The Limits of Reaction of Radioactive Dicyclohexylcarbodiimide with Amino Groups during Solid-Phase Peptide Synthesis[1,2]

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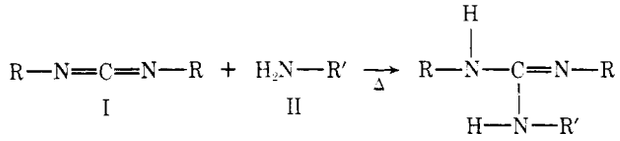
[1-^{14}C]Dicyclohexylcarbodiimide ([^{14}C]DCC) was synthesized and the extent of its reaction with N^{α}-amino groups during solid-phase peptide synthesis was determined by radioisotopic analysis. The limit of detection was below 0.2%. No measurable reaction occurred between [^{14}C]DCC and unprotonated valine which was esterified to a polystyrene–divinylbenzene resin (Val-Res). Similarly, no reaction occurred during normal [^{14}C]DCC-mediated coupling reactions such as those between Boc-Gly and Val-Res, Boc-Ala and Gly-Val-Res, or Boc-Lys(2,4-Cl_2Z) and Pro-Ala-Ile-Arg(Tos)-Arg(Tos)-Leu-Res. Therefore, this is not a significant side reaction and cannot account for the rises in picrate monitoring values that have been observed in some syntheses. [^{14}C]DCC reacted extensively with the protonated form of Val-Res to give [1-^{14}C]*N,N'*-dicyclohexylamidino-valine-resin (Dca-Val-Res). Cyclization and cleavage by diisopropylethylamine (DIEA) gave [2-^{14}C]-1-cyclohexyl-2-cyclohexylamino-4-isopropyl-4,5-dihydro-5-imidazolone. Reaction of [^{14}C]DCC with HCl·Gly-Val-Res gave the amidino peptide, which was stable to DIEA in CH_2Cl_2 but was transesterified with DIEA in methanol to give [^{14}C]Dca-glycyl-valine methyl ester hydrochloride. [^{14}C]Dca-Gly-Val-Res·HCl took up 1 equiv of picrate by the picrate monitoring method. It did not react with Boc-Ala + DCC following treatment with DIEA, but in the presence of a quaternary ammonium hydroxide (Triton B) it was acylated by Boc-Ala + DCC or by the symmetrical anhydride of Boc-Ala.

Monitoring data have suggested the occurrence of a side reaction during solid-phase peptide synthesis[3,4] that leads to a product which will titrate by the picrate method,[5,6] but which is terminated for further chain growth. Thus, in a long synthesis, the titration after coupling continued to rise slowly but the total titration after deprotection remained constant. It seemed possible that a reaction of dicyclohexylcarbodiimide (DCC, I) with the α-amino group could account for this effect.

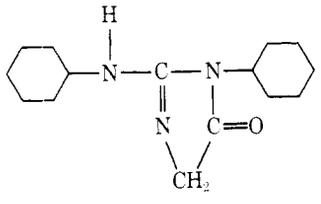
It has been known from early work[7-13] that diimides can react with amines to give guanidines, III. If this were to occur



I II III

during the coupling step of a solid-phase synthesis, in competition with the reaction of DCC with the carboxyl component, it would give a basic, titratable guanidine group which might remain protonated after the neutralization step with a tertiary amine and be resistant to further acylation reactions, just as in the case of unprotected arginine.

Muramatsu et al.[8] showed that glycine ethyl ester reacts with DCC in triethylamine to form 1-cyclohexyl-2-cyclohexylamino-4,5-dihydro-5-imidazolone (IV), and DeTar et al.[11]



IV

found that HBr·Gly-ONp reacted with DCC in acetonitrile to give the corresponding hydrobromide, whereas HCl·Gly-OEt and HBr·Ser-Gly-ONp reacted with DCC, but did not cyclize in the absence of tertiary amine. They predicted that the reactions were fast enough to be a source of by-products in certain peptide syntheses.

Soon after the DeTar publications we[14] tested the possibility that this might occur during solid-phase syntheses. Glycyloxymethyl-copoly(styrene–divinylbenzene) (Gly-Res) was treated with 4 equiv of DCC for 2 h, followed by addition of 4 equiv of Boc-Ala for 2 more h. After hydrolysis, the amino acid analysis showed equal amounts of glycine and alanine. Thus, the coupling had gone essentially to completion and no significant amount (<5%) of the side reaction appeared to have occurred. In a further test, Boc-Gly-Res (0.178 mmol/g) was deprotected with HCl and HOAc, neutralized with Et_3N, and treated for 2 h with 4 equiv of DCC in dimethylformamide. After acid hydrolysis, 0.178 mmol/g of glycine was found, indicating no formation of the acid-stable *N,N'*-dicyclohexylamidinoglycine (Dca-Gly) by reaction with DCC. In contrast, when HCl·Gly-Res was treated (without neutralization) with DCC in dimethylformamide the glycine recovered after hydrolysis decreased by 11% and when the reaction was carried out in dichloromethane glycine was reduced to 0.058 mmol/g, consistent with the presence of 67% of the guanidine derivative. Since the reaction did not occur to a measurable extent under normal coupling conditions or during treatment of the neutral amino acid-resin with DCC it was concluded at that time that this was not a significant side reaction of solid-phase synthesis.

A more sensitive way to detect and quantitate low levels of incorporation of DCC was necessary before the new evidence from monitoring data could be tested. For that purpose [1-^{14}C]dicyclohexylcarbodiimide ([^{14}C]DCC) was synthesized and examined for reaction with resin-bound amino acids and peptides under a variety of conditions.

Results and Discussion

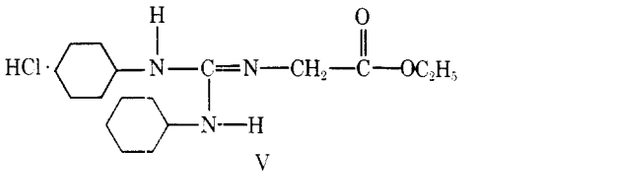
Experiments with Nonradioactive DCC. In order to compare the extent of reaction of DCC with amino groups in solution or on a resin support and to confirm some of the earlier work, a number of exploratory experiments were performed using unlabeled reagent.

It was found that reaction of DCC with unprotonated Gly-OEt in CH_2Cl_2 was very slow while in the case of Gly-Res no reaction was observed within the accuracy of the assay (<5%, amino acid analysis after hydrolysis). On the other hand, the hydrochlorides of amino acid esters reacted readily, either in solution or on a solid support, although the solution reaction appeared to be more rapid and complete. The product of the reaction of HCl·Gly-OEt with DCC, Dca-Gly-OEt·HCl[2] (V), cyclized at a rapid rate when base was added, to give IV. This reaction could also be adapted for the cleavage of Dca-amino acids esterified to a resin support. Thus, when

Table I. Reaction of [^{14}C]DCC with the Amino Component during a Solid-Phase Synthesis

Run	Amino component[a] (1 equiv)	Carboxyl component (4 equiv)	[^{14}C]DCC,[b] equiv	% reaction[c] Resin	Acid hydrol	DIEA filtrate
1	Boc-Val-R		4	0.2		
2	Val-R		4	0.2	0.1	0.1
3	Val-R	Boc-Gly	2	0.1	0.1	0.2
4	Val-R	Boc-Gly	4	0.1	0.1	0.2
5	Val-R	Boc-Gly	6	0.1		
6	Val-R	Boc-Ile	4	0.2	0.1	0.1
7	Gly-Val-R	Boc-Ala	4	0.3	0.1	
8	Ala-Gly-Val-R	Boc-Leu	4	0.2	0.1	

[a] These experiments were carried out on 200-mg samples of resin containing 0.076 mmol Val/g. [b] Specific activity 10.8 mCi/mol. [c] The reactions were for 2 h at room temperature. The percent reaction in each case is calculated from the radioactivity and is expressed as moles of DCC/100 mol Val.



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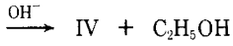
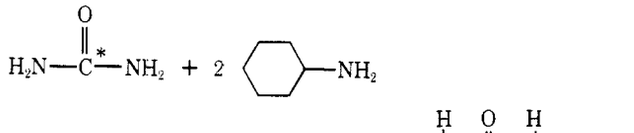
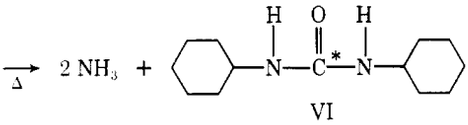


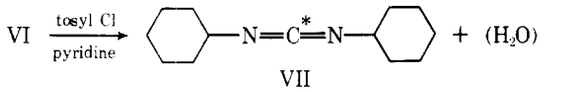
HCl·Dca-Gly-Res was suspended in CH_2Cl_2 and DIEA was added, IV was released into solution where it was readily identified by thin layer chromatography. When HCl·Val-Gly-Res (Val:Gly, 0.96:1) was treated for 18 h with 1 equiv of DCC in dichloromethane and then hydrolyzed, a Val:Gly ratio of 0.49:1 was found. This indicated that DCC also could react with peptide-resin hydrochlorides to a significant extent.

Experiments with Radioactive DCC. A sample of [1-^{14}C]DCC was prepared from [^{14}C]urea and cyclohexylamine according to the procedure of Amiard and Heymes:[15,16]





VI



VII

The purified distilled product had the correct chemical and physical properties and a specific activity of 10.8 mCi/mol.

The potential incorporation of [^{14}C]DCC into a resin-bound product was assessed by scintillation counting in three ways: (1) the resin was suspended in Aquasol and counted directly; (2) the resin was hydrolyzed and the filtrate was counted; (3) the resin was treated with diisopropylethylamine (DIEA) and the filtrate was counted. The latter was a useful diagnostic method for detecting the presence of the addition product between DCC and the α-amino group of an amino acid-resin because amidino amino acid esters are rapidly and selectively cyclized to give dihydroimidazolones.[8,11] The three methods gave essentially the same results, although at very low levels of reaction the variability was rather large.

Lack of Reaction of [1-^{14}C]DCC with Unprotonated Resin-Bound Amino Groups. It was first shown that [^{14}C]DCC could be satisfactorily removed from the resin beads by washing. Boc-Val-Res (200 mg) was shaken for 2 h in CH_2Cl_2 containing [^{14}C]DCC (2×10^6 cpm). After six washes with 4 mL of CH_2Cl_2 the recovery of radioactivity was essentially quantitative and the counts in subsequent washes had dropped to background levels. The washed Boc-Val-Res contained only 4 cpm/mg, equivalent to 0.2% of the valine (Table I). This value tends to set the lower level of sensitivity of the method.

The reaction of DCC with an amino acid resin was tested by treating Val-Res with 4 equiv of [^{14}C]DCC in CH_2Cl_2 for 2 h. The mixture was filtered and washed thoroughly and a weighed aliquot was counted. A second aliquot was hydrolyzed in 6 N HCl–dioxane for 24 h and the filtrate was counted. A third aliquot was treated with 10% DIEA in CH_2Cl_2 for 24 h and the filtrate was counted (Table I). The reaction of DCC with an amino acid resin under normal coupling conditions was then examined by mixing Val-Res with 4 equiv of Boc-Gly, followed in 5 min by 4 equiv of [^{14}C]DCC. After 2 h the resin was filtered, thoroughly washed, and examined for incorporation of counts. The data from this and related experiments are summarized in Table I.

The results showed no significant incorporation of ^{14}C above background and therefore less than 0.2% of the α-amino group of the amino acid resin could have reacted with DCC alone or under the normal conditions of a coupling reaction with a Boc-amino acid. These data lower the level of detectability of the potential side reaction by a factor of 10 below that which we could estimate previously by amino acid analyses, and they allow the conclusion that it probably is not a side reaction of practical importance.

The possibility that a hindered, and therefore much slower, coupling reaction might favor a competing reaction of the amine component with the DCC was tested by substituting Boc-Ile for Boc-Gly. We had shown previously that the rate of coupling of Boc-Ile to Val-Res was six times slower than the coupling of Boc-Gly to Val-Res.[17] The data of runs 4 and 6, Table I, showed no significant uptake of [^{14}C]DCC during either of these two coupling reactions. There was no difference in ^{14}C incorporation when the ratios of [^{14}C]DCC to Boc-Gly to Val-Res were 2:4:1, 4:4:1, or 6:4:1 and, furthermore, it was found that addition of triethylamine or pyridine did not change the extent of [^{14}C]DCC incorporation. It was also found that DCC did not react significantly with the amino component during normal coupling reactions between Boc-amino acids and resin-bound di- or tripeptides (runs 7 and 8).

Finally, a direct comparison was made between the extent of DCC incorporation and the picrate monitoring value during the synthesis of a heptapeptide. First the protected peptide, Boc-Pro-Ala-Ile-Arg(Tos)-Arg(Tos)-Leu-Res, corresponding to residues 32–37 of calf thymus histone H4, was synthesized. The picrate value was equal to 3.9% of the total peptide chains. The peptide was deprotected, neutralized, and coupled with 4 equiv each of Boc-Lys(2,4-Cl_2Z)[18] and [^{14}C]DCC. The re-

Table II. Reaction of [^{14}C]DCC with Protonated Valine Resin[a]

Run	Resin (1 equiv)	Amino acid (4 equiv)	Other, equiv	[^{14}C]DCC, equiv	Time, h	% reaction Resin	Hydrol	DIEA
1	HCl·Val-R			4	24	86	84	93
2	HCl·Val-R			4	2	23		
3	TFA·Val-R			4	2	19		
4	Val-R		0.1 TFA	4	2	3.6	3.3	3.3
5	Val-R		1.0 TFA + 1.0 DIEA	4	2	0.9	0.7	0.6
6	Val-R	Boc-Gly	0.1 TFA	4	2	0.1		
7	Val-R	Boc-Gly	1.0 TFA	4	2	0.1		

[a] These experiments were carried out on 200-mg samples of resin containing 0.076 mmol Val/g. See Table I.

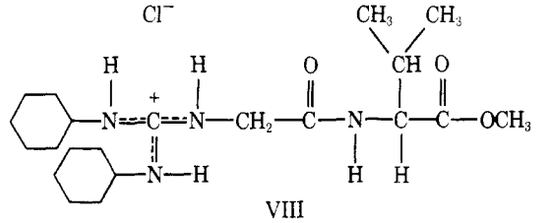
sulting protected heptapeptide, Boc-Lys(2,4-Cl_2Z)-Pro-Ala-Ile-Arg(Tos)-Arg(Tos)-Leu-Res, showed a picrate value of 6.8%, indicating an increase of 2.9% in the number of chains that could be titrated with picric acid. However, the ^{14}C counts corresponded to less than 0.1% of the peptide chains and could not account for the apparent rise in titratable groups that was indicated by the picrate method.[19]

Reaction of [1-^{14}C]DCC with Protonated Val-Res. Boc-Val-Res was deprotected with 50% TFA[2] in CH_2Cl_2, washed, treated with 4 equiv of [^{14}C]DCC for 2 h, washed thoroughly, and counted. In separate experiments the deprotected TFA·Val-Res was converted into the hydrochloride and then treated as before with [^{14}C]DCC for 2 h and for 24 h. The results of these runs are summarized in Table II. In 2 h both the trifluoroacetate and the hydrochloride of Val-Res reacted significantly with DCC (19 and 23%) and in 24 h an 86% yield was obtained from the hydrochloride. The product of the reaction was deduced to be *N,N'*-dicyclohexylamidinovaline-resin (Dca-Val-Res). All of the ^{14}C counts found on the resin beads were released into solution by acid hydrolysis. The hydrolysate contained 15% free valine, accounting for the fraction of Val-Res that had not reacted with DCC and supporting the expectation that the addition product does not give rise to the free amino acid upon acid hydrolysis.

The presence of only 0.1 equiv of TFA catalyzed the reaction of DCC with Val-Res to the extent of about 3% in 2 h (run 4) and the addition of 1 equiv of TFA plus 1 equiv of DIEA produced 0.6–0.9% reaction. In contrast, neither 0.1 nor 1.0 equiv of TFA was effective in catalyzing the reaction of DCC with Val-Res when excess Boc-Gly was present (runs 6 and 7). In these two runs the measured counts were not significantly above background. This is an important observation because it shows that a carryover, by failure to neutralize or by mechanical means, of even as much as 1 equiv of acid into the coupling reaction would not give rise to a termination reaction by DCC during a normal solid-phase synthesis.

Rate of Cyclization of [^{14}C]Dca-Val-Res·HCl by DIEA. Samples of [^{14}C]Dca-Val-Res·HCl were treated with 10% DIEA in CH_2Cl_2 for periods of 1 min to 5 h; 83% of the ^{14}C counts were released from the resin in 1 min by cyclization with the amine and the release was essentially quantitative within 1 h. The cyclization product was crystallized and characterized as 1-cyclohexyl-2-cyclohexylamino-4-isopropyl-4,5-dihydro-5-imidazolone. Since the cyclization reaction is very fast it was not possible to neutralize the HCl·Dca-Val-Res and study its susceptibility to coupling with Boc-amino acids or other acylating agents. However, those studies could be carried out on the corresponding amidino derivatives of di- and tripeptide resins.

[^{14}C]Dca-Gly-Val-Res and [^{14}C]Dca-Ala-Gly-Val-Res. Synthesis, Structure, and Properties. [^{14}C]Dca-Gly-Val-Res was synthesized by the reaction of [^{14}C]DCC with Gly-Val-Res·HCl. The structure of the product was deduced from the properties of the HF-cleavage products and by isolation and identification of the product of cleavage by amine-catalyzed transesterification: *N,N'*-dicyclohexylamidinoglycyl-valine methyl ester hydrochloride (VIII).



VIII

Picrate Titration. A sample of resin containing a mixture of HCl·Gly-Val-Res and HCl·Dca-Gly-Val-Res gave a picrate titration value[5] equal to the sum of the two basic groups, and similarly a sample containing HCl·Ala-Gly-Val-Res plus HCl·Dca-Ala-Gly-Val-Res gave a titration equal to the sum of the two components. These results showed that both the α-amino and α-guanidino groups will titrate.

Acylation. When a preparation of HCl·Dca-Gly-Val-Res was washed with DIEA and then treated with an excess of Boc-Ala and DCC the picrate value was equal to the amidine content. Following treatment with benzoyl chloride in pyridine the picrate value was unchanged. Similar results were obtained with HCl·Dca-Ala-Gly-Val-Res. It was concluded that amidinopeptide resins, after washing with DIEA, are resistant to acylation by DCC-activated Boc-amino acids or by benzoyl chloride, but that they take up 1 equiv of picric acid.

When a strong base such as Triton B[20] was used to deprotonate the amidinopeptide resins, acylation became possible with a DCC-activated Boc-amino acid or with a preformed symmetrical anhydride. Thus, HCl·Dca-Gly-Val-Res gave 63% of Ala-Dca-Gly-Val-Res.

Conclusions

Dicyclohexylcarbodiimide does not react to a measurable extent (<0.2%) with the amino group of resin-bound amino acids or peptides during solid-phase synthesis. Therefore this is not a significant side reaction and it cannot be responsible for the rises in picrate titration that have been observed. Dicyclohexylcarbodiimide will react with the hydrochloride or trifluoroacetate salts of resin-bound amino acids or peptides to give guanidines.

Experimental Section

[^{14}C]Urea, 55 mCi/mol, and dicyclohexylcarbodiimide were obtained from Schwarz/Mann; Boc-amino acids were from Beckman; chloromethylcopoly(styrene–1% divinylbenzene) 200–400 mesh resin beads were from Bio-Rad Laboratories. Quantitative amino acid analyses were performed on a Beckman Model 121 analyzer and ^{14}C was measured on a Beckman scintillation counter Model LS-355, using Aquasol scintillation fluid (New England Nuclear) and a 30–1000 channel. All samples were counted after standing for 24 h in the dark to allow phosphorescence to decay. Radioactivity on paper electropherograms was counted on a Packard radio chromatogram

scanner model 7201. Elemental analyses were by Mr. T. Bella. ^1H NMR spectra were obtained on a Varian 220-MHz instrument by Dr. Thomas Witherup and ^{13}C spectra were obtained on a Bruker 90-MHz instrument by Dr. William Wittbold.

Experiments with Nonradioactive DCC. Dca-Gly-OEt·HCl (V). Gly-OEt·HCl (1.4 g, 10 mmol) and 10 mL of a 1 M solution of unlabeled DCC in CH_2Cl_2 were combined and agitated in a sealed vessel at room temperature. After 20 h a clear solution had resulted and TLC on silica gel G (1-butanol–acetic acid–water, 4:1:1 v/v) indicated that HCl·Gly-OEt (R_f 0.24) had almost completely disappeared in favor of a single new spot (R_f 0.50) staining yellow with ninhydrin (10 min, 140 °C) or with the I_2/tolidine reaction. After evaporation of the solvent and treatment with ether the crude product was crystallized from 12 mL of acetone, filtered, and dried to give 2.8 g (81%) of Dca-Gly-OEt·HCl, mp 174–176 °C.

Anal. Calcd for $C_{17}H_{32}N_3O_2Cl$: C, 59.03; H, 9.33; N, 12.15. Found: C, 59.13; H, 9.21; N, 12.08.

Titration of an aqueous solution of V with NaOH to phenolphthalein gave an equivalent weight of 350 (calcd, 335). Hydrolysis (6 N HCl, 110 °C) did not release glycine from this compound. When the reaction of HCl·Gly-OEt and DCC described above was carried out with addition of 1 equiv of NEt_3, no clear solution resulted after 20 h. TLC showed that most of the starting material had remained unreacted and that three new products, one of them corresponding to V, had formed.

Conversion of V to 1-Cyclohexyl-2-cyclohexylamino-4,5-dihydro-5-imidazolone (IV). To 100 mg (0.3 mmol) of V in water, 0.1 N NaOH was added to pH ~8 (2.85 mL). The precipitate that formed instantaneously was collected, washed with water, and dried. This product was homogeneous by TLC (R_f 0.80) with $CHCl_3$–MeOH (9:1 v/v) staining yellow with the I_2/tolidine reaction, yield 64 mg (81%), after crystallization from EtOH–H_2O (1:1 v/v) mp 156–157 °C (lit.[8] 156 °C).

Anal. Calcd for $C_{15}H_{25}N_3O$: C, 68.40; H, 9.57; N, 15.96. Found: C, 68.38; H, 9.60; N, 16.03.

As judged by TLC, cyclization to give IV took place also when Dca-Gly-OEt·HCl was heated in water or 0.1 N HCl (100 °C, 14 h). Compound IV was stable under these condition and even 6 N HCl at 110 °C did not release glycine from it.

Reaction of DCC with HCl·Gly-Resin and Gly-Resin. Two 100-mg samples (A, B) of Boc-Gly-resin (770 μmol/g) were deprotected with 50% TFA, neutralized with 5% DIEA–CH_2Cl_2, and washed thoroughly with CH_2Cl_2. While A was converted to HCl·Gly-Res with a saturated solution of pyridine hydrochloride in CH_2Cl_2 and washed with CH_2Cl_2, B was left as the free base, Gly-Res. To each sample a 1 M solution of unlabeled DCC in CH_2Cl_2 (85 μL, 1.1 equiv) was added followed by enough CH_2Cl_2 to allow good mixing. The samples were agitated at room temperature and ~20-μL aliquots of the supernatants were taken periodically. As judged by TLC, nothing was released into solution in B, while in A small but increasing amounts of IV were detected. After 28 h, sample A contained 185 μmol/g Gly and B 736 μmol/g. Thus, under identical conditions, DCC had reacted with the hydrochloride to the extent of 76%, and with the free base to the extent of less than 5%.

Cleavage of Dca-Gly-Res Hydrochloride with DIEA and with HBr. A small sample of HCl·Dca-Gly-Res was treated with 5% DIEA in CH_2Cl_2 for 30 min and the supernatant was analyzed by TLC. Only one spot was detected, which cochromatographed with and stained like compound IV, R_f 0.65 with 1-butanol–acetic acid–pyridine–water, 15:10:3:2 (v/v). HCl·Dca-Gly-Res was also subjected to acidolytic cleavage conditions (30% HBr in AcOH, 30 min). In this case, two spots of about equal intensity were found in addition to Gly (R_f 0.21). One of these (R_f 0.65) was identical with IV, the other stained in the same way as IV (yellow with ninhydrin after heating to 140 °C, yellow with I_2/tolidine) but migrated slower (R_f 0.55).

Experiments with [^{14}C]DCC. Synthesis of [1-^{14}C]Dicyclohexylcarbodiimide (VII). [^{14}C]Urea (1 mCi, 55 mCi/mmol) was mixed with unlabeled urea (6.0 g, 0.10 mol), and cyclohexylamine (27.7 mL, 0.24 mol) was added. The suspension was heated to reflux (134 °C). The urea slowly dissolved and copious evolution of ammonia occurred. Refluxing was continued for a total of 2 h, while slowly increasing the temperature to 160 °C. The mixture, which solidified when removed from the bath to cool, was extracted with 1 N HCl, H_2O, ethanol, and ether. The solid residue of *N,N'*-dicyclohexylurea (VI) was recrystallized from 200 mL of absolute alcohol: yield 7.15 g (0.032 mol, 32%) (lit. 50%), total counts 7.52×10^8 cpm (36% radioactive yield); mp 229–230 °C uncorrected (lit.[15,16] 234–235 °C); mmp with authentic DCU 229–230 °C; NMR ($CDCl_3$) δ 1.09, 1.35, 1.64, 1.93 (m, ~20 H), 3.46 (m, 2 H, tertiary CH), 4.08 (d, 2 H, J = 8.2 Hz, NH).

[1-^{14}C]Dicyclohexylurea (VI, 7.3 g, 0.032 mol), 13 mL of dry pyridine, and 5.9 g (0.032 mol) of *p*-toluenesulfonyl chloride were stirred and heated at 70–75 °C for 4 h. After cooling, the product was dissolved in 20 mL of petroleum ether and the pyridine salts were filtered off and washed with petroleum ether. Diethylamine (1.2 mL) was added to the combined extracts and the mixture was heated to reflux. The solvent was removed on a rotary evaporator and the [^{14}C]DCC was distilled. The fraction distilling at a constant temperature of 134 °C (0.5 mm) [lit.[15,16] 140 °C (5 mm)] weighed 1.42 g (22%); total counts 1.49×10^8 cpm (21% radioactive yield from urea); specific activity 10.8 mCi/mol.

Anal. Calcd for $C_{13}H_{22}N_2$: C, 75.67; H, 10.75; N, 13.58. Found: C, 75.54; H, 10.70; N, 13.50.

1-Cyclohexyl-2-cyclohexylamino-4-isopropyl-4,5-dihydro-5-imidazolone. DCC (2.31 g, 11.2 mmol) in 50 mL of CH_2Cl_2 was added to HCl·Val-Res (10 g, 2.8 mmol Val) and the mixture was shaken for 24 h at 25 °C. After filtering and washing, 100 mL of 10% DIEA in CH_2Cl_2 was added with shaking for 1 h. The filtrate and washes were collected, evaporated to 10 mL, and cooled. The resulting solid was dissolved in methanol and crystallized by the addition of water; yield 230 mg. The product was recrystallized in the form of short needles from methanol–water, mp 50–51 °C (sinters), which analyzed as the monohydrate.

Anal. Calcd for $C_{18}H_{31}N_3O \cdot H_2O$: C, 66.83; H, 10.28; N, 12.99. Found: C, 66.82; H, 10.16; N, 12.95.

Amino acid analysis of a 6 N HCl hydrolysate showed no valine (<0.5 mol %).

Synthesis of [^{14}C]Dca-Gly-Val-Res. HCl·Gly-Val-Res (0.98 g, 0.074 mmol/g) was treated with 4 equiv of [^{14}C]DCC (61 mg, 2.17×10^7 cpm/mmol) for 24 h. After filtering and washing six times with CH_2Cl_2 the peptide resin contained 1430 cpm/mg (corrected for a blank of 4.7 cpm/mg), and an acid hydrolysate contained 1403 cpm/mg, giving an average of 0.062 mmol of incorporated DCC/g. An amino acid analysis of the hydrolysate showed Val, 0.074 mmol/g; Gly, 0.012 mmol/g.

Acylation of Amidino Peptide Resins. A. A sample of resin containing, by amino acid analysis, 0.012 mmol/g of HCl·Gly-Val-Res and 0.064 mmol/g of HCl·Dca-Gly-Val-Res and giving a picrate titration value of 0.076 mmol/g was washed with DIEA and treated with 4 equiv each of Boc-Ala and DCC. The picrate value was now 0.065 mmol/g, and Ala was 0.02 mmol/g.

B. A resin containing 0.034 mmol/g of HCl·Ala-Gly-Val-Res and 0.033 mmol/g of HCl·Dca-Ala-Gly-Val-Res (picrate value, 0.072 mmol/g) was washed with DIEA and treated with Boc-Leu and DCC. The picrate titration of the product was 0.043 mmol/g. Leu-Ala-Gly-Val (0.030 mmol/g) was identified chromatographically[21] following cleavage from the resin with HF, and 0.031 mmol/g of Leu was found in an acid hydrolysate of the peptide resin.

C. A sample containing 0.139 mmol/g of HCl·Dca-Gly-Val-Res and 0.034 mmol/g of HCl·Gly-Val-Res was mixed with 4 equiv of Triton B in CH_2Cl_2, and then treated for 2 h with the symmetrical anhydride made from 4 equiv of Boc-Ala and 2 equiv of DCC. An acid hydrolysate of the product gave 0.174 mmol/g Val, 0.035 mmol/g Gly, and 0.123 mmol/g Ala, indicating the presence of 0.035 mmol/g Ala-Gly-Val-Res, 0.051 mmol/g Dca-Gly-Val-Res, and 0.088 mmol/g Ala-Dca-Gly-Val-Res.

Cleavage of [^{14}C]Dca-Gly-Val-Res with HF. A 900-mg sample of HCl·Dca-Gly-Val-Res was stirred in 10 mL of HF containing 1 mL of anisole. After 1 h at 0 °C only 7% of the ^{14}C counts were released, but after 2 h at 25 °C 93% of the counts were removed from the resin. After evaporation of the HF, the crude product was extracted into TFA, evaporated, lyophilized from H_2O, and dissolved in 1 mL of water. The mixture was fractionated on a 1 × 10 cm column of Aminex 50W-X4 in pyridine–0.2 M acetate, pH 3.2. Two radioactive components were obtained with peaks at 65 and 71 mL. Analytical paper electrophoresis in formic acid–acetic acid–H_2O (15:10:75), pH 1.5, 1000 V, 3 h, showed radioactive spots at R_{Val} 0.69 and 0.62, respectively. At pH 5.0 and 8.5 both compounds remained at the origin. Their acid hydrolysates contained 2.42 and 2.54 μmol Val/mg and no Gly. The ratios of ^{14}C/Val were 1.05 and 1.03, respectively. These data and the ^1H NMR data were consistent with the assignment of isomeric forms of Dca-Gly-Val to the two products.

[^{14}C]Dca-Gly-Val-OMe·HCl (VIII). HCl·Gly-Val-Res (5.00 g, 0.19 mmol/g) was stirred for 48 h in 50 mL of CH_2Cl_2 containing 4 equiv of [^{14}C]DCC (900 mg, 2.17×10^6 cpm/mmol), filtered, washed, and dried. The product contained 530 cpm/mg (0.24 mmol/g) of incorporated DCC. Amino acid analysis of an acid hydrolysate showed Val, 0.196 mmol/g, and Gly, 0.010 mmol/g, indicating that 95% of the Gly had been guanidinated. The HCl·Dca-Gly-Val-Res (2.0 g) was transesterified by shaking with 60 mL of 1 M diisopropylethylamine

in methanol for 24 h. The filtrate and washes were evaporated to dryness: yield 77 mg, 36% by weight; 127 μmol Val, 7.7 μmol Gly (yield 32%). Total ^{14}C counts recovered in the filtrate indicated a yield of 36%. The solid was recrystallized from ether–petroleum ether. Paper electrophoresis at pH 1.5 gave a single radioactive spot at R_{Val} 0.48 (standards of Gly-Val, Asp, and cyclic Dca-Val appeared at 0.86, 0.74, and 0.66, respectively); ^1H NMR (Varian HF, 220 MHz, Me_2SO-d_6) δ 0.91 (m, 6 H), 1.3–1.4 (broad m, cyclohexyl axial), 1.6–1.8 (broad m, cyclohexyl equatorial), 2.06 (m, 1 H, Val β-CH), 3.1 (m, tertiary CH), 4.1–4.2 (m, 3 H, α-CH), 3.8 (s, 3 H, OCH_3), 7.32 (d, 2 H, J = 8 Hz, cyclohexyl NH), 7.68 (t, 1 H, J = 7 Hz, Gly NH), 8.64 (d, 1 H, J = 8 Hz, Val NH); ^{13}C NMR (Bruker HX-90, 22.6 MHz, proton decoupled, Me_2SO) (Me_4Si δ 0) δ 19.1, 19.8 (Val, C_γ), 25.5, 33.2, 51.6 (cyclohexyl), 31.0 (Val C_β), 44.8 (Gly C_α), 54.4 (OCH_3), 58.7 (Val C_α), 155 (Guan), 167.8 (Gly C=O), 169.7 (Val C=O). ^{13}C assignments were based on the standards, cyclohexylamine, Gly-Val, and Arg·HCl.

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Registry No.—IV, 5874-68-0; V, 61364-31-6; VI, 61348-54-7; VII, 57944-26-0; VIII, 61364-32-7; DCC, 538-75-0; Gly-OEt HCl, 623-33-6; Gly HCl, 6000-43-7; Gly, 56-40-6; Boc-Gly, 4530-20-5; Dca-Gly HCl, 61348-85-8; DIEA, 7087-68-5; [^{14}C]urea, 594-05-8; cyclohexylamine, 108-91-8; 1-cyclohexyl-2-cyclohexylamino-4-isopropyl-4,5-dihydro-5-imidazolone, 61348-56-9; Val HCl, 17498-50-9; [^{14}C]Dca-Gly-Val, 61348-57-0; Gly-Val HCl, 61348-59-2; Dca-Gly-Val HCl, 61348-60-5; Boc-Ala, 15761-38-3; Ala-Gly-Val HCl, 61348-58-1; Boc-Leu, 13139-15-6; Boc-Ile, 13139-16-7; Boc-Val, 13734-41-3; Boc-Gly-Val, 28334-73-8; Boc-Ile-Val, 61348-61-6; Boc-Ala-Gly-Val, 56133-97-2; Boc-Leu-Ala-Gly-Val, 61165-83-1.

References and Notes

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(2) Abbreviations: [^{14}C]DCC, [1-^{14}C]dicyclohexylcarbodiimide; Boc, *tert*-butyloxycarbonyl; Lys(2,4-Cl_2Z), N^{ϵ}-2,4-dichlorobenzyloxycarbonyllysine; Tos, *p*-toluenesulfonyl; DIEA, diisopropylethylamine; Res, 200–400 mesh resin beads of a 1% cross-linked copolymer of styrene and divinylbenzene; Dca, *N,N'*-dicyclohexylamidino; TFA, trifluoroacetic acid; TLC, thin layer chromatography. Other nomenclature and symbols follow the Tentative Rules of the IUPAC–IUB Commission on Biochemical Nomenclature, *J. Biol. Chem.*, **241**, 2491 (1966); **242**, 555 (1967); **247**, 977 (1972).
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Cytotoxic C-Benzylated Flavonoids from *Uvaria chamae*[1]

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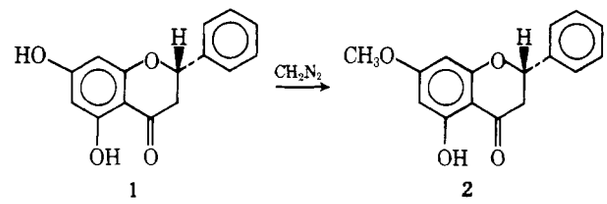
Ethanolic extracts of *Uvaria chamae* have demonstrated activity in vivo against P-388 lymphocytic leukemia in the mouse and in vitro against cells derived from human carcinoma of the nasopharynx (KB). Fractionation of these extracts yielded the known flavanones pinocembrin (**1**) and pinostrobin (**2**), the C-benzylated flavanones chamanetin (**3**), isochamanetin (**4**), and dichamanetin (**5**), and the C-benzylated dihydrochalcones uvaretin (**6**), isouvaretin (**7**), and diuvaretin (**8**). The structures were established by spectroscopic methods, chemical synthesis, and degradations.

In a previous communication[2] the isolation and structure elucidation of the cytotoxic C-benzylated flavanones chamanetin[3] (**3**) and isochamanetin (**4**) from the stem bark of *Uvaria chamae* were reported. We now wish to describe the total structure determination of these compounds. In addition, we wish to describe the isolation and structure elucidation of the known flavanone pinocembrin (**1**) and the dibenzylated flavanone dichamanetin (**5**) isolated from stem bark extracts as well as the known flavanone pinostrobin (**2**) and C-benzylated dihydrochalcones uvaretin (**6**), isouvaretin (**7**), and diuvaretin (**8**), which were isolated from root bark extracts.

Cytotoxicity[5] residing in ethanolic extracts of the stem bark was concentrated in the ethyl acetate fraction of an ethyl acetate–water partition. Silicic acid chromatography of this fraction starting with initial eluent benzene followed by ether–benzene mixtures resulted in the isolation of four flavanones (**1**, **3**, **4**, and **5**).

Scheme I



1 **2**

Compound **1** demonstrated UV, IR, and ^1H NMR[6] data consistent with a 5,7-dihydroxylated flavanone lacking B-ring[7] substituents. Comparison of the isolated product with an authentic sample of pinocembrin verified structure **1**.

Based on spectroscopic evidence (^1H NMR data in Table I) chamanetin (**3**) and isochamanetin (**4**) were designated as isomeric *o*-hydroxybenzyl derivatives of pinocembrin.[2] Support for these assignments followed from their formation of