5-(2-Aminoacetyl)-1,3-dibenzyl-1*H*,3*H*-2,1,3-benzothiadiazole 2,2-Dioxide (6d). Hexamethylenetetramine (0.9 g) was added to a solution of **6b** (3.0 g, 0.0064 mol) in THF (200 mL). The mixture was stirred for 3 h at room temperature and filtered, the residue (crude **6c**) was dissolved by heating in a mixture of methanol (50 mL) and 48% aqueous HBr (5 mL), and the solution was refluxed for 3 h. The hot solution was cooled and water (25 mL) was added. The precipitate formed was filtered off and recrystallized from methanol to yield pure **6d** as the hydrobromide (1.55 g, 52%) as white crystals, mp 210–222 °C. Anal. ($C_{22}H_{21}N_3O_3S$·HBr) C, H, N.

***N*-Benzyl-*N*-(3-acetylphenyl)trifluoromethanesulfonamide (9m).** *N*-(3-Acetylphenyl)trifluoromethanesulfonamide[16] (**8m**; 6.75 g, 0.025 mol), benzyl bromide (4.7 g), and anhydrous potassium carbonate (13.8 g) in acetone (125 mL) were refluxed for 2 h, evaporated to low bulk, and partitioned between ether (50 mL) and water (50 mL); the organic layer was evaporated to yield the product **9m** (9.7 g, 112%) containing a little occluded solvent. Prolonged drying gave a pure sample, with satisfactory spectra. Anal. ($C_{16}H_{14}F_3NO_3S$) C, H, N.

***N*-Benzyl-*N*-(4-acetylphenyl)trifluoromethanesulfonamide (9p)** was obtained similarly to **9m**, starting from **8p**:[16] mp 55–57 °C (from petrol, bp 60–80 °C); yield 86%. Anal. ($C_{16}H_{14}F_3NO_3S$) C, H, N.

***N*-Benzyl-*N*-[3-(bromoacetyl)phenyl]trifluoromethanesulfonamide (10m).** Compound **9m** (0.025 mol) in chloroform (80 mL) was treated with 40% hydrogen bromide in acetic acid (1 mL) and then bromine (4 g in 20 mL chloroform) was added dropwise over 3 min. (The bromine was decolorized instantaneously; if the HBr/AcOH initiator was omitted, the reaction was unreliable.) After 5 min, the mixture was evaporated to dryness to give the desired product (quantitative) as an oil. Side-chain bromination was confirmed by NMR ($CDCl_3$), showing CH_2Br at δ 4.26 (compare **9m** CH_3 at δ 2.45). Anal. TLC, accurate MS.

The 4-(bromoacetyl) isomer (10p) was obtained similarly from **9p**. Anal. TLC, MS.

***N*-Benzyl-*N*-[3-(2-bromo-1-hydroxyethyl)phenyl]trifluoromethanesulfonamide (11m).** Bromo ketone **10m** (9.6 g, 0.022 mol) was stirred in methanol (100 mL) at 0–5 °C and sodium borohydride (0.83 g, 0.022 mol) was added portionwise over 5 min. The mixture was allowed to warm to ambient temperature over 0.50 h. The mixture was evaporated to dryness and the residue was triturated with chloroform (2 × 50 mL); the chloroform layer was evaporated to low bulk and filtered, and the filtrate (~5 mL) was diluted with petrol, bp 40–60 °C (30 mL), depositing product (6.2 g, 64%) in two crops. This product slowly solidified (mp 61–63 °C) and was of adequate purity for conversion to **12m**. The mother liquors slowly deposited a pure sample of **11m**, mp 66–67 °C. Anal. ($C_{16}H_{15}BrF_3NO_3S$) C, H, N.

***N*-Benzyl-*N*-[4-(2-bromo-1-hydroxyethyl)phenyl]trifluoromethanesulfonamide (11p)**, an oil, was prepared (analogously to **11m**) from **10p** in 77% yield. Anal. TLC, accurate MS.

***N*-Benzyl-*N*-[3-(2-oxiranyl)phenyl]trifluoromethanesulfonamide (12m).** A methanolic solution (100 mL) of crude bromohydrin **11m** (0.025 mol) was treated with aqueous 2 N sodium hydroxide (25 mL to pH 11), then diluted with water (200 mL), and extracted with ether (200 mL). The ether extracts on evaporation gave the crude product as an oil (8.0 g), which was chromatographed over Kieselgel (Merck 60H no. 7736, 200 g), developing and eluting with dichloromethane, collecting product at R_f ~0.75. Evaporation of the fractions containing product gave pure **12m** (4.0 g 45%), mp 36–40 °C. Anal. ($C_{16}H_{14}F_3NO_3S$) C, H, N.

***N*-Benzyl-*N*-[4-(2-oxiranyl)phenyl]trifluoromethanesulfonamide (12p).** Bromohydrin **11p** (3.6 g, 0.00826 mol) in methanol (30 mL) was treated with 4 N sodium hydroxide (4.3 mL) over 5 min, stirred for a further 5 min, and diluted with water (60 mL), and the mixture was extracted with petrol, bp 40–60 °C (4 × 20 mL), and ether (2 × 30 mL); the organic extracts on evaporation yielded product **12p** (an oil, 2.5 g, 85%) of high purity. Anal. ($C_{16}H_{14}F_3NO_3S$) H, N; C: calcd, 53.78; found, 53.30.

4,4′-Dimethoxybenzhydrylamine (15). From Chloride 17b. 4,4′-Dimethoxybenzhydrol (Aldrich; 7.33 g, 0.03 mol) in ether (100 mL) was saturated with hydrogen chloride. The mixture was evaporated to dryness, and the residue was redissolved in ether (80 mL), decanted from ~1 mL aqueous phase, and reevaporated to yield 4,4′-dimethoxybenzhydryl chloride (7.7 g, 98%) as an oil, which quickly solidified, mp 79–81 °C (lit.[21] 83–84 °C). This chloride (0.5 g, 0.00191 mol) in chloroform (5 mL) was added to chloroform (100 mL) saturated with ammonia gas (1.1 g) dropwise with stirring; the mixture was stirred for 2 h and then washed with water (2 × 20 mL); the chloroform layer on evaporation yielded product **15** (0.5 g, 100%) as a clear oil, which solidified (mp 50–54 °C): previously reported[22] as an oil.

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Supplementary Material Available: Biological data on series **7**, **14**, and **17** (1 page). Ordering information is given on any current masthead page.

Alkylating Angiotensin II Analogues:[1] Synthesis, Analysis, and Biological Activity of Angiotensin II Analogues Containing the Nitrogen Mustard Melphalan in Position 8

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Melphalan derivatives suitable for peptide synthesis, i.e., Boc-Mel and Mel-OBzl·HCl, have been prepared, and the integrity of their nitrogen mustard alkylating groups was examined by NMR, Volhard chlorine analysis, and colorimetric assay with 4-(*p*-nitrobenzyl)pyridine. By using the sensitive colorimetric assay, the stability of melphalan toward conditions commonly used for peptide synthesis, purification, and bioassay was evaluated. Further qualitative and quantitative assessment of the integrity of nitrogen mustard groups in angiotensin II was attempted in order to evaluate the significance of the observed biological results. [Ac-Asn[1],Mel[8]]angiotensin II was a potent competitive antagonist of angiotensin II in vitro (rat uterus) but a transient and reversible inhibitor in vivo.

Although a wide scope of effects has been shown for many peptide hormones, their clinical application has been fairly limited,[2] partially due to the rapid inactivation of these peptides in vivo. One approach to counter this lim-

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itation has been accomplished by introducing enzymatic-resistant features into strategic positions of the peptides.[3,4] Another direction to develope ultra-long-acting therapeutics in general has been the use of alkylating analogues, such as those of oxymorphone[5] and naltrexone.[6] The latter inhibitor produced selective and sustained blockade (over 3 days) of the mice opioid receptors at a significantly lower dosage than the parent naltrexone. These findings illustrate the potential benefits of affinity-labeling ligands,[7] which can associate with the receptors irreversibly on a one to one basis. In contrast, a large excess of competitive ligands is required to occupy a receptor because of their reversible nature of binding. Unfortunately, affinity labeling requires the presence of a suitable receptor nucleophile and its proper alignment with the alkylating group in order for alkylation to occur. Although this limitation can be partially overcome by the photoaffinity labeling agents,[8-10] which have found wide application in in vitro systems due to their unusual reactivity upon irradiation, a generalized method for photoactivation in vivo has yet to be developed.

In angiotensin II, Asp-Arg-Val-Tyr-Val-His-Pro-Phe, prolonged inhibition of the target tissues of guinea pig ileum[11] and rat uterus[12] had been demonstrated for [chlorambucil[1]]angiotensin II, which specifically blocked further tissue response toward angiotensin II for over 6 h. More recently, similarly prolonged inhibition (2–6 h) of the rabbit aorta and rat stomach receptors was elicited by photoaffinity labeling analogues of antiotensin II.[13,14] These results clearly demonstrate that the affinity-labeling approach can prolong the transient effect of peptides, which in the case of angiotensin II normally lasts for several minutes.[15,16] However, irreversible blockade of the angiotensin response in vivo was not demonstrated by either inhibitor.[11,14] Because position 1 of angiotension II does not participate strongly in either binding or activation of the receptor,[17] it is possible that while [chlorambucil[1]]angiotensin II might bind tightly to the tissues either covalently or noncovalently, it did not alkylate the receptor. Since receptor activation is primarily by position-8 phenylalanine and, to a lesser extent, by position-4 tyrosine,[17-21] these residues become inviting candidates for introducing a specific affinity label because of their access to the angiotensin II receptor active center.

Among the clinically and experimentally tested alkylating groups,[22-24] some are only suitable for NH_2 substitution of a peptide, i.e., chlorambucil and bromoacyl and chloroacyl, while others are exclusively for COOH derivatization, i.e., aldehyde, chloromethyl ketone, isocyanate, imido ester, and methanesulfonate. For side-chain modification, melphalan (Mel), which contains a bulky nitrogen mustard attached to a para position of phenylalanine, is attractive in that bulky substituents in position 8 of angiotensin II have been shown to produce angiotensin inhibitors with low residual agonistic activities.[18,19]

Table I. Relative Stability of Melphalan toward Various Solvent Mixtures

solvent	approximate time, h, for 50% dec	approximate time, h, for 25% dec
CH_2Cl_2	ND[a]	ND
DMF	ND	ND
4 N HCl/dioxane	ND	ND
25% F_3AcOH/CH_2Cl_2	ND	ND
5% Et_3N/CH_2Cl_2	66	38
BAW (4:1:5)[b]	ND	90
BPAW (8:1:2:9)[c]	ND	72
1 N NH_4OAc	10	4
10% AcOH	20	12
normal saline	42	31

[a] Not detectable in 6 days. [b] The upper organic phase of the mixture of 1-butanol-acetic acid-water in the ratio of 4:1:5. [c] The upper organic phase of the mixture of 1-butanol-pyridine-acetic acid-water in the ratio of 8:1:2:9.

(1) (a) The abbreviations used to denote amino acids and peptides are those recommended by the IUPAC–IUB Commission on Biochemical Nomenclature [*J. Biol. Chem.*, **247**, 977 (1972) and *Biochemistry*, **14**, 499 (1975)]. (b) This report was presented in part. See "Abstracts of Papers", 172nd National Meeting of the American Chemical Society, San Francisco, CA, 1976, American Chemical Society, Washington, DC, 1976, Abstr MEDI 015.

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Previously, [melphalan[8]]angiotensin II was reported by Park et al. to have insignificant pressor activity (0.3%),[25] although it was not shown whether the nitrogen mustard remained intact. Neither the inhibitory activity nor the detailed chemistry of this analogue was described. In order to address these questions, we extended their study and prepared [Ac-Asn[1],Mel[8]]AII, as well as its benzyl and methyl ester analogues, in the hope to minimize potential undesirable interaction of the nitrogen mustard with the amino and the carboxylate termini. Furthermore, the integrity of the alkylating group in melphalan and its derivatives was established by NMR spectroscopy and chlorine analyses, and the results were further corroborated by a sensitive colorimetric assay. This assay was used to evaluate the stability of melphalan toward various conditions used for peptide synthesis, purification, and bioassay and was further applied to thin-layer chromatography to detect nitrogen mustard containing compounds in general. Finally, the inhibitory activities of these angiotensin analogues both in vitro and in vivo were evaluated.

Results and Discussion

Synthesis of melphalan and its derivatives was extensively investigated by Bergel and Stock and their associates.[26-28] However, in these early studies, the NH_2 and the COOH termini of melphalan were protected by groups which were not readily removable, such as the phthalyl, the ethyl ester, and the amide. We, therefore, introduced the widely used *tert*-butyloxycarbonyl (Boc) and benzyl ester (OBzl) groups into melphalan. In addition, the methyl ester of melphalan was prepared and incorporated into angiotensin II.

Although esterification of melphalan[27] proceeded smoothly and nearly quantitatively, separation of the methyl and benzyl esters of melphalan from the unreacted amino acid or from benzyl alcohol and other side products could not be easily achieved by countercurrent distribution or by recrystallization. We observed that dry column chromatography using vacuum-packed silica gel efficiently purified the melphalan derivatives and gave homogeneous products containing the theoretical amount of nitrogen mustard.

In accordance with findings of Bergel et al.,[29] condensation of the Ac-Asn-Arg-Val-Tyr-Val-His-Pro heptapeptide with melphalan ester derivatives (2–3 mol excess) by dicyclohexylcarbodiimide and 1-hydroxybenzotriazole[30] (DCC/HOBt) in dimethylformamide (DMF) presented no difficulty. Amino protection of melphalan also proceeded smoothly to Boc-Mel upon treatment with Boc-azide and triethylamine in acetonitrile.[31]

However, removal of various protection groups can be problematic. Although the nitrogen mustard group of chlorambucil appeared stable toward HF treatment and AcOH extraction,[32] TLC analysis showed that storage (24 h) of melphalan in either trifluoroacetic acid (F_3AcOH) or triethylamine (Et_3N) produced a significant amount of side products. Further evaluation by the sensitive colorimetric assay indicated that the nitrogen mustard of melphalan was stable toward F_3AcOH/CH_2Cl_2 (25%) but was decomposed to a large degree by Et_3N/CH_2Cl_2 (Table I). These findings suggest that repetitive deprotection by F_3AcOH/CH_2Cl_2 in peptide synthesis may be less preferable than by 4 N HCl/dioxane, which, because of its mineral acid nature, did not require excessive neutralization by Et_3N/CH_2Cl_2. While deprotection (1.5 h) of Mel-OBzl·HCl by HBr-saturated trifluoroacetic acid or HBr-saturated acetic acid[33] resulted in incomplete removal of the ester group, alternative treatment (0.5 h) of Mel-OMe·HCl and Mel-OBzl·HCl with boron tribromide[33] was equally unsatisfactory. When catalytic hydrogenation of Mel-OBzl·HCl in DMF was examined, the benzyl group was removed completely within 10–20 min, and excessive hydrogenolysis was not observed within 3 h. Nevertheless, prolonged hydrogenation or excessive hydrogen pressure was found to convert the melphalan generated in situ to additional ninhydrin-positive side products, probably through reductive elimination[34] of the chlorine molecules in the mustard.

Physicochemical Analyses of Melphalan and Its Derivatives. A variety of alkylating ethylenimine compounds were thoroughly studied by NMR,[35,36] titration,[37-39] and colorimetric assays.[40-43] Of the various methods, NMR and titration analyses are suitable for simple derivatives of melphalan but are less useful for the more complex peptides. The small amount of mustard in these compounds can not be adequately measured because of the low sensitivity of these methods in addition to potential interference by other functional groups. On the other hand, the colorimetric assay using 4-(*p*-nitrobenzyl)pyridine appears to be well suited for melphalan-containing peptides because of its specificity for alkylating halogens but not for ionic halides. In addition, its high sensitivity[40,41] (10^{-8} mol) in theory will permit small samples of a peptide, i.e., as little as 0.01 mg for a peptide with a molecular weight of 1000, to be analyzed accurately for its molecular content of nitrogen mustard, if standard curves for the respective melphalan derivatives can be obtained.

In order to explore this possibility, we first examined the presence of reactive nitrogen mustard in melphalan and its ester derivatives by NMR according to reported procedures.[35,36] Although the characteristic triplet pair (A_2B_2) of the nitrogen mustard was observed for Mel·HCl (δ 3.68, 4.03) and Mel-OMe·HCl·H_2O (δ 3.5, 4.1), Mel-OBzl·HCl was too sparingly soluble in deuterated water to permit meaningful analysis. Subsequent neutralization of the samples by NaO^2H further reduced their solubility, and formation of the cyclic aziridinium intermediates could

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not be monitored adequately. When Boc-Mel or its well-characterized dicyclohexylamine salt (Boc-Mel·DCHA) was dissolved in deuterated chloroform instead, an 8-proton singlet at 3.60 ppm was observed for their bis(2-chloroethyl) groups. Because identification of the reactive nitrogen mustard based on NMR assignments proved tenuous, the various derivatives were subsequently characterized by elemental analyses, which were supplemented by Volhard titration to quantitatively determine the amount of ionic and covalent chlorine present in these compounds. For example, direct titration (method A) of 10–20 mg samples indicated the lack of free chloride in Mel and Boc-Mel·DCHA but gave 1 mol of chloride per mole of melphalan ester derivatives. Simultaneous treatment of the samples with excess NaOH (method B) released the covalently bound chlorine quantitatively, and subsequent titration gave the total moles of chloride and chlorine in each mole of melphalan derivative, i.e., 2 for Mel, 2 for Boc-Mel·DCHA, 3 for Mel-OMe·HCl·H_2O, and 3 for Mel-OBz·HCl.

Stability of Melphalan. As the integrity of the melphalan derivatives was thus demonstrated, establishment of the colorimetric standard curves for Mel, Mel-OMe·HCl·H_2O, Mel-OBzl·HCl, and Boc-Mel was attempted by using the procedures of Klatt, Griffin, and Stehlin.[43] The results were unsatisfactory in that the color yields of melphalan always decreased with time, even when the standard solutions were stored at −20 °C, while the color yields of the ester derivatives also varied. Further analysis of these solutions by TLC indicated partial hydrolysis of the esters as well as gradual decomposition of the nitrogen mustard in 25% ethanol/saline or ethanol/water. Since significant (20%) and rapid (30 min) loss of nitrogen mustard was similarly observed in aqueous acetone[44] and in blood,[43] it became important to examine the stability of melphalan toward various conditions commonly used for peptide synthesis, purification, and bioassay.

This was accomplished by using the colorimetric assay to evaluate the amount of residual nitrogen mustard from melphalan stored in the solvent CH_2Cl_2, DMF; the deprotection reagent F_3AcOH/CH_2Cl_2, HCl/dioxane; the neutralization reagent Et_3N/CH_2Cl_2; the countercurrent distribution system BAW, BPAW; NH_4OAc solution for ion exchange; aqueous AcOH for gel filtration or lyophilization; or normal saline for bioassay. The results (Table I) indicated that while melphalan was relatively stable toward the organic mixtures, it was rapidly decomposed by aqueous protic solvents. Evaporation of the melphalan solutions (after 6 days) to dryness in a vacuum dessicator, followed by TLC analysis of the residues, further corroborated these findings. Thus, substantial loss of nitrogen mustard is possible during conventional column chromatography, countercurrent-distribution purification, and lyophilization of melphalan-containing peptides. In addition, the surprisingly rapid loss of melphalan in normal saline suggests that solutions containing melphalan peptides should be prepared immediately before the bioassay experiment.

Standard Curves of Melphalan and Derivatives. Since the nitrogen mustard compounds were poorly soluble in most nonaqueous and aprotic solvents, except in dimethyl sulfoxide (Me_2SO), the compatibility of this solvent with various melphalan derivatives was investigated next. In addition to the colorimetric analysis which indicated that the nitrogen mustard contents did not diminish upon prolonged storage (−20 °C) with frequent thawing in the readily freezable Me_2SO (mp 18.5 °C) for over a month, subsequent examination of the solutions by TLC further indicated the presence of the appropriate melphalan derivatives only.

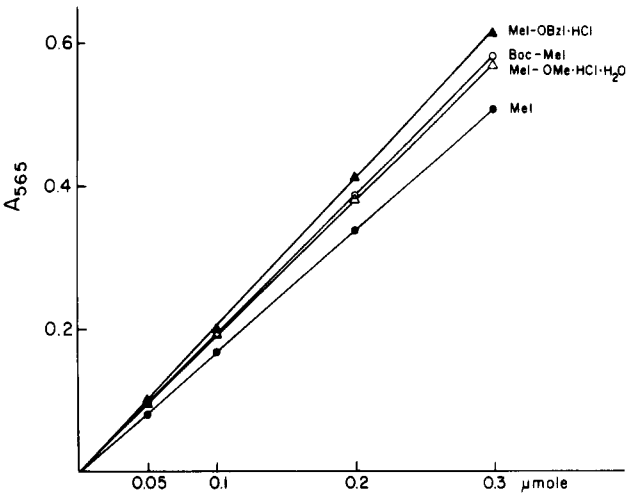

Figure 1. Determination of Mel, Mel-OMe·HCl·H_2O (1), Mel-OBzl·HCl (2), and Boc-Mel (3) with 4-(*p*-nitrobenzyl)pyridine.

Although storage of melphalan compounds in Me_2SO considerably improved their stability, the Klatt procedure[43] was still unsatisfactory in that duplicate samples often exhibited variations in their color yields. When the procedure was further modified by dissolving 4-(*p*-nitrobenzyl)pyridine in a mixture of Me_2SO, aqueous AcOH, and 2-butanone, thus permitting chromogenic reaction with the nitrogen mustard compounds in a homogeneous rather than a two-phase system, the reproducibility of the assay was significantly improved, and the resultant standard curves are shown in Figure 1.

When we used the modified procedure, [Ac-Asn[1],Mel[8]]AII was shown to contain 62% of the theoretical amount of nitrogen mustard, whereas [Ac-Asn[1],des-Phe[8]]AII lacking melphalan was indistinguishable from the reagent blank. This value is in reasonable agreement with the peptide content (75%) found in the same sample, which was shown to contain minor decomposition products by TLC.

TLC Detection of Melphalan and Melphalan-Containing Peptides. Since thin-layer chromatography (TLC) is often used to monitor the progress of chemical reactions and to assess the purity of various compounds, combination of its high resolution power with the unusual sensitivity of the colorimetric assay can, in theory, distinguish closely related melphalan derivatives in microgram quantities. We tested this possibility by applying the colorimetric procedure to TLC and observed that melphalan derivatives were detectable at the 1–5 μg level, a sensitivity equal to those of the Pauly (1 μg of histidine) and chlorine–toluidine (10 μg) sprays for peptides.[45] Conjunctive application of fluorescamine–melphalan sprays with the Pauly and chlorine–toluidine sprays on different sections of a TLC plate, containing both simple and complex melphalan derivatives developed in an appropriate solvent, enabled sensitive and selective detection of the amino- and melphalane-containing compounds from the mixture of tyrosine- and histidine-bearing peptides.

Biological activity for angiotensin II analogues containing melphalan was evaluated in the oxytocic and pressor assays. In isolated rat uterine strips, [Ac-

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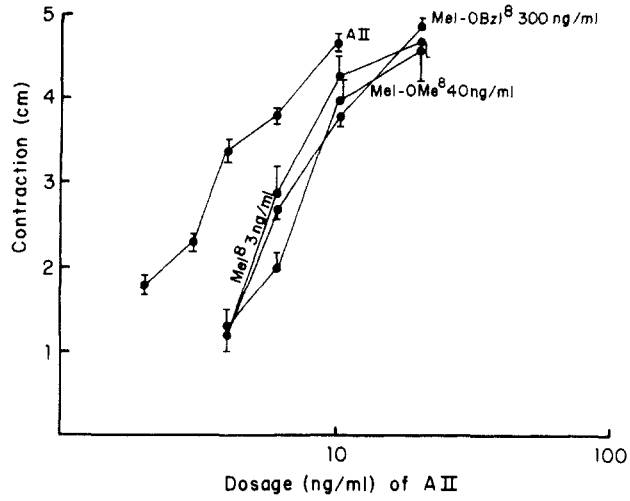

Figure 2. Inhibition of [Asn^1]angiotensin II induced rat uterine contraction by analogues containing melphalan in position 8: [Ac-Asn^1,Mel^8]AII, [Ac-Asn^1,Mel-OMe^8]AII, and [Ac-Asn^1,Mel-OBzl^8]AII.

Asn^1,Mel^8]AII antagonized the effect of angiotensin II (Figure 2) in a dose-dependent manner, with a pA_2 of 8.50 ± 0.11. Exposure of the tissue preparations to a large dosage (20 ng/mL) of this analogue, followed by thorough washing (4 times), reduced the tissue responsiveness toward AII, although maximal contraction could still be elicited. Such reduced sensitivity lasted for 2–3 h, a duration longer than that reported for tissue recovery from tachyphylaxis (30 min).[12] Thus, the melphalan analogue appeared to inhibit angiotensin II reversibly but with a prolonged effect similar to that of an irreversible inhibitor. Similarly, [Ac-Asn^1,Mel-OMe^8]AII and [Ac-Asn^1,Mel-OBzl^8]AII inhibited the oxytocic effect of AII with the respective antagonistic potencies of about one-tenth and one-hundredth of that for [Ac-Asn^1,Mel^8]AII.

In pentobarbital-anesthesized, phenoxybenzamine- and propanolol-treated rats, bolus injection of [Ac-Asn^1,Mel^8]AII inhibited the pressor response toward AII (data not shown). However, this inhibition was both reversible and transient, indicating possible rapid degradation of the inhibitor, as well as its lack of irreversible inhibition.

Conclusion

Our results with the physicochemical analyses of various melphalan derivatives suggested that the colorimetric assay using 4-(*p*-nitrobenzyl)pyridine could be a useful tool for qualitative or quantitative assessment of microgram levels of the nitrogen mustard moiety in peptides. Furthermore, certain aqueous solvents, as well as alkaline or nucleophilic environments encountered in peptide synthesis, purification, or bioassay, were shown detrimental to the alkylating group.

Because a major application of the affinity labels is to probe receptoral conformation and topography in addition to their potential for selective and irreversible receptor blockade, it is important to demonstrate the integrity of the alkylating group in the probe prior to its biological analyses. By using the colorimetric assay in conjunction with TLC, the presence of the nitrogen mustard group in [Ac-Asn^1,Mel^8]AII was established, yet results of the oxytocic and the pressor assays clearly indicated that this analogue did not alkylate the receptor. This finding, supplementing the previous proposal that the receptor sites essential for biological activity are primarily of a hydrophobic and aromatic nature,[21] further suggests the absence in these sites of an appropriate nucleophile adjacent to the alkylating nitrogen mustard. However, [Ac-Asn^1,Mel^8]AII appeared to inhibit angiotensin II on a one to one basis in rat uterine preparations, suggesting high affinity of the inhibitor to the receptor with slow dissociation, a finding which was not observed in vivo. Similarly, Park et al. reported insignificant agonistic activity (0.3%) for [Ile^5,Mel^8]AII in vivo,[25] while Paiva et al. reported irreversible inhibition in vitro but not in vivo for [chlorambucil^1]AII.[11] Since plasma is abundant in proteolytic enzymes, the significantly reduced potency of [Ac-Asn^1,Mel^8]AII in vivo suggests that the affinity-labeling approach alone may be insufficient to produce long-acting peptide inhibitors and that combination of proteolytic resistance with affinity labeling may be necessary for the development of therapeutically useful peptides.

Experimental Section

All chemicals were of reagent grade. N^{α}-(*tert*-Butyloxycarbonyl)-substituted L-α-amino acids were supplied by Bachem, Inc., Torrance, CA. Melphalan was a generous gift from Burroughs Wellcome Co., Research Triangle Park, NC. Anhydrous HCl in dioxane (4 N) was obtained from Pierce Chemical Co., Rockford, IL. 1-Acetylimidazole and 2-butanone were obtained from Aldrich Chemical Co., Milwaukee, WI, and 4-(*p*-nitrobenzyl)pyridine was from Sigma Chemical Co., St. Louis, MO. Silica gel (CC-7, neutral, 100–200 μm) for dry column chromatography of melphalan-containing compounds was supplied by Mallinckrodt, MO, and was washed with the appropriate solvents and dried in vacuo prior to column packing. Melting points (Thomas-Hoover Uni-melt) are uncorrected. Microanalyses were performed by the Robertson Laboratory, Florham Park, NJ. Proton magnetic resonance spectra in deuterated water using sodium 3-(trimethylsilyl)-proprionate-*2,2,3,3-d*$_4$ as internal reference or in deuterated chloroform containing tetramethylsilane were obtained on a Varian Associates T-60 (60 MHz). Absorbance measurements were obtained on a Gilford Model 240 spectrophotometer. Homogeneity of melphalan derivatives and of the synthetic peptides was assessed by thin-layer chromatography (TLC) on Merck precoated silica gel glass plates (Type G60-F254) in different solvent systems: I, chloroform–methanol (9:1); II, 1-butanol–acetic acid–water (4:1:5, upper phase); III, 1-butanol–pyridine–acetic acid–water (8:1:2:9, upper phase); IV, *sec*-butyl alcohol–3% NH_4OH (100:44). The products were identified by a combination of UV, fluorescamine, chlorox-toludine, and Pauly sprays, and by the melphalan spray as described under Experimental Section. For melphalan content analysis, stock solutions of the melphalan-containing peptides (10 μmol/mL of Me_2SO) were used for the colorimtric assay and for obtaining their peptide contents by hydrolyzing samples containing norleucine as an internal standard in 6 N HCl containing 0.2% phenol at 110 °C for 24 h in sealed tubes. Amino acid analyses were performed on a Beckman Model 119 analyzer equipped with a Beckman system AA computing integrator.

Melphalan Methyl Ester Hydrochloride Salt Monohydrate (Mel-OMe·HCl·H_2O; 1). Melphalan (5 g, 14.7 mmol) was suspended in 50 mL of methanol, and the suspension was saturated with anhydrous HCl for 2 h at room temperature, followed by evaporation to dryness. The residue was washed with anhydrous ether, and dissolved in CH_3OH. The methanolic solution was applied immediately on a vaccum-packed, dry column (2 × 100 cm) of silica gel and eluted with a mixture of 9:1 $CHCl_3/CH_3OH$. The eluates were analyzed by TLC, and the appropriate fractions were combined and evaporated to dryness. The residue was crystallized from CH_3OH–$CHCl_3$–Et_2O to give 2.9 g (55% yield) of 1: TLC R_f (I) 0.41, R_f (II) 0.46, R_f (III) 0.53, R_f (IV) 0.64; mp 165.5–166.5 °C; ^{1}H NMR (^2H_2O) δ 3.35 (d, 2 H, J = 7 Hz, C_β H_2), 3.60 (t, 4 H, J = 6 Hz, 2 CH_2N), 3.80 (s, 3 H, $COOCH_3$), 4.10 (t, 4 H, J = 6 Hz, 2 CH_2Cl), 4.43 (t, 1 H, J = 7 Hz, C_αH), 7.53 (s, 4 H, C_6H_4). ^{1}H NMR for the reference melphalan in ^2H_2O–^{2}HCl showed resonance bands at δ 3.35 (d, 2 H, J = 7 Hz, C_β H_2) 3.68 (t, 4 H, J = 5 Hz, 2 CH_2N), 4.03 (t, 4 H, J = 5 Hz, 2 CH_2Cl), 4.37 (t, 1 H, J = 7 Hz, C_α H), 7.48 (s, 4 H, C_6H_4). Anal. ($C_{14}H_{23}N_2O_2Cl_3$) C, H, N; Cl: Calcd, 28.4; found, 27.8.

Preliminary attempts to purify this compound by methods other than dry column chromatography, such as acid–base extraction or countercurrent distribution (4:1:5, 1-butanol–acetic acid–water), produced heterogeneous material.

Melphalan benzyl ester hydrochloride salt (Mel-OBzl·HCl; 2) was prepared from melphalan (5 g, 14.7 mmol) and benzyl alcohol (50 mL, 450 mmol) and purified by dry column chromatography on silica gel as described for 1. The product was recrystallized from methanol–2-propanol–chloroform–ether to give 2.4 g (38% yield) of 2: TLC R_f (I) 0.55, R_f (II) 0.57, R_f (III) 0.68, R_f (IV) 0.72; mp 209–210 °C. Anal. ($C_{20}H_{25}N_2O_2Cl_3$) C, H, N, Cl.

Coumpound 2 was only sparingly soluble in ^2H_2O or in 1:1 deuterated water–methanol. No meaningful NMR analysis was obtained.

N^{α}-(*tert*-Butyloxycarbonyl)melphalan (Boc-Mel; 3). Melphalan (5 g, 14.7 mmol), *tert*-butyoxycarbonyl azide (2.6 g, 18 mmol), and triethylamine (9 mL, 65 mmol) were stirred in 60 mL of acetonitrile at room temperature for 3 days. The mixture was evaporated to dryness, dissolved in ether, and washed with 1 N HCl, H_2O, and dried (Na_2SO_4). The ether solution was evaporated to dryness, and the residue was chromatographed on a dry column (2 × 50 cm) of silica gel as described for 1. Evaporation of the appropriate eluate fractions gave 5.12 g (86% yield) of an oil, which solidified upon prolonged freezing: TLC R_f (I) 0.31, R_f (II) 0.80, R_f (III) 0.84, R_f (IV) 0.36; ^1H NMR ($CDCl_3$) δ 1.4 (s, 9 H, C_4H_9OCO), 3.02 (d, 2 H J = 6 Hz, $C_\beta H_2$), 3.60 [s, 8 H, $N(CH_2CH_2Cl)_2$], 4.43 (1 H, $C_\alpha H$), 4.88 (1 H, $N_\alpha H$), 6.48 and 7.02 (2 d, 4 H, J = 9 Hz, C_6H_4), 10.30 (s, 1 H, COOH).

Neutralization of a sample of 3 in ether with dicyclohexylamine in petroleum ether (bp 30–60 °C) produced a crystalline precipitate, which was recrystallized from ether–petroleum ether to give N^{α}-(*tert*-butyloxycarbonyl)melphalan dicyclohexylammonium salt (4), mp 134–136 °C; the ^1H NMR ($CDCl_3$) spectrum of 4 was identical with that for 3, except for the absence of a COOH resonance and the presence of bands for the dicyclohexylammonium salt. Anal. ($C_{30}H_{49}N_3O_4Cl_2$) C, H, N, Cl.

Ac-Asn-Arg-Val-Tyr-Val-His-Pro (5) was prepared by Merrifield's stepwise solid-phase method.[46] Boc-Pro was attached to the chloromethylated polymer of polystyrene–1% divinylbenzene through the cesium salt procedure.[47] F_3AcOH/CH_2Cl_2 deprotection of Boc-Pro-resin (8 g, 4 mmol), followed by neutralization and sequential incorporation of Boc-His(Tos) with HOBT, Boc-Val, Boc-Tyr(Bzl), Boc-Val, Boc-Arg(Tos), and Ac-Asn with HOBT, by dicyclohexylcarbodiimide gave the fully protected peptide-resin. Cleavage of the peptide from the resin by HBr/F_3AcOH, followed by HF/anisole treatment of the peptide, gave 3.04 g of product.

A portion (2.7. g) of this was purified by countercurrent distribution in 1-butanol–pyridine–acetic acid–water (8:1:2:9) for 1200 transfers, and the appropriate fractions were combined to give 900 mg (36% yield) of 5: TLC R_f (II) 0.15, R_f (III) 0.16. An acid hydrolysate had the following amino acid ratios: Asp, 1.11; Arg, 0.98; Val, 2.02; Tyr, 0.93; His, 1.01; Pro, 0.92.

Ac-Asn-Arg-Val-Tyr-Val-His-Pro-Mel-OMe (6). Mel-OMe·HCl·H_2O (36 mg, 100 μmol), the heptapeptide 5 (50 mg, 50 μmol), and hydroxybenzotriazole monohydrate (9 mg, 60 μmol) were dissolved in 5 mL of dimethylformamide, and dicyclohexylcarbodiimide (12 mg, 60 μmol) was added. The mixture was stirred at 60 °C for 20 h and evaporated to dryness in vacuo. The residue was washed with CH_2Cl_2, EtOAc, and Et_2O and chromatographed on a dry column (1 × 12 cm) of silica gel. The peptide was eluted with the organic phase of the solvent mixture of 8:1:2:9 of 1-butanol–pyridine–acetic acid–water, and the eluate was analyzed by TLC. The appropriate fractions were combined, evaporated to dryness, and lyophilized from aqueous dioxane to give 25 mg (40% yield) of 6 containing traces of contaminants: TLC R_f (II) 0.27, R_f (III) 0.36. Melphalan analysis of a sample indicated the presence of 32% of reactive nitrogen mustard according to the standard curve for Mel-OMe·HCl·H_2O. An acid hydrolysate had the following amino acid ratios: Asp, 1.07; Arg, 0.98; Val, 2.00; Tyr, 1.02; His, 0.95; Pro, 0.93; peptide content 34%. Melphalan subjected to either acid hydrolysis or NH_4OH treatment did not elute from the column during regular amino acid analysis. Based on the observed melphalan content and peptide content, the ratio of melphalan present in the peptide was 0.32/0.34 or 0.94.

Ac-Asn-Arg-Val-Tyr-Val-His-Pro-Mel-OBzl (7). Mel-OBzl·HCl (216 mg, 0.5 mmol), the heptapeptide 5 (200 mg, 0.2 mmol), and hydroxybenzotriazole monohydrate (38 mg, 0.24 mmol) were dissolved in 10 mL of dimethylformamide, and dicyclohexylcarbodiimide (102 mg, 0.25 mmol) was added. The mixture was stirred at 60 °C for 7 h and evaporated to dryness in vacuo. The residue was washed with CH_2Cl_2, EtOAc, and Et_2O and chromatographed on a dry column (1 × 12 cm) of silica gel as described for 6. The appropriate eluate fractions were combined and evaporated to dryness, and the residue was precipitated from DMF–Et_2O to give 235 mg (82% yield) of 7 containing traces of contaminants: TLC R_f (II) 0.27, R_f (III) 0.38. Melphalan analysis of a sample indicated the presence of 70% of reactive nitrogen mustard according to the standard curve for Mel-OBzl·HCl. An acid hydrolysate had the following amino acid ratios: Asp, 1.06; Arg, 0.98; Val, 0.99; Tyr, 1.04; Val, 0.99; His, 0.95; Pro, 0.95; peptide content 81%; with a calculated ratio of 0.86 for melphalan.

Ac-Asn-Arg-Val-Tyr-Val-His-Pro-Mel (8) was prepared by hydrogenolysis of a suspension of 220 mg of 7 and 85 mg of 10% Pd/C in 5 mL of DMF at 1 atm of H_2 for 1.5 h. The suspension including the Pd/C catalyst was evaporated to dryness in vacuo, and the residue was immediately chromatographed on a dry column (1 × 12 cm) of silica gel as described for 6. The appropriate fractions were combined and evaporated to dryness, and the residue was precipitated from DMF-Et_2O to give 142 mg (51% yield) of 8 containing traces of contaminants: TLC R_f (II) 0.23, R_f (III) 0.27. Melphalan analysis of a sample indicated the presence of 62% of reactive nitrogen mustard according to the standard curve for Boc-Mel. An acid hydrolysate had the following amino acid ratios: Asp, 1.03; Val, 0.98; Tyr, 1.02; Val, 0.98; His, 0.98; Pro, 0.99; peptide content 75%; with a calculated ratio of 0.83 for melphalan.

Chloride and Chlorine Analyses by Volhard Titration. Method A. Approximately 10- to 20-mg samples were dissolved in 1 mL of HNO_3 and sequentially added 10 mL of 0.02 N $AgNO_3$ and 5 drops of $FeNH_4(SO_4)_2 \cdot 12H_2O$ indicator solution, and the mixtures were titrated with 0.02 N KSCN until a reddish-brown color appeared. The amount of ionic chloride present in the samples could be evaluated from the amount of $AgNO_3$ consumed, which was the difference between the milliequivalents of KSCN used and the milliequivalents of $AgNO_3$ added.

Method B was identical with method A, except that samples were first treated with 0.5 mL of 10 N NaOH and 0.5 mL of glycerin at 130 °C for 20 min to release covalently bound chlorine from the nitrogen mustard group. The samples were subsequently neutralized with 3 mL of AcOH and 1 mL of HNO_3 and titrated as above.

Calibration curves for Mel, Mel-OMe·HCl·H_2O, Mel-OBzl·HCl, and Boc-Mel were established for standard solutions containing, respectively, 0.1, 0.2, 0.4, and 0.6 mM compounds in Me_2SO. The chromogenic reagent was prepared by mixing 12 mL of 5% 4-(*p*-nitrobenzyl)pyridine in 2-butanone with the mixture of 30 mL of Me_2SO and 50 mL of 0.2% AcOH. For the colorimetric assay, samples (0.5 mL) were vortexed with the chromogenic reagent (4 mL) in capped test tubes, which were then heated at 100 °C for 30 min and cooled by centrifugation at 4 °C for 15 min. Mixing of this solution with an equal volume (e.g., 1 mL each) of 50% triethylamine in ethanol–2-propanol (2:1:1, v/v) produced a purple solution, the absorbance of which was read immediately at 565 nm. For a reagent blank, 0.5 mL of Me_2SO was reacted with the chromogenic reagent as above.

This modified procedure gave highly reproducible results (within ±3% variation) not only among duplicate samples but also between different experiments, probably because errors produced by multiple pipettings of the original procedure[43] were significantly reduced by dispensing all reagents in a single solution and through a single dispension. In addition, the resultant solution permitted subsequent chromogenic reaction to proceed homogeneously to a steady state under a more vigorous condition than that of the reported procedure[43] (100 °C for 30 min vs. 80 °C for 15 min).

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Sensitivity of melphalan spray was evaluated by applying, respectively, 5-, 1-, and 0.5-μg samples of melphalan and ester derivatives on TLC plates, which were developed immediately and dried with cold air. Spraying of the plate with 5% 4-(*p*-nitrobenzyl)pyridine in 2-butanone, followed by heating at 110 °C for 5 min, and spraying with either 1 N NaOH or 50% Et_3N in acetone produced an intense but rapid-fading blue color. This spray can be used sequential to fluorescamine identification of primary amines but is not compatible with either of ninhydrin, chlorox–toluidine, or Pauly reagents. The detection limits (in micrograms of melphalan) for these sprays were 0.5 for fluorescamine, 1 for ninhydrin, and 5 for melphalan.

Bioassays for angiotensin II and analogues were performed on anesthetized rats (Zivic-Miller) and on uterine preprations (albino Wistar rats) suspended in de Jalon's solution (calcium 0.5 mM, pH 7.4) according to reported procedures,[48,49] with 1 g of contractile tension in the oxytocic assay equivalent to 3 cm on the recorder scale.

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Synthesis and Antibacterial Activity of Some Esters, Amides, and Hydrazides of 3-Carboxyrifamycin S. Relationship between Structure and Activity of Ansamycins

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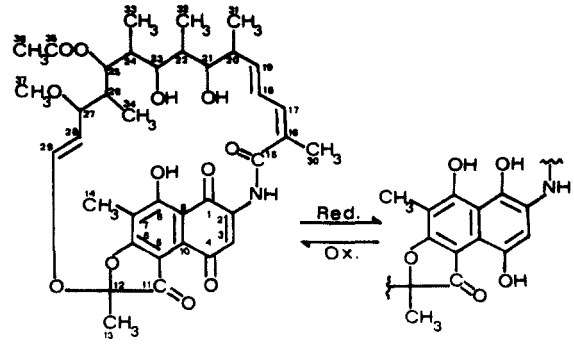
Esters, amides, and hydrazides of 3-carboxyrifamycin S were synthesized by oxidizing the cyanohydrin of 3-formylrifamycin SV to 3-(cyanocarbonyl)rifamycin S, followed by treatment with alcohols, amines and hydrazines. The in vitro microbiological activity of the derivatives was quite low, especially toward Gram-negative bacteria. This poor activity was not shown to be due to the inadequate inhibiting action on the bacterial DNA-dependent RNA polymerase but to the poor penetration of the compounds through the bacterial cell wall. The microbiological activity was correlated to the chemical properties of the substituent on C_3.

It is known that rifamycins S and SV (**1a,b**) form a very



rifamycin S (1a) rifamycin SV (1b)

stable complex with the bacterial DNA-dependent RNA polymerase (RNAP). The formation of this complex prevents the transcription process in bacterial cells.[1,2]

From structure–activity relationship studies carried out on rifamycins and related compounds,[3,4] the hypothesis was advanced that the essential requirements of the antibiotic to form the RNAP–antibiotic complex are the presence of at least a naphthalenic nucleus with a hydroxyl group on C_8 and an oxygen atom in C_1, either in the quinone or hydroquinone form, and two free hydroxyl groups on C_{21} and C_{23}. Moreover, the steric characteristics of the ansa chain must be such that the two hydroxyls on C_{21} and C_{23} can take a specific orientation with respect to the naphthalenic nucleus.[3]

To test this hypothesis, we tried to relate the differences of in vitro microbiological activity in a series of 3-substituted derivatives of rifamycin S and related compounds[5] to the influence on the ansa conformation of the steric characteristics of the substituents.

We suggest that the substituents introduced in position 3 may influence the ansa conformation and, consequently, the stability of the RNAP–antibiotic complex.

Dampier and Whitlock,[6] on the other hand, examined the relationship between the activity of a series of 3-substituted rifamycin S derivatives on RNAP isolated from *Escherichia coli* and the electronic nature of the substituents. They concluded that the activity is increased by electron-withdrawing substituents and decreased by electron-donating substituents. This conclusion is in agreement with the hypothesis that the formation of the complex between RNAP and the aromatic nucleus involves a donor–acceptor π complex.

Assuming the conformational stabilization produced by the substituents at position 3 is due to a direct interaction between this substituent and the amide group on C_2 of the ansamycin,[5,7] we synthesized a series of esters, amides, and

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