amide (14) was obtained (20% yield) as a colorless powder: mp 94–96 °C (gas evolution above 110 °C); ^{1}H NMR (200 MHz, Me_2SO-d_6) δ 10.22 (s, exchanges, 1 H, guanidino NH), 7.77 (s, 1 H, H-2), 7.5–6.7 (b, exchanges, 2 H, guanidino NH_2), 7.12 (s, exchanges, 1 H, $CONH_a$), 6.90 (s, exchanges, 1 H, $CONH_b$), 5.49 (d, J = 4.5 Hz, 1 H, H-1′), 5.30 (m, exchanges, 1 H, OH), 5.05 (m, exchanges, 1 H, OH), 5.00 (m, exchanges, 1 H, OH), 4.20–4.00 (m, 4 H, H-2′, H-3′, and CO_2CH_2), 3.81 (m, 1 H, H-4′), 3.70–3.40 (m, 2 H, 5′-CH_2), 1.22 (t, J = 7.1 Hz, 3 H, CH_3); IR (KBr) 1653, 1701, 1734, 2964, 3417 cm^{-1}; UV λ_{max} nm (ε × 10^4) (pH 1) 237 (sh) (1.0), (CH_3OH) 263 (1.0); (pH 11) 261 (0.9); FAB–mass spectrum, m/z 373 (MH^+), 395 (MNa^+).

1*H*-2-Amino-9-(β-D-ribofuranosyl)purin-6-one (Guanosine, 16). A solution of **15** (25 mg, 0.07 mmol) in 1.5 mL of concentrated NH_4OH and 0.5 mL of pyridine was allowed to stand at 45 ± 1 °C in a sealed flask for 48 h. The reaction mixture was rotary evaporated to dryness in vacuo, and the residue was dissolved in 5 mL of water and again rotary evaporated. The residue was pumped dry at 50 °C in vacuo overnight to afford 19 mg (95%) of **16** as a white powder: mp 248–251 °C dec (lit. mp 235 °C[29]). A mixture melting point with authentic guanosine showed no depression. The infrared and ultraviolet (pH 1, H_2O, and pH 11) spectra were identical with those obtained from an authentic sample; TLC analysis (solvent system I[30]) showed the product to be homogeneous, and identical in R_f value (0.12) with authentic guanosine. FAB–mass spectrum, m/z 284 (MH^+).

Acknowledgment. This work was supported by PHS Research Grant CA 28381 awarded by the National Cancer Institute, DHHS. We acknowledge the NIH Biomedical Research Support (RR 01437) for funds toward the purchase of an IBM WP-270SY NMR spectrometer. We thank Ms. Deanna VanSickle for assistance in the preparation of this manuscript and Professor Nelson J. Leonard for kindly allowing us to complete this work in his laboratory at the University of Illinois, Urbana, IL.

Registry No. **7** (R^1 = Et), 16182-04-0; **7** (R^1 = $PhCH_2$), 63220-36-0; **8a**, 54035-70-0; **8b**, 100313-31-3; **8c**, 100313-32-4; **8d**, 100313-33-5; **9a**, 100313-34-6; **9b**, 100313-35-7; **9c**, 100313-36-8; **9d**, 100313-37-9; **10**, 100313-38-0; **11a**, 100313-39-1; **11b**, 100334-11-0; **11c**, 100313-40-4; **12**, 100313-41-5; **13**, 100313-42-6; **14**, 100313-44-8; **15**, 100313-43-7; **16**, 118-00-3; H_2NCH_2Ph, 100-46-9; $H_2NCH_2C_6H$-*p*-OMe, 2393-23-9; $H_2NCH(C_6H_4$-*p*-OMe)2, 19293-62-0; ethyl chloroformate, 541-41-3; benzyl chloroformate, 501-53-1; benzyl thiocyanate, 3012-37-1; 5-amino-1-(β-D-ribofuranosyl)imidazole-4-carboxamide, 2627-69-2.

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Synthesis of Azotomycin[1]

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Total synthesis of the *Streptomyces ambofaciens* anticancer constituents (−)-azotomycin (1) from γ-OBzl-*N*-Boc-L-Glu (3) has been accomplished in nine steps. Selective protection with *N*-*tert*-butoxycarbonyl and *N*-trifluoroacetyl for the amino groups and benzyl or methyl esters for carboxyl groups, combined with the mixed carbonic anhydride peptide bond forming procedure, comprised the general strategy. Synthesis of bis diazo ketone 9 from dicarboxylic acid **8a** proved challenging and required development of precise experimental conditions for treating the diacid chloride intermediate with diazomethane. The (−)-azotomycin synthesis presents a useful alternative to the original fermentation–isolation route.

The biosynthetic virtuosity of *Streptomyces ambofaciens* began to be revealed over 30 years ago with discovery[2] of the spiramycin antibiotics (against gram-positive bacteria and rickettsia) of the erythromycin–carbomycin group. By 1960–1962 Rao had isolated and characterized, from the same microorganism, the anticancer constituents azotomycin (1)[3] and DON (2).[4] Evaluation of azotomycin against experimental neoplasms began in 1963, and Duvall[5a] reported consistent activity against a wide variety of animal tumors (predominantly murine leukemias). By 1968 azotomycin had exhibited excellent antineoplastic activity against the mouse sarcoma 180, carcinoma–755, L1210 lymphocytic leukemia, and the rat Walker 256 carcinoma.[6,7] In this period Brockman and co-workers[8] reported that azotomycin (1) was activated by in vivo conversion to DON (2). That route was further substantiated by a 1971 study.[9] And this was consistent with a more recent report[10] that azotomycin and DON have nearly identical antitumor activity against murine tumor systems such as the L1210 and P388 lymphocytic leukemias, C-26 and C-38 colon tumors, and $CD8F_1$ mammary carcinoma.

(1) (a) Part 114 of the series Antineoplastic Agents. For contribution 113, see: Pettit, G. R.; Singh, S.; Cragg, G. M. *J. Org. Chem.* **1985**, *50*, 2654. (b) Abstracted in part from the Ph.D. dissertation submitted by P.S.N. to the Graduate School, Arizona State University, May 1983.

(2) For leading references, see: Pinnert-Sindico, S.; Ninet, L.; Preud'homme, J.; Cosar, C. *Antibiotics Annu.* **1954–1955**, *2*, 724–727; *Chem Abstr.* **1955**, *49*, 10436*a*. Kulhne, M. E.; Benson, B. W. *J. Am. Chem. Soc.* **1965**, *87*, 4660.

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(7) Weiss, A. J.; Ramirez, G.; Grage, T.; Strawitz, J.; Goldman, L.; Downing, V. *Cancer Chemother. Rep.* **1968**, *52*, 611.

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(9) Brockman, R. W.; Pittillo, R. F.; Wooley, C.; Ho, Dah Hsi. *Cancer Chemother. Rep.* **1971**, *55*, 47.

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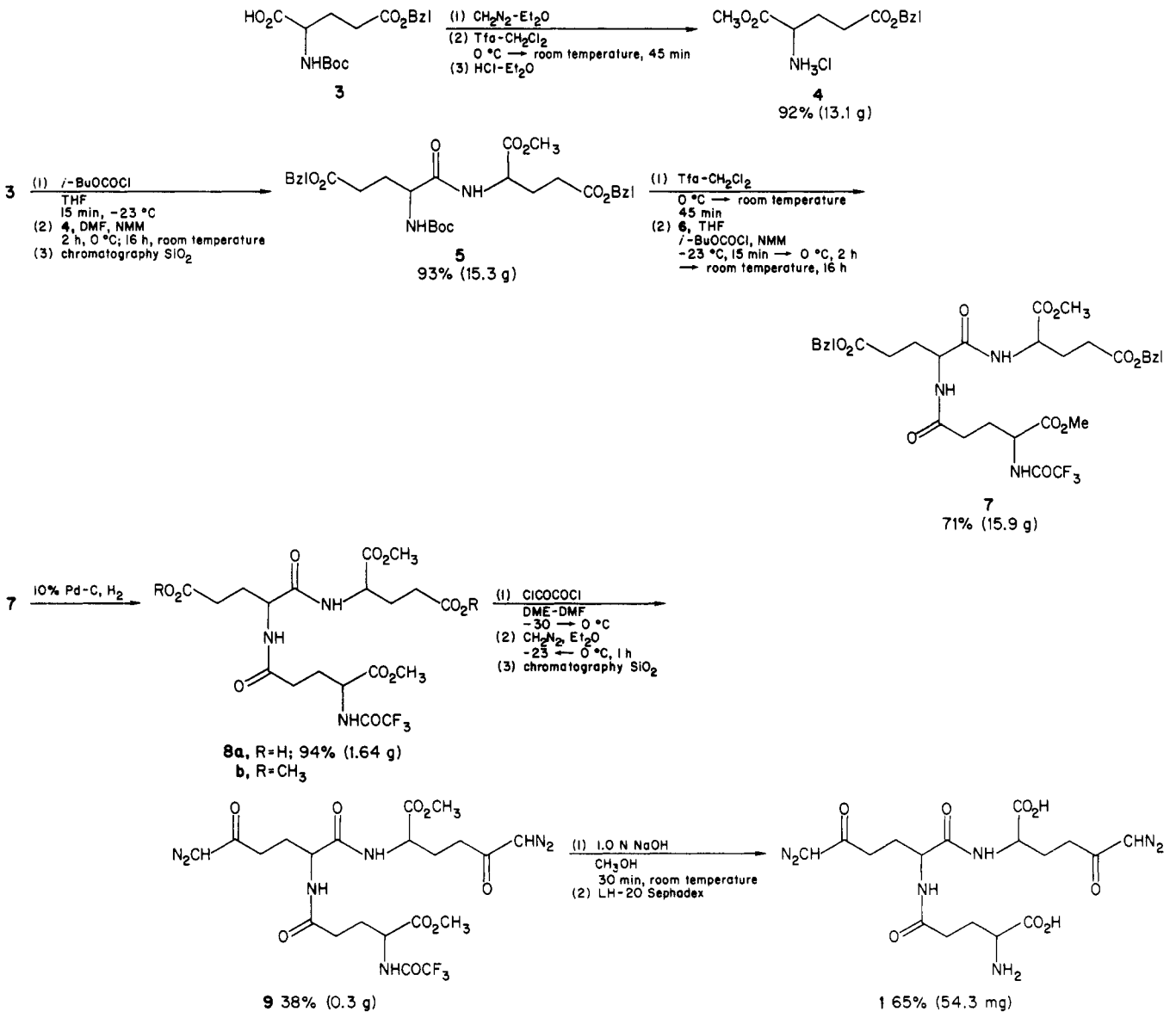
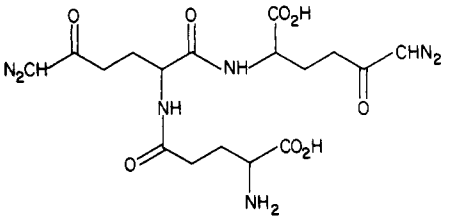
0022-3263/86/1951-1282$01.50/0

Scheme I



Clinical investigations of azotomycin were begun in 1965[11] and later indicated some promise against carcinomas of the gastrointestinal tract, certain sarcomas, melanoma, and lung cancer.[7] Despite less than optimal doses and schedules, definite antitumor activity was repeatedly established.[12] But extensive Phase II clinical trials have never been conducted.[13-16] By 1979 azotomycin was again under consideration by the U.S. National Cancer Institute for additional clinical studies[12] based on new experiments showing remarkable activity against a series of human tumor xenografts in nude mice. The feasibility of further trials has been enhanced by recent evidence that the anticancer activity of such glutamine antimetabolites can be increased and the dosage reduced by cotreatment with a glutamine-depleting enzyme.[17] Since the supply of azotomycin in 1979 was essentially exhausted, fermentation production was presenting problems,[14,18,19] and no synthetic route was known, we undertook a total synthesis to increase availability and now report a practical solution to this problem.

To devise a reasonable synthesis of azotomycin, development of appropriate protecting groups and diazo ketone



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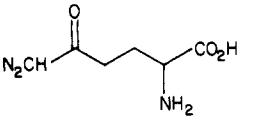

2

(11) Ansfield, F. *Cancer Chemother. Rep.* **1965**, *46*, 37.
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(16) Leventhal, B. G.; Henderson, E. S. *Cancer* **1971**, *28*, 825.
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(18) Earhart, R. H.; Koeller, J. M.; Davis, H. L. *Cancer Treat. Rep.* **1982**, *66*, 1215. Cantane, R.; Ovejera, A. A.; Houchens, D. P.; von Hoff, D. D.; Davis, H. L., Jr.; Wolpert, M. K.; Rozencweig, M.; Muggia, F. M. *Proc. Am. Assoc. Cancer Res. ASCO* **1978**, *19*, 317.
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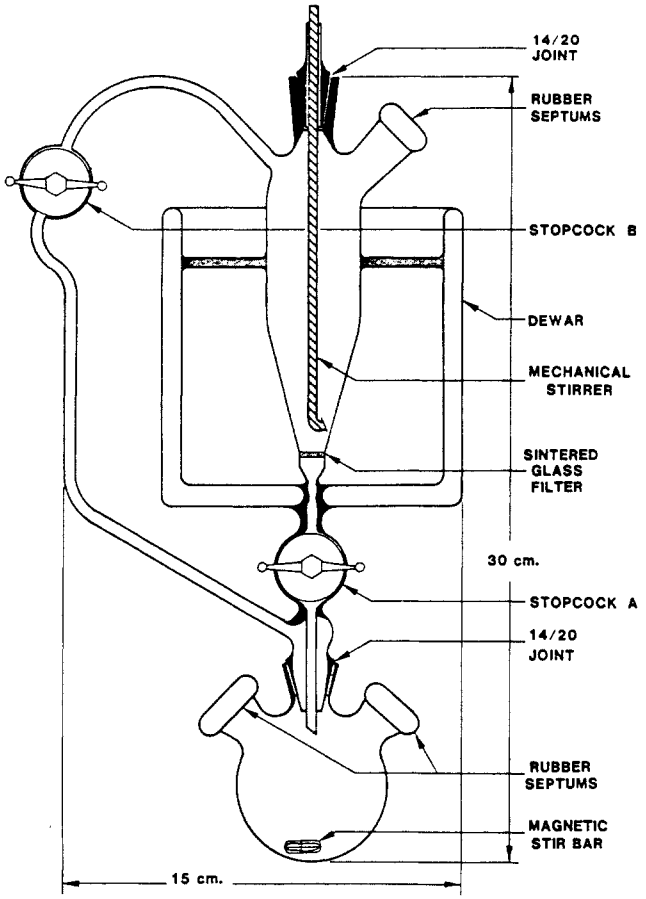

Figure 1. Apparatus for diazo ketone preparation.

formation techniques were necessary. For this purpose, synthesis of 6-diazo-5-oxo-L-norleucine (DON, **2**) was selected as a useful model compound as azotomycin (**1**) contains two molecules of DON (**2**). The synthesis of DON proved to be an excellent selection, and we subsequently reported[4] a convenient synthesis employing *N*-trifluoroacetyl and methyl ester protecting groups with a modified Arndt–Eistert method for diazo ketone formation. Similar experimental methods seemed to present a plausible strategy for synthesis of azotomycin (**1**) and this was realized as outlined in Scheme I. While initially attractive, a series of other synthetic approaches were found impractical especially at the diazo ketone stage.

Because of proven simplicity, reliability (against racemization), and efficiency, the mixed carbonic anhydride (MCA) method was chosen for peptide bond formation.[20] In practice (cf., Scheme I), commerically available γ-OBzl-*N*-Boc-L-Glu (**3**) was esterified with ethereal diazomethane, deprotected with trifluoroacetic acid–methylene chloride (1:1), and converted to hydrochloride **4** by treatment with dry hydrogen chloride in ethyl ether. Reaction of γ-OBzl-*N*-Boc-L-Glu(**3**) with isobutyl chloroformate and *N*-methylmorpholine followed by addition of amino acid hydrochloride **4** afforded dipeptide **5** in good yield. After cleaving (trifluoroacetic acid–methylene chloride) the Boc group of dipeptide **5**, *N*-Tfa-L-Glu-OMe (**6**)[4] was condensed with the product to afford tripeptide **7**. Following hydrogenolysis of the benzyl esters using palladium-on-carbon (10%), diacid **8a** was obtained in high yield.

Conversion of the selectively protected diacid **8a** to bis diazo ketone **9** proved challenging. A number of carboxylic acid → diazo ketone procedures were explored and found unsatisfactory for the azotomycin synthesis. A summary of these studies will appear in a separate treatment of this subject.[21] Eventually a modification of the acid chloride approach utilized in our synthesis[4] of 6-diazo-5-oxo-L-norleucine (**2**) indicated that the diacid chloride derivative of **8a** was obtainable in about 50% yield. Evidence for this observation was established by treating diacid **8a** with 2.1 equiv of oxalyl chloride, triethylamine (2.1 equiv), and a catalytic amount of dimethylformamide (at 0 °C) followed by addition of dry methanol to yield (47%) tetramethyl ester **8b**. A number of related procedures caused extensive yield losses, presumably to competing cyclization reactions.[21] The structure of tetramethyl ester **8b** was confirmed by an alternate synthesis (92% yield) from diacid **8a** and diazomethane.

A new apparatus (Figure 1) was designed and utilized to perform the diazo ketone forming reaction. Reapplication of the preceding acid chloride synthesis to diacid **8a** followed by diazomethane treatment afforded (39%) protected azotomycin **9** as pale yellow crystals. Chromatographic analysis of the crude reaction mixture indicated tetramethyl ester **8b** as the major side product. Simultaneous removal of the three protecting groups of bis diazo ketone **9** with 1.0 N sodium hydroxide at ambient temperature followed by steric exclusion chromatography (Sephadex LH-20) provided (−)-azotomycin (**1**). Overall, the total synthesis of azotomycin was completed in 14% yield from *N*-Boc-L-Glu-γ-OBzl (**3**). The synthetic (−)-azotomycin physical properties proved to be consistent with corresponding data previously reported.[3] While a pure specimen of authentic natural (−)-azotomycin for a definitive comparison was no longer available (due to decomposition), the identical agreement of natural and synthetic (−)-azotomycin ultraviolet and infrared spectra combined with ^1H and ^{13}C NMR spectral[22] studies of the synthetic product provided unequivocal evidence that the original structure proposal[3] for (−)-azotomycin was correct and total synthesis achieved.

The preceding nine-step synthesis of natural azotomycin provides a practical alternative to isolation from *Streptomyces ambofaciens*. The experimental techniques developed for preparation of such bis diazo ketones should now allow related substances[26] to be readily prepared and

(20) For reviews, see: Pettit, G. R. "Synthetic Peptides"; Elsevier Scientific Publishing Co., The Netherlands, 1982, 1980, 1976; Vols. 6–4. Gross, E., Meienhofer, J., Eds.; Academic Press: New York, 1979; Vol. 1.

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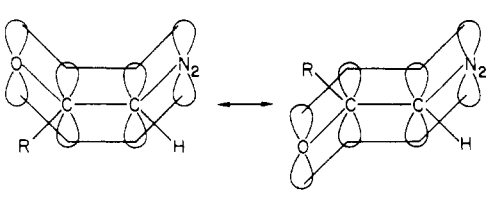
(22) ^1H NMR spectra of primary diazo ketones display the methine proton ($COCHN_2$) singlet in the 5.5 ppm region and diazo ketone derivatives of amino acids show the same characteristic resonance.[23] The methine proton singlet is believed to result from rapid interconversion of both the cis and trans isomers as shown. For example, diazoacetone



at −38 °C shows two methine proton resonances (5.32 and 5.80 ppm), indicating the existence of cis and trans isomers.[24] The isomers of diazoacetone were calculated to be in a 9:1 ratio (rapid interchange) at 38 °C and appeared as a single peak at δ 5.53. Furthermore, the methine proton can readily exchange with deuterium oxide.[25] With azotomycin (in Me_2SO-d_6) the two diazo ketone methine protons appeared as overlapping singlets at δ 6.06 and 6.11.

(23) Hall, I. H.; Loeffler, L. J.; Sajadi, Z.; Kashani, M. *J. Med. Chem.* **1980**, *23*, 275.

(24) Kaplan, F.; Meloy, G. *Tetrahedron Lett.* **1964**, 2427.

(25) Morrison, H.; Yates, P. *Chem. Ind. (London)* **1962**, 331.

(26) Cf.: Sava, G.; Giraldi, T.; Baldini, L. *Cancer Treat. Rep.* **1982**, *66*, 179.

measured as glutamine antimetabolites. Antineoplastic evalutation of DON and azotomycin synthetic intermediates has so far uncovered significant activity with 6-diazo-5-oxo-*N*-(*Z*)-L-norleu-OBzl[4] against the National Cancer Institute's murine L-1210 lymphocytic leukemia (30–78% life extension at 25–100 mg/kg) and MX-3 breast xenograft (58–84% tumor regression at 150–600 mg/kg).

Experimental Section

Both (*Z*)-L-Glu and γ-OBzl-L-Glu were obtained as earlier noted.[4] Tetrahydrofuran and dimethoxyethane were distilled from lithium aluminum hydride. All solvent extracts of aqueous solutions were dried over anhydrous sodium sulfate. Analtech silica gel GF (0.25 mm) plates were used for thin-layer chromatography (TLC) and viewed with ultraviolet light or developed with concentrated sulfuric acid (or a 1% ninhydrin spray). Silica gel (70–230 mesh) and neutral alumina supplied by E. Merck (Darmstadt) or Sephadex LH-20 (Pharmacia Fine Chemicals, AB, Uppsala, Sweden) were used for column chromatography.

All melting points were determined with a Kofler type melting point apparatus. Optical rotation data were obtained with a Perkin-Elmer 241 polarimeter. The ultraviolet spectra were recorded with a Hewlett-Packard 8450A UV–vis spectrophotometer. A Perkin-Elmer 299 infrared spectrophotometer and/or a Nicolet MX-1 FT-IR spectrometer were used for infrared measurements. Nuclear magnetic resonance spectra were collected with a Varian XL-100, a Bruker WH-90, or a Varian T-60A spectrometer (by Dr. V. Witschel) and tetramethylsilane was used as an internal standard. The mass spectra (70 eV, EI, and SP-SIMS from a glycerol solution phase) were recorded by D. Adams employing a MAT 312 instrument. Elemental analyses were determined by Dr. Spang of the Spang Microanalytical Laboratory, Eagle Harbor, MI, or by MicAnal, Tucson, AZ.

The ethereal diazomethane solutions (and standardization) used for synthesizing diazo ketones or methyl esters were prepared from *N*-(nitrosomethyl)urea by the procedure of Arndt.[27] A new apparatus (Figure 1) was designed for the preparation of diazo ketones from acid chlorides. By use of rubber septums the reaction was conducted in an inert atmosphere of nitrogen or argon. The appropriate reaction temperature (in the addition funnel) for preparing the acid chloride could be obtained by carefully adjusting a dry ice/isopropyl alcohol bath inside the Dewar flask. The newly formed acid chloride was added at the selected temperature (depending on the amount of dry ice added to the Dewar flask, usually −78 °C) slowly through stopcock A into a flask containing excess ethereal diazomethane. Since the acid chlorides were prepared in situ from oxalyl chloride and a hydrogen chloride neutralizer (triethylamine or dicyclohexylamine to retard α-chloro ketone formation), it was necessary to install a sintered-glass filter to separate the hydrochloride salt byproduct. The addition funnel was also equipped with a tube for pressure equilibration (stopcock B). When the sintered-glass funnel became blocked with salt a positive pressure of nitrogen or argon was applied (by closing stopcock B) to the addition funnel to speed the addition.

γ-OBzl-L-Glu-α-OMe·HCl (4). A solution of γ-OBzl-*N*-Boc-L-Glu (**3**; 12.9 g) was dissolved in dry ethyl ether (200 mL), cooled (ice bath), and stirred (magnetic bar). An ethereal solution of diazomethane (from 10 g of *N*-(nitrosomethyl)urea) was added dropwise until a slight yellow color persisted. After the mixture was stirred for 15 min at room temperature, nitrogen was passed through the solution (hood) until the yellow color disappeared. The remaining solvent was evaporated under reduced pressure. The oily residue was treated carefully with trifluoroacetic acid–methylene chloride (1:1, 135 mL) in an ice bath with stirring. The ice bath was removed and the mixture stirred for 45 min at room temperature. Carbon tetrachloride (50 mL) was added and the solvent evaporated in vacuo. The procedure was repeated three more times to assure removal of excess trifluoroacetic acid. The residue was dissolved in ethyl ether (200 mL), and dry hydrogen chloride was bubbled through the reaction mixture for 5 min with ice cooling. The colorless solid was collected and washed with ethyl ether. After recrystallization from methylene chloride–hexane, hydrochloride **4** was obtained in 92% (13.1 g) yield: mp 129–135 °C dec; $[\alpha]^{25}_D$ +13.3° ($CHCl_3$); IR (KBr) 3340 br, 2960 br, 1750, 1737, 1487, 1282, 1160, 741, 700 cm^{-1}; ^1H NMR ($CDCl_3$) δ 2.22–2.92 (m, 4 H), 3.76 (s, 3 H, OMe), 4.20–4.52 (m, 1 H, asymmetric α-H), 5.14 (s, 2 H, CH_2Ph), 7.40 (s, 5 H, Ar), 8.54–9.14 (br s, 3 H, NH_3); SP–SIMS mass spectrum, *m/e* 252 ($[M - Cl]^+$).

Anal. Calcd for $C_{13}H_{18}ClNO_4$: C, 54.26; H, 6.31; N, 4.87; Cl, 12.32. Found: C, 54.13; H, 6.17; N, 4.89; Cl, 12.51.

γ-OBzl-*N*-Boc-L-Glu-γ-OBzl-L-Glu-OMe (5). To a solution of γ-OBzl-*N*-Boc-L-Glu (**3**; 9.97 g, 29.5 mM) in tetrahydrofuran (150 mL, cooled to −23 °C, dry ice–carbon tetrachloride) under nitrogen was added *N*-methylmorpholine (3.25 mL, 29.5 mM) and isobutyl chloroformate (3.83 mL, 29.5 mM). The mixture was stirred for 15 min at −23 °C. To a solution of γ-OBzl-L-Glu-α-OMe·HCl (**4**; 8.51 g, 29.5 mM) in dry dimethylformamide (50 mL) was added *N*-methylmorpholine (3.25 mL, 29.5 mM) dropwise, and the reaction flask was transferred to an ice bath. After the mixture was stirred for 2 h, the ice bath was removed and the mixture allowed to stir at room temperature for 16 h. The solution was filtered (Celite) and the solid rinsed with tetrahydrofuran. Following evaporation (in vacuo) of the filtrate, a solution of the residue in ethyl acetate (500 mL) was washed with water (2 × 175 mL), 0.5 M citric acid (2 × 175 mL), 5% sodium bicarbonate (2 × 175 mL), and brine (1 × 175 mL). Solvent was removed and the light straw-colored oily residue was chromatographed in 9:1 methylene chloride–ethyl acetate on a column of silica gel (200 g). Fractions eluted by 2:1 methylene chloride–ethyl acetate showing TLC R_f 0.62 were combined, and solvent was evaporated (in vacuo) to give 15.3 g (93%) of dipeptide **5** as a colorless oil, which solidified on standing at room temperature (all attempts to recrystallize failed): mp 58.5–60 °C; $[\alpha]^{25}_D$ +7.1° ($CHCl_3$); IR 3330 br, 2980, 1745 br, 1680, 1502 br, 1460, 1370, 1170, 753, 740, 700 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.75–2.66 (m, 17 H), 1.43 (s, 9 H, Boc), 3.73 (s, 3 H, OMe), 4.15–4.78 (m, 2 H, assymetric hydrogens), 5.17 (s, 4 H, CH_2Ph), 5.22 (d, J = 8 Hz, 1 H, NH), 7.10 (d, J = 8 Hz, 1 H, NH), 7.41 (s, 10 H, Ar); ^{13}C NMR ($CDCl_3$) δ 27.0 (t), 27.8 (t), 28.3 (q), 30.2 (t), 30.3 (t), 51.6 (d), 52.4 (d), 53.6 (q), 66.5 (t), 80.0 (s), 128.2 (d), 128.5 (d), 135.8 (s), 155.6 (s), 171.7 (s), 171.9 (s), 172.5 (s), 173.0 (s); SP–SIMS mass spectrum, *m/e* 593 ($[M + Na]^+$), 571 ($[M + H]^+$).

Anal. Calcd for $C_{30}H_{38}N_2O_9$: C, 63.15; H, 6.71; N, 4.91. Found: C, 63.23; H, 6.73; N, 4.86.

***N*-(γ-*N*-Tfa-L-Glu-α-OMe)-γ-OBzl-L-Glu-γ-OBzl-L-Glu-OMe (7).** The Boc group was cleaved from γ-OBzl-*N*-Boc-L-Glu-γ-OBzl-L-Glu-OMe (**5**; 18.3 g) with methylene chloride (90 mL) trifluoroacetic acid (90 mL) as summarized for preparing (see above) Glu derivative **4**. The resulting clear oil, γ-OBzl-L-Glu-γ-OBzl-L-Glu-OMe·TFA, was placed under high vacuum for 1 h, dissolved in dry dimethylformamide (60 mL), and added, followed by *N*-methylmorpholine (3.46 mL, 31.5 mM), to a solution prepared (15 min at −23 °C) from *N*-Tfa-L-Glu-OMe (**6**; 8.1 g, 31.5 mM)[4] in dry tetrahydrofuran (160 mL cooled to −23 °C, magnetic stirring under nitrogen), *N*-methylmorpholine (3.46 mL, 31.5 mM), and isobutyl chloroformate (4.08 mL, 31.5 mM). After being stirred for 2 h at ice bath temperature, the mixture was stirred at room temperature for 16 h. The solution was filtered and the solid phase rinsed with tetrahydrofuran. After the filtrate was concentrated, the oily residue was dissolved in ethyl acetate (1 L) and washed with water (2 × 250 mL), 1% citric acid (2 × 250 mL), 1% sodium bicarbonate (2 × 250 mL), and brine (2 × 250 mL). Removal of solvent gave a solid, which recrystallized from ethyl acetate–hexanes to provide 15.9 g (71%) of tripeptide **7** as colorless crystals: mp 120–122 °C; $[\alpha]^{25}_D$ +7.7° ($CHCl_3$); IR (KBr) 3315, 1759, 1734, 1713, 1645, 1550, 1190, 755, 700 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.86–2.56 (m, 12 H), 3.76 (s, 3 H, OMe), 3.80 (s, 3 H OMe), 4.38–4.75 (m, 3 H, asymmetric hydrogens), 5.19 (s, 4 H, CH_2Ph), 6.81 (d, J = 8 Hz, 1 H, NH), 7.22 (d, J = 8 Hz, 1 H, NH), 7.44 (s, 10 H, Ar), 8.66 (d, J = 8 Hz, 1 H, $NHCOCF_3$); ^{13}C NMR ($CDCl_3$) δ 26.1 (t), 26.8 (t), 27.9 (t), 30.3 (t), 31.7 (t), 51.9 (d), 52.4 (q), 52.8 (d), 66.7 (t), 116.1 (q, J = 287 Hz, F coupling) 128.3 (d), 128.4 (d), 128.6 (d), 135.7 (s), 157.4 (q, J = 38 Hz, F coupling), 170.7 (s), 171.0 (s), 171.8 (s), 172.4 (s), 172.6 (s), 173.4 (s); SP–SIMS mass spectrum, *m/e* 722 ($[M + Na]^+$), 710 ($[M + H]^+$).

Anal. Calcd for $C_{33}H_{38}F_3N_3O_{11}$: C, 55.85; H, 5.38; N, 5.92. Found: C, 55.61; H, 5.28; N, 5.87.

***N*-(γ-*N*-Tfa-L-Glu-α-OMe)-L-Glu-L-Glu-OMe (8a).** To a

(27) Arndt, F. "Organic Syntheses"; Wiley: New York, 1943; Collect. Vol. 2, p 165.

solution of N-(γ-N-Tfa-L-Glu-α-OMe)-γ-OBzl-L-Glu-γ-OBzl-L-Glu-OMe (7; 2.35 g, 3.3 mM) in ethanol (250 mL) was added palladium-on-carbon (10%, 4.7 g, wet with 10 mL of water) and ethanol (50 mL). While the mixture was stirred at room temperature, a current of hydrogen was bubbled through the reaction flask for 1.5 h. The catalyst was removed by filtration and the solvent evaporated under reduced pressure (35 °C) until approximately 5–10 mL remained. Crystallization was accomplished by adding methylene chloride (300 mL) and refrigeration overnight. Diacid **8a** was obtained in 94% (1.64 g) yield as colorless crystals: mp 181–184 °C; $[\alpha]^{25}_D$ −37.6° (CH_3OH); IR (KBr) 3680–2800 br (max at 3325 and 3460), 1790–1695 br (max at 1743 and 1715), 1632, 1562 br, 1449, 1232, 1212, 1175 cm^{-1}; ^1H NMR (acetone-d_6) δ 1.72–2.70 (m, 12 H), 3.71 (s, 3 H, OMe), 3.76 (s, 3 H, OMe), 4.41–4.79 (m, 3 H, asymmetric hydrogens), 4.90–5.82 (br, s, 2 H), 7.65 (d, J = 7 Hz, 1 H, NH), 7.85 (d, J = 7 Hz, 1 H, NH), 9.41 (d, J = 7 Hz, 1 H, $NHCOCF_3$); ^{13}C NMR (D_2O) δ 26.8, 26.9, 27.5, 31.1, 32.3, 53.3, 53.6, 54.1, 54.5, 116.9 (q, J = 285 Hz, F coupling), 160.1 (q, J = 38 Hz, F coupling), 173.4, 174.6, 174.7, 175.7, 178.2; SP-SIMS mass spectrum, m/e 552 ($[M + Na]^+$).

Anal. Calcd for $C_{19}H_{26}F_3N_3O_{11}$: C, 43.11; H, 4.95; N, 7.94. Found: C, 42.89; H, 4.80; N, 7.86.

N-(γ-N-Tfa-L-Glu)-L-Glu-L-Glu-tetra-OMe (8b). Method A. To a solution of N-(γ-N-Tfa-L-Glu-α-OMe)-L-Glu-L-Glu-α-OMe (**8a**; 0.18 g) in dry acetone (20 mL cooled to 0 °C) was added (with magnetic stirring) ethereal diazomethane (0.8 M) until a yellow color persisted. After evaporation of the solvent, the colorless solid was recrystallized from methylene chloride–hexanes to afford 0.17 g (92%) of colorless crystals melting at 150–152 °C: $[\alpha]^{25}_D$ −8.5° ($CHCl_3$); IR (KBr) 3310, 1737, 1708, 1635, 1535 br, 1176 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.87–2.58 (m, 12 H), 3.68 (s, 3 H, OMe), 3.70 (s, 3 H, OMe) 3.75 (s, 3 H, OMe), 3.77 (s, 3 H, OMe), 4.25–4.58 (m, 3 H, asymmetric hydrogens), 6.96 (d, J = 7.5 Hz, 1 H, NH), 7.40 (d, J = 7.5 Hz, 1 H, NH), 8.70 (d, J = 6.5 Hz, 1 H, $NHCOCF_3$); SP-SIMS mass spectrum, m/e 580 ($[M + Na]^+$), 558 ($[M + H]^+$).

Anal. Calcd for $C_{21}H_{30}F_3N_3O_{11}$: C, 45.25; H, 5.42; N, 7.54. Found: C, 45.06; H, 5.11; N, 7.65.

Method B. Oxalyl chloride (0.16 mL, 1.8 mM) was added to a solution prepared from N-(γ-N-Tfa-L-Glu-α-OMe)-L-Glu-L-Glu-α-OMe (**8a**; 0.443 g, 0.84 mM), dry dimethoxyethane (15 mL), triethylamine (0.24 mL, 1.7 mM), and dry dimethylformamide (1 drop) cooled to −78 °C. The reaction temperature was increased to 0 °C while a white solid separated and gas evolved. After the mixture was stirred for 30 min at 0 °C, dry methanol (1.5 mL) was added and solvent evaporated. The residue was dissolved in chloroform (50 mL) and washed with water (2 × 12 mL) and brine (1 × 12 mL). A solution of the residue in ethyl acetate was chromatographed on a column of silica gel (50 g). The fractions with TLC R_f 0.57 (1:24 methanol–ethyl acetate) were collected and solvent evaporated to give 0.22 g (47%) of a colorless solid, which recrystallized from methylene chloride–hexanes: mp 158–160 °C; $[\alpha]^{25}_D$ 11.6° ($CHCl_3$). The product (**8b**) was found to be identical with the specimen of tetramethyl ester **8b** prepared by method A (above).

N-Tfa-(−)-azotomycin-di-OMe (9). A 1.00-g specimen of N-(γ-N-Tfa-L-Glu-α-OMe)-L-Glu-L-Glu-α-OMe (**8a**; 1.00 g, 1.9 mM) was dissolved (under argon) in dry dimethoxyethane (30 mL) with warming (magnetic stirring), and triethylamine (0.55 mL, 3.97 mM) was added. The reaction mixture (in the apparatus of Figure 1) was cooled to −30 °C (dry ice–isopropyl alcohol), and oxalyl chloride (0.35 mL, 3.97 mM) was added followed by dimethylformamide (2 drops). The reaction mixture was warmed to 0 °C by addition of hot isopropyl alcohol to the dry ice bath, stirred for 40 min, and then cooled to −78 °C. At this point the cold acid chloride solution was slowly (30 min) added through the sintered-glass filter into an ethereal solution of diazomethane (0.5 M, 30 mL) cooled to −23 °C (dry ice–carbon tetrachloride). After the mixture was stirred 30 min at −23 °C and 30 min at 0 °C, solvent was evaporated by a stream of argon, and the residue was chromatographed in 19:1 ethyl acetate–methanol on a column of silica gel (70 g). The fractions (24:1 ethyl acetate–methanol) with TLC R_f 0.16 were collected and solvent evaporated to give 0.30 g (38%) of light yellow crystals, which, upon recrystallization from chloroform–ethyl ether, melted at 134–136 °C: $[\alpha]^{25}_D$ 22.9° ($CHCl_3$); IR (KBr) 3330, 2116, 1760, 1720, 1651, 1550, 1395, 1198, 1185 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.70–2.85 (m, 12 H), 3.76 (s, 3 H, OMe), 3.79 (s, 3 H, OMe), 4.34–4.70 (m, 3 H, asymmetric protons), 4.51 (d, 2 H, two overlapping CHN_2 s), 7.62 (d, J = 7 Hz, 1 H, NH), 7.96 (d, J = 7 Hz, 1 H, NH), 9.45 (d, J = 7 Hz, 1 H, $NHCOCF_3$); SP-SIMS mass spectrum, m/e 600 ($[M + Na]^+$), 588, 566, 544.

Anal. Calcd for $C_{21}H_{26}F_3N_7O_9$: C, 43.68; H, 4.54; N, 16.98. Found: C, 43.53; H, 4.51; N, 16.72.

(−)-Azotomycin (1). To a mixture of N-Tfa-(−)-azotomycin-di-OMe (**9**; 106 mg, 0.18 mM) and methanol (0.12 mL) was added 1.0 N sodium hydroxide (0.83 mL, 8.3 mM) at room temperature. After being stirred for 30 min (room temperature), the solution was acidified to pH 6.9 with 0.1 N hydrochloric acid and extracted with chloroform (2 × 25 mL). The aqueous phase was dissolved in methanol and passed through a column of Sephadex LH-20 (250 g), and fractions containing the major product (TLC R_f 0.31, methanol, tailing) were collected. Solvent was evaporated and the residue dissolved in water (10 mL). The aqueous phase was freeze-dried to yield 54.3 mg (65%) of (−)-azotomycin (**1**) as a light yellow solid, which slowly decomposed at ambient temperature: $[\alpha]^{25}_D$ −4.3° (c 0.4, H_2O); UV λ_{max} (H_2O) 245 and 275 nm (ε 14 850 and 25 790) [lit.[3] λ_{max} 245 and 275 nm (ε 15 400 and 24 290)]; IR (KBr) 3300, 3071, 2111, 1648, 1539, 1385, 1226 cm^{-1}; ^1H NMR (Me_2SO-d_6)[28] δ 1.46–2.16 (m, 6 H), 2.16–2.60 (m, 6 H), 3.29–3.64 (m, 1 H, asymmetric proton), 6.06 and 6.11 (d, 2 H, two overlapping CHN_2 s), 8.18–8.55 (m, 3 H, sharp s at δ 8.38); ^1H NMR (1:3 D_2O–D_3COD)[29] δ 1.70–2.37 (m, 6 H), 2.37–2.78 (m, 6 H), 3.59–3.80 (m, 1 H, asymmetric proton), 4.22–4.53 (m, 2 H, asymmetric protons); ^{13}C NMR (3:1 D_2O–D_3CCN, 5 °C) δ 26.9, 27.0, 27.4, 31.19, 36.4, 36.5, 53.1, 53.9, 54.6, 58.4, 173.9, 174.1, 175.1, 175.8, 198.9, 199.0.

Anal. Calcd for $C_{17}H_{23}N_7O_8 \cdot H_2O$: C, 43.31; H, 5.35; N, 20.80. Found: C, 43.78; H, 5.51; N, 19.35 (lit.[3] C, 43.15; H, 5.59; H, 19.22).[30]

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(28) The spectrum was uninterpretable in the 3.6–5.3 ppm region due to a large water proton signal.

(29) All other expected protons exchanged with the D_2O including the diazo ketone methine protons.

(30) Since azotomycin is hydroscopic and sensitive to light and ambient temperatures it was not surprising that elemental analysis for nitrogen gave a low value essentially as reported for the original natural product (see ref 3).