115. Identification of the Bitter Principle of Cocoa

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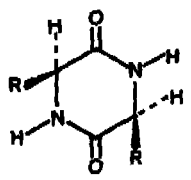
Summary. The following diketopiperazines have been identified in roasted cocoa: Cyclo(-Pro-Leu-), Cyclo(-Val-Phe-), Cyclo(-Pro-Phe-), Cyclo(-Pro-Gly-), Cyclo(-Ala-Val-), Cyclo(-Ala-Gly-), Cyclo(-Ala-Phe-), Cyclo(-Phe-Gly-), Cyclo(-Pro-Asn-), Cyclo(-Asn-Phe-).

The typical bitterness of cocoa is due to an interaction of the purine theobromine (**2**) and diketopiperazines, which are formed during the roasting of the coca beans.

Introduction. – The volatile components of roasted cocoa have been studied thoroughly but although more than 300 different compounds have now been identified [1], the substances causing the typical bitterness of cocoa have not yet been isolated. Because of the importance of these bitter compounds for the overall flavour of cocoa, we analyzed the non-volatile part of roasted beans in order to isolate them. A series of diketopiperazines **1** was identified, which, in the presence of theobromine (**2**), were shown to be responsible for the bitter taste of cocoa.

Results. – Cocoa beans contain two principal purine bases, theobromine (1.5%), and caffeine (**3**) (0.15%). The bitter taste of cocoa has often been attributed to the presence of theobromine or both purines and their proportions [2]



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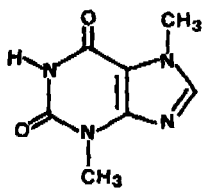

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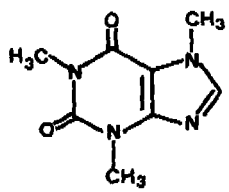

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Comparative organoleptic analyses[2]) of aqueous extracts of cocoa and of theobromine have, however, shown that quality and degree of bitterness are very different. Pure theobromine has a metallic bitterness, which is not immediately perceptible and which is relatively stable, whereas the bitterness of cocoa is rapidly detected but disappears quickly. An important phenomenon is the 'mouth feel', *i.e.* the bitterness is felt in the whole mouth, whereas for theobromine the sensation is only recognized by the hind part of the tongue. Furthermore, the bitterness of cocoa is more intense than that of a concentrated solution of theobromine in water.

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[2]) The organoleptic evaluations during the analysis were performed as described in the experimental part.

Isolation and Identification of Diketopiperazines. – After preliminary concentration of the bitter principles by extraction of defatted cocoapowder with aqueous acetone, a precipitate of theobromine was obtained by treating the concentrate with methanol. The methanol-soluble part contained only 3% of the initial amount of theobromine and had less than half of the original bitterness, but when pure theobromine – which by itself has not the typical bitterness – was added to the soluble part, the full bitterness of cocoa was restored. This showed that the bitter taste of cocoa is due to the simultaneous presence of theobromine and some other, methanol-soluble, products. For further concentration, the polyphenols were eliminated by chromatography on a polyamide column. Of the residue thus obtained, 25% was soluble in chloroform, and this part contained the whole amount of the bitter cofactors.

The remaining purines (traces of theobromine and the caffeine) could be separated by chromatography on silica gel. Chromatography on silica gel of the part soluble in chloroform and now free of purines allowed the isolation of three products, which were identified by mass-spectrometry as being Cyclo(-Asn-Pro-) and Cyclo(-Ala-Gly-) and Cyclo(-Asn-Phe-), in agreement with quantitative amino acid analysis after total hydrolysis.

The purine-free chloroform-soluble part (the average molecular weight of which was determined to be 211 $\pm$ 5 by osmometry) was analyzed by gas chromatography on a QF 1 column at 230°. This allowed the identification of the following diketopiperazines by comparison of the retention times with synthetic products[3]: Cyclo(-Pro-Leu-), Cyclo(-Val-Phe-), Cyclo(-Pro-Phe-), Cyclo(-Pro-Gly-), Cyclo(-Ala-Val-), Cyclo(-Ala-Gly-), Cyclo(-Ala-Phe-) and Cyclo(-Phe-Gly-)[4]).

These results were confirmed by comparison of mass spectra and thin-layer chromatography.

Organoleptic Properties of Diketopiperazines. – Despite the fact that diketopiperazines have been known since 1849 [3], there is little information in the literature about their organoleptic properties and about their presence in foodstuffs. *Emil Fischer* noted their bitterness [4] but only recently *Matoba & Hata* [5] reported that diketopiperazines have a stronger bitterness than the corresponding dipeptides or the amino acids. After completion of our work, a paper by *Shiba & Nunami* [6] appeared proving that a bitter peptide isolated from an enzymatic casein digest and thought to be a cyclic tetrapeptide was, in fact, Cyclo(-L-Leu-L-Trp-). We judged the bitterness of this compound very different from that of cocoa. It is sharp metallic, and does not give the typical mouth feel. Another diketopiperazine, Cyclo(-Pro-Leu-), was recently isolated from the bitter fraction of aged sake [7].

We tasted the diketopiperazines, either extracted from cocoa or synthesized, in water at concentrations between 30 and 50 ppm to compare their organoleptic properties with the cocoa-bitterness.

The bitterness of the diketopiperazines was quantitatively weaker than that of comparably dilute solutions of cocoa, but when 100 ppm of theobromine was added to the solution, the bitterness became stronger than the sum of the bitterness of both constituents tasted alone. However, a solution of 200 ppm of H-L-Val-L-PheOH and

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[3]) For the preparation of diketopiperazines see experimental part.

[4]) Neither Cyclo(-Asn-Phe-) nor Cyclo(-Asn-Pro-) were eluted under those conditions.

100 ppm of theobromine was found to have a much weaker bitterness than 50 ppm of the corresponding diketopiperazine with 100 ppm theobromine. (This proportion corresponds to a molar ratio of 1:2).

By comparing the bitterness of diketopiperazine-theobromine mixtures with those of cocoa, we found that the diketopiperazines containing phenylalanine had the closest resemblance.

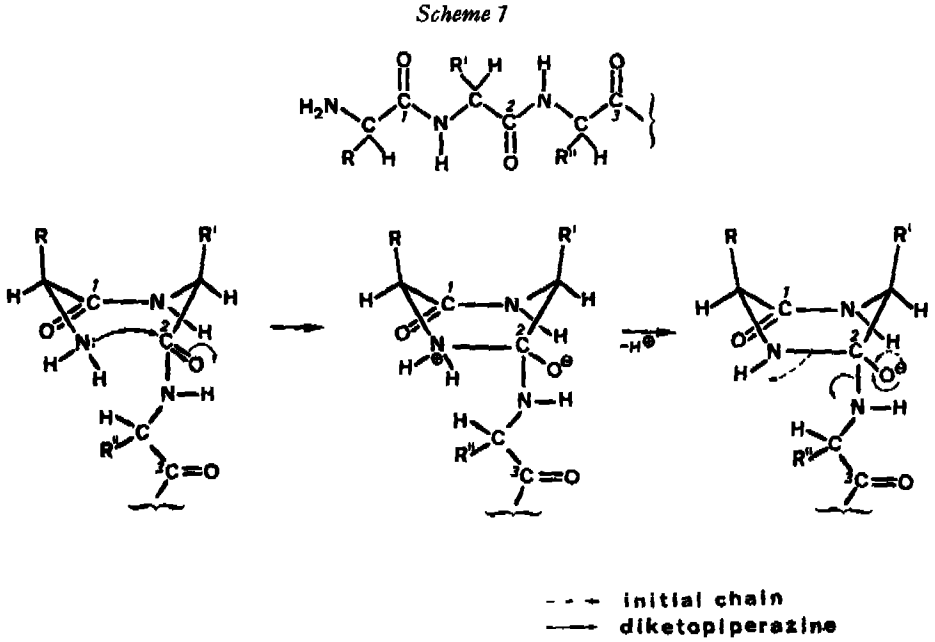
The configuration of the chiral center of the constituent amino acids does not seem to be important for the bitter taste, because all stereoisomers of Cyclo(-Phe-Val-) (L-L, L-D, D-L, D-D), which we prepared in a pure state, had the same taste in the presence of theobromine. The same result was found for the four Cyclo(-Ala-Val-) stereoisomers. To reconstitute the typical cocoa-bitterness one thus needs **a diketopiperazine** that preferentially contains phenylalanine **and theobromine**, in molar ratios of 1:2.

The Origin of Diketopiperazines in Roasted Cocoa. – By treating unroasted cocoa beans as described above, no diketopiperazines could be isolated nor did the extract have the typical cocoa bitterness, thus indicating that part of the bitter complex is formed during roasting.

It is known that diketopiperazines can be formed by heating proteins to temperatures above 100°. Two authors [9] [10] have recently proposed a method for sequencing peptides by either heating them for 25 sec. to 400°, or by boiling them for 12 h in acetic acid, followed by determination of the diketopiperazines formed.

Using synthetic tripeptides, we have checked whether diketopiperazines can be formed under the conditions of cocoa roasting. Thus 2 mg of H-L-Ala-L-Leu-Gly-OH were moistened with 5 μl of water and heated for 2 h to 130°. The brown residue was extracted with chloroform. TLC. of the chloroform-soluble part showed one spot which had the same Rf as synthetic Cyclo(-L-Ala-L-Leu-). After treating H-L-Leu-Gly-GlyOH

Scheme 7



under the same conditions, the chloroform-soluble product was purified by TLC. on silica gel and identified as Cyclo(-Leu-Gly-) by ^{1}H-NMR., combustion-analysis and comparison with authentic material. The formation of diketopiperazines under those conditions proceeds *via* an intramolecular aminolysis of the second N-terminal peptide bond by the free amino group of the peptide (see *Scheme 1*).

Physico-Chemical Mechanism for the Taste-Modifying Properties of a Mixture of Theobromine and Diketopiperazines. – There are few indications in the literature about the mechanism of the taste modification by a mixture of compounds [11].

In our case, we suppose that a physico-chemical interaction between theobromine and diketopiperazine is responsible for the characteristic bitter taste of the mixture. Since a complex is chemically different from its components, it might sensitize the receptors differently.

Microcalorimetric measurements[5]) of the mixing of solutions of theobromine and Cyclo(-Gly-L-Phe-) in molar ratios of 2:1 at concentrations that we used to match the cocoa bitterness, indicated unambiguously an interaction between these two compounds, which was not the case when N,N-dimethyl-cyclo(-Gly-Phe-) was used. This indicates the possibility of hydrogen-bonding between the amide groups of both molecules. The quantitatively small effect of 20 ± 2 cal mol^{-1} is not surprising. *Johannsen et al.* [12] predicted by *ab initio* calculations that water-amide hydrogen bonding should at least be equo-energetic with amide-amide hydrogen bonding, so that the effect of the latter should be very small at low amide concentrations in water.

So far as we know, this is the first case where the reasons for a taste modification of a mixture of compounds can be directly demonstrated by physico-chemical measurements.

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Experimental Part

General. Instruments used: ^{1}H-NMR. spectroscopy: 90 MHz, *Bruker* HX 90 with *Fourier* transform; mass spectrometry: *A.E.I.* MS. 9; amino acid determination: *Beckman* autoanalyzer Unichrom; gas chromatography: *Carlo Erba* Fractovap T 2400.

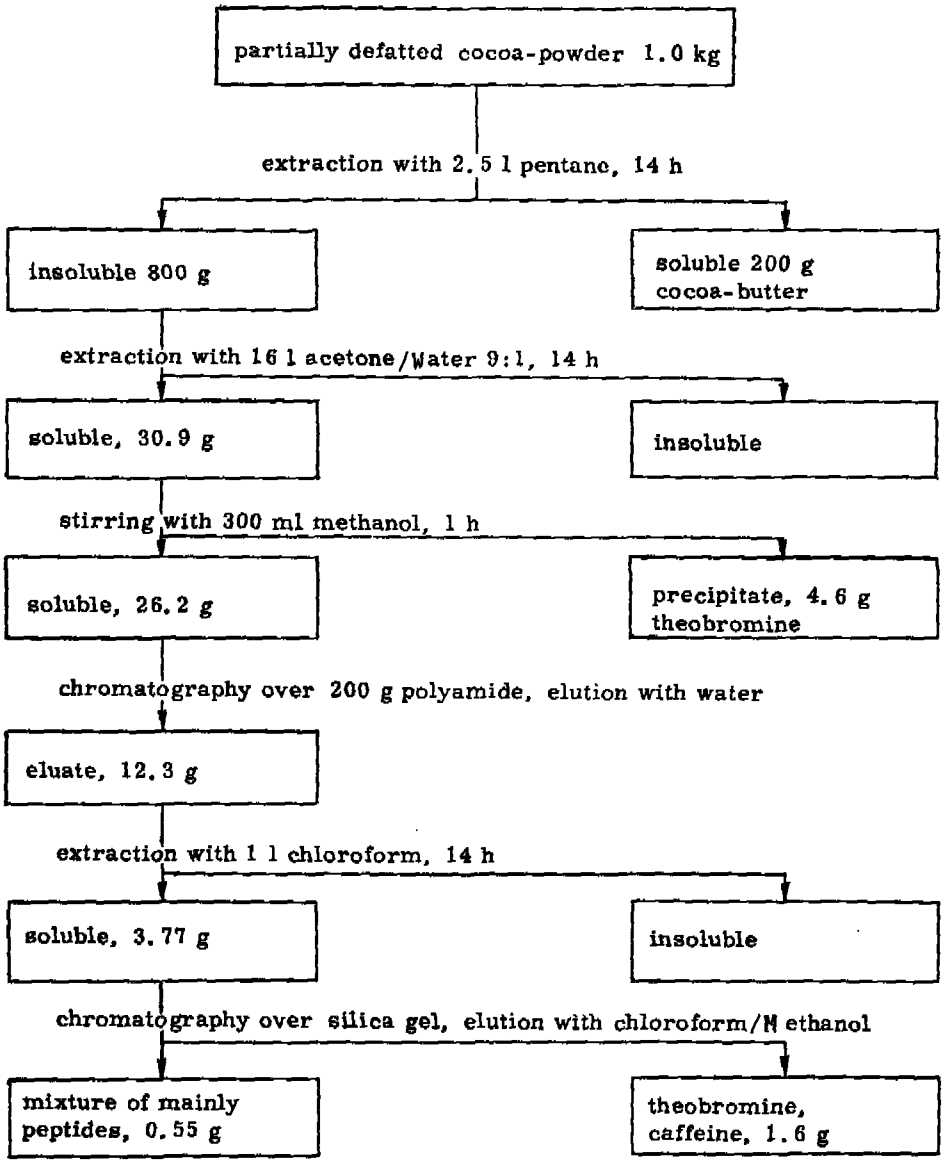
Analysis. The starting material was a partially defatted cocoa-powder obtained from roasted beans of the *genus Bahia*. In a typical separation as described in *Scheme 2*, we obtained from 1 kg of cocoa-powder 0.5 g of a mixture of mainly peptidic material, which was used for further separation. All fractions obtained during the analysis were diluted to an aqueous solution corresponding to 4 g of starting material, then compared organoleptically with a cocoa solution in the same way as described for the organoleptic analysis of synthetic diketopiperazines.

Isolation and Identification of the Diketopiperazines. 0.55 g of the mixture of peptides thus obtained were chromatographed over 90 g of silica gel (mixture of 65% silica gel *Merck* 0.05–0.2 mm and 35% under 230 mesh; this allows a flow rate of 15 ml/h) with a mixture chloroform/methanol 8:1, to obtain three fractions of 110, 90 and 45 mg respectively. Those fractions were named L-1, L-2, L-3, and were shown by TLC. to be mixtures.

Chromatography of L-3. 30 mg of the fraction L-3 were separated into its components by paper chromatography on 30 cm of *Whatman-1*, using acitic acid/pyridine/*n*-butanol/H_2O 12:40:60:48

[5]) Effected and interpreted by Dr. *J.-J. Schaer*, Department of Physicochemistry, University of Geneva (Switzerland). For a more detailed study: *J.-J. Schaer & W. Pickenhagen*, to be published.

Scheme 2



(all operations were executed at room temperature)

as solvent. The three main spots (Rf: 0.68, 0.58, 0.50 resp.) were eluted with water, to obtain respectively 2, 1, and 8 mg. Mass spectra and quantitative amino acid analysis after hydrolysis for 24 h at 105° with 6N HCl gave the following results:

	MS. (*m/e*)	Amino acids	proportions
Spot 1 (0.68)	261	Asp, Phe	1:1
Spot 2 (0.58)	128	Ala, Gly	1:1
Spot 3 (0.50)	211	Asp, Pro	1:1

The fractions L-1 and L-2 were combined and dissolved in DMF to a 10% solution, which was analyzed gas chromatographically on a 1.1 m × 2 mm column, stationary phase: QF-1 5% on

Chromosorb W 60/80. The peaks were directly integrated with a *Hewlett-Packard* Integrator 3370B.

The following diketopiperazines were identified by comparison of the retention time with synthetic samples:

Diketopiperazine	% Integration
Cyclo(-Ala-Pro-)	6.1
Cyclo(-Pro-Leu-)	8.0
Cyclo(-Val-Phe-)	1.6
Cyclo(-Pro-Phe-)	1.2
Cyclo(-Pro-Gly-)	6.9
Cyclo(-Ala-Val-)	23.7
Cyclo(-Ala-Gly-)	25.4
Cyclo(-Ala-Phe-)	3.0
Cyclo(-Gly-Phe-)	1.4

The same results were obtained qualitatively by comparison of the TLC. retention indices with synthetic material. Conditions: Thin layer plates: *Merck* silica gel; solvent: chloroform/methanol 8:1. The *Reindel-Hoppe* [28] method was modified by leaving the dried plates for 10 min in a chlorine atmosphere, followed by spraying with the toluidine-I_2 solution to give dark-blue spots.

Synthesis of the Diketopiperazines. All diketopiperazines were synthesized by preparing the dipeptide methyl ester by the method of *Boissonnas* [13] and cyclization as described by *Nitecki et al.* [14].

The description of the preparation of Cyclo(-Val-Phe-) is representative for all diketopiperazines.

To a solution of 2.51 g (10 mmol) Z-Val in 40 ml of dry tetrahydrofurane are added 1.1 g (10.1 mmol) ethyl chloroformate at – 5°. After 15 min at this temperature, a dispersion of 2.15 g (10 mmol) Phe-OMe · HCl in 20 ml N,N-dimethylformamide and 1.6 g (14.5 mmol) N-methylmorpholine are added.

The whole reaction mixture is stirred 4 h at room temperature, filtered, and concentrated *in vacuo*. The residue was taken up in 1 l of ethyl acetate, washed twice with 20 ml of 10% citric acid, then with 30 ml of 7% sodium hydrogencarbonate, and water. After drying over sodium sulfate, the solvent was distilled, to leave a solid residue, which was recrystallized from ethyl acetate/hexane. Yield: 3.47 g of white crystals (73%), m.p. 142.5–143°, $[\alpha]_D(CHCl_3)$ + 34.7°.

For the cleavage of the N-protecting-group, the crystals were dissolved in 800 ml of methanol and hydrogenated over 0.5 g of palladium 10% on charcoal. The hydrogen uptake was finished after 10 min. After filtration of the catalyst, the solvent was evaporated to 2.3 g of a white solid, which showed only one spot on TLC. Recrystallisation from ethanol gave 2.0 g of white crystals: m.p. 267°, $[\alpha]_D(H_2O)$ – 69.9°.

In cases where the dipeptide methyl ester was not completely cyclized, the whole reaction mixture was heated for 20 h at reflux in 10 times the amount of *n*-butanol/toluene 3:1 [14]. For the synthesis of the four stereoisomers of Cyclo(-Ala-Phe-) as well as of Cyclo(-Ala-Val-), 5 mmol of a mixture of two diastereomers were prepared in the same way as described above. Those

diastereomers were then separated by chromatography over silica gel using chloroform/methanol 6:1 as eluant. The following mixtures were prepared:

From Z-L-Ala and racemic Phe-OMe: Cyclo(-L-Ala-L-Phe-) Cyclo(-L-Ala-D-Phe-).

From Z-D-Ala and racemic Phe-OMe: Cyclo(-D-Ala-L-Phe-) Cyclo(-D-Ala-D-Phe-).

From Z-L-Ala and racemic Val-OMe: Cyclo(-L-Ala-L-Val-) Cyclo(-L-Ala-D-Val-).

From Z-D-Ala and racemic Val-OMe: Cyclo(-D-Ala-L-Val-) Cyclo(-D-Ala-D-Val-).

For the preparation of Cyclo(-Asn-Phe-), 1.0 g of L-aspartyl-L-phenylalanyl-methylester was dissolved in 10 ml of HCl 1.25N in methanol. After 1 h at room temperature the solvent was evaporated and the residue redissolved in 10 ml HCl 1.25N in methanol. After 20 h the solvent was distilled to leave 1.15 g of a white solid. After solution in 10 ml methanol, 0.45 ml of NH_3 7.5N in methanol were added, and the solution was concentrated. The residue was stirred for 1 h at room temperature in 10 ml of $CHCl_3$ and the insoluble NH_4Cl was removed by filtration. After concentration of the solution, the 1.1 g residue showed by TLC. to be homogenous. For cyclization, the whole residue was heated for 24 h at 90° *in vacuo*. TLC.-analysis showed that more than 90% of the starting material was converted. The white powder was then stirred with 5 ml methanol and filtered off. The insoluble part showed one spot on TLC. This product was stirred for 4 days at room temperature with 10 ml of NH_3 7.5N in methanol. After filtration, the residue was dried *in vacuo* to leave 0.61 g of a white powder which consisted of a mixture of two diastereomeric Cyclo(-Asn-Phe-). Chromatography on silica gel using a chloroform/methanol mixture 8:1 allowed isolation of 0.42 g of pure Cyclo(-L-Asn-L-Phe-).

The following diketopiperazines were prepared:

Diketopiperazine[a]	m.p.	$[\alpha]_D$ (H_2O)	Lit.
Cyclo(-Ala-Pro-)	178-180	- 147.9°	15
Cyclo(-Val-Leu-)	282-284	- 11.7°	16
Cyclo(-Val-Phe-)	266-267	- 69.9°	18
Cyclo(-Leu-Phe-)	267-268	- 15.3°	14
Cyclo(-Leu-Pro-)	168-172	- 91.3°	17
Cyclo(-Phe-Pro-)	144-146	- 67.7°	19
Cyclo(-Asn-Phe-)	268-270	- 1.7°	27
Cyclo(-Gly-Ala-)	244-246	- 4.5°	20
Cyclo(-Gly-Val-)	208-210	- 215.3°	16
Cyclo(-Gly-Leu-)	242-249	+ 22.1°	21
Cyclo(-Gly-Phe-)	271-272	+ 100.5°	22
Cyclo(-Gly-Pro-)	210-213	- 147.2°	17
Cyclo(-Ala-Val-)	265-266	- 70.0°	16
Cyclo(-Ala-Leu-)	255-260	- 13.0°	21
Cyclo(-Ala-Phe-)	276-280	+ 31.4°	23
Cyclo(-D-Ala-D-Phe-)	266-268	- 60.0°	
Cyclo(-D-Ala-L-Phe-)	253-256	+ 80.1°	
Cyclo(-L-Ala-D-Phe-)	253-255	- 80.0°	
Cyclo(-D-Ala-D-Val-)	268-270	+ 48.9°	
Cyclo(-L-Ala-D-Val-)	273-276	- 26.7°	
Cyclo(-D-Ala-L-Val-)	272-275	+ 38.6°	
Cyclo(-Gly-Gly-)	268-270	---	24
Cyclo(-L-Ala-L-Ala-)	272-275	- 24.5°	25
Cyclo(-L-Leu-L-Leu-)	274-276	- 23.17°	3
Cyclo(-L-Phe-L-Phe-)	296-299	b)	26

a) The amino acids are of L-configuration unless otherwise stated.

b) Unsufficiently soluble in water to determine the rotation. For all compounds mentioned, satisfactory combustion analysis and mass spectra were obtained.

Organoleptic Analysis of the Diketopiperazines. The taste-panel for the organoleptic analysis consisted generally of 6 to 9 professional flavourists of the Flavour Development Division of *Firmenich SA*, Geneva.

50 ppm of the diketopiperazines and 100 ppm of theobromine were dissolved in water and the bitterness of this solution was compared qualitatively and quantitatively with a solution of 4 g cocoa-powder in 100 ml water after filtration. The resemblance of the organoleptic impression was judged as very good, good, or less good.

Diketopiperazine L-L	Judgement
Cyclo(-Phe-Ala-)	very good
Cyclo(-Phe-Leu-)	very good
Cyclo(-Phe-Val-)	very good
Cyclo(-Phe-Phe-)	very good
Cyclo(-Phe-Asn-)	very good
Cyclo(-Phe-Gly-)	good
Cyclo(-Leu-Leu-)	good
Cyclo(-Leu-Val-)	good
Cyclo(-Leu-Ala-)	good
Cyclo(-Val-Ala-)	good
Cyclo(-Pro-Asn-)	good
Cyclo(-Phe-Pro-)	less good
Cyclo(-Leu-Gly-)	less good
Cyclo(-Leu-Pro-)	less good
Cyclo(-Val-Gly-)	less good
Cyclo(-Ala-Ala-)	less good
Cyclo(-Ala-Gly-)	less good
Cyclo(-Ala-Pro-)	less good
Cyclo(-Gly-Gly-)	less good
Cyclo(-Gly-Pro-)	less good
Cyclo(-Pro-Asp-)	less good

Diketopiperazines containing D-amino acids	Judgement
Cyclo(-D-Phe-Val-)	very good
Cyclo(-D-Val-Phe-)	very good
Cyclo(-D-Phe-D-Val-)	very good
Cyclo(-D-Val-Ala-)	good
Cyclo(-D-Ala-Val-)	good
Cyclo(-D-Ala-D-Val-)	good

Synthesis of Cyclo(-Leu-Gly-) by Peptide-Pyrolysis. 50 mg of H-L-Leu-Gly-GlyOH (*Fluka*) were heated with 50 µl of 30% acetic acid for 2 h to 130° in an open tube. The brown residue was extracted by stirring with 20 ml chloroform overnight. After filtration, the solution was concentrated to leave 19 mg of a brown solid, giving a single spot on TLC. After purification by preparative TLC., 9 mg of a white solid were obtained, which had the same H-NMR. spectra, as well as identical melting point and combustion-analysis as synthetic Cyclo(-Leu-Gly-).

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116. Selektive Abspaltung säurelabiler Aminoschutzgruppen von Peptiden in Trifluoräthanol[1][2]

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Chemische Forschungslaboratorien der Division Pharma der CIBA-GEIGY AG., Basel

(25. III. 75)

Summary. In building up large polypeptides, it has become the established practice to use acide-labile protecting groups of the *t*-butyl type. Up to now, only one step of selectivity under acidic conditions has been used, consisting in the cleavage of Trt, Bpoc or Ppoc from N(α) without attacking the *t*-butyl protecting groups. We have found that the use of 90% trifluoroethanol as solvent permits the selective cleavage of Trt in the presence of Bpoc or Ppoc under controlled

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[1] Auszugsweise vorgetragen an der Herbstversammlung der Schweiz. Chem. Gesellschaft in Lugano, 19.–20. Okt. 1973.

[2] Zu der hier verwendeten abgekürzten Schreibweise von Aminosäuren, Peptiden und deren Derivate vgl. [1]. Ferner bedeuten: Bpoc-: [2-(*p*-Biphenylyl)-isopropyl]-oxycarbonyl, Ppoc-: [2-Phenylisopropyl]-oxycarbonyl, TFEt: 2,2,2-Trifluoräthanol.