Ames, Berkeley, CA, while strains TA98NR and TA100NR were obtained from H. S. Rosenkranz, Cleveland, OH. CHO-K_1-BH_4 cells were provided by A. W. Hsie, Oak Ridge, TN.

Appendix

Elemental Analytical Data. 1-Nitrobenzo[*a*]pyrene (1). Anal. Calcd for $C_{20}H_{11}NO_2$: C, 80.80; H, 3.73; N, 4.71. Found: C, 80.69; H, 3.80; N, 4.80. **3-Nitrobenzo[*a*]pyrene (2).** Anal. Calcd for $C_{20}H_{11}NO_2$: C, 80.80; H, 3.73; N, 4.71. Found: C, 80.59; H, 3.60; N, 4.63.

Registry No. 1, 70021-99-7; 2, 70021-98-6; 3, 63041-90-7; 4, 17750-93-5; 5, 88598-56-5; 6, 88598-57-6; 7, 88598-58-7; benzo[*a*]pyrene, 50-32-8.

Potential Antitumor Agents: Synthesis and Biological Properties of Aliphatic Amino Acid 9-Hydroxyellipticinium Derivatives

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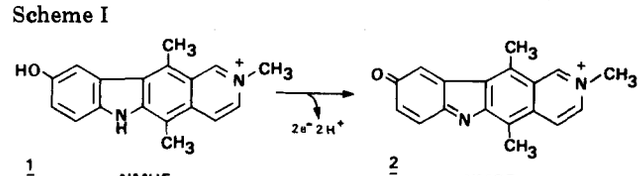
Aliphatic amino acids glycine, alanine, valine, and leucine were conjugated to the antitumor drug N^2-methyl-9-hydroxyellipticinium (NMHE) through a peroxidase-catalyzed oxidation reaction. NMR studies of the adducts so obtained have indicated (i) that the amino acids were linked to NMHE between the nitrogen of their primary amine and the C-10 position of the ellipticine ring and (ii) that a double bond was present between the nitrogen and the α-carbon of the amino acid moiety. All amino acid–NMHE adducts exhibit a higher *lipophilic* property than the parent compound (NMHE) directly correlated with the length of the aliphatic chain of the amino acids. The adducts interact with DNA through an intercalating process with apparent binding constant ranging from 2 $\times 10^5$ to 5 $\times 10^5$ M^{-1} at pH 7.40. The presence of the amino acid moiety linked to NMHE results (i) in a slight decrease of the cytotoxicity on L1210 cells in vitro (ID_{50} ranged from 0.20 to 0.50 μM) as compared to NMHE (ID_{50} = 0.05 μM), (ii) in a decrease of the antitumor efficiency in vivo against L1210 leukemia for leucine–NMHE and valine–NMHE (ILS at $LD_0/2$ = 35% and 31%, respectively), (iii) in a suppression of the antitumor activity for alanine–NMHE and glycine–NMHE (ILS < 25%), (iv) in a strong increase in the bacteriostatic activity on the quaternary ammonium sensitive *Escherichia coli* BL101 strain and on *Salmonella typhimurium* TA98 strain. The bacteriostatic effect is directly correlated with the lipophilic property of the drugs. These finding are discussed in terms of a structure–activity relationship.

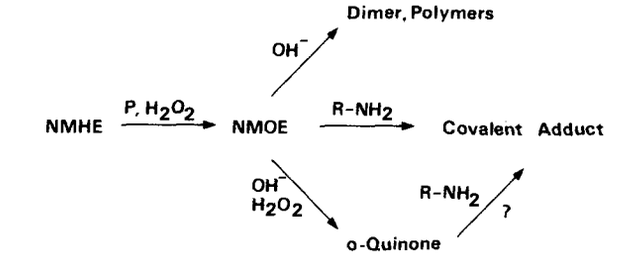
N^2-Methyl-9-hydroxyellipticinium (NMHE) (see Scheme I) exhibits a high cytotoxic activity against various experimental tumor cells[1] and is actually used in the treatment of osteolytic breast cancer metastases.[2] In the series of ellipticines, previous works have suggested that the limiting factor in the antitumor efficiency of these drugs was the transport through tumor cell membranes.[3,4] In connection with this finding, it must be noted that the clinically active drug NMHE is a charged, large, and hydrophilic compound exhibiting consequently the most unfavorable structure in terms of membrane transport.[5] A possible strategy suitable to increase the transport of such a molecule across cell membranes is the conjugation with lipophilic compounds of biological interest. Promising results have been obtained in this way with daunorubicine bound to leucine and leucine-containing dipeptides.[6,7] Taking advantage of the ability of the oxidized form of NMHE to readily undergo a nucleophilic addition with N donors,[8] we have prepared the homologous series of aliphatic amino acid–NMHE adducts, namely, glycine–, alanine–, valine–, and leucine–NMHE. The present article describes the preparation and the study of some physicochemical and pharmacological properties of these compounds.

Scheme I



1 NMHE 2 NMOE

Scheme II



Results

Reaction between N^2-Methyl-9-oxoellipticinium and Amino Acids. In aqueous medium, NMHE can be readily oxidized by an enzymatic system such as peroxidase–hydrogen peroxide (P–H_2O_2) to the corresponding quinone imine[9] N^2-methyl-9-oxoellipticinium (NMOE, compound **2** in Scheme I) as indicated in eq 1.

$$\text{NMHE} + H_2O_2 \xrightarrow{P} \text{NMOE} + 2H_2O \qquad (1)$$

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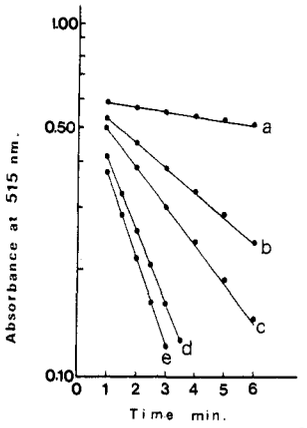


Figure 1. Kinetics of the reaction between N^2-methyl-9-oxoellipticinium (NMOE) and glycine. Experiments were performed at 26 °C in 0.05 M phosphate buffer (pH 7.40) containing 10^{-4} M NMHE, 10^{-4} M H_2O_2, and 10^{-8} M HRP. The oxidation reaction of NMHE to NMOE was started by the addition of HRP. After the complete oxidation of NMHE, increasing amounts of glycine were added as follows: (a) none, (b) 0.5 mM, (c) 1 mM, (d) 2 mM, (e) 3 mM. The change in absorbance was continuously recorded, and absorbance vs., time curves were converted to x and y coordinates and computed. The reaction was second order in nucleophile (glycine) and the observed second-order rate constant is 5.2 M^{-1} s^{-1} in the experimental conditions used.

The quinone NMOE appears to be a strong electrophilic molecule that may react with various nucleophiles, resulting in covalent binding.[8-10] As previously described for various quinone compounds,[11-12] such a reaction occurs with the primary amine of amino acids. Figure 1 indicates the kinetics of the reaction between glycine and NMOE determined by the decrease in absorbance of the quinone in the 500-nm range. The reaction exhibits second-order kinetics with a rate constant k = 5 M^{-1} s^{-1} at pH 7.40. HPLC experiments (data not shown) indicate that the addition of a P-H_2O_2 system in a phosphate buffer mixture (pH 7.40) containing NMHE and glycine results effectively in the formation of a more hydrophobic compound than NMHE and related to a glycine-NMHE adduct. Similar reactions are observed with other amino acids and related compounds. The formation of an amino acid-NMHE adduct competes with other possible reactions as shown in Scheme II (from ref 8-10).

In the presence of an excess of amino acid and near neutral pH, the formation of the amino acid-NMHE adducts is highly favored (yield from 55% to 75%). The adducts can be easily purified from the reaction mixture by the use of hydrophobic resin chromatography. The aliphatic amino acid-NMHE adducts (AA-NMHE) Gly-NMHE, Ala-NMHE, Val-NMHE, and Leu-NMHE have been prepared according to these procedures as described in the Experimental Section.

Identification of the AA-NMHE Adducts. AA-NMHE adducts have been identified by ^1H NMR and mass spectrometry. The common feature for all these adducts is the absence of hydrogen atom at the C-10 position, indicating that these compounds are produced

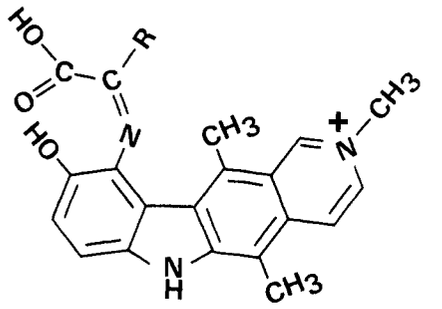
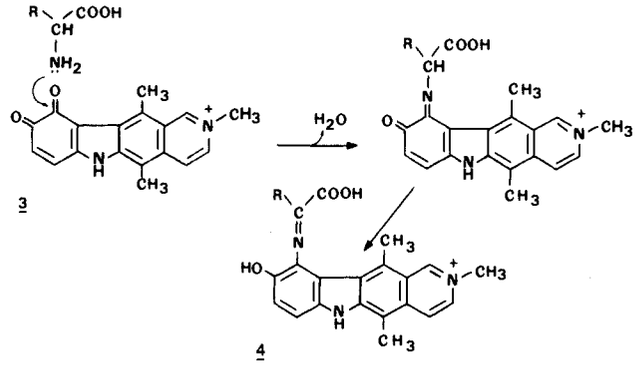


Figure 2. Structure of amino acid-NMHE derivatives: Gly-NMHE, R = H; Ala-NMHE, R = CH_3; Val-NMHE, R = $CH(CH_3)_2$; Leu-NMHE, R = $CH_2CH(CH_3)_2$.

Scheme III



from a nucleophilic addition of the amino acids at this position on the quinone imine NMOE. In all these adducts, the absence of the Cα-H of the amino acid moiety should be noted, clearly indicated by the presence of a singlet in the NMR spectrum at S = 2.41 for the methyl resonance of alanine, for example (only one Cα-H proton is observed for Gly-NMHE). This fact has been confirmed as well for the valine and leucine adducts by homonuclear decoupling experiments for Cβ and Cγ protons. In these compounds, the Cβ proton(s) appear(s) as a singlet when the C_γ protons are irradiated.

Mass data obtained by the desorption chemical ionization (DCI) method show a similar behavior for all these adducts. The highest observed peak always corresponds to the mass of the cation minus 46 mass units:Gly-NMHE (M cation = 348, observed 302), Ala-NMHE (362, 316), Val-NMHE (390, 344), and Leu-NMHE (404, 358). This difference can be explained by the release of carbon dioxide of the carboxylic function (-44) and the oxidation of the 9-hydroxy function to the corresponding quinone imine (-2). For all these adducts, the possible lactone structure resulting from the reaction between the OH group of NMHE and the acid function of the amino acid moiety is not observed. In such a benzoxazine structure, the infrared spectra should exhibit an absorption band near 1770 cm^{-1} corresponding to the lactone carbonyl. In the case of AA-NMHE adducts, a carbonyl band is present at 1670 cm^{-1} in agreement with the presence of a free carboxylic acid. From these spectroscopic data, the structure given in Figure 2 can be attributed for the amino acid-NMHE adducts.

It is known that near neutral pH, amines react with quinones either by nuclear substitution or by quinone imine formation.[11] Since the o-quinone 9,10-dioxo-NME is one of the secondary products of the peroxidase-catalyzed oxidation of NMHE,[10] these two mechanisms can be involved in the formation of AA-NMHE adducts. Scheme III shows the possible reaction between the primary amine of amino acids and the o-quinone 3 yielding a quinone

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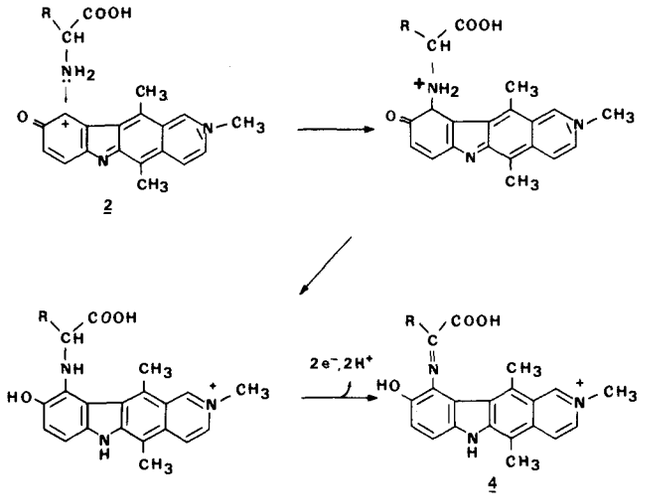
Table I. Pharmacological Properties and Interaction Parameters with DNA of NMHE, NME, and AA-NMHE Adducts

compd	$10^{-5}K_{app}$,[a] M^{-1}	slope[b]	ID_{50},[c] μM	LD_0,[d] mg/kg	ILS at $LD_0/2$,[e] %	ED_{50},[f] μM	
						BL101	TA98
NME	2.3	2.4	1.68	12.5	22		
NMHE	13.0	2.18	0.05	5	53	3.55	NT
Gly–NMHE	0.8	2.38	0.54	20	0	3.15	100
Ala–NMHE	4.7	2.71	0.20	20	23	1.95	50.3
Val–NMHE	2.1	2.31	0.31	15	31.5	0.18	1.65
Leu–NMHE	2.6	2.63	0.28	10	35	0.76	2.35

[a] Apparent binding constant (K_{app}) of the drugs for calf thymus DNA were measured by competition with ethidium bromide in 0.2 M Tris-HCl buffer (pH 7.40). Scatchard plots were calculated with a 9810 A Hewlett Packard calculator. [b] The slope indicated is that of the curve $\log \eta/\eta_0 = f \log (1 + 2r)$ measuring the length increase of sonicated calf thymus DNA in the presence of increasing amounts of drugs, determined by viscosimetry as indicated in the Experimental Section. [c] Dose that reduces by 50%, after 48 h, the L1210 cell growth in vitro as compared to controls. [d] Highest nonlethal dose (ip treatment). [e] Increase in life span over controls (10^5 L1210 cells): mouse by ip route, single injection 2 h after cell grafting. Significant antitumor effect for ILS > 25%. Results indicated for NME and NMHE were taken from ref 23. [f] ED_{50} indicates the dose reducing by 50% the bacteria growth rate as compared to controls. NT = nontoxic.

Scheme IV



imine that rearranges to the terminal product 4. In Scheme IV, NMOE undergoes a nucleophilic attack from the nitrogen of the primary amine of the amino acid at the C-10 position (Michael addition). The redistribution of electrons and protons yields the more stable aromatic structure. The highly oxidizable *o*-aminophenol moiety loses two electrons and two protons, yielding the stable product 4 likely via the formation of an *o*-quinone imine as transient intermediate. This latter mechanism seems to be very likely since near neutral pH, the *o*-quinone 9,10-dioxo-NME does not react with primary amines with an appreciable rate (data not shown).

Lipophilic Properties of AA–NMHE Adducts. The presence of the aliphatic chain in the structure of AA–NMHE adducts should increase the lipophilic property of compounds of this series as compared to NMHE. The HPLC retention index of the drugs on reverse-phase columns can be considered as the reflect of the lipophilic property and is closely related to the octanol/water partition coefficient.[13] As indicated on the chromatogram shown in Figure 3A, the retention time of each AA–NMHE adduct increases as expected with the length of the aliphatic chain of the linked amino acid. The capacity factor K_D of the drugs was determined from the observed retention time (t_R) by using the following equation:

$$K_D = \frac{t_R - t_0}{t_0}$$

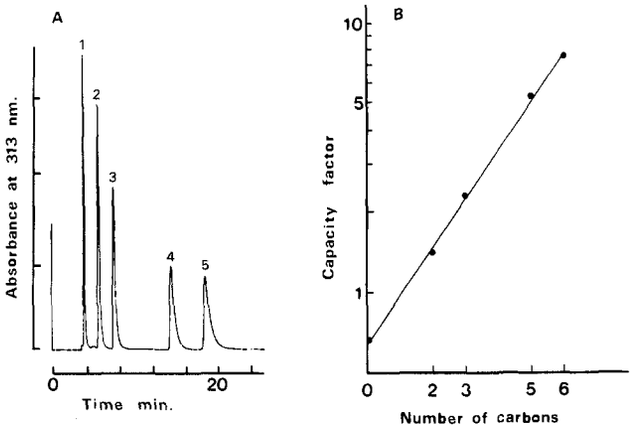


Figure 3. Lipophilic properties of NMHE and AA–NMHE. (A) HPLC of purified compounds: 1 = NMHE, 2 = Gly–NMHE, 3 = Ala–NMHE, 4 = Val–NMHE, 5 = Leu–NMHE. Mobile phase: methanol/water (70:30) containing 1 g of heptanesulfonate and 3 mL of acetic acid. Flow rate: 1.2 mL/min. (B) Capacity factor vs. the number of carbons of the linked amino acid. 0 = NMHE.

where t_0 is the retention time of the solvent. The values of K_D are directly correlated to the number of carbons of the amino acid as indicated in Figure 3B.

Interactions with DNA

It is known that NMHE interacts with DNA through an intercalating process[15] with an apparent binding constant of 1.3×10^6 M^{-1}. The presence of the amino acid moiety on the ellipticine ring should modify the interaction with DNA. DNA binding constants of the AA–NMHE derivatives have been determined by measuring their ability to compete with the binding of ethidium bromide as previously described.[17] Table I indicates the values of the different apparent binding constants of AA–NMHE as compared with other molecules in the ellipticine series. The presence of the amino acid on NMHE results in a tenfold decrease in the DNA binding constant. The binding constant value is slightly affected by the nature of the amino acid and does not increase with the hydrophobicity of the aliphatic chain. In order to determine whether the AA–NMHE were able to intercalate between DNA base pairs, we have used viscosimetry to determine the DNA length increase resulting from the intercalation process. Theoretical treatment shows that if $\log \eta/\eta_0$ is plotted vs. $\log (1 + 2r)$, where η and η_0 are the intrinsic viscosities of sonicated DNA in the absence and in the presence of the tested drug, and r is the number of drug molecules bound per nucleotide, a slope value near 2.2 is

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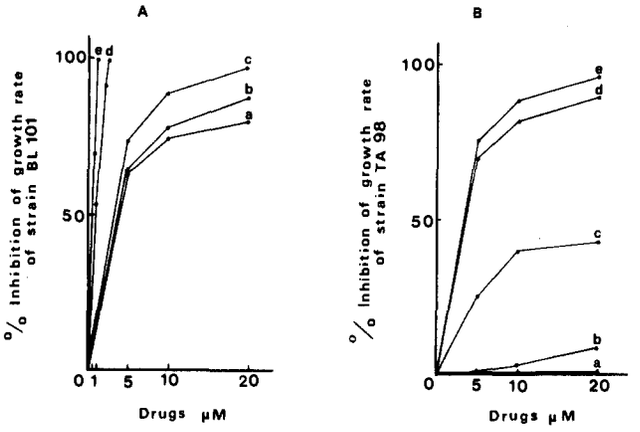

Figure 4. Effect of NMHE and AA–NMHE on the growth rate of *Escherichia coli* BL101 in A and *Salmonella typhimurium* TA98 in B. Fifty percent inhibition corresponds to a twofold increase in the generation time. Experiments were performed as described in Experimental Section. a = NMHE, b = Gly–NMHE, c = Ala–NMHE, d = Leu–NMHE, e = Val–NMHE.

expected for the monofunctionnal intercalating agents.[16]

Table I indicates the value of the slopes obtained with AA–NMHE as compared with those obtained with NME and NMHE. Both NME and NMHE are known to be perfect monofunctional intercalating agents. Accordingly, the value of the slopes obtained with these molecules is near 2.20. With AA–NMHE, the slopes obtained are significantly higher, suggesting that the viscosity increment results in these cases from at least two different causes: an increase in the length of DNA helix due to intercalation and an increase in the hydrodynamic volume due to outside binding. That was expected if one considers that only NMHE ring of AA–NMHE is intercalated inside the helix whereas the amino acid part remains on the outside.

Pharmacological Studies. (1) In Vitro Cytotoxicity on L1210 Leukemia Cells. Cytotoxicity toward L1210 tumor was determined as described in ref 23. Dose–effect relationships of the various compounds tested were determined from the regression line drawn as the percentage of cell growth inhibition plotted as a function of the logarithm of the dose. From these curves was estimated the dose of drug that reduces by 50% after 48 h the cell growth as compared to controls. The values of ID_{50} indicated in Table I show that the presence of the amino acid moiety on NMHE results in a decrease in the cytotoxicity. However, these values ranging from 0.20 to 0.50 μM indicate that AA–NMHE remain markedly cytotoxic against the L1210 cells especially as compared with the other quaternarized ellipticine NME (ID_{50} = 1.68 μM).

(2) Antitumor Activity against L1210 Leukemia. Antitumor properties of AA–NMHE adducts were determined in vivo on the L1210 leukemia as described in Experimental Section. Tests were performed at the highest nontoxic dose LD_0 and at $LD_0/2$. Results indicated in Table I show that Gly–NMHE and Ala–NMHE are devoid of any significant antitumor activity. In contrast Val–NMHE and Leu–NMHE were active at $LD_0/2$, but ILS did not exceed 40%. Moreover, at LD_0, these compounds exhibit a toxic effect on mice affected with leukemia. It must be noted that the two active compounds are those exhibiting the highest lipophilic property.

(3) Antibacterial Action. Antibacterial action of NMHE and AA–NMHE adducts was estimated by the inhibiting effect of the drugs on bacterial growth as indicated in the Experimental Section. Figure 4 shows the effect of graded concentrations of drugs on the growth rate of *Escherichia coli* BL 101 strain (Figure 4A) sensitive to quaternary ammonium compounds and on *Salmonella typhimurium* TA 98 strain (Figure 4B). From these data, the regression line drawn as the percentage of bacterial growth inhibition plotted against the logarithm of the dose allows the estimation of ED_{50}, which is the dose of drug reducing by 50% the bacterial growth rate as compared to controls. Table I summarizes the different values of ED_{50} obtained. These data show that the presence of amino acids on NMHE markedly increases the antibacterial action on the quaternary ammonium sensitive strain BL 101. The antibacterial efficiency is clearly correlated with the lipophilic property of the drugs, as measured by the capacity factor (r = –0.904). On TA 98 strain, NMHE is devoid of antibacterial activity, whereas AA-NMHE are active. The most efficient drugs are the most hydrophobic adducts Val–NMHE and Leu–NMHE.

Discussion

Conjugation of NMHE to aliphatic amino acids has been performed in hopes of obtaining ellipticine congeners exhibiting an increase in transport across cell membranes that may result in a concomitant increase in the cytotoxicity against tumor cells. This approach was supported by the following considerations: (i) amino acids are substances of biological interest known to be involved in facilitated diffusion processes, (ii) the presence of a lipid soluble moiety of aliphatic amino acids should increase the rate of passive diffusion of the adducts.[5] In fact, it must be pointed out that the nucleophilic addition of the primary amine of amino acids to the ellipticine ring yields covalent adducts that can be considered as the conjugation products between N^2-methyl-9-hydroxy-10-aminoellipticinium and saturated fatty acids. Moreover, the presence of a double bond between the nitrogen and the α-carbon of the amino acid destroys the optical center of the molecule. From a pharmacological point of view, AA–NMHE adducts exhibit the following properties as compared to the parent compound NMHE: a lower cytotoxicity on L1210 cells in vitro, a lower toxicity to mice, a lower antitumor efficiency for Val–NMHE and Leu–NMHE, and the disappearance of the antitumor activity for Ala–NMHE and Gly–NMHE. In contrast, the presence of the amino acid moiety linked to NMHE strongly increases the bacteriostatic effect on *E. coli* BL 101 and *S. typhimurium* TA 98. It is of interest to note that there is a significant correlation between the bacteriostatic effect of AA–NMHE and their lipophilic character. Moreover, Val–NMHE and Leu–NMHE, which exhibit a significant antitumor activity, are the most lipophilic compounds. These observations indicate that in the series of AA–NMHE, the hydrophobicity is a beneficial factor for the expression of the cytotoxicity of these drugs. Questions arise about the reason(s) for the decrease of the cytotoxic activity and antitumor efficiency of AA–NMHE against L1210 leukemia as compared to NMHE. It must be noted that in addition to the increase in the lipophilic properties, the presence of the amino acid moiety on NMHE results in major changes in two physicochemical properties possibly involved in the mechanism of action of NMHE: (i) a marked decrease of DNA binding constant and (ii) the inability of AA–NMHE to undergo the oxidative bioactivation (experimental data not shown) as described in ref 13. The nonhydroxylated N^2–methylellipticinium (NME), which exhibits a quite similar DNA affinity to AA–NMHE and which is not oxidizable, is endowed with a very low cyto-

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toxic efficiency against L1210 cells in vitro (ID_{50} = 1.68 μM) and is inactive against L1210 leukemia in vivo (ILS < 25%). The relative low DNA affinity of NME and its inability to be oxidized are due to the lack of the OH group at the C-9 position. The inability of AA–NMHE to be oxidized indicates the loss of the phenolic character of the NMHE moiety. That may result from the steric protection of the phenolic hydroxyl due to the presence of a bulky group in the C-10 position.

Conclusion

The presence of an hydrophobic side chain at the C-10 position of NMHE resulting from the conjugation between NMHE and aliphatic amino acids results in the following: (i) a strong increase in the bacteriostatic activity of the drug directly correlated with the hydrophobicity of the linked amino acid and (ii) a marked decrease in the cytotoxic activity and antitumor activity against L1210 leukemia related to the loss of the phenolic character of the NMHE moiety.

Therefore, attempts to link NMHE to amino acids or peptides remain an attractive approach but will have to be done in such a way that the phenolic character of the adducts remains unchanged. Conjugation of amino acids to NMHE at the C-1 position seems to be suitable for this purpose since amino-substituted ellipticines can be obtained by substitution of the 1-chloroellipticine derivatives with primary amines as described in ref 18.

Experimental Section

Absorption spectra as well as other spectrophotometric studies were performed on a Beckman Acta III spectrophotometer. Mass spectra were performed on a Riber 10-10 apparatus by using the DCI (NH_3) method. NMR spectra were performed on a Brucker WH90 (90 MHz) spectrometer in the FT mode. Chemical shifts are expressed in parts per million with Me_4Si as internal standard. HPLC was monitored on a Waters apparatus using a μBondapak C18 column.

Chemicals. Horseradish peroxidase (HRP) and amino acids were purchased from Sigma Chemical Co. (St. Louis, MO). Pronase was from Calbiochem (San Diego, CA). Ellipticine derivatives N^2-methyl-9-hydroxyellipticine (NMHE) and N^2-methylellipticine (NME) were synthesized by the SANOFI Co. (Sisteron, France).

Aliphatic amino acid–NMHE adducts were prepared as follows: 50 mg of NMHE and 250 mg of the amino acid were dissolved together in 50 mL of distilled water. After filtration, the solution was mixed with 50 mL of 0.01 M phosphate buffer (pH 7.40) and the solution maintained under continuous stirring at room temperature. A 2-mg sample of HRP was then added to the mixture. After the dissolution of HRP, 5 mL of 40 mM H_2O_2 solution was added dropwise. After the end of the reaction, the solution was allowed to stand in the dark for 30 min and then filtered. The filtered solution was applied on a Biobeads SM_2 hydrophobic column (3 × 50 cm) previously equilibrated with a 0.1 M acetate buffer (pH 5.40) containing 1% methanol.

Excess amino acid remaining in the solution was removed from the column by an extensive elution with the acetate buffer. The compounds absorbed on the column were eluted with 100% methanol. This methanolic solution was carefully evaporated at a temperature below 40 °C. The solid material so obtained was redissolved in the acetate buffer composed as indicated above. After filtration the acetate mixture was applied to a XAD2 column (2.5 × 30 cm) previously equilibrated with the same buffer. Various fractions were eluted from the column by a linear gradient of 0.1 M acetate buffer (pH 7.40) containing from 1% to 70% methanol. Amino acid–ellipticine adducts is the last fraction easily detectable by the yellow fluorescence (λ_{max} 525 nm). The mixture containing the pure adduct was evaporated at a temperature below 40 °C, and the solid material was redissolved in distilled water, filtered, and lyophilized.

Identification of Aliphatic Amino Acids–NHME Adducts. 10-[(Carboxymethylene)amino]-9-hydroxy-2-methylellipticinium acetate (salt) (Gly–NMHE): DCI mass spectrum (NH_3), 348 (M cation), 302; UV (H_2Oe λ 437 nm (ε 2550 M^{-1} cm^{-1}), 360 (3000), 300 (31 500), 243 (18 360); ^1H NMR (D_2O, CD_3OD, 50/50, v/v) δ 1.94 (s, 3 p, Ac, 2.19 (s, 3 p, Me_5), 2.82 (s, 3 p, Ml_1), 4.21 ns, 3 p, N^+–Me), 7.00 (d, 1 p, J = 8.5 H, H7 or H8), 7.48 (d, 1 p, H7 or H8), 7.k6 (d, 1 p, J = 7.2 Hz, H4), 7.68 (d, 1 p, H3), 8.34 (s, 1 p, Cα-H Gly), 8.84 (s, 1 p, H1). Anal. Calcd for $C_{22}H_{21}N_3O_5$): C, 64.86; H, 5.15; N, 10.31. Found: C, 62.54; H, 5.05; N, 9.17.

10-[(1-Carboxyethylidene)amino]-9-hydroxy-2-methylellipticinium acetate (salt) (Ala–NMHE): MS, 362 (M cation), 316; UV (H_2O) λ_{max} 440 nm (ε 2730 M^{-1} cm^{-1}), 360 (3000), 300 (31 050), 243 (18 350); ^1H NMR (D_2O, CD_3OD, 50/50, v/v) δ 1.95 (s, 3 p, OAc), 2.41 (s, 3 p, Me Ala), 2.54 (s, 3 p, Me_5), 3.35 (Me_{11} partially masked by the solvent), 4.08 (s, 3 p, N^+–Me), 6.67 (d, 1 p, J = 8.0 Hz, H7 or H8), 7.07 (d, 1 p, H7 or H8), 7.36 (d, 1 p, J = 7.0 Hz, H3 or H4), 7.64 (d, 1 p, H3 or H4, 8.53 (s, 1 p, H1). Anal. Calcd for $C_{23}H_{23}N_3O_5$: C, 65.55; H, 5.46; N, 9.97, Found: C, 62.92; N, 5.08; N, 8.61.

10-[(1-Carboxy-2-methylpropylidene)amino]-9-hydroxy-2-methylellipticinium acetate (salt) (Val-NMHE): MS, 390 (M cation), 344; UV (H_2O) λ_{max} 440 nm (ε 3150 M^{-1} cm^{-1}), 360 (3350), 300 (32 850), 243 (19 800); ^1H NMR (D_2O, CD_3OD, 50/50, v/v) δ 1.58 (d, 6 p, J = 6.0 Hz, 2 Me, Val), 1.94 (s, 3 p, OAc), 2.44 (s, 3 p, Me_5), 3.14 (s, 3 p, Me_{11}), 3.39 (m, 1 p, J = 6.0 Hz, Cβ-H Val), 4.37 (s, 3 p, N^+–Me, 7.17 (d, 1 p, J = 8.8 Hz, H7 or H8), 7.52 (d, H7 or H8), 7.89 (AB, 2 p, J = 7.0 Hz, H3 and H4, 9.12 (s, 1 p, H1). Anal. Calcd for $C_{25}H_{27}H_3O_5$: C, 66.81; H, 6.01; N, 9.35. Found: C, 64.92; H, 5.85; N, 8.71.

10-[(1-Carboxy-3-methylbutylidene)amino]-9-hydroxy-2-methylellipticinium acetate (salt) (Leu–NMHE): MS, 404 (M cation), 358; UV (H_2O) λ_{max} 440 nm (ε 3000 M^{-1} cm^{-1}), 340 (3000), 300 (32 400), 243 (18 750); ^1H NMR (D_2O, CD_3OD, 50/50, v/v) δ 1.15 (d, 6 p, J = 6.4 Hz, 2 Me Leu), 1.93 (s, 3 p, OAc), 2.37 (m, 1 p, Cγ-H Leu), 2.66 (s, 3 p, Me_5), 3.00 (d, 2 p, J = 6.7 Hz, Cβ-H2 Leu), 3.50 (s, 3 p, Me_{11}), 4.48 (s, 3 p, N^+–Me), 7.44 (d, 1 p, H7 or H8), 8.18 (br s, 2 p, H3 and H4), 9.46 (s, 1 p, H1). Anal. ($C_{26}H_{29}N_3O_5$) C, H, N.

Determination of the Affinity for DNA. DNA binding constants of the various compounds have been determined at pH 7.40 by measuring the ability to compete with the binding of ethidium bromide according to Le Pecq and Paoletti.[17]

Viscosimetric Studies. Viscosimetric measurements were performed at 25 °C in a semimicro dilution capillary viscosimeter with a suspended level (Cannon Instrument Co., State College, PA) mounted in a thermostated bath. Flow times were measured to ±0.1 ms by the combined use of photometric sensors connected to an electronic timer as described in ref 19.

Cytotoxicity and Antitumor Tests. Inhibition of cell growth was determined on L1210 lymphocytic leukemia cells as previously described. The inhibitory efficiency against cell multiplication is expressed in terms of ID_{50}, which represents the drug concentration that reduces the rate of multiplication by 50% as compared to control. The highest nonlethal dose (LD_0) was determined for each compound after a single injection into DBA/2 mice by an intraperitoneal route. The antitumor tests were performed on DBA/2 mice that had been inoculated with 10^5 L1210 cells and treated 24 h later by the same route. Antitumor efficiency is expressed in term of ILS (increase in life span) over controls: $(T - C)/C \times 100$.

Bacteriostatic Tests. Bacterial strains *E. coli* AB 1157 thr-1, leu-6, pro A_2, his-4, thi-1, $argE_3$, lacYi, $GalK_2$, $araI_4$, Xyl-5, mtl-1, tsx33, Str-A31, Sup37 were a gift of Dr. N. Otsuji, *E. coli* BL 101 was a selected strain from AB1157 for its sensitivity to ethidium bromide and ellipticinium derivatives[20] and was a gift of Dr. B. Lambert.[21] *Salmonella typhimurium* TA 98 his-D 3052, uvrB, rfa, R. was a gift of Dr. B. N. Ames.[22]

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For bacteriostatic tests, inocula from master plates were grown overnight at 37 °C and then diluted 1000-fold in the same medium. Cultures were grown at 37 °C in *Erlenmeyer* flasks on a rotary shaker, and the growth was follwed by measuring the turbidity at 650 nm. Drugs were added after 2 h of growth.

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Registry No. 1, 58337-34-1; 4 (R = H), 89020-24-6; 4 (R = H)·acetate, 89683-28-3; 4 (R = CH_3), 89683-31-8; 4 (R = CH_3)·acetate, 89683-32-9; 4 (R = $CH(CH_3)_2$), 89683-26-1; 4 (R = $CH(CH_3)_2$)·acetate, 89683-27-2; 4 (R = $CH_2CH(CH_3)_2$), 89702-38-5; 4 (R = $CH_2CH(CH_3)_2$)·acetate, 89702-39-6; Gly, 56-40-6; Ala, 56-41-7; Val, 72-18-4; Leu, 61-90-5.

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Synthesis and Kinetic Studies of Protease Substrates Containing the 1-Methyl-6-aminoquinolinium Ion as a Fluorogenic Leaving Group

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Several sensitive substrates for porcine pancreatic elastase, chymotrypsin, and trypsin were prepared that utilize the permanently charged, fluorogenic cation 1-methyl-6-aminoquinoline (MAQ^+) as the leaving group. Kinetic rates for the hydrolysis of substrates were determined fluorimetrically and compared with analogues having 6-aminoquinoline (6-AQ) as an uncharged leaving group. It was found that substrates containing the quaternized leaving group generally have a higher k_{cat}/K_m ratio. An exception to this trend was noted with a trypsin substrate, Bz-DL-Arg-MAQ^+. During the course of this investigation, several significant advantages of the MAQ^+ ion as a fluorogenic leaving group in protease substrates were found: (a) its appearance can be measured fluorimetrically using wavelengths of light that result in its *maximal* fluorescence, while under these conditions, the unhydrolyzed substrate is essentially nonfluorescent, (b) it confers a high degree of water solubility to hydrophobic peptides, thereby eliminating the need for organic cosolvents to dissolve substrates, and (c) quaternized substrates can be prepared readily and in good yield from the corresponding 6-(peptidylamido)quinolines. These positively charged synthetic fluorogenic substrates are, therefore, useful probes for investigating the steric and electronic properties of the active-site environment of proteolytic enzymes.

Proteolytic enzymes play a key role not only in the regulation of cellular protein turnover but also in the control of many other physiological functions. Examples of these are digestion, blood coagulation, fibrinolysis, inflammation, control of blood pressure, maturation of peptide hormones, ovulation, fertilization,[1] and morphogenic changes during development.[2] Uncontrolled destruction of intra- or extracellular proteins by proteases is associated with many pathological conditions, such as the breakdown of articular cartilage by elastase[3] and collagenase[4,5] during rheumatoid arthritis, the destruction of pulmonary elastin by elastase during emphysema,[6] and the activation of plasminogen during the invasion of healthy tissues by tumor cells.[7] The information concerning the catalytic and regulatory mechanisms of proteases is, therefore, crucial to the understanding of these pathophysiological states. Moreover, such information will be useful for a rational design of medicinal agents that will modulate or prevent unwanted proteolysis.

Synthetic chromogenic or fluorogenic substrates composed of peptidyl amides of aromatic amines have been widely used to detect and quantify proteases and to define their amino acid specificities.[8] Because of the exceptional sensitivity of fluorimetry, fluorogenic substrates are particularly ideal as probes of enzyme structure and mechanism. Three problems, however, are generally encountered that limit the use of these agents. First, the fluorescence spectra of the substrate and product often overlap to a significant degree, and it becomes necessary, therefore, to use wavelengths longer than the excitation maximum to excite the product in order to avoid high levels of background emission from the excess unhydrolyzed substrate. This compromise decreases the fluorescence of the product, thereby lowering the sensitivity of the assay. Second, the aromatic amines are usually hydrophobic and thus require organic cosolvents (e.g., dimethylformamide or dimethyl sulfoxide) to solubilize them. The presence of these solvents produces unpredictable inhibitory or stimulatory effects on the enzyme.[9,10] In addition, such solvents are inappropriate for the in situ assay of proteases in cell culture. Third, the currently available chromogenic or fluorogenic groups are not readily amenable to derivatization. In order to study the steric and electronic properties of the portion of the active site on the enzyme that binds the amino acids of the substrate extending toward the C terminus from the scissile bond (the leaving group subsite), a leaving group of the substrate that can readily be derivatized into a series of congeners is required. Such studies have so far received only minor attention.[11,12]

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