be of great importance for such studies.[9]

We previously described mild and versatile strategies for simultaneous arming (with a reactive functional group or array) and SAR studies that facilitate mechanism of action studies of natural products.[10,11] One such method involves chemosite selective[12] and site nonselective O−H insertions with rhodium carbenoids derived from hexynyl-α-*p*-bromophenyl diazo acetate (**1**, HBPA) as a donor/acceptor carbenoid precursor.[10] The arming process leads to natural product derivatives that are equipped with tethered alkynes for subsequent conjugation to various tags *via* Sharpless−Hüisgen cycloaddition[13] to generate cellular probes. The utility of this strategy was demonstrated with a panel of natural products including FK506. An FK506-HBPA-biotin conjugate was employed to successfully pull-down the entire "immunosuppressive complex" consisting of calcineurins A/B, calmodulin, and FKBP12. However, the large steric size of the *p*-bromophenyl group and its close proximity to the point of attachment to the natural product was a potential liability for retaining bioactivity and subsequent affinity chromatography experiments. We thus sought to develop sterically smaller diazo reagents with similar reactivity to the *p*-bromophenyl substituted reagent (HBPA) that may improve retention of bioactivity. Herein, we describe the development of an α-trifluoroethyl (HTFB) substituted carbenoid precursor that leads to greatly reduced steric footprints yet provides similar reactivities, including chemosite selectivities, as the previously described *p*-bromophenyl reagent **1**. Differences in IL-2 reporter assay activity and affinity chromatography experiments with the derived FK506 conjugates highlight

Received: February 2, 2011
Accepted: August 26, 2011
Published: September 06, 2011

dx.doi.org/10.1021/cb2002686 | *ACS Chem. Biol.* 2011, 6, 1175–1181

Scheme 1. Strategy for Bioactive Polyol Natural Product Derivatization and Synthesis of Required Diazo Reagents with Varying Steric Footprints[a]



[a] (a) Simultaneous arming/SAR studies *via* O−H insertion of polyol natural products using diazo reagents with varied steric footprints and subsequent tag conjugation. (b) General synthesis of new diazo alkyne reagents **3a**−**d**. For full details of the synthesis of diazo reagents **3a**−**d**, see Supporting Information.

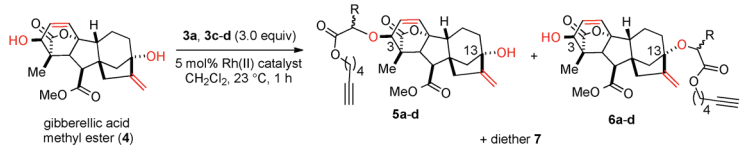
the profound changes in protein binding and capture. Importantly, the α-trifluoroethyl (HTFB) substituted conjugates may offer greater utility for identifying cellular receptors of bioactive natural products due to their smaller steric size as further demonstrated by comparison of HBPA- *versus* HTFB-fumagillol derivatives in the hMetAP2 inhibition assay and affinity chromatography experiments.

We sought diazo reagents with substantially reduced steric footprints (*i.e.*, smaller α-substituents) compared to the previously described *p*-bromophenyl substituted reagent **1** due to the proximity of these substituents to the native natural product. We therefore set out to study the α-trifluoroethyl (**3a**), α-trifluoromethyl (**3b**), and α-cyano (**3c**) diazo reagents that possess different α-substituents with varied steric sizes and electron-withdrawing potential, which would also alter the reactivity of the derived rhodium carbenoids (Scheme 1a). While the proposed diazo reagents would lead to acceptor/acceptor carbenoids,[14,15] the fluorinated hex-5-ynyl 2-diazo-4,4,4-trifluorobutanoate (**3a**, HTFB) and hex-5-ynyl 2-diazo-3,3,3-trifluoropropanoate (**3b**) have the added advantage of enabling ^{19}F NMR analysis of crude natural product derivatization reactions. In contrast, the hex-5-ynyl 2-cyano-2-diazoacetate (**3c**) was expected to provide a highly reactive metallocarbenoid species.[16] Computational studies supported the significant steric size difference based on calculated molecular volumes that could be expected between the derived natural product derivatives (cyano, 35.10 Å^3; 1,1,1-trifluoroethyl, 65.51 Å^3; *p*-bromophenyl, 117.02 Å^3). Molecular volumes were calculated using DFT-B3LYP/6-31++G(2d, 2p) level of theory (Spartan '08 v1.2.0). In addition, the hex-5-ynyl 2-(4-*p*-azidophenyl)-2-diazoacetate (**3d**)[17] was prepared for its potential as a trifunctional linker for photoaffinity labeling of low affinity binding proteins,[18] and the derived carbenoid was expected to exhibit reactivity similar to that of the *p*-bromophenyl reagent **1**. To enable studies of their reactivity, these new diazo reagents were successfully prepared on gram scale (~5 g) by esterification and subsequent base-promoted diazo transfer (Scheme 1b, see Supporting Information for complete details). We found that diazo reagents **3a**, **3c**, and **3d** are stable at RT and storable at −10 °C with no decomposition after 1 year. However, the α-trifluoromethyl reagent **3b** is highly unstable and decomposed at 23 °C.

The reactivity of diazo reagents **3a**, **3c**, and **3d** were initially studied with gibberellic acid methyl ester (**4**), which contains several potentially reactive sites (Figure 1a, highlighted in red) including an internal and a terminal disubstituted olefin, and two sterically differentiated alcohols (secondary and tertiary). This natural product derivative thus serves as an ideal substrate to study chemosite selectivity. Under the standard O−H insertion conditions with $Rh_2(OAc)_4$, diazo reagents **3a** and **3d** exhibited stability and reactivity similar to that of the *p*-bromophenyl

dx.doi.org/10.1021/cb2002686 |*ACS Chem. Biol.* 2011, 6, 1175–1181

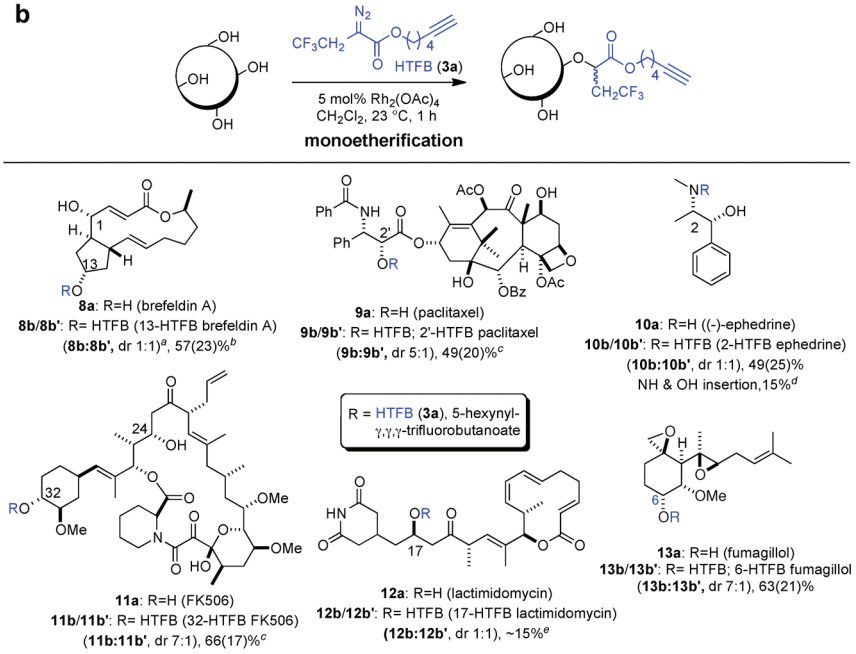
a



entry	diazo reagent	catalyst	monoether ratio 5:6 [b]	diether 7	% yield [d]	dr (5) [e]
1 [a]	**1**	$Rh_2(OAc)_4$	>95:5	<5 [c]	55 (38)	1:1
2	**3a**	$Rh_2(OAc)_4$	95:5	<5 [c]	45 (32)	2:1
3	**3d**	$Rh_2(OAc)_4$	70:30	0	57 (25)	1:1
4	**3a**	$Rh_2(esp)_2$	87:13	25	66 (7)	4:1
5	**3a**	$Rh_2(OCOCF_3)_4$	50:50	0	28 (44)	2:1
6	**3a**	$Rh_2(4S\text{-MEAZ})_4$	N/A	0	<5 [c] (74)	N/A
7	**3a**	$Rh_2(4R\text{-MEAZ})_4$	N/A	25	<5 [c] (68)	N/A
8	**3a**	$Rh_2(4S\text{-MEOX})_4$	50:50	0	41 (29)	1:1
9	**3a**	$Rh_2(S\text{-DOSP})_4$	85:15	0	75	3:1

[a]Data from ref. 10. [b]Ratios were determined by ^{1}H NMR (500 MHz). [c]Ethers **5-7** were detected by LC-MS but not ^{1}H NMR (500 MHz). [d]Yields refer to isolated yields and numbers in parentheses refer to recovered starting material. [e]Diastereomeric ratios of **5** at ether α-carbon were determined by ^{1}H NMR (500 MHz).

b



[a]Diastereomeric ratios (dr) were determined by ^{1}H-NMR (500 MHz) and refer to the mixtures of diastereomers at the ether α-carbon. [b]Values refer to isolated yields and number in parentheses refer to recovered starting material. [c]6.0 equiv of HTFB (**3a**) was added via syringe pump over 2 h. [d]Bifunctional derivatization involving both O–H and N–H insertion was detected by LC-MS. [e]Run on a 2 mg-scale reaction therefore yield is approximate (microbalance) following purification by preparative HPLC with some unreacted lactimidomycin recovered.

Figure 1. Rh(II)-catalyzed O−H insertion reactions using diazo reagents **3a**, **3c-d** with various natural products. (a) Comparison of reactivity and chemosite selectivity of novel diazo reagents **3a**, **3c-d** with various Rh(II) catalysts (potential reactive sites highlighted in red). (b) Scope of $Rh_2(OAc)_4$-catalyzed chemosite selective O−H insertion of HTFB (**3a**) with various natural products (reactive alcohols and amines highlighted in blue).

diazo **1** (Figure 1a, entry 1), providing monoethers **5a** and **5d** derived from O−H insertion with the more accessible secondary alcohol along with recovered ester **4** (Figure 1a, entries 2, 3). However, the more reactive α-cyano reagent **3c** gave a complex mixture likely resulting from both O−H insertion and cyclopropanation[19] with no recovery of starting material (not shown). Overall, the α-trifluoro diazo reagent **3a** provided the best results with regard to stability, reactivity, and selectivity of derived rhodium carbenoid compared to the *p*-bromophenyl diazo **1**. With respect to catalysts, $Rh_2(esp)_2$ described by DuBois[20] gave optimal conversion to the monoether **5a** (66%) and higher diastereoselectivity (4:1); however, this was accompanied by 25% of diether **6** (Figure 1a, entry 4). $Rh_2(OAc)_4$ was the most chemosite selective catalyst, favoring the less hindered and nucleophilic secondary alcohol at C3 (Figure 1a, entry 2).

dx.doi.org/10.1021/cb2002686 |*ACS Chem. Biol.* 2011, 6, 1175–1181

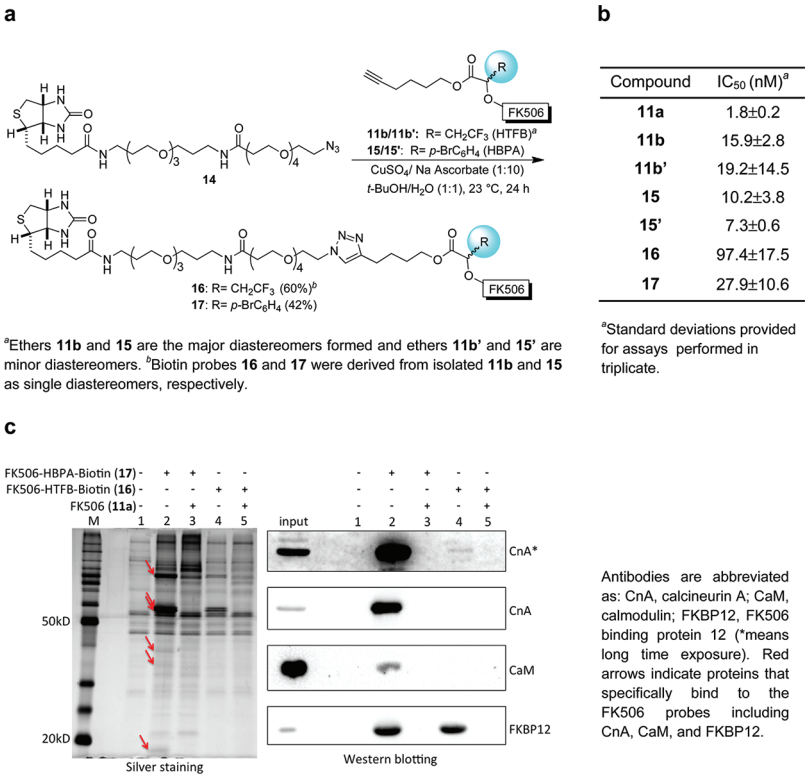


Figure 2. Comparative affinity experiments with HTFB and HBPA-FK506-biotin conjugates. (a) Synthesis of FK506-HTFB-biotin (**16**) and FK506-HBPA-biotin (**17**) conjugates. (b) IC_{50} values for IL-2 inhibition by FK506 derivatives of Jurkat T cells transiently transfected with an IL-2 promoter-driven luciferase reporter. (c) Affinity chromatography experiments with biotin conjugates **16** and **17** (left panel: silver staining of all retained proteins; right panel: Western blotting by indicated antibodies).

We next investigated various known chiral Rh(II) catalysts in conjunction with the α-trifuoroethyl diazo reagent **3a** as a means to alter chemosite selectivity of the O−H insertion. Several commercially available chiral rhodium catalysts including those of Davies[21,22] and Doyle[23] were investigated. The use of chiral catalysts enables a type of "double diastereoselectivity"[24,25] that could in principle alter chemosite selectivity. Of the four chiral catalysts studied (Figure 1a, entries 6−9), Rh_2(*S*-DOSP)$_4$ provided the highest yield of monoetherification (75%) of ester **4** with good diastereoselectivity (dr, 3:1) and a high degree of chemosite selectivity (Figure 1a, entry 9). However, $Rh_2(OCOCF_3)_4$ and $Rh_2(MEOX)_4$, which are known to lead to more reactive carbenoids compared to $Rh_2(OAc)_4$, gave lower chemosite selectivity as expected. Thus, these catalysts are ideal for obtaining the greatest number of derivatives for initial SAR studies of a novel polyol natural product.

The scope of this O−H insertion with the new α-trifluoroethyl diazo ester (**3a**) was assessed with several commercially available natural products and derivatives (Figure 1b). In general, O−H insertion with metallocarbenoids is governed by both steric and electronic effects.[26] Despite the smaller steric size of the α-trifluoroethyl group, $Rh_2(OAc)_4$ catalyzed O−H insertions with diazo reagent **3a** with the natural products studied showed chemosite selectivity similar to that of the *p*-bromophenyl reagent **1**,[10] suggesting that electronic effects play the predominant role for chemosite selectivity. Brefeldin A (**8a**) presented two potentially reactive alcohols; however, the more accessible secondary alcohol (C13) was selectively alkylated over the more electron-rich allylic alcohol (C1). Paclitaxel (**9a**) has two electronically and sterically distinct secondary alcohols and a tertiary alcohol. The more accessible secondary alcohol (C2′) was selectively derivatized with no reaction detected at the other alcohols or the amide N−H. To determine the reactivity of amines in comparison to alcohols, ephedrine (**10a**), which bears a secondary alkyl amine and a secondary alcohol was studied. As expected, the major adduct isolated was that derived from N−H insertion due to greater nucleophilicity; however, some bis-derivatization derived from both N−H and O−H insertion was also detected. In the case of FK506 (**11a**), O−H insertion led to chemosite selectivity at the more accessible cyclohexyl secondary alcohol (C32) in 66% yield and with good diastereoselectivity (dr, 7:1). No O−H insertion at the macrocyclic secondary alcohol (C24) or dietherification was observed. Previously, dietherification of FK506 was observed when HBPA (**1**) was added in one portion;[10] therefore, slow addition of 6 equiv of HTFB (**3a**) over 2 h again prevented dietherification. Overall, these results are consistent with chemoselectivities previously observed for O−H insertion with Rh(II)-carbenoids derived from HBPA, and while not all O−H environments were explored, the same reactivity patterns (primary ROH ≈ secondary alkyl NH > secondary alkyl OH > secondary allylic OH ≥ aryl NH > phenolic OH > tertiary alkyl OH > indole NH and no reaction with amide NH or alkenes) would be expected on the basis of observations to date.[10]

Lactimidomycin (**12a**) is a macrocyclic natural product with a pendant cycloheximide that has gained much interest due to its potent inhibitory effect on eukaryotic translation elongation properties leading to dramatic antitumor activities.[27,28] However, lactimidomycin is highly acid- and base-sensitive, which limits

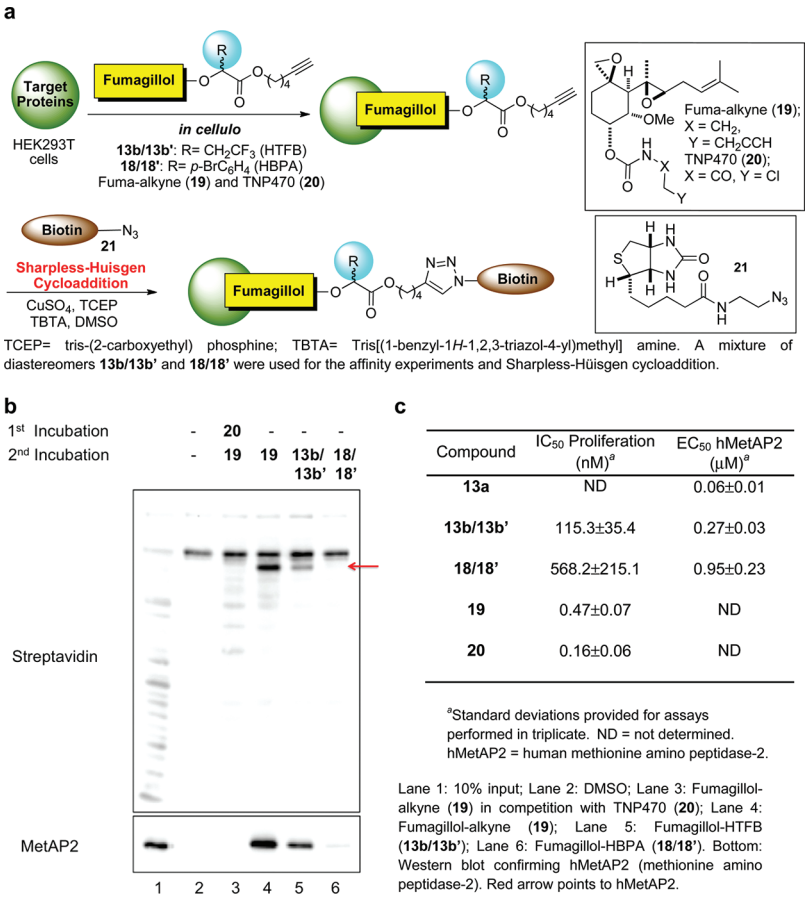


Figure 3. Comparative affinity experiments with HTFB and HBPA-fumagillol derivatives *in cellulo*. (a) Synthesis of fumagillol derivatives. (b) Affinity chromatography experiments were performed using azide **21** to afford biotinylated fumagillol dervatives, and the target of fumagillol was analyzed by a blotting membrane using streptavidin horseradish peroxidase or an antibody against hMetAP2. (c) IC_{50} values of fumagillol derivatives against HUVEC cells and EC_{50} values in the hMetAP2 assay.

derivatization strategies. Thus, this became an excellent substrate for testing the mildness of this O−H insertion process. Use of HTFB (**3a**) provided ∼15% yield of isolated, purified (preparative HPLC) ether **12b** on a reaction scale of 2 mg at 50% conversion with some loss of material attributed to degradation during purification. The successful derivatization of such a sensitive natural product demonstrates the utility of the described derivatization strategy as it proceeds under essentially neutral conditions, works efficiently on small scale (∼1−2 mg), and allows for the recovery of unreacted starting material.

We were next interested in studying the effect of having different α-substituents (*i.e.*, α-trifluoroethyl *vs* *p*-bromophenyl) on ethers obtained from O−H insertion of natural products in a cellular assay and also affinity chromatography experiments with derived biotin conjugates. For these studies, the diastereomeric FK506-derived ethers **11b**/**11b′** and **15**/**15′** were separated by preparative TLC. An FK506-HTFB-biotin probe (**16**) was prepared by Sharpless−Hüisgen cycloaddition of the major diastereomeric HTFB ether **11b** and biotin azide **14**. The FK506-HBPA-biotin conjugate **17** was also prepared in a similar fashion (Figure 2a). In our previous study, the use of an FK506-HBPA-biotin probe led to pull-down of the entire immunosuppressive complex including FKBP12, calcineurin A/B, and calmodulin.[10] In the IL-2 reporter assay, the FK506-HTFB derivatives **11b** and **11b′** showed diminished inhibition of IL-2 production (∼2 fold, IC_{50} 15.9 ± 2.8 and 19.2 ± 14.5 nM respectively) compared to the FK506-HBPA derivatives **15** and **15′** (IC_{50} 10.2 ± 3.8 and 7.3 ± 0.6 nM, respectively) (Figure 2b). A similar trend was observed when the biotin conjugates were assayed in the IL-2 reporter assay with the FK506-HTFB-biotin probe **16** showing a >4-fold decrease in IC_{50} value compared to the corresponding FK506-HBPA-biotin conjugate **17**. In the most dramatic demonstration of the impact of a smaller steric footprint, side by side comparison of the two FK506-biotin probes **16** and **17** showed that the smaller α-trifluoroethyl conjugate led only to pull-down of FKBP12, whereas the *p*-bromophenyl conjugate led once again to pull-down of the entire ternary complex containing both FKBP12 and calcineurin (Figure 2c). These results demonstrate the profound effect of a smaller α-trifluorethyl *versus* a larger *p*-bromophenyl substituent in pull-down experiments and is consistent with C32-aryl substituted FK506 derivatives, which were previously reported to increase binding to calcineurin.[29] This previous study also reported increased IL-2 inhibition by C32-aryl *versus* C32-alkyl substituted FK506 derivatives proposed to be due to favorable π-π interactions with calcineurin A.

Fumagillol (**13a**, Figure 3b) is a natural product known to inhibit angiogenesis through irreversible inhibition of human type 2 methionine aminopeptidase (hMetAP2).[30] Previous SAR studies have shown that the structure of the C6-substituent can

dramatically affect bioactivity.[31] We thus set out to evaluate the difference between α-trifluoroethyl and *p*-bromophenyl substituted fumagillol derivatives obtained from O−H insertion of the C6-alcohol of fumagillol. Docking experiments with HTFB- and HBPA-fumagillol using the X-ray structure of hMetAP2 suggested that the *p*-bromophenyl derivative would suffer from greater unfavorable interactions upon binding to hMetAP2 compared to the α-trifluoroethyl derivative (see Supporting Information for details). The O−H insertions proceeded in good yield to provide HTFB-fumagillol **13b**/**13b**′ (63%) and HBPA-fumagillol **18**/**18**′ (58%) without competing cyclopropanation or epoxide degradation. The mixture of diastereomeric fumagillol ethers **13b**/**13b**′ and **18**/**18**′ were not readily separated; therefore, they were used as a mixture of diastereomers for *in cellulo* protein profiling (Figure 3a). Side by side assays with fuma-alkyne (**19**) and TNP470 (**20**) were performed for comparison. As seen in Figure 3b, affinity experiments with fumagillol-HTFB (**13b**/**13b**′) led to pull-down of hMetAP2 greater than that of fumagillol-HBPA (**18**/**18**′) (Figure 3b). Consistent with these results, fumagillol-HTFB **13b**/**13b**′ also showed greater inhibitory activity in a HUVEC proliferation assay compared to the fumagillol-HBPA derivatives **18**/**18**′ (∼5-fold decrease, IC_{50} 115.3 ± 35.4 and 568.2 ± 215.1 nM, respectively). A similar trend was observed when **13b**/**13b**′ and **18**/**18**′ were assayed in the hMetAP2 enzymatic assay (Figure 3c). The fumagillol-HTFB **13b**/**13b**′ showed ∼4-fold increase in EC_{50} value compared to fumagillol-HBPA **18**/**18**′ (EC_{50} 0.27 ± 0.03 and 0.95 ± 0.23 nM, respectively). Taken together, the comparative results of both fumagillol and FK506-HBPA and HTFB alcohol derivatives highlight the significance of the smaller steric footprint and demonstrate the utility of the novel α-trifluoroethyl diazo reagent **3a** for natural product derivatization.

In conclusion, we developed two new diazo reagents, a α-trifluoroethyl diazo reagent **3a** (HTFB) and a *p*-azidophenyl diazo reagent **3d**, for simultaneous arming and SAR studies of bioactive natural products *via* O−H insertion. HTFB (**3a**) possesses a reduced steric footprint compared to that of the *p*-bromophenyl reagent (**1**) and enables the use of ^{19}F NMR to facilitate small-scale, crude derivatization reaction analysis. Furthermore, this reagent showed comparable reactivity and good chemosite selectivity compared to HBPA (**1**); secondary (and likely primary) amines, if present, exhibit greater reactivity over alcohols diminishing to an extent the degree of chemoselectivity. The difference in steric footprint and binding affinity of an FK506-HTFB derivative for FKBP12 was demonstrated by measurement of IC_{50} values in the IL-2 reporter assay; in addition, affinity chromatography experiments in side by side comparisons of FK506-HTFB-biotin (**16**) and FK506-HBPA-biotin (**17**) led to dramatic differences in proteins captured. Furthermore, HTFB- and HBPA-fumagillol derivatives prepared by these methods also demonstrated the advantage of the α-trifluoroethyl substituent in terms of smaller steric footprint leading to increased binding to hMetAP2. The *p*-azidophenyl diazo reagent **3d** should prove useful for photoaffinity experiments with low affinity natural product receptors. Further applications of these reagents to natural product derivatization and their subsequent use for receptor isolation are under active investigation.

METHODS

General Procedure for Rh-Catalyzed O−H Insertion for the Synthesis of Natural Product/HTFB Ethers. The natural product (1.0 equiv) and rhodium catalyst (0.05 equiv) were placed into a flame-dried, round-bottomed flask under a nitrogen atmosphere at 23 °C . Dry dichloromethane was added to make the final concentration of natural product 0.01 mM, providing a slurry. A solution of HTFB (**3a**, 3.0 equiv) in dry dichloromethane (0.05 mM) was slowly added *via* syringe pump over a 1 h period. Following complete addition, the reaction mixture was stirred at 23 °C for an additional 1 h. The solvent was evaporated under reduced pressure (rotary evaporator), and the residue was purified by preparative thin layer chromatography to afford the desired HTFB-ethers.

ASSOCIATED CONTENT

Supporting Information. Experimentals and full characterization of all new compounds including ^{1}H and ^{13}C NMR spectra. Experimental details for IL-2 reporter, hMetAP2, and proliferation assays in addition to affinity chromatography and modeling/docking experiments. This material is available free of charge *via* the Internet at http://pubs.acs.org.

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ACKNOWLEDGMENT

We thank the NIH (GM 086307 to D.R. and CA 78743 to J.O.L.) for generous support of this work. We thank the Royal Thai Government for a Graduate Fellowship to S.C. We also thank Ms. Mingchien Li for performing preparative HPLC and Mr. Mikail Abbasov for performing the molecular volume calculations.

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