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[10] X-ray crystal structure determination of **2a**: $C_{90}H_{72}Cl_2Ir_2O_2P_4 \cdot CH_3OH$, $M_r = 1796.70$; crystal dimensions $0.43 \times 0.23 \times 0.13\ mm^3$; monoclinic, space group $C2$, $a = 17.722(3)$, $b = 16.201(4)$, $c = 16.424(3)$ Å; $\beta = 113.604(12)^\circ$, $V = 4321.0(16)$ Å^3, $Z = 2$, $\rho_{calcd} = 1.381\ g\,cm^{-3}$, $\mu = 3.258\ mm^{-1}$. Single crystals suitable for X-ray measurements were obtained by repeated recrystallization of **2a** from anhydrous methanol under argon. Because the surface of the pale yellow crystals became rough immediately when isolated from the mother liquor, presumably as a consequence of loss of the solvate methanol, the crystals were coated with silicon grease and sealed in glass capillaries containing methanol. Cell constants and an orientation matrix for data collection were determined by the least-squares refinement, using the setting angles of 25 reflections in the range $23^\circ < 2\theta < 25^\circ$ on a Rigaku diffractometer (AFC-7R) equipped with a Rotaflex rotating anode X-ray generator (50 kV, 200 mA) with monochromated $Mo_{K\alpha}$ radiation. For the data collection, reflections in the range 0 to $+h$, 0 to $+k$, $-l$ to $+l$, and the corresponding Friedel pairs (matrix: $(-1\ 0\ 0)$, $(0\ -1\ 0)$, $(0\ 0\ -1)$) were measured by the $\omega - 2\theta$ scan technique (scan speed $16.0^\circ\,min^{-1}$, scan width $(1.57 + 0.30\tan\theta)^\circ$). Three standard reflections were monitored every 200 reflections and showed a 7.19 % intensity decrease over the collection period. Intensity data were corrected for Lorentz and polarization effects. The absorption corrections ($T_{min} = 0.4874$, $T_{max} = 0.6545$) based on psi-scan data and a linear correction factor were applied for the raw data. A total of 10642 reflections ($3.0^\circ < 2\theta < 55.12^\circ$) were collected at 293(2) K, of which 9955 unique reflections ($R_{int} = 0.0171$) were used for the structure solution and refinement. The structure was solved by direct methods (SHELXS-86)[11] and weighted Fourier techniques[12] and refined on F^2 by full-matrix least-squares methods using SHELXL-93.[13] Non-hydrogen atoms of the cationic part were refined anisotropically. The chlorine atom of the anionic part and non-hydrogen atoms of methanol were refined isotropically. Hydrogen atoms of aromatic and methoxy groups were included in the refinement on calculated positions riding on their carrier atoms ($C{-}H_{sp^2} = 0.93$ Å, $C{-}H_{sp^3} = 0.96$ Å, $U_{iso}(H) = 1.2\,U_{eq}(C)$). The hydrogen atoms of the methyl group of the solvate methanol were located on a difference-Fourier map and fixed in the refinements. The OH hydrogen atoms of the methanol molecule were not included in the calculations. The function minimized was $[\Sigma w(F_o^2 - F_c^2)^2]$ ($w = 1/[\sigma^2(F_o^2) + (0.0534\,P)^2 + 1.6777\,P]$), where $P = (\max(F_o^2,0) + 2F_c^2)/3$ with $\sigma^2(F_o^2)$ from counting statistics. The functions $R1$ and $wR2$ were $(\Sigma\,||F_o| - |F_c||)/\Sigma\,|F_o|$ and $[\Sigma w(F_o^2 - F_c^2)^2/\Sigma(wF_o^4)]^{1/2}$, respectively; $R1 = 0.0290$ and $wR2 = 0.0838$ for 9226 reflections with $I > 2.0\sigma(I)$. Final R factors: $R1 = 0.0351$ and $wR2 = 0.0866$ for 9955 reflections (all data). The number of parameters is 455. Flack χ parameter shows $-0.018(7)$, and the absolute configuration of the binap ligand is R. GOF values are 1.153 for $I > 2.0\sigma(I)$ and 1.146 for all data. The maximum shift to esd ratio in the last full-matrix least-squares cycle was 0.000. The final difference Fourier map revealed a number of areas of residual electron density, the greatest of which corresponds to 1.109 e Å^{-3}. The minimum hole of the final difference Fourier map is -1.270 e Å^{-3}. For more details refer to the supporting information. Crystallographic data (excluding structure factors) for the structure reported in this paper have been deposited with the Cambridge Crystallographic Data Center as supplementary publication no. CCDC-101027. Copies of the data can be obtained free of charge on application to CCDC, 12 Union Road, Cambridge CB2 1EZ, UK (fax: (+44) 1223-336-033; e-mail: deposit@ccdc.cam.ac.uk). All calculations were carried out on Silicon Graphics SGI Power Challenge workstation at the Research Center for Protein Engineering, Institute for Protein Research, Osaka University.

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Micro-Scale Frontal Affinity Chromatography with Mass Spectrometric Detection: A New Method for the Screening of Compound Libraries

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The application of combinatorial chemistry to accelerated drug discovery has generated new problems in screening for potential therapeutics. Classical approaches of a one-compound, one-assay variety are often untenable because of time and resource constraints since the goal of combinatorial chemistry is to produce large libraries of compounds. These libraries are either generated on a solid phase (beads) or in solution.[1] A binding assay must be performed against a target at some point, and this usually means assaying either a large

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number of beads or a mixture of compounds in solution. The identification and isolation of receptors has allowed for radioligand binding and ELISA-type assays as primary screening strategies for such mixtures.[2] Iterative synthesis and analysis are often required in the determination of structure – activity relationships. It would be ideal to examine an entire library (or sublibrary) to identify and rank the constituent ligands, and to ascertain the absolute binding constants for each in a single analysis. Current assays do not provide this level of analysis.

Mass spectrometry is emerging as an important tool for the interrogation of combinatorial libraries. It has been used to assess library quality[3, 4] and, when coupled with molecular recognition technologies, has allowed for some success in the isolation and characterization of active library compounds.[5] Most of the approaches to combining molecular recognition with mass spectrometry have incorporated a "capture and release" methodology, where compound mixtures are applied to immobilized receptors, washed, and the high-affinity ligands stripped with a denaturing solution and presented to the mass spectrometer.[6] As an alternative, we have investigated the direct coupling of frontal affinity chromatography (FAC) with mass spectrometry.

In FAC a receptor is immobilized on a suitable support material and packed in a column.[7] A mixture containing potential ligands is *continuously* infused through the column, rather than injected in the conventional "spike" form. Active ligands will bind to the column, but eventually the capacity of the column will be exceeded, which results in the ligands breaking through at their infusion concentration. All non-retained compounds will break through earlier in the void volume of the system. Traditionally, this technique has been used for the characterization of chromatographic stationary phases, and the determination of thermodynamic and kinetic binding data of individual compounds.[8] There are several advantages to miniaturizing FAC columns and coupling them to an electrospray mass spectrometer: The requirement for the precious receptor is greatly reduced and the demands on the amounts of ligand are proportionally decreased. Electrospray mass spectrometry allows for the sensitive detection of compounds that break through the column (less than 1 pmol μL^{-1}), but more importantly it provides an extra dimension to the analysis, namely the m/z ratio. Conventional FAC columns are most often interfaced with a single wavelength UV/Vis spectrophotometer. Such a detector is one-dimensional in the sense that all solution components contribute to the measured intensity at a given point in time. In this case, trace components would be indistinguishable from the background created by the excess of other compounds. By using a mass spectrometer as a detector the opportunity is provided for a two-dimensional analysis, where even a trace component can be identified provided it appears at a m/z value measurably distinct from the other solution components.

To illustrate the potential of FAC/MS for the screening of mixtures we prepared a microcolumn containing an immobilized monoclonal antibody and coupled it to an electrospray mass spectrometer (Figure 1). The carbohydrate-binding antibody recognizes the 3,6-dideoxy-D-xylohexose (abequose) epitope in *Salmonella paratyphi* B O-antigens.[9, 10] The affinity-purified antibody was biotinylated, and then incubated with beads bearing covalently-coupled avidin. The beads were then slurry-packed into a 11.5 cm long poly(ether ether ketone) (PEEK) column with an internal diameter of 500 μm (ca. 23 μL column volume). In this design a tee served a dual role as a column end-fitting and mixing chamber for the column eluent and organic make-up flow. The column was then directly connected to an electrospray mass spectrometer. For operation in frontal analysis chromatography mode, the column was first flushed with ammonium acetate buffer (NH_4OAc, 2 mM, pH 6.7). The flow was then switched to a second solution containing a mixture of six oligosaccharides (Scheme 1) in NH_4OAc buffer, each present at 1 μM . This solution was chosen to model a simple combinatorial "library" of compounds. Three of these oligosaccharides (**1**–**3**) have no specificity for the antibody. The remaining three (**4**–**6**) contain the minimal requirement for recognition (abequose) and span a range of affinity for the antibody. The K_d values for the active ligands were determined independently by titration microcalorimetry (Table 1).

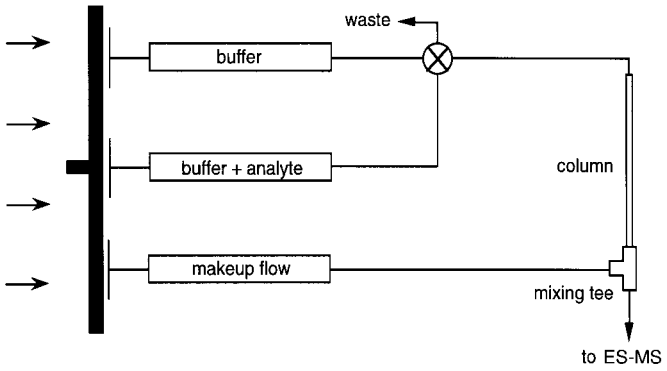

Figure 1. Schematic representation of the FAC/MS apparatus.

A total-ion chromatogram (TIC) was constructed from a 50 minute run time of this mixture through the column (Figure 2, top), which represents the consumption of only 400 pmol of each oligosaccharide. Peaks at specific m/z values could then be identified through the analysis of the mass spectra that give rise to the TIC, and selected ion chromatograms reconstructed from the TIC (Figure 2, middle). Mass spectra were generated from time-slices of the chromatogram and chart the progression of the various oligosaccharides through the column (Figure 2, bottom). Compounds **1**–**3** break through at the void volume (V_0), while compounds **4**–**6** break through later at volumes V_x, which relate to their concentrations and K_d values. The equation governing the breakthrough volume $V_x - V_0$ for the infusion of a single ligand can be determined by Equation (1), where B_t represents the dynamic binding capacity of the column and X the

$$V_x - V_0 = \frac{B_t}{[X]_0 + (K_d)_x} \quad (1)$$

compound with its corresponding dissociation constant K_d.[7] This simple equation indicates that once B_t and the concentration of the ligand are known, the dissociation constant can

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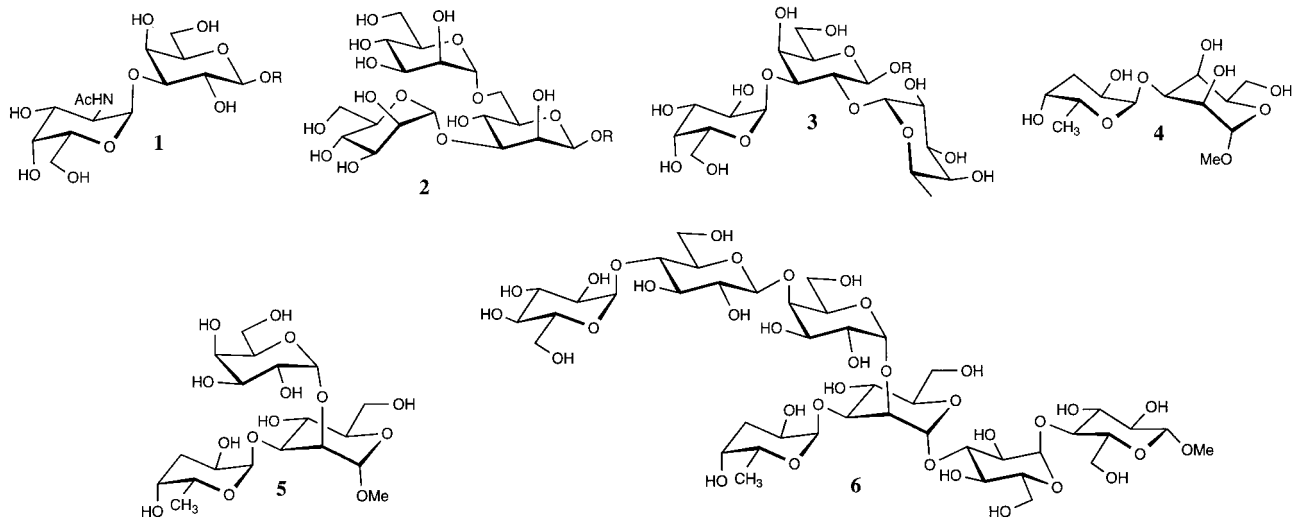

Scheme 1. Structures of the oligosaccharides used ($R = O(CH_2)_8COOMe$).

Table 1. Molecular weight and dissociation constants of the oligosaccharides **1**–**6**.

Oligosaccharide ($R = O(CH_2)_8CO_2Me$)	m/z $[(MNa)]^{+}$[a]	$K_d \pm s$ [μM][b] microcalorimetry[c]	FAC/MS individual[d]	FAC/MS mixture[e]
αGalNAc(1→3)βGal-OR (**1**)	576.3	–	–	–
αGal(1→3)[αFuc(1→2)]βGal-OR (**2**)	681.3	–	–	–
αMan(1→3)[αMan(1→6)]βMan-OR (**3**)	697.3	–	–	–
αAbe(1→3)αTal-OMe (**4**)	347.0	190	185 ± 17	178 ± 23
αGal(1→2)[αAbe(1→3)]αMan-OMe (**5**)	509.2	6.3	12.6 ± 1.3	10.2 ± 1.1
αGlc(1→4)βGlc(1→4)αGal(1→2)[αAbe(1→3)]-αMan(1→3)αGlc(1→4)βGlc-OMe (**6**)	1157.4	0.88	1.79 ± 0.20	1.71 ± 0.16

[a] Monoisotopic molecular weight of the singly charged sodium adduct. [b] Dissociation constant with the corresponding standard deviation, calculated from Equation (1). All values are determined from triplicate experiments, with the exception of the first FAC/MS entry for **5** (determined from Figure 3). The standard deviations s include the uncertainty in B_t. [c] Unpublished data, as determined by methods discussed in ref. 9. [d] Values determined from infusion of individual ligand, with compounds **1**–**3**. [e] Values determined from the infusion of the six-compound mixture.

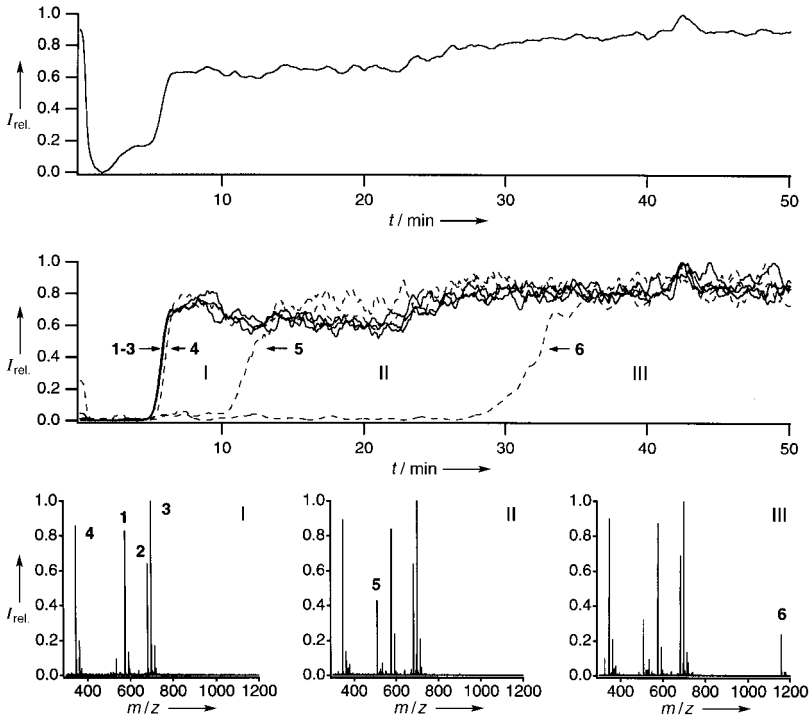

Figure 2. Extraction of data from a frontal chromatogram. Top: Total-ion chromatogram (TIC). Middle: Selected ion chromatograms of all six compounds. Compounds **1**–**3** break through simultaneously, as indicated by the solid line. Bottom: Spectra extracted from time-slices of the TIC. A spectrum representing the onset of break through of **4** is not shown.

be determined from a single measurement of its $V - V_0$. In order to determine B_t compound **5** was infused through the column at various concentrations and the corresponding $V - V_0$ values measured. A plot of $([\mathbf{5}](V - V_0))^{-1}$ versus $[\mathbf{5}]^{-1}$ was generated (Figure 3), where the reciprocal of the y intercept indicates a B_t of 554 pmol (analogous to a Lineweaver–Burk plot). Each antibody molecule contains two binding sites, therefore this corresponds to an active capacity of 277 pmol of protein (which represents ca. 100% of the total amount of protein bound). The negative reciprocal of the x intercept indicates a K_d of 12.6 μM for this compound, which is only a factor of two different from the value determined by microcalorimetry (Table 1).

Knowledge of the column capacity prior to the screening of a mixture, and control over the concentration of the mixture, allows the determination of dissociation constants for all ligands from a single frontal analysis chromatogram. For compounds with [X] much less than $(K_d)_x$, the K_d can be determined simply from Equation (1) even in the presence of stronger binders. For example, the K_d of

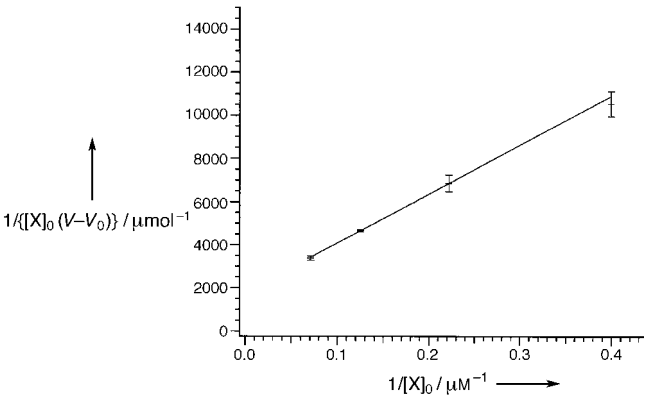

Figure 3. Determination of the column capacity and ligand binding affinity of **5** by FAC/MS, with a linear fit to the experimental data. Data points represent the average of three replicates, and the fit is weighted by the inverse of their standard deviations.

compound **4** as determined from the chromatogram of the multi-ligand mixture (Figure 2) is $178 \pm 23\,\mu M$, which is equivalent to the K_d determined individually ($185 \pm 17\,\mu M$, Table 1). The concentration of **5** was also sufficiently lower than its K_d ($12.6\,\mu M$) such that the equation can be used to calculate K_d from runs of the multi-ligand mixture. This value agrees with the one determined individually as well at the 95% confidence limit (Table 1). Furthermore, both compounds **4** and **5** are not present at concentrations high enough to compete with the binding of the strongest ligand (**6**), therefore its K_d could be determined from the mixture in a straightforward fashion from Equation (1). This is also verified by the equivalency between the values determined from the mixtures and individually (Table 1). It should be noted that if multi-ligand competition is expected the mixture can be diluted and re-run, such that conditions equivalent to this test case can usually be duplicated.

Of course, compounds that bind nonspecifically to column elements (walls, beads, or protein) would also demonstrate breakthrough volumes greater than the void volume of the system, and might give a false indication of activity. This can be easily ascertained. Library compounds that bind in a manner that affects the activity of the immobilized receptor will reduce the value of B_t. A known ligand that is flowed through the column, after the column has reached dynamic equilibrium with the mixture of library compounds, will generate a reduction in $V - V_0$ for this ligand, as it "sees" a lower B_t. The known ligand can be run through a fully active column to determine its expected $V - V_0$ value. Nonspecifically bound species would not give rise to a reduction in the $V - V_0$ value for this ligand, therefore an observed reduction indicates the presence of ligand(s) in the library.

The general FAC/MS approach is particularly advantageous in that a protein–ligand denaturing step is not required for the screening of the mixture or for the determination of dissociation constants. Column regeneration is achieved off-line by washing with a large volume of binding buffer, with or without a competitive ligand, thereby preserving the target receptor. The column used in this study was subjected to over 100 runs with no observable loss of activity and/or leaching of the antibody. Furthermore, not having to switch to a harsh denaturing buffer for compound elution has practical advantages for mass spectrometry. Electrospray detection only has to be optimized for one buffer system, rather than two or more.

This newly developed technique can also be used to accelerate the screening of compound libraries. Active components in mixtures can be both identified and ranked by their K_d value in a single chromatogram. An additional and very important advantage of this technique, relative to the traditional inhibition experiments, is that the molecular weight of the active compound is established, which effectively eliminates false positives that arise from reaction by-products or impurities. This approach should be widely applicable to other systems, and requires only the biotinylation of the receptor under investigation. The presence of isobaric compounds and/or discrimination between compounds in the generation of gas-phase ions[11] might limit the size of the library open to this approach; this will be investigated in future work.

Experimental Section

Affinity-purified antibody[10] (0.5 mg) was biotinylated with a biotin reagent containing a long chain spacer arm (NHS-LC-biotin, Pierce). The extent of biotin incorporation was monitored by matrix-assisted laser desorption/ionization, and the reaction was terminated at 14 biotins per immunoglobulin G (average).[12] The biotinylated antibody was then incubated with Ultralink immobilized avidin (25 μL; Pierce, cat. no. 53119) in bicarbonate buffer (pH 8.5) for 1 hour. The beads were thoroughly washed. A UV quantitation indicated that an immobilization of approximately 45 μg antibody per 25 μL beads was achieved.

All solutions were infused concurrently with a multi-syringe pump (PHD 2000, Harvard Apparatus) at a flow rate of $8\,\mu L\,min^{-1}$ per syringe (1-mL syringes). A Rheodyne valve (model 9725) was used for flow switching. The column eluent was combined with the make-up flow (10 vol% NH_4OAc buffer in acetonitrile) in the tee, to give a total flow rate of $16\,\mu L\,min^{-1}$ to the Hewlett–Packard series 1100 MSD electrospray single quadrupole mass spectrometer, operating in positive-ion mode. For the analysis the spectrometer was scanned from m/z 100–1500 in 2.5 seconds and the data stored in the standard peak maxima (condensed) mode. A chamber voltage of -4000 V with a grounded electrospray needle, N_2 drying gas flow rate of $4\,L\,min^{-1}$, and N_2 nebulizer pressure of 480 mbar were used. Breakthrough volumes were measured as midpoints in the selected ion chromatograms for each m/z value.

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A Convenient and General Method for Pd-Catalyzed Suzuki Cross-Couplings of Aryl Chlorides and Arylboronic Acids**

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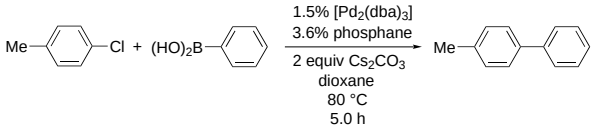
In memory of George Büchi

The Suzuki reaction has emerged as an extremely powerful method for the cross-coupling of aryl bromides, iodides, and triflates with arylboronic acids.[1] As compared with the Stille cross-coupling,[2] the Suzuki reaction possesses the practical advantages that the boron-containing by-products, unlike the tin-containing by-products of the Stille process, are nontoxic and are easily separated from the desired product.[3] One current limitation in the scope of the Suzuki reaction is its inefficiency when aryl chlorides are employed as substrates. Although there have been several accounts of Suzuki reactions of electron-poor aryl chlorides,[4–6] to the best of our knowledge there has been only one report of a coupling of an electron-neutral or an electron-rich aryl chloride (41% yield).[5i] In view of the increased availability and decreased expense of aryl chlorides relative to aryl bromides and iodides,[4a] this methodological gap becomes all the more significant. Herein we describe a solution to this problem: specifically, the development of conditions under which Suzuki cross-couplings of an array of electronically and sterically different aryl chlorides and arylboronic acids proceed in excellent yield with commercially available reagents (see Table 3).

In early experiments we established that 4-chlorotoluene and phenylboronic acid are efficiently cross-coupled in the presence of 1.5% $[Pd_2(dba)_3]$ (dba = dibenzylideneacetone), 3.6% P*t*Bu$_3$, and two equivalents of Cs_2CO_3 (dioxane, 80 °C; 86% yield by GC after 5.0 h; Table 1, entry 9). We observe little or no coupling in the absence of phosphane (entry 1) or in the presence of triarylphosphanes (entries 2 – 5) and chelating phosphanes (entries 6 and 7). Of the ligands that we have examined to date, only PCy_3 approaches P*t*Bu$_3$ in effectiveness (entries 8 and 9). , The use of a phosphane:Pd ratio between 1 and 1.5 appears to be optimal with P*t*Bu$_3$ as the ligand, an observation that suggests that the active palladium catalyst bears a single P*t*Bu$_3$ group. Thus, we believe that the steric bulk and the electron-richness of P*t*Bu$_3$ are critical for this unprecedented reactivity.[4, 7]

Table 1. Effect of phosphane on the palladium-catalyzed Suzuki cross-coupling of aryl chlorides.



Entry	Phosphane	Yield [%] (GC)
1	–	0
2	PPh_3	0
3	BINAP[a]	0
4	dppf[b]	0
5	P(*o*-tol)$_3$	10
6	$Ph_2P(CH_2)_3PPh_2$	0
7	$Cy_2P(CH_2)_2PCy_2$	0
8	PCy_3	75
9	P*t*Bu$_3$	86

[a] BINAP = 2,2′-bis(diphenylphosphanyl)-1,1′-binaphthyl. [b] dppf = 1,1-bis(diphenylphosphanyl)ferrocene.

Among the bases listed in Table 2, Cs_2CO_3 has proved to be the base of choice (entry 6).[8, 9] In the absence of base (entry 1) or in the presence of other bases commonly used in Suzuki reactions (entries 2 – 5), cross-coupling proceeds with diminished efficiency. In contrast to most Suzuki cross-couplings,[1] only one equivalent of base is required for our process (95% GC yield after five hours with 1.2 equiv Cs_2CO_3). We have found $[Pd_2(dba)_3]$ and dioxane to be best among the palladium sources (for example, $Pd(OAc)_2$) and solvents (for example, THF) that we have surveyed thus far.

A wide array of electronically and structurally diverse aryl chlorides and arylboronic acids can be cross-coupled very efficiently under these conditions (1.5% $[Pd_2(dba)_3]$, 3.6% P*t*Bu$_3$, 1.2 equiv Cs_2CO_3).[10] Thus, with respect to the aryl chloride, both electron-neutral 4-chlorotoluene and electron-

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