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GAS–LIQUID CHROMATOGRAPHY OF N-ACYL AMINO ACID ALKYL ESTERS*

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SUMMARY

Columns of polar and non-polar nature were chosen for studies of N-acyl amino acid alkyl esters in which systematic changes of the acyl and alkyl ester were made. Procedures for N-acylation of amino acids followed by esterification were found to give clean preparations of acyl amino acid esters, the identity and purity of which were established. The N-*n*-butyryl *n*-propyl esters of twenty-four amino acids were subjected to gas–liquid chromatography. By pairing the two columns, all the amino acid derivatives give distinct peaks, except arginine, ornithine and citrulline. The elution patterns on the polar and non-polar columns are similar but not identical.

Using glycine as a standard unit, N-acylglycine methyl esters, N-acetylglycine alkyl esters and N-acylglycine alkyl esters have been chromatographed on both columns at isothermal temperatures. The effect on retention time shown by the N-acyl group is of the same magnitude as that shown by the alkyl ester. The plot of log retention time *vs.* number of carbons of substituent X was found to be linear. The sum of the slopes of N-acylglycine methyl ester and of the N-acetylglycine alkyl ester equals the slope of N-acylglycine alkyl ester, which demonstrates that the effects of substituent X are additive.

INTRODUCTION

N-Acyl amino acid esters have been used in gas–liquid chromatography (GLC) for amino acid analysis[1–8]. The amino acid derivatives investigated were chosen on the basis of their volatility and ease in conversion from the amino acids. In the literature, a large proportion of communications are in the form of short notes or preliminary reports. The derivatives converted as well as the experimental conditions have varied widely. Furthermore, many investigations appear to be variations on a general theme with little discrimination in selection of derivatives or in design of experiments. It has been found impossible in this laboratory to correlate the effects of weight, size, geometric shape, polarity, etc. of the N-acyl and O-ester substituents of amino acids in relation to the patterns of the chromatogram.

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N-Acyl and O-alkyl ester substitutions on aliphatic amino acid have been chosen in this investigation on account of their versatility in structural variation, all of which nevertheless are of the basic structure as below.

Fig. 1.

Series of N-acyl amino acid alkyl esters have been chromatographed in both polar and non-polar columns. The identical substituents X by design, on both ends of the molecule, assure simplicity in evaluating and calculating the effect on the chromatogram.

This communication consists of studies on series of amino acids with systematic change of N-acyl and O-alkyl ester substituents. The shape of the N-acyl amino acid esters approaches the form of a dumb-bell as the size of X increases. Experiments have also been performed on N-acyl amino acid alkyl esters of unequal X substituents.

It is hoped that this investigation may contribute further understanding to the relationship of substituents on amino acids and their GLC patterns. This study and further extension may provide more rationale for selection of derivatives for analysis of amino acids in the most effective manner and perhaps also in the shortest possible time.

EXPERIMENTAL

Reagents and materials

All amino acids were from Takara Kohsan Co., Ltd., Tokyo, Japan, and were of pure grade with no other amino acid or nucleic acid detectable chromatographically in a 30 μg sample. DL-α-Aminobutyric acid (DL-butyrine)* was from Sigma Co., St. Louis, Mo., U.S.A. Their purity was confirmed in this laboratory by paper chromatography in two solvent systems: water–formic acid–2-butanol (150:30:20, v/v) and 2-butanol–3 % ammonium hydroxide (150:60, v/v).

Compressed gases were from Hong Kong Oxygen & Acetylene Co., Ltd., Hong Kong; N_2 99.9991 %, H_2 99.5 % and air with oil and hydrocarbons filtered.

Instrument and columns

The instrument used was an F&M Model 5750, Hewlett and Packard Co. gas chromatograph with dual coil columns, dual flame ionisation detectors and dual-pen recorder. Column A was a 1.83 m (6 ft.) × 3.2 mm (1/8 in.) O.D. stainless steel tube, with 10 % Carbowax 20M on Chromosorb W, 60–70 mesh, acid washed, dimethylchlorosilane treated, preconditioned for 10 h at 225°. Column B was a 1.83 m (6 ft.) × 3.2 mm (1/8 in.) O.D. stainless steel tube, 1 % silicone gum GE XE-60, on Chromosorb W, 60–70 mesh AW, DMCS, preconditioned as for column A. The column was later coated with hexamethyldisilazane by injecting 10 μl of 10 % of the silylation reagent in anhydrous hexane and again preconditioned at 225° for 1 h. The gas flow rates were 28 ml/min, 32 ml/min, and 296 ml/min (20 mm, 30 mm, and 100 mm on the gauges of the instrument) for H_2, N_2, and air, respectively.

* Butyrine for α-amino-n-butyric acid was first suggested by GREENSTEIN AND FU[9]. The term was adopted by GREENSTEIN AND WINITZ[10].

Preparation of N-acyl amino acid esters

(*I*) *Esterification of amino acid followed by N-acylation*

Amino acid, 1 mg, was suspended in 2 ml of the appropriate alcohol and the reaction mixture was saturated with anhydrous hydrogen chloride for 5 min. The excess hydrogen chloride and alcohol were removed under reduced pressure in a Flash evaporator. The residual oil, sometimes crystallized, of amino acid ester hydrochloride was treated with 1 ml of acid anhydride at 25–30° for 10 min with vigorous stirring. The reaction mixture was again evaporated under reduced pressure to remove the excess reagent, etc. The resulting sirup of the N-acyl amino acid ester was dissolved in either benzene or the alcohol for chromatographic studies.

(II) *N-Acylation of amino acids followed by esterification*

N-Acyl amino acids. (a) To 1 mg of amino acid in 1 ml of water, 1 ml of acid anhydride was added, and mixed with vigorous stirring first at room temperature and then at 30° for a total of 10–15 min. The resulting white crystalline N-acyl amino acid precipitated. The aliphatic acid formed and water were removed under reduced pressure in a Flash evaporator. The white crystals were treated three times each with 2 ml of benzene and evaporated under reduced pressure to remove the trace amounts of impurities and water. This procedure was effective only for acetic, propionic and butyric anhydrides.

(b) In a 5-ml centrifuge tube, 1 mg of amino acid, 1 ml of acid anhydride (or acid chloride) of a higher aliphatic acid and 1 ml of 10 % sodium hydroxide were mixed with vigorous stirring at 5° and then at room temperature. The oil drops dissolved in 10–15 min indicating completion of the reaction. 1 mole equivalent of 6 *N* hydrochloric acid was added to neutralize the sodium hydroxide. The crystallized N-acyl amino acid was isolated by centrifugation. The N-acyl amino acid obtained was dried over P_2O_5 and solid sodium hydroxide in a vacuum desiccator and used for subsequent esterification. As in the Schoetten-Baumann reaction, acetic and propionic chlorides cannot be used.

Esterification of N-acyl amino acids. (c) To the dried N-acyl amino acid residue, 2 ml of the appropriate alcohol and 0.5 ml of benzene were added in the presence of approximately 1 mg of Amberlite IR-120(H) which was first thoroughly washed successively with abs. ethanol and benzene. This reaction mixture was heated under reflux for 10 min. The resin was separated from the solution by filtration and was washed three times each with 1 ml of benzene. The washings were combined with the filtrate. The excess of the alcohol and benzene were removed under reduced pressure in a Flash evaporator. The residue, N-acyl amino acid ester, remaining in the flask was dissolved in either 1 ml of benzene or 1 ml of the alcohol. The solution was immediately used for chromatographic studies.

(d) Alternatively, the dried N-acyl amino acid residue could be esterified in 2 ml of the alcohol by passing a stream of dry hydrogen chloride for 5–10 min or until the acyl amino acid dissolved. The reaction mixture was then heated under reflux for another 10 min. The excess alcohol and hydrogen chloride were removed by evaporation in a Flash evaporator under reduced pressure. The resulting sirup was dissolved as described in (c).

(III) Isolation of N-acyl amino acids and N-acyl amino acid esters

In some instances, pure N-acyl amino acid or N-acyl amino acid ester were isolated. The preparative procedures used were the same as given above, except 0.1–0.5 g of amino acid was used. Some of the known compounds prepared were used as reference to verify the conversion procedures and as standards for quantitative estimation of N-acyl amino acid esters. The compounds given below are new compounds which were identified and analyzed.

N-*n*-Valerylglycine was prepared from glycine and valeric anhydride by procedure IIb; m.p. 81–81.5° (benzene). Anal.*: calcd. for $C_7H_{13}NO_3$: C, 52.81; H, 8.23. Found: C, 53.02; H, 7.99.

N-*n*-Valerylglycine methyl ester was prepared from N-valerylglycine and abs. methanol by procedure IId; $b.p._{2.8}$ 120°. Anal.: calcd. for $C_8H_{15}NO_3$: C, 55.49; H, 8.73. Found: C, 55.82; H, 8.84.

N-Enanthylglycine was prepared from glycine and enanthic chloride by procedure IIb; m.p. 88° (H_2O). Anal.: calcd. for $C_9H_{17}NO_3$: C, 57.75; H, 9.16. Found: C, 58.10; H, 9.16.

N-Enanthylglycine methyl ester was prepared from N-enanthylglycine and abs. methanol by procedure IId; $b.p._{3.5}$ 160°. Anal.: calcd. for $C_{10}H_{19}NO_3$: C, 59.68; H, 9.51. Found: C, 59.91; H, 9.32.

Chromatographic procedure

Starting from 1 mg of the amino acid, the benzene or the alcohol solution of the N-acyl amino acid ester prepared was diluted to 1 ml in a calibrated volumetric tube. It gave a concentration of approximately 1×10^{-5} mole/ml, *i.e.*, 1×10^{-8} mole per μl or 1 μg/μl, assuming the molecular weight of amino acid is 100. A Hamilton syringe No. 701N, 10 μl or No. 75, 5 μl was used. Either 0.5 μl or 1.0 μl of the solution was injected into the columns. For higher dilution, the N-acyl amino acid ester was diluted to 5 to 30 ml in order to measure at various concentrations. The injection was never less than 0.5 μl. The N-acyl amino acid alkyl esters were chromatographed individually and as a mixture under isothermal and programmed conditions.

RESULTS AND DISCUSSION

Columns

Commercially packed columns were chosen for this investigation on account of availability and minimum variation in column characteristics due to packing, composition of stationary and liquid phase, etc. Column A of 10% Carbowax 20M liquid phase and column B of 1% silicone gum rubber liquid phase were used. In Fig. 2, the chromatogram of N-isobutyryl amino acid isopropyl esters was used as an illustrative example. The retention time of the acyl esters eluted from column B was 3 min and offered relatively good separation except that glycine and valine appeared as one peak. Column A, however, showed good separation of all the esters under the same conditions.

Both columns were tested for recognizable signals at minimum amount of the acyl ester. The observable minimum quantity was 0.01 μg on both columns but the

*The elemental analyses were performed by the Microanalytical Laboratory, University of Singapore.

noise level was too high to be useful without a signal analyzer/averager. The noise to signal ratio was 1:2.5. However, 0.02 μg would give desirable peaks in height and shape for normal measurement. Measurements were made in most cases at concentrations of 0.1–5 μg. The column performance in terms of plate number (N) was also determined. A modification[11] of the equation suggested by JAMES AND MARTIN[12] was used for calculations. The average N calculated for column B was 1000 at isothermal temperatures 150° and 165°. When the column was operated at higher temperatures, the resolution and column behavior became increasingly poor. At 200°, the average N dropped as low as 256. However, column A showed an average N of 1207 and 1091 for 165° and 200°, respectively.

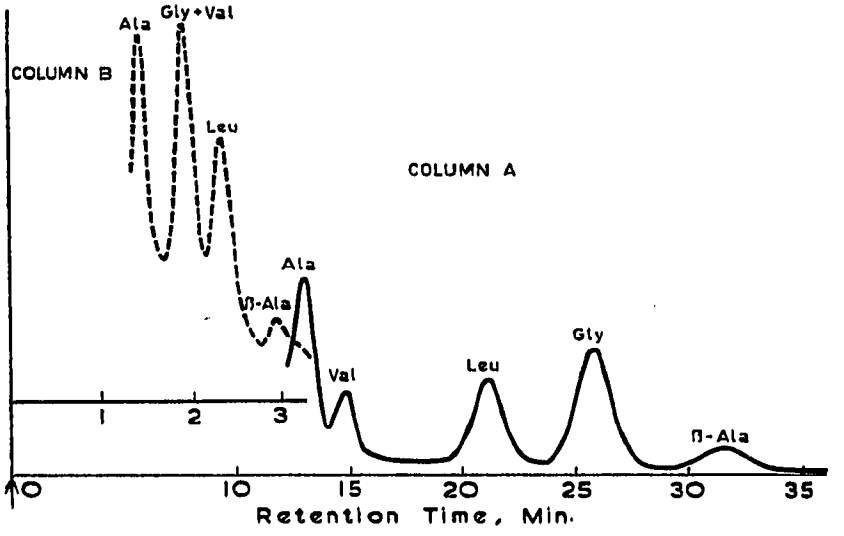

Fig. 2. Gas–liquid chromatograms of N-isobutyryl amino acid isopropyl esters. Sample: A mixture of approx. 1 μg each of the amino acids converted, in 1 μl benzene. Conditions: isothermal at 165°, inj. port 230° and det. 210°. (———), column A: attenuation 2 × 10^2; chart speed 0.25 in./min. (– – – –), column B: attenuation 8 × 10^2; chart speed 1 in./min.

N-Acyl amino acid alkyl esters

The conversion of amino acid to its N-acyl amino acid ester generally was accomplished by esterification of the amino acid in alcohol in the presence of a hydrogen halide. The amino acid ester was then N-acylated by reacting with excess of acid anhydride or acid chloride. However, the total percentage of conversion was not quantitative. Particularly, in this investigation, when higher aliphatic acid anhydrides or chlorides were used, the removal of aliphatic acid formed and the excess acid anhydride or chloride, by distillation *in vacuo*, was found too difficult. Decomposition often took place even at very low pressure.

The N-acylation of the amino acid followed by esterification had to be investigated, from which procedure II was established. The esterification of N-acyl amino acid with alcohol in the presence of Amberlite IR-120 (H) or hydrogen chloride was found to be quantitative. The removal of alcohol was accomplished by distillation at comparatively low temperature. Acyl esters which showed two or three components when prepared by procedure I were obtained in GLC pure forms. The esters converted by procedure IIc or d were compared with the corresponding authentic samples of N-acyl esters at the same concentration. Their retention time, peak height and peak

shape on the chromatogram were indistinguishable in all aspects. Since the esterifications proved to be quantitative, it was possible to test the percentage conversion of the N-acylation step. If the resulting N-acyl amino acid ester was not quantitative, the percentage yield must be due to the N-acylation procedure employed. Some of the standard N-acyl amino acid esters prepared were isolated and purified by micro fractional distillation or recrystallization. In some cases, elementary analyses were performed to ascertain identity and purity. These standard N-acyl amino acid esters have been stored at 0° in the dark for more than a year without deterioration. These pure samples were also used as standards to determine minor variations of new columns. In no instance was N-diacylation of the amino acid observed in any of the procedures described.

GLC studies

The aliphatic amino acids: glycine, alanine, butyrine*, valine, leucine and β-alanine were chosen on account of their gradual increase in size of the side chain R group on the amino acid structure, as shown in Fig. 1. Symmetric substitutions on the N and C terminals of the amino acid were made; for example, the CH_3 of the acetyl and the CH_3 of the methyl ester, in N-acetyl amino acid methyl ester, which straddled the $-CONH\overset{R}{\overset{|}{C}}HCOO-$ unit, assumed a form of symmetry; as did the C_2H_5 of *n*-propionyl and the C_2H_5 of the ethyl ester in N-*n*-propionyl amino acid ethyl ester, etc.

The N-acylating groups were from acetyl to enanthyl and the esters were from methyl to *n*-hexyl. Among all amino acid derivatives studied, the N-butyryl amino acid propyl esters give the most desirable pattern. Further, 24 amino acids were chromatographed in both column A and B, shown in Table I. Although column B showed very fast elution of the six aliphatic amino acids, it gave desirable retention times and patterns for acidic, basic and some heterocyclic amino acids whose retention times on column A would be too long to be practical for routine analytical use. Their elution patterns were further improved at temperature-programmed conditions. The non-polar column apparently offered the most advantages for amino acids of complex structure. By pairing these two columns, all the amino acids gave distinct peaks, except arginine, ornithine and citrulline. The order of elution on the polar and non-polar columns was not identical but there was a common elution pattern.

Effect of substituents on the N and C terminals of amino acids

The effects of N-acyl and alkyl ester substituents were studied individually and in combination. Glycine was used as a standard unit in the acyl alkyl esters. N-Acylglycine methyl esters, N-acetylglycine alkyl esters and N-acylglycine alkyl esters were chromatographed on both columns at isothermal temperatures. The effect of the N-acyl group on the retention time is shown in Table II. On changing the ester group from methyl to *n*-hexyl on acetylglycine, the retention time was systematically prolonged, as shown in Table III. Although the effect of the alkyl ester appeared to be slightly greater than that of the N-acyl, the difference is so slight that they may actually be considered of the same order of magnitude. The combined effects of N-acyl

* Butyrine was eluted from the columns with valine in most cases. Therefore, it was not used as a component when a mixture of amino acids was converted for chromatographic studies.

TABLE I

RETENTION TIME (min) OF *n*-BUTYRYL AMINO ACID *n*-PROPYL ESTERS

Temperatures: injection port 230°, detector 210°.

Column A			*Column B*		
Amino acid	*Isothermal 165°*	*Programmed 100–185° (4°/min)*	*Isothermal 150°*[a]	*Isothermal 165°*	*Programmed 100–180° (4°/min)*[a]
Ala	36.6	30.0	6.3	2.6	13.9
But[b]	40.6	31.3	6.8	3.1	14.5
Val	41.0	31.3	7.8	3.5	14.9
isoLeu	51.6	40.6	10.2	4.4	16.6
Leu	61.2	37.6	10.4	4.8	16.9
Gly	67.2	41.4	7.8	3.6	15.4
β-Ala	82.0	47.4	11.7	5.0	17.2
Pro	109.6	67.4	17.7	7.2	18.9
γ-But[c]	[d]	97.0	26.6	10.6	21.1
Thr	[d]	140.4	38.0	14.0	24.4
Asp	[d]	[d]	41.6	17.0	26.6
Ser	[d]	[d]	47.2	18.2	25.6
Met			60.0		29.6
Phe			66.0		28.8
Cys			96.4		34.8
Glu			105.2		33.7
Hypro			149.6		46.0
Lys			[d]		21.9
Tyr			[d]		22.2
His			[d]		33.6[e]
Try			[d]		53.6[e]
Arg			[d]		[d]
Orn			[d]		[d]
Cit			[d]		[d]

[a] Injection port 240° and detector 220°.
[b] See footnote on p. 210.
[c] γ-Aminobutyric acid, *cf.*[b].
[d] No peak appeared in additional 120 min.
[e] Programmed column temperature 100–230 (4°/min), injection port 270° and detector 250°.

TABLE II

RETENTION TIME (min) OF N-ACYLGLYCINE METHYL ESTERS

Temperatures: injection port 230°, detector 210°.

N-Acyl group	*Column A*		*Column B 165°*	*Ester group*
	165°	*200°*		
Acetyl	28.8	8.8	1.5	Methyl
Propionyl	30.1	9.5	1.6	Methyl
n-Butyryl	39.5	11.9	2.0	Methyl
n-Valeryl	57.8	16.8	2.8	Methyl
n-Caproyl	83.6	21.4	4.4	Methyl
Enanthyl	117.4	31.0	6.4	Methyl

TABLE III

RETENTION TIME (min) OF N-ACETYLGLYCINE ALKYL ESTERS

Temperatures: injection port 230°, detector 210°.

N-Acyl group	*Column A*		*Column B*	*Ester group*
	165°	*200°*	*165°*	
Acetyl	28.8	8.8	1.5	Methyl
Acetyl	31.9	9.0	1.6	Ethyl
Acetyl	44.6	12.2	2.3	*n*-Propyl
Acetyl	66.4	16.8	3.2	*n*-Butyl
Acetyl	96.0	23.4	4.4	*n*-Amyl
Acetyl	148.0	35.0	6.2	*n*-Hexyl

TABLE IV

RETENTION TIME (min) OF N-ACYLGLYCINE ALKYL ESTERS

Temperatures: injection port 230°, detector 210°.

N-Acyl group	*Column A*		*Column B*	*Ester group*
	165°	*200°*	*165°*	
Acetyl	28.8	8.8	1.5	Methyl
Propionyl	32.2	10.2	1.9	Ethyl
n-Butyryl	67.2	18.1	3.6	*n*-Propyl
n-Valeryl	145.4	34.8	7.2	*n*-Butyl
n-Caproyl	—	58.8	14.0	*n*-Amyl
Enanthyl	—	118.2	29.4	*n*-Hexyl

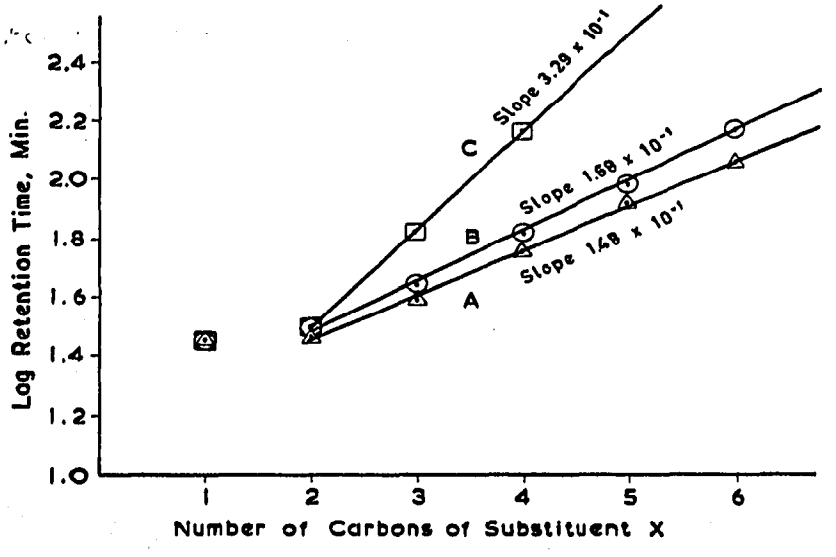

Fig. 3. Log retention time *vs.* number of carbons of substituent X on column A, isothermal at 165°. A (△—△), X-$CONHCH_2COO-CH_3$, B (⊙—⊙), CH_3-$CONHCH_2COO$-X, C (⊡—⊡), X-$CONHCH_2COO$-X.

and alkyl esters on the retention time are shown in Table IV. When the data in the three tables were plotted, log retention time *vs.* number of carbons of the substituents X (see Fig. 1), a linear relationship was revealed. It was particularly noteworthy that the slope of N-acylglycine methyl esters plus the slope of N-acetylglycine alkyl esters equaled the slope of N-acylglycine alkyl esters, in all cases, which demonstrated that the effects of the substituents X were additive; an example is shown in Fig. 3.

Substituents X used were all normal (straight chain) aliphatic groups. The retention time was found to be proportionally prolonged with increasing size of X. The size increment of X accompanying the increase in molecular weight is a factor which should not be overlooked. In order to distinguish between these two effects, the chromatograms were made of N-isobutyryl amino acid isopropyl esters which are the structural isomers of N-*n*-butyryl amino acid *n*-propyl esters. If the molecular weight increment was the dominating factor affecting the retention time, the corresponding isomers should have nearly identical GLC patterns and retention times. The fact was that the retention time of the N-isobutyryl amino acid isopropyl esters shown in Table V, which was approximately one third that of the *n*-isomers, shown in Table I, was found to fall near their corresponding N-acetyl amino acid methyl esters, shown in Table VI. This indicates that the shape of the substituent X rather than other factors dominated the GLC patterns and retention times.

TABLE V

RETENTION TIME (min) OF N-ISOBUTYRYL AMINO ACID ISOPROPYL ESTERS

Temperatures: injection port 230° and detector 210°.

Amino acid	*Column A*		*Column B*
	165°[a]	*200°*	*165°*[a]
Ala	13.2	4.5	1.5
Val	15.3	5.1	1.9
But	15.6	5.0	1.3
Leu	21.3	6.6	2.3
Gly	26.0	7.8	1.9
β-Ala	31.8	9.6	2.8

[a] See also Fig. 2.

TABLE VI

RETENTION TIME (min) OF N-ACETYL AMINO ACID METHYL ESTERS

Temperatures: injection port 230°, detector 210°.

Amino acid	*Column A*		*Column B*
	165°	*200°*	*165°*
Ala	15.2	5.0	1.2
Val	17.8	5.8	1.5
But	18.2	5.9	1.5
Leu	26.2	8.2	2.1
Gly	28.0		1.6
β-Ala	34.0	10.3	2.1

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