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An Efficient Combinatorial Method for the Discovery of Glycosidase Inhibitors**

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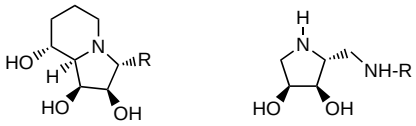
The specific inhibition of *N*-linked glycoprotein-processing α-mannosidases may provide a useful anticancer strategy.[1] Clinical trials have shown that swainsonine (**1**; Scheme 1), a natural α-mannosidase inhibitor that contains a 4-amino-4-deoxy-manno-



1 R = H
2 R = CH_2CH_2Ar

3 R = Bn
4 R = H

Scheme 1. *Structure of swainsonine and α-mannosidase inhibitors. Bn = benzyl.*

furanoside moiety,[2, 3] reduces solid tumor and hematological malignancies.[4] In order to avoid problems that arise from co-inhibition of lysosomal mannosidases, analogues of swainsonine such as **2** have been prepared and shown to have interesting properties.[5] Simpler synthetic analogues are also potent α-mannosidase inhibitors.[3, 6] We have reported that (2*R*,3*R*,4*S*)-3,4-dihydroxypyrrolidin-2-yl derivatives such as **3** are selective α-mannosidase inhibitors.[7, 8] The search for better inhibitors implies the multistep synthesis of a large number of analogues

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[**] *The concept and part of the text of this work were presented at the XIth Eurocarb Conference in Lisbon on September 7, 2001, under the title "An Efficient Combinatorial Method for the Discovery of Glycosidase Inhibitors. Imines Equilibrating with (2R,3R,4S)-2-Aminomethyl pyrrolidine-3,4-diol and Aldehydes are Inhibitors of α-Mannosidases and Models for the Inhibitory Activity of Corresponding Amines".*

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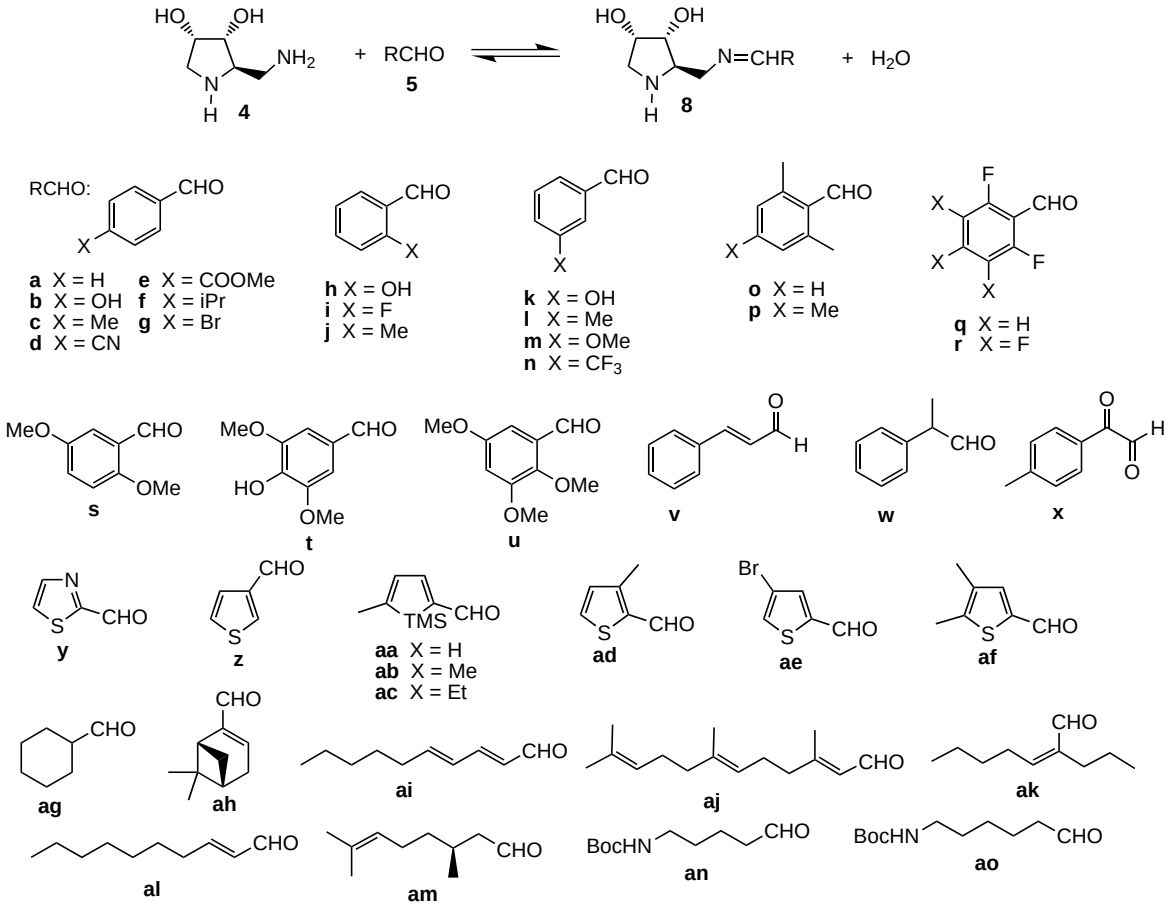
and derivatives and their individual testing. Since diamines of type **3** have shown promising properties, we wondered whether the corresponding imines, obtained by mixing the unprotected diamine **4** with aldehydes, would have α-mannosidase inhibitory activities and could eventually act as models for the corresponding diamines. An efficient discovery method would thus be realized if the imines could be generated in a few hours under the high dilution conditions typical of the assays (<1 mM, 0.2 mL of solution) used to detect glycosidase inhibitors. Such an assay would allow one to evaluate a large number of imines rapidly by using minute amounts of amines and aldehydes mixed in the wells of multiwell plates.

One of the difficulties with this plan is that equilibrium constants for the formation of imines might be too small.[9] Thus, in the reaction of amines with aldehydes to form imines and water, equilibrium would have to be obtained at relatively high amine and aldehyde concentrations. Although this method would require larger amounts of each amine and aldehyde, it would not be a problem if, under the pH conditions of the enzymatic assay, dilution did not lead to fast re-equilibration with the consequence of near disappearance of the imines.

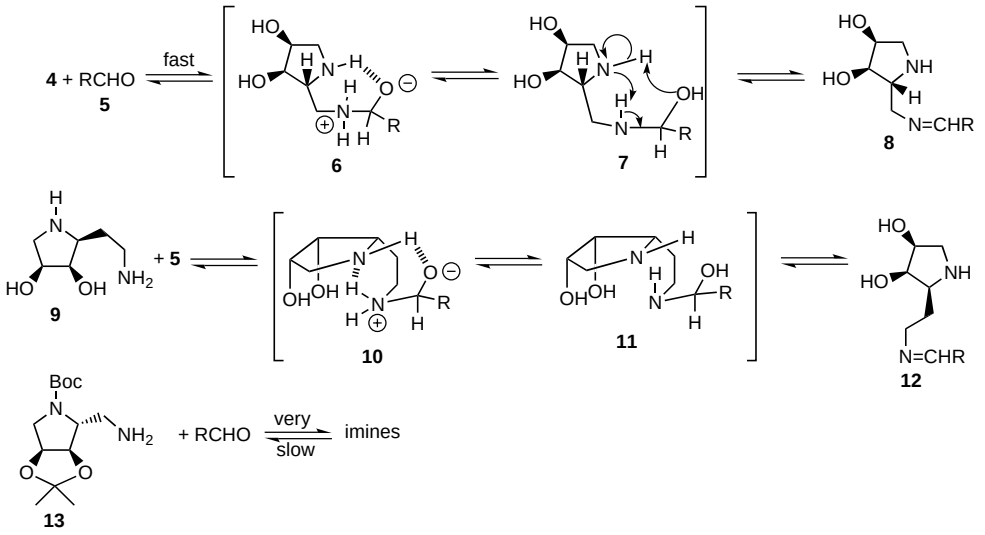
Alternatively, if equilibria are established rapidly under dilute conditions, the mixtures of imines that result from combinations of amine and aldehyde sublibraries could be incubated with the enzyme. The enzyme is expected to bind preferentially to the imine that is the best inhibitor (the solution forms a dynamic library[10] of imines) and thus a rapid assay of a large number of imines should be possible. We have observed that the unprotected diamine **4**[7] reacts rapidly with all kinds of aldehydes **5** (Scheme 2) at pH 8 and these reagents equilibrate with the corresponding imines **8**. For instance, a solution of **4** (5 mM) and benzaldehyde (5 mM) in water generates imine **8a** in less than one hour at 25 °C. ^{1}H NMR spectroscopy showed that less than 5% of the solution consists of each of the reagents **4** and PhCHO, which suggests a minimum equilibrium constant $K_{8a} > 4.2 \times 10^6$ [Eq. (1)].

$$K = \frac{[\text{imine}][H_2O]}{[\text{amine}][\text{aldehyde}]} \quad (1)$$

Under similar conditions, a solution of diamine **9** (2 mM) and benzaldehyde (8 mM) in water equilibrates with imine **12a** after 24 hours at 25 °C (Scheme 3). The ^{1}H NMR spectrum of this solution shows less than 10% compound **9** to be present and thus suggests a minimum equilibrium constant of $K_{12a} > 8 \times 10^4$. In contrast, the semiprotected diamine **13** did not react with aldehydes in solution at pH 8. For instance, a solution of **13** (5 mM) and benzaldehyde (10 mM) in D_2O did not give a trace of



Scheme 2. *Mixture of diamine **4** with a sublibrary of aldehydes **5**. These aldehydes did not inhibit jack bean α-mannosidase at 1 mM concentration. Boc = tert-butoxycarbonyl.*



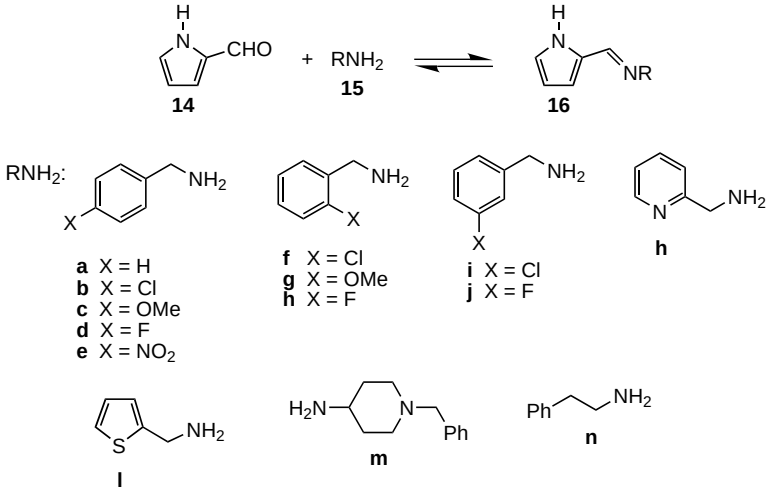
Scheme 3. *Interpretation of the relative rates of imine formation through autocatalysis by proximal secondary amines.*

the corresponding imine after 24 h at 25 °C. As expected,[11] the unprotected secondary amine β-amino and δ-amino moieties in diamines **4** and **9**, respectively, autocatalyze their addition to aldehydes to give first α-hydroxylamines **7** and **11** and then the products **8** and **12**, respectively, by water elimination from these intermediates (Scheme 3). We also examined the reactivity of pyrrol-2-carbaldehyde (**14**) towards all kinds of primary amines **15** and found that it equilibrates with the corresponding imines **16** in D_2O at 25 °C (Scheme 4). ^{1}H NMR spectroscopy after 24 h gave $K > 2.2 \times 10^4$ for **14** and benzylamine.

A 0.25 mM solution of diamine **4** leads to 58% inhibition of α-mannosidase from jack beans[12] at the optimal pH value (pH = 5; 0.02 U mL^{-1} enzyme, substrate: *p*-nitrophenyl α-mannopyranoside[13]). A mixture of **4** (0.25 mM) and ethanal, propanal, butanal, pentanal, and hexanal (0.25 mM each) was left at 25 °C for 24 h (pH 8) and then buffered (pH 5). The inhibitory activity of this mixture (about 65%) was not significantly better than for pure **4**. When mixtures containing aromatic aldehydes (0.25 mM) were prepared, significantly higher inhibitory activity was detected. For the aldehydes **5** listed in Scheme 2, mixtures composed of only one aldehyde and **4** were prepared in the wells of 96-well plates and analyzed after 24 h as above. The activities shown in Figure 1 were measured. These values were the same after 5 minutes or 30 minutes of incubation with the enzyme. The highest activities were found for benzaldehyde and its substituted derivatives. None of the aldehydes used in this work were inhibitors of jack bean α-mannosidase at concentrations of less than 1 mM. We repeated the same assays with mixtures that contained diamine **9** (0.21 mM) and each of the aldehydes (0.21 mM) listed in Scheme 2. Pure **9** showed 13% inhibition (Figure 2). The best mixture was that with salicylaldehyde, which led to 32% inhibition, still a much smaller inhibitory activity than that observed for pure **4** and for mixtures of **4** with aromatic aldehydes.

We also evaluated the inhibitory activities of a mixture of pyrrol-2-carbaldehyde (**14**; 0.5 mM) and the primary amines **15** (0.5 mM) listed in Scheme 4. Significant inhibitory activities towards jack bean α-mannosidase were not found. The highest inhibitory activity (13%) was observed with 2-fluorobenzylamine (**15h**). No activity was detected with 4-nitrobenzylamine (**15e**) or 2-phenylethylamine (**15n**). Neither aldehyde **13** nor amine **14** was an inhibitor of jack bean α-mannosidase at a concentration of 1 mM .[14]

We had previously found that diamines **3** and **17** (Scheme 5) are competitive inhibitors of α-mannosidases.[7] At 1 mM concentration, 92% inhibition (inhibition equilibrium constant $K_i = 7$ μM) and 89% inhibition ($K_i = 26$ μM) of jack bean α-mannosidase by these diamines was observed, respectively. These inhibitory activities seem to parallel those observed for the related imines **8a** and **8aa**, respectively. To test this hypothesis further, we prepared diamines **20c** and **20m** from aldehyde **23**[15] (as shown in Scheme 5). The related imines **8c** and **8m** in equilibrium with **4**, 4-methylbenzaldehyde, and



Scheme 4. *Mixture of pyrrol-2-carbaldehyde **14** and primary amines **15**.*

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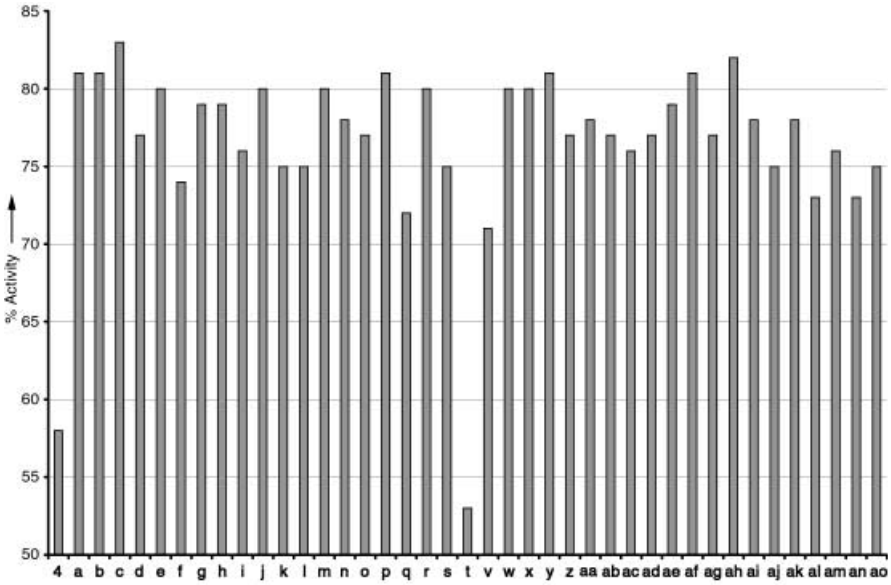


Figure 1. *Inhibitory activities toward jack bean α-mannosidase (pH 5, 25 °C) measured for imines **8a–ae** (0.25 mM) mixed with diamine **4** (0.25 mM) and aldehydes **5** (0.25 mM).*

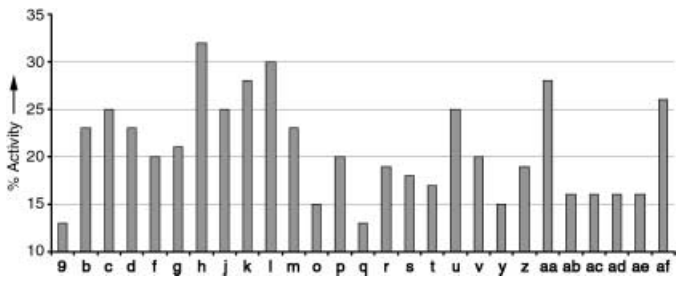
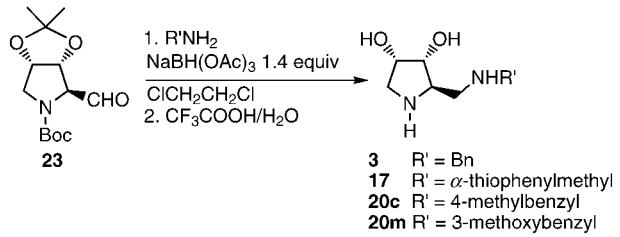
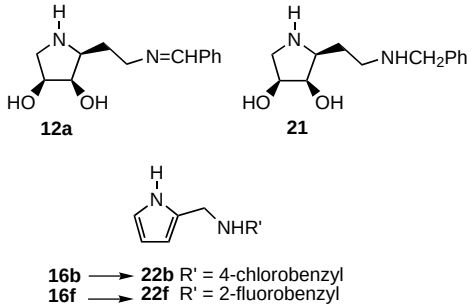


Figure 2. *Inhibitory activities toward jack bean α-mannosidase (pH 5, 25 °C) measured for imines **12b–af** (0.21 mM) mixed with diamine **9** (0.21 mM) and aldehydes **5** (0.21 mM).*



Scheme 5. *Preparation of α-mannosidase inhibitors.*

3-methoxybenzaldehyde gave high inhibitory activities (Figure 1). Both diamines **20c** and **20m** were assayed for their inhibitory activities with respect to jack bean α-mannosidase and were found to be competitive inhibitors with $K_i = 3.0$ μM and 9.2 μM, respectively.[12] Imine **8c** was also independently prepared, isolated, and assayed for its inhibitory activity toward jack bean α-mannosidase. 87% inhibition was observed (concentration required for 50% inhibition $IC_{50} = 130$ μM, $K_i = 29.1$ μM). This experiment confirms that our method establishes a parallel between the activity of the imine and that of the related diamine. Moreover, under the concentration and pH conditions used for the test, equilibrium is reached within the incubation time. In order to test whether our method of discovery of glycosidase inhibitors would lead us to miss potential inhibitors, we prepared pure imine **12a** from diamine **9** and benzaldehyde and assayed it. At 1 mM concentration, it showed 70% inhibition of jack bean α-mannosidase ($IC_{50} = 350$ μM, $K_i = 148$ μM, competitive). The diamine **21** that results from the reduction of **12a** ($NaBH_4$, MeOH, RT) also inhibited the same enzyme (29% inhibition at 1 mM concentration). Pure imines **16b**, **16f**, and the corresponding diamines **22b** and **22f**, were made and assayed (Scheme 6). None of these products showed inhibitory activity toward jack bean α-mannosidase at 1 mM concentration.



Scheme 6. *Reduction of inhibitory imine **12a** gives a diamine **21** which is also an inhibitor of jack bean α-mannosidase. The inactive imines **16b** and **16f** give the corresponding diamines **22b** and **22f**, which show no such inhibitory activity.*

We expect the method presented in this report to be applicable to the rapid discovery of inhibitors of all kind of enzymes. Imines can model the inhibitory activities of the corresponding amines. If the imines are formed rapidly from sublibraries of amines and aldehydes, minute amounts of reagents are required in each enzymatic assay.

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A Three-State Mechanism of Integrin Activation and Signal Transduction for Integrin $\alpha_v\beta_3$

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KEYWORDS:

docking · drug research · integrins · signal transduction · molecular modeling

Integrins are membrane-spanning heterodimeric receptors which couple signal transduction with cell matrix adhesion. Several integrins recognize the tripeptide sequence in RGD-containing peptides and proteins. However, the atomic detail of the ligand binding site is currently unknown. Starting from the crystal structure of the extracellular domains of the integrin $\alpha_v\beta_3$, we develop a three-state mechanism of integrin activation based on Ca^{2+} displacement upon ligand binding. We present a detailed working model of the highly important but poorly understood mechanism of an integrin signaling event. Additionally, we describe the ligand binding site in atomic detail, which might be a first step in future rational drug design attempts.

In a recent pioneering work, Xiong et al. solved the crystal structure of the extracellular domains of $\alpha_v\beta_3$ (Protein Data Bank (pdb) entry 1JV2),[1] which provides the possibility of looking at integrin-activating processes at the atomic level (Figure 1 A). Currently, the consensus model for integrin activation and signal transduction is a two-state mechanism: a low-affinity resting state (state one) is activated by intracellular events that lead to a high-affinity binding state (state two) after a conformational change. This activation process involves the separation of the α_v propeller domain and the β_3 βA domain[2–4] (the so-called head groups, Figure 1 B). However, biochemical investigations also support a model with multiple activation states.[5, 6] Here, we start from the crystal structure of the extracellular domains of $\alpha_v\beta_3$ and use automated docking and molecular modeling to develop a three-state mechanism involving allosteric effects triggered by a ligand-induced shift of a Ca^{2+} ion. We introduce an additional step before dissociation of the head groups, which corresponds to the crystal structure and constitutes a high-affinity state.

In our model, the inactive state (state one, Figure 1 C, left) is locked in its position by intracellular interactions.[7] Proteins binding at intracellular domains[4, 8] trigger a rotational movement of an integrin subunit, which activates the integrin. This activated or preconditioned state (state two, Figure 1 C, middle)

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