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FURTHER STUDIES ON THE CHROMATOGRAPHIC BEHAVIOUR OF DINUCLEOSIDE MONOPHOSPHATES

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SUMMARY

The separation of 2′-5′ dinucleoside monophosphates from their corresponding 3′-5′ isomers on QAE-Sephadex A-25 (bicarbonate form) is described. The column is loaded with a mixture of the two isomers and eluted with a linear gradient of ammonium bicarbonate (from 0.02 to 0.2 *M*). The two isomers appear in two different peaks, and the nucleotidic material is isolated by lyophilization. In addition, the separation of 2′-5′ dinucleoside monophosphates from the corresponding 3′-5′isomers by paper chromatography and by thin-layer chromatography on aluminium cards coated with cellulose powder is described.

INTRODUCTION

UKITA *et al.*[1] reported on the synthesis of dinucleoside monophosphates containing a mixture of 2′-5′ and 3′-5′ linkages and the separation of the two isomers by chromatography on a Dowex 1 X2 (formate form) column. The elution was carried out with formic acid, and special care had to be taken in order to avoid phosphoryl migration. Recently, we showed that 2′-5′ dinucleoside monophosphates can be separated from the corresponding 3′-5′ isomers by chromatography on a DEAE-Sephadex A-25 column (bicarbonate form)[2]. The elution was carried out with ammonium bicarbonate, pH 8.6, and the nucleotidic material was isolated by lyophilization.

In this paper we describe the separation of 2′-5′ dinucleoside monophosphates from the corresponding 3′-5′ isomers on a QAE-Sephadex A-25 column (bicarbonate form). In addition, the separation of 2′-5′ dinucleoside monophosphates from the corresponding 3′-5′ isomers by paper chromatography and by thin-layer chromatography (TLC) on aluminium cards coated with cellulose powder is described.

MATERIALS AND METHODS

QAE-Sephadex A-25 with the capacity of 3.0 mequiv./g was purchased from Pharmacia, Uppsala, Sweden. 2′-5′ and 3′-5′ dinucleoside monophosphates were prepared as described elsewhere[2]. UV absorption was measured on a Cary 14 spectrophotometer.

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The chromatographic apparatus consisted of a 30 × 2.5 cm column (Chromaflex, Kontes, N.J., U.S.A.), a 254-nm UV flow-through cell (LKB Uvicord I), a recorder (LKB 6520) and an LKB fraction collector.

Mixtures of 0.2 ml of 2′-5′ and 0.2 ml of 3′-5′ dinucleoside monophosphates were applied (the concentration of the solutions was 10 mg/ml) and the column was eluted with a linear gradient of ammonium bicarbonate. The mixing vessel contained 2 l of 0.02 *M* ammonium bicarbonate, and the reservoir contained an equal volume of 0.2 *M* ammonium bicarbonate. The flow-rate was 6 ml/min, and fractions of 22.3 ml were collected. The identification of each peak was carried out by high-voltage paper ionophoresis on DEAE-cellulose paper, together with the appropriate markers[3].

Paper chromatography was carried out on Whatman No. 1 paper by the descending technique. TLC was carried out on 20 × 20 cm aluminium cards (Riedel de Haen, Seelze-Hannover, G.F.R.) coated with cellulose powder and fluorescent indicator (DC-Karten CEF). The paper and the thin-layer chromatograms were developed with saturated ammonium sulphate–1 *M* sodium acetate–isopropanol (80:18:2)[4].

RESULTS AND DISCUSSION

The behaviour of the different dinucleoside monophosphates* on the QAE-Sephadex A-25 column is shown in Figs. 1–4. Fig. 1 shows the separation of A (2′-5′) N from the corresponding A (3′-5′) N. In Fig. 1a, A (2′-5′) A is eluted before A (3′-5′) A; in Fig. 1b, A (2′-5′) C is eluted before A (3′-5′) C; in Fig. 1c, A (2′-5′) G is eluted before A (3′-5′) G; and in Fig. 1d, A (2′-5′) U is eluted before A (3′-5′) U.

Figs. 2–4 show the separation of C (2′-5′) N, G (2′-5′) N and U (2′-5′) N from the

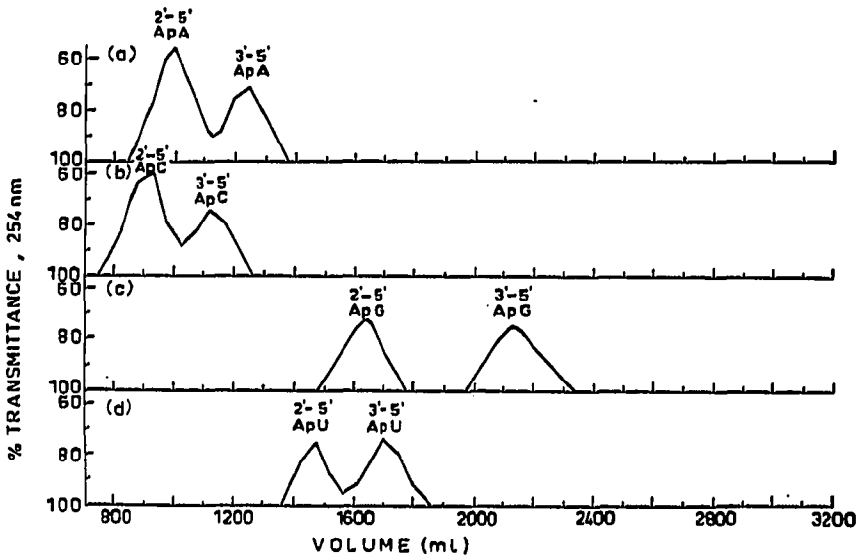

Fig. 1. Chromatography of 2′-5′ and 3′-5′ ApN on a QAE-Sephadex A-25 column. For details see MATERIALS AND METHODS.

* Abbreviations used: A = adenosine; C = cytidine; G = guanosine; U = uridine. NpA, NpC, NpG, NpU = dinucleoside monophosphates; N > p = nucleoside-2′,3′-cyclic phosphate; N = A, C, G and U.

corresponding C (3'-5') N, G (3'-5') N and U (3'-5') N. The separations shown in Figs. 2–4 are similar to those in Fig. 1. CpU was the only dinucleoside monophosphate for which no separation of the two isomers could be detected. As a rule, all the dinucleoside monophosphates containing the unnatural 2'-5' linkage were eluted from the QAE-Sephadex column before the corresponding natural 3'-5' isomers.

From the results in Figs. 1–4, a general rule can be deduced concerning the differences between the 2'-5' and 3'-5' isomers of the different dinucleoside mono-

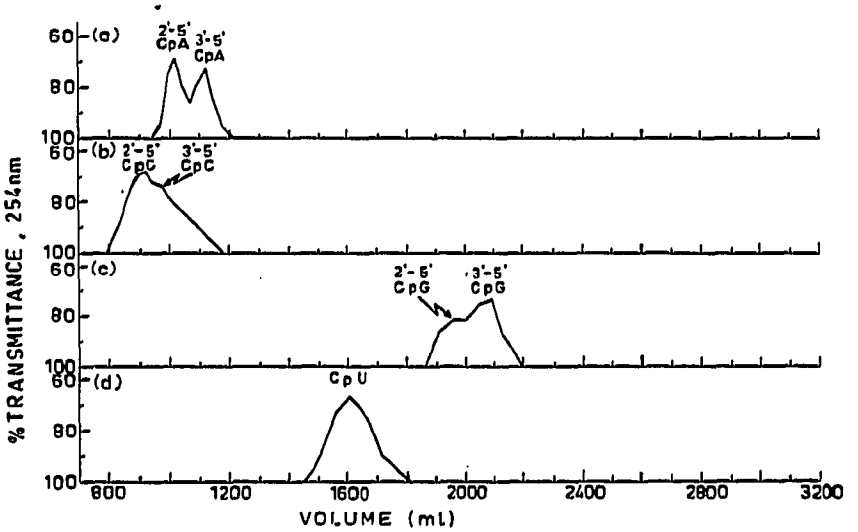

Fig. 2. Chromatography of 2'-5' and 3'-5' CpN on a QAE-Sephadex A-25 column. For details see MATERIALS AND METHODS.

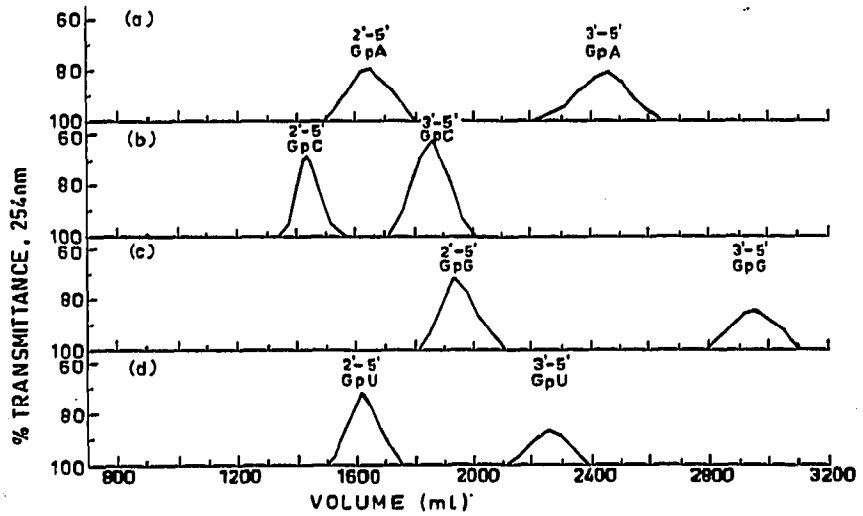

Fig. 3. Chromatography of 2'-5' and 3'-5' GpN on a QAE-Sephadex A-25 column. For details see MATERIALS AND METHODS.

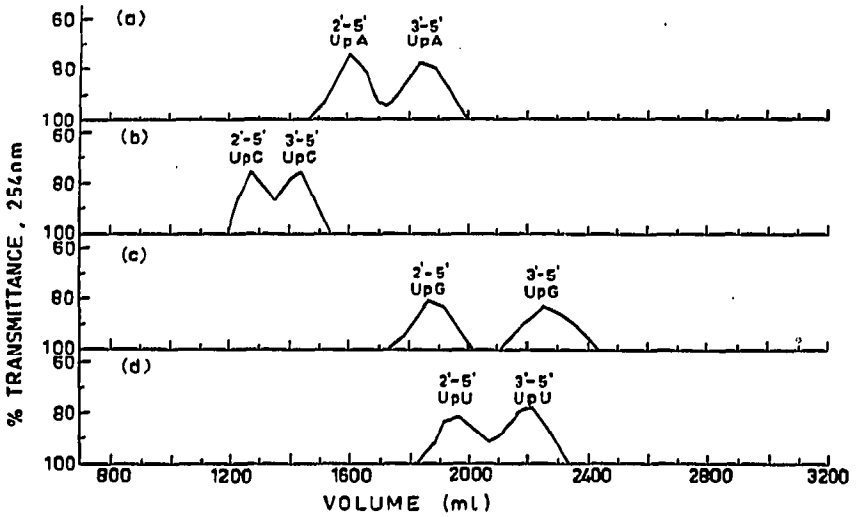

Fig. 4. Chromatography of 2'-5' and 3'-5' UpN on a QAE-Sephadex A-25 column. For details see MATERIALS AND METHODS.

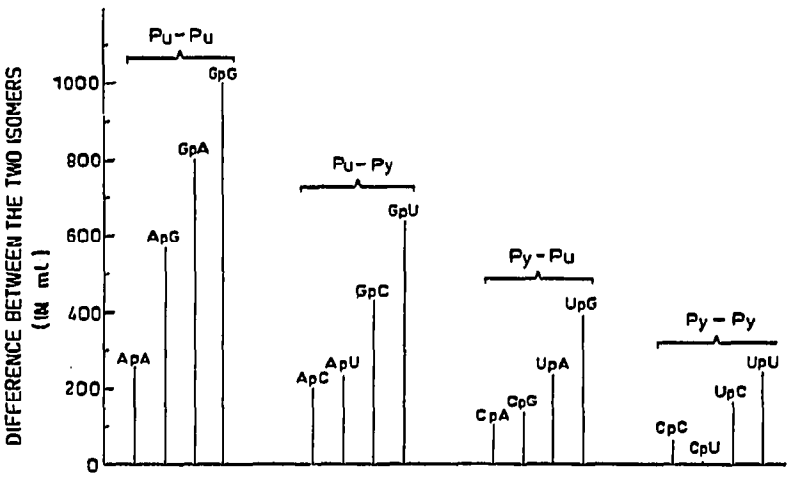

Fig. 5. Separation of 2'-5' and 3'-5' isomers of dinucleoside monophosphates containing the same base sequence on a QAE-Sephadex A-25 column under conditions as in Figs. 1–4. The ordinate corresponds to the difference in millilitres between the peaks of the 2'-5' and the 3'-5' isomers.

phosphates. The differences between the two isomers are given in Fig. 5, and can be summarized by the following equations:

$$GpN > ApN \geqq UpN > CpN \quad (1)$$

$$NpG > NpA \geqq NpU > NpC \quad (2)$$

The differences between the two isomers in eqn. 1 are much greater than those in eqn. 2.

If the elution profile of all the dinucleoside monophosphates containing the same phosphodiester linkage (all 3'-5' or all 2'-5') is drawn, it can be seen in Fig. 1, for

example, that ApC is eluted first, then ApA, then ApU, and finally ApG. The same rule applies to the elution profiles shown in Figs. 2–4, and in general, NpC is eluted first, then NpA, then NpU, and finally NpG. Similarly, CpN is eluted first, then ApN, then UpN, and finally GpN (the only exception being GpU, which is eluted before GpA). In other words, the retardation of a given dinucleoside monophosphate depends on its base composition. Those containing guanine are more strongly retarded than those containing uracil, and the latter are more retarded than those containing adenine. The least retarded are those containing cytosine. Thus, the order of retardation is G > U > A > C. A similar order of retardation was found when mononucleotides were chromatographed on a QAE-Sephadex A-25 column[5], when the order of elution was Cp, then Ap, then Up, and finally Gp.

Recently, we reported on the separation of 2′-5′ dinucleoside monophosphates from the corresponding 3′-5′ isomers by ion-exchange TLC and paper ionophoresis[3]. We have extended this work to paper chromatography and TLC on aluminium cards coated with cellulose powder.

MARKHAM AND SMITH[6] found that purine-2′-phosphate can be separated from purine-3′-phosphate by paper chromatography, using saturated ammonium sulphate–water–isopropanol (79:19:2) as solvent. No separation was found between pyrimidine-2′-phosphate and pyrimidine-3′-phosphate. In 1968, WIGLER[4] reported on the separation of cytidine-2′-phosphate from cytidine-3′-phosphate and uridine-2′-phosphate from uridine-3′-phosphate on an Avicel SF thin-layer chromatogram, using saturated ammonium sulphate–1 *M* sodium acetate–isopropanol (80:18:2) as solvent. In the present work, the same solvent was used for the separation of 2′-5′ dinucleoside monophosphates from the corresponding 3′-5′ isomers by paper chromatography and TLC.

TABLE I

PAPER CHROMATOGRAPHY OF DINUCLEOSIDE MONOPHOSPHATES AND MONONUCLEOTIDES

Nucleotide	R_F *value*	*Nucleotide*	R_F *value*
A (2′-5′) A	0.09	C (2′-5′) A	0.25
A (3′-5′) A	0.04	C (3′-5′) A	0.21
A (2′-5′) C	0.28	C (2′-5′) C	0.57
A (3′-5′) C	0.14	C (3′-5′) C	0.49
A (2′-5′) G	0.22	C (2′-5′) G	0.45
A (3′-5′) G	0.08	C (3′-5′) G	0.35
A (2′-5′) U	0.26	C (2′-5′) U	0.57
A (3′-5′) U	0.14	C (3′-5′) U	0.52
A2′p	0.30	C2′(3′)p	0.78
A3′p	0.20		
A > p	0.12	C > p	0.51
G (2′-5′) A	0.23	U (2′-5′) A	0.26
G (3′-5′) A	0.10	U (3′-5′) A	0.21
G (2′-5′) C	0.44	U (2′-5′) C	0.57
G (3′-5′) C	0.26	U (3′-5′) C	0.48
G (2′-5′) G	0.34	U (2′-5′) G	0.41
G (3′-5′) G	0.17	U (3′-5′) G	0.34
G (2′-5′) U	0.50	U (2′-5′) U	0.50
G (3′-5′) U	0.27	U (3′-5′) U	0.44
G2′p	0.54	U2′(3′)p	0.72
G3′p	0.44		
G > p	0.31	U > p	0.46

The R_F values of the different compounds on the paper chromatogram are given in Table I, and the chromatographic behaviour of the different dinucleoside monophosphates on aluminium cards coated with cellulose powder is demonstrated in Fig. 6. All the 2′-5′ dinucleoside monophosphates separated from the corresponding 3′-5′ isomers in both the paper and the thin-layer chromatograms. However, the separation between the isomers of the type ApN and GpN was better than that of the type CpN and UpN. The only exception was GpG, which could not be chromatographed on the thin-layer cards, probably because of aggregation.

A good separation was found on the thin-layer chromatograms (Fig. 6) between cytidine-2′-phosphate and cytidine-3′-phosphate, and between uridine-2′-phosphate

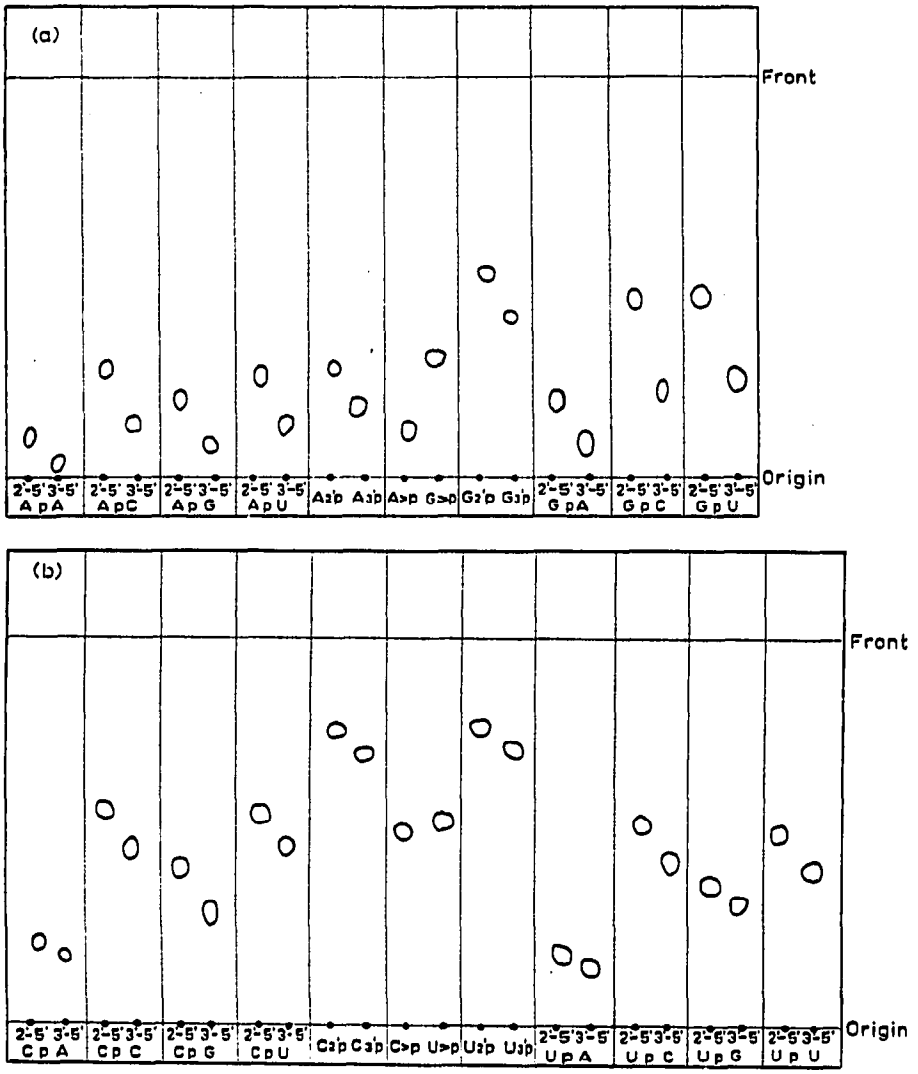

Fig. 6. TLC of (a) ApN and GpN and (b) CpN and UpN, on aluminium cards coated with cellulose powder.

and uridine-3'-phosphate. It is interesting to note that no separation between the two isomers of the pyrimidine monophosphate could be detected on the paper chromatogram when the same solvent system was used.

The paper chromatography and TLC described can be used not only for the separation of 2'-5' dinucleoside monophosphates from the corresponding 3'-5' isomers, but also for the separation of the different 3'-5' dinucleoside monophosphates (and the different 2'-5' dinucleoside monophosphates) from each other. Thus, CpA, CpG and CpC (or CpU); UpA, UpG and UpC (or UpU); ApA, ApG and ApC (or ApU); and GpA, GpG and GpC (or GpU) are separated from each other. However, no separation between dinucleoside monophosphates of the type NpC and NpU could be detected (CpC migrates as CpU; UpC migrates as UpU; etc.).

As a rule, all dinucleoside monophosphates having a 2'-5' linkage move faster than the corresponding 3'-5' isomers. This phenomenon is similar to the behaviour of the dinucleoside monophosphates on ion-exchange thin-layer chromatograms[3], on paper ionophoresis[3], on a DEAE-Sephadex column[2], on a Dowex 1 X2 column[1], and on a Sephadex LH-20 column[7]. As suggested elsewhere[7], the difference in the behaviour of the 2'-5' dinucleoside monophosphates and the 3'-5' isomers is due to differences in conformation.

If the migration of the dinucleoside monophosphates containing the same phosphodiester linkage (all 2'-5' or all 3'-5') is considered, it is found that ApC (or ApU) migrates faster than ApG, and the latter migrates faster than ApA; CpC (or CpU) migrates faster than CpG, and the latter migrates faster than CpA. The same order of migration also applies to the dinucleoside monophosphates of the type GpN and UpN. The migration of all the dinucleoside monophosphates can be summarized as NpC = NpU > NpG > NpA. In a similar way, the migration of the mononucleotides can be summarized as Cp = Up > Gp > Ap. In other words, the retardation of the nucleotidic material to the cellulose is dictated by its base composition. Nucleotides containing adenine are more retarded than those containing guanine; and nucleotides containing pyrimidines (cytosine or uracil) are less retarded than those containing guanine.

In addition to the chromatographic behaviour, the UV spectra ($\lambda_{max.}$ and $\lambda_{min.}$) at pH 1.0 and 7.0 of all the different dinucleoside monophosphates were measured (Table II). Almost no differences were found between the $\lambda_{max.}$ and the $\lambda_{min.}$ of the 2'-5' dinucleoside monophosphates and the corresponding 3'-5' isomers. However, a significant difference was found between the ratio $E\ \lambda_{max.}/\lambda_{min.}$ of the 2'-5' and the 3'-5' isomers of a given dinucleoside monophosphate. For example, $E\ \lambda_{max.}/\lambda_{min.}$ for G (2'-5') C at pH 1.0 is 2.97 and at pH 7.0 is 1.58, while the ratio for G (3'-5') C at pH 1.0 is 3.73 and at pH 7 is 1.94 .In general, the differences in the ratio $E\ \lambda_{max.}/\lambda_{min.}$ at pH 1.0 are greater than the differences at pH 7.0.

The separation of the different dinucleoside monophosphates on a QAE-Sephadex A-25 column described here is similar to the separation on a DEAE-Sephadex A-25 column, and can be used for both analytical and preparative purposes. In addition, the separation of the dinucleoside monophosphates on aluminium cards coated with cellulose powder is very sensitive and can be used for analyzing a given dinucleoside monophosphate including the nature of the phosphodiester bond (2'-5' or 3'-5' linkage).

TABLE II

UV SPECTRA ($\lambda_{max.}$ AND $\lambda_{min.}$) OF THE DIFFERENT DINUCLEOSIDE MONOPHOSPHATES

Nucleotide	*pH 1.0*			*pH 7.0*		
	$\lambda_{max.}$	$\lambda_{min.}$	$E\lambda_{max.}/\lambda_{min.}$	$\lambda_{max.}$	$\lambda_{min.}$	$E\lambda_{max.}/\lambda_{min.}$
A (2'-5') A	256	236	2.15	258	235	4.33
A (3'-5') A	257	230	4.00	257	228	3.63
A (2'-5') C	267	235	3.32	262	231	1.78
A (3'-5') C	266	235	3.41	261	227	1.82
A (2'-5') G	256	229	4.34	255	226	3.43
A (3'-5') G	257	229	7.87	255	225	4.08
A (2'-5') U	258	230	4.47	261	230	4.70
A (3'-5') U	258	230	5.08	260	229	5.14
C (2'-5') A	263	233	2.72	259	229	2.16
C (3'-5') A	265	233	2.46	261	228	2.10
C (2'-5') C	279	241	9.47	269	251	1.25
C (3'-5') C	280	241	9.25	269	250	1.37
C (2'-5') G	274	231	3.41	251	225	1.90
C (3'-5') G	277	232	4.07	256	226	1.98
C (2'-5') U	270	235	2.61	265	230	2.26
C (3'-5') U	270	236	4.76	265	231	1.68
G (2'-5') A	258	230	3.89	255	225	3.58
G (3'-5') A	258	230	4.43	256	225	4.23
G (2'-5') C	276	232	2.97	254	225	1.58
G (3'-5') C	277	233	3.73	255	224	1.94
G (2'-5') G	255	227	5.11	253	225	3.72
G (3'-5') G	256	236	2.03	252	237	1.36
G (2'-5') U	258	230	5.00	256	228	4.00
G (3'-5') U	258	229	5.80	254	226	4.10
U (2'-5') A	258	229	5.52	259	229	5.15
U (3'-5') A	257	230	4.38	259	229	5.23
U (2'-5') C	268	235	3.47	263	230	1.75
U (3'-5') C	269	235	4.30	263	230	1.87
U (2'-5') G	257	228	5.14	254	225	3.75
U (3'-5') G	258	229	4.72	254	225	4.12
U (2'-5') U	261	235	2.50	261	232	2.42
U (3'-5') U	260	230	5.40	261	230	4.41

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