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DIRECT RESOLUTION OF ENANTIOMERS OF 2- AND 3-HYDROXY ACID ALKYL ESTERS BY FUSED-SILICA CAPILLARY GAS CHROMATOGRAPHY

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SUMMARY

The gas chromatographic resolution of enantiomers of eighteen hydroxy acids as their alkyl esters on the optically active stationary phase Chirasil-Val is described. The problem of peak tailing due to the free hydroxy group is overcome by the application of fused-silica capillary columns. Five different alcohol components of the esters were examined, the best overall results being obtained with 3-pentanol. As a rule, the L-enantiomers of 2-hydroxy acid esters are eluted first from the stationary phase containing L-valine, whereas with the 3-hydroxybutanoic acid ester the order of elution is reversed. The separation factors are closely related to structural features.

INTRODUCTION

Hydroxy acids play an important role in the metabolism of biological systems[1]. Therefore, the simultaneous determination of the enantiomeric composition of a large number of compounds of this class is highly desirable. In addition, the method should be readily accessible.

The gas chromatographic separation of diastereomers[2,3] on an achiral stationary phase as compared with the resolution of enantiomers on an optically active phase[4], is handicapped by well known shortcomings. Several efforts have been made to solve the problem according to the latter approach, as follows.

The three-step conversion to O-acylated hydroxy acid amides permitted a baseline resolution[5,6] on the optically active stationary phase Chirasil-Val (Applied Science Labs., College Station, PA, U.S.A.). However, mandelic acids showed an unacceptably high degree of racemization during the derivatization procedure, as is demonstrated by an alternative method, *viz.*, reduction with lithium aluminium hydride to phenylglycols which were resolved as their di-O-pentafluoropropionates[7].

Derivatization of the hydroxy group by isocyanates yields urethanes that can be resolved on chiral polysiloxanes of the Chirasil-Val type[8–10], but in our hands the course of the reaction was not satisfactory owing to low conversion yields and the formation of large amounts of side products[11]. Although successful resolutions of

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enantiomers of nitrogen-free substrates on Chirasil-Val are well known[5,7], the O-acylated hydroxy acid esters exhibit only small separation factors. Slightly better results were obtained by König *et al.*[12], who examined a variety of optically active stationary phases, thus achieving sufficient separation in special cases using highly efficient glass capillaries. Nevertheless, this approach is not a practical route.

Our target is the applicability of a single stationary phase to almost any class of compound. For this reason, we focused our attention on the direct resolution of hydroxy acid alkyl esters on Chirasil-Val, without resorting to any derivatization of the hydroxy group. In contrast to Ôi *et al.*[13], who reported moderate resolutions of lactic acid ester enantiomers on several triazine-linked peptide phases, we generally observed considerable separation factors. The problem of peak tailing due to the free hydroxy group was overcome by the application of fused-silica capillary columns, thus permitting a baseline resolution in most instances. Further, we were not faced with peak broadening due to kinetic effects, as obtained by Ôi *et al.*[14] in the resolution of hydroxy acid alkyl esters on optically active copper complexes.

Having a convenient system to hand, we worked towards the simultaneous resolution of a large number of substrates. The methodical variation of the alcohol component of the esters in order to optimize the system should also yield a tentative mechanistic rationale.

EXPERIMENTAL

Materials

RS- and *S*-lactic acid, *RS*-2-hydroxybutanoic acid (sodium salt), *RS*-2-hydroxy-3-methylbutanoic acid, *RS*-2-hydroxypentanoic acid, *RS*- and *S*-2-hydroxy-4-methylpentanoic acid, *RS*-3-phenyllactic acid and *RS*- and *S*-malic acid were obtained from Sigma (St. Louis, MO, U.S.A.). *RS*-2-Hydroxy-2-methylbutanoic acid, *RS*- and *S*-mandelic acid and *S*-3-phenyllactic acid were purchased from EGA. *RS*-2-Hydroxyhexanoic acid, *RS*-2-hydroxyoctanoic acid, *RS*-2-phenyllactic acid and *RS*-3-hydroxybutanoic acid were supplied by Fluka. Aliphatic alcohols were obtained from EGA.

RS-2-Hydroxy-3,3-dimethylbutanoic acid[15], *RS*- and *S*-hexahydromandelic acid[16], *RS*- and *S*-2-hydroxy-4-(methylmercapto)butanoic acid[17], 2*RS*,3*RS*-, 2*R*,3*S*- and 2*S*,3*S*-2-hydroxy-3-methylpentanoic acid[17] were prepared according to literature procedures.

Esterification of carboxylic groups

Anions were converted into the corresponding acids by micro-scale cation-exchange chromatography before esterification.

Typically, 0.1–1 mg of the sample was placed in a "conic vial" (Macherey, Nagel & Co., Düren, G.F.R.), volume 1 ml, sealed with a PTFE-coated septum, and 0.5 ml of the reagent was added. After reaction, the solvent and excess of reagents were removed in a stream of dry nitrogen. The residue was dissolved in 0.5 ml of dry diethyl ether and used for gas chromatography.

Reaction conditions. For methyl esters, a solution of diazomethane in diethyl ether (*ca.* 3%), 2 h at ambient temperature; for *n*-propyl-, isopropyl-, *tert.*-butyl- and 3-pentyl esters, a solution of hydrochloric acid in the appropriate alcohol [prepared by addition of 10% (v/v) acetyl chloride to the alcohol], 1 h at 110°C.

Gas chromatography

Two Chirasil-Val batches (phase I and phase II)[18], with different ratios of chiral to non-chiral siloxane units, were employed.

Fused-silica capillary columns (20 m × 0.25 mm I.D.) were supplied by Applied Science Labs. After deposition of colloidal silicic acid[19], the columns were coated statically with 0.3% (w/w) of Chirasil-Val in pentane.

Gas chromatography was performed in Carlo Erba Model 2101 gas chromatographs with hydrogen as the carrier gas (typically 0.6 bar, splitting ratio 1:10). Net retention times (from solvent peak) were measured with a Spectra-Physics System I electronic integrator. Linear regressions were calculated by a computer program according to the method of Deming[21], with some modifications[7].

RESULTS AND DISCUSSION

First, the methyl esters of some representative 2-hydroxy acids were explored briefly. Although the separation factors (α) were satisfactory, the practical value of this method was diminished by several restrictions. Owing to the high volatility of the methyl esters of short-chain hydroxy acids (*e.g.*, lactic acid), part of these compounds was lost during the concentration procedure and, in addition, their retention times at 50°C were too short. In multi-functional compounds, the methyl esters turned out to be less favourable in terms of their polarity.

At this point, some general considerations became necessary, as the well aimed choice of the alcohol part of the ester was expected to harbour a significant potential for the optimization of the method. An enlargement of the alcohol should lead to opposing effects: the polarity of the derivative is reduced, thus improving the chromatographic properties, while at the same time decreasing the volatility of short-chain acid esters. On the other hand, long-chain acid esters may be excessively retarded. The higher temperature required for elution in an acceptable time, however, leads to lower enantiomeric separation factors (α). Hence, it follows that branched-chain alcohols are preferable, but additional chiral structures should be omitted. Further, the chemical behaviour during the derivatization procedure should be taken into account, e.g., *tert.*-butanol yields the *tert.*-butyl cation in an acidic medium, giving rise to side reactions.

Beyond that, the chiral recognition of the esters is an important factor. To gain an insight into this problem, we examined the separation factors for a characteristic set of esters at different temperatures. Fig. 1 shows the results for an almost complete matrix of esters of five different alcohols with seven selected hydroxy acids. Thirty-three different compounds are shown, as the methyl ester and the *tert*-butyl ester of lactic acid are omitted because of their too small retention times. The accuracy of the data was improved by recording each racemate at several temperatures, the number of data points being five to eight. Linear regression permitted the calculation of α values by interpolation (at 70°C) or extrapolation (at 25°C).

Some important conclusions can be drawn. As expected, the α value is influenced strongly by the residue R of the hydroxy acid. The effect of a homologous series of *n*-alkyl chains, from R = methyl (*i.e.*, lactic acid) up to R = *n*-butyl (*i.e.*, 2-hydroxyhexanoic acid) is instructive: a maximum in α occurs for R = ethyl. It is apparent that the addition of an alkyl group at C-3, being in a β-position relative

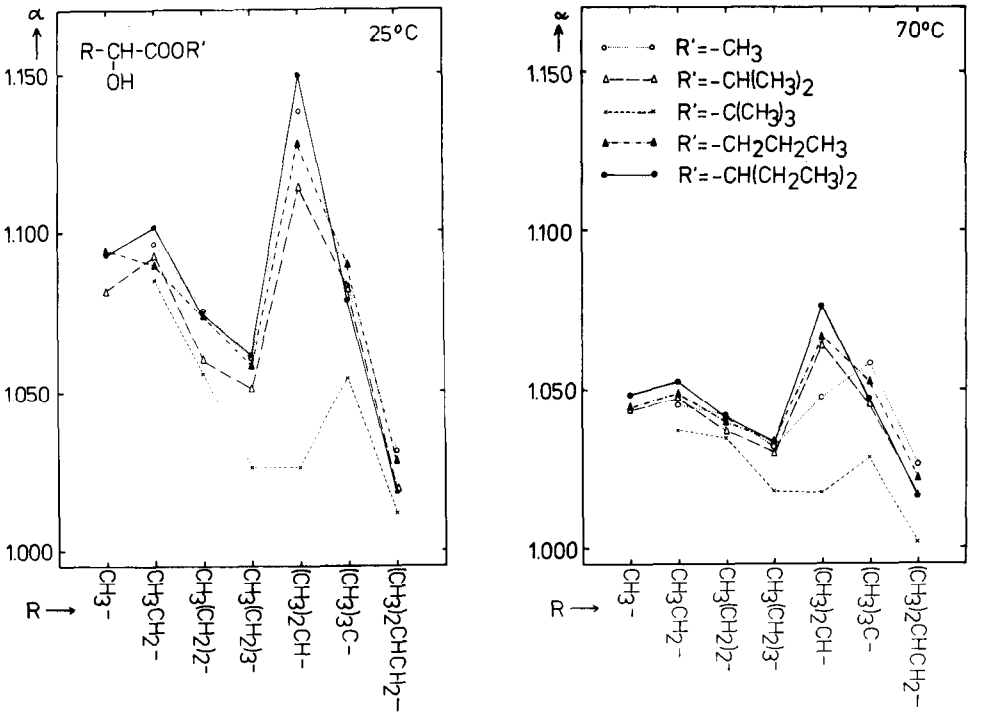


Fig. 1. Separation factors (α) of a characteristic set of 33 2-hydroxy acid alkyl esters on Chirasil-Val (phase I) at two different temperatures.

to the asymmetric centre (see Fig. 2), is favourable for the resolution, whereas the γ-position is unfavourable and the effect of larger *n*-alkyl chains is unclear owing to their conformational flexibility. This hypothesis receives strong support from the findings with branched alkyl chains. Attachment of a second methyl group in the β-position (*i.e.*, 2-hydroxy-3-methylbutanoic acid) greatly increases the chiral recognition, while the effect of two methyl groups in the γ-position (*i.e.*, 2-hydroxy-4-methylpentanoic acid) is a large decrease in the resolution. It is interesting that the addition of a third methyl group in the favourable β-position (*i.e.*, 3,3-dimethylbutanoic acid) does not increase the separation. The *tert.*-butyl group is not able to contribute to an induced fit in the same manner as the isopropyl group. This explanation is corroborated by the strong temperature dependence of the α value in the latter instance, owing to the high entropy contribution.

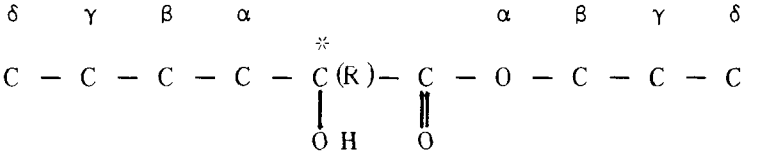


Fig. 2. Assignment of positions in 2-hydroxy acid esters.

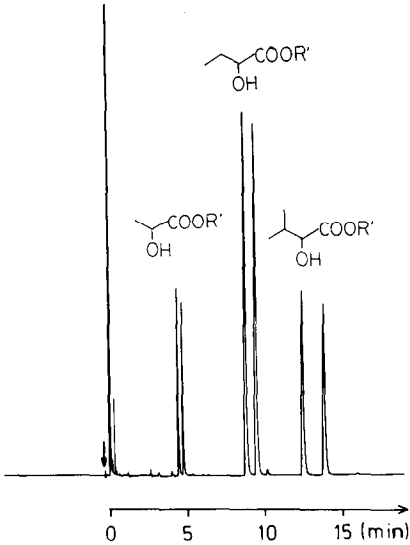


Fig. 3. Resolution of enantiomers of 2-hydroxy acid 3-pentyl esters on a fused-silica capillary column (20 m × 0.25 mm I.D.), coated with Chirasil-Val (phase I), at 50°C isothermal, illustrating the influence of methyl substituents in the β-position (in relation to the asymmetric centre) on the separation factors (α).

An analogous trend is seen in the alkyl group R'. Being in the β-position (with respect to the carbonyl group; *cf.*, Fig. 2), the methyl esters exhibit considerable α values. These were decreased continually by the attachment of two or three methyl groups in the less favourable γ-position, thus ruling out the isopropyl and *tert.*-butyl esters for practical use. This is not only a consequence of sheer steric bulk, as is seen from the 3-pentyl esters. Compared with the isopropyl esters, the addition of two methyl groups in the favourable δ-position results in a significant improvement of chiral recognition, following the trend that has been already observed with the *n*-propyl esters. It should be added that these conclusions are confined to the range of temperature examined.

Bearing all these facts in mind, we decided to focus our attention on the 3-pentyl esters. An instructive example is given in Fig. 3.

The next aim was to optimize the separation of a series of compounds. During these investigations, it was found that the separation factors for 2-hydroxy acid esters could be improved significantly by increasing the charge of the silicon chain of Chirasil-Val with diamide groups from one seventh (phase I) to one fifth (phase II) of each silicon atom. The results are recorded in Fig. 4. For all 2-hydroxy acids investigated as the 3-pentyl esters on phase II, a satisfactory resolution was observed. Esters of aliphatic hydroxy acids up to 2-hydroxyhexanoic acid (15,16) were eluted within 40 min. The trend in the separation factors as demonstrated in Fig. 1 was maintained, yet the differences between the distinctive hydroxy acids were less pronounced.

Aryl-substituted hydroxy acids were resolved even better, mandelic acid (21,22) exhibiting the maximum separation factor (*i.e.*, $\alpha = 1.12$ at 50°C). With the homologous 3-phenyllactic acid, the separation is only moderate, in complete analogy with the findings with simple alkyl substituents.

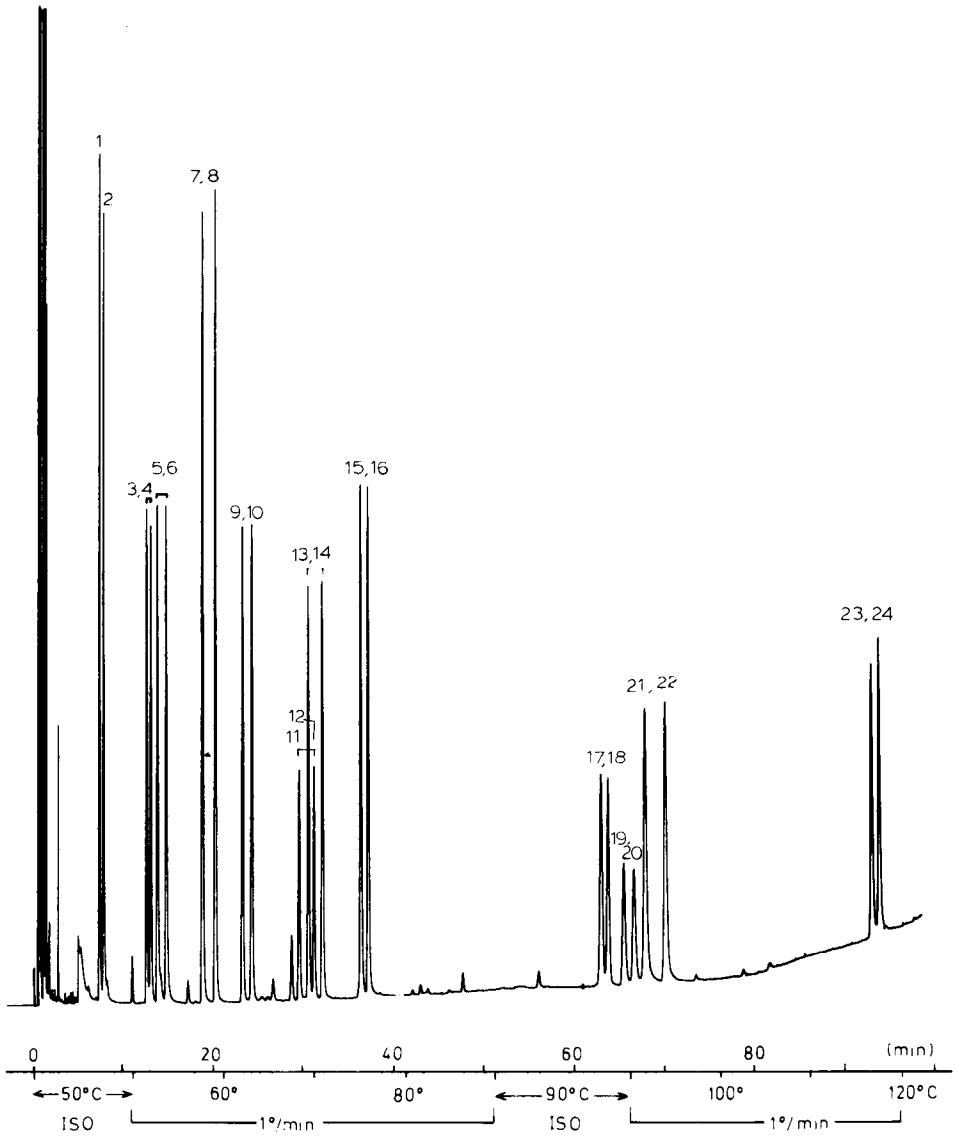


Fig. 4. Resolution of the enantiomers of 2-hydroxy acid 3-pentyl esters on a fused-silica capillary column (20 m × 0.25 mm I.D.), coated with Chirasil-Val (phase II). Peak numbering according to Table I.

Examples of multi-functional compounds are malic acid (23,24) and 2-hydroxy-4-(methylmercapto)butanoic acid (19,20), derived from methionine. As expected, the analogy between the 2-(methylmercapto)ethyl group and the approximately isosteric *n*-butyl group (15,16) is shown well by the α values.

Branched 2-hydroxy acids bearing two different hydrocarbon residues at the asymmetric center, *i.e.*, atrolactic acid (2-phenyllactic acid) (17,18), and 2-hydroxy-2-methylbutanoic acid (3,4), were also amenable to our method, although the sep-

aration factors were significantly decreased in comparison with the parent compounds with only one of the two residues.

It has been pointed out that the separation factor is closely related to structural features. Therefore, it is not surprising that a relatively high specificity was observed in the retention behaviour. Thus we were able to resolve the 3-pentyl esters of nine isomeric 2-hydroxy acids of the formula $C_6H_{12}O_3$ completely from each other. The side chains are *tert.*-butyl (9,10), *S*- and *R-sec.*-butyl (11,12 and 13,14, derived from *allo*-isoleucine and isoleucine, respectively), isobutyl (*S*-2-hydroxy-4-methylpentanoic acid, derived from leucine) and *n*-butyl (15,16). Only the *R-sec.*-butyl residue (14) was not completely discriminated from the isobutyl residue in *R*-2-hydroxy-4-methylpentanoic acid 3-pentyl ester under these conditions.

The results are summarized in Table I. Seventeen 2-hydroxy acids were examined. In ten instances, the absolute configuration was assigned to the peaks by injection of the enantiomerically enriched compounds. An illustration is given for 2-hydroxy-3-methylpentanoic acid. The 2-hydroxy acid was synthesised from the corresponding 2-amino acid by diazotization, which is known to proceed with overall retention at C-2 due to double inversion[17,21]. Starting from a racemic mixture of isoleucine and *allo*-isoleucine, four peaks were obtained (11–14). The reaction of L-isoleucine with *erythro*-configuration (2*S*,3*S*) yielded mainly one isomer (13). From D-*allo*-isoleucine with *threo*-configuration (2*R*,3*S*), peak 12 was derived. The allocation of peaks 11 and 14 was possible by comparing the peak areas in the racemates (*cf.*, Fig. 4), *e.g.*, 11 was enantiomeric to 12 and therefore assigned to the opposite configuration (2*S*,3*R*).

With respect to C-2, the absolute configuration of the 2-hydroxy acid 3-pentyl ester enantiomer that is eluted first from Chirasil-Val (with L-valine) is L in all examples studied up to now (usually this corresponds to the *S*-configuration). It can concluded from this result that the D-enantiomers enter into the stronger interactions with the optically active stationary phase. Interestingly, the order of emergence is opposite to the behaviour of N-acetylated 2-amino acid esters[4,22]. Obviously, there are some differences between the two classes of compounds concerning the mechanism of interaction with the stationary phase.

Encouraged by the promising findings with 2-hydroxy acids, we extended our investigations to 3-hydroxybutanoic acid (Table II). The separation factors of different alkyl esters did not correspond exactly with the pattern observed with 2-hydroxy acid esters. The methyl ester was not resolved at all and the *n*-propyl and isopropyl esters only partially. Whereas the bulky *tert.*-butyl ester was not resolved, the larger 3-pentyl ester furnished the best overall results for separation factor, volatility and polarity. Hence it is gratifying that the same procedure is applicable to both 2- and 3-hydroxy acids.

Surprising at first glance, *R*-3-hydroxybutanoic acid 3-pentyl ester is eluted before its enantiomer from Chirasil-Val (containing L-valine), the order of emergence being reversed, compared with the 2-hydroxy acid esters. This discovery can be explained by the observation that a similar trend, but with opposite sign, occurs on going from the N-acylated esters of 2-amino acids to the derivatives of 3-amino acids[23]. In order to corroborate this result, the absolute configuration of 3-hydroxybutanoic acid was correlated unambiguously with the series of 2-hydroxy acids, as is outlined in Fig. 5. *S*-3-(1′-Ethoxyethoxy)butyronitrile, synthesized in four steps

TABLE I

RESOLUTION OF 2-HYDROXY ACID 3-PENTYL ESTERS ON CHIRASIL-VAL (PHASE II)

*No**	*R''*	*R*	*Absolute configuration*	*Compound*	*RRT*** *at 70°C*	α***	
						70°C	*50°C*
1	–H	$-CH_3$	*S* (L)	Lactic acid	0.0260	1.0565	1.0708
2			*R* (D)		0.0274	±0.0022§	±0.0043§
3	$-CH_3$	$-CH_2CH_3$	§§	2-Hydroxy-2-methyl-butanoic acid	0.0441	1.0273	1.0377
4					0.0455	±0.0017	±0.0020
5	–H	$-CH_2CH_3$	§§	2-Hydroxybutanoic acid	0.0473	1.0589	1.0778
6					0.0501	±0.0032	±0.0047
7	–H	$-CH(CH_3)_2$	*S* (L)	2-Hydroxy-3-methyl-butanoic acid	0.0664	1.0810	1.1070
8			*R* (D)		0.0718	±0.0016	±0.0027
9	–H	$C(CH_3)_3$	§§	2-Hydroxy-3,3-dimethyl-butanoic acid	0.0887	1.0552	1.0725
10					0.0936	±0.0013	±0.0022
§§§	H	$-(CH_2)_2CH_3$	§§	2-Hydroxypentanoic acid	0.0910	1.0536	1.0651
§§§					0.0959	±0.0042	±0.0054

11	-H	$CH(CH_3)CH_2CH_3$	2*S*,3*R* (L)	2-Hydroxy-3-methyl-pentanoic acid	0.1260	1.0957	1.1190
12			2*R*,3*S* (D)		0.1383	±0.0079	±0.0106
13			2*S*,3*S* (L)		0.1352	1.0895	1.1112
14			2*R*,3*R* (D)		0.1475	±0.0074	±0.0102
§§§	-H	$CH_2CH(CH_3)_2$	*S* (L)	2-Hydroxy-4-methyl-pentanoic acid	0.1413	1.0327	1.0361
§§§			*R* (D)		0.1459	±0.0032	±0.0038
15	-H	$-(CH_2)_3CH_3$	§§	2-Hydroxyhexanoic acid	0.1870	1.0480	1.0610
16					0.1963	±0.0033	±0.0044
§§§	-H	$-(CH_2)_5CH_3$	§§	2-Hydroxyoctanoic acid	0.813	1.0467	1.0591
§§§					0.851	±0.0012	±0.0018
17	$-CH_3$	$-C_6H_5$	§§	2-Phenyllactic acid	0.838	1.0335	1.0472
18					0.862	±0.0014	±0.0023
19	-H	$-CH_2CH_2SCH_3$	*S* (L)	2-Hydroxy-(4-methyl-mercapto)butanoic acid	0.904	1.0477	1.0627
20			*R* (D)		0.953	±0.0027	±0.0042
§§§	-H	$-C_6H_{11}$	*R*(L)	Hexahydromandelic acid	0.911	1.0795	1.1049
§§§			*R* (D)		0.984	±0.0051	±0.0075
21	-H	$-C_6H_5$	*S*(L)	Mandelic acid	1.000	1.0956	1.1228
22			*R* (D)		1.096	±0.0024	±0.0032
§§§	-H	$-CH_2C_6H_5$	*S* (L)	3-Phenyllactic acid	2.340	1.0228	1.0288
§§§			*R* (D)		2.387	±0.0009	±0.0014
23	-H	$-CH_2COOCH(CH_2CH_3)_2$	*S* (L)	Malic acid	3.284	1.0587	1.0711
24			*R* (D)		3.478	±0.0044	±0.0062

* *cf.*, Fig. 2.

** RRT = relative retention time, calculated from the linear regression; reference compound is *S*-mandelic acid 3-pentyl ester, net retention time = 61.67 min at 70°C and 0.6 bar H_2.

*** α = Separation factor of the enantiomers, calculated from the linear regression = net retention time of the second peak/net retention time of the first peak.

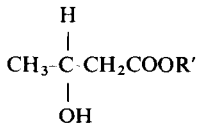
§ Standard deviation of α, calculated as described previously[7].

§§ Absolute configuration not determined.

§§§ Not recorded in Fig. 2.

TABLE II

RESOLUTION OF ENANTIOMERS OF 3-HYDROXYBUTANOIC ACID ALKYL ESTERS ON CHIRASIL-VAL (PHASE I)



R'	*Compound*	*RRT* at 50°C*	*α at 50°C*
CH_3	Methyl ester	0.089	Not resolved
$CH(CH_3)_2$	Isopropyl ester	0.216	1.012
		0.219	
$C(CH_3)_3$	*tert.*-Butyl ester	0.244	Not resolved
$CH_2CH_2CH_3$	*n*-Propyl ester	0.367	1.010
		0.371	
$CH(CH_2CH_3)_2$	3-Pentyl ester *R* (D)	0.981	1.020
	S (L)	1.000	

* RRT = Relative retention time; reference compound is *S*-3-hydroxybutanoic acid 3-pentyl ester, net retention time = 37.34 min at 50°C and 0.6 bar H_2.

from *S*-lactic acid ethyl ester[24], was deprotected and converted into *S*-3-hydroxybutanoic acid 3-pentyl ester in a single step by applying the general derivatization procedure for 3-pentyl esters (Fig. 5).

For the accurate determination of the enantiomeric purity of a given substrate, the stereochemical integrity has to be maintained during the preparation of the sample and also during the chromatographic process. In order to attack at the most vulnerable point, the racemization of mandelic acid was scrutinized. After fractional recrystallization[25] of the diastereomeric salts of *RS*-mandelic acid with *S*-1-phenylethylamine (enantiomeric purity almost 100%)[7], mainly *S*-mandelic acid was obtained. A further enantiomeric enrichment was achieved by fractional recrystallization of the free acid from benzene and from water. After derivatization with hydrochloric acid in 3-pentanol for 1 h at 110°C, the amount of *R*-mandelic acid 3-pentyl ester did not exceed 0.1%. As it is probable that the enantiomeric enrichment was not complete, the extent of racemization during the acid-catalysed esterification appears to be negligible. A comparison with other derivatization methods reveals that esterification causes the smallest amount of racemization[11].

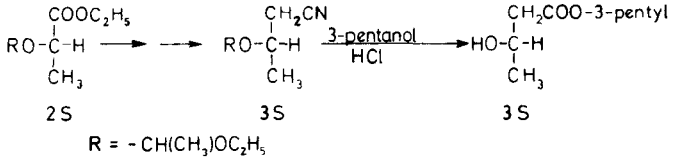


Fig. 5. Chemical correlation of the absolute configuration of *S*-3-hydroxybutanoic acid 3-pentyl ester with *S*-lactic acid ethyl ester.

CONCLUSIONS

Free hydroxy acid alkyl esters are well suited to mechanistical studies owing to their simple composition, thus limiting the possibilities of interaction with the diamide core of Chirasil-Val. As the separation factors are dependent not only on the structures of both the acid and the alcohol part but also on the number of valine residues in the optically active silicone, further improvements are conceivable. A comparison of the chromatographic behaviour of esters of 2- and 3-hydroxy acids with appropriate derivatives of 2- and 3-amino acids deserves attention, but more detailed studies are necessary in order to gain a deeper insight into the mechanism of the resolution.

In conclusion, it has been shown that fused-silica capillary columns, coated with the thermostable, commercially available stationary phase Chirasil-Val, are a powerful tool for the chromatographic resolution of hydroxy acid enantiomers, and additional applications may be expected.

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