Automated Combinatorial Chemistry in the Organic Chemistry Majors Laboratory

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The use of combinatorial chemistry to generate libraries of compounds with identical core functionality is ubiquitous within the pharmaceutical research industry *(1−3)*. This approach, combined with modern rapid-throughput biological assays *(4)*, allows for efficient identification of lead structures. This *Journal* has published several laboratory exercises that involve combinatorial chemistry, including the synthesis of esters *(5, 6)*, amides *(7)*, oligopeptides *(8)*, and hydrazones *(9)*. Modern industrial combinatorial chemistry also incorporates a significant amount of automated instrumentation in the preparation, purification, and analysis of products. Some effort has been made to implement automation in the undergraduate laboratory experience *(10, 11)*, and experiments using automated flow-injection techniques for protein analysis *(12)* and chemiluminescence detection *(13)* have recently been developed. Since many of our chemistry majors will work in industry, an opportunity to become familiar with this type of instrumentation is valuable.

A reaction that is taught in a one-year introductory course in organic chemistry is the generation of a hydrazone **3** by the reaction of a hydrazine **1** with an aldehyde **2** (Scheme 1). Formation of the hydrazones is typically rapid, and equilibrium significantly favors the product *(14)*.

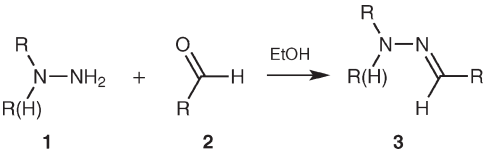
In this experiment *(15)*, students use a Gilson-215 liquid-handling robot to make an array of 48 hydrazones from a combination of 8 aldehydes and 6 hydrazines. They then test the compounds for antibacterial activity in a Kirby−Bauer disk diffusion assay *(16)*, a simple and common assay found in typical microbiology textbooks that involves growing bacteria on an agar plate in the presence of disks impregnated with test compounds *(17)*. If the organism is susceptible to the compound being tested, a large circular "zone of inhibition" with no bacterial growth will appear on the agar plate after incubation. Since the test results can be affected by factors such as the diffusion rate of the compound through agar, the diameter of the zone of inhibition cannot be directly correlated with antibiotic activity. Nevertheless, the assay works well enough to classify an organism as susceptible, intermediate, or resistant to a particular antibiotic compound.

Timetable

This experimental sequence has been implemented in a laboratory course specifically designed for chemistry majors who are taking the second-semester of organic chemistry. It has a typical enrollment of 7−12 students, and the class meets for two 3 h periods each week. The entire experimental sequence took four complete laboratory periods plus the first 20 min of another period:

- Period 1: Working as a group, the students prepare 38 stock solutions of the starting materials, and some students start using the robot to make their libraries.
- Period 2: The remaining students use the robot to make libraries.
- Period 3: The students assay their libraries with thin-layer chromatography (TLC). They use premade agar plates (2 each) to run antibiotic tests on the 14 starting materials and prepare more agar plates (7−8 each) for use in the next period.
- Period 4: The students measure and record zones of inhibition for the starting materials. Using the agar plates they prepared in period 3, they run Kirby−Bauer tests on their library of 48 compounds.
- Period 5: Students record the zones of inhibition for their libraries.

Scheme 1. Formation of a Hydrazone **3**



1 **2** **3**

Experimental Procedure

Before making the libraries, stock solutions of the 17 aldehydes and 21 hydrazines (Table 1) had to be prepared. As a group, students weighed out 0.900 mmol of each compound (plus or minus 1%) into a 100 mL Qorpak screw-cap bottle. Working as a group allowed each student to prepare 3−6 stock solutions to share, rather than 14 for their individual use. The Gilson-215 liquid-handling robot was then used to add 30.00 mL of ethanol to all but four of the starting materials. Four of the hydrazines were not soluble in ethanol, so either a 50:50 ethanol/water mixture or distilled water were used to dissolve them. The bottles were then manually shaken to produce a 0.0300 M solution of each compound.

Each student then selected 8 aldehydes and 6 hydrazines to make the 48 hydrazones in their library. They transferred 5 mL

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Table 1. Hydrazines and Aldehydes Used in the Combinatorial Laboratoy

Hydrazines	Soluble in	Aldehydes	Soluble in
Ethyl carbazate	Ethanol	2-Furaldehyde	Ethanol
1-Amino-4-methylpiperazine	Ethanol	*p*-Tolualdehyde	Ethanol
Aminoguanidine bicarbonate	Eater	*o*-Tolualdehyde	Ethanol
Phenylhydrazine·HCl	Ethanol	2-Fluorobenzaldehyde	Ethanol
4-Nitrophenylhydrazine	Ethanol	*p*-Anisaldehyde	Ethanol
p-Tolylhydrazine·HCl	Ethanol	*o*-Anisaldehyde	Ethanol
m-Tolylhydrazine·HCl	Ethanol	3-Chlorobenzaldehyde	Ethanol
o-Tolylhydrazine·HCl	Ethanol	2-Chlorobenzaldehyde	Ethanol
4-Cyanophenylhydrazine·HCl	1:1 E/W[a]	4-Chlorobenzaldehyde	Ethanol
2-Ethylphenylhydrazine·HCl	Ethanol	5-Nitro-2-furaldehyde	Ethanol
2,5-Dichlorophenylhydrazine	Ethanol	*p*-Dimethylaminobenzaldehyde	Ethanol
2-Chlorophenylhydrazine·HCl	Ethanol	Piperonal	Ethanol
4-Chlorophenylhydrazine·HCl	Ethanol	3-Nitrobenzaldehyde	Ethanol
4-Isopropylphenylhydrazine·HCl	Ethanol	2-Nitrobenzaldehyde	Ethanol
2-Hydrazinobenzoic acid·HCl	Ethanol	1-Naphthaldehyde	Ethanol
3-Nitrophenylhydraazine·HCl	1:1 E/W[a]	3,4-Dimethoxybenzaldehyde	Ethanol
4-Chloro-*o*-tolylhydrazine·HCl	Ethanol	4-Chloro-3-nitrobenzaldehyde	Ethanol
Pentafluorophenylhydrazine	Ethanol		
1,1-Diphenylhydrazine·HCl	1:1 E/W[a]		
2-Bromophenylhydrazine·HCl	Ethanol		
4-Bromophenylhydrazine·HCl	Ethanol		

[a] E/W is ethanol/water.

	1	2	3	4	5	6
A	A1 + H1	A1 + H2	A1 + H3	A1 + H4	A1 + H5	A1 + H6
B	A2 + H1	A2 + H2	A2 + H3	A2 + H4	A2 + H5	A2 + H6
C	A3 + H1	A3 + H2	A3 + H3	A3 + H4	A3 + H5	A3 + H6
D	A4 + H1	A4 + H2	A4 + H3	A4 + H4	A4 + H5	A4 + H6
E	A5 + H1	A5 + H2	A5 + H3	A5 + H4	A5 + H5	A5 + H6
F	A6 + H1	A6 + H2	A6 + H3	A6 + H4	A6 + H5	A6 + H6
G	A7 + H1	A7 + H2	A7 + H3	A7 + H4	A7 + H5	A7 + H6
H	A8 + H1	A8 + H2	A8 + H3	A8 + H4	A8 + H5	A8 + H6

Figure 1. Layout of microtiter plate (A1−A8 are aldehydes and H1−H6 are hydrazines).

of each of the 14 stock solutions to a screw-cap test tube, and these were put onto the robot's deck. The robot then filled half of a 96-well microtiter plate with 0.250 mL of each hydrazine and each aldehyde, making the 48 different combinations by adding the aldehydes across 8 rows and the hydrazines down 6 columns (Figure 1). The plate was sealed and shaken overnight.

To assess the success of the reactions, students ran TLC on the solution in each of the 48 wells, visualizing the results with a UV lamp.

To test the antibiotic activity of their products, students perform a Kirby−Bauer disk diffusion assay on each of the 48 products and 14 starting materials, using 10 μL of solution (5 μL for the starting materials, since they are twice as concentrated) pipetted onto a 5 mm disk of sterile filter paper. Those were placed onto the surface of a tryptic soy agar Petri dish that had been inoculated with one of two test organisms, the Gram-negative *Escherichia coli* or the Gram-positive *Staphylococcus epidermidis*. After incubation at 37 °C for 24 h, the plates were refrigerated to arrest growth, and the zones of inhibition for each compound were measured during the next available class.

Hazards

All of the hydrazines are either toxic, harmful, or irritants. 4-Isopropylphenylhydrazine·HCl and 4-nitrophenylhydrazine·HCl can explode if heated. Both *o*- and *p*-tolylhydrazine are listed as possible carcinogens. Nearly all of the aldehydes are either toxic, harmful, or irritants, and the liquid aldehydes are flammable. 2-Chlorobenzaldehyde is corrosive, and 2-furaldehyde is listed as a possible carcinogen. 3-Nitrobenzaldehyde is listed as a possible teratogen. Ethanol is flammable and an irritant. Powdered tryptic soy agar is a mild irritant.

Results

Once the reactions were complete, students noticed a colorful array of wells, some of which contained precipitates. These were presumably the hydrazone products, and thus their

Table 2. Zones of Inhibition for the Combinatorial Laboratory[a]

	Staphylococcus epidermidis (2007)	Aldehyde only	1 Ethyl carbazate	2 1-Amino-4-methylpiperazine	3 Aminoguanidine bicarbonate	4 Phenylhydrazine HCl	5 4-Nitrophenylhydrazine	6 *p*-Tolylhydrazine HCl	7 *m*-Tolylhydrazine HCl	8 *o*-Tolylhydrazine HCl	9 4-Cyanophenylhydrazine HCl	10 2-Ethylphenylhydrazine HCl	11 2,5-Dichlorophenylhydrazine	12 2-Chlorophenylhydrazine HCl	13 4-Chlorophenylhydrazine HCl	14 4-Isopropylphenylhydrazine HCl	15 2-Hydrazinobenzoic acid HCl	16 3-Nitrophenylhydraazine HCl	17 4-Chloro-*o*-tolylhydrazine HCl	18 Pentafluorophenylhydrazine	19 1,1-Diphenylhydrazine HCl	20 2-Bromophenylhydrazine HCl	21 4-Bromophenylhydrazine HCl
	Hydrazine only	—	—	7,0,0	6,0	7,0,0	0	6	—	8,0,0	0,0	—	10,8,7	7	0	0	7	0	6,0	8,7	20,6	6,0	0
1	2-Furaldehyde	0	—	0	—	0	0	0	—	0	—	—	9	9	—	—	—	—	7	8	0	7	—
2	*p*-Tolualdehyde	—	—	—	—	—	—	0	—	—	—	—	—	0	—	—	—	—	9	7	0	7	—
3	*o*-Tolualdehyde	—	—	—	—	—	—	7	—	—	—	—	—	0	—	—	—	—	9	0	7	9	—
4	2-Fluorobenzaldehyde	0	—	—	—	—	—	9,0	—	—	—	—	—	10,0	—	—	—	—	8,6	10,0	15,7	8,0	—
5	*p*-Anisaldehyde	0,0	—	0	—	0	0	18,7	—	7	—	—	0	7,7	—	—	—	—	15,7	8,0	19,7	14,0	0
6	*o*-Anisaldehyde	9,7	—	0,0	9,0	8,0	—	10	—	7,7	8,7	—	10,9	10	—	—	—	—	10	7	0	11	—
7	3-Chlorobenzaldehyde	0	—	0	—	0	0	—	—	0	—	—	9	—	—	—	—	—	—	—	—	—	0
8	2-Chlorobenzaldehyde	0	—	0	—	0	0	—	—	7	—	—	0	—	—	—	—	—	—	—	—	—	0
9	4-Chlorobenzaldehyde	11,6,6,0	—	7,7,0	0,0	0,0,0	0	8	—	9,7,0	7,0	—	10,9,8	11	—	—	—	—	10	12	21	9	0
10	5-Nitro-2-furaldehyde	23,21,20,19	—	18,17	17,14	14,0	—	11	—	7,0	7,0	—	8,8	8	0	16	0	0	6,0	13,7	18,0	9,0	—
11	*p*-Dimethylaminobenzaldehyde	7,6,0,0,0	—	7,6,0	0,0	0,0,0	0	0	—	13,7,0	7,0	—	8,7,0	—	13	10	0	0	0	7	0	0	0
12	Piperonal	6,0,0,0	—	7,6	0,0	8,0	—	6	—	8,7	0,0	—	8,0	6	15	0	0	0	7,6	6,0	16,0	12,0	—
13	3-Nitrobenzaldehyde	8,7,6,0	—	12,8	10,0	0,0	—	7,7	—	7,6	7,6	—	8,6	6,0	16	0	10	0	9,7,0	7,7,0	22,7,0	8,0,0	—
14	2-Nitrobenzaldehyde	8,8,7,0	—	7,6,0	0,0	8,0,0	0		—	9,7,6	6,0	—	6,0,0	—	9	7	0	0	7	0	0	0	0
15	1-Naphthaldehyde	8,6	—	—	—	—	—	12,0	—	—	—	—	—	8,0	17	0	7	0	6,6,0	6,0,0	22,7,0	6,0,0	—
16	3,4-Dimethoxybenzaldehyde	n/a	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
17	4-Chloro-3-nitrobenzaldehyde	8,7,0	—	11,8,0	9,7	10,8,6	0	—	—	8,6,0	10,0	—	7,6,0	—	—	—	—	—	—	—	—	—	9

[a]The numbers are the diameters in mm of the inhibition zone on the agar plate. There can be multiple diameters if more than one student examined the combination. Zeros represent no inhibition observed, and the dash represents that no data was obtained.

actual concentrations in solution were less than the 0.0150 M that they were for those that did not form precipitates. This could have resulted in false negatives in the antibiotic screen.

TLC analysis of the 48 wells was done efficiently with 6 or 8 small TLC plates and with 1:1 hexane/ethyl acetate as the mobile phase. In general, over 80% of the TLC experiments showed only one spot, which is not surprising given the quantitative nature of the reaction. In some cases, the aldehydes were impure (contaminated with some of the corresponding carboxylic acid), so some unreacted hydrazine was present and TLC showed multiple spots. All of the aldehydes and all but two of the hydrazines were aromatic and thus easy to visualize due to their UV activity.

Students were asked to prepare two reports for this experiment. The preliminary report included a 6 × 8 grid that represented their particular library: the structure of each hydrazone was drawn in the correct space. This helped students understand the basic idea of combinatorial chemistry in that they were making a large number of compounds with the same core functionality with the same basic reaction. The report also included a table of zones of inhibition for their 14 starting materials and 48 products in a Microsoft Excel worksheet. After receiving the student data, the instructor compiled a large table (e.g., Table 2) of the diameters of the zones of inhibition (zero means no such zone was observed) for each organism. In their final report, the students used this table to discern any structure−activity relationships.

Student data showed some major inconsistencies, such as a zone of inhibition range of 0−22 mm (column 19, row 13) for the same compound. Also, since the students randomly selected their 14 starting materials, the global results include large gaps as well as overlap. Some of the starting materials had strong activity (e.g., 5-nitro-2-furaldehyde, row 10) and in many cases so did all or most of the hydrazones made from them; the activity may have been due to the hydrazone or from interference by the strongly active starting material still present. In a follow-up experiment, students could confirm a "hit" from this initial assay by doing a traditional hydrazone synthesis and purification *(19)* and then determining a minimum inhibitory concentration (MIC) using a dilution assay *(20)*.

One known but clinically obsolete antibiotic is included in the library: guanofuracin *(18)* is formed from the reaction of aminoguanidine and 5-nitro-2-furaldehyde (column 3, row 10 in Table 2). It showed one of largest zones of inhibition against *S. epidermidis* (Table 2) and *E. coli* (see the supporting information).

This experiment can be run successfully even if no liquid-handling robot is available. In fact, in one semester, the robot was out of order, and the students did the work of the robot: adding solvent to form the 38 stock solutions with a 30 mL volumetric pipet and using a calibrated Eppendorf pipet with disposable tips to dispense 250 μL of their starting materials into the 6 × 8 array of cells in the microtiter plate. This work, while somewhat repetitive, was not particularly time-consuming: eight students finished in one lab period even with only two Eppendorf pipets available.

Whether this experiment is carried out with the use of the automation technology or by using traditional pipetting techniques, the students gain valuable experience working at the chemistry−biology interface, functioning as a group, working with automation, and understanding the basics of combinatorial chemistry.

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Literature Cited

1. Borman, S. *Chem. Eng. News* **2004**, *82* (40), 32–40.
2. Borman, S. *Chem. Eng. News* **2003**, *81* (43), 45–56.
3. Pohl, N. L. *J. Chem. Educ.* **2000**, *77*, 1421–1423.
4. Armstrong, J. W. *J. Assoc. Lab. Automation* **1999**, *4*, 28–29.
5. Birney, D. M.; Starnes, S. D. *J. Chem. Educ.* **1999**, *76*, 1560–1561.
6. Nielsen, J. T.; Dragojlovic, V. *J. Chem. Educ.* **2004**, *81*, 1010–1015.
7. Miles, W. H.; Gelato, K. A.; Pompizzi, K. M.; Scarbinsky, A. M.; Albrecht, B. K.; Reynolds, E. R. *J. Chem. Educ.* **2001**, *78*, 540–542.
8. Truran, G. A.; Aiken, K. S.; Fleming, T. R.; Webb, P. J.; Markgraf, J. H. *J. Chem. Educ.* **2002**, *79*, 85–86.
9. Wolkenberg, S. E.; Su, A. I. *J. Chem. Educ.* **2001**, *78*, 784–785.
10. Legrand, M.; Foucard, A. *J. Chem. Educ.* **1978**, *55*, 767–771.
11. Bell, S. *J. Chem. Educ.* **2000**, *77*, 1624–1626.
12. Wolfe, C. A. C.; Oates, M. R.; Hage, D. S. *J. Chem. Educ.* **1998**, *75*, 1025–1028.
13. Economou, A.; Papagyris, D.; Stratis, J. *J. Chem. Educ.* **2004**, *81*, 406–410.
14. Nguyen, R.; Huc, I. *Chem. Commun.* **2003**, 942–943.
15. Nichols, C. J.; Hanne, L. F. Synthesis and Antibacterial Analysis of Hydrazone Libraries in the Organic Chemistry Laboratory. Poster presented at the CSUPERB Symposium, Los Angeles, CA, Jan 14, 2005.
16. Bauer, A. W.; Kirby, W. M. M.; Sherris, J. C.; Turck, M. *Am. J. Clin. Pathol.* **1966**, *45*, 493–496.
17. For example, see Forbes, B. A.; Sahm, D. F.; Weissfeld, A. S. *Bailey and Scott's Diagnostic Microbiology*, 10th ed.; Mosby: St. Louis, MO, 1998; pp 257−266.
18. World Health Organization. http://www.who.int/medicinedocs/es/d/Js4902e/4.1.181.html (accessed Oct 2009).
19. For example, see; Pasto, D.; Johnson, C.; Miller, M. *Experiments and Techniques in Organic Chemistry*; Prentice Hall: Englewood Cliffs, NJ, 1992; pp 328−333.
20. Forbes, B. A.; Sahm, D. F.; Weissfeld, A. S. *Bailey and Scott's Diagnostic Microbiology*, 10th ed.; Mosby: St. Louis, MO, 1998; pp 253−257.

Supporting Information Available

Student handout, student data tables, and instructor notes. This material is available via the Internet at http://pubs.acs.org.

