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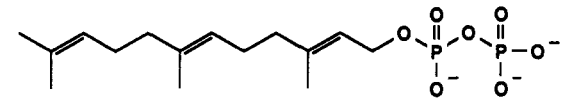
Communications to the Editor

Isoprenoid (Phosphinylmethyl)phosphonates as Inhibitors of Squalene Synthetase

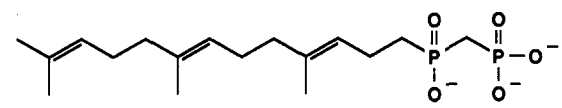
Sir:

In this paper, we describe our initial findings on the preparation and biological evaluation of isoprenoid (phosphinylmethyl)phosphonates (PMPs), stable analogues of farnesyl diphosphate (FPP, 1), as inhibitors of squalene synthetase. Squalene synthetase[1-4] catalyzes the reductive dimerization of two molecules of FPP to form squalene at the final branchpoint in the cholesterol biosynthesis pathway. Considering the recent clinical successes achieved with the mevinic acid family of HMG CoA reductase inhibitors[5] in the treatment of hypercholesterolemia and atherosclerosis,[6] the inhibition of squalene synthetase has received relatively little attention.[7]

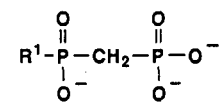
In designing inhibitors of squalene synthetase based on the structure of FPP, strategies for dealing with the allylic diphosphate moiety had to be devised. The diphosphate is essential for binding and catalysis, but is unsuitable as an inhibitor component due to its chemical and enzymatic lability toward both allylic C–O bond cleavage and phosphoric anhydride hydrolysis. We have synthesized analogues of FPP, PMPs **2a–c** and **3a,b**, where the allylic and anhydride oxygen atoms are replaced with carbon.[8-10] The PMP subunit thereby serves as a stable surrogate for the diphosphate. We demonstrate herein that isoprenoid



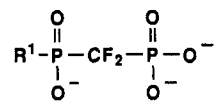
1 FPP



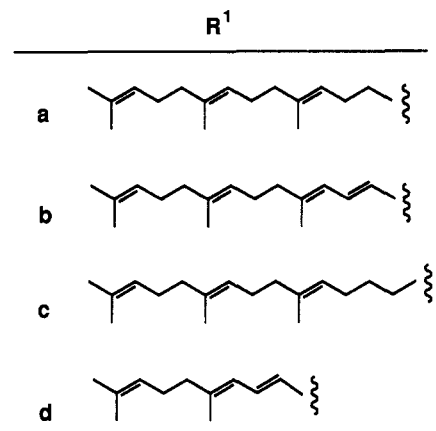
2a



2a-d



3a,b



PMPs are effective inhibitors of squalene synthetase, binding to the enzyme with affinity comparable to FPP itself.

(1) Reviews: (a) Poulter, C. D.; Rilling, H. C. In *Biosynthesis of Isoprenoid Compounds*; Porter, J. W., Spurgeon, S. L., Eds.; Wiley: New York, 1981; Vol. 1, Chapter 8. (b) Popják, G.; Agnew, W. S. *Mol. Cell. Biochem.* **1979**, *27*, 97–116.
(2) Agnew, W. S. *Methods Enzymol.* **1985**, *110*, 359–373.
(3) Dewar, M. J. S. *Tetrahedron* **1987**, *43*, 2661–2674.
(4) Squalene synthetase from yeast has recently been solubilized and purified: (a) Kuswik-Rabiega, G.; Rilling, H. C. *J. Biol. Chem.* **1987**, *262*, 1505–1509. (b) Sasiak, K.; Rilling, H. C. *Arch. Biochem. Biophys.* **1988**, *260*, 622–627.
(5) Hoeg, J. M.; Brewer, H. B., Jr. *J. Am. Med. Assoc.* **1987**, *258*, 3532–3536.
(6) Reviews: (a) Newton, R. S.; Krause, B. R. *Annu. Rep. Med. Chem.* **1986**, *21*, 189–200. (b) Blum, C. B.; Levy, R. I. *Ration. Drug Ther.* **1986**, *20*, 1–4.
(7) (a) Corey, E. J.; Volante, R. P. *J. Am. Chem. Soc.* **1976**, *98*, 1291–1293. (b) Ortiz de Montellano, P. R.; Castillo, R. *Tetrahedron Lett.* **1976**, 4115–4118. (c) Ortiz de Montellano, P. R.; Wei, J. S.; Castillo, R.; Hsu, C. K.; Boparai, A. *J. Med. Chem.* **1977**, *20*, 243–249. (d) Bertolino, A.; Altman, L. J.; Vasak, J.; Rilling, H. C. *Biochim. Biophys. Acta* **1978**, *530*, 17–23. (e) Sandifer, R. M.; Thompson, M. D.; Gaughan, R. G.; Poulter, C. D. *J. Am. Chem. Soc.* **1982**, *104*, 7376–7378.
(8) Reviews on C–P analogues of biologically relevant phosphate containing molecules: (a) Engel, R. *Chem. Rev.* **1977**, *77*, 349–367. (b) Engel, R. In *The Role of Phosphonates in Living Systems*; Hilderbrand, R. L., Ed.; CRC: Boca Raton, FL, 1983; Chapter 5.
(9) (a) Vargas, L. A.; Miao, L. X.; Rosenthal, A. F. *Biochim. Biophys. Acta* **1984**, *796*, 123–128. (b) Blackburn, G. M.; Kent, D. E.; Kolkmann, F. *J. Chem. Soc., Perkin Trans. 1* **1984**, 1119–1125. (c) Blackburn, G. M.; Perrée, T. D.; Rashid, A.; Bisbal, C.; Lebleu, B. *Chem. Scr.* **1986**, *26*, 21–24. (d) Stremler, K. E.; Poulter, C. D. *J. Am. Chem. Soc.* **1987**, *109*, 5542–5544.
(10) After the completion of this work, McClard and co-workers reported the preparation and biological activity of the related PMP analogues of dimethylallyl and isopentenyl diphosphate. These substances were found to be inhibitors of FPP synthetase. In addition, the reaction of the latter analogue with geranyl diphosphate catalyzed by avian liver FPP synthetase gave a product tentatively identified to be **2a**. No biological data was reported for the putative **2a**. See: McClard, R. W.; Fujita, T. S.; Stremler, K. E.; Poulter, C. D. *J. Am. Chem. Soc.* **1987**, *109*, 5544–5545.

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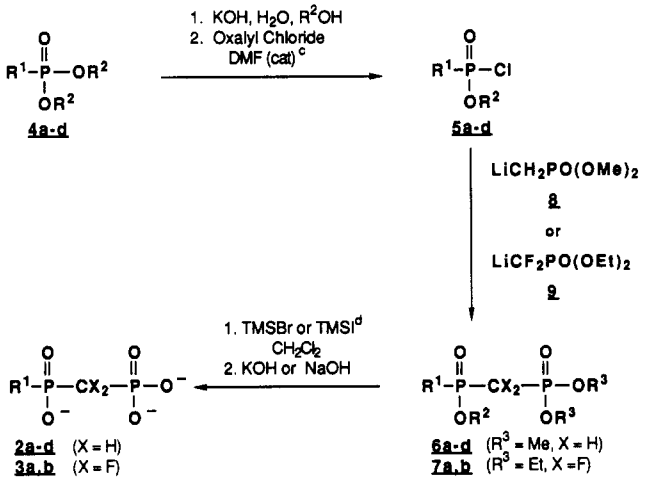
Table I. Preparation of PMP Analogues According to Scheme I

triester[a]	% yield from 4	triacid[a]	% yield from 6 or 7	formula[b]
6a	64	**2a**	60	$C_{17}H_{29}O_5P_2K_3 \cdot 2.8H_2O$
6b	71	**2b**	81	$C_{17}H_{27.6}O_5P_2K_{2.4}$[c]
6c	65	**2c**	71	$C_{18}H_{31}O_5P_2Na_3$
6d	59	**2d**	73	$C_{12}H_{19}O_5P_2Na_3$[d]
7a	42	**3a**	82	$C_{17}H_{27}F_2O_5P_2K_3$
7b	30	**3b**	74	$C_{17}H_{25}F_2O_5P_2K_3$

[a] All compounds had IR, ^1H NMR, ^{13}C NMR, ^{31}P NMR, ^{19}F NMR, and mass spectra consistent with the structures. Spectral data for representative PMP salt **2a** can be found in the footnotes.[30] [b] All compounds had C, H, F, and P analyses consistent with the proposed formula except where indicated. All analytical samples except **2a** were dried to a constant weight at 50 °C under vacuum. [c] H: calcd, 5.95; found, 6.47. This compound is a 1.5:1 mixture of di- and tripotassium salts. [d] C: calcd, 38.52; found, 38.96. P: calcd, 16.55; found, 17.11.

Scheme I. Preparation of PMP Analogues **2** and **3**[a,b]



[a] R^2 = Me except for series c, where R^2 = Et. [b] See Table I for yields and analytical data. [c] See ref 13. [d] TMSBr was employed for X = H, TMSI for X = F.

We developed a general route for the synthesis of PMPs (Scheme I, Table I) that utilizes the reaction of phosphonate-stabilized carbanions with phosphonochloridates[11] in the key coupling step. Phosphonates **4a–d**[12] were hydrolyzed to the corresponding monoacids (4–10 equiv of KOH, H_2O, R^2OH, reflux) and then converted to acid chlorides **5a–d** (3 equiv of oxalyl chloride, catalytic DMF, PhH, room temperature).[13] The phosphonochloridates **5a–d** were reacted with anion **8** (2.15 equiv)[14] in THF at −78 °C for 2 h to provide triesters **6a–d**. Cleavage of the esters with TMSBr[15] (4–4.5 equiv) in the presence of 2,4,6-collidine (1.5–2 equiv) in CH_2Cl_2 at room temperature followed by salt formation (>3 equiv of NaOH or KOH) gave inhibitors **2a–d**. Similarly, **5a** and **5b** were coupled with anion **9** (1.05 equiv, THF, −78 °C, 18 h)[16] to provide **7a** and **7b**. For the fluorinated esters **7a,b**, TMSI[17] (4 equiv; 2 equiv bis(trimethylsilyl)trifluoroacetamide, CH_2Cl_2, 0 °C) was required to effect deprotection to **3a,b**. The PMP salts were purified by MPLC on CHP20P gel,[18] eluting with water/acetonitrile or water/methanol gradients, and were isolated as amorphous, hygroscopic lyophilates. This route to PMPs readily accommodates variations in the structure of R^1 and the substituents on the P–C–P carbon and thus has advantages over prior methods.[9a,10,19]

Table II. Inhibition of Squalene Synthetase by PMP and Related Analogues[21,23]

compd	I_{50}, μM	compd	I_{50}, μM
2a	31.5	**3b**	15.5
2b	12.2	**10**	42
2c	67	**11**	177
2d	−40% at 600	**12**	inactive at 200[a]
3a	29.9	**13**	inactive at 100[a]

[a] Compounds were insoluble at greater concentrations.

In order to evaluate the inhibitory potency of the PMP analogues, we devised a new procedure to measure squalene biosynthesis from FPP with rat liver microsomes.[20] The assay conditions[21] employed were a modification of those reported by Agnew[2] for the yeast enzyme. Squalene production was quantitated by automated GLC analysis[23] of the nonsaponifiable lipid extract. This me-

(11) Aboujaoude, E. E.; Lietjé, S.; Collignon, N.; Teulade, M. P.; Savignac, P. *Tetrahedron Lett.* **1985**, *26*, 4435–4438. Teulade, M. P.; Savignac, P.; Aboujaoude, E. E.; Liêtge, S.; Collignon, N. *J. Organomet. Chem.* **1986**, *304*, 283–300.

(12) Phosphonic diester **4a** was prepared according to the literature procedure.[7a] Analogues **4b** and **4c** were prepared from (*E*,-*E*)-farnesol (Aldrich Chemical Co.) via the following reaction sequences. **4b**: (a) oxalyl chloride, DMSO, CH_2Cl_2, −60 °C; Et_3N, room temperature; (b) $CH_2(PO(OMe)_2)_2$, NaH, THF. **4c**: (a) PBr_3, Et_2O, 0 °C; (b) $CH_2(CO_2Et)_2$, NaH, THF; (c) LiCl, H_2O, DMSO, 160 °C; (d) LAH, Et_2O; (e) $MeSO_2Cl$, Et_3N, CH_2Cl_2, 0 °C; LiBr, THF; (e) $P(OEt)_3$, 150 °C. Compound **4d** was prepared from (*E*)-geraniol analogously to **4b**.

(13) For the preparation of **6c**, **6d**, and **7b**, the intermediate phosphonic acid monoesters were first treated with $TMSNEt_2$ (2 equiv, CH_2Cl_2, room temperature) followed by solvent evaporation, prior to reaction with oxalyl chloride (1.8 equiv, catalytic DMF, CH_2Cl_2, 0 °C to room temperature) to form the acid chlorides. This procedure minimizes contact with HCl.

(14) Corey, E. J.; Kwiatkowski, G. T. *J. Am. Chem. Soc.* **1966**, *88*, 5654–5656.

(15) McKenna, C. E.; Higa, M. T.; Cheung, N. H.; McKenna, M. C. *Tetrahedron Lett.* **1977**, 155–158.

(16) Obayashi, M.; Ito, E.; Matsui, K.; Kondo, K. *Tetrahedron Lett.* **1982**, *23*, 2323–2326.

(17) Blackburn, G. M.; Ingleson, D. *J. Chem. Soc., Perkin Trans. 1* **1980**, 1150–1153.

(18) CHP20P is a highly porous styrene–divinylbenzene copolymer available from Mitsubishi Chemical Industries.

(19) Novikova, Z. S.; Prishchenko, A. A.; Skorobogatova, S. Ya.; Martynov, V. I.; Lutsenko, I. F. *J. Gen. Chem. USSR* **1980**, *50*, 787–790. Farrington, G. K.; Kumar, A.; Wedler, F. C. *J. Med. Chem.* **1987**, *30*, 2062–2067.

(20) The rat hepatic microsomes were prepared according to Slakey and co-workers and were frozen in homogenization buffer and maintained at −78 °C for 2 months with little loss of activity: Slakey, L. L.; Craig, M. C.; Beytia, E.; Briedis, A.; Feldbruegge, D. H.; Dugan, R. E.; Qureshi, A. A.; Subbarayan, C.; Porter, J. W. *J. Biol. Chem.* **1972**, *247*, 3014–3022.

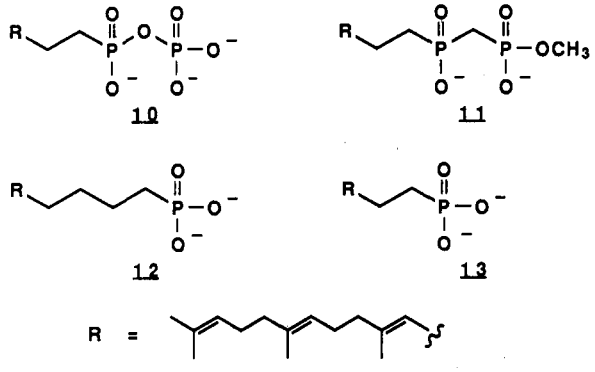
(21) Reaction mixtures composed of potassium phosphate buffer (0.36 mL, 0.275 M, pH 7.4), KF (0.36 mL, 55 mM), NADPH (0.36 mL, 5 mM), $MgCl_2$ (0.36 mL, 27.5 mM), rat liver microsomes[20] (0.20 mL of a suspension containing 0.48 mg of microsomal pellet protein in homogenization buffer), and 0.16 mL of water or a water solution of the test compound were prepared at 4 °C. The reaction mixtures were equilibrated under nitrogen at 4 °C for 5–15 min and warmed to 30 °C, and the reaction was initiated by adding aqueous FPP[22] (0.2 mL, 219 μM). Under these conditions, enzyme activity was linear with respect to microsomal protein concentration up to 1 mg per reaction mixture, and squalene production was linear for 3 h at 0.48 mg of protein per reaction mixture. After 60 min at 30 °C, the reaction was terminated with 1 mL of 40% KOH and 1 mL of 95% ethanol, and docosane (5 nmol in 10 μL of hexane) was added as an internal standard. The mixture was saponified at 65 °C for 30 min and extracted with two 10–mL portions of hexane.

(22) FPP was prepared according to Poulter and co-workers: Davisson, V. J.; Woodside, A. B.; Poulter, C. D. *Methods Enzymol.* **1985**, *110*, 130–144. Davisson, V. J.; Woodside, A. B.; Neal, T. R.; Stremler, K. E.; Muehlbacher, M.; Poulter, C. D. *J. Org. Chem.* **1986**, *51*, 4768–4779.

thod is rapid and accurate and does not require radiolabeled FPP.

The ability of PMP analogues to inhibit squalene production vs controls are expressed as I_{50} values in Table II. PMPs **2a**, **2b**, **3a**, and **3b**, which are isosteric with FPP, are effective inhibitors of squalene synthetase. The difluoro-PMP surrogate of **3a** and **3b** was expected to closely resemble the diphosphate of FPP with regard to acidity, favoring the fully ionized form at physiological pH.[9b-d] Despite this factor, no difference in potency is observed between the corresponding PCH_2P (**2a,b**) and PCF_2P (**3a,b**) analogues. This suggests that both **2** and **3** can achieve the ionization state appropriate for binding to the enzyme. 1,3-Dienyl PMPs **2b** and **3b** are somewhat more active than **2a** and **3a**, respectively, indicating that a vinyl group may be a superior substitute for the allylic C–O linkage.[24] Homologation of **2a** to give **2c** results in a 2-fold loss of activity. The geranyl derivative **2d** is at least 20-fold less active than the corresponding farnesyl counterpart **2b**. The full FPP chain length is apparently required for maximal inhibitory potency. This is consistent with the observation that geranyl diphosphate is both a poor substrate[25] and inhibitor[7c] for squalene synthetase.

Corey and Volante reported that phosphinylphosphate **10** inhibited the conversion of mevalonic acid to squalene in a 10000*g* supernatant from rat liver homogenate.[7a] We have prepared **10** and have shown it to possess comparable potency to **2a** in our assay. We surmise, therefore, that the anhydride oxygen does not contribute significantly to the binding of FPP to the enzyme. The 6-fold lower activity displayed by the monomethyl ester **11** relative to the parent acid **2a** suggests that a trianionic species may be required for a full expression of activity. In addition, the poor inhibition achieved by monophosphonates **12**[26] and **13** indicates that both phosphorous moieties are essential for good inhibition.



10 **11** **12** **13**

The inhibition of microsomal squalene synthetase by **2a** was studied at increasing FPP (2.5–100 μM)[27] and saturating NADPH (0.9 mM) concentrations. Double-reciprocal analysis of the kinetic data (Figure 1) indicates that **2a** is a competitive inhibitor with an apparent K_i of 10 μM. Since the apparent K_m for FPP under these conditions is 14.8 μM,[29] **2a** binds to the enzyme with an affinity comparable to FPP. We infer from this that neither the allylic nor anhydride oxygens of FPP are critical in substrate binding.

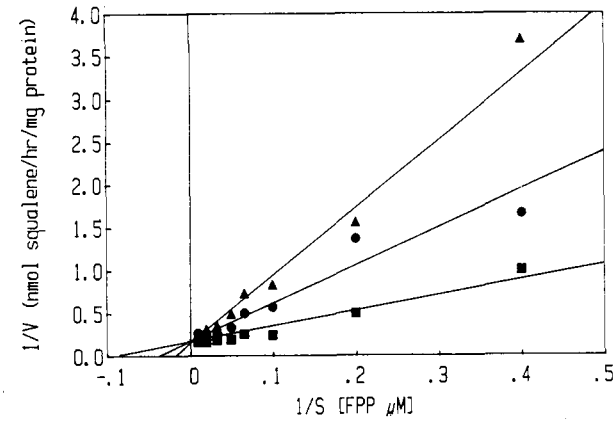


Figure 1. Lineweaver–Burk analysis of the inhibition of microsomal squalene synthetase by PMP **2a**. Concentration of **2a**: 0 (■), 10 (●), and 30 μM (▲).

In conclusion, we have demonstrated that the PMP moiety can serve as a stable surrogate for the allylic diphosphate of FPP in the construction of effective inhibitors of squalene synthetase. Prototypical PMP **2a** is a competitive inhibitor of squalene synthetase, binding to the enzyme as strongly as the natural substrate, FPP.

Registry No. **1**, 372-97-4; **2a**, 115731-53-8; **2b**, 115731-55-0; **2c**, 115731-57-2; **2d**, 115731-58-3; **3a**, 115731-54-9; **3b**, 115731-56-1; **4a**, 115705-13-0; **4a** (monoacid), 115705-27-6; **4b**, 115705-17-4; **4b** (monoacid), 115705-28-7; **4c**, 115705-21-0; **4c** (monoacid), 115705-29-8; **4d**, 115705-24-3; **4d** (monoacid), 115705-30-1; **5a**, 115705-14-1; **5b**, 115705-18-5; **5c**, 115705-22-1; **5d**, 115705-25-4; **6a**, 115705-15-2; **6b**, 115705-19-6; **6c**, 115705-23-2; **6d**, 115705-26-5; **7a**, 115705-16-3; **7b**, 115705-20-9; **8**, 58648-56-9; **9**, 83567-74-2; $CH_2(PO(OMe)_2)_2$, 756-79-6; $CH_2(CO_2Et)_2$, 105-53-3; squalene synthetase, 9077-14-9; (*E,E*)-farnesol, 106-28-5; (*E,E*)-farnesal, 502-67-0; (*E,E*)-farnesyl bromide, 28290-41-7; diethyl (*E,E*)-farnesyl malonate, 58456-69-2; ethyl (*E,E*)-farnesyl acetate, 59822-16-1; (*E,E*)-dihomofarnesol, 67858-77-9; (*E,E*)-dihomofarnesyl mesylate, 115705-31-2; (*E,E*)-dihomofarnesyl bromide, 115705-32-3; (*E*)-geraniol, 106-24-1; (*E*)-geranial, 141-27-5.

(23) Squalene production was measured by concentrating the hexane extract to 50 μL and injecting 2 μL of each sample onto a fused silica mega-bore DB-17 column (15 M × 0.525 mm, J and W Scientific) with use of a splitless injection mode and 15 mL/min flow rate of helium carrier gas. The following temperature program was employed, with a 20 °C/min ramp between each temperature: 180 °C for 10 min, 250 °C for 10 min, 260 °C for 10 min. The docosane internal standard had a retention time of 3.6–4.0 min, and squalene had a retention time of 14.7–15.2 min. All reaction mixtures were run in duplicate. Potential inhibitors were tested at several different concentrations in at least two independent assays, and the I_{50} values were determined by linear regression analysis of the combined data. Average standard error = 14% of reported I_{50} values (range = 7–19%).

(24) Hullar, T. L. *J. Med. Chem.* **1969**, *12*, 58–63.

(25) Koyama, T.; Ogura, K.; Seto, S. *Biochim. Biophys. Acta* **1980**, *617*, 218–224.

(26) A related observation was made by Corey and Volante.[7a]

(27) Higher levels of FPP decreased enzyme velocity, as observed for the yeast enzyme.[28]

(28) Agnew, W. S.; Popják, G. *J. Biol. Chem.* **1978**, *253*, 4566–4573.

(29) A similar apparent K_m was reported for the yeast microsomal enzyme,[4b,28] and more recently for the homogeneous, solubilized yeast enzyme.[4b]

(30) NMR data for **2a** were recorded in 1:1 CD_3OD/D_2O: ^1H NMR (400 MHz) δ 5.06, 5.08, 5.20 (3 t, 1 H each, J = 7 Hz, =C*H*), 2.22 (m, 2 H, PCH_2CH_2), 1.9–2.1 (m, 10 H, =CCH_2, PCH_2P), 1.64 (s, 3 H, =CCH_3), 1.62 (s, 3 H, =CCH_3), 1.56 (s, 6 H, =CCH_3) (an additional CH_2 is buried beneath the =CCH_3's); ^{13}C NMR (67.8 MHz) δ 136.0, 135.9, 132.6, 126.3 (d, J = 7 Hz), 125.3, 125.1, 40.5, 40.4, 33.5 (dd, J = 81.4, 117.3 Hz), 33.3 (d, J = 92.7 Hz), 27.5, 27.3, 25.9, 22.0, 17.8, 16.2, 16.1; ^{31}P NMR (109 MHz, ^1H-decoupled, 85% H_3PO_4 external reference) δ 43.9 (d, J = 8 Hz, C*P*C), 17.2 (d, J = 8 Hz, C*P*O_3). MS (FAB, + ions), m/e 531 (M + K), 493 (M + H), 455 (M + 2H – K).

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