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Formation and Verification of the Structure of the 1-Fluorenylmethyl Chloroformate Derivative of Sulfamethazine

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Sulfamethazine (SMZ) is derivatized with 1-fluorenylmethyl chloroformate (FMOC) to form the fluorescent adduct SMZ−FMOC. Conditions for formation are optimized with respect to pH, reagent concentration, and reagent ratio. Reagent and product profiles (including the hydrolysis byproduct FMOC-OH) versus time are followed by reversed phase HPLC with UV absorbance detection. FMOC−SMZ has been crystallized, its composition confirmed by microanalysis, and its structure corroborated by IR and NMR spectroscopy. From 10 down to 1 ppm, there is clear gentle curvature in the fluorescence intensity of SMZ−FMOC. The linear response range extends from above 100 ppb down to about 100 ppt, and an increase in sensitivity for the fluorescent detection of FMOC−SMZ (over the usual UV absorbance detection of SMZ) is calculated to be better than 3 orders of magnitude.

Sulfonamides (SFAs) are widely used for the prevention of infectious diseases in livestock animals. However, there is a consumer expectation of residue-free food, and residues of SFAs in foods derived from treated animals could lead to the development of drug-resistant strains of microorganisms.[1] Consequently, the SFAs have been under surveillance for several years. The U.S. Department of Agriculture,[2,3] the Australian Meat and Livestock Research and Development Corporation,[4−6] the European Economic Community,[7] and the Food and Agriculture Organization of the United Nations[8] have all concerned themselves with veterinary drug residues. The SFAs in general have been the principal class targeted, and sulfamethazine (SMZ, alternately called sulfadimidine) has been preeminent due to a study indicating possible carcinogenicity.[9] Hence, there has been a huge analytical effort in SFA residue analysis worldwide. Bovine milk in the USA,[10] salmon flesh in Canada,[11] beef, pork, chicken, ham, sausage, bacon, roast beef,[12,13] and milk[14] in Japan, and pork in the Netherlands[15] have been the subjects of SFA analysis in recent years. However, the dependence of sectors of the meat producing industry on the SFAs[16] indicates that the SFAs will continue in their prophylactic veterinary role. Hence, there is the continued need for sensitive methods of analysis of these antiinfective drugs.

Most instrumental methods of separation have been tried for the SFA,[17] but specific and quantitative methods are limited to GC,[18−22] GC/MS,[7,22] HPLC (for some recent examples, see refs 17, 18, 23−32), thermospray LC/MS,[33−35] CZE,[36] MECC,[37] and CZE-MS.[34,38] HPLC has been the preferred basis for analysis.

(1) Pratt, W. B.; Fekety, R. *The Antimicrobial Drugs*; Oxford University Press: Oxford, UK, 1986.
(2) *Compound Evaluation and Analytical Capability*; 1985 National Residue Program Plan; Food Safety and Inspection Service, Science, U.S. Dept. of Agriculture, US GPO: Washington, DC, 1985.
(3) Guest, G., Director, Center for Veterinary Medicine, USFDA. 47th Annual Meeting of the Animal Health Institute, Tucson, AZ, May 10−13, 1987.
(4) McCausland, I.; Healey, K.; Wells, R. *Emerging Technologies for Testing Chemical Residues in Meat*; AMLRDC Study Team to the USA, 1987.
(5) Pryor, W. J. *Study of Important Meat Residue Chemicals. Their Use and Alternatives*; Australian Meat and Live-Stock Research and Development Corp., Sydney, Australia, 1987.
(6) Pryor, W. J. *Potential Meat Contaminants*; Australian Meat and Live-Stock Research and Development Corp., Sydney, Australia, 1988.
(7) Heitzman, R. J., Ed. *Veterinary Drug Residues. Residues in food producing animals and their products. Reference materials and methods*; Commission for the European Communities, EUR 14126 EN, 1992.
(8) *Residues of Some Veterinary Drugs in Animals and Foods*, 34th Meeting, Joint FAO/WHO Expert Committee on Food Additives, Geneva, January 30−February 8, 1989.
(9) *Cronic Toxicity and Carcinogonicity of Sulphamethazine in B_6CF_1 Mice*. Technical report for experiment no. 418, National Center for Toxilogical Research, Jefferson, AR; *Fed. Regist.* **1988**, *53*, FR9492.
(10) Smedley, M. D.; Weber, J. D. *J. Assoc. Off. Anal. Chem.* **1990**, *73*, 875−9.
(11) Pleasance, S.; Blay, P.; William, M. A. Q.; O'Hara, G. *J. Chromatogr.* **1991**, *558*, 155−73.
(12) Takeda, N.; Akiyama, Y. J. *J. Chromatogr.* **1991**, *558*, 175−80.
(13) Kishihara, S.; Shimokawa, C.; Izumi, H. *Ishikawa-ken Eisei Kogai Kenkyusho Nenpo* **1983**, 198−204.
(14) Takeda, N.; Akiyama, Y. J. *J. Chromatogr.* **1992**, *607*, 31−5.
(15) Ackermans, M. T.; Beckers, J. L.; Everaerts, F. M.; Hoogland, H.; Tomassen, M. J. H. *J. Chromatogr.* **1992**, *596*, 101−9.
(16) Australian Pig Industry Policy Council, Annual Report, 1991−2.
(17) Ricci, M. C.; Cross, R. F. *J. Microcolumn Sep.* **1993**, *5*, 207−15.
(18) Duan, R. J. *J. Assoc. Off. Anal. Chem.* **1971**, *54*, 1277−82.
(19) Goodspeed, D. P.; Simpson, R. M.; Ashworth, R. B.; Shafer, J. W.; Cook, H. R. *J. Assoc. Off. Anal. Chem.* **1978**, *61*, 1050−3.
(20) Holder, C. L.; Thompson, H. C., Jr.; Bowman, M. C. *J. Chromatogr. Sci.* **1981**, *19*, 625−33.
(21) Holtmannspotter, H.; Thier, H. P. *Dtsch. Lebensm. Rundsch.* **1982**, *78*, 347−50.
(22) Matusik, J.; Sternal, S.; Barnes, J.; Sphon, J. A. *J. Assoc. Off. Anal. Chem.* **1990**, *73*, 529−33.
(23) Ikai, Y.; Oka, H.; Kawamura, N.; Hayakawa, J.; Yamada, M.; Makoto, K.-I.; Suzuki, H.; Nakazawa, H. *J. Chromatogr.* **1991**, *541*, 393−400.
(24) Horie, M.; Saito, K.; Hoshino, Y.; Nose, N.; Hamada, N.; Nakazawa, H. *J. Chromatogr.* **1990**, *502*, 371−8.
(25) Cox, B. L.; Krzeminski, L. F. *J. Assoc. Off. Anal. Chem.* **1982**, *65*, 1311−5.
(26) Long, A. R.; Hsieh, L. C.; Malbrough, M. S.; Short, C. R.; Barker, S. A. *J. Agric. Food Chem.* **1990**, *38*, 425−8.
(27) Lindsay, H. J.; Smith, P. J.; Love, P. J. *Food Analysis Conference*, Brisbane, Australia, 1989.
(28) Aerts, M. M. L.; Beek, W. M. J.; Brinkman, V. A. T. *J. Chromatogr.* **1988**, *435*, 97−112.
(29) Weber, J. D.; Smedley, M. D. *J. Assoc. Off. Anal. Chem.* **1989**, *72*, 445−7.
(30) Smedley, M. D.; Weber, J. D. *J. Assoc. Off. Anal. Chem.* **1990**, *73*, 875−9.
(31) Long, A. R.; Short, C. R.; Barker, S. A. *J. Chromatogr.* **1990**, *502*, 87−9.
(32) Agarwal, V. K. *J. Liq. Chromatogr.* **1990**, *13*, 3531−7.
(33) Horie, M.; Saito, K.; Hoshino, Y.; Nose, N.; Tera, M.; Kitsuwa, T.; Nakazawa, H.; Yamane, Y. *Eisei Kagaku* **1989**, *4*, 283−9.
(34) Perkins, J. R.; Parker, C. E.; Tomer, K. B. *Am. Soc. Mass Spectrom.* **1992**, *3*, 139−49.
(35) Abian, J.; Churchwell, M. I.; Korfmacher, W. I. *J. Chromatogr.* **1993**, *629*, 267−76.

0003-2700/96/0368-0086$12.00/0

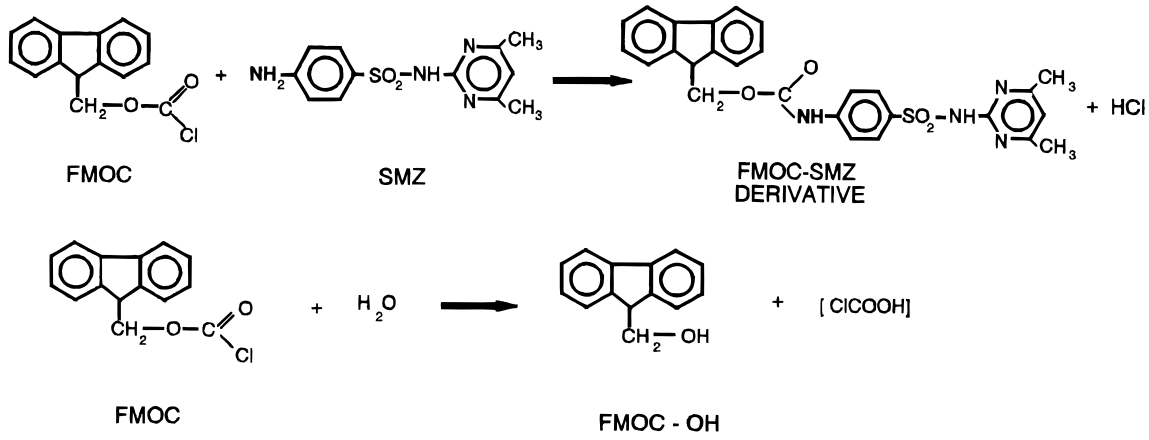

Figure 1. FMOC reactions with SMZ and water.

Even with derivatization, HPLC, especially reversed phase (RP) HPLC, does not require as much cleanup as is needed for GC analyses. Water-based biological systems are inherently compatible with the aqueous mobile phases of RP-HPLC and the gentler conditions of analysis (lower temperatures) than in GC. The alternative solution technique of CE is very attractive due to its higher efficiency, but the inferior concentration sensitivity is counterproductive in quantitation.

Most HPLC determinations of the SFA have used UV absorption detection. However, derivatization with a strongly fluorescent moiety would clearly enhance detection limits. 9-Fluorenylmethyl chloroformate (FMOC) was first introduced in HPLC fluorescence methods based on precolumn derivatization and RP separation of FMOC-amino acids by Einarsson et al.,[39] was later commercialized by Cunico and others,[40] and has found wide application to the analysis of amino acids and their derivatives.[41−46] Under mild conditions, it reacts with primary and secondary amines and yields highly fluorescent derivatives. FMOC has also been used for the derivatization and analyses of other types of amines including biogenic amines[47] and polyamines[48−50] and more recently—during the course of this study—has been used for the analysis of catecholamines[51] and amphetamines.[52] FMOC should react with the amine group of SMZ to yield a highly fluorescent FMOC−SMZ derivative according to the first reaction in Figure 1. (FMOC also hydrolyzes[39,40] according to the second reaction in Figure 1.)

We have examined this derivatization reaction, the optimum conditions for formation of FMOC−SMZ, and the spectral properties of the derivative. The proposed structure has been verified. Concurrent with this study, the fluorescamine derivative has also been formed and used for the the HPLC analysis of sulfonamides.[12−14]

EXPERIMENTAL SECTION

Chemicals. All solvents used were analytical reagent quality or spectroscopic grade. SMZ was purchased from Sigma Chemical Co. (St. Louis, MO), and standard solutions of it were prepared by dissolution in 50% acetone−50% water. FMOC (97%) was obtained from Fluka Chemika (Buchs, Switzerland) and dissolved in acetone. The standard HPLC buffer was prepared from NaH_2PO_4 and Milli-Q water and adjusted to pH 3.5 with H_3PO_4. The pH 8 reaction buffer was 0.1 M $NaHCO_3$. To study the effect of pH, reaction solutions were prepared from NaAc−HAc (pH 3−5), Na_2HPO_4−NaH_2PO_4 (pH 6−9), and NaOH (pH 9−10).

Instrumentation. Two HPLC systems were used to monitor reactions. The first was a conventional system from Waters (Milford, MA) comprising Model 450 pumps, a 441 fixed-wavelength (254 nm) UV absorbance detector with a 10 mm path length flow cell, a 721 data system printer/plotter, and a 730 gradient controller. A Rheodyne (Cotati, CA) six-port injector with a 20 μL sample loop was used, and the TMS-capped Micro Park C18 5 μm, 15 cm × 4 mm i.d. column was supplied by Varian (Walnut Creek, CA). The stationary phase support was Separations Group (Hesperia, CA) Vydac IDI TP 80 $m^2\ g^{-1}$ silica.

The alternate system was a Varian Vista 5560 HPLC modified for fast analyses. The conventional flow cell in the Varian UV 200 detector was replaced with the 0.5 μL, 0.5 mm path length option, and the detector was mounted on the column oven of a cut-down Perkin-Elmer (Norwalk, CT) LC 65-T UV detector. A TMS-capped, Varian Micro Pak SP C18 3 μm, 4 cm × 4 mm column, with PhaseSep 200 $m^2\ g^{-1}$ silica, was located directly below the detector inlet and was connected via 5 cm of 0.13 mm (0.005 in.) i.d. stainless steel tubing to minimize dispersion. The Valco (Houston, TX) C14M air-actuated automatic injector was mounted on the side of the oven and connected through the oven wall by 10 cm of 0.13 mm i.d. tubing. The wavelength of maximum absorbance was chosen for detection with this system (264 nm), and the data system was a Varian CDS 401/2.

UV spectra were recorded on a Varian (Springvale, Australia) Cary 3, IR spectra were run on a Perkin-Elmer Model 781

(36) Ackermans, M. T.; Beckers, J. L.; Everaerts, F. M.; Hoogland, H.; Tomassen, M. J. H. *J. Chromatogr.* **1992**, *596*, 101−9.
(37) Dang, Q. X.; Sun, Z. P.; Ling, D. K. *J. Chromatogr.* **1992**, *603*, 259−66.
(38) Pleasance, S.; Thibault, P.; Kelly, J. *J. Chromatogr.* **1992**, *591*, 325−39.
(39) Einarsson, S.; Josefsson, B.; Lagerkvist, S. *J. Chromatogr.* **1983**, *282*, 609−18.
(40) Cunico, R. L.; Mayer, A. G.; Dimson, P.; Zimmerman, C. *BioChromatography* **1986**, *1*, 6−14.
(41) Naesholm, T.; Sandberg, G.; Ericsson, A. *J. Chromatogr.* **1985**, *396*, 225−36.
(42) Korte, E. *GIT-Suppl.* **1987**, *3*, 44−6.
(43) Kisby, G. E.; Roy, D. N.; Spencer, P. S. *J. Neurosci. Methods* **1988**, *26*, 45−54.
(44) Blankenship, D. T.; Krivanek, M. A.; Ackermann, B. L.; Cardin, A. D. *Anal. Biochem.* **1989**, *178*, 227−32.
(45) Teerlink, T.; Boer, D. E. *J. Chromatogr.* **1989**, *491*, 418−23.
(46) Guo, Y. J.; Huang, Y. L.; Yao, Z. *J. Sepu* **1989**, *7*, 219−21.
(47) Harduf, Z.; Nir, T.; Juven, B. J. *J. Chromatogr.* **1988**, *437*, 379−86.
(48) Price, J. R.; Metz, P. A.; Veening, H. *J. Chromatogr.* **1987**, *424*, 795−9.
(49) Yun, Z.; Zhang, R. N. *Biomed. Chromatogr.* **1987**, *2*, 173−6.
(50) Sabri, M. I.; Soiefer, A. I.; Kisby, G. E.; Spencer, P. S. *J. Neurosci. Methods* **1989**, *29*, 27−31.
(51) Descombes, A. A.; Haerdi, W. *Chromatographia* **1992**, *33*, 83−6.
(52) Maeder, G.; Pelletier, M.; Haerdi, W. *J. Chromatogr.* **1992**, *593*, 9−14.

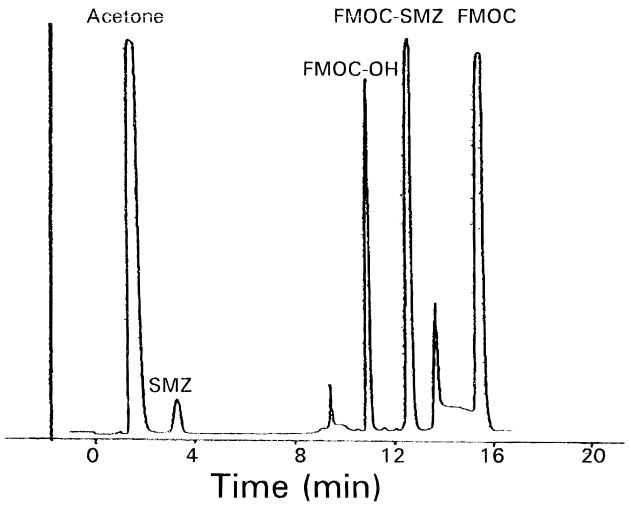

Figure 2. Chromatogram of the reaction mixture at 15 min. Peak assignments are acetone (at 1.9 min), SMZ (3.7 min), FMOC-OH (11.5 min), FMOC−SMZ (12.3 min), and unreacted FMOC (at 15.5 min).

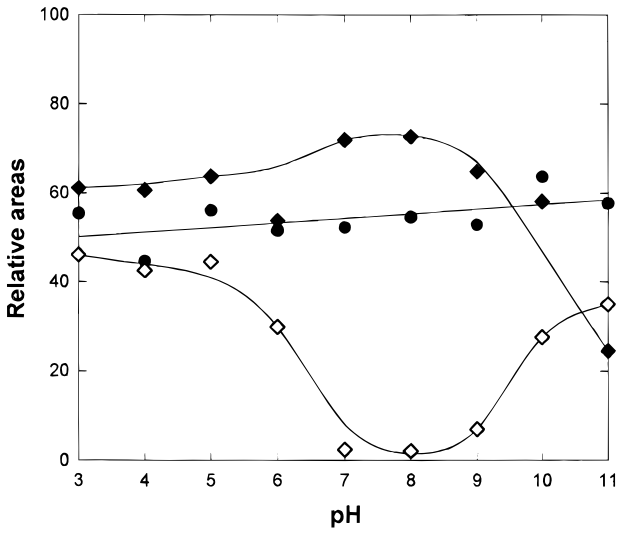

Figure 3. Effect of pH on the reactions of FMOC: ◇, SMZ; ◆, FMOC−SMZ; ●, FMOC-OH. The relative areas were determined at 254 nm.

(Norwalk, CT), fluorescence measurements were performed on a Shimadzu (Kyoto, Japan) RF-500 spectrofluorophotometer, and NMR spectra were obtained from a Varian (Palo Alto, CA) NMR Gemini 200 H/C. Operating frequencies were 200.0 and 50.0 MHz.

Procedures. As SMZ is not fluorescent, all of the studies of rates and yields were necessarily performed using UV absorbency. All reactions and analyses were performed at ambient temperatures.

RESULTS AND DISCUSSION

HPLC Analysis of Reaction Mixtures. Using the conditions found to be optimal for the reaction of FMOC and amino acids as determined by Einarsson's study,[39] and confirmed by Cunico et al.,[40] the reaction of FMOC and SMZ was first tested at pH 8. One milliliter of 1 mg/mL (3.59 mM) SMZ solution, 1 mL of pH 8 buffer, and 2 mL of 10 mM FMOC in acetone were mixed. Preliminary studies indicated that the pH for RP-HPLC analyses was not critical in the low end of the available range (2.5−4.5). pH 3.5 was chosen. Isocratic analysis was impractical since the weak elution conditions required for the separation of SMZ from the large acetone solvent peak yielded unacceptably long retention times for the other reagents and products, especially FMOC. The analysis conditions found to be most satisfactory on the conventional HPLC system were a medium concave gradient from 65% A (0.05 M NaH_2PO_4, pH 3.5)/35% B (methanol) to 15% A/85% B over 4 min, followed by isocratic elution with 85% methanol. The flow rate was 1 mL min^{-1}. From comparison with SMZ and FMOC blanks, prepared by substitution of the other reagent with an equal volume of the appropriate solvent, unambiguous assignments are possible for all reagents and products. Figure 2 shows the chromatogram after 15 min of reaction. It can be seen that baseline resolution was achieved for acetone, SMZ, the FMOC hydrolysis product (FMOC-OH) formed during reaction, and FMOC−SMZ. Unreacted FMOC at 15.5 min can be seen to have undergone on-column hydrolysis in both the FMOC blank and the reaction mixture, giving rise to the fronting structure comprising the sharp peak at 13.6−13.7 min and the area up to 15.5 min.

Conditions for Derivatization. With the exception of the 1 mL of buffer, for which the pH was varied, the same reaction conditions were used as described in the previous section. After 20 min at each pH, the reaction mixture was analyzed at 254 nm. Figure 3 shows the measured chromatographic areas for SMZ, FMOC−SMZ, and FMOC-OH. It should be noted that the relative areas of SMZ and FMOC−SMZ are not in the ratio of the molar absorptivities stated in the last section of this paper due to measurement at 254 nm rather than 264 nm. (Areas for FMOC are not shown due to the overlap of the bands in the 13.4−15.8 min area of the chromatogram and the irreproducible integration of the area.) The coincidental minimum in the SMZ residue and maximum in the FMOC−SMZ adduct indicate about 8 to be the optimum pH for reaction of FMOC with SMZ. A further study at half pH unit intervals between pH 6 and pH 10 confirmed that the sulfonamide amine group had maximum reactivity with FMOC in the range pH 7.8−8.0, as do the amino acids.

Under the typical reaction conditions used above—2 mL of aqueous phases and 1 mL of acetone solution—FMOC reagent solutions above 15 mM led to precipitation of that reagent upon mixing. Hence, the required stoichiometry of reaction was established in a series of experiments in which the water content of the reaction mixture was reduced. One milliliter of 4 mM SMZ in pH 8 bicarbonate buffer was mixed with 4 mL of 1−15 mM FMOC in acetone. Figure 4 shows the relative chromatographic areas (at 254 nm) for FMOC−SMZ and FMOC-OH after 30 min for concentrations of FMOC up to 8 mM and demonstrates the need for a minimum ratio of FMOC/SMZ as 7:1. Reaction can be seen to be complete in 30 min.

A further constraint upon reaction also involves the FMOC concentration. For the typical reaction conditions, initial concentrations of FMOC below 4 mM clearly led to the preeminence of the hydrolysis reaction and incomplete derivatization of SMZ, irrespective of its concentration. Hence, there are upper (solubility) and lower (rate of hydrolysis) limits to the acceptable FMOC concentrations with respect to the water content of the reaction mixture, as well as the need for a minimum ratio of FMOC/SMZ of 7:1.

Following isolation of the FMOC reaction products (see below), a mass balance study versus time was undertaken. Using the fast LC system, but with the same phases in reservoirs A and

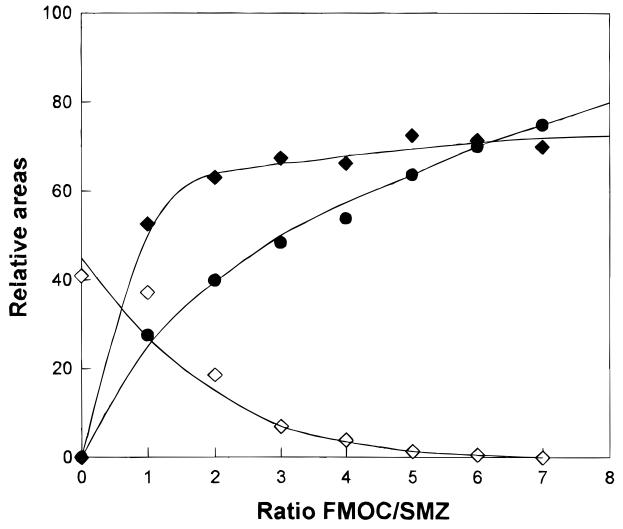

Figure 4. Effect of the molar ratio of FMOC/SMZ on the extent of reaction. Symbols and the absorption wavelength are as in Figure 3.

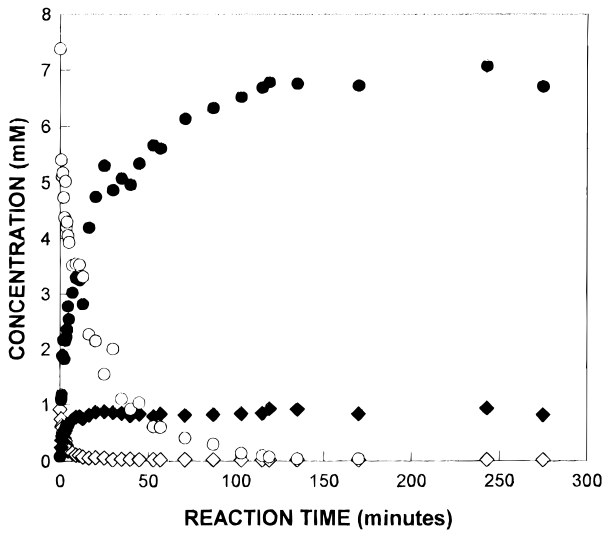

Figure 5. Variations in concentrations of the reagents and products with time: ◇, SMZ; ◆, FMOC−SMZ; ○, FMOC; and ●, FMOC-OH.

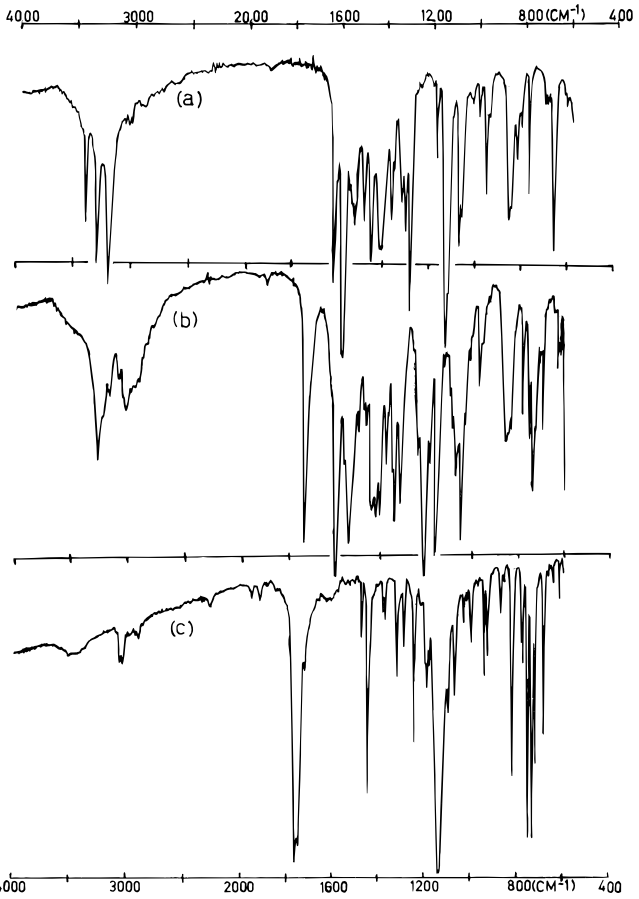

Figure 6. Infrared spectra of (a) SMZ, (b) FMOC−SMZ, and (c) FMOC.

B, the acetone solvent and SMZ were baseline resolved in 1.2 min at 30% methanol, and a jump to 70% methanol was effected over 1.25 min. Chromatography similar to that shown in Figure 2 was obtained, and all peaks were eluted in 12 min. One milliliter of ~2 mM SMZ in bicarbonate buffer was reacted with 1 mL of ~15 mM FMOC in acetone. Calibration curves for each of the reagents and products were obtained concurrently with the analyses. Good linear plots resulted: for SMZ (11 points, 0.02−2.00 mM), $R = 0.9999$; for FMOC−SMZ (6 points, 0.1−1.2 mM), $R = 0.9984$; for FMOC (14 points, 0.075−15.00 mM), $R = 0.9989$; and for FMOC-OH (11 points, 0.5−10 mM), $R = 0.9992$. For the residual FMOC concentration, only the chromatographic area due to the unhydrolyzed reagent was used. The results are shown in Figure 5. From the calibration curve and the SMZ blank, the initial concentration of SMZ in the reaction mixture was determined as 0.91 mM. It is clear that the SMZ is quantitatively converted to its FMOC derivative. As the reaction mixture was initially 7.4 mM in FMOC and the final solution was 0.9(1) mM in FMOC−SMZ, the expected final concentration of FMOC-OH was 6.5 mM. Figure 5 indicates a final FMOC-OH concentration around 6.7 mM. Given that there are several minor impurities in the FMOC (that may be seen in the blank) and that the FMOC-OH solid undergoes degradation, within experimental error, mass balance does appear to be have been achieved.

Isolation and Purification of FMOC−SMZ and FMOC-OH. FMOC−SMZ crystals were produced when 1 mL of pH 8 buffer was mixed with 4 mL of 2 mM SMZ (in 50% acetone/50% water), added to 5 mL of 15 mM FMOC in acetone, mixed well, and left overnight. The solution was filtered with No. 4 filter paper, and the crystals were washed with small amounts of acetone and then distilled water (three times each) and left to dry in a vacuum desiccator overnight.

FMOC-OH was produced by mixing 15 mM FMOC in acetone with water in equal volumes and leaving overnight. The solution was blown down to half its volume, filtered with No. 4 paper, and then washed and dried as for FMOC−SMZ.

Verification of Composition and Structure of FMOC−SMZ. *Microanalysis.* Commercial microanalysis of the SMZ derivative confirmed the composition to be $C_{27}H_{24}O_4N_4S$ and the crystals to have a clear-cut melting point of 258 °C. The microanalytical (and calculated) elemental compositions were C, 65.07% (64.79%); H, 4.95% (4.83%); N, 11.19% (11.19%); S, 6.20% (6.40%); and O (by difference), 12.59% (12.79%).

IR Spectroscopy. Figure 6, parts a−c show the IR spectra in KBr disks for SMZ, FMOC−SMZ, and FMOC, respectively. Reference spectra in Nujol for SMZ and FMOC[53] appear to be identical other than for the dispersant.

(53) Pouchert, C. J. *The Aldrich Library of FT-IR Spectra*; Aldrich Chemical Co.: Milwaukee, WI, 1985.

Reference to Colthup, Daly, and Wiberley[54] permits a full interpretation of the spectral changes observed. First, there are clear changes to all absorbances associated with SMZ vibrational modes near the reaction site. Above 3100 cm^{-1}, SMZ dislays the typical structure of three sharp, medium-intensity peaks associated with primary aromatic amines. Absorption at 3435 cm^{-1} is due to the N−H asymmetric stretch. The other two absorbances at 3335 and 3235 cm^{-1} are a result of Fermi resonance between the N−H symmetric stretch (expected at 3400−3200 cm^{-1}) and the weak overtone of the NH_2 deformation (at 1635 cm^{-1}) that would be expected around 3270 cm^{-1}. Because these three peaks are all dependent on the primary amine stretching fundamentals, all are missing from the FMOC−SMZ FT-IR spectrum. They are replaced by a broad, medium-intensity band at 3280 cm^{-1} (which is compatible with the absorbance expected at 3400−3070 cm^{-1} for monosubstituted amides) and a weak, medium-intensity broad band centered on 3000 cm^{-1}. Again, this structure is probably due to Fermi resonance between an overtone (of the 1550 cm^{-1} band in this case) and a stretching fundamental (3400−3070 cm^{-1}).

For a primary aromatic amine, the NH_2 deformation is expected at 1650−1590 cm^{-1}. Hence, the absence of the 1635 cm^{-1} SMZ peak in the FMOC−SMZ spectrum confirms that NH_2 is the reacted SMZ functional group. Primary aromatic amines also absorb strongly at 1330−1260 cm^{-1} due to stretching of the phenyl (Ph) carbon−nitrogen bond, in this case at 1300 cm^{-1} in the SMZ spectrum. In FMOC−SMZ, the strong Ph−N stretching absorbance would be expected to move to higher wavenumbers.[55] It is observed at 1310 cm^{-1} and is again consistent with the derivatization.

At 675 cm^{-1} in SMZ only, the strong absorption is due to the $-NH_2$ wag or rock (but is not broad, as is often shown for the spectrum of the liquid). The removal of this peak again indicates derivatization of the primary amine in SMZ. The comparable out-of-plane −NH wag for N-substituted amides absorbs broadly near 700 cm^{-1}.[54] This appears to be centered on 730 cm^{-1} beneath various FMOC absorbances.

The second series of spectral changes that support the proposed reaction are those observed between FMOC and FMOC−SMZ. In chloroformates, carbonyl absorbances are expected at 1860−1760 cm^{-1}; in FMOC, this (split) peak is at 1775−1750 cm^{-1}. The relative effects of chloro and amino substitution upon (C═O) absorbances in ketones are well known.[56] Relative to an unsubstituted ketone, chloro substitution causes a shift to higher frequencies, while amino substitution lengthens and weakens the bond, leading to lower frequencies. Thus, the C═O absorbance frequency should be lower in the derivative. The FMOC−SMZ C═O absorbance occurs at 1730 cm^{-1} and is consistent with the observed range for phenyl N-substituted carbamates (1730−1700 cm^{-1}).

Chloroformates have a C−O stretch at 1172−1134 cm^{-1}. It is observed at 1140 cm^{-1}. In the SMZ derivative, this very strong peak can be seen to have shifted to a higher frequency, 1200 cm^{-1}. This is again consistent with the derivatization. The adjacent (Cl) group in the FMOC reagent would withdraw electrons, weaken the C−O bond, and shift it to lower frequencies (relative to an adjacent C), while the adjacent N in the derivative is known to have a net electron donation effect, thus strengthening the C−O bond and shifting it to a higher absorbance frequency.

A band in the 694−689 cm^{-1} range is stated to be the C−Cl stretch in chloroformates. Examples of other chloroformates confirm the presence of a medium-intensity peak just below 700 cm^{-1}. However, allowing for dilution of the FMOC in the derivative, there appears to be little loss of intensity at this frequency. The matter is further complicated by the presence of a medium-intensity absorbance in about the same place in the spectrum of fluorene.[53]

The third and final source of IR absorption evidence for the reaction of SMZ as shown in Figure 1 is provided by a comparison with spectra of molecules with the same structure as FMOC−SMZ in the region of reaction. Monosubstituted amides in general provide a reference pattern; phenyl-substituted amides provide a more specific reference, and, as indicated above, carbamates provide the model compounds.

The weak SMZ band at 1545 cm^{-1} (of the sulfonamide N−H out-of-plane bend) is swamped in FMOC−SMZ (1535 cm^{-1}) due to the superimposition of the complex CNH vibration that is very characteristic for monosubstituted amides (near 1550 cm^{-1}). The next lowest absorbance frequency of significance is centered about 1045 cm^{-1} in FMOC−SMZ. This medium-strong absorbance is absent in both reagents and would therefore be expected to be closely associated with the newly formed C−N bond, especially as this falls within the normal range of absorbance for C−N stretching. Inspection of carbamate $R_1NHCOOR_2$ IR spectra[54] rules out this possibility, and the authors assign the absorbance to the O−R_2 bond. The correctness of this appears to be verified by the spectrum of the compound $PhNHCONH_2$, in which there are no significant absorbances below 1160 cm^{-1}. For the five carbamate spectra given, the O−C stretching frequency varies from 1120 to 1030 cm^{-1}. Of greatest interest is the spectrum of ethyl *N*-phenylcarbamate, $PhNHCOOC_2H_5$. This compound is the closest analogue to FMOC−SMZ and has the O−C stretch assigned at 1070 cm^{-1}. It also has the CO−O absorption assigned to a very strong band at about 1240 cm^{-1}. This is equivalent to the FMOC−SMZ absorbance at 1200 cm^{-1}. It appears that the two C−O absorbances in FMOC−SMZ (CO−O, 1200 cm^{-1} and O−CH_2, 1045 cm^{-1}) are merged in the FMOC reagent. This would also be consistent with the very high intensity of the FMOC 1140 cm^{-1} absorbance.

One further feature of the ethyl *N*-phenylcarbamate spectrum is the N−H stretch. It is a broad, medium-intensity band centered just above 3300 cm^{-1} and is very similar to the FMOC−SMZ band centered just below 3300 cm^{-1}.

NMR Spectroscopy. The NMR spectra of SMZ, FMOC−SMZ, and FMOC were obtained in dimethyl sulfoxide and are shown in Figure 7, parts a−c, respectively. For SMZ, the NMR spectrum is in excellent agreement with the literature.[57] For FMOC, a reference spectrum was not located, but that of fluorene[57] confirmed the majority of absorbances. (The broad absorbance centered on 8.25 ppm is assumed to be due to the readily formed hydrolysis product.)

(54) Colthup, N. B.; Daly, L. H.; Wiberley, S. E. *Introduction to Infrared and Raman Spectroscopy*, 3rd ed.; Academic: London, 1990.

(55) Pecsok, R. L.; Shields, L. D.; Cairns, T.; Mcwilliam, I. G. *Modern methods of chemical Analysis*, 2nd ed.; John Wiley: New York, 1976; p 70.

(56) Kemp, W. *Organic spectroscopy*, 2nd ed.; Macmillan: London, 1987.

(57) Pouchert, C. J.; Campbell, J. R. *The Aldrich Library of NMR Spectra*; Aldrich Chemical Co.: Milwaukee, WI, 1974.

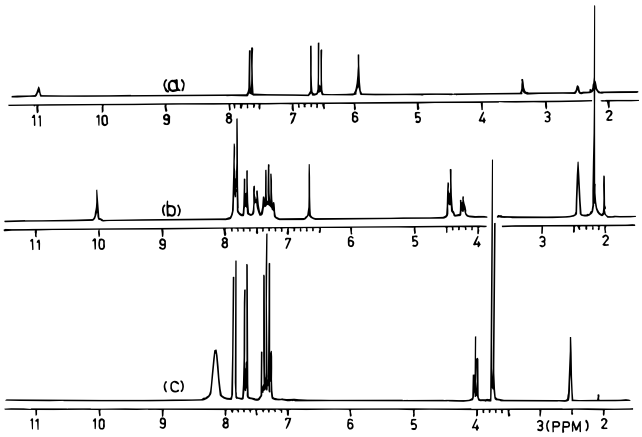

Figure 7. ^{1}H NMR spectra of (a) SMZ, (b) FMCO−SMZ, and (c) FMOC.

In the SMZ spectrum, the singlet equivalent in area to two protons at $\delta = 5.94$ is due to the two amine protons.[58] This absorbance is missing in the spectrum of the derivative. The remaining proton at this site (−OCONH−) is downshifted by the deshielding from the OCO group and from the areas is seen to be located within the aromatic group at $\delta = 7.2-7.4$. This is also consistent with the tabulated range of chemical shifts for phenyl-substituted amides[58] at 7.5−8.5 ppm. The other revealing assignment is for the SMZ two-proton doublet at $\delta = 6.5-6.6$, which is also absent in the spectrum of the FMOC adduct. Taking benzene as the standard substance with $\delta = 7.27$, the protons ortho to the amine group may be assigned via substituent effects:[58] $\delta = 7.27 - 0.8$ [*o*-NH_2] + 0.15 [*m*-SO_2] = 6.62 ppm. Replacement of the amine substituent by a monosubstituted amide (as an approximation) leads to $\delta = 7.27 + 0.4$ [*o*-NCOR] + 0.15 [*m*-SO_2] = 7.82 ppM, and comparison of the FMOC−SMZ spectrum with that of FMOC shows a 2-fold increase in area of the FMOC two-proton doublet at $\delta = 7.2-7.4$.

In the FMOC spectrum, for the 9-fluorenyl proton, $\delta = 1.54$ [CH] + 1.3 [Ph] × 2 = 4.14 ppm, and a triplet equivalent to one proton is located between 4.0 and 4.1 ppM. For the adjacent methylene protons, $\delta = 1.25$ [CH_2] + 2.7 [OCO] = 3.95 ppM, and the required doublet equivalent to two protons is found between 3.7 and 3.8 ppM. As these protons are adjacent to the reaction site, their chemical shifts would be expected to change. From the peak areas and splittings, it is clear that the signals generated around 4.5 ppM in the FMOC−SMZ spectrum are due to the CH_2 protons, and the CH proton absorbs at 4.4 ppM. This inversion of order of the chemical shifts is not surprising, since the downfield shift caused by SMZ is weakened by distance.

In summary, the structure of FMOC−SMZ analyzed from the NMR spectrum is coincident with the expected one.

UV Absorption and Fluorescence Spectroscopy. UV absorbance spectra were determined for both of the reagents and both of the products at 0.04 mM in the mobile phases in which they were monitored during the mass balance study. In 30% pH 3.5 phosphate buffer/70% methanol, the wavelengths of maximum absorbance (λ_{max})/nm and the molar absorptivities (ϵ)/mol^{-1} L cm at those wavelengths were determined as 264 and 19 810 for FMOC, 262 and 54 250 for FMOC−SMZ, and, 265 and 19 110 for FMOC-OH. In 70% pH 3.5 phosphate buffer/30% methanol, the values were 264 and 20 250 for SMZ. In addition, ϵ was measured at exactly 264 nm in the 70% methanol phase for SMZ (20 180 mol^{-1} L cm) and FMOC−SMZ (53 760 mol^{-1} L cm). These data show that for UV absorbance detection in the vicinity of the peak maxima, the sensitivity for SMZ may be increased by a factor of 2.7 by derivatization with FMOC and that the absorptivity of the derivative (53 760) is enhanced relative to those of the reagents (20 180 + 19 810 = 39 990). As the absorbance centers are independent in the derivative and the fluorene moiety is distant from the reaction site, this enhancement must arise from an increase in absorbency of the sulfonamide phenyl system.

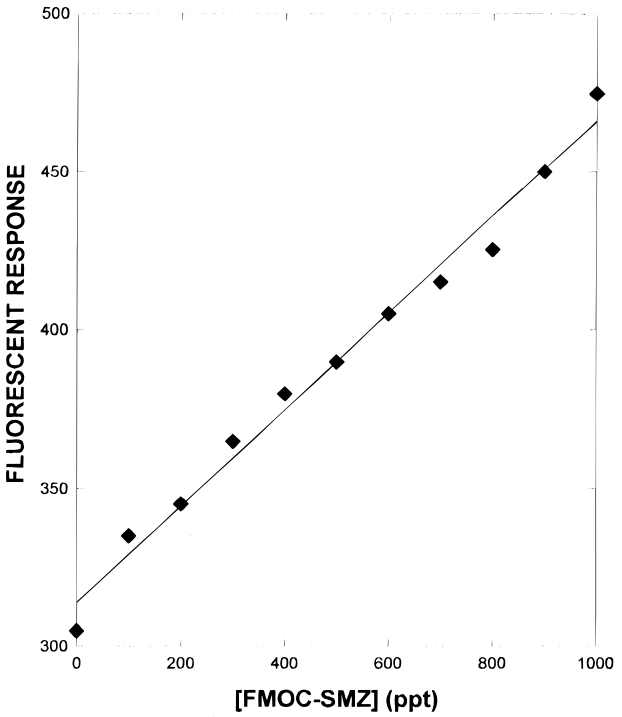

Figure 8. Fluorescent response of FMOC−SMZ in the 100−1000 ppt range.

The fluorescence spectra of FMOC and its derivatives are well known.[40] Maximum absorbance in excitation occurs at 264 nm, and in emission a maximum is observed around 307 nm. FMOC−SMZ was diluted in the 70% methanol buffer. Figure 8 shows the fluorescence response in the lowest concentration range examined. A linear response is evident, $R = 0.924$. In the ppb range (10 points, 10−100 ppb, $R = 0.9976$), less scatter was observed and linearity maintained. However, when the concentration is in the 1−10 ppm range, gentle curvature begins to appear in the fluorescence response. Above this, the response rapidly maximizes (at 20 ppm) and then "exponentially" decreases up to 100 ppm.

Without detection limit studies, exact sensitivities cannot be determined. However, an estimate of the increase in sensitivity is obtained from spectrofluorometric detection of the FMOC−SMZ derivative and spectrophotometric UV absorbance of SMZ when standard 1 cm path length/3 cm^3 cells were used. Taking the 0.81 absorbance of 0.04 mM SMZ, assuming linearity and the minimum detectable absorbance to be 0.005, the minimum detectable concentration of SMZ by UV absorbance would be 60 ppb. From Figure 8, it is clear that an improvement of 3−4 orders

(58) Kemp, W. *NMR in Chemistry. A Multinuclear Introduction*; Macmillan: London, 1986.

of magnitude is possible by fluorence detection of the FMOC derivative.

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