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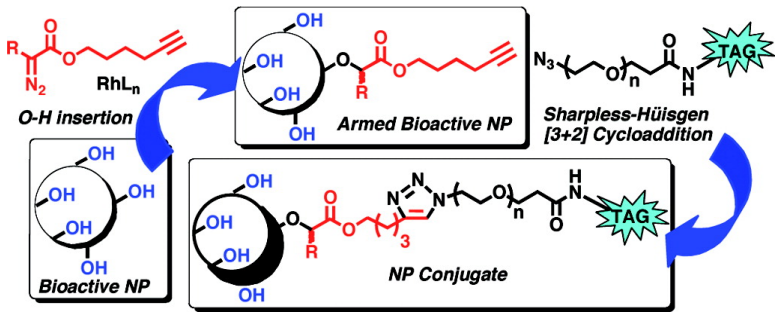

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Simultaneous Arming and Structure/Activity Studies of Natural Products Employing O−H Insertions: An Expedient and Versatile Strategy for Natural Products-Based Chemical Genetics

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Abstract: The identification of "druggable" targets is an immediate opportunity and challenge in the post-genomic era. Natural products are enduring tools for basic cellular studies and leads for identifying medically relevant protein targets. However, their use for these studies is often hampered by limited quantities and a lack of selective and mild monofunctionalization reactions. The development of selective methods that could simultaneously equip the natural product with a reactive group for subsequent conjugation to reporter tags and provide important structure−activity relationship (SAR) information, requiring only a knowledge of functional groups present in the natural product, could significantly decrease the time between bioactive natural product isolation and target identification. Herein, we report such a strategy that enables simultaneous arming and SAR studies of alcohol-containing natural products involving both chemo- and site-selective ("chemosite" selective) and site-nonselective O−H insertion reactions with rhodium carbenoids derived from alkynyl diazo acetates. This strategy was applied to a diverse set of natural products, and general guidelines for predicting chemosite selectivity were formulated. A subsequent Sharpless−Hüisgen [3 + 2] cycloaddition reaction with the appended alkyne allows for attachment of a variety of reporter tags. Using this strategy, we synthesized a novel FK506−biotin conjugate that enabled pull-down of the entire "immunosuppressive complex" including FKBP12, calcineurins A and B, and calmodulin. In addition, the potential for a chemoselective but site-nonselective process was shown with both gibberellic acid methyl ester and brefeldin A using only achiral rhodium catalysts.

Introduction

Understanding the function of encoded proteins and identifying "druggable" targets among the large number of disease-related genes in the human genome is a formidable task.[1] Current pharmaceuticals are thought to access only 483 out of an estimated 3,000−10,000 potential therapeutic targets.[2] Natural products are designed with high chemical diversity, cellular target specificity, and cell permeability and thus, not surprisingly, form the basis of more than half the drugs currently in clinical use.[3,4] This realization has recently spurred a renaissance of natural products as potential drug candidates.[5] In addition, there is enormous continued potential of natural products as probes for chemical genetics research, a field that seeks to identify small molecule activators and inhibitors for the majority of proteins encoded in the human genome.[6] One approach toward exploiting the inherent, information-rich content of natural products requires the synthesis of natural product conjugates wherein a reporter tag, such as a fluorophore or biotin, is covalently attached via a flexible linker of appropriate length at a position that does not significantly abrogate binding with a putative cellular receptor(s).[7] While multistep synthesis of an appropriate natural product derivative can provide novel and unique sites for tag attachment without loss of bioactivity, this approach can be both challenging and time-consuming.[7,8] Alternatively, random techniques such as photo-cross-linking of natural products to an affinity matrix are rapid; however,

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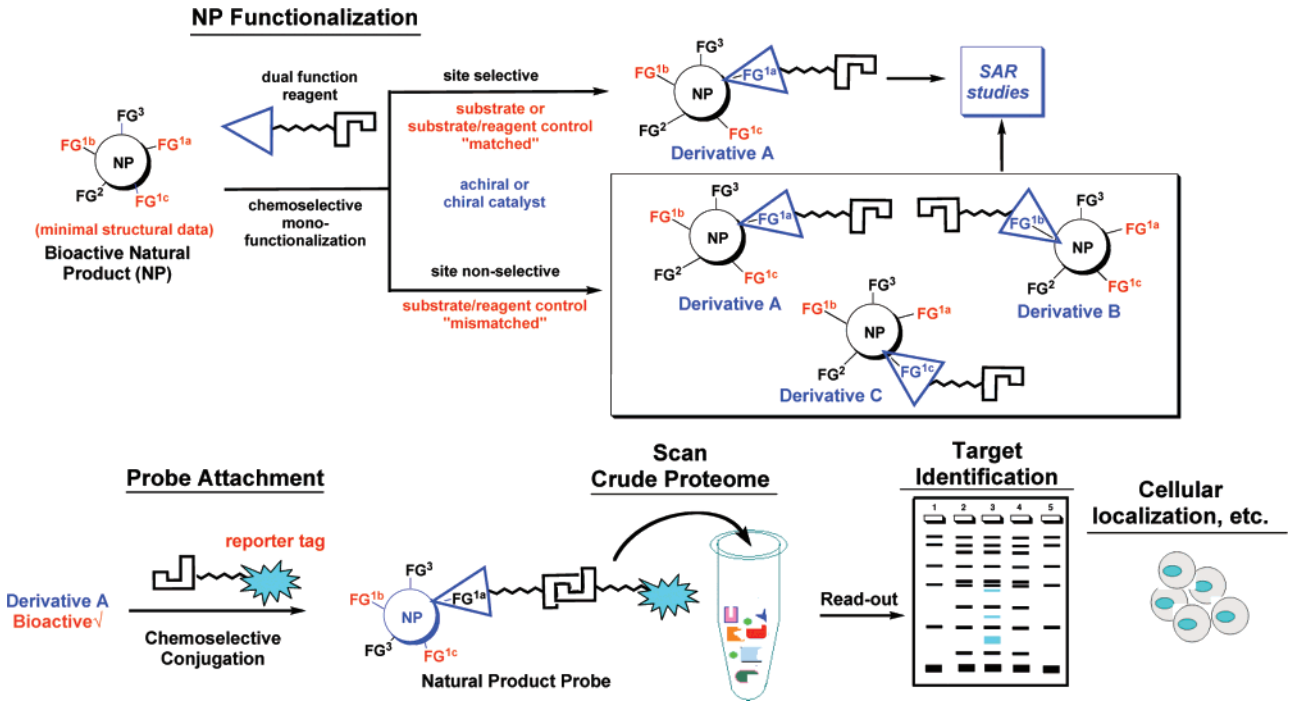


Figure 1. Generalized two-step derivatization/conjugation strategy for simultaneous arming and structure−activity studies of natural products for use in chemical genetics employing substrate or substrate/reagent control, a type of "double diastereodifferentiation". FG = Functional group.

they cannot relay any information on the site-of attachment, on degree of retained bioactivity after immobilization, nor any design problems in case of failure to identify the target.[9] Furthermore, the potential for degradation of the natural product on UV irradiation must be considered.

Natural products are often challenging to functionalize in a chemo- and site- ("chemosite")[10] selective manner because of their structural complexity, dense functionality, and often limited quantities. However, enzymatic methods have recently been applied successfully for this purpose achieving high site selectivity, in some cases, with a limited set of enzyme-catalyzed reactions.[11] Our overall strategy toward chemosite-selective and site-nonselective functionalizations of natural products enables simultaneous structure−activity studies and arming of a natural product for subsequent bioactive probe synthesis. Furthermore, the strategy relies on metal catalysis which will facilitate variation of chemosite selectivity based on catalyst/natural product interactions, that is, a type of "double diastereodifferentiation"[11a] and makes use of dual-function reagents with two orthogonally reactive groups (i.e., an α-diazo ester and an alkyne) for initial arming and then subsequent conjugation (Figure 1). Requirements for reactions to be utilized in this strategy are those readily performed on small scale and providing monofunctionalization, high chemosite selectivity, and the potential for some degree of site-nonselectivity. The latter requirement becomes particularly important when initial derivatives generated on the basis of the inherent natural product reactivity (e.g., "substrate control" leading to derivative A) lose significant bioactivity. In this case, other derivatives must be obtained by a site-nonselective process leading to derivatives B and C (Figure 1). Therefore, using reactions that are amenable to chiral catalysis would enable a type of "mismatched double diastereodifferentiation" that could alter chemosite selectivity.[11a] Derivatives B and C could then be assayed for bioactivity to obtain structure−activity relationship (SAR) data and determine an appropriate site for probe attachment. A two-step rather than a single-step process for derivatization was chosen to enable the same functionalized natural product (e.g., derivative A) that retains biological activity to be utilized for the synthesis of a variety of probes (e.g., biotin, fluorophore, radiolabel, etc.) and subsequent scanning of select proteomes for binding proteins via affinity experiments, activity-based profiling, cellular localization studies, and etc. (Figure 1). Importantly, this approach theoretically requires minimal structural information for the natural product (i.e., only functional groups present) and thus may enable natural product protein receptor isolation to precede or proceed in parallel with complete structure elucidation, a common bottleneck in natural products-based chemical genetics.

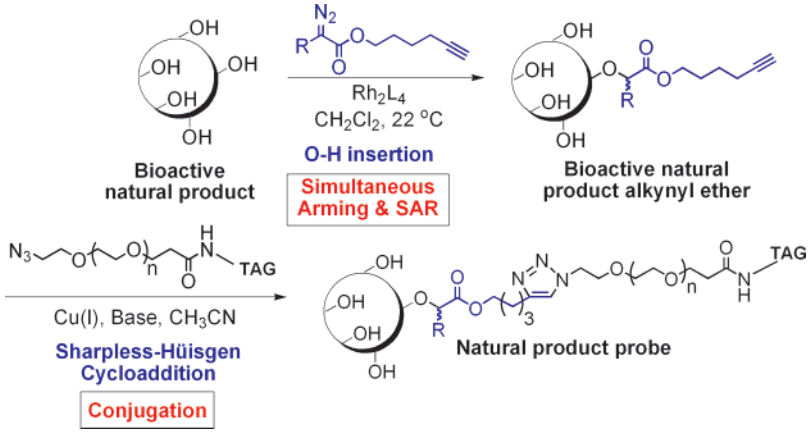
Herein, we describe our first approach toward implementing this general strategy involving Rh-catalyzed O−H insertions

(9) Kanoh, N.; Honda, K.; Simizu, S.; Muroi, M.; Osada, H. *Angew. Chem. Int. Ed.* **2005**, *44*, 3559−3562.

(10) The term chemoselectivity is used to describe reactions that discriminate between two functional group types in a given molecule. Site selectivity has been utilized to describe selective reaction at one position in a natural product (see ref 11) or one amino acid of a protein over another but it does not inherently imply chemoselective. Therefore, we propose use of the term "chemosite selective" to describe a reaction that exhibits both chemo and site selectivity.

(11) For a recent example of minimal peptide-mediated acylation of erythromycin aglycone, see: (a) Lewis, C. A.; Miller, S. J. *Angew. Chem. Int. Ed.* **2006**, *45*, 5616−5619. This article, which appeared when our work was in progress, adequately describes the concept of "double diastereodifferentiation" employing chiral peptide catalysts and natural products. For examples of glycorandomization of natural products, see: (b) Thibodeaux, C. J.; Melancon, C. E.; Liu, H.-W. *Nature* **2007**, *446*, 1008−1016. (c) Minami, A.; Eguchi, T. *J. Am. Chem. Soc.* **2007**, *129*, 5102−5107. (d) Zhang, C.; Griffith, B. R.; Fu, Q.; Albermann, C.; Fu, X.; Lee, I.-K.; Li, L.; Thorson, J. S. *Science* **2006**, *313*, 1291−1294 and references therein. (e) Yang, M.; Proctor, M. R.; Bolam, D. N.; Errey, J. C.; Field, R. A.; Gilbert, H. J.; Davis, B. G. *J. Am. Chem. Soc.* **2005**, *127*, 9336−9337. (f) Minami, A.; Uchida, R.; Eguchi, T.; Kakinuma, K. *J. Am. Chem. Soc.* **2005**, *127*, 6148−6149. For examples of prenylation of natural products, see: (g) Kuzuyama, T.; Noel, J. P.; Richard, S. B. *Nature* **2005**, *435*, 983−987. (h) Cui, G.; Li, X.; Merz, K. M., Jr. *Biochemistry* **2007**, *46*, 1303−1311.

Scheme 1. Two-Step O−H Insertion/Sharpless−Hüisgen Cycloaddition Sequence for Simultaneous Arming and SAR Studies of Natural Products



with alcohol-containing natural products. The strategy leads to natural products armed with a tethered alkyne for subsequent copper-catalyzed, Sharpless−Hüisgen [3 + 2] cycloaddition[12,13] which allows for attachment of various probes useful for chemical genetics experiments (Scheme 1). The utility of this methodology was demonstrated in "proof of principle" studies on a diverse set of natural products including FK506 leading to a novel biotin−FK506 conjugate that enabled pull-down of the entire FKBP−calcineurin−calmodulin A and B complex. Furthermore, the potential for site-nonselectivity was unexpectedly found with the use of various achiral rhodium catalysts with gibberellic acid methyl ester and brefeldin A (BFA).

Results and Discussion

Alcohols are the most prevalent functional group found in natural products;[14] however, not all alcohols in a natural product are crucial for biological activity and thus can serve as sites for derivatization. Standard acylation is one of the most commonly employed methods for direct derivatization, but the precise conditions are typically selected in an ad hoc manner often guided by only limited knowledge of the reactivity and stability of a natural product.[7,15] However, these reactions are conducted typically under predominantly acidic or basic conditions that may be too harsh for many natural products.[16] Thus, as described earlier, we became interested in developing an alcohol derivatization method that was chemosite selective, mild (i.e., neutral reaction conditions), and amenable to variation in chemosite selectivity using reagent control, thus enabling application to natural products with minimal structural information and being complementary if not superior to standard acylation chemistry. A further requirement was reactions that would allow for not only derivatization of alcohols but also simultaneously arming with a unique functional group handle for subsequent chemoselective conjugation to appropriate proteomic tags. For these reasons, we elected to study metal-catalyzed decomposition of diazo esters[17] and insertion of the resulting metallocarbenes into alcohol O−H bonds since diazo esters are stable, easy to prepare, and react under mild, neutral conditions that would be compatible with potentially sensitive natural products. Since the pioneering work of Teyssié et al. in 1973,[18] the selectivity of metal-catalyzed insertion reactions of α-diazo esters with alcohols has received only scant attention.[19,20] In these early studies, alcohol substrates were typically used in excess or as solvents to compete with dimerization of the diazo reagents, a situation not suitable for sample-limited natural products. Moreover, chemosite selectivity issues in O−H insertion processes are largely unexplored especially in the context of polyfunctional compounds, and thus these studies would provide fundamental knowledge regarding such issues. Furthermore, a number of chiral catalysts[21,22] have been employed for C−H insertions, and recently a chiral catalyst was described for the first enantioselective O−H insertion,[23] opening possibilities for control of chemosite selectivity during the O−H insertion process.

Monoetherification and Chemosite Selectivity: Model Studies of the O−H Insertion Process with Gibberellic Acid Methyl Ester. Using $Rh_2(OAc)_4$ as catalyst, we initially prepared diazo esters bearing a distal alkynyl group for subsequent Sharpless−Hüisgen cycloaddition and various α-substituents[24] and screened them for selectivity toward monoetherification of gibberellic acid methyl ester (**1**). This ester

(12) (a) Rostovtsev, V. V.; Green, L. G.; Fokin, V. V.; Sharpless, K. B. *Angew. Chem. Int. Ed.* **2002**, *41*, 2596−2599. For mechanistic studies of this cycloaddition, see: (b) Bock, V. D.; Hiemstra, H.; van Maarseveen, J. H. *Eur. J. Org. Chem.* **2005**, *1*, 51−68. (c) Torne, C. W.; Christensen, C.; Meldal, M. *J. Org. Chem.* **2002**, *67*, 3057−3064.
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(20) Cox, G. G.; Kulagowski, J. J.; Moody, C. J.; Sie, E.-R. H. B. *Synlett* **1992**, *51*, 975−976.
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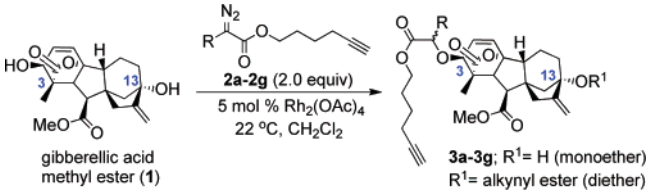
(23) Maier, T. C.; Fu, G. C. *J. Am. Chem. Soc.* **2006**, *128*, 4594−4595.
(24) For details regarding the preparation of alkynyl diazo esters utilized in this study, see Supporting Information.

Table 1. Screening of Alkynyl Diazo Esters for Monoetherification with Gibberellic Acid Methyl Ester (**1**)[a]



entry	R	mono/diether	% yield[b]
1	H (**a**)	n.d.	10
2	PhCH=CH (**b**)	<5:95	90[c]
3	Ph (**c**)	50:50	80[c]
4	benzyl (**d**)	>95:5	12 (80)
5	4-nitrophenyl (**e**)	>95:5	17 (76)
6	$(CH_3)_2C$=CH (**f**)	>90:10	32 (55)
7	4-bromophenyl (**g**)	>95:5	55 (38)

[a] Reaction of various alkynyl diazo esters **2a**−**2g** with gibberellic acid methyl ester (**1**). [b] Values refer to isolated yields and are ∼1:1 mixtures of diastereomers at the ester α-carbon. [c] Both mono- and diether are formed, and yields refer to the mixture (ratios were determined by 500 MHz ^{1}H NMR). Numbers in parentheses refer to recovered starting material.

possesses two distinct alcohols from both steric and electronic perspectives, namely a secondary allylic C_3−OH and a tertiary C_{13}−OH, making it a useful initial model system (Table 1).

The stability of diazo esters toward decomposition by rhodium catalysts is critically dependent on the α-substituent,[19] and the use of donor and acceptor α-substituted carbenoids pioneered by Davies and Nikolai has led to excellent control of reactivity and thus selectivity for C−H insertions.[25] As expected, unsubstituted diazo ester **2a**, which is prone to dimerization, resulted in poor conversion (Table 1, entry 1). However, use of resonance-stabilized diazo esters such as styryl and phenyl diazo esters, **2b** and **2c**, respectively, resulted in high conversion but poor or no selectivity for monoetherification presumably due to increased rates of metallocarbenoid formation and subsequent indiscriminate O−H insertion (Table 1, entries 2 and 3).[26] Completely removing resonance stabilization or decreasing resonance stabilization by addition of electron-withdrawing groups slows the formation of metal carbenoids,[25] and thus, use of benzyl and 4-nitrophenyl diazo esters, **2d** and **2e**, respectively, resulted in exclusive monoetherification of the secondary alcohol (Table 1, entries 4 and 5). However, these diazo esters gave low conversion, and therefore efforts to identify a compromise between reactivity and stability led to the use of a α-dimethylvinyl diazo ester **2f**, which provided good selectivity for monoetherification and good conversion (Table 1, entry 6). Further improvements were obtained with the hexynyl-α-4-bromophenyl (HBP) diazo acetate **2g**, which gave good conversion (55%) while preserving high chemosite selectivity (Table 1, entry 7). Moreover, excellent recovery of unreacted starting material was also possible in this case (Table 1, entries 4−7), an important consideration and preferable to bis-etherification with sample-limited natural products. This result was especially gratifying since the 4-bromophenyl substituent provides opportunities for synthesis of trifunctional probes through subsequent metal-catalyzed processes with the bromoarene including attachment of protein-reactive labels (e.g., photoaffinity tags)[27] in close proximity to the natural product binding site which may aid with isolation of low-affinity protein receptors. In addition, the presence of an ester linkage in the conjugate, which was shown not to be a liability during affinity experiments (vide infra), provides opportunities for subsequent cleavage of the reporter tag which facilitates mass spectral analysis of tagged proteins during activity-based profiling experiments.[28,29] It should be noted that an ∼1:1 mixture of diastereomers is obtained in these OH insertions and each diastereomer might be expected to display differential bioactivity. However, in general the introduction of this new stereocenter is expected to be overshadowed by the attachment of a relatively large group (i.e., bromophenyl ester) to this position, and thus such differences would not be expected to significantly affect initial qualitative SAR studies to determine a suitable site for derivatization.

Scope of the $Rh_2(OAc)_4$-Catalyzed Chemosite-Selective O−H Insertion with a Diverse Set of Natural Products and Derivatives. The scope of this chemosite-selective process was assessed on several commercially available natural products and derivatives employing diazo ester **2g** (Table 2) and compared and contrasted to published methods for derivatization of alcohols in natural products. Alkylation by insertion of carbenoids into O−H bonds is known to be dependent on both steric and electronic effects (i.e., nucleophilicity) of the alcohol.[30] This was reflected both in the preferential insertion into the primary alcohol (C_{21}) of dexamethasone (**4**) over the 2° and 3° alcohols and in the higher yield obtained with respect to reaction with gibberellic acid methyl ester (Table 2, **5** vs Table 1, **3g**). In an example that clearly demonstrates the complementarity of the present method to standard acylation, the secondary alcohol (C_{17}) of *β*-estradiol (**6**) reacted preferentially under typical OH insertion conditions over the less nucleophilic phenolic hydroxyl to give the 5-hexynyl-(α-4-bromophenyl) acetate (HPBA) ether **7** exclusively (Table 2). In contrast, previous acylations of *β*-estradiol using acid chlorides and anhydrides under basic conditions led to predominant or exclusive acylation at the phenolic hydroxyl, attributed to the formation of the more nucleophilic phenolate ion under basic conditions.[31] In addition, this reaction operates under essentially neutral conditions, an important consideration for commonly labile natural products, and in this case preferentially reacts with the most nucleophilic alcohol. Brefeldin A (**8**) presents two potentially reactive secondary alcohols; however, the more sterically accessible cyclopentyl alcohol (C_{13}) was selectively

(25) For an excellent review on donor- and acceptor-substituted carbenoids, see: Davies, H. M. L.; Nikolai, J. *Org. Biomol. Chem.* **2005**, *3*, 4176−4187.

(26) Lu, C.-D.; Liu, H.; Chen, Z.-Y.; Hu, W.-H.; Mi, A.-Q. *Org. Lett.* **2005**, *7*, 83−86.

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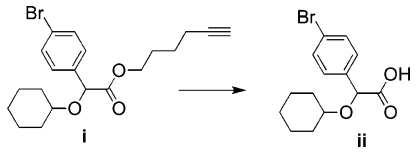
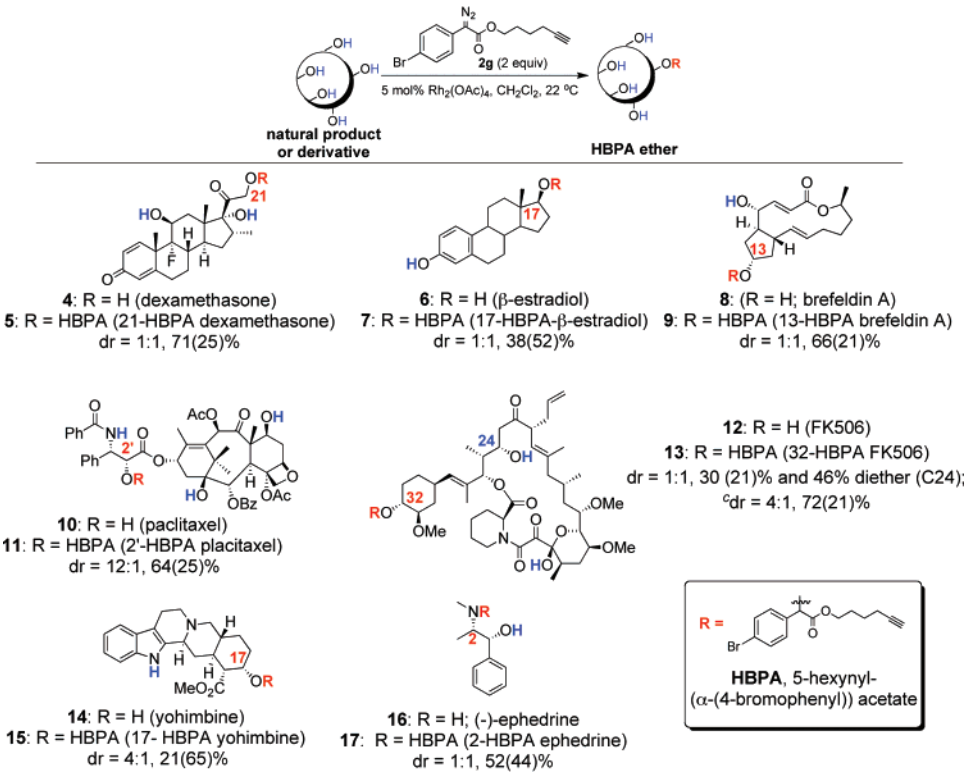
(29) Hydrolysis experiments were performed with a simple model ester **i** to determine the stability of the bromobenzyl acetate esters. Complete hydrolysis of ester **i** required a pH = 13 at 22 °C in 48 h and delivered the bromobenzylacetic acid **ii**. See Supporting Information for details.



(30) Cox, G. G.; Haigh, D.; Hindley, R. M.; Miller, D. J.; Moody, C. J. *Tetrahedron Lett.* **1994**, *35*, 3139−3142.

(31) (a) Srivastava, V.; Tandon, A.; Ray, S. *Synth. Commun.* **1992**, *22*, 2703−2710. (b) Yamada, S. *Tetrahedron Lett.* **1992**, *33*, 2171−2174.

Table 2. Scope of $Rh_2(OAc)_4$-Catalyzed Chemosite-Selective O−H Insertion of HBP−Diazo Ester **2g** with Various Natural Products and Natural Product Derivatives[a,b]



[a] Alcohols and amines highlighted in blue were unreactive under the conditions described with the exception of FK506 at C24. [b] Numbers in parentheses refer to recovered starting material. [c] HBP−diazo ester **2g** added in three portions.

alkylated over the electron-rich allylic alcohol (C_1), a reactivity pattern similar to that seen in acylation (Table 2, HBPA-**9**). However, this example shows the mildness of the current method as previous acylations of BFA required harsh conditions (110 °C, 2,6-lutidine, 12−36 h), leading to modest yields of the C_{13} and C_1 monoesters accompanied by bis-esterification.[32] Paclitaxel (**10**) has two electronically and sterically distinct secondary alcohols and a tertiary alcohol. The more accessible side chain, secondary alcohol (C_2'), which can be selectively acylated,[33] not unexpectedly underwent selective etherification in preference to the two other alcohols and the amide NH to provide 2′-HBPA paclitaxel (**11**), which was verified by X-ray analysis.[34] Regioselective acylation at the C_{32} hydroxyl and subsequent conjugation has proven to be difficult in the case of FK506 and rapamycin because of competitive reaction at the C_{24} hydroxyl and the sensitive functionalities present. These difficulties led to the development of specialized acylations including carbamoylation[35] and enzymatic acylations[36] for these natural products. Application of the typical OH insertion conditions to FK506 (**12**) led to chemosite-selective reaction at the cyclohexyl secondary alcohol (C32) over the secondary alcohol on the macrocyclic periphery (C24). However, diether formation (42% isolated yield) was also observed under the usual single portion addition of diazo ester **2g**. This undesired outcome was readily avoided by portionwise addition of the diazo ester to provide 32-HBPA FK506 (**13**), providing a modification to the procedure, which could prove useful in other settings. In the case of yohimbine (**14**), the sterically hindered cyclohexyl secondary alcohol (C_{17}) was preferentially alkylated over the indole NH as previously observed leading to C_{17} HBPA yohimbine (**15**).[30] Complete chemoselectivity was observed with ephedrine (**16**) leading to reaction at the more nucleophilic 2° amine to provide 2-HBPA ephedrine (**17**). Finally, to demonstrate that this process could be performed on a small scale, OH insertion with BFA was performed on ∼1-mg scale and provided comparable conversion as judged by ^{1}H NMR of the crude reaction mixture. The HBPA esters could be separated and isolated by preparative thin layer chromatography (TLC).[34]

^{1}H and ^{13}C NMR analysis of derivatized natural products revealed that the etherification of alcohols leads to the following observed trends in chemical shifts: $\Delta\delta_H \approx -0.06$ to 0.60 and $\Delta\delta_C \approx -4.0$ to 10.0. Diastereomeric mixtures were obtained in all cases (dr ≈ 1:1 → 12:1), and while the impact of this new stereocenter can be ascertained during SAR studies, it is generally expected that this will be overshadowed by the effect of attaching a large substituent to the site of derivatization. The

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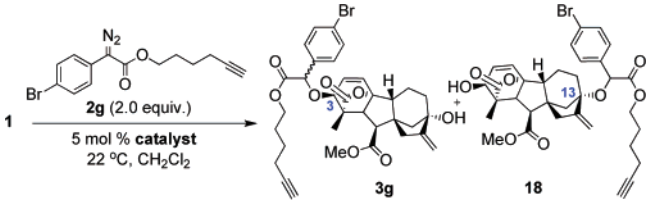
Table 3. Screening of Catalysts To Randomize the Site of OH Insertion



entry	catalyst	**3g**/**18**	% yield[a]
1	$Rh_2(OAc)_4$	>95:5	55 (38)
2	$Rh_2(oct)_4$	>95:5	42 (50)
3	$Rh_2(NHCOCF_3)_4$	50:50	27 (55)
4	$Rh_2(esp)_2$	50:50	38 (52)
5	Cu(NHC)Cl	>95:5	5
6	$Cu(OTf)_2$	NA	0

[a] Yields refer to isolated, purified products. oct: octanoate; $Rh_2(tfacam)_4$ (tfacam = NHC(O)CF_3); esp: α,α,α′,α′-tetramethyl-1,3-benzenedipropionic acid; NHC: [1,3-bis(diisopropylphenyl)imidazole-2-ylidene].

site of O−H insertion for all derivatives was determined by detailed NMR analysis and comparison to published spectral data for the starting natural products and derivatives.[34] These studies enable initial general guidelines to be posited for predicting chemosite selectivity for novel natural products and can be summarized in decreasing order of reactivity as follows: 1° ROH ≈ 2° alkyl NH > 2° alkyl OH > 2° allylic OH ≥ aryl NH > phenolic OH > 3° alkyl OH > indole NH. Furthermore, under the described conditions, no reaction with amide NH or alkenes was observed.

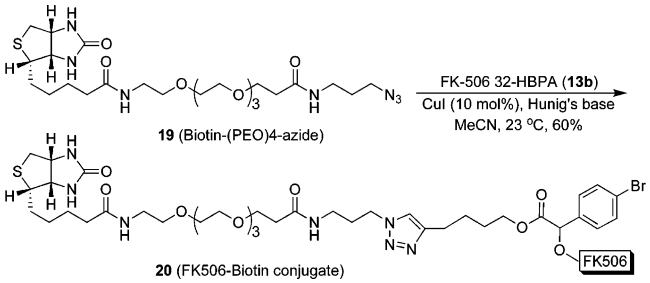
Screening Catalysts for Randomizing Site Selectivity of the OH Insertion with Gibberellic Acid Methyl Ester. Although rhodium acetate provided good conversion and chemosite selectivity to deliver gibberellic HBPA ester **3g**, we screened a small set of rhodium and copper catalysts with the goal of altering or randomizing the innate selectivity of OH insertion with this substrate (Table 3). While rhodium octanoate resulted in reduced yield but comparable chemosite selectivity (Table 3, entry 2), the more reactive $Rh_2(tfacam)_4$ (tfacam = NHC(O)CF_3) catalyst afforded a 1:1 mixture of the chemosite isomers **3g** and **18** with only traces of bis-etherification (Table 3, entry 3). This is an important finding as it suggests that some degree of site-nonselectivity during OH insertion can be achieved with even achiral catalysts. This will facilitate SAR studies with novel natural products and confirms earlier findings that secondary and tertiary alcohols can exhibit similar reactivity in OH insertion reactions.[18] Use of the sterically demanding DuBois catalyst, $Rh_2(esp)_2$ bearing a tethered dicarboxylate ligand, also gave an ∼1:1 mixture of chemosite isomers with improved conversion (Table 3, entry 4).[37] Copper-based catalysts led to poor or no O−H insertion under our standard conditions (Table 3, entries 5 and 6).[38]

Proof of Principle Affinity Experiments with a Novel FK506−Biotin Conjugate Obtained via OH Insertion/Sharpless−Hüisgen Cycloaddition Leading to Pull-Down of the Entire "FK506−Immunosuppressive Complex". To demonstrate the utility of this derivatization sequence, an

Table 4. IC_{50} Values for Diastereomeric FK506 32-HPBA Ethers **13a**/**13b** and Biotin Conjugate **20** in the IL-2 Reporter Gene Assay

compd	IC_{50} (nM)
12	0.1 ± 0.01
13a	1.7 ± 0.2
13b	0.4 ± 0.03
20	1.7 ± 0.4

Scheme 2. Synthesis of FK506−Biotin Conjugate **20** via Sharpless−Hüisgen [3 + 2] Cycloaddition



FK506−biotin conjugate **20** was synthesized and utilized in an affinity pull-down experiment. FK506 is useful in this respect, as it has commonly been employed for evaluation of new methods for receptor isolation due to its high potency and the abundance of FK506 binding proteins (FKBPs) in numerous cell lines.[39] First, the potency of the separable (HPLC), diastereomeric 32-HBPA FK506 ethers **13a** and **13b** was compared individually to that of FK506 (**12**) using an interleukin 2 (IL-2) promoter driven luciferase reporter assay. Compared with FK506, derivatives **13a** and **13b** exhibited an ∼4- and 17-fold increase in IC_{50} values, respectively, but maintained activity in the low nanomolar range (Table 4).[40] The more active FK506 ether diastereomer **13b** was then converted to the FK506−biotin conjugate **20** via the Sharpless−Hüisgen cycloaddition with biotin-$(PEO)_4$-azide **19**, which proceeded in 60% yield (Scheme 2).[12]

The biotin conjugate retained significant activity (IC_{50} = 1.7 ± 0.4 nM) in the IL-2 assay and was therefore used in conjunction with streptavidin sepharose in a pull-down experiment using RKO cell lysates (Figure 2). A nonspecific protein of ∼12 kDa, likely streptavidin cleaved from the affinity resin, concealed the band for FKBP12. However, Western blot analysis with anti-FKBP12 antibody revealed and confirmed that the FK506−biotin conjugate **20** successfully captures FKBP12 in a competitive manner (Figure 2A,B, lane 3 vs 4 and lane 6 vs 7 and bottom panel). However, a striking result is that an additional three bands at ∼17, 19, and 61 kDa were also

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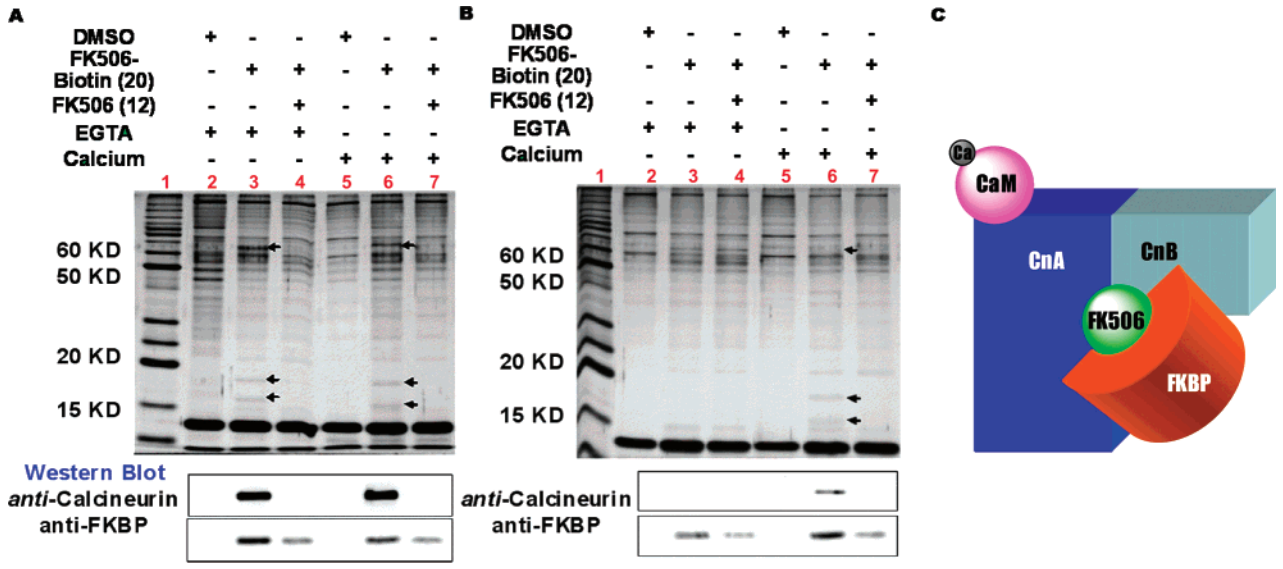

Figure 2. Pull-down experiment using RKO cell lysates. Bound proteins were captured using streptavidin-conjugated agarose. (A) Before proteins were eluted from beads by boiling, 5 mM EGTA (lanes 2−4) or 2 mM calcium (lanes 5−7) was added into sample buffer. (B) Lysis buffer with 5 mM EGTA (lanes 2−4) or 2 mM calcium (lanes 5−7) was used for lysing cells and incubating with drugs. After elution, all proteins were visualized by denaturing SDS-PAGE followed by silver staining or transferring onto nitrocellulose membrane followed by Western blotting and probed with anti-calcineurin or anti-FKBP12 antibodies. (C) Schematic showing the binding of FK506 to FKBP12 and the ternary complex that is formed with calcineurin and calmodulin.

observed in the pull-down assay, which were initially surmised to be calcineurin B, calmodulin, and calcineurin A, respectively (Figure 2A,B, lanes 3 and 6). Binding of FK506 to FKBP12 is known to lead to formation of a ternary complex with calcineurin A and B and calmodulin (Figure 2C).[41] The 61 kDa band was confirmed through western blot analysis to be calcineurin A (Figure 2A,B, bottom panels). Calmodulin and calcineurin B are known to bind to the FK506−FKBP12 complex in a calcium-dependent fashion leading to a calcium-dependent gel mobility shift.[41] The three bands at ∼17, 19, and 61 kDa show a clear shift in their position when EGTA, which chelates calcium, and additional calcium were added separately into sample buffer before analysis by SDS-PAGE (Figure 2A, lane 3 vs 6). Additionally, compared to experiments with calcium included in the lysis buffer, the bands at ∼19 and 17 kDa proteins disappeared when EGTA was included in the lysis buffer during pull-down assays confirming that these bands indeed correspond to calmodulin and calcineurin B (Figure 2B, lanes 3 vs 6). The disappearance of calcineurin A was also confirmed through Western blot analysis (Figure 2A,B, bottom panels). These results indicate that the C_{32} HBPA FK506−biotin conjugate **20** was able to pull down the entire "immunosuppressive complex" involving FKBP, calcineurins A and B, and calmodulin; the latter three proteins were secondary targets.[42] It is interesting to note that only FKBP12 was initially identified as the FK506 binding protein using a conventional affinity chromatography approach with derivatization at C_{32},[39c,43] and calcineurin was not discovered as a relevant target for FK506 until a glutathione-S-transferase/FKBP fusion protein was used together with FK506 as an affinity reagent a few years later.[41] Furthermore, this result experimentally confirms the previous hypothesis based on docking studies that aryl ether substituents at C_{32} of FK506 might stabilize the interaction with calcineurin and provide a potential way to separate cytotoxicity and immunosuppresive activity of FK506.[44] These experiments also indicate the robustness of the ester linkage in the FK506−biotin conjugate **20** during affinity chromatography.[29]

Proof of Principle Studies for Chemosite-Selective and Site-Nonselective O−H Insertions Employing an Achiral Catalyst: Simultaneous Arming and SAR Studies of Brefeldin A. To further illustrate the concept of simultaneous arming and SAR by site-nonselective O−H insertions, we chose BFA (**8**), a macrolide antibiotic bearing two secondary alcohols with one being allylic. This natural product is known to disrupt the Golgi complex and display a wide variety of biological properties, including antitumor, antiviral, antifungal, apoptotic, and antimitotic effects.[45,46] However, undesirable properties including solubility and neurotoxicity have limited its use as a therapeutic agent. Thus, there has been continued interest in the synthesis of analogues for SAR and improvement of the therapeutic properties of BFA.[32]

Of the two alcohols present in BFA, the more accessible cyclopentyl alcohol (C_{13}) was selectively alkylated (**9a**) over the electron-rich allylic alcohol (C_1) with $Rh_2(OAc)_4$ and 4-bromophenyl diazo ester **2g** (Figure 3; for HPLC trace see Figure 3C).[47] Use of the more reactive acetamide catalyst, $Rh_2(tfacam)_4$, led to a moderate degree of site-nonselectivity leading to reaction at the less accessible alcohol in ∼3% isolated yield. While this degree of site randomness is quite modest, it should be

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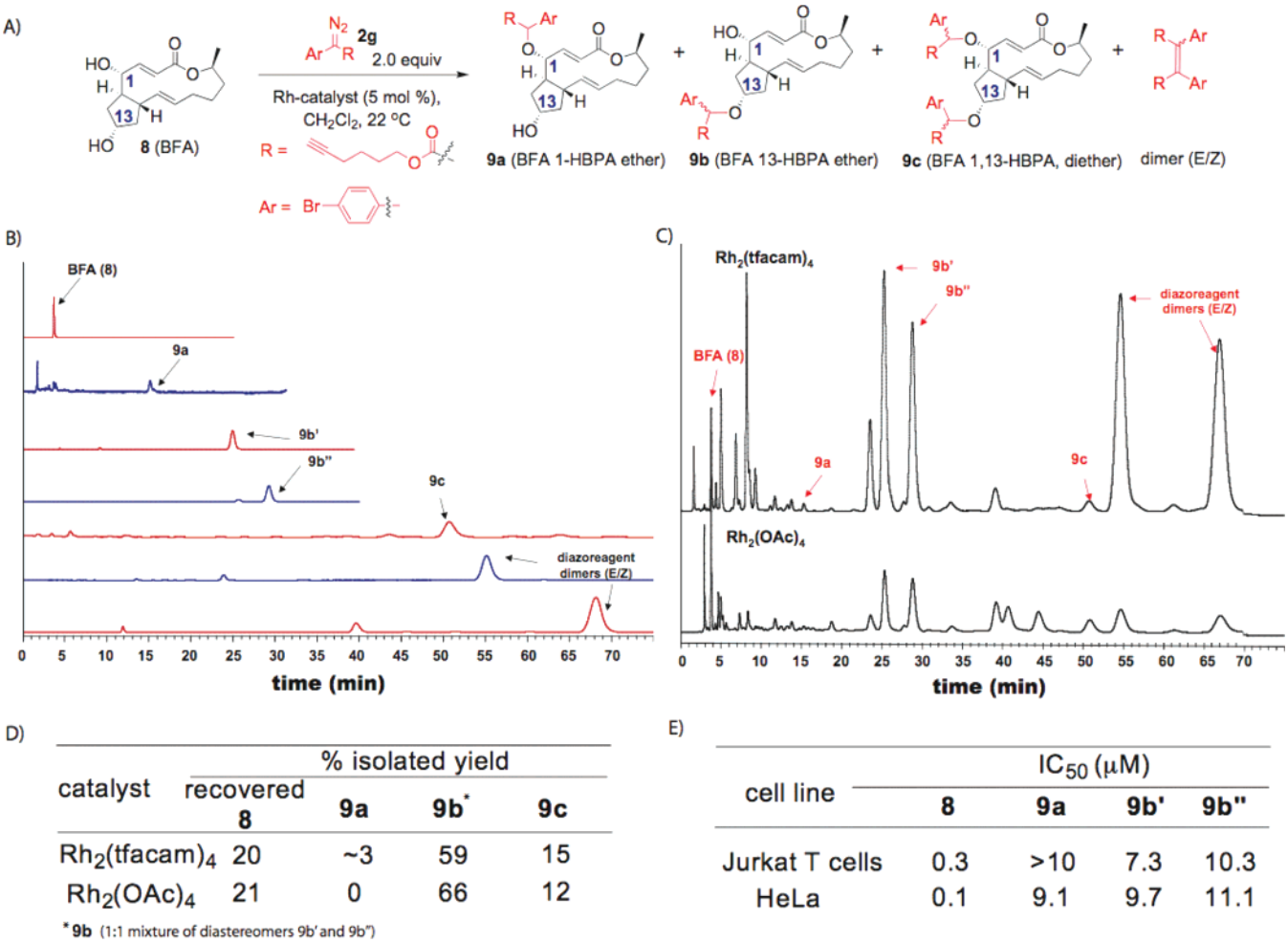
(47) The cyclopentyl alcohol (C13) has previously been shown to be the most readily functionalized. See ref 32.



D)

catalyst	recovered 8	% isolated yield 9a	9b*	9c
$Rh_2(tfacam)_4$	20	~3	59	15
$Rh_2(OAc)_4$	21	0	66	12

*9b (1:1 mixture of diastereomers 9b′ and 9b″)

E)

cell line	IC_{50} (μM) 8	9a	9b'	9b''
Jurkat T cells	0.3	>10	7.3	10.3
HeLa	0.1	9.1	9.7	11.1

Figure 3. Simultaneous arming and SAR studies of BFA via chemosite-selective and -nonselective O−H insertions. (A) Rh(II)-catalyzed O−H insertion with BFA and diazo acetate **2g** leading to chemosite isomers **9a**/**9b′**/**9b″** (diastereomers), bis-functionalized BFA **9c**, and diazo reagent-derived dimers (*E*/*Z* isomers) formed during. (B) Reverse-phase (RP)-HPLC traces showing retention times for isolated, pure components BFA, **9a**, **9b**, **9c**, and dimers (*E*/*Z* mixture of olefin isomers) of diazo reagent **2g**. (C) RP-HPLC based screening of two rhodium catalysts for simultaneous arming and SAR of BFA; $Rh_2(tfacam)_4$ leads to derivatization of both alcohols providing monoethers **9a** and **9b**; $Rh_2(OAc)_4$ leads to near-exclusive regioselectivity for the cyclopentyl alcohol (C13). (D) Isolated yields (preparative RP-HPLC) of OH insertion products with BFA including recovered starting material. (E) IC_{50} values of cytotoxicity for chemosite isomeric HPBA monoethers **9a** and purified diastereomers **9b′** and **9b″** against HeLa and Jurkat cell lines based on incorporation of [^{3}H]-thymidine.

recognized that, for initial SAR studies of a novel natural product, a key requirement is simply the ability to isolate and purify the minor chemosite isomer to enable its bioactivity to be measured. Subsequent optimization of this minor chemosite isomer may require screening of chiral catalysts to achieve a type of "matched double diastereodifferentiation" (*vide supra*) and would be highly case specific. Purification of both BFA isomeric ethers (**9a**) and (**9b**) was achieved by preparative HPLC in sufficient quantities to enable characterization by MS and ^{1}H NMR and determination of their differential cytotoxicity compared to BFA using [^{3}H]-thymidine uptake in Jurkat and HeLa cells (Figure 3E). Proliferation studies with HeLa cells gave comparable IC_{50} values of 9.1, 9.7, and 11.1 μM for the 1-HBPA derivative (**9a**) and 13-HBPA diastereomers **9b′** and **9b″**, respectively, corresponding to a ~100-fold drop in activity in comparison to BFA (reported $IC_{50} = 0.55$ μM, MTT assay).[48] In Jurkat cells, the IC_{50} of 13-HPBA BFA diastereomers **9b′** and **9b″** was ~30-fold less than BFA. Interestingly, the 1-HBPA BFA derivative **9a** is almost completely inactive in Jurkat T cells with only marginal antiproliferative effects at the highest concentrations used ($IC_{50} \gg 20$ μM, Figure 3E). In agreement with previous observations with methyl ethers, etherification of the alcohols at C_1 or C_{13} significantly diminishes cytotoxicity of BFA with the alcohol at C_1 being most crucial for cytotoxicity consistent with our results with 1-HBPA BFA (**9a**).[32b] It is well known that BFA-mediated apoptosis is independent of Apaf-1 and p53.[49] However, it remains controversial as to whether apoptosis induced by BFA is a secondary effect upon disruption of the Golgi apparatus.[50] Thus, we determined the apoptotic effects of BFA and chemosite isomeric HBPA ethers **9a**, **9b′**, and **9b″** and found that they do induce apoptosis as evidenced by activation of caspase 8, caspase 9, and cleavage of caspase 3 at high concentrations consistent with results from the cell

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proliferation assay using Jurkat T cells.[34] These derivatives were also found to lead to disruption of the Golgi complex using confocal fluorescence microscopy indicating a similar mechanism of action as BFA.[34]

Conclusion

A mild, chemosite-selective and site-nonselective O−H insertion process using alkynyl diazo esters and $Rh_2(OAc)_4$ was developed that enables simple, two-step, simultaneous arming and SAR studies of bioactive natural products. A subsequent conjugation step involving the Sharpless−Hüisgen [3 + 2] cycloaddition allows attachment of various probes including biotin. Importantly, variation in chemosite selectivity was possible even with achiral catalysts between a secondary and tertiary alcohol in gibberellic acid methyl ester and two secondary alcohols in BFA. This method was applied to the synthesis of HPBA ethers of a structurally diverse set of natural products and derivatives bearing alcohols with varied steric environment and electronics, enabling initial guidelines for applying this process to other natural products and derivatives. The described method is demonstrated in several cases to be superior in terms of mildness of reaction conditions and to be flexible in terms of modifying site selectivity based on reagent control (i.e., modifying catalysts or diazo reagent). These studies open possibilities for further modifying chemosite selectivity through the agency of chiral ligands employing a type of "double diastereodifferentiation". An O−H insertion/[3 + 2] cycloaddition sequence with FK506 provided access to a novel FK506−biotin conjugate **20**, which was successfully employed to pull-down the ternary complex involved in immunosuppression by FK506 from RKO cell lysates. The concept of simultaneous arming and SAR was demonstrated with BFA in this case simply by modifying achiral ligands on rhodium. These studies initiate an important area in the biological chemistry of natural products, involving the development of both chemosite-selective and also site-nonselective monofunctionalizations toward a rapid, systematic approach for fully exploiting natural products for chemical genetics. The described overall strategy (1) enables rapid arming and SAR studies of novel natural products, requiring minimal structural information (e.g., presence of alcohols) and (2) provides a mild, versatile synthesis of natural product conjugates that should expedite the identification of natural product cellular receptors, facilitate their continued use as modulators of cellular function, and contribute to their continued potential as enduring leads for drug discovery. The application of this method to novel natural products including the use of chiral rhodium catalysts to further modulate site-selectivity constitutes our ongoing studies in this area, and the results of these studies will be reported in due course.

Experimental Section

Reactions were carried out in flame-dried glassware under a nitrogen or an argon atmosphere, unless otherwise noted. Commercial solvents and reagents were used as received. Anhydrous solvents were dried over neutral alumina (MBraun system). All reactions were magnetically stirred and monitored by TLC, performed using glass-backed silica gel 60_{F254} (Merck, 250-μm thickness). Yields refer to chromatographically and spectroscopically pure compounds unless otherwise stated. Flash column chromatography was performed using 60 Å silica gel (Baker, 230-400 mesh, or Silacycle, 230-400 mesh) as a stationary phase. Preparative TLC was performed using Partisil PKL5F silica gel plate (150 Å, 20 × 20, 1000 μm) and developed with appropriate solvent. Bands were identified by UV, and silica was scraped off the plate and placed into 20-mL scintillation vials. Organic residues were extracted by suspending the silica gel in 5:95 methanol/ethyl acetate. This was followed by two more elutions with 5:95 methanol/ethyl acetate. The combined washings were filtered through Gelman Nylon Acrodisc (0.45 μm) to remove fine particles of silica gel. Unless indicated otherwise, deuterochloroform ($CDCl_3$) served as an internal standard (7.26 ppm) for all ^{1}H spectra and (77.0 ppm) for all ^{13}C spectra. Mass spectra were obtained at the Center for Chemical Characterization and Analysis (Texas A&M University).

Representative Procedure for the Rh(II)-Mediated O−H Insertion As Described for the Preparation of Gibberellic Acid Methyl Ester 3-HBPA Ether, 3g. Gibberellic acid methyl ester (20 mg, 0.056 mmol) and rhodium acetate (1.3 mg, 0.003 mmol, 0.05 equiv) were weighed into a flame-dried Schlenk flask under a nitrogen atmosphere. The solids were suspended in dry CH_2Cl_2 (4 mL), and the mixture was stirred for 5 min to dissolve the methyl ester (Note: the rhodium catalyst does not dissolve) at 23 °C. A solution of 5-hexynyl-α-(4-bromophenyl)-diazo acetate **2g** (36 mg, 0.112 mmol) in dry CH_2Cl_2 (2 mL) was prepared and added via syringe. The reaction mixture was stirred for 1 h at 22 °C. The solvent was evaporated, and the residue was purified by flash chromatography on SiO_2 (pentane/ether, 20:80) to afford ether **3g** (20 mg, 55% yield) and recovered gibberellic acid methyl ester **1** (8 mg, 38%). R_f 0.39 (hexanes/Et_2O, 20:80). IR (thin film) cm^{-1}: 2117, 1772, 1733. LRMS (MALDI): calculated for $C_{34}H_{37}BrO_8$ (M + Na) = 675.2 and observed (M + Na) = 675.2 (see Supporting Information for ^{1}H and ^{13}C NMR line listings and NMR spectra).

Synthesis of FK506−Biotin Conjugate 20 by Sharpless−Hüisgen Cycloaddition. (+)-Biotin− $(PEO)_4$-propionyl azide **19** (2.3 mg, 0.004 mmol) was dissolved in 0.2 mL of dry acetonitrile in a vial flushed with nitrogen and equipped with a magnetic stir bar. FK506 32-HBPA ether **14b** (4.0 mg, 0.004 mmol) was dissolved in 0.2 mL of dry acetonitrile and then transferred to the reaction vial followed by CuI (0.1 mL, 0.004 μM, stock solution in dry acetonitrile) and Hünig's base (10 μL, 0.6 μM, stock solution in dry acetonitrile), and the mixture was stirred at 22 °C overnight. The solvent was evaporated, and the residue was purified by preparative TLC (20 × 10 cm^2, 1000-μm thickness SiO_2, MeOH/EtOAc, 30:70) to afford the FK506−biotin conjugate **20** (3.7 mg, ~60% yield): R_f 0.35 (EtOAc/MeOH, 50:50). HRMS (MALDI): calcd for $C_{82}H_{125}BrN_8O_{21}S$ (M + H) = 1691.7942; found (M + H) = 1691.7959 (see Supporting Information for ^{1}H and ^{13}C NMR line listings and NMR spectra).

Cell Culture. HeLa, Jurkat, and RKO cells were grown in DMEM or RPMI1640 medium with 10% fetal bovine serum and cultured at 5% CO_2 and 37 °C in a humidified atmosphere.

Interleukin 2 Reporter Gene Assay. The transient transfection was performed by electroporation using minimal IL-2 promoter driven luciferase reporter plasmid (Promega). Jurkat T cells (1×10^7) were harvested, washed once in RPMI-1640 medium, resuspended in 300 μL of RPMI, and mixed with 2 μg of IL-2 plasmid. The electroporation was carried out by applying an electric pulse (150 V, 960 μF using the Bio-Rad Gene Pulser II). The cells were allowed to rest for 10 min before they were transferred back to the culture medium (RPMI with 10% fetal calf serum and 2 μM glutamine) and incubated at 37 °C for 24 h, and then aliquoted into 24 well plates. FK506 (**12**), 32-HBPA FK506 **13a**,**b**, and biotin−FK506 conjugate **20** dissolved in DMSO were added to cultured cells and incubated with the cell cultures for 1 h before PMA (final concentration 40 nM) and ionomycin (final concentration of 1 μM) were added. After another 6 h of incubation, cells were harvested and prepared for luciferase assay according to manufacturer's protocol (Promega).

Affinity Pull-Down Experiment. RKO cells were cultured in RPMI-1640 with 10% FBS medium and treated with or without PMA (final concentration 40 nM) and ionomycin (final concentration of 1 μM) before harvesting. Cells were homogenized using a dounce homogenizer

in 1X lysis buffer (20 mM Tris HCl, pH 7.5, 100 mM KCl, 5 mM β-mercaptoethanol, 0.1% Triton X-100 and protease inhibitors). The mixture was centrifuged at 16000*g*, and the supernatant was further centrifuged at 37000*g*. The supernatant was collected and precleared with Streptavidin conjugate agarose (20 μL, Pierce) for 30 min, centrifuged, and divided into equal aliquots (250 μL), which were then incubated with DMSO or 32-HBPA FK506 **13b** for 1 h, and further with DMSO or FK506−biotin conjugate **20** as indicated in Figure 3 and incubated for 1 h. Streptavidin agarose was then added, and samples were incubated for 30 min to capture the natural product−protein complexes. The samples were centrifuged at 5000*g* for 1 min, and supernatant was discarded. The pellets were then washed 4 times with lysis buffer, and bound proteins were eluted by heating at 95 °C for 5 min in the presence of 2X Lammli sample buffer. The resulting supernatants were loaded onto SDS-PAGE gels, and the separated proteins were visualized by silver staining or Western blot analysis.

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Supporting Information Available: General procedures for synthesis of diazo esters **2a**−**2g** and HBPA ethers **5**−**11**, **17**, azides **19** and **23**, NMR spectra, and X-ray crystallographic data. This material is available free of charge via the Internet at http://pubs.acs.org.

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