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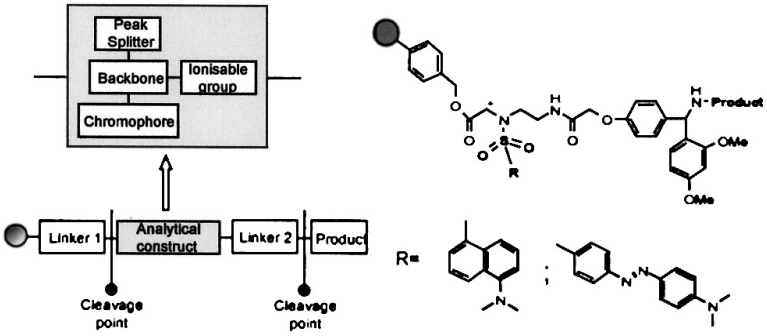

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Dansyl and Dabsyl Analytical Constructs as Tools for the Accurate Estimation of Compounds in Solid-Phase Synthesis

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The presence of dansyl or dabsyl chromogenic moieties in a solid-phase analytical construct, an assembly of linkers/spacers/sensitizers for improving analytical characterization, allows the accurate estimation of products from solid-phase synthesis by UV detection during liquid chromatography−mass spectrometry analysis in the cleavage solution. The spectroscopic properties of dansylated molecules have been evaluated to verify the "compound-independent UV absorption" necessary for using the chromophore in the accurate estimation. First, measurements on commercial dansylated compounds were made, then a series of construct-like molecules were prepared by solution-phase synthetic procedures and their UV properties were determined. Compound calibration curves were determined, and UV absorption was shown to be both proportional to the compound concentration and compound-independent. An example of a dansyl construct derivative was then prepared on a polymeric matrix, and an accurate estimation using the calibration curves was carried out in the cleavage solution. Good agreement was found between the calculated amount of released compound using the UV calibration curves and the calculated amount using both ^{1}H NMR and LC/chemiluminescent nitrogen detection quantitative techniques. Preliminary studies using the dabsyl moiety as an improved chromophore with higher wavelength and extinction coefficient are also reported.

Introduction

Solid-phase synthesis has been known for many years, mostly in the field of peptide and oligonucleotide synthesis. Recently, interest was renewed by combinatorial chemistry and preparation of small-molecule chemical libraries on solid phase as potential sources of new leads for drug discovery.[1−4]

Combinatorial chemistry methods enable very large numbers of compounds to be prepared from limited numbers of building blocks in a relatively small number of reactions on solid phase. Such a high-throughput synthesis has had a big impact on the drug discovery process.[5−8] Hundreds of new drug candidates can be produced simultaneously by a single chemist with the aid of laboratory robotic equipment to automate solid-phase synthetic protocols.[9,10]

The growing interest in this field has required the development of solid-phase analytical techniques suited for high-throughput analysis of small organic molecules.[11−14]

A universally applicable and reliable solid-phase analytical method would massively increase the effectiveness and efficiency of solid-phase chemistry and its use in library synthesis. Mass spectrometry (MS) has a suitable throughput, but not all compounds produce the same response under mass spectrometric conditions.[15] Moreover, MS is not generally suitable for measuring either the absolute quantity of products or the relative concentrations of products in a mixture.

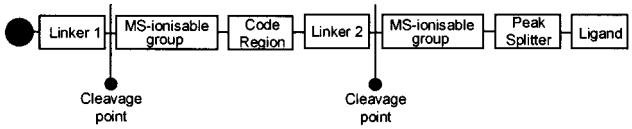

Figure 1. Analytical construct scheme proposed by Geysen et al.

Some attempts to obtain uniform mass spectral responses have been performed by various groups introducing solid-phase linkers with ionizable groups to give a detectable mass spectral response. Geysen et al.[16] introduced a readily ionizable basic lysine into a solid-phase linker and applied it to determine the yield of solid-phase reactions. A schematic representation of their solid-phase analytical construct is illustrated in Figure 1.

The construct is characterized by two portions: the code and the ligand region separated by two identical solid-phase linkers. Each region contains a mass sensitizer (lysine fragment) and a peak splitter (MS fingerprint). Cleavage for analysis affords the two fragments, namely, the mass-sensitized code (tracking the chemical history of the bead

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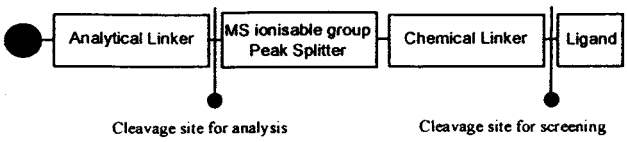


Figure 2. Analytical construct scheme proposed by McKeown et al.

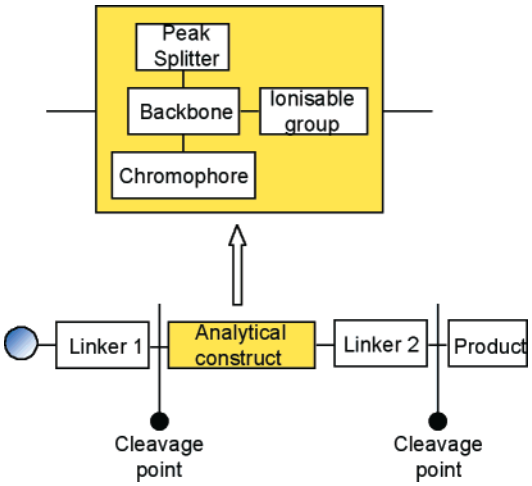


Figure 3. Generic UV-based analytical construct.

when a mix and split protocol is used) and the mass-sensitized ligand. When the areas of the code (internal standard) and the ligand are compared, the efficiency of chemical transformations occurring on solid phase can be determined.

A similar analytical construct has been published by Carrasco.[17] A tetrapeptide chain, called "ionization tag", provided an already ionized group, enabling the construct to be detected by matrix-assisted laser desorption ionization (MALDI) mass spectrometry.

McKeown et al.,[18] inspired by Geysen's analytical constructs, developed a system in which an analytical construct sits between the conventional solid-phase linker (chemical linker) and the solid support. The construct is attached to the resin via a linkage (analytical linker) orthogonal to the conventional linker (Figure 2). The two fragments can be released either at the analytical linker level for analysis purposes or at the chemical linker level for biological screening. Some applications of this construct for the qualitative analysis by mass spectrometry of the compound released from solid-phase synthesis were presented.[19]

Our objective, pursued also elsewhere in our company,[20] is to provide a method to quantitatively analyze products from solid-phase synthesis. Our concept adds to the analytical construct a chromogenic moiety that can be easily quantified by UV detection in the cleavage solution using a conventional liquid chromatography−mass spectrometry (LC−MS) system coupled to an UV detector.

An analytical construct has the following essential features: a reliable MS response ensured by the presence of an ionizable group; an isotopically enriched fragment, called "peak splitter", as the signature for compounds cleaved from solid phase and two orthogonal commonly used linkers for solid-phase combinatorial synthesis.

We have also included a chromophore in the design, summarized in Figure 3. The chromophore must typically

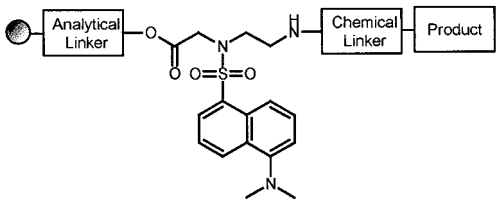


Figure 4. Dansyl analytical construct.

be selected with a characteristic absorption band that distinguishes it from any absorption of the substrate.

Different products synthesized on solid support and released in solution should be quantified using the properties of the strong chromophore inserted in the analytical construct, providing that the cleaved fragment (product + construct) has a constant UV absorbance.

A number of factors were considered in selecting the chromophore to be incorporated into the construct: (1) it should possibly be commercially available; (2) it should be easily introduced into the analytical construct; (3) it must be inert under a wide range of reaction conditions; (4) it must have one of its maximum of absorption occurring in a high-wavelength region, free from absorbance due to resin-bound products (library compounds).

The dansyl moiety, extensively used for amino acid analysis, seemed to have most of the desired features: (1) a useful precursor, dansyl chloride, is commercially available; (2) it is easily insertable into an analytical construct; (3) it is pretty inert under a wide range of chemical conditions; (4) it has the second maximum of absorption at about 335 nm; (5) it also contains a dimethylamino group that, acting as an MS sensitizer moiety, guarantees a detectable mass spectral response.

Therefore, we designed the UV-dansyl analytical construct depicted in Figure 4.

We started by validating if the construct would give UV linear responses independent of influence from the chemical environment around the chromophore by analyzing eight commercial *N*-dansyl derivatives in solution. The following aspects, later discussed in detail, characterized our study: (a) evaluation of UV properties of commercially available dansyl compounds; (b) preparation of dansylated construct-like derivatives by solution-phase chemistry as standards and determination of their spectroscopic properties (calibration curves construction and data elaboration); (c) determination of the UV detection limits using solid-phase analytical constructs; (d) solid-phase synthesis and characterization of dansylated constructs; (e) accurate estimation of a selected compound prepared by solid-phase synthesis and its comparison with results obtained from assessed quantitative analytical techniques (e.g., HPLC/chemiluminescent nitrogen detection (CLND)); (f) preliminary investigation of other, more sensitive chromophores having both a higher λ_{max} (>350 nm of absorption) and a higher extinction coefficient with respect to the dansyl group.

Results and Discussion

UV Response of Eight Commercially Available Dansylated Derivatives. Preliminary work was carried out on eight commercially available dansyl compounds to evaluate

Table 1. Values of Slopes (a) and Intercepts (b) Obtained from Linear Regression of the Experimental Data from the Solutions of the Eight Commercial Dansyl Derivatives

compound	a	Δa	b	Δb	R^2
N-dansylphenylalanine	4.21×10^{-3}	0.04×10^{-3}	3.1×10^{-3}	2.2×10^{-3}	0.9998
N-dansyl-4-hydroxyproline	4.66×10^{-3}	0.01×10^{-3}	1.2×10^{-3}	0.7×10^{-3}	1.0000
N-dansylasparagine	4.29×10^{-3}	0.05×10^{-3}	5.4×10^{-3}	3.1×10^{-3}	0.9998
N-dansylmethionine	4.38×10^{-3}	0.02×10^{-3}	-0.4×10^{-3}	0.9×10^{-3}	1.0000
N-dansylaspartic acid	4.48×10^{-3}	0.07×10^{-3}	3.7×10^{-3}	2.5×10^{-3}	0.9995
N-dansyltryptophan	4.38×10^{-3}	0.02×10^{-3}	1.5×10^{-3}	1.0×10^{-3}	1.0000
N-dansyllysine	4.20×10^{-3}	0.04×10^{-3}	3.3×10^{-3}	1.8×10^{-3}	0.9998
dansylamide	4.77×10^{-3}	0.05×10^{-3}	2.2×10^{-3}	4.1×10^{-3}	0.9997

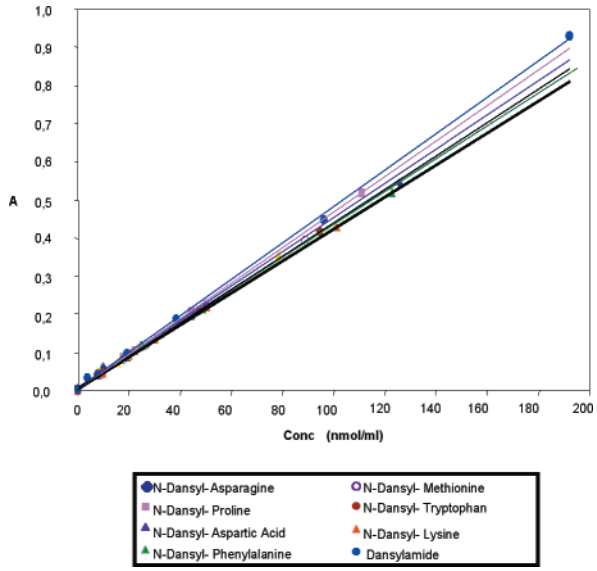


Figure 5. UV absorbance values at 334 nm of eight commercial dansyl derivatives.

both the linearity and the compound-independence of their UV response. For each compound a set of solutions at known concentrations was prepared and the absorbance values at 334 nm were collected. The wavelength corresponding to the second λ_{max} value (334 nm) of the dansyl was chosen to be sufficiently far away from other chromophore absorptions present in the majority of the organic molecules. The concentrations of the solutions used for these measurements were between 5 and 190 nmol/mL. The absorbance data were plotted versus the concentration values, and the calibration curves are reported in Figure 5.

Linear regression of the experimental values was calculated to evaluate the linearity of the data obtained and the differences between the regression parameters a and b (indexes of the compound independent response), where a is the slope of the curve and b is the intercept. The general equation used is the following:

$$y = ax + b \tag{1}$$

Data obtained from the dansylated compounds were processed according to the general form of eq 1. The values of parameters a and b are reported in Table 1.

Mean linear regression was calculated using the mean of the slopes and the mean of the intercepts from Table 1. The following equation resulted:

$$y = (4.42 \times 10^{-3})x + 2.50 \times 10^{-3} \tag{2}$$

From the data reported in Table 1 and from the mean curve constructed from eq 2 some observations can be made: (1) data from the eight commercial dansylated compounds showed a linear UV response, as demonstrated by the correlation factors (R^2), which are close to 1; (2) the slopes from the linear regression are in the range of ±10%, and six out of eight are within the range of ±5% with respect to the calculated mean curve slope; (3) the differences among intercepts values are within the experimental error.

Additional experiments using the same dansyl compounds solutions were carried out using a HPLC−UV method, and the peak area data were plotted versus the concentration values. The results, as expected, were superimposable.

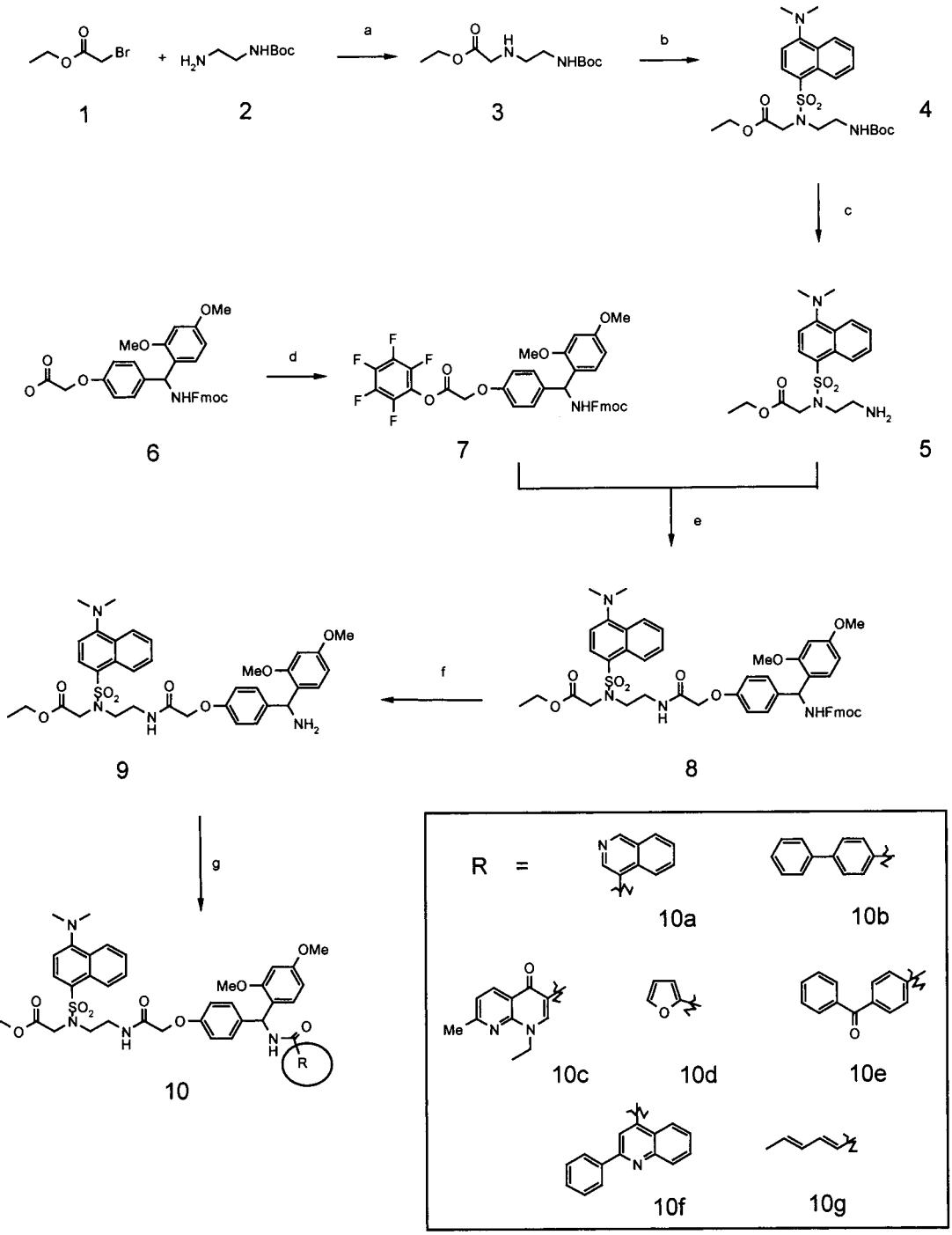
Determination of the concentration of the compounds in the standard solutions using the calculated mean curve gave values within the ±10% range of error with respect to the known concentration. This range of error in view of accurate estimation of compounds from solid-phase synthesis was considered acceptable, and we moved to transfer UV−HPLC−MS analytical constructs to the solid-phase.

UV Response of Nine Dansylated Construct Derivatives. Dispite the multistep synthetic sequence necessary to access construct-like derivatives, it was considered convenient to approach the preparation of these compounds by classical solution-phase synthesis (Scheme 1). Nine construct-like derivatives were prepared in relatively large amounts (more than 50 mg after purification), and each compound was purified by semipreparative HPLC to obtain high-quality compounds (**10a**, 87%; **10b**, 96.3%; **10c**, 97.04%; **10d**, 99.0%; **10e**, 97.8%; **10f**, 93.3%; **10g**, 96.0% a/a by HPLC/diode array detector (DAD) analysis; for more details on the characterization and UV spectra, see Supporting Information) used for the preparation of a number of standard solutions at various concentrations. Polyconjugated derivatives and nitrogen-containing heterocycles were chosen as acid monomers for making real construct-like compounds. Possible spectroscopic interference at the wavelength of interest (~340 nm) was forecasted using some of these monomers, but we also wanted to include troublesome structures with the aim of fully assessing the scope of our approach.

Representative UV spectra of construct-like derivatives are reported in Figure 6. Solutions of known concentrations were prepared for each of the nine compounds to evaluate their UV response.

Concentrations were between 0.83 and 569.5 nmol/mL. For each solution the UV chromatogram was recorded at 342 nm (second λ_{max}). For each concentration the experiment was repeated three times and the mean value of the peak

Scheme 1[a]



[a] (a) TEA, DCM, room temp; (b) dansyl-Cl, K_2CO_3, dioxane/H_2O; (c) 5% trifluoroacetic acid in dry DCM; (d) pentafluorophenyltrifluoroacetate, pyridine, anhydrous DMF; (e) anhydrous DMF; (f) DBU, MeCN; (g) RCOOH, pentafluorophenyltrifluoroacetate, pyridine, anhydrous DMF.

area was calculated. The peak area values (mAU's) for each compound at 342 nm were plotted vs concentrations (nmol/mL) (Figure 7).

The linear regression of the experimental values for each compound was calculated to evaluate the linearity of the data obtained. The values of these parameters are reported in Table 2.

The mean linear regression was calculated using the mean of the slopes and the mean of the intercepts from Table 2, according to the following equation:

$$y = 2.0488x + 0.088 \quad (3)$$

The data generated from these compounds showed a linear response, with correlation factors (R^2) close to 1 (see Table 2). Seven out of nine curves were sufficiently close to each other. Two outliers, **10c** (nalidixic acid derivative) and **10f** (phenyl quinolinic acid derivative), were identified. This deviation could be expected because both the phenyl quinolinic and the nalidixic moieties have a maximum absorption close to 340 nm, which disturbs the monitored dansyl signal at 342 nm. This finding represents a limitation of the dansylated constructs approach, which could not be applied to libraries of compounds having strong chromophores at

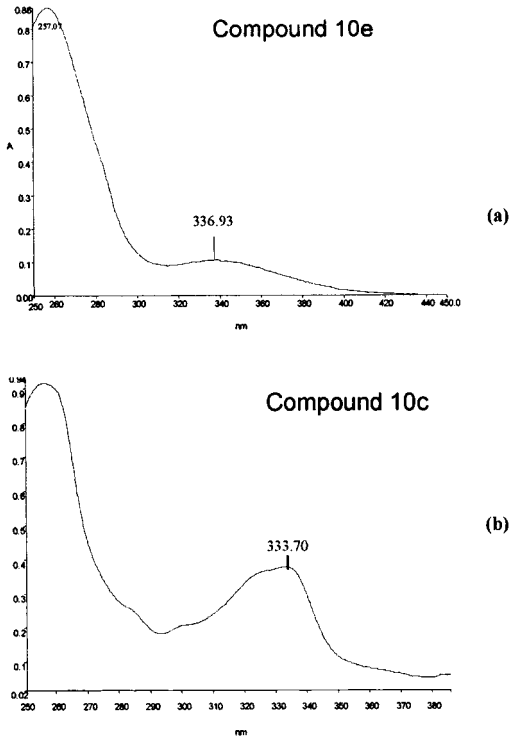


Figure 6. Representative UV spectra of construct-like derivatives: (a) **10e**; (b) **10c**.

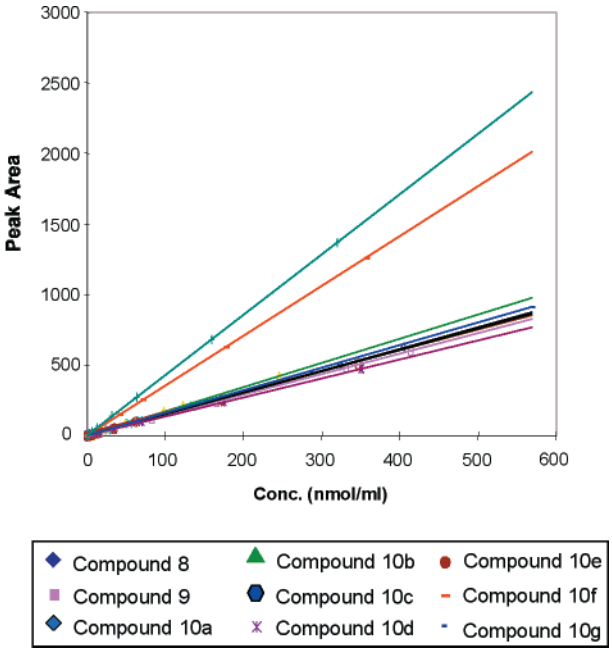


Figure 7. Peak area, at 342 nm, versus concentration of nine dansyl construct-like derivatives.

long wavelengths (>340 nm). Improved chromophores (dabsyl analogues (see below) or, even better, antracenyl derivatives[20]) have been considered in order to address this issue.

It was thus decided to discard **10c** and **10f** from the calculation of the mean linear regression curve. The resulting equation is reported below.

$$y = 1.5194x + 0.110 \quad (4)$$

From the data reported in Table 2 and from the mean curve according to eq 4, some observations can be made. (1) A total of 34 out of 50 data points are in the range ±10% with respect to the mean calculated curve, 11 out of 50 data points are in the range ±15%, and the remaining 5 data points are in the range ±25%. These last data points correspond to the lowest concentrations at which the method sensitivity is very low. (2) The intercept values are within the limits of the experimental error. These data confirmed the preliminary results obtained from eight dansyl amino acids, namely, linearity and a reasonable compound-independence of the UV responses. The UV method proved to be reliable dispite suffering from low sensitivity, using the dansyl moiety as chromophore.[20]

Table 2. Values of Slopes (*a*) and Intercepts (*b*) Obtained from the Linear Regression of the Experimental Data from the Solutions of the Nine Dansylated Construct-like Compounds

compound	*a*	Δ*a*	*b*	Δ*b*	R^2
8	1.5369	0.0056	0.390	1.091	1.0000
9	1.4563	0.0046	−0.726	1.164	1.0000
10a	1.5160	0.0033	0.234	0.251	1.0000
10b	1.7158	0.0020	0.135	0.215	1.0000
10c	4.2763	0.0031	−0.740	0.377	1.0000
10d	1.3411	0.0032	0.070	0.421	1.0000
10e	1.4681	0.0127	1.038	1.764	0.9998
10f	3.5270	0.0042	0.767	0.569	1.0000
10g	1.6016	0.0018	−0.372	0.374	1.0000

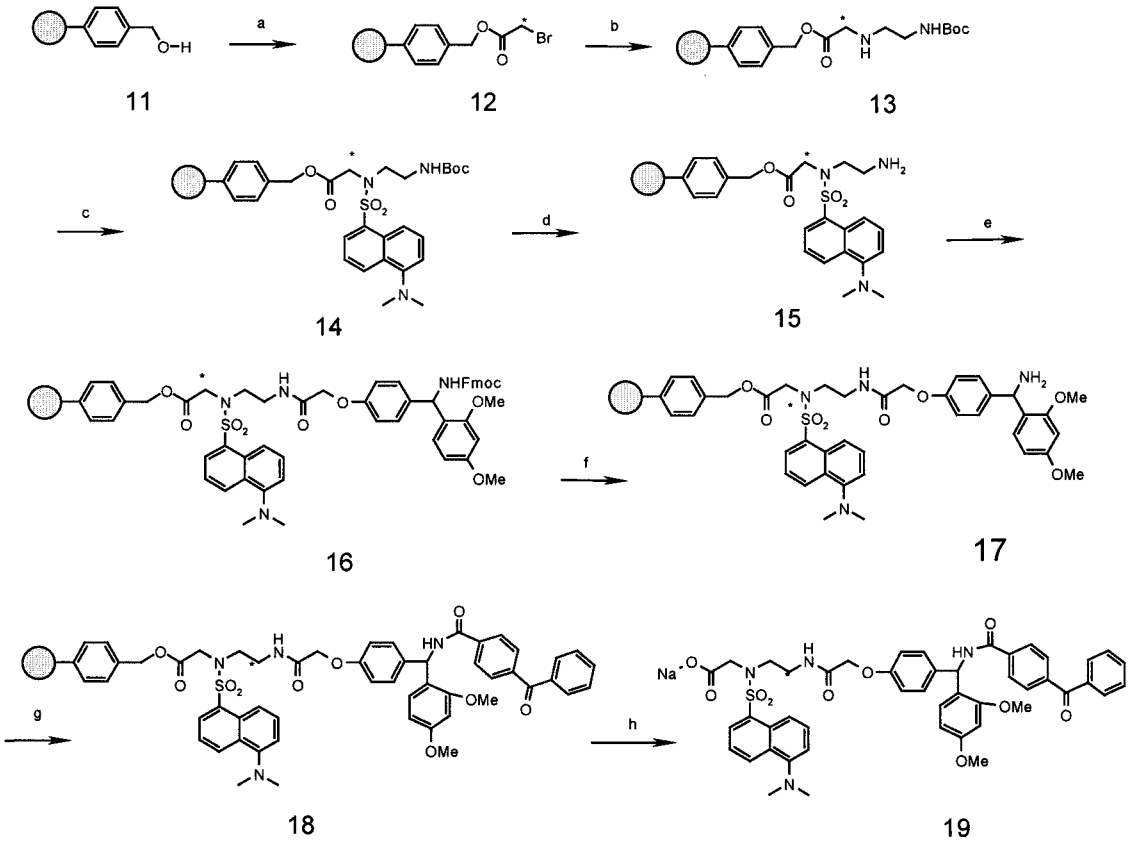
Accurate Estimation of a Dansyl Construct from Solid-Phase Synthesis. Solid-phase synthesis of **18** (supported **10e**) was performed by introducing only minor modifications (benzyl ester in **18** versus the ethyl ester in **10e**) to the final compound with respect to the solution-phase method (Scheme 2). All the solid-phase transformations performed very well as monitored by the conventional solid-phase analytical methods (colorimetric assays (e.g., chloranyl and ninhydrine Kaiser tests) and NMR spectroscopy techniques (e.g., ^{13}C gel-phase NMR and ^{1}H MAS NMR)). An overall 70% w/w corrected (by NMR and HPLC/DAD assays) yield (>95% yield *per* step) has been assigned to the eight-step process used to prepare compound **19** (cleavage recovery from resin **18**; see below).

The accurate estimation of the obtained dansylated derivative **19** released in solution after cleavage was attempted. The previously calculated calibration curve for the corresponding compound **10e** and the mean curves from the panel of solution-phase construct-like molecules were used.

Resin **18** (45 mg, theoretical loading 0.5017 mmol/g) was submitted to the cleavage conditions (1 M aqueous NaOH in THF, 2 equiv). Compound **19** was filtered and washed off the resin, obtaining theoretically 22.58 μmol of compound **19** in 4 mL of aqueous tetrahydrofuran (solution A). A total of 1 mL of this solution was concentrated to dryness, obtaining 5.2 mg of crude compound **19** (quantitative recovery). Furthermore, 1 mL of solution A was diluted to 50 mL with a mixture of 50:50 H_2O/MeCN to obtain a theoretical concentration of 112.9 nmol/mL (solution B).

HPLC/DAD analysis was performed on solution B in order to estimate the % a/a assay of compound **19**, and the chromatogram at 225 nm was selected for the determination. Both the UV chromatograms acquired at 225 and 342 nm are reported in Figure 8.

Scheme 2[a]



[a] (a) 1/1 bromoacetic acid $^{12}C/^{13}C$, DMAP, DIC, DMF; (b) *N*-Boc-diaminoethylene, DMSO; (c) dansyl-Cl, DIPEA, THF; (d) 20% trifluoroacetic acid in DCM; (e) HOBT, DIC, Rink linker, DMF; (f) 20% piperidine in DMF; (g) 4-benzoylbenzoic acid, HOBT, DIC, DMF; (h) NaOH, THF.

The assay of solution B was estimated as 69.3% a/a at 225 nm (average of 3 measurements); this lower wavelength with respect to 342 nm was chosen with the aim to better identify low levels of possible impurities and to assign an as-realistic-as-possible assay to compound **19**. The corrected concentration of compound **19** was thus defined as 78.24 nmol/mL. A good agreement with the ^{1}H NMR analysis results that assigned a 70% mol/mol assay of compound **19** from the cleavage mixture was observed.

To confirm the concentration of compound **19**, in accordance with the measurement at 225 nm, the peak area value of the UV chromatogram at 342 nm (Figure 8b, λ_{max} of dansylated construct) was then considered in the diluted cleavage solution. The target compound (t_R = 2.6 min) showed a peak area of 127 mAU's. This value was used to calculate the concentration of the solution using the equations related to the curve of the benzoylbenzoic-construct **10e** and the equation related to the mean curve, obtained as reported previously (see Figure 7 and eq 4).

The calculated concentration for solution B using the equation related to **10e** ($y = 1.4681x + 1.038$) was 85.79 nmol/mL. The same determination made with the mean curve equation ($y = 1.5194x + 0.110$) gave a concentration value of 83.51 nmol/mL. Deviations of 8.8% and 6.3%, respectively, were calculated comparing these concentrations at 342 nm and the concentration (78.24 nmol/mL) obtained from HPLC % a/a at 225 nm and ^{1}H NMR.

This experiment demonstrates how the use of UV constructs allows an accurate estimation of crude compounds derived from solid-phase synthesis. Therefore, quantitation is theoretically possible even if improvements (higher λ_{max} and a higher extinction coefficient) are still necessary in order to increase sensitivity and to have a more general applicability (inclusion of library compounds having strong chromophores at long wavelengths, >340 nm).

Comparison between UV Calibration Curves and HPLC/CLND Estimation. An aliquot of the solution A was diluted with water, filtered to discard insoluble particles, and freeze-dried. The sample obtained was submitted to HPLC/CLND quantitative analysis. The HPLC/CLND 8060 system was calibrated using nitrobenzene as standard. The freeze-dried sample was dissolved in methanol, obtaining solution C, and analyzed by HPLC/CLND; the chromatograms obtained are reported in Figure 9. Total nitrogen concentration measured in solution C by HPLC/CLND was 2.38 mmol/mL. On the basis of the four nitrogen atoms present in the molecule, the concentration of the sample was determined to be 0.595 mmol/mL (595 nmol/mL).

Solution C was analyzed by UV−HPLC−MS using the UV calibration curves to compare the concentration data obtained by HPLC/CLND. The target peak (t_R = 9.0 min) gave an area of 873 mAU's at 342 nm (mean of three injections). This value was used to calculate the concentration of the solution using the equations related to the curve of the benzoylbenzoic-construct **10e** and to the mean curve.

From the equation of the benzoylbenzoic-construct **10e** ($y = 1.4681x + 1.038$) the calculated concentration was

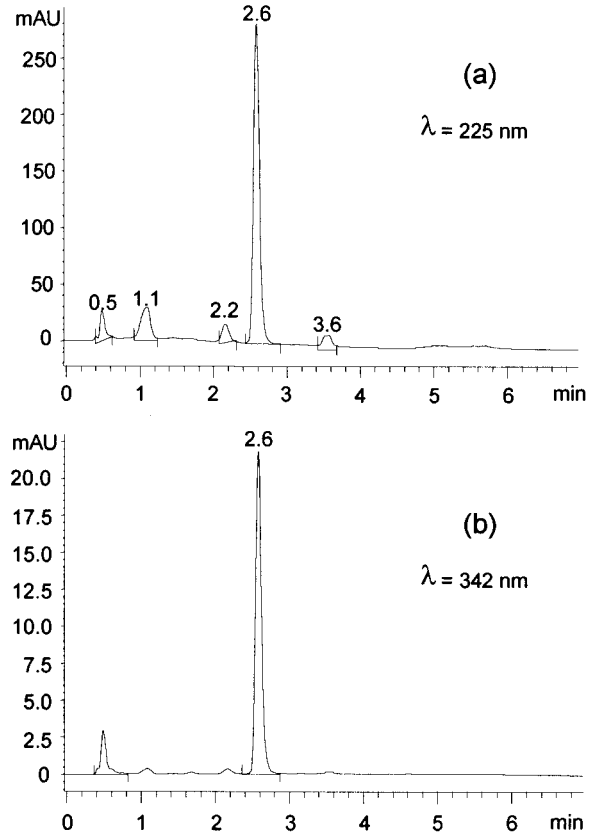


Figure 8. HPLC/DAD analysis of compound **19** with t_R = 2.6 min: (a) UV chromatogram acquired at 225 nm; (b) UV chromatogram acquired at 342 nm.

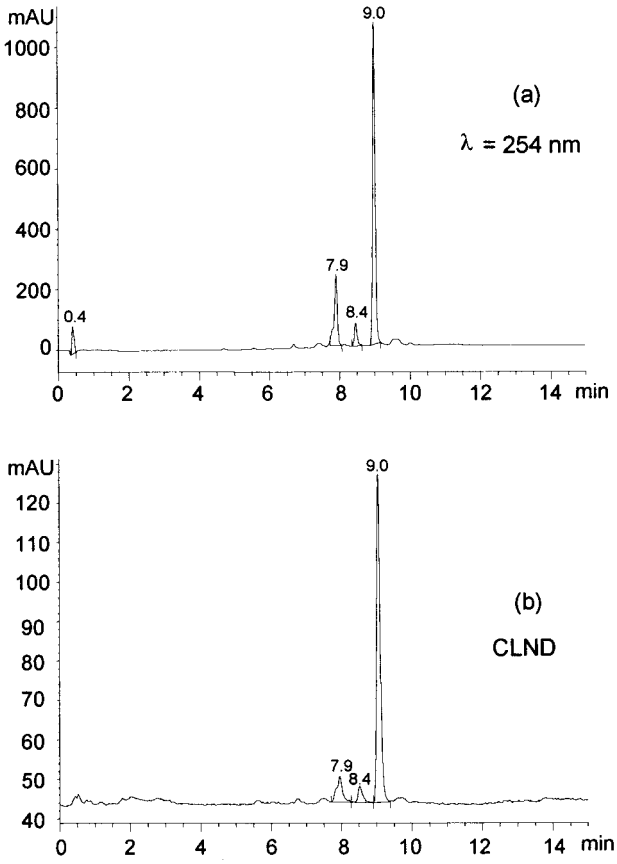


Figure 9. HPLC/DAD/CLND analysis of compound **19** with t_R = 9.0 min: (a) UV chromatogram acquired at 254 nm; (b) CLND chromatogram. On the basis of this chromatogram, the peak area, 873 units, was determined.

593.9 nmol/mL. The value, compared with the HPLC/CLND concentration of 595 nmol/mL, gave a deviation of −0.2%.

The same determination made using the mean curve equation ($y = 1.5194x + 0.110$) gave a concentration of 574.5 nmol/mL with a deviation of −3.4% from the HPLC/CLND concentration.

The assessed HPLC/CLND quantitation method and the UV calibration curves showed good accordance, confirming the validity of the dansyl analytical constructs as a tool for an accurate estimation of compound in solid-phase synthesis.

Preliminary Investigation on a Dabsyl Analytical Construct. The dabsyl group was considered to be an improved chromogenic moiety, taking into consideration its nonideal chemical inertness. The dabsyl group has a λ_{max} at higher wavelengths (452 vs ∼340 nm) and a higher extinction coefficient (ϵ) with respect to the dansyl group. A single example of dabsylated construct derivative **20** (Figure 10) was prepared by solid-phase synthesis (the same synthetic pathway shown in Scheme 2 was followed; see Supporting Information for details) and evaluated.

A set of solutions at known concentrations was prepared to evaluate the UV response and to determine the detection limit value for the dabsyl construct derivative **20**. A comparison with the dansyl analogue was then made. The concentrations of the solutions were between 0.0908 and 90.82 nmol/mL. For each solution the UV chromatogram was recorded at 452 nm (second λ_{max} for dabsyl), each sample was injected three times, and the mean value of the peak area was calculated. The peak area (mAU's) versus concentration values (expressed as nmol/mL) were plotted. The linear regression of the experimental values was calculated in order to evaluate the linearity of the data obtained.

For compound **20** the linear regression gave the equation

$$y = 5.355x - 0.096 \quad (5)$$

with $R^2 = 1.000$.

The detection limit value for this experiment corresponded to a concentration of 0.0908 nmol/mL, based on a signal-to-noise ratio of 3:1. The dansylated compound **10e** had a detection limit of 3.46 nmol/mL; therefore, the substitution of the dansyl moiety (**10e**) with dabsyl (**20**) increased the sensitivity of the method by 38 times.

Considering the UV properties of dabsyl derivatives (very high λ_{max} at 452 nm and ϵ_{450}), it could be argued that a single calibration curve of any dabsyl-containing compound should be sufficient as a standard for any accurate estimation of any compound. This has been demonstrated by considering dabsyl chloride as the simplest standard. A set of solutions of dabsyl chloride at different concentrations ranging between 1.78 and 178.3 nmol/mL were prepared and used for the construction of the related calibration curve. The area values obtained from this experiment (mean of three injections) were plotted versus concentration, and the linear regression of the experimental data gave the following equation:

$$y = 5.408x - 4.372 \quad (6)$$

with $R^2 = 0.9999$.

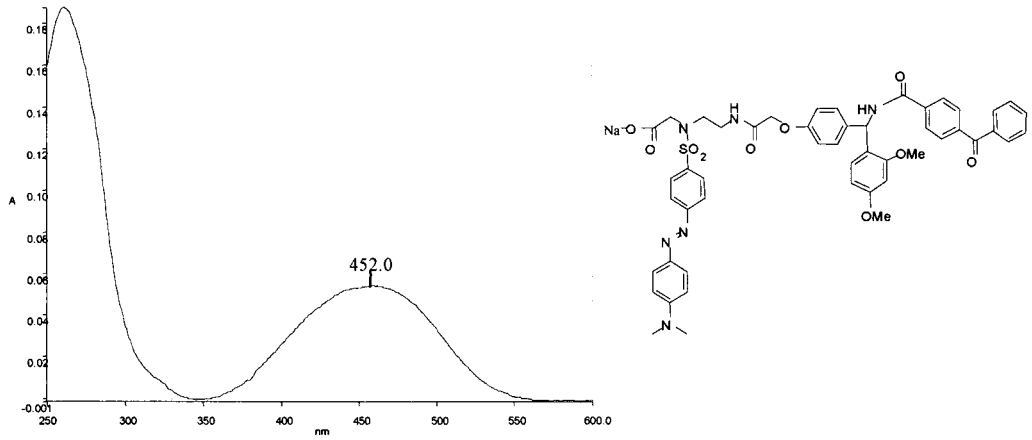


Figure 10. Dabsyl analytical construct **20**.

A solution of compound **20** giving a peak area of 100 mAU's was processed using the two different calibration curves. From eq 5 (specific for compound **20**) and from eq 6 (for a "generic" dabsyl-containing derivative, the chloride) the deviation of the two results was only 3.1%.

These encouraging results confirmed that the dabsyl group is significantly better than dansyl from a spectroscopic point of view and deserves further investigation.

Conclusions

The reported results indicate linearity of the UV responses and acceptable compound-independent UV absorbance of dansyl construct molecules. The preparation of a dansyl construct derivative on solid phase and the accurate estimation of its concentration in the cleavage solution were carried out and demonstrated to be accurate, with an estimated experimental error within ±10%. On the basis of the described findings, we considered that the proof of concept related to the accurate estimation of compounds in solid-phase synthesis using UV constructs was accomplished.

In addition, positive preliminary results related to the use of the dabsyl group as a chromophore were obtained. This improved chromophoric moiety should allow much increased sensitivity of the UV determination, and applications of the methodology to the relative quantitation at the single bead level could be envisaged. Similarly, a more chemically suitable anthranyl chromophore has been recently incorporated into amine-releasing dual linker constructs.[20]

Other applications of dansyl and dabsyl analytical constructs in the quantitative estimation of compounds from solid phase are under evaluation in our group to further increase their usefulness as tools for facile quantitation using standard UV−HPLC−MS analysis.

Experimental Section

Materials. All solution-phase reactions were carried out using conventional glassware under an inert atmosphere. Organic solutions were dried over anhydrous magnesium sulfate and evaporated with a rotary evaporator. Thin-layer chromatography was done with precoated plates of silica gel (Merck F-254) using the indicated solvent. All the individual solid-phase reactions were carried out in glass vials (Wheaton), and the resin washings were carried out on Extract Clean Tube syringes (Alltech or IST). Reagents were purchased from Aldrich, Sigma, Fluka, and Novabiochem and used without further purification. Hydroxymethyl polystyrene resin (0.87 mmol/g, 1% cross-linking, 100−200 mesh, batch no. AI8860) was purchased from Novabiochem.

Instrumentation. UV spectra were collected using a Lambda 10 UV spectrometer (Perkin-Elmer). Analytical characterization of the synthesized compounds was made by FTIR (spectra recorded on an IFS 48 instrument), by gel-phase ^{13}C NMR (Varian unit 300MHz), by ^{1}H MAS NMR (Varian unit 400MHz using the Nanoprobe), and by ^{1}H NMR (Varian INOVA PFG 500 instrument). Mass spectra were acquired using Micromass Platform LZC and Micromass Platform II spectrometers equipped with an electrospray ion source.

LC/DAD analyses were performed using an HP1100 chromatography system (Hewlett-Packard, Germany) equipped with a quaternary pumping system, a variable-volume autosampler, and a DAD detector. The liquid chromatography/chemiluminescent nitrogen detection (HPLC/CLND) was performed with a CLND 8060 detector system (Antek Instruments).

Chromatographic Conditions. The analyses were performed at room temperature using a Supelcosil ABZ+ Plus column (3.3 cm × 0.46 cm, 3 μm) with a flow rate of 0.8 mL/min. The mobile phase was water + 0.01% acetic acid (A) and acetonitrile (B). Single-gradient conditions (80/20 of A/B to 10/90 in 10 min) were used for an analysis time of 12 min; a 10 μL injection volume was used.

Data Processing. Raw data were processed using the HP Chemstation software (revision A.05.02). The collected data were processed using the Grafit Data Analysis program (version 3.09b), and the graphs were generated using Microsoft Excel 97.

Sample Preparation for HPLC−MS. The compounds used were dissolved in 50/50 v/v water/acetonitrile and diluted to a set of solutions with different concentrations.

Preparation of Compound 3. A solution of bromoethyl acetate (1.32 mL, 11.85 mmol) in dichloromethane (10 mL) was added to a stirred solution of Boc-ethylenediamine (1.58 mL, 10 mmol) and triethylamine (1.74 mL, 12.5 mmol) in dichloromethane (15 mL) at room temperature. The reaction, monitored by TLC, was completed after 3 h. The solvent

was evaporated under reduced pressure, and the residue was purified by flash chromatography (elution with cyclohexane and ethyl acetate) to give product **3** (1.6 g, 68%). ^{1}H NMR ($CDCl_3$): δ 5.03 (1H, bm), 4.20 (2H, m), 3.42 (2H, m), 3.24 (2H,m), 2.78 (2H, m), 1.46 (9H, s), 1.29 (3H, m). MS-ES$^+$: *m*/*z* 247 $[M + H]^+$.

Preparation of Compound 4. Dansyl chloride (2.75 g, 10.2 mmol) was added portionwise to a mixture of **3** (1.6 g, 6.8 mmol) and potassium carbonate (1.65 g, 11.95 mmol) dissolved in 50/50 v/v water/dioxane (30 mL). The mixture was stirred for 2.5 h at room temperature, then water was added. The organic phase was extracted with diethyl ether (5 × 15 mL), washed with brine, and dried over sodium sulfate. Solvents were evaporated under vacuum, and the crude was purified by flash chromatography to obtain **4** (1.7 g, 53.5%). ^{1}H NMR ($CDCl_3$): δ 8.56 (1H, d), 8.28 (1H, d), 8.25 (1H, dd), 7.58 (1H, t), 7.53 (1H, dd), 7.21 (1H, d), 5.29 (1H, bs), 4.77 (1H, bs), 3.18 (2H, m), 3.02 (2H, q), 2.90 (6H, s), 1.40 (9H, s). MS-ES$^+$: *m*/*z* 480 $[M + H]^+$ and *m*/*z* 380 $[M - Boc + H]^+$.

Preparation of Compound 8. To a solution of **4** (1.8 g, 3.84 mmol) in dry dichloromethane (40 mL), a solution of trifluoroacetic acid (3 mL) was added at room temperature. The mixture was stirred for 2 h and then quenched with saturated aqueous sodium hydrogenocarbonate solution. The organic layer was separated and dried over sodium sulfate. The resulting dried solution containing crude compound **5** was immediately used without further purification. To a solution of **5** was added the pentafluorophenyl ester **7** of the Rink linker (2.6 g, 3.84 mmol) as dry DMF solution (15 mL) (see the general procedure reported after the pentafluorophenyl ester preparation section). The mixture was stirred for 2 h at room temperature with the reaction course monitored by TLC. Upon reaction completion ethyl acetate (100 mL) was added. The reaction mixture was washed with a saturated sodium hydrogenocarbonate solution (30 mL), water (2 × 20 mL), and brine (2 × 20 mL). The organic layer was dried over sodium sulfate, and the solvents were evaporated under reduced pressure to give the crude compound **8** (3.5 g). A portion of this batch (0.1 g) was purified by semipreparative HPLC and used as a standard for the calibration curve construction. ^{1}H NMR ($CDCl_3$): δ 8.53 (1H, d), 8.26 (1H, d), 8.22 (1H, d), 7.77 (2H, d), 7.60 (2H, d), 7.50 (2H, m), 7.40 (3H, m), 7.29 (2H, m), 7.15 (2H, d), 7.13 (1H, dd), 7.10 (1H, d), 6.84 (2H, d), 6.49 (1H, s), 6.48 (1H, dd), 6.06 (1H, bd), 5.82 (1H, bd), 4.44 (2H, m), 4.31 (2H, s), 4.25 (1H, t), 4.15 (2H, s), 4.05(2H, q), 3.75 (3H, s), 3.50 (4H, bs), 2.84 (6H, s), 2.82 (3H, s), 1.15 (3H, t). MS-ES$^+$: *m*/*z* 923 $[M + Na]^+$. Assay, by HPLC/DAD analysis, after semipreparative purification: l00% a/a.

Preparation of Compound 9. To a solution of compound **8** (3.06 g, 3.4 mmol) in dry acetonitrile (40 mL) at 80 °C a solution of DBU (0.510 mL, 3.4 mmol) was added. The reaction, monitored by TLC, was completed after 1 h. The mixture was cooled at room temperature and extracted with petroleum ether (6 × 20 mL). The acetonitrile layer was evaporated under vacuum to afford **9** (2 g, 87%). A portion of **9** (0.1 g) was purified by semipreparative HPLC and used as a standard for the calibration curve construction. ^{1}H NMR (DMSO-d_6): δ 8.44 (1H, d), 8.10 (2H, m), 7.94 (1H, t), 7.57 (2H, m), 7.25 (1H, d), 7.18 (3H, m), 6.76 (2H, d), 6.46 (1H, s), 6.45 (1H, m), 5.20 (1H, s), 4.22 (2H, s), 4.19 (2H, s), 3.90 (2H, q), 3.70 (6H, s), 2.77 (6H, s), 1.35−1.32 (4H, m), 1.01 (3H, t). MS-ES$^+$: *m*/*z* 679 $[M + H]^+$. Assay, by HPLC/DAD analysis, after semipreparative purification: 91.3% a/a.

General Procedure for the Preparation of Pentafluorophenyl Esters. To a solution of the carboxylic acid (0.5 mmol) in dry dimethylformamide (1 mL), pyridine (1.15 equiv) and pentafluorophenyltrifluoroacetate (1.2 equiv) were added. The mixture was allowed to react until reaction completion. The reaction was quenched by dilution with ethyl acetate (10 mL), and the mixture was washed with a 0.1 N HCl solution (3 × 5 mL) and a 5% sodium hydrogenocarbonate solution (3 × 5 mL). The organic layer was dried over sodium sulfate, and the solvents were evaporated under reduced pressure. Crude material was used without further purification.

Reaction between Compound 9 and Pentafluorophenyl Esters: General Procedure. To a solution of compound **9** (0.1 g, 0.147 mmol) in dry dimethylformamide (0.5 mL) a solution of pentafluorophenyl activated ester (1.1 equiv) was added. The mixture was allowed to react until completion monitored by TLC. The mixture was diluted with ethyl acetate (5 mL) and washed with an aqueous 5% sodium hydrogenocarbonate solution (2 × 3 mL), a saturated ammonium chloride solution (2 × 3 mL), and brine. The organic layer was evaporated under reduced pressure. Crude compounds **10a**−**g** were purified by semipreparative HPLC and characterized by NMR and HPLC−MS analysis (see Supporting Information for details).

Preparation of Compound 12. To a suspension of hydroxymethylbenzylpolystyrene resin **11** (1 g, 0.87 mmol, 1 equiv; Novabiochem 100−200 mesh, 1% DVB) in dry dimethylformamide (12 mL), bromoacetic acid ^{12}C-2 (0.6 g, 5 equiv), bromoacetic acid ^{13}C-2 (0.65 g, 5 equiv), DMAP (catalytic amount), and DIC (1.4 mL, 10 equiv) were added. The mixture was stirred for 24 h at room temperature. The resin was filtered, washed with dimethylformamide (2 × 10 mL), dichloromethane (5 × 10 mL), methanol (2 × 10 mL), and dichloromethane (2 × 10 mL), and dried under reduced pressure. Gel-phase ^{13}C NMR (C_6D_6): δ 25.82 (1C).

Preparation of Compound 13. To a suspension of resin **12** (0.95 g, 0.83 mmol, 1 equiv) in dry dimethyl sulfoxide (10 mL), neat *N*-Boc-ethylenediamine (1.3 mL, 8.3 mmol, 10 equiv) was added. The mixture was stirred at room temperature for 24 h. Resin **13** was filtered, washed with dimethyl sulfoxide (2 × 10 mL), dichloromethane (5 × 10 mL), methanol (2 × 10 mL), and dichloromethane (5 × 10 mL), and dried under reduced pressure. The colorimetric chloranyl test[21] for secondary amine detection was positive. MAS ^{1}H NMR (CD_2Cl_2): δ 5.06 (2H, bm), 3.41 (2H, bm), 3.14 (2H, m), 2.69 (2H, m), 1.42 (9H, s). Elemental analysis for N: theoretical 2.38%; found 2.28%, yield 95.8%.

Preparation of Compound 14. To a suspension of resin **13** (0.85 g, 0.74 mmol, 1 equiv) and dansyl chloride (2 g, 1.48 mmol, 10 equiv) in dry tetrahydrofuran (10 mL), diisopropylethylamine (1.93 mL, 11.1 mmol, 15 equiv) was added. The resulting mixture was stirred at room temperature

for 24 h. Then resin **14** was filtered, washed with tetrahydrofuran (2 × 10 mL), dichloromethane (5 × 10 mL), methanol (2 × 10 mL), and dichloromethane (5 × 10 mL), and dried under reduced pressure. The negative colorimetric chloranyl test confirmed the saturation of all the secondary amine sites. MAS ^{1}H NMR (CD_2Cl_2): δ 8.49 (1H, bs), 8.25 (1H, bs), 8.15 (1H, bs), 7.48 (2H, bm), 4.15 (2H, bs), 3.39 (2H, bs), 3.16 (2H, bs), 2.81 (6H, bs), 1.37 (9H, bs).

Preparation of Compound 15. To a suspension of resin **14** (0.74 mmol, 1 equiv) in dry dichloromethane (10 mL), trifluoroacetic acid (2 mL) was added. The mixture was stirred at room temperature for 24 h. Resin **15** was filtered, washed with dichloromethane (5 × 10 mL), methanol (2 × 10 mL), and dichloromethane (5 × 10 mL), and dried under reduced pressure. The colorimetric Kaiser ninidrine test[22] to reveal the presence of primary amine was positive.

Preparation of Compound 16. To a solution of Rink linker (3.9 g, 10 equiv) in dry dimethylformamide (5 mL), HOBT (0.97 g, 10 equiv) and DIC (1.15 mL, 10 equiv) were added. The mixture was stirred at room temperature for 15 min, and then a suspension of resin **15** (0.74 mmol, 1 equiv) in dimethylformamide (5 mL) and diisopropylamine (0.25 mL, 2 equiv) were added. The mixture was stirred for 24 h at room temperature. Resin **16** was filtered, washed with dimethylformamide (2 × 10 mL), dichloromethane (5 × 10 mL), methanol (2 × 10 mL), and dichloromethane (5 × 10 mL), and dried under reduced pressure. The negative colorimetric Kaiser ninhidrine test indicated a quantitative conversion.

Preparation of Compound 17. Resin **16** (0.049 g, 24.5 μmol, 1 equiv) as a suspension in a 20% piperidine/dimethylformamide solution (2 mL) was stirred for 2 h. Resin **17** was filtered, washed with dimethylformamide (2 × 10 mL), dichloromethane (5 × 10 mL), methanol (2 × 10 mL), and dichloromethane (5 × 10 mL), and dried under reduced pressure. Yield determination after this reaction was quantitative as checked by UV Fmoc reading. The colorimetric Kaiser test was positive.

Preparation of Compound 18. To a solution of 4-benzoylbenzoic acid (0.0554 g, 10 equiv) in dry dimethylformamide (0.5 mL), HOBT (0.033 g, 10 equiv) and DIC (40 μL, 10 equiv) were added. The mixture was stirred at room temperature for 15 min, and a suspension of resin **17** (24.5 μmol, 1 equiv) in dimethylformamide (0.5 mL) was added. The mixture was stirred for 20 h at room temperature and then filtered, washed with dimethylformamide (2 × 10 mL), dichloromethane (5 × 10 mL), methanol (2 × 10 mL), and dichloromethane (5 × 10 mL), and dried under reduced pressure. The negative colorimetric Kaiser test indicated quantitative conversion.

Preparation of Compound 19. To a suspension of resin **18** (0.045 g, estimated loading 0.5017 mmol/g ≥ 22.58 μmol, 1 equiv) in tetrahydrofuran (0.4 mL), 1 N sodium hydroxide aqueous solution (45 μL, 2 equiv) was added. The mixture was stirred at room temperature for 24 h. The resin was filtered and washed with tetrahydrofuran and water. An aliquot of the cleavage solution (1 mL out of 4) was evaporated to obtain compound **19** as a sodium salt (0.0052 g, quantitative recovery). ^{1}H NMR (DMSO-d_6): δ 9.62 (1H, t), 9.28 (H, d), 8.39 (H, d), 8.33 (1H, d), 8.25(1H, d), 8.05 (2H, d), 7.8−7.65 (5H, m), 7.6−7.5 (4H, m), 7.34 (1H, d), 7.16 (1H, d), 7.07 (2H, d), 6.87 (2H, d), 6.6−6.5 (3H, m), 4.27 (2H, s), 3.74 (8H, s), 3.3 (2H, m), 3.25 (2H, m), 2.78 (6H, s). MS-ES$^+$: *m*/*z* 881−882 $[M + Na]^+$ peak split present.

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Supporting Information Available. Synthetic procedures for the solid-phase synthesis of **20** and the related intermediates **21**, **22**, **23**, **24**, and **25**; analytical data (^{1}H NMR, MS, and UV) for **10a**−**g**, **19**, and **20**. This material is available free of charge via the Internet at http://pubs.acs.org.

References and Notes

(1) Czarnik, A. W. Combinatorial Chemistry. *Anal. Chem.* **1998**, *70*, 378A−386A.
(2) Gallop, M. A.; Barrett, R. W.; Dower, W. J.; Fodor, S. P. A.; Gordon, E. M. Application of Combinatorial Technologies to Drug Discovery. 2. Combinatorial Organic Synthesis, Library Screening Strategies, and Future Directions. *J. Med. Chem.* **1994**, *37*, 1384−1401.
(3) Brown, D. Future Pathways for Combinatorial Chemistry. *Mol. Diversity* **1997**, *2*, 217−222.
(4) Lam, K. S.; Lebl, M.; Krchnak, V. The "One-Bead−One-Compound" Combinatorial Library Method. *Chem. Rev.* **1997**, *97*, 411−448.
(5) Kauvar, L. M.; Laborde, E. The Diversity Challange in Combinatorial Chemistry. *Curr. Opin. Drug. Discovery Dev.* **1998**, *1*, 66−70.
(6) Thomson, L. A.; Ellman, J. A. Synthesis and Applications of Small Molecule Libraries. *Chem. Rev.* **1996**, *96*, 555−600.
(7) Terrett, N. A.; Gardner, M.; Gordon, D. W.; Kobylecki, R. J.; Steele, J. Combinatorial Synthesis—The Design of Compound Library and Their Application to Drug Discovery. *Tetrahedron* **1995**, *51*, 8135−8173.
(8) Gallop, M. A.; Barrett, R. W.; Dower, W. J.; Fodor, S. P. A.; Gordon, E. M. Application of Combinatorial Technologies to Drug Discovery. 1. Background and Peptide Combinatorial Libraries. *J. Med. Chem.* **1994**, *37*, 1233−1251.
(9) Antonenko, V. V. Automation in Combinatorial Chemistry. *Combinatorial Chemistry and Technologies—Principles, Methods, and Applications*; Marcel Dekker, Inc.: New York, 1999; pp 205−232.
(10) Bondy, S. S. The Role of Automation in Drug Discovery. *Curr. Opin. Drug Discovery Dev.* **1998**, *1*, 116−119.
(11) Fitch, W. L.; Look, G. C.; Detre, G. Analytical Chemistry Issues in Combinatorial Organic Synthesis. In *Combinatorial Chemistry and Molecular Diversity in Drug Discovery*; Gordon, E. M., Kervin, J. F., Jr., Eds.; Wiley: New York, 1998, pp 349−368.
(12) Fitch, W. L. Analytical Methods for Quality Control of Combinatorial Libraries. *Mol. Diversity* **1999**, *4*, 39−45.
(13) Anderson, R. C.; Jarema, M. A.; Shapiro, M. J.; Stokes, J. P.; Ziliox, M. Analytical Techniques in Combinatorial Chemistry: MAS-CH Correlation in Solvent-Swollen Resins. *J. Org. Chem.* **1995**, *60*, 2650−2651.
(14) Taylor, E. V.; Quian, M. G.; Dollinger, G. D. Simultaneous On-Line Characterization of Small Organic Molecules Derived from Combinatorial Libraries for Identity, Quality, and Purity by Reverse-Phase HPLC with Chemiluminescent Nitrogen, UV, and Mass Spectrometric Detection. *Anal. Chem.* **1998**, *70*, 3339−3347.

(15) Zeng, L.; Kassel, D. B. Development of a Fully Automated Parallel HPLC/MS Spectrometry System for the Analytical Characterization and Preparative Purification of Combinatorial Libraries. *Anal. Chem.* **1998**, *70*, 4380−4388.
(16) Geysen, H. M.; Wagner, C. D.; Bodnar, W. M.; Markworth, C. J.; Parke, G. J.; Schoenen, F. J.; Wagner, D. S. Kinder, D. S. Isotope Mass Encoding of Combinatorial Libraries. *Chem. Biol.* **1996**, *3*, 679−688.
(17) Carrasco, M. R.; Fitzgerald, M. C.; Oda, Y.; Kent, S. B. H. Direct Monitoring of Organic Reactions on Polymeric Supports. *Tetrahedron Lett.* **1997**, *40*, 2407−2410.
(18) McKeown, S. C.; Watson, S. P.; Carr, R. A.; Marshall, P. A Photolabile Carbamate-Based Dual Linker Analytical Construct for a Facile Monitoring of Solid-Phase Chemistry: "TLC" for Solid-Phase? *Tetrahedron Lett.* **1999**, *40*, 2407−2410.
(19) Murray, P. J.; Kay, C.; Scicinski, J. J.; McKeown, S. C.; Watson, S. P.; Carr, R. A. Rapid Reaction Scanning of Solid-Phase Chemistry Using Resins Incorporating Analytical Constructs. *Tetrahedron Lett.* **1999**, *40*, 5609−5612.
(20) Dual linker analytical constructs incorporating anthracene as a chromophore have also been prepared in our company. Williams, G. M.; Carr, R. A.; Congreve, M. S.; Kay, C.; McKeown, S. C.; Murray, P. J.; Scicinski, J. J.; Watson, S. P. Analysis of Solid-Phase Reactions: Product Identification and Quantification by Use of UV-Chromophore-Containing Dual-Linker Analytical Constructs. *Angew. Chem., Int. Ed.* **2000**, *39*, 3293−3296.
(21) Vojkovsky, T. Detection of Secondary Amines on Solid-Phase. *Pept. Res.* **1995**, *8*, 236−237.
(22) Kaiser, E.; Colescott, R. L.; Bossinger, C. D.; Cook, P. I. Color Test for Detection of Free Terminal Amino Groups in the Solid-Phase Synthesis of Peptides. *Anal. Biochem.* **1970**, *34*, 595−598.

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