Table I. Per Cent of Optically Active Tripeptide Derivatives Obtained after Reaction between Oxazolone and Various Amino Acid Esters

Amino acid ester	Toluene	Chloroform	Ethyl acetate	Dioxane
Ethyl glycinate	78	66	38	14
Methyl DL-alaninate	52	32	11	0
Methyl α-aminoisobutyrate	0	0	0	0

is allowed to react with equimolar solutions of various amino acids esters in a number of solvents. The per cent retention of optical activity in the tripeptide product in each instance was obtained by direct comparisons with the optical rotations of the same tripeptides synthesized by the nonracemizing azide-coupling route.

The figures show that for any given solvent the least amount of racemization occurs when ethyl glycinate is the attacking nucleophile.[6] The ratio of nucleophilicity to basicity of a particular amino acid ester governs the amount of racemization observed. Our data indicate that for ethyl glycinate the nucleophilicity-to-basicity ratio is most favorable for retention of optical activity. Schnabel[7] prepared a partially racemized oxazolone from carbobenzoxyglycyl-L-phenylalanine and allowed it to react with ethyl glycinate. The product he obtained exhibited some optical activity.

In the case of methyl DL-alaninate the nucleophilicity-to-basicity ratio is less favorable and more racemization is observed than with ethyl glycinate. For methyl α-aminoisobutyrate, the ratio favors basicity because of steric hindrance. As a result, when this amino acid ester is allowed to react with the pure oxazolone, only complete racemization is encountered. Hence, in a specific peptide coupling procedure, if an optically pure product results when methyl α-aminoisobutyrate is used as the attacking agent, one could state with certainty that the technique is free from oxazolone formation, the major route for racemization during peptide synthesis.

As can be seen from Table I, the solvent plays an important role in the racemization process. Toluene, the least polar of the four solvents, gives the best retention results. Chloroform is also a good solvent, perhaps because it contains an acidic proton. Dioxane, the most basic of the solvents, gives the highest extent of racemization probably because it solvates the proton leaving the oxazolone ring.

Our findings do not appear to agree with those of Vaughan.[8] He found that in using the mixed anhydride coupling method, dioxane as a solvent led to less racemization than chloroform as a solvent. This difference in results could arise from the fact that in dioxane less oxazolone forms, or that Vaughan used amino acid ester hydrochlorides and tertiary amines, a combination known to give rise to the "chloride ion" effect[4] in chloroform. We employed the free amino acid esters in all cases.

In addition to the above findings, we also wish to present direct chemical evidence to explain why optically pure hydrazides are obtained when esters are allowed to react with hydrazine hydrate. Nowak and Siemion[9] reported that the noncrystalline 2-methyl-L-4-isobutyloxazolone reacts with hydrazine hydrate to give optically active products.

When we allowed oxazolone I to react with a huge excess of hydrazine hydrate in methanol, we obtained a product identical with the hydrazide made by the traditional method of allowing dipeptide ester to react with hydrazine hydrate in refluxing methanol. The hydrazide Z-Aib-L-Phe-$NHNH_2$,[10] obtained from the oxazolone reaction, had mp 51–56°, $[\alpha]^{25}D$ −33.8° (*c* 1.0, $CHCl_3$). *Anal.* Calcd for $C_{21}H_{26}N_4O_4$: C, 63.31; H, 6.53; N, 14.07. Found: C, 63.24; H, 6.61; N, 13.89.

We expected the dipeptide oxazolone to be much more susceptible to racemization than the corresponding methyl ester in the strongly basic medium. Since identical products were obtained by both methods, it is clear that even if the oxazolone formed from the ester, no racemization would be encountered. Thus this key displacement step in the azide method does not involve racemization.

In an analogous reaction, Czonka and Nicolet[11] observed that an amino acid in acetic anhydride solution reacts with ammonium thiocyanate to form an optically active thiohydantoin. They postulated that an oxazolone intermediate was involved. We have evidence that hydroxylamine reacts with oxazolone I to give an optically active hydroxamic acid.[12] These extraordinarily enhanced nucleophilicities are largely due to what Edwards[13] has called "the α effect" and are apparently characteristic of those nucleophiles containing unshared pairs of electrons on the atom attached to the nucleophilic center.

Acknowledgment. We gratefully acknowledge support of this research by a grant from the National Institutes of Health (AM 03868).

(6) We define the ability of an amino acid ester to ring open the oxazolone as its nucleophilicity and the capacity to racemize the oxazolone as its basicity.

(7) E. Schnabel, *Ann.*, **688**, 238 (1965).

(8) J. R. Vaughan, Jr., *J. Am. Chem. Soc.*, **74**, 6137 (1952).

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(10) The abbreviations Aib and Phe refer to aminoisobutyryl and phenylalanyl residues, respectively.

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(12) It is interesting to point out that anhydrous ammonia under conditions identical with the hydrazide reaction leads to almost complete racemization.

(13) J. O. Edwards and R. E. Pearson, *J. Am. Chem. Soc.*, 84, 16 (1962).

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Plant Antitumor Agents. I. The Isolation and Structure of Camptothecin, a Novel Alkaloidal Leukemia and Tumor Inhibitor from *Camptotheca acuminata*[1,2]

Sir:

Camptothecin (I), an alkaloid with a novel ring system exhibiting potent antileukemic and antitumor activities in animals, has been isolated from the tree *Camptotheca acuminata*,[3] *Nyssaceae*. The stem wood

(1) This investigation was conducted under Contract SA-43-ph-4322, Cancer Chemotherapy National Service Center, National Cancer Institute, National Institutes of Health.

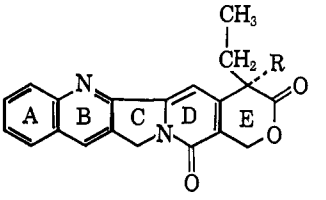
(2) X-Ray investigations at the University of Illinois were supported under Grant GB2878, National Science Foundation.

(3) The only previous chemical examination of *C. acuminata* is

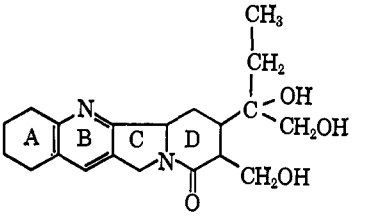
was extracted by our standard method[4] using leukemia L1210 assay.[5] Silica gel chromatography of the methanol-insoluble material from the chloroform extract followed by crystallization from methanol–acetonitrile gave camptothecin as pale yellow needles:[6] M^+ at *m/e* 348.1117, calcd for $C_{20}H_{16}N_2O_4$, 348.1111; mp 264–267° dec;[7] $[\alpha]^{25}D$ +31.3° ($CHCl_3$–MeOH, 8:2); λ_{max} 220 mμ (ε 37,320), 254 (29,230), 290 (4980), and 370 (19,900);[8] ν_{max} 3440 (hydroxy), 1760–1745 (lactone), 1660 (lactam), and 1610, 1585 cm^{-1} (aromatic C=C); nmr[9] (60 Mhertz, CD_3SOCD_3): 0.91 (3 H, triplet, $-CH_2CH_3$), 1.90 (2 H, multiplet, $>C(OH)-CH_2CH_3$), 5.45 (2 H, $ArCH_2O-$), and 5.28 (2 H, $ArCH_2-N<$); aromatic proton signals were poorly resolved. Camptothecin does not form stable salts with acids. Prolonged treatment of I with acetic anhydride–pyridine at 25° yielded the acetate, Ia,[10] M^+ at *m/e* 390.1217, calcd for $C_{22}H_{18}N_2O_5$, 390.1216; mp 271–274° dec; λ_{max} 220 mμ (ε 39,010), 254 (28,740), 290 (6160), and 360–370 (22,000); ν_{max} 1770–1750 cm^{-1} (acetate and lactone). I with chloroacetic anhydride gave the chloroacetate Ib, $C_{22}H_{17}N_2O_5Cl$; mp 245–248° dec; λ_{max} and ν_{max} similar to Ia; nmr (100 Mhertz, $CDCl_3$): 1.08 (3 H, triplet, $-CH_2CH_3$), 2.26 (2 H, multiplet, $-(ClCH_2COO)CCH_2CH_3$), 4.24 (2 H, $ClCH_2COO-$), 5.26 (2 H, $ArCH_2N<$), 5.54 (2 H, quartet, J_{AB} = 17 cps, $ArCH_2O-$),[11] and 7.22–8.36 (6 H, aromatic). The iodoacetate Ic was prepared from Ib using sodium iodide in acetone, $C_{22}H_{17}N_2O_5I$, mp 238–240° dec; λ_{max} and ν_{max} similar to Ia. I with thionyl chloride and pyridine in benzene yielded chlorocamptothecin (Id), $C_{20}H_{15}N_2O_3Cl$, mp 248–250° dec; λ_{max} 224 mμ (ε 28,610), 255 (24,580), 290 (6020), and 370 (20,910); ν_{max} 1755, 1660, and 1610 cm^{-1}; nmr (60 Mhertz, $CDCl_3$): 1.03 (3 H, triplet, $-CH_2CH_3$), 2.66 (2 H, multiplet, $>C(Cl)CH_2CH_3$), 5.30 (2 H, $ArCH_2N<$), and 5.56 (2 H, $ArCH_2O-$).[11] Exhaustive hydrogenation of I in acetic acid with Adams catalyst gave the dodecahydro derivative II, $C_{20}H_{28}N_2O_4$, M^+ at *m/e* 360, λ_{max} 234 mμ (ε 4700) and 303 (7410); ν_{max} 3460, 3250, and 1650 cm^{-1}; nmr (60 Mhertz, CD_3CO_2D): 5.25 (2 H, $ArCH_2N<$) and 7.20 (1 H, aromatic), pK_a = 6.8.[12] The unusual reactivity of the lactone in I (as shown by the immediate formation of a sodium salt with base[13] and the rapid reduction with sodium borohydride, both at room temperature) is probably due to intramolecular hydrogen bonding.[14] This may also account at least in part for the biological activity of I. The acetate Ia does not form a sodium salt under the same conditions as I and unlike I and its sodium salt has no antileukemic activity.

X-Ray analysis was conducted on the iodoacetate Ic.

Crystals of camptothecin iodoacetate are orthorhombic, space group $P2_12_12_1$, with four molecules of $C_{22}H_{17}IN_2O_5$ in a cell of dimensions a = 12.77, b = 22.70, c = 7.07 A. X-Ray intensity data were collected by means of equiinclination Weissenberg photographs. The initial position of the iodine atom was obtained from the three-dimensional Patterson synthesis and the other atoms (apart from hydrogen) were then located by evaluating a three-dimensional electron



I, R = OH
a, R = $OCOCH_3$
b, R = $OCOCH_2Cl$
c, R = $OCOCH_2I$
d, R = Cl



II

density distribution with Fourier coefficients weighted according to the method proposed by Sim.[15] For the atoms other than hydrogen, coordinates and temperature factors (anisotropic for the iodine atom, isotropic for the rest) were subsequently adjusted by least-squares calculations, and the present value of R for 1257 independent X-ray reflections is 9.8%.

We distinguished the carbon and nitrogen atoms on the basis of the magnitudes of the atomic temperature

recorded by one of us (M. E. Wall, *et al.*, AIC Bulletin 367, June 1954, p 24 under family *Nyssaceae*). This extract showed preliminary activity in Carcinoma 755,[5] and was the basis for further studies with this rare plant, a native of China. We thank Dr. Robert E. Perdue, New Crops Research Branch, Plant Industry Station, Beltsville, Md., who obtained the plant material.

(4) "Standard fractionation" consists of continuous, hot hexane–heptane extraction of dried plant material followed by similar extraction with 95% ethanol. The concentrate from the ethanol extract is partitioned between water and chloroform.

(5) We thank Dr. John M. Venditti and Miss Betty J. Abbott, CCNSC, for their assistance in obtaining the necessary bioassay data; for procedures *cf. Cancer Chemotherapy Rept.*, 25, 1 (1962).

(6) Camptothecin tested against leukemia L1210 in mice gives life prolongation as high as 100% on a daily dose of 0.25–1.0 mg/kg; against Walker 256 (intramuscular) tumor (rats) concentrations as low as 1.25 mg/kg gave significant inhibitions of growth; it also shows moderate cytotoxicity against KB cell culture, ED_{50} = 0.07 μg/ml.[5]

(7) Melting points were obtained with a Kofler hot stage, infrared spectra as "Nujol" mulls, and ultraviolet spectra in 95% ethanol; mass spectra were determined with the A.E.I. MS-9 mass spectrometer. All compounds gave satisfactory elemental analyses.

(8) The ultraviolet spectra of I and derivatives were not appreciably changed in acidic or alkaline solutions.

(9) Nmr results are given as δ units with TMS as an internal standard.

(10) Alkaline hydrolysis of Ia followed by acidification regenerates I.

(11) The compounds I and Ia–d give a signal at 5.25–5.27 (singlet, 2 H). On deuterium exchange of I under alkaline conditions or oxidation (of I) with alkaline permanganate this singlet disappears. In I and its chloro derivative Id, a second singlet (2 H) at 5.50 is observed whereas in Ia and Ib this signal is an AB quartet, J = 17 cps, centered at 5.56, which is more clearly observed after deuterium exchange. Only the 5.25 signal remains after borohydride reduction of I. The 5.25 signal is therefore due to $ArCH_2N<$ and that at 5.5–5.6 to $ArCH_2O-$.

(12) The nmr spectrum of II which still shows the characteristic signal at 5.25 ($ArCH_2N<$) and the presence of only *one aromatic proton* conclusively proves that ring A is reduced and that the nitrogen is in ring B. The pK_a value of 6.8 is also consistent with the formulation of II as a tetrasubstituted pyridine; *cf.* A. Albert, "Physical Methods in Heterocyclic Chemistry," Vol. I, A. R. Katritzky, Ed., Academic Press Inc., New York, N. Y., 1963, Chapter I.

(13) The sodium salt, which has the same order of antileukemic activity as I, is converted to camptothecin on acidification.

(14) The conformation of the lactone ring E may well be affected by hydrogen bonding.

(15) G. A. Sim, *Acta Cryst.*, **12**, 813 (1959); **13**, 511 (1960); "Computing Methods and the Phase Problems in X-ray Crystal Analysis," R. Pepinsky, J. M. Robertson, and J. C. Speakman, Ed., Pergamon Press, Oxford, 1961, p 227.

factors and the diagonal elements of the least-squares normal equation matrix obtained when scattering factors appropriate to carbon atoms were used for both carbon and nitrogen in the least-squares refinement. The location of the hydrogen atoms of rings A–E in a (F_o-F_c) synthesis unequivocally confirmed our assignment of the heteroatoms; the hydrogen atoms of the ethyl and iodoacetate side chains were only diffusedly visible, presumably because of the thermal motion of this part of the molecule.

The results of the X-ray analysis establish that the iodoacetate ester has structure Ic, and it follows, therefore, that camptothecin has structure I. The absolute configuration shown was determined by Bijvoet's method,[16] based on the anomalous dispersion by the iodine atom of the Cu Kα radiation.

We are actively pursuing the synthesis of camptothecin and simpler analogs and the effects of structure modification on biological activity. These and other results will be presented in future communications.

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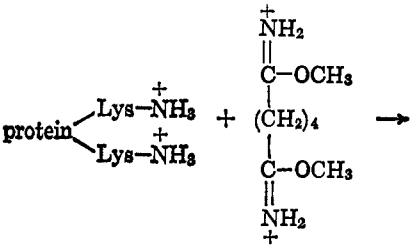
Bifunctional Reagents. Cross-Linking of Pancreatic Ribonuclease with a Diimido Ester[1]

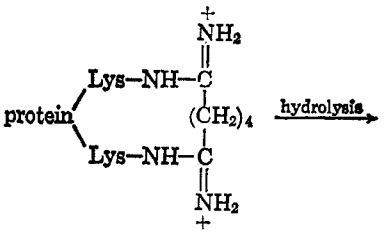
Sir:

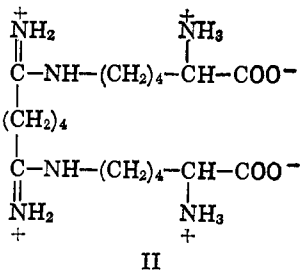
Hunter and Ludwig[2] have shown that water-soluble imido esters react specifically, under mild conditions, with protein amino groups. Since the resulting amidines have pK_a's slightly higher than those of ϵ-amino groups, the amidination should not alter the net charge of the protein in the acid or neutral pH range. Diimido esters might thus provide suitable bifunctional protein reagents, useful in the study of interresidue distances in proteins.[3]

We have investigated the reaction of dimethyladipimidate (I) with bovine pancreatic ribonuclease A (RNAase) according to



I





II

[^{14}C]Adiponitrile, prepared by the reaction of 1,4-dichlorobutane with [^{14}C]NaCN under the conditions of Smiley and Arnold,[4] was converted to [^{14}C]-dimethyl adipimidate (I) as described by McElvain and Schroeder.[5] The diimido ester dihydrochloride, obtained in over-all 85% yields, had a specific activity of 270,000 dpm/μmole and was converted to adipamide, mp 218–220°, during attempted melting point determinations.[6]

N_α,N_ϵ'-Adipamidinobislysine (II) was prepared by the reaction of α-N-formyllysine[7] with I and purified by ion-exchange chromatography on Dowex 50. The purified material moves as a single component, R_f 0.12, during paper chromatography (BuOH–HOAc–H_2O, 7:2:5). It can be measured quantitatively on the automatic amino acid analyzer by elution, as a single peak, from the short column with 0.1 *M* borate buffer (0.35 *M* in acetate), pH 9.7. The observed ninhydrin color yield is equal to that of lysine.

The modification of RNAase was conducted at room temperature in the following manner. [^{14}C]-Dimethyl adipimidate (27 mg, 110 μmoles) was added in 2-mg portions at 5-min intervals to a continuously stirred solution of RNAase (500 mg, 36.7 μmoles) in 50 ml of 0.1 *M* phosphate, pH 10.5. One hour after the addition of reagent was completed, the reaction mixture was subjected to gel filtration on a Sephadex G-75 column previously calibrated with RNAase which had been lyophilized from 50% HOAc to form dimers and higher aggregates.[8] Three fractions corresponding to monomers, dimers, and higher aggregates

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