11·$C_2H_2O_4$, 90791-69-8; 12, 67562-48-5; 12·HCl, 67562-49-6; 13, 67562-67-8; 13·HCl, 67562-68-9; 14, 67562-71-4; 14·HCl, 67562-72-5; 15, 67562-69-0; 15·$C_4H_6O_6$, 90791-70-1; 16, 67562-73-6; 16·$C_2H_2O_4$, 90822-51-8; 17, 67562-61-2; 17·HCl, 67562-62-3; 18, 67562-63-4; 18·$C_4H_6O_6$, 90822-52-9; 19, 67562-65-6; 19·$C_4H_6O_6$, 90791-71-2; bromobenzene, 108-86-1; 2-bromopyridine, 109-04-6; 4-bromoanisole, 104-92-7; 3-bromoanisole, 2398-37-0; 2-bromoanisole, 578-57-4; 4-bromotoluene, 106-38-7; 1-bromo-2-nitrobenzene, 577-19-5; 4-fluoro-1-bromobenzene, 460-00-4; 3-fluoro-1-bromobenzene, 1073-06-9; 2-fluoro-1-bromobenzene, 1072-85-1.

Synthesis and Antitumor Evaluation of Some Nitrosourea and Nitrogen Mustard Amino Acid Derivatives

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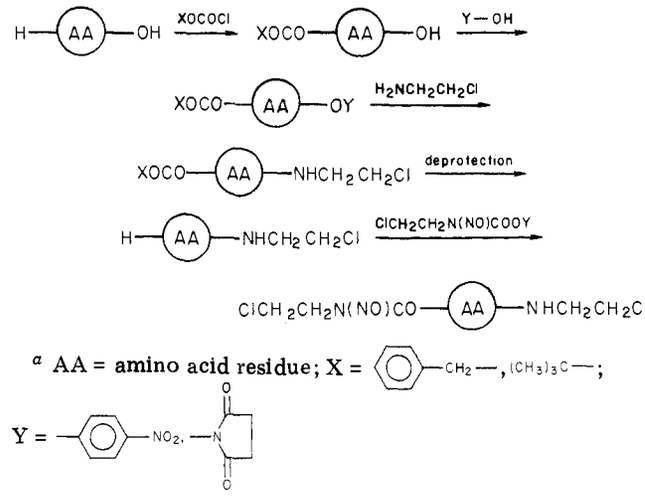
Received November 1, 1983

A series of (2-chloroethyl)nitrosourea and nitrogen mustard amino acid derivatives have been synthesized for antitumor evaluation. Reaction of an appropriate N-protected amino acid with 2-chloroethylamine followed by removal of the N-protecting group and condensation with an active (2-chloroethyl)nitrosocarbamate yielded *N*-[(2-chloroethyl)nitrosocarbamoyl]amino acid (2-chloroethyl)amides. Antitumor evaluation was performed against leukemia L1210, in vivo, in mice. These derivatives exhibited very interesting activities, particularly the sarcosine and γ-aminobutyric acid derivatives.

Many nitrosoureas and nitrogen mustard derivatives have been investigated for antitumor activity.[1] Of these compounds, several have proven to be potent antineoplastic agents, in particular, 2-haloethyl derivatives and some of their metabolites showed great promise.[2] For example, 2-[[[(2-chloroethyl)nitrosoamino]carbonyl]-amino]-2-deoxy-D-glucose (CZT, chlorozotocin),[3] *N*-(2-chloroethyl)-*N'*-[2,3-*O*-(1-methylethylidene)-5-*O*-(4-nitrobenzoyl)-D-ribofuranosyl]-*N*-nitrosourea (RFCNU),[4] *N*-(2-chloroethyl)-*N'*-cyclohexyl-*N*-nitrosourea (CCNU),[5] or *N,N'*-bis[(2-chloroethyl)nitrosocarbamoyl]cystamine (CNCC)[6] are some of the most important nitrosourea derivatives in the treatment of tumors. We recently described the synthesis and antitumor evaluations of some "pseudo-peptide" compounds containing covalently bonded cytotoxic units, alkylnitrosoureas at the N-terminus, and/or nitrogen mustard at the C-terminus.[7] These derivatives showed interesting cytotoxic activity on L1210 leukemia, in vivo, in mice. Tang and Eisenbrand reported the preparation of various *N*-[(2-chloroethyl)nitrosocarbamoyl]amino acid derivatives.[8] Suami et al. published the synthesis and antitumor evaluation of (2-chloroethyl)nitrosourea amino acid amide congeners[9] and Montero et al. of their methyl ester derivatives.[10] The antineoplastic activity of these derivatives against L1210 leukemia was quite significant. We herein report the synthesis and antitumor evaluations of some amino acid derivatives bearing two (or three, for polyfunctional amino acids) functional groups—a (2-chloroethyl)nitrosoureido group at the N-terminus end and a (2-chloroethyl)amide group at the C-terminus end.

Chemistry. The synthesis of amino acid (2-chloroethyl)amide derivatives was carried out according to Scheme I. Treatment of a N-protected amino acid active ester (either *p*-nitrophenyl ester[11] or *N*-hydroxysuccinimide ester[12]) with 2-chloroethylamine yielded the N-protected amino acid (2-chloroethyl)amide. In the case of the synthesis of the aspartic acid derivative, a 2 molar excess of 2-chloroethylamine was allowed to react with N-(benzyloxycarbonyl)-L-aspartic acid with use of dicyclohexylcarbodiimide as condensing reagent.[13] Partial deprotection by hydrogenolysis or by trifluoroacetic acid (depending

Scheme I[a]



[a] AA = amino acid residue; X = $C_6H_5CH_2$—, $(CH_3)_3C$—;

on the nature of the N-protecting group, benzyloxycarbonyl, Z, or *tert*-butyloxycarbonyl, BOC) and treatment

† This work is part of the Ph.D. thesis of Marc Rodriguez.
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0022-2623/84/1827-1222$01.50/0

Table I. Physical and Analytical Data of N-Protected Amino Acid (2-Chloroethyl)amides (R-AA-$NHCH_2CH_2Cl$)

amino acid[b]	R[d]	yield, %	recryst solvent[a]	mp, °C	$[\alpha]^{20}_D$ (c 1, DMF)	R_f, solv[c]	anal. C, H, N
Gly	Z	89	AcOEt Hex	107–108		0.60 A	$C_{12}H_{15}N_2O_3Cl$
β-Ala	Boc	65	CH_2Cl_2 PE	85–87		0.40 A	$C_{10}H_{19}N_2O_3Cl$
GABA	Z	63	AcOEt PE	97–99		0.35 A	$C_{14}H_{19}N_2O_3Cl$
Sar	Boc	80	AcOEt Hex	119		0.41 A	$C_{10}H_{19}N_2O_3Cl$
Ala	Boc	76	Et_2O PE	101–103	−8.1	0.68 B	$C_{10}H_{19}N_2O_3Cl$
Ile	Z	78	EtOH	175–177	+3.5	0.68 C	$C_{16}H_{23}N_2O_3Cl$
Leu	Z	91	AcOEt PE	101–103	−8.8	0.65 C	$C_{16}H_{23}N_2O_3Cl$
Phe	Z	86	AcOEt Hex	138–140	−14.2	0.68 C	$C_{19}H_{21}N_2O_3Cl$
Pro	Z	90	oil			0.52 A	
Asn	Z	73	EtOH	185–187	−2.0	0.81 D	$C_{14}H_{18}N_3O_4Cl$
Met	Boc	90	oil			0.65 A	
Thr	Z	67	AcOEt Hex	122–125	+3.7	0.40 A	$C_{14}H_{19}N_2O_4Cl$
Trp	Z	73	Et_2O	144–146	−23.0	0.36 A	$C_{21}H_{22}N_3O_4Cl$
Tyr	Z	85	EtOH Et_2O	175–176	−15.6	0.32 E	$C_{19}H_{21}N_2O_4Cl$
Asp*	Z	31	*i*-PrOH	174–176	+2.6	0.43 F	$C_{16}H_{21}N_3O_4Cl_2$
Lys†	Zα, Zε	88	Acet Hex	123–125	+4.2	0.52 A	$C_{24}H_{30}N_3O_5Cl$

[a] CH_2Cl_2, methylene chloride; AcOEt, ethyl acetate; Et_2O, diethyl ether; PE, light petroleum ether; Hex, hexane; MeOH, methanol; EtOH, ethanol; *i*-PrOH, 2-propanol; Acet, acetone. [b] (*) (2-chloroethyl)amide on the side chain; (†) Z on the side chain. [c] A (AcOEt), B (CH_2Cl_2/Et_2O, 2/1), C (CH_2Cl_2/AcOEt, 1/1), D (acetone), E (AcOEt/CH_2Cl_2, 1/2), F (CH_2Cl_2/MeOH, 96/4). [d] Z = benzyloxycarbonyl, Boc = *tert*-butyloxycarbonyl.

with an active (2-chloroethyl)nitrosocarbamate[14] gave the expected compounds, *N*-[(2-chloroethyl)nitrosocarbamoyl]amino acid (2-chloroethyl)amides. Amino acids bearing two amino functions (such as lysine) yielded, after deprotection, by reaction with a 2 molar excess of an active (2-chloroethyl)nitrosocarbamate bis-substituted (2-chloroethyl)nitrosocarbamoyl derivatives. The expected compounds were usually purified by chromatography on a silica gel column and most of them were crystalline materials. Physical and analytical data of these derivatives are given in Tables I and II.

Biological Results and Discussion

The compounds synthesized in the present study were evaluated for antitumor activity against leukemia L1210, in vivo, in mice. Results are shown in Table III. Their activities were compared with those of most significant other nitrosourea derivatives: CCNU, CZT, RFCNU, and CNCC.

Almost all the derivatives presented were highly active against leukemia L1210 in mice. It should be noted that the lethal median doses (LD_{50}) of these congeners for mice, as well as their therapeutic indexes, seem to increase with the length of the side-chain amino acid. GABA and sarcosine derivatives **3** and **4** are among the most interesting: LD_{50} = 125 and 400 mg/kg, respectively. Maximum effective dose (MED) range = 20–50 mg/kg, for **3**; MED range = 130–280 mg/kg, for **4**. Therapeutic index (TI) = 3.6 for **3** and 2 for **4**. One of the most attractive members in this series is the sarcosine derivative **4**. All mice treated with **4** (at doses between 140 and 225 mg/kg) were cured by day 60. Results arising from comparison of Met **11**, Leu **7**, and Ile **6** derivatives showed the effect of the amino acid side chain steric hindrance. Johnston et al.[15] mentioned that bis-substitution of the nitrogen atom of the (2-chloroethyl)nitrosourea lead to less active compounds. The weak cytotoxic activity of the proline derivative **9** is in agreement with these findings. It is interesting to note the weak toxicity of compounds **15** and **16** bearing both an additional active unit [(2-chloroethyl)nitrosocarbamoyl or (2-chloroethyl)amide] as compared to other derivatives. These two congeners **15** and **16** showed very good therapeutic indexes, 3.5 and 4.5, respectively, as well as interesting MED range (25–50 mg/kg, 65–160 μM/kg for **15** and 20–40 mg/kg, 40–85 μM/kg for **16**).

In summary, almost all of the derivatives synthesized and tested in this work exhibited low toxicity as compared with other significant nitrosourea derivatives and are highly active compounds against leukemia L1210, in vivo, in mice. Some of them, **3**, **4**, **15**, and **16** exhibited especially high antitumor activity and low acute toxicity. These compounds can compete with the best nitrosourea derivatives actually used, like CCNU, RFCNU, CNCC, or CZT.

Experimental Section

All capillary melting points were determined on a Büchi apparatus and are reported uncorrected. Thin-layer chromatography (TLC) experiments were carried out on Merck silica gel GF_{254}

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Table II. Physical and Analytical Data of (2-Chloroethyl)nitrosourea Amino Acid (2-Chloroethyl)amides [$ClCH_2CH_2N(NO)CO$-AA-$NHCH_2CH_2Cl$]

amino acid	no.	yield, %	recryst solvent	mp, °C	$[\alpha]^{20}_D$ (c 1, DMF)	R_f, solv[a]	IR, cm^{-1} [ν(NNO)]	anal. C, H, N
Gly	1	78	AcOEt PE	121–122		0.50 A	1485	$C_7H_{12}N_4O_3Cl_2$
β-Ala	2	66	AcOET PE	84–85		0.58 A	1470	$C_8H_{14}N_4O_3Cl_2$
GABA	3	79	Et_2O Hex	63–64		0.45 A	1485	$C_9H_{16}N_4O_3Cl_2$
Sar	4	63	Et_2O Hex	63–65		0.36 C	1480	$C_8H_{14}N_4O_3Cl_2$
Ala	5	60	CH_2Cl_2 Hex	112–114	+42.3	0.43 B	1475	$C_8H_{14}N_4O_3Cl_2$
Ile	6	67	Et_2O	103–104	+37.2	0.58 C	1480	$C_{11}H_{20}N_4O_3Cl_2$
Leu	7	71	AcOEt PE	117–118	+18.5	0.52 D	1480	$C_{11}H_{20}N_4O_3Cl_2$
Phe	8	75	AcOEt PE	93–95	−24.6	0.80 C	1480	$C_{14}H_{18}N_4O_3Cl_2$
Pro	9	51	Acet Hex	129–131	−39.0	0.48 A	1470	$C_{10}H_{16}N_4O_3Cl_2$
Asn	10	55	AcOET Et_2O	140–141	+37.5	0.16 A	1475	$C_9H_{15}N_5O_4Cl_2$
Met	11	63	Et_2O PE	53–55	+9.5	0.64 A	1485	$C_{10}H_{18}N_4O_3SCl_2$
Thr	12	60	CH_2Cl_2 PE	108–110	+40.3	0.55 A	1480	$C_9H_{16}N_4O_4Cl_2$
Trp	13	70	Acet Hex	144–146	−48.1	0.50 B	1480	$C_{16}H_{19}N_5O_3Cl_2$
Tyr	14	70	CH_2Cl_2 Hex	107–109	−33.2	0.43 B	1480	$C_{14}H_{18}N_4O_4Cl_2$
Asp	15	70	Acet Hex	156–157	+23.0	0.29 F	1475	$C_{11}H_{18}N_5O_4Cl_3$
Lys	16	52	AcOET PE	69	+12.3	0.40 G	1470–1480	$C_{14}H_{24}N_7O_5Cl_3$

[a] A (AcOEt), B (CH_2Cl_2/AcOET, 2/1), C (CH_2Cl_2/AcOEt, 1/1), D (AcOET/hexane, 2/1), E (Et_2O), F (CH_2Cl_2/AcOET, 3/1), G (AcOEt/hexane, 1/1).

Table III. Effects of (2-Chloroethyl)nitrosourea Derivatives on L1210 Leukemia in vivo

deriv	no.	DL_{50}, mg/kg	T/C max, %	dose, mg/kg	dose, μM/kg	D_{opt}, mg/kg	D_{opt}, μM/kg	TI
CCNU		30	∞	10				
RFCNU		90	∞	10–30		20		4.5
CNCC		75	∞	20–50		30		2.5
CZT		35	∞	10–25		15		2.5
Gly	1	25	∞	5–20	20–75	12	45	2
β-Ala	2	100	∞	20–50	70–175	35	120	2.9
GABA	3	125	∞	20–50	70–170	35	120	3.6
Sar	4	400	∞	130–280	470–1000	200	730	2
Ala	5	35	∞	10–30	35–105	20	70	1.75
Ile	6	130	∞	30–75	90–230	50	155	2.6
Leu	7	64	∞	15–50	45–155	35	110	1.8
Phe	8	80	∞	40–60	110–165	50	140	1.6
Pro	9	80	194	35	115			
Asn	10	35	∞	10–30	30–90	20	60	1.75
Met	11	32	∞	10–30	30–90	20	60	1.6
Thr	12	25	∞	10–20	30–65	15	50	1.6
Trp	13	65	∞	20–35	50–85	27	70	2.4
Tyr	14	40	211	25	65			
Asp	15	130	∞	25–50	65–130	37	95	3.5
Lys	16	135	∞	20–40	40–85	30	65	4.5

[a] $TI = DL_{50}/D_{opt}$.

plates. Column chromatography was on Merck silica gel, 60–230 mesh, ASTM. Elemental analyses were performed by "Le Service Central de Microanalyse du CNRS", Montpellier. IR spectra were determined on a Beckmann Acculab 4 spectrophotometer in KBr pellets.

General Procedure for the Synthesis of (2-Chloroethyl)amide of N-Protected Amino Acids. To a cold (0 °C), stirred solution of 2-chloroethylamine hydrochloride (0.02 mol, 2.30 g) and *N*,*N*-diisopropylethylamine (DIEA) (0.02 mol, 3.45 mL) in 30 mL of DMF was added the active ester of a N-protected amino acid (0.01 mol). The reaction mixture was stirred overnight.

The solution was concentrated in vacuo, and the residue was dissolved in ethyl acetate (200 mL). This solution was washed with 10% citric acid solution (2 × 100 mL), water (2 × 100 mL), a saturated sodium bicarbonate solution (2 × 100 mL), and water (2 × 100 mL) and dried over sodium sulfate.

The solvent was removed in vacuo and the residue was recrystallized from the appropriate solvent (see Table I).

***N*-(Benzyloxycarbonyl)-L-aspartic Acid α,β-Bis(2-chloroethyl)amide.** To a cold (0 °C), stirred solution of *N*-(benzyloxycarbonyl)-L-aspartic acid (0.02 mol, 5.43 g) in 70 mL of DMF were added 2-chloroethylamine hydrochloride (0.08 mol, 9.28 g), DIEA (0.08 mol, 13.8 mL), and dicyclohexylcarbodiimide (DCC) (0.04 mol, 8.24 g). The reaction mixture was stirred during 20 h at room temperature and filtered, and the precipitate was washed twice with 10 mL of DMF. The filtrate and the washings

were concentrated in vacuo. The residue was dissolved in 200 mL of ethyl acetate and treated as described in the general procedure. Recrystallization from 2-propanol: yield 2.4 g (31%); mp 174–176 °C; R_f 0.43 (dichloromethane/methanol, 96/4); $[\alpha]^{20}_D$ 2.6° (c 1, DMF). Anal. ($C_{16}H_{21}N_3O_4Cl$) C, H, N.

N^α,N^ϵ-Bis(benzyloxycarbonyl)-L-lysine (2-chloroethyl)amide: from N^α,N^ϵ-bis(benzyloxycarbonyl)-L-lysine *N*-hydroxysuccinimide ester;[16] recrystallized from a mixture of acetone and hexane: yield 88%; mp 123–125 °C; R_f 0.52 (ethyl acetate); $[\alpha]^{20}_D$ 4.2° (c 1, DMF). Anal. ($C_{24}H_{30}N_3O_5Cl$) C, H, N.

General Procedure for the Synthesis of [(2-Chloroethyl)nitrosocarbamoyl]amino Acid (2-Chloroethyl)amide. Method A. The *N*-(benzyloxycarbonyl)amino acid (2-chloroethyl)amide (0.01 mol) was hydrogenated in 100 mL of ethanol with an equivalent of a 10% HCl aqueous solution and 10% Pd/C as catalyst, at room temperature and under atmospheric pressure. The reaction was monitored by TLC.

The catalyst was removed by filtration and the filtrate concentrated in vacuo to give an oily residue, which was dried under reduced pressure.

To a cold solution of that compound in 20 mL of DMF was added DIEA (0.01 mol, 1.72 mL) and (2-chloroethyl)nitrosocarbamic acid 2,4,5-trichlorophenyl ester (0.012 mol, 3.98 g). The mixture was stirred for 3 h at room temperature and concentrated in vacuo. The residue was quickly chromatographed through a silica gel column and the compound was crystallized in the appropriate solvent (see Table II) or purified by chromatography on a silica gel column.

In the case of the lysine derivative, a twofold excess of reactants was used.

Method B. The *N*-(*tert*-butyloxycarbonyl)amino acid (2-chloroethyl)amide (0.01 mol) was dissolved in 5 mL of trifluoroacetic acid containing 2% of anisole or thioanisole under nitrogen and at room temperature. The reaction, monitored by TLC, was over in half an hour.

The solution was concentrated in vacuo at room temperature to give an oily residue, which was triturated in ether to give a white, amorphous solid, which was dried in a dessicator.

The subsequent operations were the same as those described for method A.

Antitumor Evaluation. The oncostatic activities and acute LD_{50} values were evaluated on L1210 leukemia. The method used for this evaluation is described: on day 0, adult F1 (DBA/2 × C57B1/6) mice were inoculated ip with 10^5 L1210 leukemia cells. A group of 8–10 mice was used for each concentration of every compound. On day 1, the mice received various doses of compound to be tested in olive oil (2–150 mg/kg). On days 5 and 9, drug or solvent injections were repeated only in mice with no signs of toxicity. The mortality of mice was monitored daily and autopsies were performed to find out whether or not deaths were due to leukemia or to a toxic action of the drug. The acute LD_{50} of each compound was determined graphically (98% confidence limits). For each compound, the oncostatic index, $T/C \times 100$ (T = median survival time in the treated group of mice, C = median survival time in the control group) was calculated. This index expressed prolongation of survival. When this oncostatic index > 125 and the difference between treated and control groups was statistically significant according to the Wilcoxon nonparametric W test, the agent was considered active at the given dose. The value ∞ means that more than 50% of treated animals in the group had been cured. Antitumor activity evaluations were performed in Villejuif, France (ICIG, Dr. Maral and Chenu, Service of Professeur Mathe).

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Acknowledgment. These investigations were supported by grants from CNRS and DGRST. We thank Dr. Maral and E. Chenu for antitumoral evaluations.

Registry No. $ClCH_2CH_2N(NO)CO$-Gly-$NHCH_2CH_2Cl$, 90764-33-3; $ClCH_2CH_2N(NO)CO$-β-Ala-$NHCH_2CH_2Cl$, 90790-68-4; $ClCH_2CH_2N(NO)CO$-GABA-$NHCH_2CH_2Cl$, 90764-34-4; $ClCH_2CH_2N(NO)CO$-Sar-$NHCH_2CH_2Cl$, 90764-35-5; $ClCH_2CH_2N(NO)CO$-Ala-$NHCH_2CH_2Cl$, 90764-36-6; $ClCH_2CH_2N(NO)CO$-Ile-$NHCH_2CH_2Cl$, 90764-37-7; $ClCH_2CH_2N(NO)CO$-Leu-$NHCH_2CH_2Cl$, 90764-38-8; $ClCH_2CH_2N(NO)CO$-Phe-$NHCH_2CH_2Cl$, 90764-39-9; $ClCH_2CH_2N(NO)CO$-Pro-$NHCH_2CH_2Cl$, 90764-40-2; $ClCH_2CH_2N(NO)CO$-Asn-$NHCH_2CH_2Cl$, 90764-41-3; $ClCH_2CH_2N(NO)CO$-Met-$NHCH_2CH_2Cl$, 90764-42-4; $ClCH_2CH_2N(NO)CO$-Thr-$NHCH_2CH_2Cl$, 90764-43-5; $ClCH_2CH_2N(NO)CO$-Trp-$NHCH_2CH_2Cl$, 90764-44-6; $ClCH_2CH_2N(NO)CO$-Tyr-$NHCH_2CH_2Cl$, 90764-45-7; $ClCH_2CH_2N(NO)CO$-Asp-$NHCH_2CH_2Cl$, 90764-46-8; $ClCH_2CH_2N(NO)CO$-Lys-$NHCH_2CH_2Cl$, 90764-47-9; Z-Gly-$NHCH_2CH_2Cl$, 4815-70-7; BOC-β-Ala-$NHCH_2CH_2Cl$, 90764-48-0; Z-GABA-$NHCH_2CH_2Cl$, 90764-49-1; BOC-Sar-$NHCH_2CH_2Cl$, 90790-69-5; BOC-Ala-$NHCH_2CH_2Cl$, 90764-50-4; Z-Ile-$NHCH_2CH_2Cl$, 15190-34-8; Z-Leu-$NHCH_2CH_2Cl$, 83510-60-5; Z-Phe-$NHCH_2CH_2Cl$, 90821-95-7; Z-Pro-$NHCH_2CH_2Cl$, 15054-24-7; Z-Asn-$NHCH_2CH_2Cl$, 90821-96-8; BOC-Met-$NHCH_2CH_2Cl$, 90790-70-8; Z-Thr-$NHCH_2CH_2Cl$, 90764-51-5; Z-Trp-$NHCH_2CH_2Cl$, 15164-96-2; Z-Tyr-$NHCH_2CH_2Cl$, 90764-52-6; Z-Asp($ClCH_2CH_2NH$)-$NHCH_2CH_2Cl$, 90764-53-7; Z^α,Z^ϵ-Lys-$NHCH_2CH_2Cl$, 15295-80-4; 2-chloroethylamine hydrochloride, 870-24-6; *N*-(benzyloxycarbonyl)-L-aspartic acid, 1152-61-0; N^α,N^ϵ-bis(benzyloxycarbonyl)-L-lysine *N*-hydroxysuccinimide ester, 21160-83-8; (2-chloroethyl)nitrosocarbamic acid 2,4,5-trichlorophenyl ester, 80354-51-4.

Synthesis and Antibacterial Activity of 2-[(Methoxycarbonyl)methylene]cephalosporins

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Received January 30, 1984

The synthesis and in vitro activities of a series of 2-[(methoxycarbonyl)methylene]-3-cephem-4-carboxylic acids with methyl or acetoxymethyl at the 3-position are described. The key step in the synthesis includes the stereospecific formation of the 2-[(*Z*)-(methoxycarbonyl)methylene] group by Pummerer rearrangement of the sulfoxides **3a** and **3b**. It was also possible to isomerize photochemically the C-2 olefin of **4a** to its *E* isomer, **9**. The new derivatives exhibited significant in vitro Gram-positive antibacterial activity.

The incorporation of substituents at the C-2 position of the cephalosporin nucleus has been of considerable interest in the search for cephalosporin analogues with interesting antibacterial activity.[1] Recently, a number of C-2 ethy-

0022-2623/84/1827-1225$01.50/0