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Structure–activity studies on *seco*-pancratistatin analogs: Potent inhibitors of human cytochrome P450 3A4

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ABSTRACT

Two total syntheses of fully functionalized *seco*-analogs of the anticancer compound pancratistatin are reported. Structure–activity relationship (SAR) studies identified potent and selective inhibitors of human cytochrome P450 3A4 (CYP3A4) and revealed several core pharmacophoric elements. These studies identify potential roadblocks and will guide the further development of a viable selective clinical pancratistatin derivative.

Several groups have been interested in the isolation, synthesis, semi-synthesis and biological evaluation of various amaryllidaceae alkaloids (**1**–**7**, Scheme 1) over the last few years.[1] The potent anticancer activity displayed by amaryllidaceae constituents such as pancratistatin **5** and narciclasine **6** has fuelled an immense amount of synthetic work in many laboratories.[2] The amaryllidaceae alkaloids are all derived biosynthetically from norbelladine **1** (Scheme 1).[3] Sub-classification is readily discernible within the family on structural grounds forming the crinane, lycorane and galanthamine-type compounds (**2**–**7**), although at least six other minor groups are known to occur.[3b]

Galanthamine **7** is the first of these alkaloids to receive FDA approval, in this case for the treatment of Alzheimer's disease due to its unique ability to reversibly inhibit acetylcholinesterase.[3c] Crinane compounds are known for a host of biological properties, including antimalarial and antiproliferative action as well as protein synthesis inhibition.[4a–e] We have recently shown that α-bridged crinanes (such as **3**) are distinguishable from β-forms (such as **2**) at selectively initiating apoptosis in rat liver hepatoma (5123tc), but not in normal HEK293t cells.[4f,g] Lycorine **4** is the most abundant of the amaryllidaceae alkaloids and its broad spectrum of biological activities is well documented.[4d,e,5a–k] As a potential chemotherapeutic, it has shown most promise as an antiproliferative agent in a number of cancer cell lines.[4e] Recent work in our laboratories uncovered the apoptosis-initiating ability of lycorine in human leukemia (Jurkat) cells, and explored some elements of this remarkable pharmacophore.[5k]

Returning to the lycorane derivatives pancratistatin **5** and narciclasine **6**, significant efforts have revealed crucial core elements of the anticancer pharmacophore.[1,2] In particular, the stereochemically defined 2,3,4-trihydroxy functionalized ring C is essential for potent apoptosis-inducing activity.[1] The apoptotic mode of death initiated by pancratistatin is indicated by early activation of caspase-3 followed by flipping of phosphatidyl serine to the outer leaflet of the plasma membrane.[6] This was shown by us to occur selectively in cancer cells but not in normal cells and that mitochondria were the site of action.[6] In view of the potential clinical development of an anticancer agent within the series,[2a–c] we recently documented the human cytochrome (CYP3A4) inhibitory activity of a compound library consisting of 26 amaryllidaceae alkaloids and derivatives.[7] From this analysis we determined that pancratistatin **5** exhibited low interaction with CYP3A4 while narciclasine **6** proved to be a potent inhibitor, raising issues with regard to its ultimate clinical efficacy. In addition, we determined that lipophilic substitution at positions 1 and 2 in the lycorine series **4** resulted in the formation of potent CYP3A4 inhibitors one magnitude less active than the clinically used antifungal P450 inhibitor ketoconazole.

Human cytochromes P450 (CYP450) constitute a diverse superfamily of hemoproteins which use a plethora of both exogenous and endogenous compounds as substrates in enzymatic reactions, usually forming part of multicomponent electron transfer chains. Human CYP450s consist of four major families (CYP1 to CYP4) of

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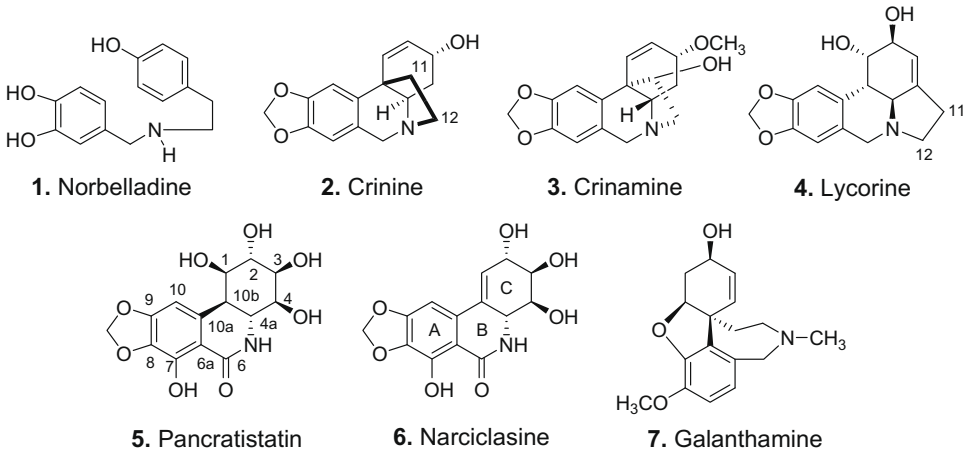
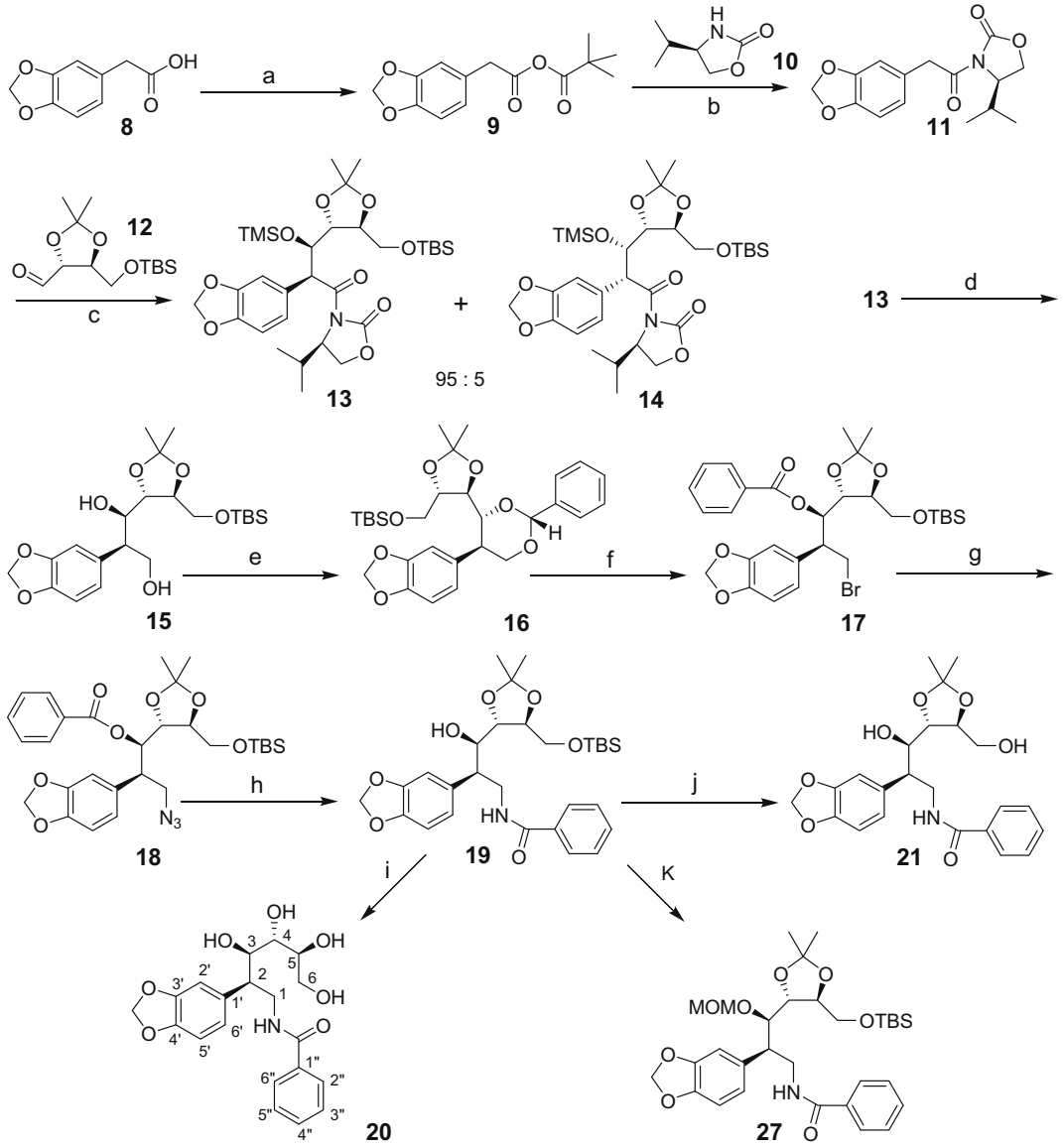


Scheme 1. Structurally-diverse alkaloid representatives of the plant family amaryllidaceae.



Scheme 2. Synthesis of *seco*-pancratistatin analogs via Evans' catalytic *anti*-aldol reaction and radical-mediated benzylidene fragmentation as key steps. Reagents and conditions: (a) PivCl, TEA, DEE, −78 °C to rt, 2 h; (b) LiHMDS, THF, −78 °C to rt, 6 h, 84%; (c) $MgCl_2$, TEA, TMSCl, EtOAc, rt, 12 h, 95%; (d) $LiBH_4$, THF/MeOH (1:1), 0 °C to rt, 3 h, 88%; (e) BDMA, TsOH (cat), DCM, rt, 1 h, 91%; (f) NBS, AIBN, Ph, 60 °C, 1 h, 70%; (g) NaN_3, DMF, rt, 5 h, 85%; (h) 5% Pd/C, H_2, THF, rt, 2 h, 94%; (i) 2 M HCl, THF, rt, 2 h, 87%; (j) TBAF, THF, rt, 1 h, 96%; (k) DIPEA, MOMCl, DCM, 0 °C to rt, 10 h, 95%.

mono-oxygenase enzymes which are expressed primarily on smooth ER membranes of hepatocytes and by cells along the intestinal tract mucosal surface.[8a] They are involved in the detoxification of a wide variety of xenobiotics such as drugs, biogenic amines from food sources, environmental toxins, and chemical carcinogens, and in the oxidation of steroids, fatty acids, prostaglandins, leukotrienes, and fat-soluble vitamins.[8b] The CYP3A subfamily comprises about 30% of the total liver cytochrome P450 enzyme pool in humans, and the isoenzyme CYP3A4 accounts for approximately 60% of drugs metabolized.[8b] In addition, an estimated 70% of CYP protein in the small intestinal epithelium is formed by this isoenzyme.[8c] Furthermore, it is widely known that co-administration of multiple CYP3A4 substrates, inducers or inhibitors, including compounds from food sources, may alter pharmacokinetic and pharmacodynamic parameters of many prescribed drugs. Thus, evaluation of CYP3A4-drug interactions is critical to the furthering of a clinical candidate. In view of the selective lycorane-CYP interactions identified[7] and the potential of pancratistatin in the anticancer field, we now report the extension of this CYP3A4 assay to a wide array of conformationally flexible semisynthetic derivatives uncovering valuable structural information concerning CYP3A4 inhibitory activity.

We recently reported the total synthesis of ring-B, C *seco*-pancratistatin analogs such as **20** (Scheme 2).[1g] While these derivatives possess all of the essential elements of the known anticancer pharmacophore, including the stereochemically defined 1,2,3,4-tetrahydroxy motif and the amide function, they were shown to be devoid of anticancer activity.[1g] Key steps towards the synthesis of **20** include: (i) A highly efficient and diastereoselective catalytic Evans' aldol leading to **13** (Scheme 2, step c); (ii) an oxidative regiospecific radical-mediated fragmentation of a 1,3-benzylidene (Scheme 2, step f); and (iii) an intramolecular *O*- to *N*-acyl migration (Scheme 2, step h). The stereochemical outcome of the aldol was confirmed by single-crystal X-ray structural determination of benzylidene **16** (Fig. 1),[1f] indicating that the desired non-Evans *anti*-adduct was highly favoured in this process. The above data confirm the stringent configurational requirements that are necessary for anticancer activity. The differences between the anticancer pharmacophore and CYP3A4 inhibitory pharmacophore are exemplified by the data reported for narciclasine (potent anticancer, potent CYP3A4 inhibitory) and pancratistatin (potent anticancer, CYP3A4 inactive) leading us to hypothesize that conformationally flexible *seco*-derivatives might unravel further attributes of the CYP3A4 interaction. In order to gain further insights into this cytochrome inhibitory pharmacophore, the *seco*-analogs **18** to **20** depicted in Scheme 2 were screened for CYP3A4 activity, see Table 1. This analysis revealed the hydroxybenzamide **19** as a potent inhibitor of CYP3A4 (K_i 0.17 μM). These results are consistent with and extend our previous findings in the lycorine derivatives[7] for which CYP3A4 inhibition required a free hydroxyl group or a small acyl group (acetate but not benzoate) at C3 (C1 in the lycoranes), enhanced by lipophilic substitution at C4 (C2 in the lycorane series). This is consistent with the C3 substituent functioning as an H-bond acceptor, subject to moderate steric interactions with the enzyme. The present results indicate that CYP3A4 inhibition is enhanced by lipophilic substituents at positions 4, 5 and 6 (corresponding to C2, C3 and C4 in the lycoranes), given the lack of inhibitory activity of derivative **21**.

Our next goal was to probe these CYP3A4 SAR studies further by extension of the substituent at C3 (C1 in the lycorane series) to a conformationally flexible H-bond acceptor for which we selected a methoxymethyl (MOM) substituent. We also wished to be able to differentially functionalize the amino group in these *seco*-analogs to circumvent the *O*- to *N*-benzoyl migration on reduction of azide **18** (Scheme 2). The new synthetic approach outlined in Scheme 3 was developed that allowed us to achieve both of these goals and proved crucial to our unraveling of two potent inhibitors of CYP3A4, with activities similar to the clinical antifungal cytochrome inhibitor ketoconazole.

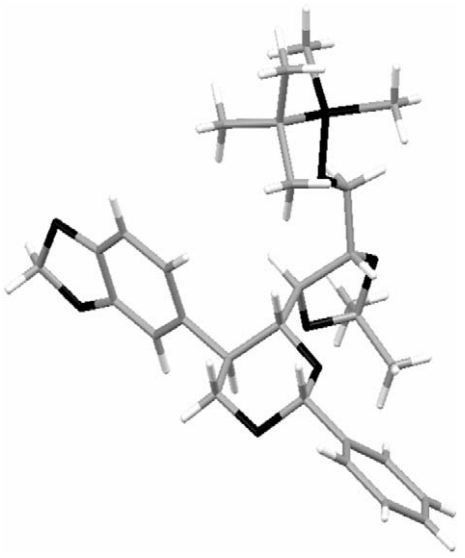


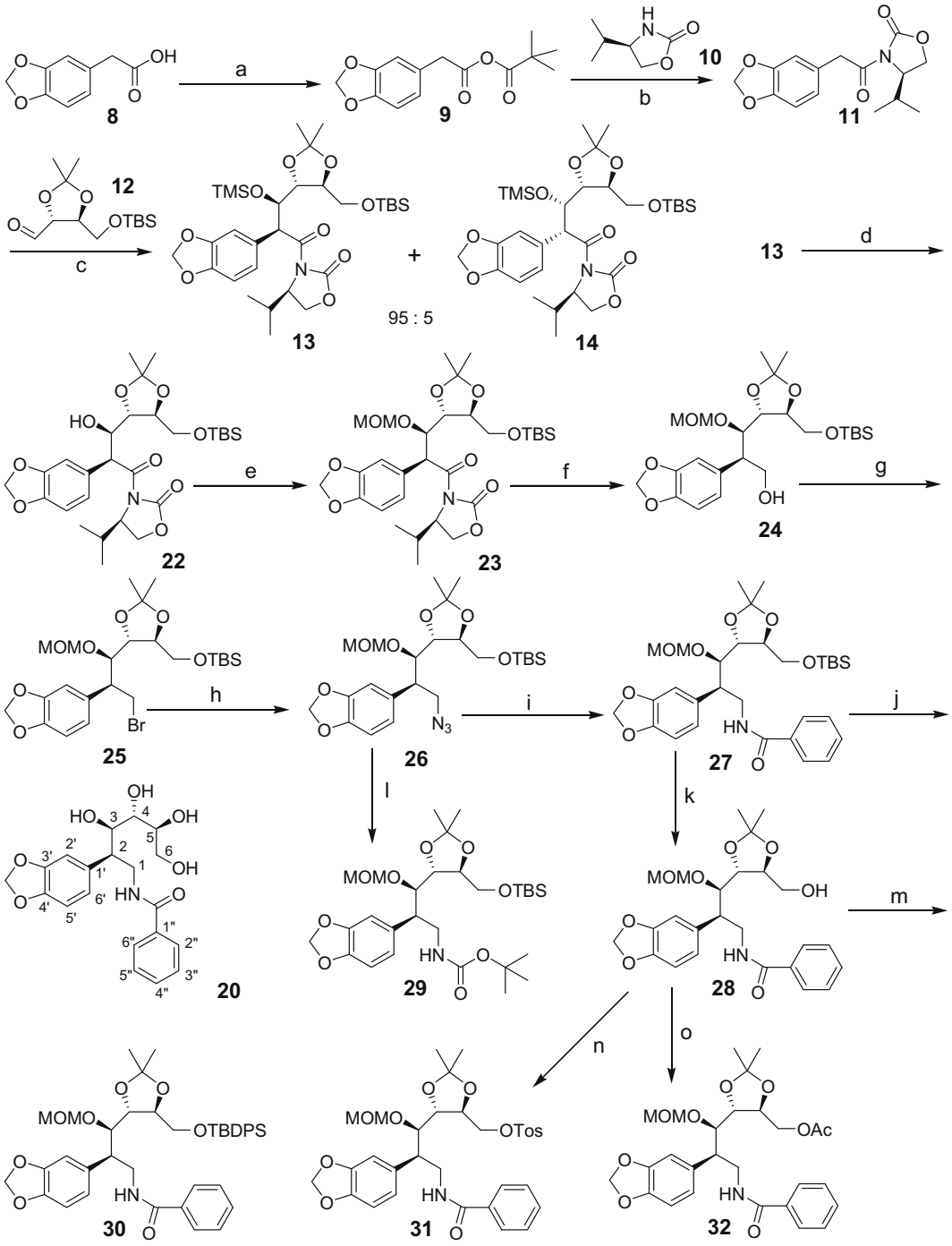
Figure 1. X-ray structure of 1,3-benzylidene **16**.

Table 1
Inhibitory activity against the biotransformation of 7-benzyloxyquinoline by cDNA-expressed human CYP3A4

Compound	K_i (μM)	pK_i[a] (M)
18	na	—
19	0.17	6.78 (±0.05)
20	na	—
21	na	—
26	na	—
27	0.03	7.50 (±0.10)
28	1.55	5.81 (±0.09)
29	0.32	6.50 (±0.05)
30	0.58	6.24 (±0.10)
31	0.07	7.15 (±0.02)
32	1.01	5.99 (±0.03)
Ketoconazole	0.03	7.48 (±0.02)

[a] Values are means of three experiments, standard deviation is given in parentheses (na = not active at 10 μM).

This fully controlled approach is outlined in Scheme 3. Activation of 3,4-methylenedioxyphenylacetic acid **8** with pivaloyl chloride under basic conditions gave the mixed anhydride **9** which was reacted further without purification with the lithium salt of (*R*)-(+)-oxazolidinone **10** providing chiral imide **11** in 84% yield. Evans' $MgCl_2$-catalyzed aldol process[9] was next invoked to couple imide **11** with L-threose aldehyde **12** (derived in four steps from L-tartaric acid), leading to the non-Evans *anti*-adduct **13** as the major diastereomer (95:5) in 95% yield.[1f,g] This single step is remarkable in that it connects the ring A aromatic moiety with the nascent ring B fragment and, notably, sets the stereochemistry at two (C1 and C10b) of the six contiguous stereocentres in pancratistatin **5**, with a further two (C2 and C3) preset by chiron **12**. The selective formation of the non-Evans *anti*-adduct **13** is mechanistically of significance as it indicates that the facial selectivity of the chiral auxiliary has an overriding influence on the stereochemical outcome of this reaction, overcoming any Felkin-Ahn bias on the part of the aldehyde.[1f] Furthermore, the efficiency of the reaction conducted with enolizable aldehyde **12** under the conditions outlined in Scheme 3 (step c) is remarkable as we readily confirmed[1f] that enolizable aliphatic aldehydes in general suffer from low conversions as noted



Scheme 3. Evans' catalytic *anti*-aldol reaction as key step in the synthesis of *seco*-pancratistatin analogs. Reagents and conditions: (a) PivCl, TEA, DEE, −78 °C to rt, 2 h; (b) LiHMDS, THF, −78 °C to rt, 6 h, 84%; (c) $MgCl_2$, TEA, TMSCl, EtOAc, rt, 12 h, 95%; (d) HOAc (cat), MeOH, rt, 4 h, 96%; (e) DIPEA, MOMCl, DCM, 0 °C to rt, 10 h, 92%; (f) $LiBH_4$, THF/MeOH (1:1), 0 °C to rt, 2 h, 90%; (g) NBS, PPh_3, TEA, DCM, 0 °C to rt, 5 h, 85%; (h) NaN_3, DMF, rt, 5 h, 91%; (i) (i) 5% Pd/C, H_2, THF, rt, 3 h; (ii) BzCl, TEA, DCM, rt, 2 h, 98%; (j) 2 M HCl, THF, rt, 2 h, 88%; (k) TBAF, THF, rt, 1 h, 97%; (l) (i) 5% Pd/C, H_2, THF, rt, 3 h; (ii) Boc_2O, TEA, DCM, rt, 2 h, 98%; (m) TBDPSCl, py, DCM, rt, 7 h, 98%; (n) TosCl, py, DCM, rt, 5 h, 98%; (o) Ac_2O, py, DCM, rt, 4 h, 98%.

by Evans et al.[9] As mentioned above, single-crystal X-ray structure of benzylidene **16** (Fig. 1),[1f] derived from **13** via 1,3-diol **15** (Scheme 2), confirmed the aldol adduct as the desired non-Evans *anti*-diastereomer.

Interestingly, the minor adduct **14** was shown by us to be the Evans *anti*-diastereomer.[1f] At this stage, the two synthetic strategies (Schemes 2 and 3) diverged. With adduct **13** in hand, chemoselective removal of the TMS group with a catalytic amount of acetic acid afforded hydroxyimide **22** which was subsequently protected as the MOM ether **23**. Cleavage of the chiral auxiliary with lithium borohydride in THF/MeOH gave hydroxy-MOM ether **24** in 90% isolated yield. Nucleophilic bromination with NBS/triphenylphosphine then converted the primary hydroxyl group in **24** to bromide **25** which underwent smooth S_N2 displacement with sodium azide to give the MOM-azide **26**. Palladium on carbon reduction of azide **26** and subsequent benzoylation of the free amine afforded MOM-benzamide **27**. A full set of physical data for **27** is provided in the accompanying supplementary section of

this article. Similarly, the amine derived from **26** was also converted to Boc-derivative **29** in 98% yield by reaction with Boc-anhydride. Securing the MOM-azide **26** allowed us to circumvent the *O*- to *N*-acyl migration that compromised the prior reduction of azidobenzoate **18** in Scheme 2.[1g] Desilylated hydroxybenzamide **28** was obtained almost quantitatively from MOM-benzamide **27** by reaction with tetrabutylammonium fluoride (TBAF). A series of 6-substituted analogs **30–32** were efficiently prepared (Scheme 2, steps m, n and o) directly from hydroxybenzamide **28**. Straightforward global deprotection of **27** with 2 M HCl then led to the 3,4,5,6-tetrahydroxyhexyl-1-benzamide **20**.

The library comprising compounds **18–21** (Scheme 2) as well as **26–32** (Scheme 3) was screened for CYP3A4 inhibitory activity via kinetic monitoring of the conversion of 7-benzyloxyquinoline (BQ) to 7-hydroxyquinoline (HQ) by fluorometric measurement of emission at 538 nm after excitation at 410 nm (see Supplementary data), utilizing ketoconazole as control (Table 1).[7]

The MOM-benzamide derivative **27** (K_i 0.03 μM) was shown to be a powerful inhibitor with a potency equal to that of ketoconazole. Removal of the MOM ether group results in a ~6-fold drop in activity as shown for hydroxybenzamide **19** with K_i 0.17 μM. A pronounced reduction (~50-fold) in activity is observed in going from MOM-benzamide **27** to desilylated analogue **28** (K_i 1.55 μM). Cleavage of both MOM and TBS groups from **19** produced inactive 3,6-diol **21** while removal of all protecting groups gave the 3,4,5,6-tetrahydroxyhexylbenzamide **20** which also exhibited no inhibition. Azides **18** and **26** were void of activity while replacement of the benzamide moiety in **27** with a *t*-butyl carbamate resulted in a 10-fold decrease in activity as evident for **29**. Substitution of the 6-TBS group in **27** with other protecting groups had effects on inhibitory activity to varying degrees; tosylate **31** was highly active (K_i 0.07 μM), TBDPS-benzamide **30** was 20-fold less active than **27** while acetate **32** was mildly active with K_i 1.01 μM. The above activity data highlight several core pharmacological elements of the potent CYP3A4 inhibitor **27**. First, the 1-benzamide functionality is essential as azides **18** and **26** were inactive and since there is a ten fold difference in activity between MOM-1-benzamide **27** and MOM-1-carbamate **29**. This suggests that this part of the molecule must occupy a fairly bulky, polar pocket within the enzyme active site. There is a ~6-fold modulation in activity between hydroxybenzamide **19** and MOM-benzamide **27**, consistent with our earlier hypothesis that a relatively small hydrogen bond acceptor is required at C3. The effect of the silyl group on inhibition is observed to be dramatic as seen for **27** versus **28**, pointing at a lipophilic binding site within the enzyme. Again, these results are fully consistent with the pronounced CYP3A4 inhibitory effects of TBS-substituted derivatives of lycorine **4**.[7] Absence of both 3-MOM and 6-TBS groups results in complete lack of activity as evident with 3,6-diol **21**, while a further loss of the 3,4-isopropylidene group also produces inactive tetrahydroxyhexylbenzamide **20**. The effect of the isopropylidene group is seen to serve as an anchor within a more rigid portion of the enzyme. The bulkiness of the 6-TBDPS group in **30** is somewhat detrimental to inhibition while introduction of a small, polar group such as an acetate, as in **32**, results in further loss of activity. Interestingly, inhibition is sustained with the introduction of an *O*-tosylate group at C6 as in **31** (K_i 0.07 μM), also highlighting the requirement for heteroatom-containing functional groups (**27**, **30**, **31** vs **32**).

In conclusion, a more comprehensive view of the cytochrome P450 3A4 inhibitory pharmacophore has been developed through SAR studies with flexible, differentially functionalized pancratistatin analogs. This pharmacophore is worthy of significant consideration, given the intensive efforts to advance a viable narciclasine **6** or pancratistatin **5** analog into clinical development. The results detailed in our earlier report[7] and the structural features of compounds **27** and **31** and the lesser active compounds shown here allow three major conclusions to be drawn. (i) Significant interactions with the cytochrome CYP3A4 involve an H-bond acceptor region at C3 (C1 in the alkaloid series). A small substituent such as hydroxyl and acetoxy contributes to this cytochrome interaction. Compounds with a benzoyl group were less potent, while the flexible MOM H-bond acceptor showed strong interaction. (ii) The cytochrome interaction is enhanced when a bulky lipophilic substituent is placed at C3 or C6 (C2 or C4 alkaloid numbering),[7] indicating a strong interaction with a large hydrophobic binding pocket in the cytochrome active site. Both activities are additive in that the most potent cytochrome inhibitors have both a small, flexible H-bond acceptor at C3 and a lipophilic substituent at C3 or C6. (iii) Lastly, a double bond between C1-C10b elicits strong interaction with the cytochrome alone, as evidenced by the previous results contrasting the potency of narciclasine **6** with pancratistatin **5**. It is noteworthy that both pancratistatin **5** and B,C-*seco*-pancratistatin **20** are inactive against CYP3A4, while narciclasine **6** exhibits good activity (K_i 0.63 μM).[7] The present results are fully in accord with our earlier structure–activity CYP3A4 mapping conducted in the lycorane series.[7] Overall, these studies provide valuable insight into regions of the amaryllidaceae anticancer pharmacophore likely to interact with CYP3A4. Recent studies have focused on both narciclasine derivatives,[2a–c,s] and C1 benzoyl[2q] and other[1g,2r] derivatives. The present results highlight regions in the pancratistatin series that may affect the bioavailability of the compounds, their capacity to elicit significant drug–drug interactions and their inhibition of other crucial oxidative processes carried out by this important enzyme. Compounds that demonstrate ketoconazole-like activity may also contribute to hepatic toxicity. Lastly, we have identified two derivatives **27** and **31** that exhibit CYP3A4 inhibitory activity at the nanomolar level, similar to the known inhibitor ketoconazole. Extension of these leads towards the synthesis of potent, selective antifungal agents and further extension of the CYP3A4 studies are ongoing in our laboratories.

Acknowledgments

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Supplementary data

CCDC file 654288 contains the supplementary crystallographic data for **16**. These data can be obtained free of charge from The Cambridge Crystallography Data Centre via www.ccdc.cam.ac.uk/data_requestcif.html. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.08.032.

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