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Cross-referenced combinatorial libraries for the discovery of metal-complexing ligands: library deconvolution by LC-MS

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N-Acylthioureas are excellent ligands for a variety of heavy metals, but their metal selectivity is highly dependent on the precise nature of the substituents present. In this paper we show how combinatorial chemistry techniques can be used to establish relative affinities for copper within a mixture of 100 such thioureas. Following a straightforward synthesis, and copper extraction using standard liquid–liquid extraction techniques, LC-MS was used to identify the ligands which bind most strongly to the copper ions. Among the 100 ligands XC(O)N(Z)C(S)NHY, the most important substituent is the Y group bound to the NH: only aromatic Y substituents give strong binding to copper. The acyl X substituents are invariably aromatic, and an electron-rich X group is best; the affinity for copper seems to be less dependent on the Z substituent, although a large group such as benzyl disfavours copper binding. The five ligands from the library which bind copper most strongly have been clearly identified by a series of experiments: they all have aromatic groups in the Y position, but the X and Z substituents can be more varied. This is a very convincing demonstration of the power of combinatorial methods: to have found the same information by conventional methods would have required a lengthy and repetitive series of syntheses and investigations. In addition, our results give some preliminary evidence for synergistic binding of two different ligands, but this requires further investigation.

Introduction

N-Acylthioureas have been known for over 100 years as metal chelating agents,[1] but their potential for selective metal isolation is only now starting to be appreciated. Selective metal ion chelators have applications in several important areas, including environmental remediation of contaminated water, recycling heavy metals and extracting metals from low-grade ores. *N*-Acylthioureas have a number of advantages in these contexts. They are readily synthesized from cheap starting materials in only one or two steps. Their toxicity is relatively low, and they are stable over fairly long periods (over 1 year in our laboratory). Most important, they are selective for heavy metals, binding strongly to Cu and Ag in particular,[2,3] and do not extract such non-toxic, ubiquitous metals as the alkali and alkaline earth metals, iron or aluminium. These advantages are now beginning to be exploited. Ringmann and Schuster investigated the use of *N*-acylthioureas as precipitants for a variety of toxic metal ions, including Co, Ni, Cu, Zn, Cd and Pb; importantly, both ligands and metals can be recovered from many of the complexes.[4] However, a serious drawback for large-scale applications is that humic acids tend to interfere with precipitation of the complexes. This problem may well be overcome by immobilisation of the thioureas on a solid support, or by using liquid–liquid extraction technology. Koch and co-workers in South Africa have investigated these ligands for the selective extraction of individual platinum group metals from low-grade ores.[5] This is important since the metals tend to occur together, and current extraction techniques leave a low-grade residue which still contains valuable quantities of these expensive metals. However, the very diversity and selectivity of the ligands have created a further problem in exploiting their metal ion selectivity: that of identifying the optimum thiourea for the chosen application. Small changes in the substituent groups may have very striking, and often very subtle, effects on the affinity of the ligand for a particular metal ion, as illustrated by our own results described below. These changes may reflect the different binding modes available to these ligands.[2b,5] Thus Koch's group has made and tested over 50 individual *N*-acylthiourea ligands;[6] this is time consuming, and a better approach is urgently needed to reap the full benefits of these interesting ligands.

Combinatorial chemistry provides a solution to this problem, by allowing ligands to be tested as components of mixtures rather than as single compounds. Combinatorial approaches have thus far largely, although not exclusively,[7] been confined to biological problems,[8] and the advantages of combinatorial approaches need to be more widely recognised and applied. We have been studying the relative affinity of various *N*-acylthioureas for copper ions using combinatorial methods, and describe here how our methodology has established a structure–activity correlation among 100 *N*-acylthioureas. Our methodology involves treating mixtures or 'libraries' of ligands in solution with a sub-stoichiometric amount of copper, and using extensive LC-MS analysis for 'deconvolution', *i.e.*, to establish which ligands from the library bind most strongly to the metal. Solid-supported combinatorial libraries are much more common than libraries in solution, but the advantages of the latter are well known.[9] In our case, working with libraries in solution gives us immediate access to a range of well-established analytical techniques. The importance of the LC-MS analysis is that it enables us to work directly with the library mixtures to determine the optimum structure for metal binding. Partial separation of the mixture is achieved by the chromatography, and unambiguous identification of each ligand is then possible using both LC and MS data. Our methodology very rapidly establishes the relative affinities of the ligands; structural optimisation can thus be achieved far more rapidly than if each

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ligand had to be made and tested individually. Among the other strengths of the methodology is the possibility of discovering synergistic interactions between ligands. If optimum metal extraction occurs using a mixture of two ligands, this will be discovered very quickly using our methods, but may be missed altogether using conventional approaches. Our combinatorial method can in principle be applied to a wide range of metals and their ligands, or indeed in biological systems, provided a suitable assay is available, but is here applied to determining the optimum *N*-acylthiourea for copper complexation. Combinatorial chemistry has very rarely been applied before to ligand discovery, and the LC-MS deconvolution strategy is, to the best of our knowledge, new.

Experiment design

A combinatorial approach to problem solving breaks down into four parts: library synthesis, library analysis, assay and deconvolution. Commonly, 'combinatorial' approaches involve multiple parallel synthesis. These are ideal for applications based on high-throughput biological screens, which are amenable to automation, and can thus be performed rapidly and repetitively. Many assays, however, are time consuming, and are much more tedious to repeat numerous times. We therefore sought a method which would allow deconvolution based on a small number of assays, and which would also avoid the repeated 'iterative resynthesis' which is a common feature of truly combinatorial approaches. Our approach, which is based on indexed sub-libraries,[10] has two great strengths: it combines deconvolution with LC-MS library analysis[11] and it allows the investigation of a number of parameters following a single round of synthesis and using the same LC-MS data for deconvoluting all the results. The approach is outlined schematically in Fig. 1. The key requirements are a short, high-yielding synthesis, LC- or GC-MS analysis, an assay which physically separates the active library members from the inactive and an assay which will return the active library members unchanged. The methodology described below was carefully checked and validated by a number of control and background studies, outlined in the Results and discussion section.

Synthesis phase

Libraries of *N*-acylthioureas can be synthesised very straightforwardly in two steps, giving four points of diversity, labelled X, Y, Y′ and Z (Fig. 2). Thus treatment of an acid chloride XCOCl with ammonium thiocyanate followed by a primary or secondary amine YNH_2 or YY′NH generates thioureas **I** (Y′ = H) and **II** (Y′ ≠ H),[12] and treatment of these with sodium hydride followed by an alkylating agent introduces the final diversity into the library members **III** (Y′ = H) and **IV** (Y′ ≠ H). IR spectroscopy (see Experimental section) shows clearly that under the strongly basic conditions used, alkylation occurs on the central nitrogen, and not on sulfur as is general under non-basic conditions.[13] This conclusion was confirmed by ^{1}H NMR spectroscopy of a very wide range of thioureas of all types **I–IV**.[14]

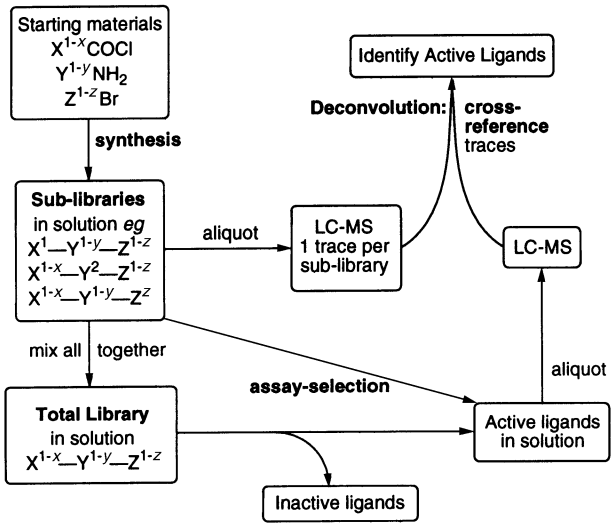

Fig. 1 Outline of cross-referencing approach.

In the first step, the main by-product is the amide derived from reaction of the amine directly with the acid chloride. Amide formation can clearly be minimised by allowing sufficient time for the acyl isothiocyanate intermediate to form; the amide can also be easily removed by column chromatography since it is considerably more polar than the desired thiourea. Although non-enolisable alkyl acid chlorides give good yields in the first step of the process, aromatic groups X proved necessary for efficient alkylation of **I** to **III** and **II** to **IV**.[15] In this step, the major by-product from thioureas **I** is the over-alkylated product **IV**, Y′ = Z. Fortunately, both this and the non-alkylated thioureas **I** and **II** are considerably more polar than the products **III**. Individual ligands **III** and also library mixtures can therefore be purified by filtration through a short (normal-phase) silica plug. The lower polarity of thioureas **III** relative to thioureas **IV** is presumably due to internal hydrogen bonding in the former.[16] The first step of the synthesis shown in Fig. 2 was generally complete after stirring at room temperature for a few hours; reactions on mixtures were therefore left stirring overnight. The second step can be driven to completion by by heating for a few hours, so reactions on mixtures were routinely heated to reflux overnight to ensure complete reaction. Individual products and small library mixtures from both steps were analysed by ^{1}H NMR in addition to LC-MS.

The orthogonal sub-libraries were synthesised as outlined in part in Fig. 3, and as described in full in the Experimental section. A split-mix technique was employed to avoid complications with kinetics which might otherwise be problematic when working with mixtures. For these initial trial studies, we used a small library of 100 compounds, derived from five acid chlorides XCOCl, five primary amines YNH_2 and four alkylating agents ZBr or ZI. We thus obtained a series of five 'X sub-libraries' each derived from a single acid chloride, a series of five 'Y sub-libraries' each derived from a single amine and a series of four 'Z sub-libraries' each derived from a single alkylating agent. Each of the 100 ligands was thus present in three sub-libraries, one X, one Y and one Z; the 14 sub-libraries each contained 20 or 25 compounds. The X, Y and Z substituents used are shown in Fig. 3. In addition to the amines shown below, a library was synthesised from a range of phenolic primary amines, but the solubility and decomplexation (see below) properties of the thioureas were not encouraging, and this library was not pursued further. Various ligands **II** and **IV** were also synthesised, but also proved unsuitable, for reasons discussed below.

Analysis of sub-libraries

The analysis of the library mixtures was investigated using GC-MS and LC-MS. Either method is appropriate to our combinato-

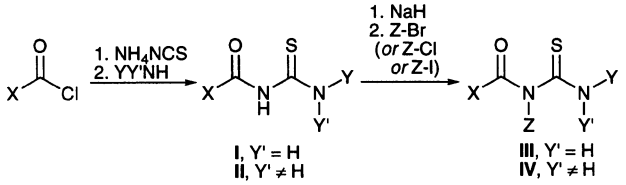

Fig. 2 Ligand synthesis.

rial approach, and some ligands **I** proved amenable to GC-MS analysis after silylation. Ligands **III**, however, were not, and LC-MS became the method of choice for analysis of these ligands. Incomplete and over-alkylation were readily detected, since the retention times of ligands **III** ($Z \neq H$) are markedly shorter than those of ligands **I** ($Z = H$). This enabled us to determine that alkylation using the four chosen alkylating agents was always complete following an overnight reflux, even with complex sub-library mixtures. Other impurities were not observed in these sub-library mixtures.

All 14 sub-libraries, of up to 25 compounds, were analysed by LC-MS. LC separation was of course incomplete, but using frit-FAB ionisation, each ligand **III** gave a protonated molecule M + H. The smallest significant fragment was attributable to the group $XCHO^+$, derived from the original acid chloride. Fragmentation occurred by several routes, most usefully by loss of the Z group, particularly when Z was benzyl or allyl, and by fragmentation of the nitro group when present. Each of the 100 ligands possesses a unique combination of retention time and molecular mass, and confirmation of a compound's identity is available from the isotope and/or fragmentation pattern. Following LC-MS analysis, the sub-libraries could be mixed in appropriate proportions, based on average molecular mass, to obtain the complete library of 100 ligands. Although the LC analyses, based on total ion current (TIC), were of low resolution and did not give quantitative measurements of the relative proportions of the individual ligands, small mixtures (*e.g.*, before the final mix in Fig. 3) could be quantitatively analysed by ^{1}H NMR spectroscopy, to check that the ligands were present in close to equimolar quantities.

Assay-selection procedure

It was necessary to establish a protocol for the physical separation of the active, copper-binding ligands from the less active or inactive components of the library mixtures. We investigated a range of possibilities for the assay-selection procedure; the chosen method is outlined in Fig. 4. Thus the library or sub-library mixtures were treated with a small quantity of aqueous copper sulfate, adjusted to pH 10,[2c] and containing acetate ions to prevent formation of insoluble copper hydroxide. The amount of Cu^{2+} was chosen to correspond to that of five ligands in the library or sub-library mixture, to ensure that only a small number of the best ligands would be selectively bound. As stated above, the first requirement on the assay-selection procedure is to separate the active from the inactive species. The simplest way to achieve this would be by filtration, but this requires that all copper complexes be insoluble even at low concentration. Instead, we preferred separation by filtration through neutral alumina: the free ligands elute very rapidly using 5–10% of ethyl acetate in light petroleum, and complete elution of the complexes can be ensured by switching to 5–10% of methanol in ethyl acetate. This caused partial dissociation of the copper from the active ligands in some cases, but this is rather an advantage than otherwise since the free ligands must be recovered for deconvolution (see below).

Deconvolution

For deconvolution, the free 'active' ligands must be recovered from their complexes. This allows analysis by LC-MS and comparison with the original sub-library analyses. For release of the ligands from the copper complexes, the complexes were dissolved in diethyl ether or dichloromethane and treated with ethylenediammonium acetate (en.2AcOH) in aqueous solution. Control studies showed minimal degradation of the free ligands under these conditions, with complete release of the copper into the aqueous phase (measured by AAS and UV). The recovered free 'active' ligands, which remained in the organic phase, were then submitted to LC-MS analysis, under the same conditions (eluent, flow rate, ionisation) as previously used for the sub-library mixtures, and the chromatograms thus obtained were

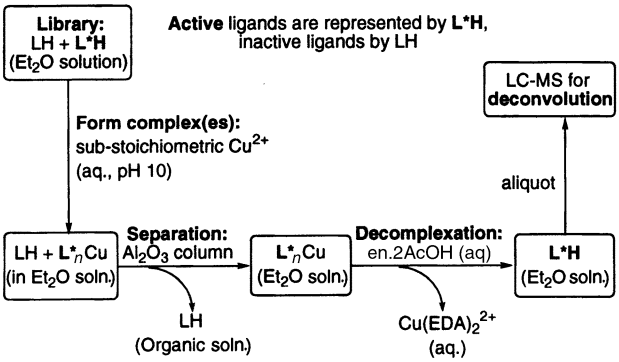

Fig. 4 Outline of assay-selection procedure.

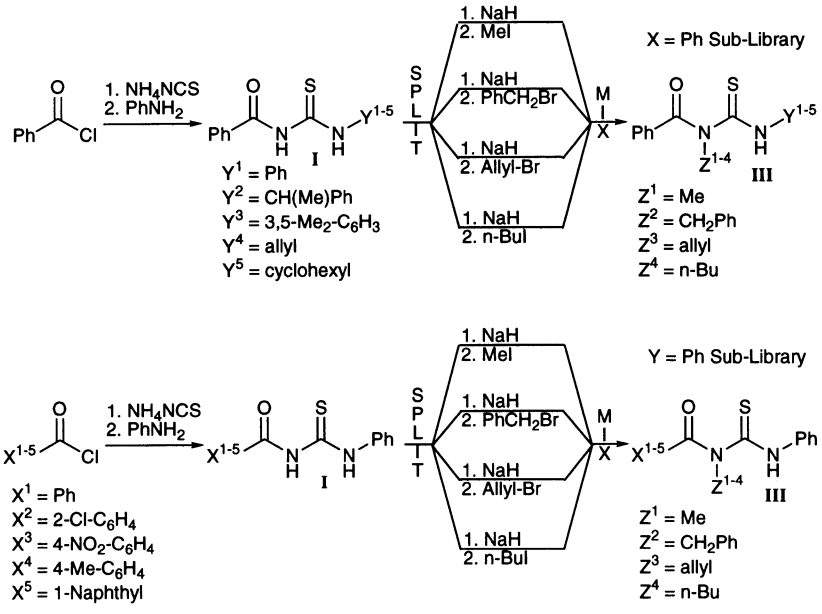

Fig. 3 Split-mix sub-library synthesis.

cross-referenced with the original sub-library results. The active ligands could thus be identified by their unique combinations of retention time and molecular mass, as previously established, and again the isotope and/or fragmentation patterns were available for confirmation.

Experimental

General experimental procedures

Tetrahydrofuran (THF) was dried by distillation from sodium wire. Dichloromethane was dried by distillation from calcium hydride. NMR spectra were obtained on a Bruker DPX 400 instrument, operating at 400 MHz for ^{1}H spectra and 100 MHz for ^{13}C spectra. Samples were dissolved in $CDCl_3$ and chemical shifts (δ) are given in ppm relative to chloroform or to added tetramethylsilane. IR spectra were obtained on KBr disks, using a Perkin-Elmer Model 410 spectrometer. HPLC was carried out using a Spectra-Physics P4000 pump used isocratically and a normal-phase Capital HPLC SI-KL5-21860 column, 15 cm × 4.6 mm id, packed with silica, with a UV detector set to 254 nm. LC-MS was carried out using a Thermo Separation Products P100 isocratic pump and a Hichrom S5CN-3287 column, 20 cm × 4.6 mm id, packed with Spherisorb S5CN, and a Jeol JMS.700 spectrometer operating in the frit-FAB mode, using glycerol as the matrix, introduced by a Shimadzu LC-6A isocratic pump. UV-VIS analysis was carried out using a Shimadzu UV-1601 spectrophotometer and AAS using a Varian SpectrAA-10 flame spectrometer.

Synthesis of single *N*-acylthioureas *I*. According to the method of Zhang *et al.*[12] the appropriate aromatic acid chloride (10 mmol) was added to a suspension of powdered, freshly recrystallised ammonium thiocyanate (1.14 g, 15 mmol) and polyethylene glycol (PEG-400) (0.18 g) in anhydrous dichloromethane (20 ml), and the suspension stirred at room temperature for 1 h. The appropriate primary amine (9.5 mmol) was added, and stirring was continued at room temperature for 1 h, or until TLC (CH_2Cl_2) showed the reaction to be complete. The suspension was filtered and the filtrate concentrated under reduced pressure. The product could be purified by crystallisation or column chromatography (on SiO_2, eluting with CH_2Cl_2) to give the desired thiourea **I**. Over 300 examples of *N*-acylthioureas of type **I** have previously been reported in the literature, including **I**, X = Ph, Y = Ph;[17] **I**, X = Ph, Y = allyl;[18] **I**, X = 2-ClC_6H_4, Y = Ph;[19] and **I**, X = 4-$O_2NC_6H_4$, Y = CH(Me)Ph.[20] Spectroscopic details (*J* in hertz) are given below for a few representative examples.

1-(2-Chlorobenzoyl)-3-(1-phenylethyl)thiourea ***I*** *[X = 2-ClC_6H_4, Y = CH(Me)Ph].* δ_H 1.67 (3 H, d, *J* 6.9, Me), 5.62 (1 H, quintet, *J* 7.1, C*H*Me), 7.28–7.70 (9 H, m, Ar), 9.10 [1 H, br s, C(O)NH], 10.90 [1 H, br d, *J* 7, C(S)NH].

1-(4-Nitrobenzoyl)-3-phenylthiourea, ***I*** *(X = 4-$O_2NC_6H_4$, Y = Ph).* δ_H 7.25 (1 H, t, *J* 7.4, H *para* to N), 7.38 (2 H, t, *J* 7.4, H *meta* to N), 7.64 (2 H, d, *J* 7.5, H *ortho* to N), 8.02 (2 H, d, *J* 4.9, H *ortho* to CO), 8.33 (2 H, d, *J* 4.9, H *meta* to CO), 8.99 [1 H, br s, C(O)NH], 12.30 [1 H, br s, C(S)NH]; ν_{max} 3432 (NH), 1670 (C=O), 1557 (NO_2), 1524 (NH bend), 1347 (NO_2), 1179 (C=S, weak).

1-(4-Nitrobenzoyl)-3-cyclohexylthiourea, ***I*** *(X = 4-O_2N-C_6H_4, Y = C_6H_{11}).* δ_H 1.28–2.13 [10 H, m, NCH(CH_2)$_5$], 4.31 (1 H, m, NCH), 8.04 (2 H, d, *J* 8.7, H *ortho* to CO), 8.38 (2 H, d, *J* 8.7, H *meta* to CO), 9.05 [1 H, br s, C(O)NH], 10.55 [1 H, br d, *J* 6, C(S)NH].

1-(4-Methylbenzoyl)-3-(2-propenyl)thiourea, ***I*** *(X = 4-MeC_6H_4, Y = $CH_2CH{=}CH_2$).* δ_H 2.37 (3 H, s, Me), 4.29 (1 H, tt, *J* 1.5, 5.5, NCH_2), 5.19 (1 H, qd, *J* 1.3, 10.3, CH=C$H_{cis}H_{trans}$), 5.21 (1 H, qd, *J* 1.6, 17.2, CH=C$H_{cis}H_{trans}$), 5.90 (1 H, tdd, *J* 5.7, 10.3, 17.2, C*H*=CH_2), 7.25 (2 H, d, *J* 8.2, H *meta* to CO), 7.66 (2 H, d, *J* 8.2, H *ortho* to CO), 8.91 [1 H, br s, C(O)NH], 10.75 [1 H, very br, C(S)NH].

1-(1-Naphthoyl)-3-cyclohexylthiourea ***I*** *(X = 1-$C_{10}H_7$, Y = C_6H_{11}).* δ_H 1.28–2.20 [10 H, m, NCH(CH_2)$_5$], 4.36 (1 H, m, NCH), 7.28–7.95 (7 H, m, Ar), 8.85 [1 H, br s, C(O)NH], 10.75 [1 H, br d, *J* 7, C(S)NH].

Synthesis of single *N*-acylthioureas III. The appropriate thiourea **I** (10 mmol) was added to a suspension of sodium hydride (11 mmol) in anhydrous THF (20 ml), and the suspension stirred for a few minutes at room temperature until evolution of hydrogen was complete. The appropriate alkylating agent (11 mmol) was added and stirring was continued until TLC showed the reaction to be complete. Water was added and the aqueous layer was extracted twice with CH_2Cl_2. The combined organic layers were dried ($MgSO_4$), filtered and concentrated under reduced pressure. The residue was purified by column chromatography (on SiO_2, eluting with 10% v/v ethyl acetate in hexane) to give the desired *N*-acylthiourea **III**. Relatively few thioureas of type **III** have been reported previously.[13,21] Spectroscopic details (*J* in hertz) are given below for a few representative examples.

1-Benzoyl-1-(2-propenyl)-3-phenylthiourea, **51**. δ_H 3.87 (2 H, d, *J* 7.0, NCH_2), 5.08 (1 H, d, *J* 10.0, CH=C$H_{cis}H_{trans}$), 5.23 (1 H, dd, *J* 0.1, 17.0, CH=C$H_{cis}H_{trans}$), 5.94 (tdd, *J* 7.0, 10.0, 17.0, C*H*=CH_2), 7.26–7.48 (8 H, m, Ar), 7.90 (2 H, d, *J* , H *ortho* to CO), 12.75 [1 H, br s, C(S)NH]; ν_{max} 3453 (NH), 1636 (C=C), 1601 (C=O), 1544 (NH bend), 1190 (C=S, weak);[17] *m/z* 297 (M + H), 105 (C_7H_5O).

1-(4-Nitrobenzoyl)-1-methyl-3-phenylthiourea, **11**. δ_H 2.54 (3 H, s, NMe), 7.19–7.40 (5 H, m, Ph), 8.21 (2 H, d, *J* 8.5, H *ortho* to CO), 8.38 (2 H, d, *J* 8.5, H *meta* to CO), 12.70 [1 H, br s, C(S)NH]; δ_C 20.7 (Me), 129.4, 132.3, 134.4, 135.6, 136.7, 142.0, 149.0, 156.0, 180.0, 181.0; *m/z* 316 (M + H), 302 (M + H − N), 386 (M + H − NO), 150 ($C_7H_4NO_3$), 136 ($C_7H_4O_3$), 120 ($C_7H_4O_2$).

1-(4-Nitrobenzoyl)-1-(2-propenyl)-3-phenylthiourea, **61**. δ_H 3.94 (2 H, d, *J* 6.9, NCH_2), 5.20 (1 H, d, *J* 10.0, CH=C$H_{cis}H_{trans}$), 5.33 (1 H, d, *J* 17.0, CH=C$H_{cis}H_{trans}$), 6.01 (1 H, tdd, *J* 6.9, 10.0, 17.0, C*H*=CH_2), 7.35-7.39 (3 H, m, Ph), 7.41–7.46 (2 H, m, Ph), 8.31 (2 H, d, *J* 7.0, H *meta* to CO), 8.45 (2 H, d, *J* 7.0, H *ortho* to CO), 12.79 [1 H, br s, C(S)NH]; ν_{max} 3443 (NH), 1615 (C=C), 1590 (C=O), 1539 (NH bend), 1190 (C=S, weak); *m/z* 342 (M + H), 328 (M + H −N), 312 (M + H − NO), 150 ($C_7H_4NO_3$), 136 ($C_7H_4O_3$), 120 ($C_7H_4O_2$).

1-(4-Nitrobenzoyl)-1-(2-propenyl)-3-cyclohexylthiourea, **65**. δ_H 1.20–2.06 [10 H, m, NCH(CH_2)$_5$], 3.64 (1 H, m, NHC*H*), 3.98 (2 H, d, *J* 6.9, NCH_2), 5.22 (1 H, d, *J* 10.0, CH=C$H_{cis}H_{trans}$), 5.36 (1 H, d, *J* 17.0, C*H*=C$H_{cis}H_{trans}$), 6.03 (1 H, tdd, *J* 7.0, 10.0, 17.0, C*H*=CH_2), 8.26 (2 H, d, *J* 8.6, H *ortho* to CO), 8.36 (2 H, d, *J* 8.9, H *meta* to CO), 11.40 [1 H, br d, *J* 7, C(S)NH]; *m/z* 348 (M + H), 334 (M + H −N), 318 (M + H − NO), 150 ($C_7H_4NO_3$), 136 ($C_7H_4O_3$), 120 ($C_7H_4O_2$).

1-(4-Methylbenzoyl)-1,3-di(2-propenyl)thiourea, **69**. δ_H 2.42 (3 H, s, Me), 3.99 (12 H, d, *J* 6.9, NCH_2), 4.03 (2 H, t, *J* 5.5, NHCH_2), 5.19 (1 H, d, *J* 10.0, $NCH_2CH{=}CH_{cis}H_{trans}$), 5.27 (1

H, d, *J* 10.3, $NHCH_2CH=CH_{cis}H_{trans}$), 5.35 (2 H, br d, *J* 17, $NCH_2CH=CH_{cis}H_{trans}$ + $NHCH_2CH=CH_{cis}H_{trans}$), 5.91 (1 H, tdd, *J* 5.5, 10.3, 17.0, $NHCH_2CH=CH_2$), 6.05 (1 H, tdd, *J* 7.0, 10.0, 17.0, $NCH_2CH=CH_2$), 7.24 (2 H, d, *J* 8.1, H *meta* to CO), 8.15 (2 H, d, *J* 8.1, H *ortho* to CO), 11.35 [1 H, very br, C(S)NH]; *m/z* 275 (M + H), 119 (C_8H_7O).

Synthesis of 'X pools' of *N*-acylthioureas I. By a modification of the above procedure, the appropriate aromatic acid chloride (25 mmol) was added to a suspension of powdered, freshly recrystallised, ammonium thiocyanate (2.85 g, 37.5 mmol) and PEG-400 (0.45 g, phase transfer catalyst) in anhydrous dichloromethane (50 ml), and the suspension was stirred at room temperature for 1 h. The appropriate mixture of five primary amines (4.75 mmol of each, 23.75 mmol) was added, and the mixture was stirred at room temperature overnight. The suspension was filtered and the filtrate concentrated under reduced pressure. The residue could be purified by filtration through a silica plug (eluting with CH_2Cl_2) to give the desired *N*-acylthiourea pool. The pools were characterised by ^{1}H NMR spectroscopy; the NH protons were most characteristic, although the high-field region was also useful.

Synthesis of 'Y pools' of *N*-acylthioureas I. By a modification of the above procedure, the appropriate mixture of five aromatic acid chlorides (5 mmol of each, 25 mmol) was added to a suspension of powdered, freshly recrystallised, ammonium thiocyanate (2.85 g, 37.5 mmol) and PEG-400 (0.45 g) in anhydrous dichloromethane (50 ml), and the suspension was stirred at room temperature for 1 h. The appropriate primary amine (23.75 mmol) was added, and the mixture was stirred at room temperature overnight. The suspension was filtered and the filtrate concentrated under reduced pressure. The residue could be purified by filtration through a silica plug (eluting with CH_2Cl_2) to give the desired *N*-acylthiourea pool. The pools were characterised by ^{1}H NMR spectroscopy; the NH protons were most characteristic, although the high-field region was also useful.

Synthesis of 'Z pools' of *N*-acylthioureas III. Each 'X' or 'Y' pool was divided into five equal portions. The 'average molecular mass' of each was calculated based on the assumption (justified by ^{1}H NMR and HPLC) that each contained equimolar amounts of the five constituents. By a modification of the above procedure, each mixture of five thioureas **I** (20 mmol of the mixture) was added to a suspension of sodium hydride (26 mmol) in anhydrous THF (40 ml), and the suspension was stirred for a few minutes at room temperature until evolution of hydrogen was complete. The appropriate alkylating agent (26 mmol) was added and the mixture was heated at reflux overnight. Water was added and the aqueous layer extracted twice with CH_2Cl_2. The combined organic layers were dried ($MgSO_4$), filtered and concentrated under reduced pressure. The residue was purified by filtration through a sillica plug (eluting with 10% v/v ethyl acetate in hexane) to give the desired *N*-acylthiourea pool. The pools were analysed by HPLC (eluting with 2% v/v ethyl acetate in hexane with a flow rate of 2.5 ml min^{-1}) to confirm presence of five components in each mixture, and the completion of the alkylation reaction. These analyses showed that alkylations using 4-methoxybenzoyl chloride were unsuccessful, so these pools were discarded. The remaining 'Z pools' (representing the four alkylating agents MeI, $PhCH_2Br$, allyl bromide and n-BuI) were used to generate the sub-libraries as described below. The pools were also characterised by ^{1}H NMR spectroscopy; the NH protons were again most characteristic, although the high-field region was also useful.

Generation of 14 'X', 'Y' and 'Z' sub-libraries from the 'Z pods'. At this stage, we had in hand 40 pools, each containing five constituents. In each pool, the Z group and one other group, either X or Y, was known. It was therefore possible to combine these in two different ways. In all cases the amounts used were based on the calculated 'average molecular mass' of the 'Z pools', using the assumption that the five constituents were present in equal amounts. This assumption was shown to be justified by HPLC. Thus each 'X sub-library' was constructed by mixing equimolar quantities (35–40 mg) of the four pools known to contain that particular X group. The 'Y sub-libraries' were constructed similarly. Each 'Z sub-library' was constructed by mixing equimolar quantities (15–25 mg) of the five pools known to contain that particular Z group and a known X group. Although this was not the original split-mix synthetic design, it proved both practical and efficient in practice, and gave accurately the five 'X sub-libraries', five 'Y-sub-libraries' and four 'Z sub-libraries' as described. The sub-libraries were characterised by LC-MS as described below. In the same way, the "total library" was constructed by mixing equimolar quantities (approximately 10 mg) of each of the 20 pools containing known Y groups.

LC-MS analysis of sub-libraries. Mass spectra of the individual ligands, and of the components of the sub-library mixtures, were obtained using an HPLC system coupled *via* a continuous flow, frit-FAB probe to a Jeol JMS.700 magnetic sector, high resolution mass spectrometer. Samples were made up in dichloromethane solution to give a concentration of approximately 0.5 mg ml^{-1} of each component. A volume of 20 μl of each sample was injected into the system, which consisted of a single isocratic pump (Thermo Separation Products P100) feeding the mobile phase of 2% ethyl acetate in hexane with a flow rate of 1 ml min^{-1} to the column described above. The output from this column was combined in a simple mixture with the FAB matrix, 2% glycerol in ethyl acetate, at 0.2 ml min^{-1} from another isocratic pump (Shimadzu LC-6A). The combined effluent was then fed to a 100 : 1 splitter to give a constant flow *via* the probe to the mass spectrometer. The LC chromatogram was generated from the total ion current (TIC) through the mass spectrometer. Positive ion spectra of each of the components were obtained by averaging 5–10 scans and then subtracting the matrix spectrum. The results of the LC-MS analysis are given in Table 1 for all 100 components of the 'total library'; the retention time quoted is the average across the three original sub-libraries in which each compound occurs.

Assay-selection procedure: sub-libraries. The appropriate sub-library (approximately 80–150 mg, 0.3–0.5 mmol of thiourea core unit) was dissolved in diethyl ether (50 ml). The aqueous layer contained Cu^{2+}, approximately 4.2 mmol dm^{-3}, from a 0.010 mol dm^{-3} solution of $CuSO_4$; AcO^-, approximately 33 mmol dm^{-3}, from a solution 0.05 mol dm^{-3} in NaOAc and 0.05 mol dm^{-3} in AcOH; and HO^-, approximately 25 mmol dm^{-3}, from a 0.1 mol dm^{-3} solution of NaOH. The total amount of copper was chosen to correspond to five ligands in the sub-library mixture. The total volume of the aqueous layer varied slightly according to the molarity of the organic layer, but was approximately 20 ml. The two layers were mixed and stirred together (vigorous magnetic stirring) for 48 h, then separated. The organic layer was not dried, since in some cases a small amount of precipitated complex was present (which was carefully retained with the organic layer during separation), but was concentrated under reduced pressure. The residue was subjected to column chromatography on neutral alumina gel, eluting first with 10% v/v EtOAc in hexane, then with EtOAc and finally with 10% v/v MeOH in EtOAc. First eluted were the 'inactive' ligands, as described in the text. The 'active' ligands and their complexes eluted as the second fraction. This second fraction, after concentration under reduced pressure, was

redissolved in CH_2Cl_2 or Et_2O, and shaken vigorously for 2–3 h with the decomplexation solution. This was a 0.25 mol dm^{-3} solution of en.2AcOH, and the quantity used gave 10 equiv. of ethylene diamine (en) relative to the quantity of Cu^{2+} originally measured. The layers were then separated, and the organic layer dried ($MgSO_4$) and concentrated under reduced pressure. The residue was dissolved in CH_2Cl_2 for LC-MS analysis as before.

Assay-selection procedure: total library. The above procedure was followed exactly, except that the total library (approximately 200 mg) was used in place of the sub-libraries.

Analysis of copper content. Copper concentration was measured using both UV-VIS spectrophotometry and AAS. The two methods were found to be in good agreement. Typically, en (10 drops) was added to the aqueous phase from an assay-selection process and the mixture was diluted to 100 ml. The resulting solution was used for AAS and/or UV-VIS analysis, relative to a standard calibration plot. UV-VIS analysis of the purple $Cu(en)_2$ complex was carried out at 550 nm. The absorptions of 5–50 mg Cu in 100 ml of solution can be measured in a 1 cm cell. This solution was diluted a further 100-fold for quantification of copper by AAS at 324.8 nm, in the range 0.5–5 mg dm^{-3}.

Experiment optimisation

Before the combinatorial studies could be implemented, many studies on individual ligands and small mixtures (of four or five ligands) were carried out. Some of these are described here.

LC-MS analysis of the sub-libraries proved much more generally applicable than the alternative GC-MS analysis. The latter was successful for some ligands **IV** and for some ligands **I** after silylation, but gave poor results for ligands **II** and **III**. Many of the thioureas proved thermally unstable above 200 °C,

Table 1 Library analysis by LC-MS

Compound No.	X	Y	Z	t_R/min	$M^+ + H$	Compound No.	X	Y	Z	t_R/min	$M^+ + H$
1	Ph	Ph	Me	8.07	271.3	**26**	Ph	Ph	CH_2Ph	8.57	347.4
2	Ph	CH(Me)Ph	Me	10.93	299.4	**27**	Ph	CH(Me)Ph	CH_2Ph	10.85	375.5
3	Ph	3,5-$Me_2C_6H_3$	Me	6.22	299.4	**28**	Ph	3,5-$Me_2C_6H_3$	CH_2Ph	6.47	375.5
4	Ph	Allyl	Me	13.59	235.3	**29**	Ph	Allyl	CH_2Ph	13.26	311.4
5	Ph	Cyclohexyl	Me	7.12	277.4	**30**	Ph	Cyclohexyl	CH_2Ph	7.43	353.5
6	2-ClC_6H_4	Ph	Me	14.59	305.8	**31**	2-ClC_6H_4	Ph	CH_2Ph	14.84	381.9
7	2-ClC_6H_4	CH(Me)Ph	Me	18.34	333.8	**32**	2-ClC_6H_4	CH(Me)Ph	CH_2Ph	17.03	409.9
8	2-ClC_6H_4	3,5-$Me_2C_6H_3$	Me	10.24	333.8	**33**	2-ClC_6H_4	3,5-$Me_2C_6H_3$	CH_2Ph	10.38	409.9
9	2-ClC_6H_4	Allyl	Me	24.65	269.8	**34**	2-ClC_6H_4	Allyl	CH_2Ph	22.88	345.9
10	2-ClC_6H_4	Cyclohexyl	Me	12.45	311.8	**35**	2-ClC_6H_4	Cyclohexyl	CH_2Ph	11.40	387.9
11	4-$O_2NC_6H_4$	Ph	Me	15.50	316.3	**36**	4-$O_2NC_6H_4$	Ph	CH_2Ph	16.49	392.4
12	4-$O_2NC_6H_4$	CH(Me)Ph	Me	21.11	344.4	**37**	4-$O_2NC_6H_4$	CH(Me)Ph	CH_2Ph	20.05	420.5
13	4-$O_2NC_6H_4$	3,5-$Me_2C_6H_3$	Me	10.17	344.4	**38**	4-$O_2NC_6H_4$	3,5-$Me_2C_6H_3$	CH_2Ph	10.43	420.5
14	4-$O_2NC_6H_4$	Allyl	Me	12.22	280.3	**39**	4-$O_2NC_6H_4$	Allyl	CH_2Ph	26.41	356.4
15	4-$O_2NC_6H_4$	Cyclohexyl	Me	10.69	322.4	**40**	4-$O_2NC_6H_4$	Cyclohexyl	CH_2Ph	11.06	398.5
16	4-MeC_6H_4	Ph	Me	8.65	285.4	**41**	4-MeC_6H_4	Ph	CH_2Ph	9.20	361.5
17	4-MeC_6H_4	CH(Me)Ph	Me	12.36	313.4	**42**	4-MeC_6H_4	CH(Me)Ph	CH_2Ph	11.94	389.5
18	4-MeC_6H_4	3,5-$Me_2C_6H_3$	Me	6.54	313.4	**43**	4-MeC_6H_4	3,5-$Me_2C_6H_3$	CH_2Ph	6.87	389.5
19	4-MeC_6H_4	Allyl	Me	15.22	249.3	**44**	4-MeC_6H_4	Allyl	CH_2Ph	14.86	325.4
20	4-MeC_6H_4	Cyclohexyl	Me	7.89	291.4	**45**	4-MeC_6H_4	Cyclohexyl	CH_2Ph	7.95	367.5
21	1-Naphthyl	Ph	Me	9.56	321.4	**46**	1-Naphthyl	Ph	CH_2Ph	9.71	397.5
22	1-Naphthyl	CH(Me)Ph	Me	11.88	349.4	**47**	1-Naphthyl	CH(Me)Ph	CH_2Ph	11.31	425.5
23	1-Naphthyl	3,5-$Me_2C_6H_3$	Me	7.23	349.4	**48**	1-Naphthyl	3,5-$Me_2C_6H_3$	CH_2Ph	7.23	425.5
24	1-Naphthyl	Allyl	Me	14.71	285.4	**49**	1-Naphthyl	Allyl	CH_2Ph	13.76	361.5
25	1-Naphthyl	Cyclohexyl	Me	7.78	327.4	**50**	1-Naphthyl	Cyclohexyl	CH_2Ph	7.27	403.5
51	Ph	Ph	Allyl	6.86	297.4	**76**	Ph	Ph	n-Bu	6.10	313.4
52	Ph	CH(Me)Ph	Allyl	8.51	325.4	**77**	Ph	CH(Me)Ph	n-Bu	7.44	341.5
53	Ph	3,5-$Me_2C_6H_3$	Allyl	5.35	325.4	**78**	Ph	3,5-$Me_2C_6H_3$	n-Bu	4.89	341.5
54	Ph	Allyl	Allyl	10.44	261.4	**79**	Ph	Allyl	n-Bu	8.96	277.4
55	Ph	Cyclohexyl	Allyl	5.94	303.4	**80**	Ph	Cyclohexyl	n-Bu	5.54	319.5
56	2-ClC_6H_4	Ph	Allyl	11.34	331.8	**81**	2-ClC_6H_4	Ph	n-Bu	9.74	347.9
57	2-ClC_6H_4	CH(Me)Ph	Allyl	12.84	359.9	**82**	2-ClC_6H_4	CH(Me)Ph	n-Bu	11.22	375.9
58	2-ClC_6H_4	3,5-$Me_2C_6H_3$	Allyl	8.22	359.9	**83**	2-ClC_6H_4	3,5-$Me_2C_6H_3$	n-Bu	7.21	375.9
59	2-ClC_6H_4	Allyl	Allyl	17.13	295.8	**84**	2-ClC_6H_4	Allyl	n-Bu	14.64	311.8
60	2-ClC_6H_4	Cyclohexyl	Allyl	9.11	337.9	**85**	2-ClC_6H_4	Cyclohexyl	n-Bu	8.24	353.9
61	4-$O_2NC_6H_4$	Ph	Allyl	12.75	342.4	**86**	4-$O_2NC_6H_4$	Ph	n-Bu	10.21	358.4
62	4-$O_2NC_6H_4$	CH(Me)Ph	Allyl	15.15	370.4	**87**	4-$O_2NC_6H_4$	CH(Me)Ph	n-Bu	11.59	386.5
63	4-$O_2NC_6H_4$	3,5-$Me_2C_6H_3$	Allyl	8.38	370.4	**88**	4-$O_2NC_6H_4$	3,5-$Me_2C_6H_3$	n-Bu	6.96	386.5
64	4-$O_2NC_6H_4$	Allyl	Allyl	21.21	306.3	**89**	4-$O_2NC_6H_4$	Allyl	n-Bu	15.85	322.4
65	4-$O_2NC_6H_4$	Cyclohexyl	Allyl	9.09	348.4	**90**	4-$O_2NC_6H_4$	Cyclohexyl	n-Bu	7.03	364.5
66	4-MeC_6H_4	Ph	Allyl	7.40	311.4	**91**	4-MeC_6H_4	Ph	n-Bu	6.58	327.4
67	4-MeC_6H_4	CH(Me)Ph	Allyl	9.39	339.5	**92**	4-MeC_6H_4	CH(Me)Ph	n-Bu	8.27	355.5
68	4-MeC_6H_4	3,5-$Me_2C_6H_3$	Allyl	5.71	339.5	**93**	4-MeC_6H_4	3,5-$Me_2C_6H_3$	n-Bu	5.22	355.5
69	4-MeC_6H_4	Allyl	Allyl	11.70	275.4	**94**	4-MeC_6H_4	Allyl	n-Bu	10.11	291.4
70	4-MeC_6H_4	Cyclohexyl	Allyl	6.58	317.4	**95**	4-MeC_6H_4	Cyclohexyl	n-Bu	6.15	333.5
71	1-Naphthyl	Ph	Allyl	7.50	347.4	**96**	1-Naphthyl	Ph	n-Bu	6.48	363.5
72	1-Naphthyl	CH(Me)Ph	Allyl	8.71	375.5	**97**	1-Naphthyl	CH(Me)Ph	n-Bu	7.33	391.5
73	1-Naphthyl	3,5-$Me_2C_6H_3$	Allyl	5.86	375.5	**98**	1-Naphthyl	3,5-$Me_2C_6H_3$	n-Bu	5.11	391.5
74	1-Naphthyl	Allyl	Allyl	10.56	311.4	**99**	1-Naphthyl	Allyl	n-Bu	8.68	327.4
75	1-Naphthyl	Cyclohexyl	Allyl	6.38	353.5	**100**	1-Naphthyl	Cyclohexyl	n-Bu	5.41	369.5

further reducing the applicability of GC. By contrast, LC analysis proved compatible with all four classes of thiourea ligands, and was particularly suitable for ligands **III**, because of their relatively low polarity (see above). Frit-FAB MS analysis proved capable, reliably, of ionising all the library members and of giving a protonated molecule for each.

The assay-selection procedure required most optimisation. Several individual ligands were used for these studies, not all of which were present in the final library; a small library of 25 ligands **I** was used for early trials of combinatorial assay-selection. Copper concentrations in aqueous en solution were measured using both UV-VIS spectrophotometry and AAS, with good correlation between the two methods. Aqueous concentrations of the ligands could also be measured by UV absorption. In all cases, removal of copper from the aqueous phase was being quantified, using ligands which remained in the organic solution. Using an excess of copper, it was established that copper extraction increased with increase in pH. Since at high pH ($\geqslant$12) the ligands became susceptible to hydrolysis, the pH selected for the combinatorial studies was 10. At this pH, a stabilising agent was necessary to prevent precipitation of copper hydroxide; acetate ion worked well, without interfering with the copper extraction by the thiourea ligands. Various organic solvents were investigated, including diethyl ether, dichloromethane, ethyl acetate and isobutyl methyl ketone; the first two proved more efficient than the last two for extractions on a small scale.

Recovery of the copper from the complexes proved the most problematic aspect. Efficient ligand recovery is crucial to the deconvolution process, and efficient recovery of both metal and ligand is highly desirable for practical application of our work.[4] We therefore isolated several copper complexes, using an excess of copper to ensure complete complexation of the ligands. Simply lowering the pH did not give efficient recovery of the free ligands; this is perhaps not surprising, since Ringmann and Schuster have shown that copper extraction is efficient with ligands **I** at pH 2.[4] In addition, they have shown that although recovery of the copper is efficient using 4 mol l^{-1} sulfuric acid, higher concentrations cause ligand decomposition. Being reluctant to risk any decomposition, we turned our attention instead to competitive chelation using amine ligands, and finally using sodium sulfide. The ligands with the highest affinity for copper proved to be ligands **I**, X = i-Pr, Y = benzyl or CH(Me)Ph. We were unable to identify conditions for the quantitative recovery of these ligands from their copper complexes. This suggests that these ligands bind very strongly to copper; we are currently investigating this, with a view to applications in environmental remediation. Similar difficulties have been encountered by Koch and co-workers in attempting to recover related ligands from their complexes with the platinum group metals; in these cases, however, the value of the metals is so high that the ligands can be regarded as expendable.[6] We therefore directed our attention towards the other classes of ligands, **II–IV**. Ligands **II** proved to have the same drawback as ligands **I**, and ligands **IV** showed very poor uptake of copper from the aqueous phase. Complete recovery of copper from the complexes of ligands **III** was achieved using chelating amine reagents, such as en. However en also caused some hydrolysis of the ligands themselves (confirmed by treating representative free ligands with en). After investigating EDTA and other ligands, we established that quantitative ligand recovery was possible using en buffered with acetic acid in the aqueous phase, with either diethyl ether or dichloromethane as the organic phase.

Finally, a protocol was required for separating the copper complexes of the 'active' ligands from the free 'inactive' ligands. Separation by precipitation requires all copper complexes to be effectively insoluble. Since this assumption could not be relied on, chromatographic separation was preferable: the complexes are considerably more polar than the free ligands. It was essential to establish chromatographic conditions which would permit elution of the free ligands, without any decomposition of the complexes. Using isolated complexes of test ligands **III**, we found that chromatography on silica gel tended to cause low levels of dissociation using a range of eluting solvents, but substituting basic alumina as the stationary phase solved this problem. With 10% ethyl acetate in light petroleum as the eluent, no dissociation of copper was observed when using the isolated complexes, and the complexes themselves moved down the column very slowly, if at all. Fortunately, this solvent system gives rapid elution of the free ligands, as established using individual compounds or sub-libraries before complexation. Switching to 10% methanol in ethyl acetate then permits elution of the 'active' ligands. Our protocol is therefore to put a mixture of free 'inactive' ligands (LH) and complexes (L^*_nCu) on to an alumina column and elute with 10% ethyl acetate in light petroleum until elution of the free ligands LH is complete (as judged by TLC). With only the complexes left on the column, the eluent is then changed to 10% methanol in ethyl acetate. This causes some dissociation of the complexes, and a mixture of 'dissociated active ligands' L*H and copper complexes L^*_nCu is collected. Decomplexation is then completed by treating this last mixture with en.2AcOH as described above.

Results and discussion

Analysis of sub-libraries

The 14 sub-libraries (five in the X, five in the Y and four in the Z dimension) were analysed by LC-MS. LC separation of these mixtures of 20 or 25 compounds was incomplete, but this is relatively unimportant, since the mass spectra clearly demonstrate when more than one compound is present in an LC peak. By comparing the retention times and protonated molecules generated from the 14 sub-libraries, we were able unambiguously to assign a unique combination of retention time (t_R) and molecular mass to each member of the total library. All isomers were clearly separated by t_R. This is illustrated by the sample results given in Fig. 5 and 6. Fig. 5(a) shows the chromatogram, generated by total ion current (TIC) and consequently of low resolution, for the X1 sub-library. Fig. 5(b) shows the MS analysis of the peak at 6.0 min (obtained, like all the mass spectra, by subtraction of a spectrum of the matrix): this peak contains three compounds, all giving (M + H) peaks. Only one compound in this sub-library has molecular mass 312, so the peak at 313 is easily assigned as ligand **76**. Similarly, the peak at *m/z* 303 can only be due to compound **55**; indeed, this is the only ligand in any sub-library with molecular mass 302. The peak at *m/z* 299 is ambiguous, however: both **2** and **3** have molecular mass 298, and both (having X = Ph) are present in the X1 sub-library. As shown in Fig. 5(c), *m/z* 299 occurs again at t_R 10.9 min, this time accompanied by a peak at *m/z* 375. This is due to ligand **27**, and shows the loss of 90 mass units characteristic of ligands having Z = CH_2Ph. Ligands **2** and **3** cannot be distinguished from these traces alone, as they differ only in the Y substituent.

By cross-refering to the Y sub-libraries (Fig. 6), the ambiguity is immediately resolved. Fig. 6(a) shows the chromatogram for the Y2 sub-library. On MS analysis, an ion with *m/z* 299 is found in the peak at 11.8 min [Fig. 6(b)]. Since compound **3** is not present in the Y2 sub-library, this peak must be due to ligand **2**, which is in fact the only compound eluting at 11.8 min. Ligand **3** is found, as expected, in the Y3 sub-library at 6.6 min (not illustrated). In every case, isomeric pairs were well separated by LC, with the same substituent (in this case Y3) generally eluting faster in every pair. This was one of several trends observed in the LC data; for example, the nitrated

X3 compounds tended to elute most slowly, whereas the naphthyl X5 derivatives were always faster.

All the data analysis was performed manually, but most LC- and GC-MS instruments have the capacity for storing and using libraries of fragmentation patterns, which would considerably ease the deconvolution. As mentioned above, the fragment XCO was observed for all the ligands. Multiple selective ion searching, for the (in our case) five possible XCO ions would therefore be a very convenient approach to analysing LC traces for the library mixtures. Considerable improvements in LC resolution could be achieved by installing an optical detector between LC and MS; greater repeatability in retention times can be expected using purpose-built LC-MS instrumentation.

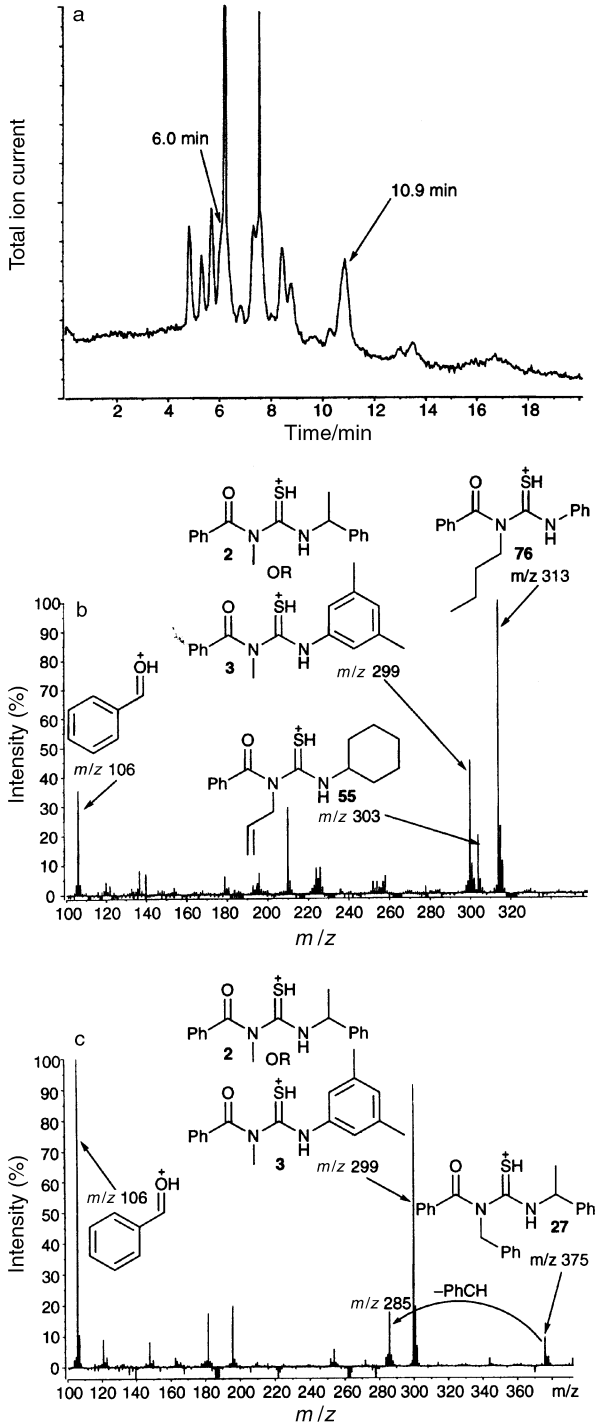

Fig. 5 LC-MS traces for X = Ph sub-library. (a) LC by TIC of X = Ph sub-library; (b) MS analysis of X = Ph sub-library at t_R = 6.0 min; (c) MS analysis of X = Ph sub-library at t_R = 10.9 min.

Assay-selection and deconvolution

We carried out the assay-selection procedure on the 14 sub-library mixtures and also on the total library. The results from the sub-library extractions will be considered first. Since each ligand was present in three sub-libraries (one X, one Y and one Z), each could be selected (by the copper-based assay-selection procedure) up to three times. It was anticipated that the best ligands would be selected three times, whereas others might be selected only once or twice, depending on competition with more strongly-binding ligands. In addition, since all the members of each sub-library shared one common group, it was expected that some sub-libraries might be much more amenable than others to copper binding. This complication is avoided when the total library is used for the assay-selection process.

The results obtained from carrying out assay-selection, decomplexation and LC-MS analysis on the X = Ph sub-library are illustrated in Fig. 7. These results, measured on the 'active ligands', allow a direct comparison with Fig. 5, measured on the same sub-library before treatment with copper. Comparison of Fig. 5(a) and 7(a), the chromatograms for library X1 before and after treatment with copper, respectively, immediately shows that some, but far from all, of the X1 ligands are active. Comparison of the mass spectra in Figures 5b and 7b reveals that, for example, ligands **3** and **76** have both been selected, but ligand **55** is clearly inactive. The absence of an LC peak after 11 min shows the inactivity (confirmed by MS analysis of this region) of ligands **2** and **27**, having Y = CH(Me)Ph. LC analysis of library Y2 [Y = CH(Me)Ph] after treatment with copper showed that, in fact, none of these ligands is active. Similar traces were obtained for all 14 sub-libraries, and referred back in the same way to the traces before copper treatment.

On correlation of the LC-MS data for all the sub-library extracts, we were pleased to observe selective recovery of the

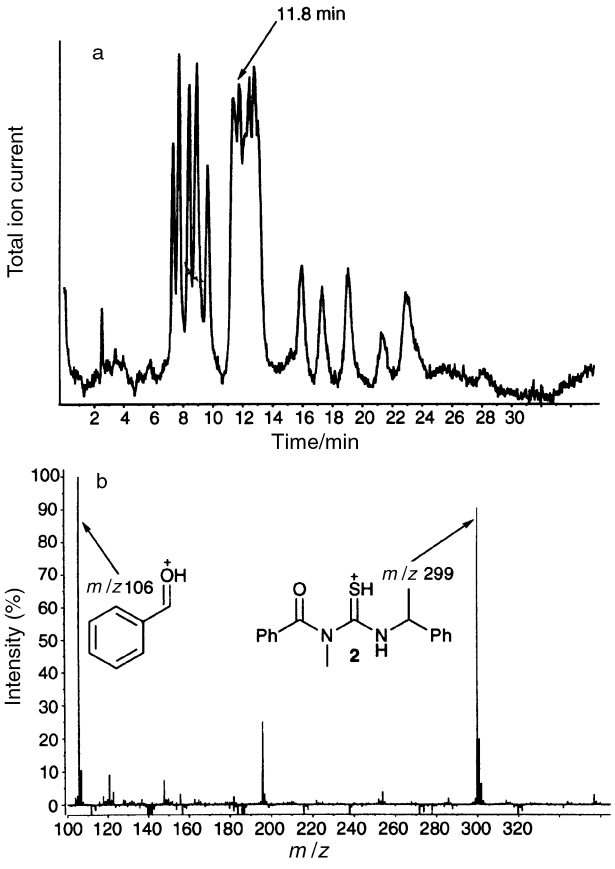

Fig. 6 LC-MS traces for Y = CH(Me)Ph sub-library. (a) LC by TIC of Y = CH(Me)Ph sub-library; (b) MS analysis of Y = CH(Me)Ph sub-library at t_R = 11.8 min.

type predicted above. Some sub-libraries removed virtually all the available copper from the aqueous solution, whereas others extracted almost none. Of the 100 compounds, 44 were never selected; 21 were selected once only, 21 twice only and just 14 on all three possible occasions. These results are summarised in Table 2. Clear patterns are evident, as illustrated by Fig. 8, which summarises the selection of each individual substituent. Since there are 20 compounds containing substituent X1, and each can be selected up to three times, the maximum possible for X1 is 60 times. The same holds for each X and Y substituent. For Z substituents, the maximum possible is 75 times, since there are 25 compounds containing each Z substituent. Substituent Y2, for example, was selected just four times. Table 2 shows that these were from sub-libraries X4 (in ligands **17** and **42**), Y2 (in compound **97**) and Z1 (in thiourea **2**).

Inspection of Table 2 and Fig. 8 shows a clear structure–activity correlation, particularly in the Y dimension. Substituents Y1 (Ph) and Y3 (3,5-$Me_2C_6H_3$) are frequently selected: they clearly confer a relatively high affinity for copper. The other groups in the Y dimension, particularly substituents Y2 [CH(Me)Ph] and Y5 (C_6H_{11}), are almost never selected: they clearly confer a relatively low affinity for copper. In the other dimensions the trends are less strongly marked, but nevertheless discernible: X4 (4-MeC_6H_4) is the best of the X groups, while X3 (4-$O_2NC_6H_4$) and X5 (1-naphthyl) are the worst; and Z4 (n-butyl) is the best of the Z groups, while Z2 (benzyl) is the worst. Moreover, on closer inspection of Table 2, it is clear that a 'good' group in one position can overcome the effect of a 'poor' group in another, and *vice versa*. Thus the two

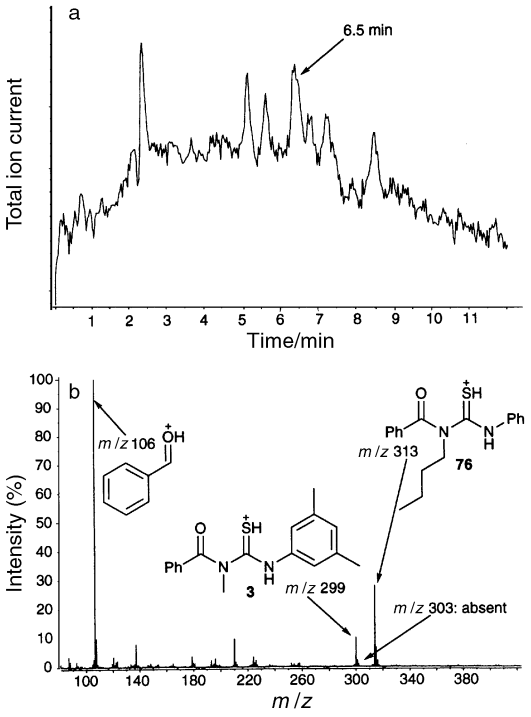

Fig. 7 LC-MS traces for active ligands from X = Ph sub-library. (a) LC analysis by TIC of X = Ph sub-library after assay, (b) MS analysis of X = Ph sub-library at 6.5 min after assay.

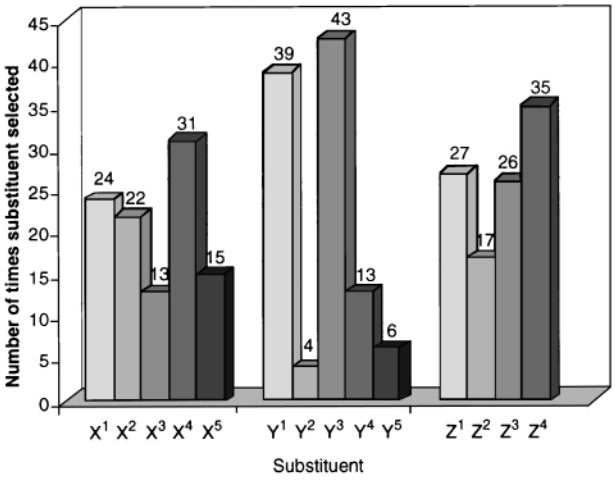

Fig. 8 Selection of substituents from sub-libraries.

Table 2 Selection of ligands from sub-libraries

Sub-library[a]			Compound[b]	Sub-library			Compound	Sub-library			Compound	Sub-library			Compound
X1	Y1	**Z1**	**1**	X1	**Y1**	**Z2**	**26**	**X1**	Y1	**Z3**	**51**	**X1**	**Y1**	**Z4**	**76**
X1	Y2	**Z1**	**2**	X1	Y2	Z2	**27**	X1	Y2	Z3	**52**	X1	Y2	Z4	**77**
X1	**Y3**	**Z1**	**3**	**X1**	Y3	**Z2**	**28**	**X1**	**Y3**	**Z3**	**53**	**X1**	**Y3**	**Z4**	**78**
X1	**Y4**	Z1	**4**	X1	Y4	Z2	**29**	X1	**Y4**	Z3	**54**	X1	Y4	Z4	**79**
X1	Y5	Z1	**5**	X1	Y5	Z2	**30**	X1	**Y5**	Z3	**55**	X1	Y5	Z4	**80**
X2	**Y1**	**Z1**	**6**	X2	**Y1**	**Z2**	**31**	**X2**	**Y1**	**Z3**	**56**	**X2**	Y1	**Z4**	**81**
X2	Y2	Z1	**7**	X2	Y2	Z2	**32**	X2	Y2	Z3	**57**	X2	Y2	Z4	**82**
X2	**Y3**	**Z1**	**8**	**X2**	**Y3**	**Z2**	**33**	**X2**	**Y3**	**Z3**	**58**	**X2**	Y3	**Z4**	**83**
X2	Y4	Z1	**9**	X2	Y4	Z2	**34**	X2	Y4	Z3	**59**	X2	**Y4**	Z4	**84**
X2	Y5	Z1	**10**	X2	Y5	Z2	**35**	X2	Y5	Z3	**60**	X2	Y5	Z4	**85**
X3	Y1	**Z1**	**11**	X3	Y1	Z2	**36**	X3	**Y1**	**Z3**	**61**	**X3**	**Y1**	**Z4**	**86**
X3	Y2	Z1	**12**	X3	Y2	Z2	**37**	X3	Y2	Z3	**62**	X3	Y2	Z4	**87**
X3	Y3	**Z1**	**13**	X3	Y3	Z2	**38**	**X3**	**Y3**	Z3	**63**	**X3**	**Y3**	**Z4**	**88**
X3	Y4	Z1	**14**	X3	Y4	Z2	**39**	X3	Y4	Z3	**64**	X3	**Y4**	Z4	**89**
X3	Y5	Z1	**15**	X3	Y5	Z2	**40**	X3	Y5	Z3	**65**	X3	Y5	Z4	**90**
X4	**Y1**	**Z1**	**16**	X4	**Y1**	**Z2**	**41**	**X4**	Y1	**Z3**	**66**	**X4**	**Y1**	**Z4**	**91**
X4	Y2	Z1	**17**	**X4**	Y2	Z2	**42**	X4	Y2	Z3	**67**	X4	Y2	Z4	**92**
X4	**Y3**	**Z1**	**18**	X4	**Y3**	**Z2**	**43**	**X4**	Y3	**Z3**	**68**	**X4**	**Y3**	**Z4**	**93**
X4	Y4	**Z1**	**19**	**X4**	**Y4**	Z2	**44**	**X4**	**Y4**	Z3	**69**	**X4**	**Y4**	Z4	**94**
X4	**Y5**	Z1	**20**	X4	Y5	Z2	**45**	X4	Y5	Z3	**70**	**X4**	**Y5**	Z4	**95**
X5	Y1	**Z1**	**21**	X5	Y1	Z2	**46**	**X5**	Y1	Z3	**71**	**X5**	Y1	**Z4**	**96**
X5	Y2	Z1	**22**	X5	Y2	Z2	**47**	X5	Y2	Z3	**72**	X5	**Y2**	Z4	**97**
X5	Y3	**Z1**	**23**	X5	**Y3**	Z2	**48**	**X5**	Y3	Z3	**73**	**X5**	**Y3**	Z4	**98**
X5	Y4	Z1	**24**	X5	Y4	Z2	**49**	X5	**Y4**	Z3	**74**	X5	**Y4**	Z4	**99**
X5	Y5	**Z1**	**25**	X5	Y5	Z2	**50**	X5	Y5	Z3	**75**	X5	**Y5**	Z4	**100**

Key to sub-library: [a] Bold indicates selection from the sub-library in the highlighted dimension. [b] Key to compound numbers: compounds selected two or three times are highlighted by underlining; compounds selected once or never selected are not underlined.

compounds having Y = Ph which were never recovered, **36** and **46**, both bear Z = CH_2Ph, and have the two poor X groups, X = naphthyl and X = 4-nitrophenyl. The one compound having Z = CH_2Ph which was recovered on all three possible occasions, **33**, has some of the "best" other substituents, X = 2-chlorophenyl and Y = 3,5-dimethylphenyl. On the other hand, a combination of X = 4-$O_2NC_6H_4$ and Z = CH_2Ph is enough to prevent any of ligands **36–40** ever being selected, regardless of the nature of Y, and a combination of X = naphthyl and Z = CH_2Ph was recovered only once, in compound **48** where Y = 3,5-dimethylphenyl.

For the assay-selection on the total library mixture, an amount of copper ions equivalent to five times that of a single ligand was again used, and all other parameters were identical with those in the earlier procedures on the sub-libraries. The ligands selected were **6**, **56**, **61**, **76**, **81**, **86** and **93**. Of these seven compounds, five (**6**, **56**, **76**, **86** and **93**) were previously selected on all three possible occasions (see Table 2). This strongly suggests that these are the best five members of the 100-compound library. Notably, these five compounds all have the Y1 (Ph) or Y3 (3,5-$Me_2C_6H_3$) groups, and three bear the Z4 (n-Bu) group, previously identified as 'best'. The X groups are more diverse, reflecting the lower dependence of Cu affinity on this substituent.

Interestingly, two of the seven selected compounds (**61** and **81**) were selected only twice from their respective sub-libraries. Although detailed testing on individual compounds will be needed to establish with certainty the reasons for this apparent anomaly, it is possible that this reflects 'synergy' in ligand binding. *N*-Acylthioureas are known to form complexes CuL_2 or $Cu_nL_{>2}$ in the presence (as here) of an excess of the ligand. They show a variety of binding modes, including monodentate,[2b,22] chelating[23] and bridging across two metal ions.[2c] The scope for 'mixed ligand' complexes, and synergistic binding, is therefore considerable. Ligand **81** was not selected from the 'Y1' (Ph) sub-library: possibly it binds strongly only in conjunction with ligand **93**, which was absent from that sub-library since it has Y = 3,5-$Me_2C_6H_3$. Ligand **61** was not selected from the 'X3' (4-$O_2NC_6H_4$) sub-library: possibly it binds strongly only as a partner ligand for one of ligands **6**, **56**, **76** and **93** which were absent from that sub-library. To the best of our knowledge, synergistic binding of two different ligands has never been investigated with *N*-acylthioureas; we therefore intend to pursue these intriguing results.

Conclusions

We have shown how combinatorial methods can be used to establish a structure–activity correlation for over 100 *N*-acylthioureas, by deducing the relative affinities of 100 ligands for copper ions. These ligands have three sites of diversity, and we have shown not only how variation at each affects the binding of the whole ligand to copper, but also how the 'best' and 'worst' groups at each site interact when present together. In particular, we have identified the 'best five' ligands, and the best group(s) in each of the three variable positions. This was achieved without the need for highly sophisticated technology, but using LC-MS instrumentation, which is now very widely available. The method is equally applicable to other metals and other groups of ligands, or even to totally unrelated problems such as enzyme inhibition, provided an assay-selection procedure can be established to separate the 'active' from the 'inactive' library members, and to recover the former unchanged. We believe this methodology will find many applications in a wide range of fields.

Acknowledgements

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