Analytical techniques for solid-phase organic and combinatorial synthesis

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In the developmental phase leading to solid-phase library generation, it is essential that reactions can be efficiently and readily optimized and quantified, and this requires reliable and sensitive techniques for the analysis of solid-phase reactions. The exploding interest in different types of solid-phase chemistry is placing increased demands on the reliability, diversity and sensitivity of such analytical methods. The authors describe the most exciting developments of the past 4–5 years.

Solid phase synthesis has, until recently, been the domain of the peptide and nucleic acid chemist. Efficient, high-yielding couplings could be forced by mass action and reliably monitored by a range of sensitive, but predominantly colorimetric, assays. In peptide chemistry this has been evidenced by the ubiquitous ninhydrin-based test to detect amines both in a quantitative and a qualitative manner[1]. For the assay of secondary and tertiary amino groups, bromophenol blue[2] (especially useful for the consumption of secondary amines), trinitrobenzenesulphonic acid[3] and chloranil are all well documented. Continuous-flow and single-point assays arising from protection group liberation have been widely utilized in both DNA and peptide chemistry[4,5], allowing monitoring of coupling and deprotection efficiency. Classic examples are the use of the methoxytrityl cation (DNA synthesis) and fulvene–piperidine adducts arising from 9-fluorenylmethoxycarbonyl (FMOC) deprotection in solid-phase peptide synthesis. Other techniques include counterion distribution monitoring[2,6–8] to visualize residual unreacted amino groups on the solid phase. This is achieved either by the formation of a coloured ion pair with an inert anionic dye, such as bromophenol blue or 1-oxo-2-hydroxydihydrobenzotriazine (Dhbt; yellow), or by measuring the displacement of the dye into solution as the acylation proceeds. Other analytical methods that are used in this field, albeit infrequently, include the use of gel-phase ^{13}C-NMR (Ref. 9) and IR spectroscopy[10].

The current surge of interest in solid-phase chemistry[11–14] has placed increasing demands on the analytical methods available for solid-phase reaction monitoring. The ever-increasing range of chemical reactions being carried out on the solid phase necessitates an array of analytical tools that are more diverse than those traditionally used in solid-phase peptide/DNA chemistry. In the developmental phase leading to solid phase library generation, it is essential that reactions can be efficiently and readily optimized and quantified, and ideally the analysis automated. This requires reliable and sensitive techniques for solid-phase reaction analysis, in essence replacing the established thin-layer chromatography (TLC) methodologies of the organic chemist. In this article we will cover the main analytical methods that have been employed in the past 4–5 years to study solid-phase reactions.

Cleavage and analysis

By far the most widely used method in solid-phase reaction analysis is small-scale cleavage and analysis by conventional methods. Using familiar analytical techniques, this usually

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provides unambiguous answers to the questions being asked. A huge number of reactions, for example the Mitsunobu[15], Stille[16], Heck[17] and Suzuki[18] reactions, and the formation of numerous heterocycles[19,20], have been analysed from the solid-phase in this manner, but the process is time-consuming and material-inefficient. It can, however, be automated using current HPLC/MS instrumentation. The use of pins in reaction optimization has been widely reported by the Chiron Mimotopes group[21,22], reactions being analysed using automated HPLC and MS following cleavage from the pin. In one such study, 56 reductive amination conditions were investigated, while in another 256 reaction conditions were used to optimize solid-phase ether formation. It is unclear whether reactions on pins are representative of those on all other solid-phase supports, but the facile methodology is highly amenable to automation and the assessment and optimization of new solid-phase transformations.

Colorimetric assays

A variety of colorimetric assays have been used in nonclassical solid-phase-based chemistry. For example, the consumption of thiols on TentaGel resins has been determined by use of bis(3-carboxy-4-nitrophenyl)disulphide (Ellman's reagent) in the synthesis of β-turn mimetics[23]. This reagent has also been used to monitor the alkylation of thiols on polystyrene-based resins in a range of other thioether formations.

Ninhydrin-based assays have been used to detect non-peptidic amines, although they appear not to be quantitative in this application. To solve this problem, 3-hydroxymethyl-4-nitrophenylisothiocyanate-*O*-trityl has been introduced as a reagent for the quantification of amino groups via the release of the trityl cation following thiourea formation and acidolysis[24]. Alternatively, in an analogous fashion to FMOC-peptide chemistry, protection of amines with a 4-nitrophenylethyloxycarbonyl group allows the subsequent cleavage to be monitored by the liberation of nitrostyrene[25].

NMR spectroscopy

NMR spectroscopy is a technique that is highly accessible to most organic chemists, providing information in an immediately interpretable format. Intensive research has therefore been directed towards the development of NMR methodology to identify materials covalently attached to resin supports.

^{19}F, ^{31}P, ^{13}C and ^{13}C-enriched materials

'Gel-phase' ^{13}C-NMR (where the resin is swollen in solvent and is hence neither solution nor solid phase) was first described in 1971 to study crosslinked polymers[26]. Its first application to peptide chemistry was in 1980 by Manatt and coworkers, who used ^{13}C-NMR to determine the extent of chloromethylation of crosslinked polymers and ^{19}F-NMR to monitor peptide synthesis using ^{19}F-labelled protecting groups[27,28]. This work was soon followed by that of Epton and coworkers, who characterized intermediates in a solid-phase peptide synthesis[29]. Four years later, in a comprehensive study by Giralt and coworkers, ^{13}C-NMR was used to monitor the homogeneity of starting polymers, to determine the success of a solid-phase peptide synthesis (including the observation of side-reactions) and to obtain an estimate of peptide mobility during synthesis[9]. In 1989, gel-phase ^{13}C-NMR was used to assess the efficiency of peptide deprotection and resin cleavage[30].

A drawback of gel-phase ^{13}C-NMR on resin-bound material is the low sensitivity which results from the small amount of compound attached to the resin. In addition, there are sometimes nonassignable resonances in the resins, which vary from batch to batch and with supplier[31]. Also, it

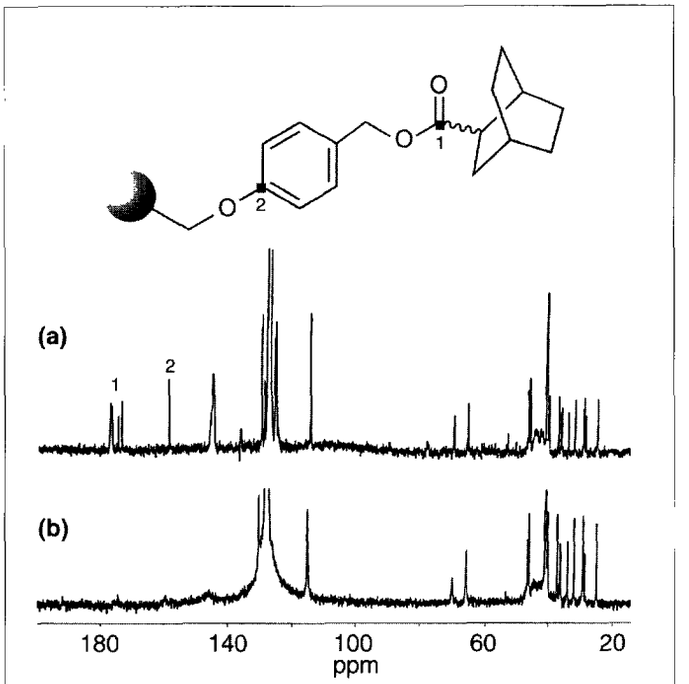

Figure 1. *Magic angle spinning (MAS) ^{13}C-NMR (a) and a conventional ^{13}C-NMR (b) of Wang-resin-linked norbornane-2-carboxylates. (a) MAS (C_6D_6 'wet sample' spun at 5 kHz, 7 mm rotor, 512 transients). (b) Gel phase (C_6D_6, 125 MHz, 5 mm probe). (Reproduced with permission from Ref. 32.)*

can take several hours to acquire a spectrum with a suitable signal-to-noise ratio (Figure 1b)[32].

On the positive side, gel-phase ^{13}C-NMR gives relatively sharp resonances (10–15 Hz) and has a favourably large chemical shift spread. (Spectra are observed because small molecules attached to the solid support are relatively mobile compared with the less mobile polymer backbone when the resin is in the gel phase; indeed, variations in compound mobility have been examined using ^{13}C- and ^{19}F-NMR and correlated to the relative success of peptide synthesis on differing resins and under differing reaction conditions, as well as in the study of peptide aggregation[33].) Most resins used for solid-phase chemistry are suitable for gel-phase ^{13}C-NMR studies. Resins such as TentaGel give rise to the narrowest line widths compared with simple polystyrene-based materials, but the difference is probably immaterial in most cases.

Gel-phase NMR has been used with a variety of different nuclei. For example, ^{19}F-NMR has been used to study the kinetics of aromatic substitution reactions with resin-linked fluoronitrobenzamides[34,35], and ^{31}P-NMR has been used to study Horner-Wadsworth-Emmons (HWE) chemistry on the solid phase[36]. The utility of these naturally high-abundance nuclei leads logically to the use of ^{13}C-enriched materials[37]. This provides a valuable increase in the utility of conventional ^{13}C-NMR methodologies for studying solid-phase reactions, allowing a dramatic decrease in the necessary acquisition times (often less than 100 transients are needed) to determine the fate of the labelled atom. The method does not require 100% labelled materials; indeed, labelling levels of 20% are sufficient for the majority of cases[38]. As in conventional gel-phase ^{13}C-NMR, a wide variety of resins can be used. The example in Figure 2 shows the 30 ppm change in the chemical shift of a bromomethyl group upon alkylation using polystyrene resin swollen in deuterated dimethylformamide and 20% ^{13}C-labelled material. The application of this method has been demonstrated in several reports[39,40].

^{13}C magic angle spinning NMR

The simple approaches outlined above have been extended with the aid of magic angle spinning (MAS) NMR (Ref. 32), and also by the use of a specially designed high-resolution MAS probe, the so-called 'nanoprobe'[41]. The major advantages to

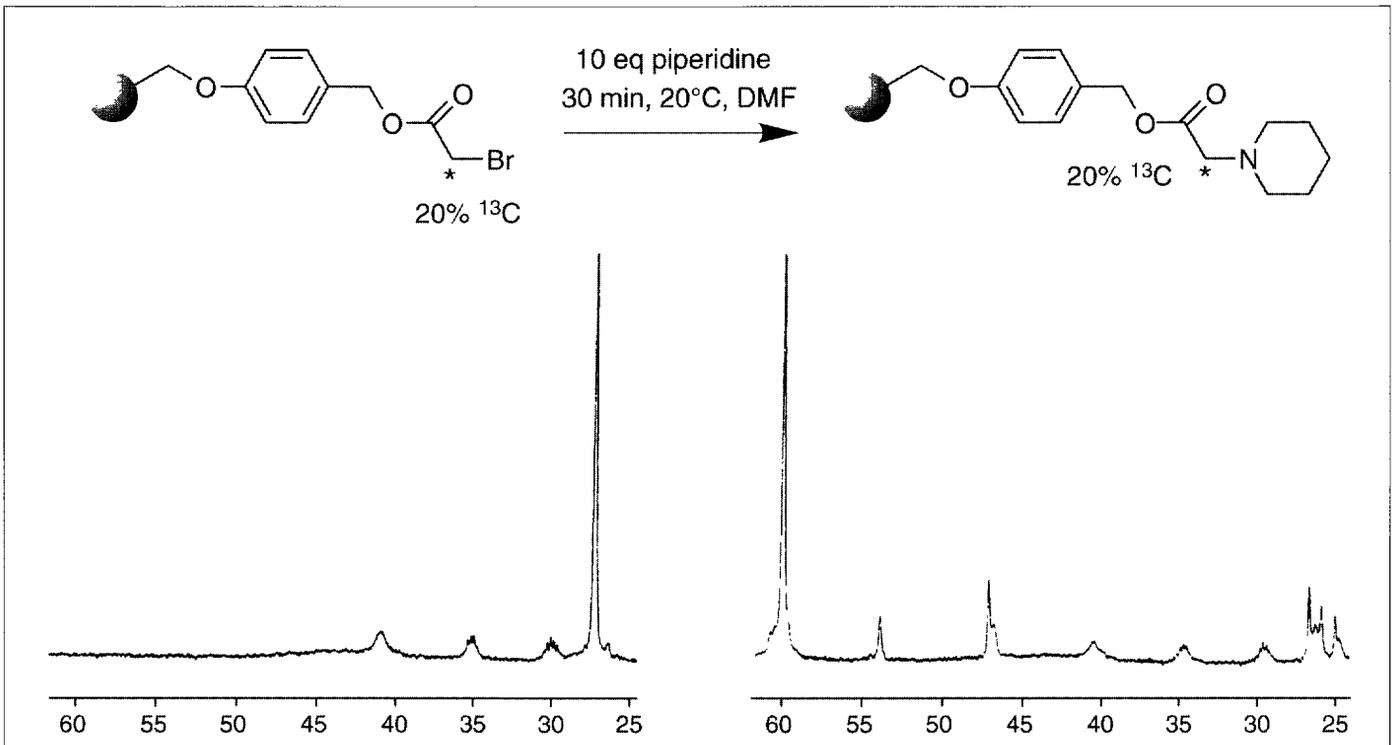

Figure 2. *^{13}C-enriched materials in the monitoring of a solid phase alkylation reaction using 20% ^{13}C enrichment. The chemical shift of the ^{13}C-enriched carbon atom (marked with an asterisk) changes 30 ppm upon alkylation (DMF, dimethylformamide). Spectra were acquired on a Brüker DRX 400 at 100.6 MHz using 526 scans. (Reproduced from Ref. 38.)*

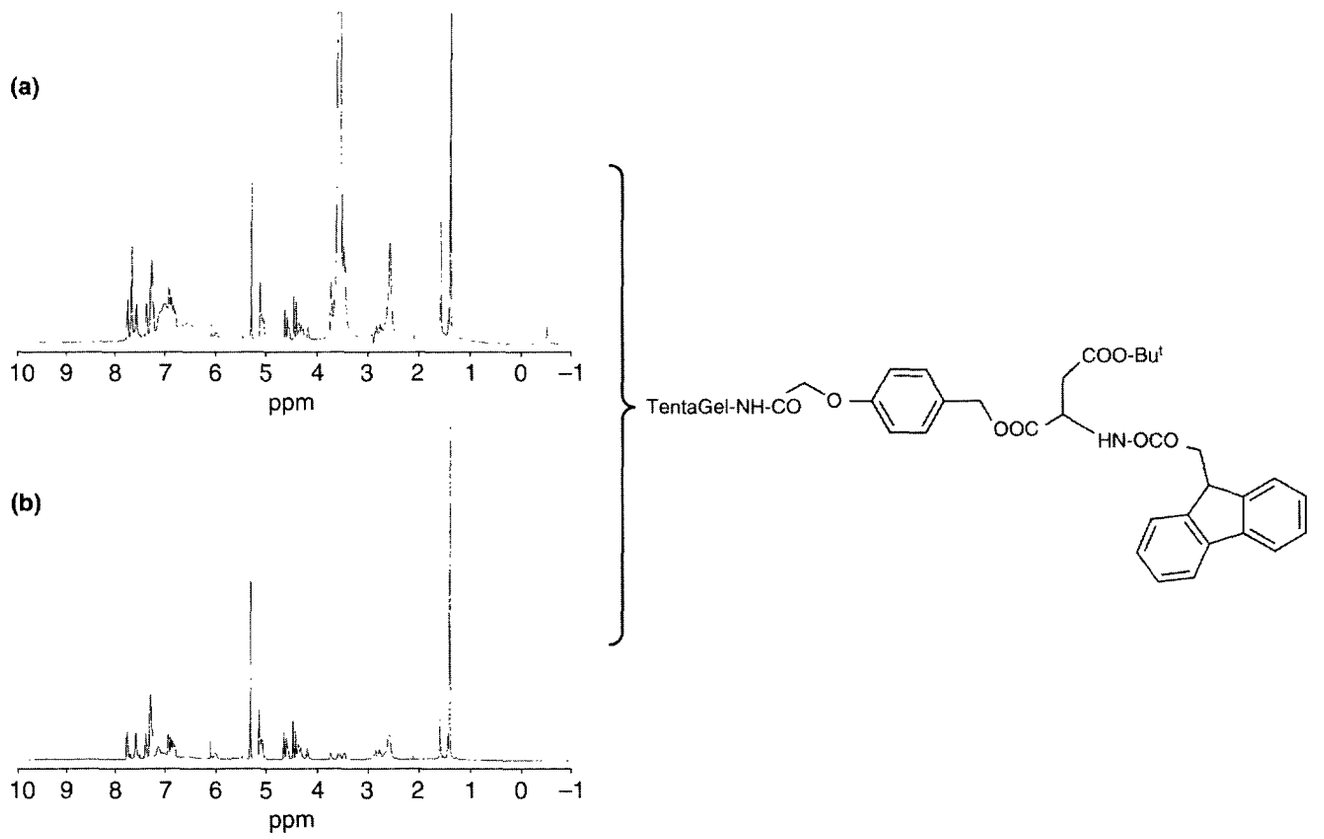

Figure 3. *^{1}H magic angle spinning (MAS) nanoprobe NMR. (a) ^{1}H MAS Varian nanoprobe NMR of FMOC-Asp(OBu^t)-O-CH_2-PhOCH_2CO-NH-TentaGel resin, swollen in CD_2Cl_2, 1 scan, spun at 2 kHz. (b) ^{1}H MAS Varian nanoprobe NMR of FMOC-Asp(OBu^t)-O-CH_2-PhOCH_2CO-NH-TentaGel resin, swollen in CD_2Cl_2, 1 scan, spun at 2 kHz, with presaturation at 3.50 ppm. (Reproduced from the Varian catalogue of ^{1}H-NMR spectra using nanoprobe NMR technology, with the permission of Paul Keifer.)*

be gained by MAS techniques are the ability to obtain information in the quaternary carbon region, the general narrowing of line widths and the smaller amounts of resin required to acquire spectra, comparable with those obtained with the traditional 5 mm probe for liquids. The spectra shown in Figure 1 illustrate the difference between MAS-NMR and conventional ^{13}C-NMR. Although ^{13}C-MAS-NMR cannot provide a fast sample turnaround for reaction analysis (without labelling), it brings solid-phase chemistry into the heartland of the organic chemist. Importantly, automation of this process is now possible.

MAS gel-phase ^{1}H-NMR, single-bead ^{1}H-NMR and C–H correlations

High-resolution ^{1}H-NMR spectra can be obtained on resin-bound materials using MAS. The best quality 1D spectra have been acquired using a specially designed MAS nanoprobe, which is 'built with magnetic-susceptibility-matched materials' to eliminate the line-broadening component[33,42] and has a highly efficient detection system by virtue of 100% of the sample being placed in the receiver coil. A variety of different resins, spacers and linkers have been thoroughly investigated for their influence on spectral quality. Presaturation of TentaGel resins for suppression of polymer-matrix resonances appears optimal (see Figure 3)[43]. This nanoprobe technology has also been used to record ^{1}H spectra from single beads[44]. It is not yet clear, however, whether such methodology will become useful and routine.

Perfectly adequate 2D spectra can be obtained using a conventional MAS probe; however, this does require more sample[45,46]. The technique is illustrated in Figure 4, where a total correlation spectroscopy (TOCSY) NMR spectrum

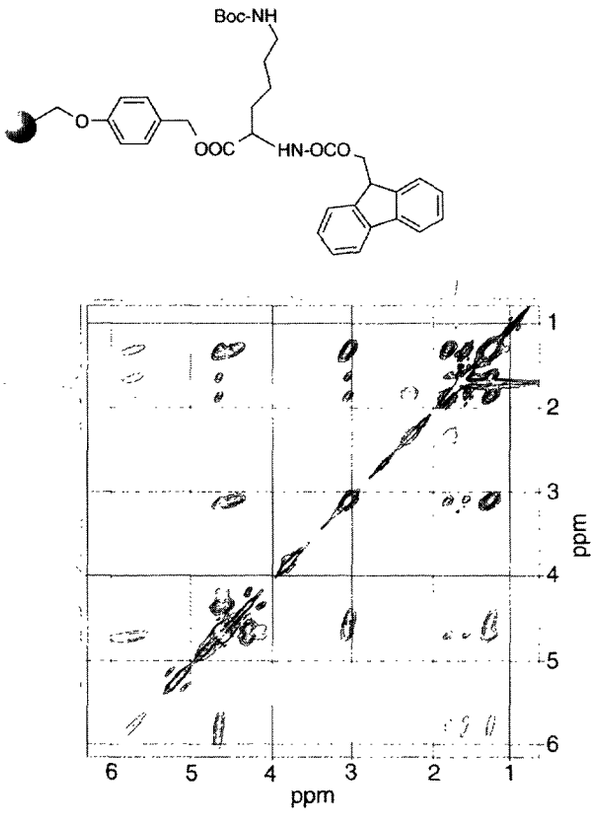

Figure 4. *Magic angle spinning TOCSY (2D ^1H) NMR of FMOC-Lys(BOC)-Wang polystyrene resin. Spectra were obtained on a Brüker DMX 400 at 400 MHz, with a 7 mm rotor, spun at 5 kHz, and a data matrix of 1024×256 scans. (Reproduced from Ref. 45.)*

of FMOC-Lys(BOC)-Wang linker resin (BOC, *tert*-butoxycarbonyl) is shown. Clearly, its utility will depend on the application, but good-quality 2D spectra (^{1}H-NMR) are rapidly obtained on traditional MAS instruments.

IR spectroscopy

Fourier transform infrared (FTIR) spectroscopy has a unique role to play in solid-phase reaction analysis. It is a particularly powerful method for obtaining detailed kinetic information about particular solid-phase reactions, and allows variables such as solvent and the influence and advantages of particular resins to be rapidly assessed. This methodology can readily be used to detect reaction completion to >90–95%. In 1971, Frechet and Schuerch used KBr disks of ground beads, with spectra being obtained in a normal transmittance-type mode[10]. Many more examples of this simple method have since been reported[47,48].

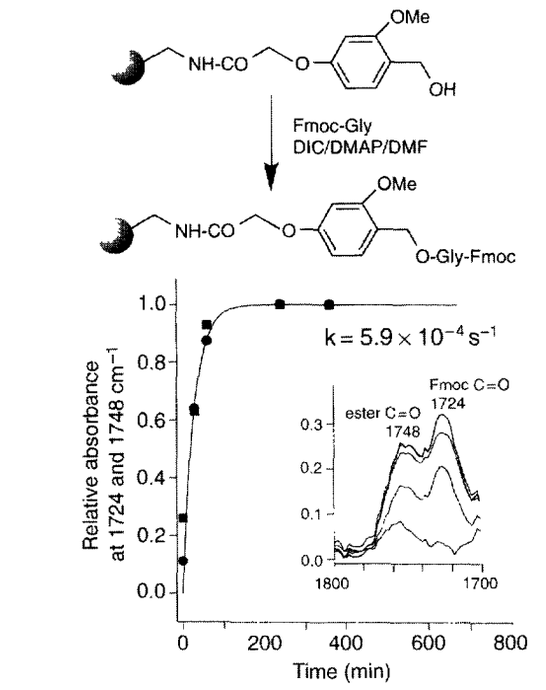

Figure 5. *Single-bead IR spectroscopy. Reaction kinetics of simple esterification of 4-hydroxymethyl-3-methoxyphenoxyacetamido-TentaGel resin with FMOC-Gly/DIC/DMAP (FMOC, 9-fluorenylmethyloxycarbonyl; DIC, diisopropylcarbodiimide; DMAP, 4-dimethylaminopyridine) in dimethylformamide (DMF). Spectra were recorded in transmission mode on a Bio-Rad FTS-40 spectrophotometer coupled with a UMA-300 IR microscope, with 4 cm^{-1} resolution and averaging 64 scans. IR bands due to ester and urethane functionalities. (Reproduced with permission from Ref. 51.)*

Single-bead FTIR

More powerful methods have recently been published that require just a single resin bead for analysis[49]. The first method uses an IR microscope to locate and focus the radiation onto the bead, and an IR spectrum can be obtained of the whole bead in either transmission or reflectance mode. In this geometry, the beads can also be slightly flattened to reduce the sample pathlength to 10–15 μm, significantly reducing spectral distortions and interference[50] (this can be achieved by placing the bead for analysis directly onto the NaCl window and using a manual pellet-maker, although certain beads, such as TentaGel, cannot be flattened as they tend to disintegrate). The second method uses attenuated total reflection (ATR)[51], where the ATR objective is in physical contact with the bead (which also slightly

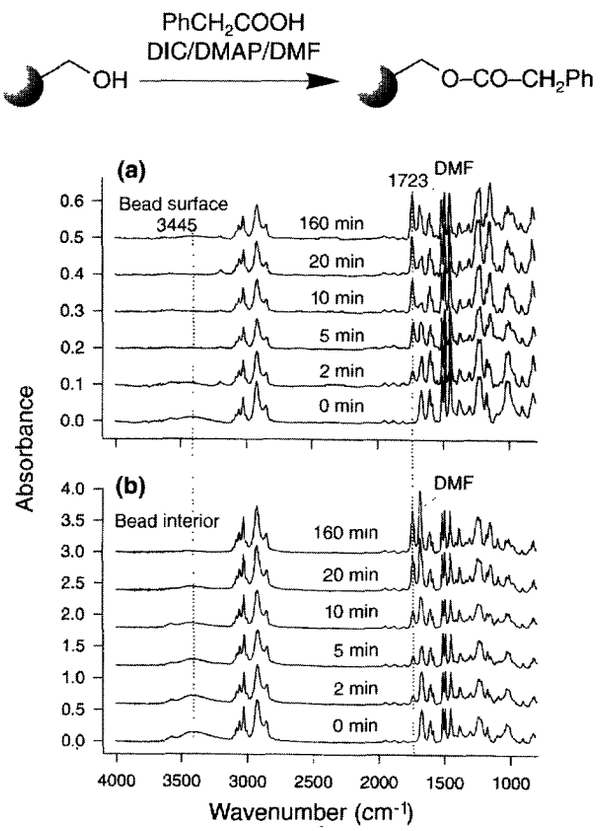

Figure 6. *Single-bead IR spectroscopy of surface and bead interior. Esterification of hydroxymethyl-polystyrene resin with phenylacetic acid/DIC/DMAP (DIC, diisopropylcarbodiimide; DMAP, 4-dimethylaminopyridine) in dimethylformamide (DMF). (a) Attenuated total reflection spectra (surface) were recorded on a Magna-IR™ System 550 coupled with a Nic-Plan™ microscope. (b) Transmission spectra. IR absorbance bands at 3445 cm⁻¹ due to disappearing hydroxyl groups and at 1738 cm⁻¹ due to appearing carbonyl groups. (Reproduced with permission from Ref. 51.)*

flattens the bead). It records an IR spectrum primarily of material on the surface of the bead (to a depth of a few micrometres), giving spectra comparable with those obtained in the transmission mode. This method also demonstrates the extraordinary sensitivity of the IR technique, with a detection limit in the femtomole region. Although single beads are all that are required for IR analysis in these two optimal methods, for ease of handling several beads are usually withdrawn from the reaction mixture for washing before analysis.

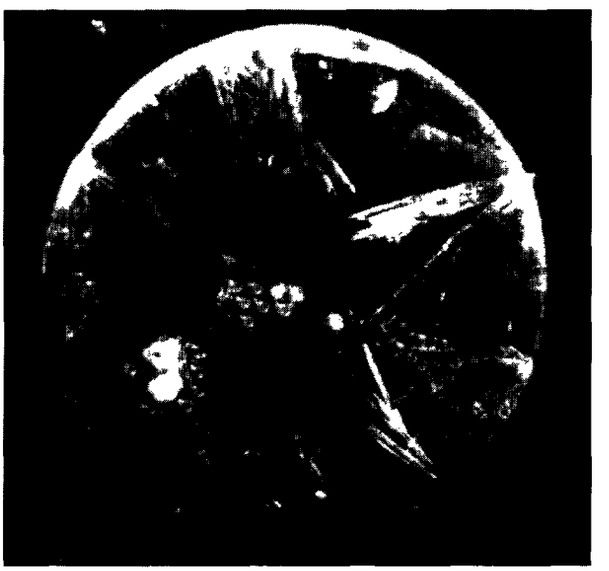

Figure 7. *Resin beads and a crystallizing matrix (2,5-dihydroxybenzoic acid) in a MALDI-TOF sample plate. Well diameter, 2 mm.*

Figure 5 shows the utility of single-bead IR for determining the kinetics of esterification. The technique has been used to demonstrate that there is little variation in rate between resin and solution reactions and has provided some relatively unexpected findings with regard to solid-phase reaction kinetics; for example, comparison of reaction rates on the surface and the interior of the beads by IR has shown little difference between them (Figure 6). In a recent report, IR microscopy was used to study the C–D bond content on resin beads as a tool for reaction analysis[52].

Mass spectrometry

Mass spectrometry (MS), although destructive in nature, is undoubtedly the most sensitive method available for studying solid-phase reactions. It necessitates an efficient and routine linker-cleavage chemistry, and to some extent lacks the accurate quantification abilities of other analytical methods. It is, however, a tool that can be used to rapidly assess solid-phase reactions and is especially useful for the solid-phase synthetic analysis of compounds with molecular weights >500 (Refs 53,54). In fact, it is one of the few methods available for the analysis of large compounds being synthesized on solid phase beads and will find a valuable niche in natural product template-based library synthesis. Three MS methods, namely matrix-assisted laser desorption ionization time-of-flight MS (MALDI-TOF-MS), imaging time-of-flight

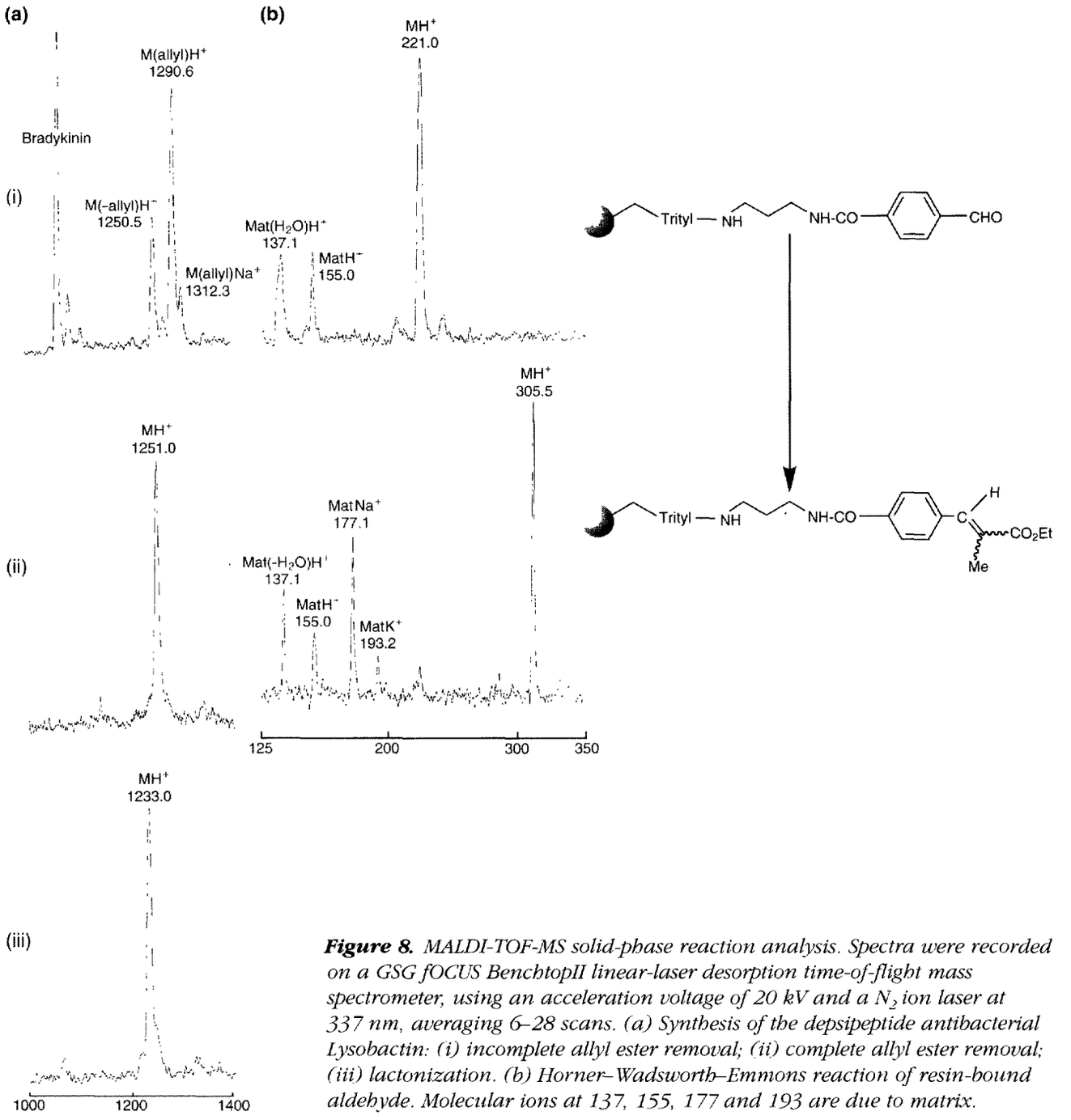

Figure 8. *MALDI-TOF-MS solid-phase reaction analysis. Spectra were recorded on a GSG fOCUS BenchtopII linear-laser desorption time-of-flight mass spectrometer, using an acceleration voltage of 20 kV and a N_2 ion laser at 337 nm, averaging 6–28 scans. (a) Synthesis of the depsipeptide antibacterial Lysobactin: (i) incomplete allyl ester removal; (ii) complete allyl ester removal; (iii) lactonization. (b) Horner–Wadsworth–Emmons reaction of resin-bound aldehyde. Molecular ions at 137, 155, 177 and 193 are due to matrix.*

secondary ion MS (TOF-SIMS) and electrospray MS (ES-MS), have been used for direct analysis of materials from small numbers of resin beads[53–55]. The use of MS for solid-phase library analysis can be conveniently divided into two categories. First, the direct monitoring of reaction progress and, second, the structural determination of an unknown compound from a single bead. Because of the nature of this review, we will concentrate on the first issue, but readers are directed to the work of Brummel and coworkers for information concerning the determination of unknowns from single beads[56].

The major obstacle to overcome in MS analysis of compounds bound to solid supports is not one of limited material (a single bead contains more than sufficient material for repeated analysis), but one of sample preparation. In our

group[53,57], we place single, or more usually (for practical reasons) several, beads into a sample well and carry out an *in situ* cleavage. This may be by trifluoroacetic acid (TFA) vapour or light, depending on the nature of the linker used (supersensitive acid-based linkers require only a couple of minutes of TFA treatment for complete cleavage). Matrix is then added, together with an internal calibrant, and the mixture is allowed to crystallize (5–10 minutes) (Figure 7) before mass analysis by MALDI-TOF-MS (5 minutes). Typically, 10–50 samples are loaded and analysed sequentially, allowing a whole range of reaction conditions to be investigated. In keeping with mass spectroscopists' acronyms, we have termed this technique SPIMS (solid-phase *in situ* mass spectrometry) because the bead(s) are not removed from the sample before analysis. Figure 8a shows the removal of an allyl ester followed by peptide lactonization with material being cleaved *in situ* with TFA vapour, while Figure 8b shows the analysis of some HWE-type chemistry.

Conclusions

Solid-phase synthesis has a major role to play in the lead identification and development process. However, its success will depend critically on the widespread acceptance and utilization of solid-phase methodologies, and on the ability to broaden the range of reactions that can be carried out routinely on the solid phase. The development of analytical methodologies for the assessment of solid-phase reactions, as discussed in this review, will be pivotal to the success of these processes. It is our belief that all the methods outlined above will continue to have widespread utility, but clearly strategies that replace the fast TLC methodologies such as single-bead IR or MS will assume major roles in the future. Akin to TLC developers and stains, there is the need for the development of a range of new colorimetric assays. The more time-consuming techniques, such as gel-phase NMR, will probably play a similar role in a modern solid-phase laboratory to that of solution-phase NMR in a traditional laboratory setting.

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